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This special issue of the JOMBA marks a significant departure from the normal content we are familiar with in the Journal. Following a period of intense and often rather focused research, Marine Biology, indeed the whole of the Marine Sciences community is undergoing a ‘sea change’ (sic!!) in its outlook and approach. We are now embracing ideas and establishing closer collaborative links with the Social Sciences (including economics and the law), and the biomedical and public health communities.

Much of this is driven by the need for us all to think more about the impact and relevance of our work to the wider public, together with the sustainability of the marine ecosystem and ecosystem services whose form and function we explore.

The collection of papers in this issue arises from a Workshop on Oceans and Human Health held in 2014 (http://www.ecehh.org/events/oceans-human-health/). The range of topics covered is impressive, from the more traditional papers on harmful marine algae to reviews of how the law impacts the use of our coastal areas, economic perspectives of oceans and human health and the 'Blue Gym'. All the papers share in their highly interdisciplinary approach, and their focus on the health of both humans and the marine environment.

This diversity of titles reflects the complexity of interactions between the seas and oceans, and human health and wellbeing. It is of interest here to consider that until recently in the social sciences and the medical and public health communities, attention has primarily focused on the negative impacts of the oceans and seas on human health (e.g. drowning and injury from extreme weather events or from fishing disasters), while in the marine biology/marine science community attention has traditionally been directed to the many benefits (e.g. healthy foods, novel drugs etc.) as well as the potentially negative environmental impacts (e.g. red tides, exotic species etc.). Now, there is increasing recognition that the 'health' of our seas and oceans is inextricably linked to human health and wellbeing in a number of other, often quite complex ways.

This has led to the realization that marine biologists/scientists, social scientists, as well as public health and medical scientists must come together if we are to address the impacts of human activities on the marine environment, and how this affects the stability and sustainability of human populations and their activities. These interactive effects can be seen at both the local scale (e.g. microbial pollution on specific beaches) and the global scale (e.g. ocean acidification associated with higher atmospheric CO2). It has become apparent that many of these anthropogenic ocean impacts will inevitably affect human health and activities now and into the future (Knap et al. 2002; Bowen et al., 2006; Fleming et al., 2006; Fleming and Laws, 2006; Walsh et al., 2008; Bowen et al., 2014; Fleming et al., 2014).

There is then a growing realization that all the interactions between humans and the oceans can have both benefits and risks. Destruction of these ecosystems, through pollution and unsustainable development for example, threatens important potential health-related discoveries. Additional, possibly more complex interactions, are being recognized by the wider population; for example, while algae and other phytoplankton form the basis of the entire marine food chain, some species produce potent natural toxins harmful to humans and other animals, and these same species may be increasing due to human activities. At the same time, we are increasingly relying on the oceans and seas for a range of important resources and human activities, from fishing and transport to future deep sea mineral mining and energy exploration (e.g. European Union Blue Growth Strategy: http://ec.europa.eu/maritimeaffairs/policy/blue_growth/). And all these human interactions impact on the health of local and (in the case of climate change) global marine ecosystems, and through ecosystem degradation, on human health and wellbeing.

While most of these kinds of interactions are quantifiable in one way or another, those surrounding human health and wellbeing need to be explored using qualitative research methods to truly understand the scope of their short- and long-term impacts. For example, the exploration of the potential positive benefits from interacting with the coasts and 'blue environment' need both qualitative and quantitative methodologies. Since the 18th century, 'taking the waters' either as medicine or immersion was promoted as being beneficial to health; this contributed to the growth of the medical 'thalassotherapy' approach and industry. In the 21st century, we are now seeing the emergence of terms such as the 'blue gym', coined to describe the sometimes indefinable, or at least difficult to quantify, mental and physical health benefits to be obtained from experiencing proximity to marine environments.

Our aim in this special issue of JOMBA is then to expose marine scientists to the extensive literature surrounding 'Health Promotion and Prevention' and other cognate areas...
of Public Health. If we are to address this, a new, integrated and truly interdisciplinary approach is needed, that embraces not only among scientists and across scientific communities, but also policymakers and other stakeholders including the general public (Laws et al., 2008; Walsh et al., 2008; European Marine Board, 2014). This new meta-discipline, Oceans and Human Health, necessarily also incorporates perspectives from medicine, psychology, public health, geography, oceanography, economics and sociology, as well as the law, humanities, business and the arts.

Supporting this vision, an increasing number of unanticipated groups are realizing they too are stakeholders in oceans and human health. For example, in addition to the millions of inhabitants of rapidly growing coastal cities, there are increasing numbers of people moving to or visiting coastal locations around the globe with a resulting growing number of businesses that serve them (e.g. tourism, aquaculture and fisheries, pharmaceuticals, transport, energy sectors), non-governmental organizations (NGOs) (e.g. Ocean Conservancy (http://www.oceanconservancy.org/), World Wildlife Fund (http://www.worldwildlife.org/), Surfrider Foundation (http://www.surfrider.org/), Pew Foundation (http://www.pewtrusts.org/en) and other similar organizations).

In this special issue, we present a range of papers which we hope provide an accessible overview of this exciting new field. Those included are representative of the diverse nature of those with an interest and concern for the marine environment. We hope that this will serve to highlight the necessary interdisciplinarity required for collaborative partnerships across wider scientific communities that will be vital if we are to work effectively towards the preservation and protection of our coasts and oceans.

REFERENCES


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DEDICATION

We would like to dedicate this special issue to two key researchers and leaders in the world of Oceans and Human Health, the late Professor Eric DeWailly (Laval University) and Professor Laurence Mee (Scottish Association of Marine Sciences). Their interdisciplinary research approach, enthusiasm, creativity, inclusiveness and international horizon scanning perspective have impacted all whose lives they touched and contributed directly to Oceans and Human Health.
The ‘Blue Gym’: What can blue space do for you and what can you do for blue space?

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The Blue Gym Initiative was created in the UK in 2009 to explore: (1) whether blue space environments might be positively related to human health and well-being; and (2) whether the public could be encouraged to preserve and protect these environments. Whilst the wider initiative considers all blue spaces including inland bodies of water (e.g. lakes, rivers and canals as well as the coasts and oceans), to date the focus has been primarily on marine and coastal environments. In this paper, we provide a brief history of the Blue Gym Initiative, and outline some of the research that has emerged to date. An important early finding was the observation that individuals living near the coast are generally healthier and happier than those living inland; much subsequent work has tried to understand why this might be. More recently we have begun to focus on how to promote pro-marine behaviours (e.g. sustainable fish choice, reduction of plastic use, avoidance of littering). This strand is still very much work in progress but highlights the importance of understanding public awareness, values and attitudes and the power of visualization in communicating the marine sustainability issues. We conclude with a brief discussion of some of the implications of the findings and future research needs.

Keywords: Blue space, Health and well-being, Pro-marine behaviours

BACKGROUND: WHERE DID THE IDEA ORIGINATE AND WHAT PROGRESS HAS BEEN MADE SO FAR?

The environment as a source of both disease and good health

Perhaps the greatest advance in public health was the realization that the environments in which people live and work are important determinants of ill health and disease (Di Giulio & Monosson, 1996; Rayner & Lang, 2013). Swamps breed malaria-carrying mosquitoes; poor sewage treatment can encourage the spread of cholera. Since these early realizations, the field of public health has taken enormous steps in identifying a range of potential sources and vectors of disease in the environment, and implementing strategies to reduce risks to people. This perspective sees the environment, natural and human-made, as a potential problem for health that needs to be managed; the emphasis is on the disease-causing aspects of the built and natural environment. Despite the obvious importance of this perspective an alternative, yet also complementary, perspective sees the environment as a potential source of good health. This salutogenic (health creating) perspective notes, for instance, the importance of exposure to sunlight for vitamin D synthesis and the role of urban design, e.g. provision of cycle paths, in facilitating health-promoting physical activity. This second perspective sees the environment, natural and human-made, as a potential way of promoting health and well-being.

A brief history of the Blue Gym Initiative

The Blue Gym Initiative developed from this second perspective (Depledge & Bird, 2009). With support from the National Health Service (NHS), Natural England and the UK Environment Agency, its first aim was to understand the potential of ‘natural’ aquatic environments (e.g. ponds, rivers, lakes, canals, coastlines etc.) to enhance and promote human health and well-being more broadly. In this respect, the initiative built deliberately on the earlier ‘Green Gym’ initiative which was trying to encourage people to engage in environmental volunteering in green spaces such as parks and woodlands (Yerrel, 2008). This programme, run by the British Trust for Conservation Volunteers (now, The Conservation Volunteers), was intended to have the dual benefits of both being good for participants’ health and well-being and also good for the environment. However, it was also clear that ‘blue space’ environments posed very different risks (e.g. drowning, harmful algal blooms [HABs], etc.) and potential benefits from terrestrial green spaces. At this early stage in the concept there was very little understanding of exactly what these benefits might be; and in particular what benefits might be lost if: (a) people began reducing their exposure to these environments in an attempt to avoid the real and perceived risks; and (b) the present rate of degradation of these ecosystems continued (through habitat destruction, pollution, etc.). However, in the last five years, significant steps forward have been made in understanding some of these issues and these are reviewed below.

The second aim of the Blue Gym Initiative Programme was to encourage a wider public debate and therefore develop and promote more public awareness and direct involvement in the more responsible use of blue space environments with benefits both to human health and to these often fragile ecosystems. For example, could public engagement be used to encourage...
enjoyment of the shoreline or riparian zones without concomitant negative impacts on species and habitats? Could marine or river ‘champions’ be identified and supported to ‘spread the word’, encourage greater participation, and help link and coordinate organizations that were already doing some of these activities but often in isolation?

To support these latter outcomes in particular, the Blue Gym Initiative established an internet-based ‘social network’, the aim of which was to foster an organic social movement to deliver these goals, and also to provide a platform for researchers in this area to promote robust, evidence-informed, practice. The early days of this network were highly encouraging with hundreds of individuals and organizations joining and contributing (see for instance early videos: https://www.youtube.com/watch?v=Jqq4KbB56QA). However, it quickly became apparent that there were many challenges to running and organizing a social network community, not least disruptive internet practices, which, in hindsight, the Blue Gym team did not have the resources to deal with. In particular, updating, monitoring and moderating the site became an ever greater challenge as its popularity grew. Since we did not have the means necessary to maintain the early high standards as the site grew, we decided to scale back this aspect and focus on our research objectives. Our decision to do this was primarily pragmatic; we still believe that there is a potential role for this kind of public engagement activity if sufficient resources are allocated and maintained over the long term.

**Two emerging strands of interconnected research**

As greater effort was focused on the research component, it also became apparent that two parallel, though interconnected, strands were emerging that were broadly in line with the two aspects noted above. In his inaugural presidential address, President Kennedy famously said ‘ask not what your country can do for you, but what you can do for your country’. Paraphrasing this, the two strands were essentially asking: (a) What can blue space do for you and (b) what can you do for blue space? The first strand looked at the potential benefits to health and well-being of different blue space environments. The second strand looked at how people could be encouraged to protect and conserve such environments by looking at issues such as public awareness of the issues and how best to communicate the problems. Our discussion below is broadly structured around these two issues.

Driven by connections with existing projects and for geographic reasons, most of the research that has been conducted under the Blue Gym banner has focused on marine and coastal environments. Based in the South West UK, the research team had (and continues to have) excellent access to a variety of coastal settings and coastal populations. It is not yet clear how some of our findings might relate to inland waterways, and we have identified this as a key research priority going forward. We are also aware of the excellent work on these topics that has been, and continues to be, carried out by others in the UK and around the world (e.g. Ronan Foley in Ireland, https://www.maynoothuniversity.ie/geography/our-people/ronan-foley; Adi Hanein and Kelly Biedenweg in the USA, http://www.epugetsound.org/articles).

**WHAT CAN BLUE SPACE DO FOR US – HEALTH AND WELL-BEING**

**Identifying the questions**

Research into landscape preferences has long documented the appeal of aquatic environments, both inland and coastal, over other landscapes (Hubbard & Kimball, 1967; Zube, 1974; Ulrich, 1983; Herzog, 1985; Kaplan & Kaplan, 1989). Indeed in some studies, aquatic landscapes are actually excluded because preferences for them are so strong as to distort the findings related to natural settings in general (Herzog et al., 2003).

These preferences manifest themselves in practical ways. Estate agents and hoteliers, for instance, are well aware that people are willing to pay more for homes and hotel rooms with blue space views (Luttik, 2000; Lange & Schaeffer, 2001). Those who cannot afford such views still seek closeness to these environments in their free time. For instance, coastal resorts are, by far, the most popular holiday destination in the EU with over 16 million tourist beds (Collett, 2010). People like blue space and are willing to spend time and money to be near it. These findings constitute the basis of our (and others’) research questions. We assumed that the reason that people like these environments – and are prepared to pay to experience them – is because they derive some kind of benefit from them. What is then important to determine is what exactly the benefits might be, how widely available they are, how they might arise, who might benefit the most, and what the relationships are between the types and quality of different blue space environment and positive outcomes. Put simply, would any coast or river do, or did, for instance, the visual aesthetic, cleanliness and biodiversity of the site also play an important role?

**Early studies – identifying the potential benefits**

The first study used a very simple paradigm adapted from environmental psychology and landscape preference research (White et al., 2010). The approach involved asking participants to sit in a research laboratory and view a series of 120 still photographs of different environments. Their task was to imagine sitting in each environment and state how they thought being there would make them feel or help to combat mental fatigue. Previous studies had shown that people are relatively good at tasks of this nature, and that...
their rank order preferences in the laboratory closely tally with their reports in situ. People know which kinds of environments stimulate which kinds of emotions they experience. What was original about this study was that the amount of water visible in the scenes was carefully controlled, along with a range of potential confounders (e.g. the number of people, animals, and human-made objects such as cars).

In addition to replicating earlier work that found that people preferred aquatic landscapes, and were willing to pay more for hotel rooms with aquatic views, we further showed that urban landscapes containing water are associated with positive outcomes broadly similar to those of rural green space environments without water. Previous work had concluded that rural environments are generally better than urban environments, but these reports had not controlled for the presence of water. To give an indication of the magnitude of these effects we report the mean preference ratings for three sets of urban images; those with no green/blue space visible (all buildings, roads etc.), those with roughly one third green space visible, and those with roughly one third blue space visible (for all nine environment types see figure 2 in White et al., 2010). Preferences were based on two items asking about how attractive the scene was and how willing to visit the scene respondents were on scales from 0 'Not at all' to 10 'Extremely'. Compared with urban settings with no natural elements (M = 3.66), preferences were significantly higher for urban scenes with both green space (M = 4.89, P < 0.001) and blue space (M = 5.96, P < 0.001). However, as can be seen from the means, preferences for urban images with some blue space were also significantly higher (P < 0.001, reflecting over a 1 point difference on an 11 point scale) than those for urban images with some green space. Moreover, these preferences for urban images with some blue space were not significantly different from images of open countryside and woodlands with no visible built content (M = 5.83), hinting at the potential importance of water for preferences in urban settings. A further novel finding was that images of aquatic margins (e.g. the coast, M = 7.68) were associated with significantly higher preferences than open water (M = 7.40; P < 0.01), suggesting an important role for the land-water interface. Three subsequent studies using the same paradigm showed that these preferences are not simply due to colour (i.e. more 'blue'); they persisted using black and white images, or different types of weather, since aquatic landscapes are still preferred in rainy/stormy weather. Furthermore, underwater scenes are also highly rated (White et al., 2014a).

We reasoned that high levels of habitual exposure to these environments should manifest itself in some way. More specifically, given the extensive evidence linking positive mental states and physical health (e.g. Pressman & Cohen, 2005), we reasoned that people who lived near the coast should be experiencing high levels of positive emotion, and in turn this should be apparent in their general levels of health. To test this hypothesis, we used the English 2001 census data (~48 million adults), and analysed the frequency with which people in ~30,000 neighbourhoods responded 'Good' to the following question: 'Over the last 12 months would you say your health has on the whole been, Good, Fairly Good or Not Good?' (Wheeler et al., 2012). Due to geographic variation in population age/sex structure, we calculated directly standardized rates of good health in these neighbourhoods. Although this self-reported health variable is a simple measure, answers to this question are strongly correlated with objective measures of health (people generally know if they are in poor health; Kyffin et al., 2004); and had previously been used in green space research (Mitchell & Popham, 2007). Due to identification restrictions in the census data it was only possible to make an approximate estimation of how close individuals lived to the sea, based on how close to the coast their neighbourhood was (each ~1500 people and 4 km² on average), rather than the actual location of their dwelling.

Allocating coastal distance into one of five categories broadly reflecting access potential (e.g. 0–1 km = walking distance; 2–5 km = cycling/short drive distance etc.), and looking at the sample overall, we found a relatively steady coastal proximity gradient. The closer a neighbourhood was to the coast, the better, on average, its residents' overall self-reported health. Although the effects were relatively small, they potentially apply to many people. For example, there was only a 1.13% increase in those reporting 'good health' among those who lived in urban areas within 1 km of the coast compared with those who lived in urban areas over 50 km from the coast. However, the number of people who live within 1 km of the English coast is ~3 million which suggests a potentially important cumulative effect. Moreover, when the level of neighbourhood deprivation (Noble et al., 2004) was stratified, we found that this proximity gradient was strongest among the most deprived neighbourhoods and weakest in the least deprived neighbourhoods. That is, living near the sea seemed to confer the greatest health-related benefit on those living in the poorest areas; a similar finding for urban green space was reported by Mitchell & Popham (2008). There are a number of possible explanations for this finding, and one of these might be that the coast offers a range of relatively cost-free, health-promoting opportunities (e.g. stress reduction and physical activity) in an attractive environment that individuals with limited economic resources could nonetheless profit from. Wealthier individuals, by contrast, may have broader access to health-promoting opportunities wherever they live and may also be more mobile, so that close proximity to the coast may be less important.

A limitation of the study is that it is open to the possibility of an 'ecological fallacy', i.e. inferring individual level association from group/area associations. A good example of this would have been to have assumed that wealthier voters were more likely to have voted Democrat in the 2004 US presidential election because John Kerry won more wealthy States than George Bush, even though wealthier individuals were actually more likely to have voted for Bush. The problem of inferring individual relationships from area associations with respect to local area greenspace has been noted previously with respect to US cities (Richardson et al., 2012). A further limitation is the cross-sectional nature of the data which meant that it was not possible to control for selection effects (i.e. healthier people might move to the coast rather than the coast promoting the health of those who live nearby).

To counter these problems, a subsequent study used data from the British Household Panel Survey (BHPS; White et al., 2013a). The BHPS is a nationally representative longitudinal survey that ran annually from 1991 to 2008 and included over 5000 households. Importantly, some people moved home during their participation in the panel survey, sometimes nearer to the coast and sometimes further from the coast. By comparing individuals’ self-reported health (and mental well-being) in years when they lived at different distances
from the sea, it was possible to control for a range of potential individual level confounds which might be affecting selection effects. Extending the census findings it was found, using a sample of 109,844 observations from 15,471 individuals, that people did indeed report better mental and physical health in years when they lived closer to the sea (see also Brereton et al., 2008). Again, although the absolute size of the effects was small, the relative effects compared with other things that are widely cited as important for health and well-being were nonetheless telling. For instance, living within 5 km of the coast (compared with further inland) was associated with 0.22 times the beneficial effect of being employed rather than unemployed, suggesting coastal living can have meaningful impacts on health. Moreover, this finding was not simply due to a ‘healthy retiree effect’ as the same pattern was found among the working age population.

Although this study reduced the possibility of a selection bias in coastal living, it still sheds little light on the mechanisms underlying these apparent benefits.

Examing the mechanisms

In their extensive review of the factors linking natural environments more broadly with health outcomes, Hartig et al. (2014) discussed four key potential mechanisms: stress reduction, physical activity, social interactions and environmental quality (in particular air quality). Broadly speaking, they argued that the reason why people who live in greener areas (e.g. near parks or woodlands) are generally healthier is that they are: (a) less stressed, (b) exercise more, (c) have more positive social interactions and (d) the environment in which they live is of a better quality for health promotion (i.e. has lower levels of air pollution). Similar indicators were used in an attempt to help explain our findings from the Census and BHPS data.

To determine whether coastal and marine environments might be especially good for stress reduction, data from Natural England’s national survey of outdoor leisure visits, the Monitor of Engagement with the Natural Environment (MENE) was examined. Focusing on 4255 visits where the Monitor of Engagement with the Natural Environment (MENE) was examined. Focusing on 4255 visits where 

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To determine whether coastal and marine environments might be especially good for stress reduction, data from Natural England’s national survey of outdoor leisure visits, the Monitor of Engagement with the Natural Environment (MENE) was examined. Focusing on 4255 visits where people reported how the visit had helped them to feel calm, relaxed, refreshed and revitalized (i.e. less stressed), emotional experiences during visits to urban green spaces, the rural countryside and the coast (White et al., 2013b) were compared. Two key findings emerged. First, people who lived nearer the coast were more likely to have visited it within the last 7 days, supporting the contention of the earlier studies that living near the coast is associated with greater exposure to the coasts (see also Schipperijn et al., 2010). Second, coastal visits were associated with significantly greater feelings of stress reduction than either of the other two environmental categories of urban parks and countryside (see also MacKerron & Mourato, 2013). Although the average differences across environment types were relatively small, they pertained to just a single visit. Given that coastal dwellers visit the coast more frequently, these small gains from any given visit are likely to accumulate over time.

Data from the MENE survey was further used to assess the potential of the coast to encourage physical activity, but this time using a much larger sample, N = 183,755 (White et al., 2014b). Using the same neighbourhood approach as in the census analysis, it was estimated how close each individual lived to the sea and to what extent they achieved the government’s recommended guidelines of at least 5 days a week of 30 minutes or more physical activity. A pronounced coastal proximity gradient was evident, even after controlling for a range of potential confounders (such as age, gender and socio-economic status). Specifically, compared with living more than 20 km from the coast, a person living within 1 km of the coast was 8% more likely, and a person living between 1–5 km from the coast was 4% more likely, to be meeting recommended weekly physical activity levels (see also Bauman et al., 1999; Humpel et al., 2004; Witten et al., 2008). Moreover, the frequency with which they visited the coast appeared to mediate this effect, that is, the increased level of physical activity did indeed seem to be occurring at the coast. Intriguingly, however, there were marked regional differences. Although a relatively strong coastal gradient was evident in both the south and north-west of England, no such gradient was seen in the north or south-east. There are no obvious historical, geographic, meteorological or socio-economic reasons for this finding, although this possibility is being investigated further.

In order to examine the potential benefits of coastal environments for social interactions, a very different approach was employed (Asbullahy et al., 2013). Specifically, in-depth interviews were conducted with 15 families including individual interviews with parents and children aged 8–11. Interviews provide extremely rich data about highly complex phenomena, e.g. social interactions, which are hard to capture with the kind of secondary survey data discussed above. The aim was to listen to parents and children (separately) about their experiences of spending time at the coast together as a family or on other occasions. What were their motivations for visiting, what were the barriers and, crucially, what kinds of interactions took place and what did they lead to? Expected barriers included the weather, time, and ‘packing up the car’. After visits had been made, one of the striking things was how the children, in particular, saw the beach as a setting where the whole family played together in ways that did not occur in other outdoor settings such as a park. As one boy aged 11 put it: ‘Instead of the adults just sitting somewhere on a bench while the kids do activities, they get up and they play frisbee or cricket and football and sometimes go swimming with them.’ Although far from providing a comprehensive overview, these interviews have offered much greater insight into the kinds of questions we need to ask in the future as we continue to explore the role of social interactions on the coast.

Finally, we have also begun to explore possible differences in the range of environmental factors at the coast that may also be contributing to better health (Cherrie et al., 2015). In particular, regional differences in ultraviolet radiation (UVR) exposure were examined. Although potentially harmful in terms of skin damage, this is nonetheless important because higher exposure UVR promotes the synthesis of vitamin D, which itself is important for a range of health outcomes (e.g. autoimmune diseases, cardiovascular disease and certain cancers; Holick, 2004). Importantly, coastal dwellers appeared to experience more UVR due to the effects of topographic forcing on clouds, meaning that more sunshine ‘gets through’ at the coast. Data from 7295 participants in the British 1958 Birth Cohort study showed that those who lived closer to the coast exhibited higher levels of 25(OH)D, a marker of vitamin D status, especially in autumn and winter when vitamin D synthesis is at its lowest level in the UK. In
other words, coastal dwellers visit the coast more often than those who live inland, and during these visits they are more likely to receive doses of UVR, especially in the autumn and winter, that help to produce vitamin D, which itself is important for a range of potentially positive health outcomes.

On-going research

Clearly, research in this area is at an early stage, and there is still much to learn. For instance, although we have started to look at environmental factors such as UVR, other, potentially more important factors such as air pollution levels and cultural factors have yet to be explored. Moreover, little is known about the relative importance of the various mechanisms or how they might operate in synergistic or antagonistic ways. It seems likely that the marginal effects on all these outcomes for any given exposure are likely to be quite small. Thus, cumulative coastal exposure over the mid to long term may be crucial. Furthermore, in the research presented above, it has been assumed that the coast is a homogeneous environment and barely considers different types and quality of coastal environment. This latter issue has been explored more recently and is discussed below.

Furthermore, the focus to date has been on what blue space, especially marine and coastal environments, can do for ‘us’ rather than on what ‘we’ can do for blue space environments.

WHAT CAN WE DO FOR BLUE SPACE – A CLOSER LOOK AT VARIATIONS IN BLUE ENVIRONMENTS, AND THE DEVELOPMENT OF PRO-MARINE LIFESTYLES

This review summarizes considerable evidence for the health and well-being benefits of spending time in blue environments. This developing research agenda raises additional questions. One critical issue is how these benefits play out in blue environments that vary in state or type, and that are increasingly threatened or damaged. Do benefits differ between sandy, muddy and rocky shores, or between tidal states? Are benefits maintained with coastlines that are littered, altered by sea level rise, or experiencing loss of biodiversity? Are there critical thresholds for these types of effects, and does it matter whether the cause is natural or human-made, whether the harm is visible (e.g. litter, harmful algal blooms (HABs), etc.) or hidden requiring communication and knowledge (e.g. microplastics, microbial and chemical pollution)?

Moreover, health benefits will need to be considered in terms of the potentially detrimental impacts on the sustainability of marine ecosystems. For example, if many more people visited a sensitive sandy shore, is this likely to damage the flora and fauna in this habitat. A closely related question concerns how people can be engaged and motivated to protect the blue environment.

Variations in blue environments

Blue environments vary over time, e.g. in terms of season, tides, erosion, litter present on the shoreline, etc. Hipp & Ogunseitan (2011) investigated psychological restoration in more than a thousand visitors to beaches near urban areas in California. They were particularly interested in linking the effects of current variations in temperature and air quality to future predicted changes due to climate change. People rated their visits as more restorative on days that were cooler than in future climate change scenarios, during low tide, and when air quality was better (i.e. ‘good’ ground level ozone according to objective environmental data – in other words, low concentrations of ozone). No relationship was observed between perceived restoration and objective water quality, wind speed or humidity. Building on this work, a laboratory study systematically manipulated photographic stimuli and found that higher restorativeness was associated with pristine rather than littered coastal scenes (Wyles et al., in press). More importantly, it was specifically visitor litter that was seen as detrimental, as opposed to fishing litter. Participants reported that visitor litter indicated lack of care and deliberate behaviour on the part of the litterers. These studies suggest that while overall weather conditions may not change overall preferences for blue space (White et al., 2014a), there are definitely variations in the state of the blue space that affect visit experiences.

Trade-offs also need to be considered between recommending visits to blue space and the potential adverse impacts these can have on these environments. Wyles et al., (2014) began investigating this by asking both regular visitors and experts to rate a range of visitor activities on rocky shores in terms of both the benefits to people and the risks to the habitat. Reasonable agreement was found between visitors and experts overall (although the former focused on littering, whereas the latter focused on rock-pooling as the biggest hazard to the habitat). By plotting activities according to risk and benefit ratings, this study attempted an integrated analysis. For example, swimming was characterized as good for people with little impact on the environment, and therefore potentially an activity to encourage. While this analysis is preliminary and perception-based, it offers a promising methodology to develop further.

Towards protecting blue space

If the state of the environment is important, it becomes vital that Blue Gym activities include protection of and reduction of harm to blue environments. Wyles et al. (2015) suggested the term ‘marine mind-set’ to describe ‘a mental readiness to address marine environmental problems’. This encompasses pro-marine awareness and attitudes (e.g. it is important to protect the ocean; the ocean is at risk), as well as intentions (e.g. ‘I will reduce my use of plastics to ultimately reduce shoreline litter’). Such self-report attitudes and intentions help to predict actual behaviour (see meta-analysis by Kormos & Gifford, 2014).

A few studies have indicated that people who live closer to the coast have greater knowledge and report higher awareness of coastal ecosystems (Steel et al., 2005a, b; Fletcher & Potts, 2007). Hartig et al. (2001) showed that feeling ‘restored’ in a freshwater environment was associated with willingness to protect this environment. Feelings of connectedness and place attachment have also been linked to environmental concern and self-report behaviour (e.g. Mayer & Frantz, 2004), although it is unclear whether there is evidence that applies to blue environments specifically. Other predictors of pro-environmental behaviour include values and norms supporting the behaviour, and ascribing responsibility to
oneself (e.g. Dietz et al., 1999), but these have typically not yet been investigated specifically for pro-marine issues.

Some evidence exists surrounding specific interventions or contexts that address marine mind-set outcomes. Wyles et al. (2013) found that visiting an aquarium was associated with enhanced attitudes and behavioural intentions for both littering and fish sustainability. In addition, giving a subset of visitors a leaflet with recommendations specifically targeting fish consumption in this study increased that group’s reported intentions further. Hartley et al. (2015) gathered data from 8- to 13-year-olds surrounding a marine litter education event. Although children were already aware of a number of marine litter issues, at a one-week follow-up, they reported greater understanding of the causes and consequences of littering, more concern, and more pro-environment actions.

The intervention studies so far have focused on the British context which is clearly a limitation. The European MARLISCO project (www.marlisco.eu) is tasked with the evaluation of the impacts of a range of activities (including video competitions with schools, training for educators, public exhibitions and national debates) on increasing awareness around the issues associated with marine litter. These results will be available soon. The final international example is a highly innovative citizen science project entitled Científicos de la Basura (Litter Scientist; Eastman et al., 2013; Hidalgo-Ruiz & Thiel, 2013). The programme works with school children throughout Chile and Easter Island who are taught how to collect and interpret samples of microplastics. Hidalgo-Ruiz & Thiel (2013) suggest that this type of involvement can enhance scientific literacy, awareness, and attitudinal change regarding microplastics. Koss & Kingsley (2010) close the circle by investigating the well-being benefits reported by marine volunteers, which include feelings of enjoyment and positive connections with other volunteers.

Overall, these examples show increasing activity, and a burgeoning field of interdisciplinary research that is beginning to quantify effects where possible and further elucidate the underlying mechanisms of health and well-being from coastal interactions for humans and marine ecosystems.

CONCLUSIONS/SUMMARY

Early results from the Blue Gym Initiative are encouraging on two fronts. First, they demonstrate that alongside the well-known risks (e.g. drowning, exposure to pathogens and pollutants) are numerous potential opportunities to improve health and well-being arising from interactions with marine, coastal and other aquatic environments. Moreover, the pathways for these effects are consistent with those seen linking other natural environments (e.g. green space) to better health (e.g. physical activity, stress reduction and positive social relations; Hattig et al., 2014). This reinforces confidence in the findings. Nevertheless, exactly why marine and coastal settings seem to be particularly beneficial, even compared with green spaces, is still unclear and needs further exploration. The photo studies that showed continued coastal preferences using monochrome images and poor weather conditions (White et al., 2014a) have begun to rule out some relatively obvious possibilities (e.g. preferences for the colour blue, or an association of coastal environments with pleasant weather) but have not managed to reveal specific mechanisms or a clear explanation. Elsewhere (Wheeler et al., 2014; White et al., 2014a), we have discussed possible evolutionary, cultural and individual factors that may be important, but these need to be evaluated systematically.

Second, early results from the second strand of research suggest that carefully designed interventions, especially those that actively engage the public in innovative ways, could foster a ‘marine mind-set’ which itself could actively encourage people to adopt increasingly pro-marine behaviours and apparently greater health and well-being benefits from marine-human interactions. Much of the degradation of marine and coastal environments is anthropogenic (e.g. European Environment Agency, 2007) and thus human action is required both at the individual and policy levels to tackle these problems.

Early data appear timely and are starting to be recognized by the meta-discipline of Oceans and Human Health (Moore et al., 2013; Bowen et al., 2014; Fleming et al., 2014; Fleming et al., 2015). In October 2014, the European Marine Board’s ‘Rome Declaration’ stated that Europe urgently needed: ‘A coordinated, cross-disciplinary and integrated programme on Oceans and Human Health, targeted at understanding and managing the risks and benefits to human physical and mental wellbeing from interactions with the seas’. The Blue Gym’s aim is help play a part in developing this programme.

The stakes are high. Approximately 43% of all EU citizens (~218 million people) live in one of its 446 ‘coastal regions’, including 194 coastal cities, across 22 member states (Collet, 2010). The coast is also the EU’s primary tourist destination, with some 16 million ‘tourist beds’ attracting large numbers of non-coastal residents, including an estimated 170 million tourists annually to the Mediterranean alone (European Environment Agency, 2007). Understanding the direct benefits to health and well-being people receive from these environments, and the impairment of their health and well-being from degradation to these same environments, is thus of high importance. Given the central role of anthropogenic influences on the changes in the marine environment, it should also be clear that we not only need to better understand what the marine environment can do for us, but also how we can encourage people to ask themselves what they can do for the marine environment. The Blue Gym Initiative will continue to link with other research programmes, stakeholders and policy makers within the EU and beyond to explore these fundamentally important issues.

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An economic perspective on oceans and human health

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Human health and wellbeing are intrinsically connected to our seas and oceans through a complex relationship comprising both positive and negative influences. Although significant public health impacts result from this relationship, the economic implications are rarely analysed. We reviewed the literature to assess current knowledge on the economic valuation and impacts of ocean and human health interactions in a European context. Quantitative analyses on the economic impacts of varying ocean–health interactions were limited. Common challenges to economic assessment included the difficulty in obtaining estimates for indirect healthcare costs, under-reporting of illness and the lack of standardization of surveillance data on illnesses, when available. It was also evident that non-market values, such as health promotion and psychological benefits are underrepresented in economic assessments, most likely because of the lack of standardized valuation methods for such non-market values. We provide recommendations to improve knowledge of ocean and human health linkages and progress future assessment of its economic implications in Europe.

Keywords: Oceans and human health, Blue Growth, wellbeing, economic assessment, Blue Gym

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INTRODUCTION

There is increasing recognition in Europe that the links between the seas and oceans on one hand, and human wellbeing on the other, are highly important but poorly understood from a public health standpoint. Understanding ocean and human health interactions is, therefore, the focus of a growing research field, applying expertise from across the natural and social sciences. From a policy perspective, there are significant potential benefits to addressing ocean–human health interactions from the point of view of reducing disease burden and increasing the quality of life for European citizens. However, there are also significant economic implications across the full spectrum of ocean and human wellbeing interactions. The economics of ocean and human health interactions is also in its infancy as a research area and is sparsely addressed in the literature. Yet, taking account of such implications is an important contributor to achieving sustainable growth as part of Europe’s Blue Growth strategy.

Coastal environments are the most valued locations globally. By calculating the economic value of goods and services provided by coastal ecosystems, Martínez et al. (2007) estimated that altogether, coastal ecosystems contribute 77% of the value of global ecosystem-services calculated by Costanza et al. (1997). Such global ecosystem services include raw materials and food, coastal protection, water purification, carbon sequestration, tourism and recreation (Barbier et al., 2011), all of which influence human health and wellbeing. Although the ocean has many positive influences on human health and wellbeing, negative influences also exist in the form of climate change and extreme weather and illness as a result of exposure to harmful algal blooms and water or vector-borne pathogens (Fleming et al., 2014; Tyson et al., 2004).

Oceans and human health is an important research area for Europe considering the proportion of the population living by the coast and the amount of low-lying coastal areas (EEA, 2006). With a coastline 89 000 km in length, Europe has an estimated coastal population of at least 200 million (Depledge et al., 2013), and in line with worldwide trends, population sizes along Europe’s coasts are increasing. At the same time, Europe’s maritime economy, with political support under the auspices of the EU Blue Growth strategy, is increasing in magnitude and in terms of its environmental impact (Boyes & Elliott, 2014), a pattern with implications for human health. Introduced in 2012, the Blue Growth strategy recognizes the importance of seas and oceans as drivers of the European economy, and their potential for growth and innovation, with a focus on the aquaculture, coastal and maritime tourism, biotechnology, mineral resources and renewable energy sectors (European Commission, 2014).

The valuation of oceans and human health in terms of economic impacts is recommended as a priority area for research in Europe, with suggested areas comprising both opportunities and risks associated with those sectors targeted for expansion by the Blue Growth strategy and cumulative direct and indirect impacts on ecosystems and on human health and wellbeing (Fleming et al., 2014). Economic studies on human health impacts which quantify and provide data on public health issues can contribute to addressing current and future challenges such as risks from marine-borne...
disease through increased understanding of the incidence and cost of disease (in terms of lost productivity and cost of treatment) so that economically optimal management strategies can be achieved (Ralston et al., 2011). Economic studies also serve to highlight gaps where further research is needed to establish the linkages and quantify population risk (e.g., burden of disease) for various environmental risk factors (Pruß-Ustün & Corvalán, 2006). Such studies also play an important role in informing and raising public awareness about risks and benefits to human health, which can alter human behaviour that in turn has economic repercussions. For the purposes of this article, we carried out a review of the literature addressing specifically the economic valuation and impacts of ocean and human wellbeing interactions in a European context.

STATE OF KNOWLEDGE

Quantitative analyses on the economic impacts of varying ocean–human health interactions are limited and this is particularly the case for Europe. Worldwide, more than 60,000 cases of poisoning by exposure to harmful algal blooms are reported each year (Van Dolah, 2000); the global burden of human disease caused by sewage pollution of coastal waters is estimated at 4 million lost ‘person-years’ annually, which equates to an economic loss of approximately US$ 16 billion a year (van de Guchte & Vandeweerdt, 2004); an estimated 250 million cases of gastroenteritis occur worldwide each year as a result of bathing in contaminated water (van de Guchte & Vandeweerdt, 2004); annually, an estimated 4 million cases of infectious hepatitis A and E result from consuming raw or lightly steamed filter-feeding shellfish harvested globally from polluted coastal waters (Shuval, 2003); and it is estimated that each year around the world, there are in excess of 50 million cases of respiratory disease caused by swimming and bathing in wastewater-polluted coastal waters (Shuval, 2003).

Despite these estimates, to date there are no good data on the overall impacts of human disease that arise from the ocean (Grimes et al., 2012). Similarly, there are few data on the extent of waterborne diseases and public health effects in Europe and a lack of economic studies that estimate associated costs, partly due to the lack of human health considerations in European marine research to date (Depledge et al., 2013). Outbreaks of seafood-borne illnesses have occasionally been reported in Europe (e.g., Martinez-Urtaza et al., 2005; Boxman et al., 2006; Le Guyader et al., 2008) and it is reported that microbial contamination of bathing water, primarily in the Mediterranean Sea, is responsible for more than 2 million cases of gastrointestinal disease annually (Stanners & Bourdeau, 1995). This is significant because the Mediterranean is currently the world’s leading tourist area, accounting for approximately 35% of all international tourist arrivals and revenues globally (Farsari & Bourdeau, 2006). This is significant because the Mediterranean is currently the world’s leading tourist area, accounting for approximately 35% of all international tourist arrivals and revenues globally (Farsari & Bourdeau, 2006).

To date, studies dealing with economic costs related to oceans and human health have predominantly been carried out in the USA with a focus on harmful algal blooms, specifically the Florida red tide blooms. Florida red tide blooms are primarily associated with the blooms of the toxic dinoflagellate, Karenia brevis. They have been documented on the Florida west coast since the 1800s and, more recently, have spread as far as the eastern coast of Mexico (Fleming et al., 2011). Hoagland et al. (2002) estimated the economic effects of harmful algal bloom events in the USA over the period of 1987–1992, focusing on public health, commercial fisheries, recreation and tourism, and monitoring and management. Noting that the type and amount of economic data available were limited and that many of the results are preliminary in nature, they estimated that nationwide, the average annual economic impact was in the order of US$ 50 million (Hoagland et al., 2002). More recent studies on the human health effects of Florida red tide blooms found that bloom events were significant predictors of emergency department visits and hospital inpatient admissions for both respiratory and digestive illnesses for residents and coastal tourists. In addition, annual costs of illness ranged from US$ 60,000 to $ 700,000 annually (Hoagland et al., 2009, 2014).

Using a different approach, Dyson & Huppert (2010) estimated the hypothetical regional economic impact of a year-long closure of four recreational razor clam beaches on the Washington coast due to harmful algal bloom outbreaks. They estimated that the reduction in expected visitor expenditures would cause a US$ 11.36 million per year reduction in coastal county incomes due to reduced recreational activity, and an almost US$ 2 million per year reduction in incomes due to lack of tribal and non-tribal commercial harvest of razor clams. Analysing seafood-borne illnesses in the USA, Ralston et al. (2011) estimated that the health consequences have annual costs of approximately US$ 650 million, including US$ 350 million due to pathogens and marine toxins specifically identified as causing food-borne disease and US$ 300 million due to seafood-borne disease with unknown aetiology.

Such calculations of the economic costs associated with ocean–human health interactions are of increasing importance as negative health impacts are projected to increase in relation to the marine environment. For example, in the past three decades, harmful algal blooms have become more frequent, more intense and more widespread. This is, in part, ascribed to climate changes (Gilbert et al., 2005; FAO, 2012), representing an increased threat to public health as coastal populations and the popularity of water-based recreational activities increase (Backer & McGillicuddy, 2006). In addition, Vibrio-related illnesses (Vibrionaceae) are a family within the Gammaproteobacteria and are common natural members of marine and estuarine bacterial plankton communities (Böer et al., 2011) are reported to be increasing worldwide including fatal acute diarrheal diseases such as cholera, gastroenteritis, wound infections and septicaemia (Vezzulli et al., 2013). Notably, higher water temperatures, a known impact of climate change, can lead to an increase in the growth rate of pathogens such as Vibrio species (Lindgren et al., 2012). Within Europe specifically, there is growing concern that V. vulnificus and V. parahaemolyticus may represent an important and increasing clinical problem as a result of increasing water temperatures and factors such as the increasing global consumption and trade of seafood produce (Baker-Austin et al., 2010). In line with these predictions, there is evidence that the number of reported Vibrio-related wound infections associated with recreational bathing in northern Europe has increased within the last decades (Böer et al., 2013). While a limited research effort aims to address the public health implications of these negative health impacts, we are entirely lacking in information on the corresponding economic impacts.
Equally important is calculation of the positive economic effects resulting from ocean and human health interactions. A significant potential contributor in this regard is the 'Blue Gym' concept which refers to using the coastal environment specifically to promote health and wellbeing by increasing physical activity, reducing stress and building stronger communities (Depledge & Bird, 2009). A number of studies from the UK are elucidating the Blue Gym concept further with recent findings indicating that good health (both mental and physical) is more prevalent the closer one lives to the coast with the positive effects of coastal proximity greater amongst more socio-economically deprived communities (Wheeler et al., 2012); feelings of restoration are greater after coastal visits compared with other categories of natural environment (White et al., 2013a); there is a positive association between self-reported health and living near the coast (White et al., 2013b); beaches encourage families to be physically active, increase social and family interaction, engage with nature and are associated with fun and stress relief (Ashbullby et al., 2013); and the likelihood of meeting recommended guidelines on physical activity per week increase with coastal proximity (White et al., 2014).

The positive effect of coastal environments on human health and wellbeing and the preference of many people to spend their leisure time at the coast have many economic implications. The EU Blue Growth strategy recognizes coastal tourism as the largest maritime sector employing over 3.2 million people and generating a total of €183 billion in gross added value across the EU. However, an area that is yet to be investigated is the translation of the Blue Gym effect into healthcare savings. Taking obesity as an example of a disease to which lifestyle factors contribute, the British foresight report ‘Tackling Obesities: Future Choices’ estimates that the UK’s National Healthcare System costs attributable to obesity and being overweight are projected to double to £10 billion per year by 2050, with wider costs to society and business estimated to reach £49.9 billion per year (Government Office for Science, 2007). As noted by Depledge & Bird (2009), there is a substantial body of evidence that indicates that taking outdoor exercise offers genuine benefits in treating or even avoiding the onset of obesity, depression and many other conditions currently on the rise and which can potentially save health services large amounts of money.

**CHALLENGES**

Further studies are needed to determine the economic implications of oceans and human health interactions in Europe. However, quantifying those economic implications is not without its challenges. A common drawback referred to in studies concerning water-borne illnesses is the issue of under-reporting. Factors contributing to under-reporting include patients not attending a doctor, misdiagnosis or failure to recognize the illness as resulting from a marine-borne agent, and illnesses not being reported to public health officials (Newell et al., 2010; Ralston et al., 2011). Furthermore, surveillance data on illnesses, when available, are not standardized and a remaining challenge is to establish surveillance systems that assess the level, source and severity of food-borne illnesses (Todd, 2006). In the case of Europe, available data on waterborne diseases and outbreaks are often incomplete and inconsistent for reasons that include differences in recording and reporting procedures, disease classification and financial restrictions, and variation in the legal basis for reporting between different countries (EEA and WHO, 2002). Current EU-wide surveillance of infectious diseases is either indicator-based (annual country-level reporting of confirmed human cases) or event-based (detection of individual disease outbreaks through ‘epidemic intelligence’ which refers to the identification, verification, assessment and investigation of potential health threats) (Lindgren et al., 2012). However, reporting of infectious diseases varies widely within Europe as some European countries rely on voluntary as opposed to mandatory reporting systems (Knowles et al., 2007).

An additional ongoing challenge to economic assessments is incorporating non-market values into studies, i.e. values for which a price cannot be found in the economic marketplace (Ciscar et al., 2014). In the most simplistic economic models, the value of the environment is reduced to resources that can be directly harvested, mined or otherwise used by humans (Hylland, 2006). Health impacts often do not lend themselves to such valuation and hence, there is a need for ways of recognizing and measuring health aspects that cannot be directly valued, such as the psychological benefits people experience as a result of the Blue Gym concept.

**OPTIONS FOR IMPROVING ECONOMIC IMPACT ASSESSMENT**

There are a number of recommended actions that can be taken to improve knowledge of oceans and human health linkages and progress future assessment of its economic implications in Europe. These include:

- **Enhancing understanding of oceans and human health:** Increasing our understanding of the links between oceans and human health, for example through further investigation of how rising water temperatures will affect the proliferation and distribution of pathogens, how to improve diagnosis of marine-related illnesses and further study on the Blue Gym concept, will improve the accuracy of economic studies assessing the benefits and risks of coastal interactions. This requires an interdisciplinary effort.

- **Increased reporting of marine-related illnesses:** A standardized reporting system in Europe for recording confirmed and suspected marine-related illnesses will improve the issue of under-reporting and, in turn, the accuracy of relevant economic studies.

- **More emphasis on non-market values:** Guidelines on measurement and inclusion of non-market values in studies, such as stress relief, will contribute to capturing benefits and risks that are currently underrepresented but have significant economic effects.

- **Increased communication:** Providing the public with information on oceans and human health impacts can influence behaviours that in turn have economic impacts, such as harnessing the Blue Gym concept to encourage increased physical activity and decrease health-care costs.

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• Implementing more comprehensive surveillance: Standardising current surveillance systems between European member states and expanding them to include potential new threats such as pathogenic Vibrio species will allow for an improvement in determining and addressing risks.

• Enhancing Europe’s leadership in the field: Aligning economic impact studies in the area of oceans and human health with Europe’s Blue Growth strategy provides an opportunity for Europe to build its research capacity and leadership in this emerging research field.

CONCLUSION

The seas and the coasts are drivers of Europe’s economy (European Commission, 2012). To meet the goal of sustainable growth it is necessary that consideration of human health impacts be taken into account in the process of implementing the Blue Growth strategy. As coastal populations continue to increase and as sectors such as tourism, aquaculture and blue biotechnology expand as part of Europe’s Blue Growth strategy, the public health and economic repercussions emanating from the interactions between oceans and human health will become increasingly prominent, requiring effective and integrated public health solutions to be developed through the formulation of politically and environmentally meaningful policies (Moore et al., 2013). However, as yet, Europe has largely failed to promote an integrated interdisciplinary and collaborative research effort in the area of oceans and human health on a scale necessary to address the public health implications of rapidly increasing human activity in European seas and oceans, especially in the coastal zones (Fleming et al., 2014). Quantifying the economic implications of oceans and human health in Europe will contribute to achieving sustainable development through providing information that can influence evidence-based environmental and public health policies, which recognize and address the positive and negative impacts of oceans on human health. Boyes & Elliott (2014) recognize the European Union as a pre-eminent player in sustainable development through its adoption of over 200 pieces of legislation underpinning marine environmental policy and management. Accounting for the economic implications of oceans and human health can only serve to maintain and enhance Europe’s reputation for supporting sustainable development.

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The marine biology of law and human health

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This review uses a multidisciplinary approach to investigate legal issues concerning the oceans and human health. It firstly seeks to define the boundaries of oceans and human health research. We use three case studies as examples: biomedical research, marine litter and human well-being. Biomedical research raises complex issues relating to coastal states’ sovereign rights to exploit their marine resources and the patenting processes. Coastal states have differing degrees of control over research at sea. There are differences in EU and US law over the status of genetic discoveries, with the US having stricter criteria to qualify for patent protection. International law sets the standard for bioprospecting in developing countries under the Nagoya Protocol. The cost and complexity of marine biomedical research mean that it cannot be left to commercial exploration and needs some public funding. The second case study highlights the rise in marine plastics pollution using Marine Conservation Society beachwatch data. It details the need to alter product design to avoid marine pollution and records an unsuccessful attempt by academics and an NGO to make contact with the manufacturers of one polluting product. It also introduces the concept that faulty design could amount to a public nuisance. The third case study highlights the potential health benefits from access to the coast and the statutory responsibility which sits with the US and UK authorities in the provision of well-being. It posits that there needs to be greater inter-agency coordination to promote access to the coast for human well-being.

Keywords: Law, conservation, well-being, oceans, human, health, biopiracy, bioprospecting, patent, litter

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INTRODUCTION

The issue of oceans and human health (OHH) requires a cross-cutting multidisciplinary team to analyse it effectively. Humans are playing an ever-increasing role in the marine ecosystem and their creation, the legal system, in its own way is intrinsic to marine biology, as the law potentially controls and defines human activity. Marine ecosystems are impacted by the maze of legal relationships we have created: shipping and insurance law have been one of the central conduits for the transoceanic passage of marine organisms; the laws of the sea dictate the shape of national and institutional controls over marine spaces; and marine property rights (or the absence of them) dictate human reactions to, and interactions with, the marine environment. It is then clear that the law relates to marine biology, but why is there a need to relate the biological-legal nexus to human health? OHH research takes a transverse slice across this relationship using a multidisciplinary team in a given parameter. An OHH analysis reveals the nature of the biological/legal/human relationship in a practical context, much in the same way that a core sample reveals the different depositional strata on the seabed. This permits the researcher to reflect upon the nature of this relationship and perhaps suggest solutions to problems, which in a more focused study might not have been revealed. Many of the issues raised in this paper came together at a conference on OHH in Bedruthan, Cornwall in March 2014, which led to the delegates agreeing the ‘message from Bedruthan’ (European Centre for Environment and Human Health, 2014). The delegates present shared the view that human health and well-being were intrinsically connected with the health of the oceans. The purpose of this paper is not to produce a systematic review of all the legislation which may affect human health and the marine environment, as this would require an assessment of water security (United Nations, online), environmental security (United Nations Environmental Programme, online) and health and safety regulation (Health and Safety Executive, online) to name but a few. Instead, the intent is to look at three case studies from different research areas and thus demonstrate how the OHH approach can lead to the generation of impactful research through the application of a multidisciplinary team including marine ecologists, lawyers and health researchers.

MATERIALS AND METHODS

Background

OHH research takes two subtly different forms on either side of the Atlantic. In the US, Congress set about defining its parameters in the 2005 Oceans and Human Health Act (online):

(A) vector- and water-borne diseases of humans and marine organisms, including marine mammals and fish;

(B) harmful algal blooms and hypoxia (through the Inter-Agency Task Force on Harmful Algal Blooms and Hypoxia);

(C) marine-derived pharmaceuticals;

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(D) marine organisms as models for biomedical research and as indicators of marine environmental health;
(E) marine environmental microbiology;
(F) bioaccumulative and endocrine-disrupting chemical contaminants; and
(G) predictive models based on indicators of marine environmental health or public health threats.

With the exception of potential useful biomedical research the US OHH approach dealt firmly with threats to the human population arising from the oceans. An attempt to develop this agenda with an Oceans and Human Health Reauthorization Bill in 2011 unsuccessfully sought to extend the programme but did not expand its scope too far.

In Europe the European Marine Board (2013, p. 5) has set a wider interpretation on the research area:

1. Innovative monitoring and surveillance techniques which allow much greater provision of relevant and accurate datasets (e.g. remote observation systems for coastal and marine ecosystems, detection of chemical/material pollutants, biogenic and microbial toxins and human pathogens, and improved testing for seafood and water safety).
2. Improved understanding of the physical, chemical and biological processes involved in the transport and transmission of toxic chemicals and pathogenic organisms through the marine environment to humans.
3. Improved understanding of the direct and indirect causal relationships between degradation of the marine environment and the incidence of human disease.
4. Improved environmental models to determine the patterns and extent of natural dispersion of sewage, agricultural effluents and industrial waste.
5. Expert systems to link existing models with our experience and knowledge of the connectivity between the marine environment and human health.
6. Appropriate indicators in support of sustainable development where environmental, social and economic measures are linked.
7. Methods and mechanisms which demonstrate the value (economic, cultural, aesthetic, etc.) to human well-being of marine environments from coastal seas to global oceans.

The European approach expands OHH research to include some important additional elements, both humans’ effects on the oceans, particularly where they negatively impact back on humans and potentially important human benefits from oceans such as well-being. The emphasis for European research proposals is on connectivity rather than straightforward cause and effect.

Research design

Legal research can take a number of forms, from a pure investigation of the law itself (known as doctrinal or expository research) to a more socio-legal approach which reflects on the law in its social context. Figure 1 sets out the various established routes of conducting legal research. All the research reviewed in this paper will be doctrinal research explaining the law in an applied context.

In understanding OHH in a legal context three distinct (and contrasting) case studies have been used:

1. Biomedical research
2. Marine litter
3. OHH and human well-being

![Fig. 1. Legal research styles (Arthurs, 1983).](image-url)
The marine litter strand will expand on a simple explanation of the legal issues, the 'black letter approach' (Chynoweth, 2009) with aspects of action research. Action research (Figure 2) permits the researcher to take an active role in the research topic, in this case by lobbying a pharmaceutical company to make changes to its product design. This demonstrates that because of the multidisciplinary skills that underpin OHH research it can have real direct impact in the world beyond academia.

The three case studies have been chosen because they demonstrate the breadth of OHH research. Biomedical research shows the opportunities for human health; marine litter shows the vulnerability of the oceans to human agency and the potential threat of marine plastics to both humans and the marine environment; and human well-being takes a purely European approach exploring some of the less-appreciated health benefits of the marine environment.

**Case study 1 – biomedical research**

There is a professional discipline around the law of intellectual property and patent medicine. The pharmaceutical industry is a colossus comprising some of the world’s largest companies. The potential for marine biomedical research is enormous. Research by the Global Oceans Commission (2013, p. 1) found:

‘Today, about 18,000 natural products have been reported from marine organisms relating to about 4800 named species. The number of natural products from marine species has been growing at 4% per year. The increase in the rate is largely due to technological advances in exploring the ocean and the genetic diversity it contains.’

These marine natural products have a greater tendency to lead to medical breakthroughs. Research by the US National Cancer Institute found that 1% of samples from marine animals tested in a laboratory had anti-tumour potential compared with 0.01% from terrestrial samples (Global Oceans Commission, 2013, p. 2). This is the foundation of an industry which now has a turnover of billions of dollars (Arnaud-Haond et al., 2011, p. 1521).

The broad principles which govern marine biomedical research are set out in the United Nations Convention on the Laws of the Sea (UNCLOS). Under Article 4, coastal states’ territorial waters extend to 12 nautical miles from their baseline (an approximation of the shoreline). As part of their territory, coastal states can regulate the practical development of patentable materials in any manner they see fit subject to international agreements. Outside the 12 mile limit to the edge of a coastal state’s exclusive economic zone (EEZ), up to 200 nautical miles from the baseline the state has sovereignty; article 56(1) of UNCLOS gives coastal states: ‘sovereign rights for the purpose of exploring and exploiting, conserving and managing the natural resources, whether living or non-living’.

Within both territorial waters and the EEZ rights to conduct bioprospecting are controlled by the coastal state. Where coastal states have a continental shelf which extends beyond the exclusive economic zone, under article 77 of UNCLOS they also have some sovereignty over natural resources but these only extend to ‘sedentary species’. Mossop (2007) makes the point that:

‘The general rule is that states must have [the relevant] coastal state[s] consent to conduct marine scientific research in the[ir] exclusive economic zone and on the continental shelf. There is an expectation that coastal states will grant consent for marine scientific research conducted for peaceful purposes and to increase the scientific knowledge of the marine environment for the benefit of humanity. If the research is of significance for resource exploitation in the coastal state’s jurisdiction, the coastal state may generally withhold consent for the project if it so wishes.’

Mossop (2007), citing a number of sources, goes on to raise particular problems associated with the ‘vexed question’ of whether bioprospecting is a scientific or harvesting activity. This has implications in the way the activity is regulated, as coastal states have less power to control marine scientific research on their outer continental shelf under article 246(6) of UNCLOS, than they do to control other commercial activities, so coastal states tend to argue that bioprospecting is a commercial operation on the continental shelf. There are limited options for a state to challenge a refusal of consent for bioprospecting.

In the areas beyond national jurisdiction (the high seas and waters over the continental shelf) there are fewer limits on bioprospecting. Article 136 of UNCLOS established that this area is part of the common heritage of mankind, but did not go on to establish an explicit management regime (as it did with mineral extraction by the International Seabed Authority under article 137(3)). So whether there needs to be a licensing regime and some form of compensation paid via a representative body for the exploitation of this global common or whether mankind is benefiting enough from the discovery and application of new products remains a live issue (Global Oceans Commission, 2014, p. 16).

The lack of governance in areas beyond national jurisdiction raises obvious issues, but, even where there is a clear national regulator, Oldham et al. (2012) argue that the current legal framework contains some major issues for the future of scientific development and appropriate human
benefits. Under article 27 of the Agreement on Trade-Related Aspects of Intellectual Property Rights there is an established framework for the development of international monopoly rights for the commercial exploitation of invention which contain an ‘inventive step’ and which are capable of ‘industrial application’. The creation of long-term monopoly rights is seen by industry as being vital to justify the enormous cost of bringing drugs to market, but the patent system significantly affects the whole development of the market in biomedical products.

However, there are some real problems in the detail. One major difficulty is whether genetic sequences are patentable. Both the EU and US accept that gene sequences may be patented but there are differences in how these patents arise and are applicable (Odling-West, 2011). Since the Supreme Court ruling in the case of Association for Medical Pathology v USPTO and Myriad and the Directors of the University of Utah Research Foundation (AMP) [2013] No. 12–398, US law has confirmed that genetic sequences per se are not patentable if they are performing the function they already performed in nature. This contrasts with EU law where the isolation of a gene sequence for a specific function is patentable, although only for that identified function (Monsanto Technology LLC v Cefetra BV (C-428/08) [2011] All E.R. (EC) 209). It is difficult to understand the importance of these cases. Inventions can attract patent protection but scientific discoveries, however important, do not, unless they attract patent protection through some form of legally established inventive step.

Oldham et al. (2012) highlight six major issues with patenting:

- Distortion of the freedom, orientation and basic cost of scientific research;
- Prohibitive property rights or ‘patent thickets’ around some areas of research;
- Ethical problems relating to the morality of the patenting and creation of life;
- Protection of indigenous communities (both for their distinctive genes and use of traditional medicines);
- Fair access to the application of research for developing countries;
- Protection from biopiracy.

These are all issues, which to a greater or lesser extent, shape marine research. Oldham et al. (2012) go on to point out that international law has made some attempt to redress this balance via The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (the Nagoya Protocol). The Nagoya Protocol established three principles:

- prior informed consent from the relevant government (article 6);
- prior informed consent from relevant indigenous and local communities (article 7); and
- benefit-sharing agreement on mutually agreed terms (article 5).

Protocols like this are not necessarily legally binding, particularly when the agents involved may be corporations rather than nations, but the expense involved in marine and pharmaceutical research means that public funding is often part of the research process. Breaching this sort of international law means that public bodies (and private corporations) face considerable reputational risk if not direct legal challenge. The Nagoya protocol and the Convention on Biological Biodiversity with which it is associated favour a conciliatory approach in respect of any breaches as parties have to explicitly agree in their accession document to submission to arbitration or the International Court of Justice. At present the protocol has only 54 signatories, which exclude inter alia the US and the UK, but includes the European Union as a whole (Convention on Biological Diversity, 2015).

Oldham et al. conclude their analysis:

'We have argued that what is required is greater attention to opening up biodiversity to research and development to serve human needs based on the principles of equitable benefit-sharing, respect for the objectives of the Convention on Biological Diversity, human rights and ethics.'

This is a justifiable conclusion as commercial biomedical research on its own will not meet all the opportunities which marine research can give human health.

Case study 2 – Marine Litter

The United Nations Environment Programme states that ‘marine litter poses a vast and growing threat to the marine and coastal environment’ (United Nations Environment Programme, 2005). If no action is taken litter will continue to accumulate and increase in the marine environment and on our beaches. Marine litter can harm and kill wildlife through entanglement and ingestion; beach litter can impact on human health and local economies and costs millions to clean up.

The Marine Conservation Society (MCS) has coordinated a UK-wide beach litter survey and clean up since 1994. Through these surveys they have recorded an increase in the amount of plastic litter on UK beaches and this trend is reflected in a number of other surveys throughout the world. Plastic items have always dominated the litter found during MCS Beachwatch surveys and consistently account for over 50% of all litter. The amount of plastic litter items $km^{-1}$ in the same period has increased by over 120% (now ~2000 items $km^{-1}$).

It should also be noted that most items of seawage-related debris (SRD) are now entirely or partially made of plastic including polystyrene. In 2013 these accounted for 43 and 9.5% of all litter respectively. Table 1 sets out that the percentage of beach litter caused by all plastics is over 70% (MCS, 2014).

Whilst plastics can be extremely useful and are now a part of everyday life, the material attributes that have led to the extensive use of plastics in the packaging, consumer and fishing industries have unfortunately also made them one of the most pervasive, persistent and hazardous forms of litter in the marine environment.

Plastics are made of long chain hydrocarbons that few micro-organisms can break down. Plastics at sea break down at a much slower rate than plastics exposed to weathering on land (Packforsk, 1989) mainly because temperatures at sea will generally be lower, thus slowing the degradation process. The rate of breakdown can be further reduced by chemical or biological fouling (Andrady, 2000).

Estimates for plastic degradation at sea range from 450 to 1000 years. However, plastics may never fully degrade; they...
PCBs, and DDEs from seawater to levels up to a million times greater than in the surrounding seawater, posing a potential hazard for birds and fish which mistake the pellets for food such as fish eggs (Ananthaswamy, 2001).

PCBs have also been linked to the masculinization of female polar bears and spontaneous abortions and declines in seal populations (Reijnders, 1982; Wiig et al., 1998). Ryan et al. (1990) obtained evidence that PCBs in the tissues of Great Shearwaters were derived from ingested plastic particles (from Derraik, 2002).

Toxins adsorbed onto plastic may be ingested by filter feeders (Thompson et al., 2004), and passed up the food chain to fish and ultimately to human consumers. The accumulation of microscopic plastic fibres in sand substrates may leach out toxins such as PCBs and heavy metals (Thompson & Hoare, 1997). These can be absorbed by microalgae and also potentially enter the food chain.

We know that plastics have been found inside a wide variety of marine organisms including invertebrates, fish, birds and mammals and that microplastics are now distributed throughout the oceans from the Arctic to the Antarctic. There is emerging evidence of transfer of chemicals from ingested plastics into tissues so whilst plastics bring us considerable benefits, our approach to their production, use and disposal are not sustainable and present serious concerns for wildlife and human health.

Article 194 of UNCLOS has some provisions relating to marine litter:

‘States shall take, individually or jointly as appropriate, all measures consistent with this Convention that are necessary to prevent, reduce and control pollution of the marine environment from any source, using for this purpose the best practicable means at their disposal and in accordance with their capabilities, and they shall endeavour to harmonize their policies in this connection.’

At the international level the International Maritime Organization has overseen the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter (the London Convention) and its subsequent protocol. However, this relates to pollution from ships, and while there are a maze of international treaties concerning water use between nations there is a real lack of specific regulations at the UN level relating to the responsibility for cleaning the oceans in the face of this vast unprecedented spread of marine plastic pollutants.

At the EU level the leading water pollution regulation is set out in the Water Framework Directive (2000/60/EU); its aim is described in article 1 and is inter alia:

‘achieving the objectives of relevant international agreements, including those which aim to prevent and eliminate pollution of the marine environment, by Community action under Article 16(3) to cease or phase out discharges, emissions and losses of priority hazardous substances, with the ultimate aim of achieving concentrations in the marine environment near background values for naturally occurring substances and close to zero for man-made synthetic substances.’

The Directive also has the broader aim:

‘at enhanced protection and improvement of the aquatic environment, inter alia, through specific measures for the progressive reduction of discharges, emissions and losses of

<table>
<thead>
<tr>
<th>Year</th>
<th>Plastic items km$^{-1}$</th>
<th>% of total litter</th>
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<tbody>
<tr>
<td>1994</td>
<td>530</td>
<td>54.8</td>
</tr>
<tr>
<td>1995</td>
<td>845.0</td>
<td>53</td>
</tr>
<tr>
<td>1996</td>
<td>1779.0</td>
<td>51.8</td>
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<td>1997</td>
<td>881.0</td>
<td>56.7</td>
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<td>1998</td>
<td>1095.1</td>
<td>56.6</td>
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<tr>
<td>1999</td>
<td>1024.6</td>
<td>53.6</td>
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<tr>
<td>2000</td>
<td>995.4</td>
<td>55.9</td>
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<tr>
<td>2001</td>
<td>853.2</td>
<td>54.1</td>
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<tr>
<td>2002</td>
<td>908.6</td>
<td>56.5</td>
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<tr>
<td>2003</td>
<td>1170.0</td>
<td>56.4</td>
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<td>2004</td>
<td>1106.7</td>
<td>58.5</td>
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<tr>
<td>2005</td>
<td>1159.3</td>
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<td>2006</td>
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<td>2008</td>
<td>1307.4</td>
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<td>2009</td>
<td>1173.6</td>
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<tr>
<td>2010</td>
<td>1246.7</td>
<td>63.3</td>
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<td>2011</td>
<td>1071.6</td>
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<tr>
<td>2012</td>
<td>1272.5</td>
<td>65.4</td>
</tr>
<tr>
<td>2013</td>
<td>1433.4</td>
<td>62.1</td>
</tr>
</tbody>
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Note: excludes polystyrene and sewage-related debris.
priority substances and the cessation or phasing-out of discharges, emissions and losses of the priority hazardous substances.

A key aim of the Directive is set out in article 4:

‘Member States shall protect, enhance and restore all bodies of surface water, subject to the application of subparagraph (iii) for artificial and heavily modified bodies of water, with the aim of achieving good surface water status at the latest 15 years after the date of entry into force of this Directive, in accordance with the provisions laid down in Annex V, subject to the application of extensions determined in accordance with paragraph 4 and to the application of paragraphs 5, 6 and 7 without prejudice to paragraph 8 . . .’

The definition of ‘surface waters’ in article 2 includes coastal waters out to one nautical mile.

As a key source of plastics Europe’s rivers (a major supplier of marine plastic pollution) should be free of plastic pollution by this year, but a glance at the legislation shows a number of important exemptions. The effectiveness of the Directive is somewhat restricted to ‘priority substances’ and ‘priority hazardous substances’, so the impetus is to prove the harm of these substances before they can be added to an ever-growing list of priority substances annexed to the Directive (European Commission, 2015). However this approach can have the effect of creating ever more complicated substances rather than simply stopping the production of polluting products at source, and of course a chemical needs to be added to the list before it is banned, rather than actively applying the precautionary principle.

In its investigation into water pollution the UK House of Commons Science and Technology Committee (2013, p. 15) heard:

‘Witnesses agreed that reducing the release of micro-plastics at source would be the most effective way of preventing their accumulation in the marine environment. We heard that in many cases there were alternatives to their use, especially in cosmetics, or there was “no need for these items to be there in the first place”.’

This is an approach echoed in the literature (Gouda, 2014). A major issue here is that there is no regulation relating to the use of plastic in many products, so when manufacturers have switched from biodegradable to plastic materials there has been no legal disincentive. For many products the transition has been unremarkable but for some this transition has significant problems. A common pollutant found on UK beaches is the plastic stalk of a cotton bud (Williams et al., 2003). Cotton buds are disposed by users via the lavatory and therefore the sewerage system. The design of the cotton bud means that they are not removed via the sewage screening process and they are a known problem around UK coasts, particularly in times of high flow through the system. There is clearly industrial awareness of the issue as Johnson & Johnson, a major manufacturer of cotton buds, has attached a logo on the packaging instructing users to ‘bag it in a bin, don’t flush it.’ The Marine Conservation Society and leading marine academics wrote to Johnson & Johnson (Appleby, 2014) pointing out that the evidence showed that substantial amounts of cotton buds were still be disposed of by the sewerage system and the resultant plastic pollution was a product design as much as a consumer issue. This correspondence raised a novel approach that there could be some legal liability attached to Johnson & Johnson via the ancient common law remedy of public nuisance:

‘An act that endangers the life, health, property, morals or comfort of the public or obstructs the public in the exercise or enjoyment of rights common to all.’ (Practical Law Company, 2014)

The accretion of cotton bud stalks on the foreshore harms property which is largely owned and enjoyed by the public (The Crown Estate, 2015). Moreover such litter is unsightly and thus interferes with the use and enjoyment of the beach by the public, so it is conceivable that a nuisance action might be constructed, particularly as the manufacturers are aware that there is a problem or they would not label their products with the ‘bag it don’t bin it’ logo.

Whether a public nuisance action can in practice be sustained remains to be seen but it is clear that there has been a failure to alter the established public practice in this area and detailed legal intervention may be necessary to remedy the problem at source.

Case study 3 – Oceans and human health and well-being

A wealth of research has developed over the last few decades regarding the ‘salutogenic’ (health creating) effects of nature (Hartig et al., 2014). Mechanisms proposed for these potential health benefits include providing opportunity and motivation for physical activity, recovery from stress, cognitive psychological restoration, and supporting social contact. Much of the research has considered ‘green space’ – primarily in urban areas – but more recently evidence has started to appear on the salutogenic effects of ‘blue space’ (environments with water as a significant component). Research carried out under a programme of work on coastal environments and human health at the European Centre for Environment and Human Health (http://www.ecehh.org) has indicated that there may well be psychological and physical health benefits of coastal environments in particular.

For example, a geographic study using data from the UK’s 2001 Census found that in areas nearer the coast, populations tended to be more likely to report ‘good’ general health, after accounting for age, sex, indices of area socio-economic deprivation and green-space density (Wheeler et al., 2012). It also suggested that the association was strongest in the most socio-economically deprived areas. A subsequent study used individual-level data from the British Household Panel Survey. Following these individuals over time, an analysis was carried out to address the question: ‘Do people report better mental health when they live closer to the coast?’ After accounting for many individual and area characteristics, the analysis suggested that in those years that people lived close to the coast, compared with living further inland, they did report better mental health (White et al., 2013).

The extent of physical activity of around 180,000 people from the Monitor of Engagement with the Natural Environment programme analysed by Natural England (2013) indicated that people living closer to the coast were more likely to report meeting government guidelines for physical activity, again after accounting for various other circumstances that could influence physical activity.
(such as socio-economic status) (White et al., 2014). More
in-depth, qualitative research has further investigated the
beach as a setting for family health promotion. This study
highlighted the complex means by which time at the beach
could provide an opportunity for healthy activities amongst
children and their families, and was consistent with the phys-
ical, psychological and social mechanisms proposed to link
health and nature (Ashbullby et al., 2013).

Under section 1 of the National Health Service Act 2006 it
is the duty of the Secretary of State for Health in England
to continue the promotion in England of a comprehensive
health service designed to secure improvement –

(a) in the physical and mental health of the people of
England, and
(b) in the prevention, diagnosis and treatment of physical and
mental illness.

This is an important definition because it means that English
health care (and almost certainly health care in other parts of
the UK) relates to the improvement of health generally rather
than just the provision of some health care services. In the US,
which has a rather more complicated approach to health care,
section 4001 of the Compilation of Patient Protection and
Affordable Care Act (Consolidated to 2010) provides for the
creation of the National Prevention, Health Promotion and
Public Health Council which inter alia:

'provides coordination and leadership at the Federal level, and
among all Federal departments and agencies, with respect to
prevention, wellness and health promotion practices, the
public health system, and integrative health care in the
United States.'

It is clear therefore that there are government agencies on both
sides of the Atlantic charged with assessing and implementing
potential benefits which arise from using the blue space.
However, while it may be a cheap and enjoyable method of
promoting health (for example, a walk on the beach) there are real issues in terms of the way government is struc-
tured. As in the USA the UK system is geared towards curative
health through surgeon-led teams in hospitals and in general practice. It may be more sensible and cost effective to pre-
scribe a walk on the beach rather than antidepressants, but
it is not traditionally the preserve of the health care system
to advocate access to the foreshore.

In the UK access to the foreshore is largely controlled via
the Crown Estate (who own most of the foreshore and
permit public access), local authorities and charities such as
the National Trust. Access is governed by the laws of property,
planning and more recently marine planning. Moreover plan-
ing laws, although often geared to the provision of public
access, also seek to regulate spaces to protect the environment
from too much human interference. To obtain real public
health benefits there will be a requirement to involve not
just traditional 'health providers' but also these other public
bodies and civil society groups which do not traditionally
view themselves as involved in health care. There are institu-
tional hurdles from getting these differing public agencies with
widely different approaches to coordinate their activities, and
so there is a real need for academia to make the case for the
provision of access to the blue space to overcome that inertia.

CONCLUSIONS

OHH research uses a cross-cutting, multidisciplinary approach
in order to understand human interactions with the marine
environment. The law is a good guide as to how that human rela-
tionship with the marine environment is managed. Not all law is
adhered to, nor is it perfect, but it does reflect a model of human
behaviour. A healthy life in a healthy ecosystem is a legitimate
human aspiration, in the same way that America’s Founding
Fathers sought life, liberty and the pursuit of happiness.
However, because of the organizational nature of humans as a
species we have had to break down the parts of this ideal to
make it attainable. The OHF strategy aims to assemble those
parts back together again and analyse how successful we have
been in achieving that aspiration. The case studies outlined
above are an attempt to begin that process.

Biomedical research seems to have an established legal
framework within which it takes place and a clear forward
structure with the continued adoption of the Nagoya
Protocol and sound principles on which such research takes
place. However, this masks deeper problems associated with
the creation of private monopoly rights and whether it is
better to fund such research publicly and make its results
open access or operate via the private sector and make later
consumers pay for the fruits of the research. It is clear that
commercial development in this area alone will not sustain
commercial investment for all the necessary research, particu-
larly in the light of Association for Medical Pathology v USPTO
and Myriad and the Directors of the University of Utah
Research Foundation (AMP) [2013] No. 12–398, as such
research requires patentable outcomes to be commercially
viable, and not all breakthroughs will be patentable.

The transition from naturally occurring biodegradable
materials to the use of cheap but complex plastics has left a
legacy of pollution in the world’s seas and oceans. The health
effects of these chemicals on human and marine life are
not always known, although in some cases damage and
toxicity are clear and it is abundantly clear that they have
negatively impacted marine ecosystems. UNCLOS places
clear duties on coastal states to manage this pollution and
although the EU’s Water Framework Directive is a useful
piece of legislation, the fact that something as ubiquitous as a
cotton bud persists as a significant plastic pollutant in the
marine environment despite the regulation is very alarming.
There is a sad irony that it is a sanitary product which has
such an unsanitary effect on the environment, and it is a great
pity that marine charities and academics are having to resort
to direct confrontation in order to redress this obvious problem.

A legal analysis of the benefits of blue space shows the dif-
ficulty inherent in public medicine of achieving change
outside of the control of the commissioning agency.
Effective access to blue space needs input to planning policy,
transport, labour legislation and laws relating to the owner-
ship of the foreshore. It means that the communication of
the scientific research needs to be beyond traditional academic
biomedical journals and directly into the spaces where policy
is formulated for those other sectors.

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Challenging the links between seafood and human health in the context of global change

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Living marine resources have strong links with human health and well-being that are complex, still not well understood and that are being modified by global change. This review attempts to illustrate how fishing activities, aquaculture and climate change are challenging these connections and the consequent health risks and benefits posed to citizens. Although global change may provide some positive aspects for human health locally, such as new sources of omega-3, overall it will exacerbate existing seafood security and safety concerns. Unsustainable fishing and aquaculture practices, and climate change, particularly sea warming, ocean acidification and changes in riverine runoff, are threatening not only the protein and fish oil/omega-3 supplies available for consumers, but also raising ecological and health concerns associated with the increase of contaminants, microbes and biotoxins. In this context, we propose a number of management measures that could mitigate the negative effects of global change on seafood, and hence on human health and well-being.

Keywords: Seafood security and safety, fishing and aquaculture, climate change, sea warming, river runoff, ocean acidification, oceans and human health

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LINKS BETWEEN SEAFOOD AND HUMAN HEALTH AND WELL-BEING

Marine living resources provide an excellent example of how human activities and the ocean environments are inseparably linked, having both benefits and risks (Bowen et al., 2006; Brunner et al., 2009; Tacon & Metian, 2013). In this review we describe the complex links that exist between seafood and human health and well-being.

Seafood’s role in food security in coastal developing countries

Seafood (particularly fish) plays an important role in food security as a key source of food and nutrition for coastal regions of developing countries, contributing almost a quarter of the animal protein consumed by people in Low Income Food Deficit Countries (FAO, 2014; United Nations, 2014). Fish is the main source of animal protein along with essential micronutrients and fatty acids for 3 billion people (United Nations, 2014). Populations in Africa and Asia rely even more on fish for their intake of animal proteins, and this contribution can reach up to 40% or more in some small developing island states (United Nations, 2014). Overall, it is estimated that fish contributes about 17% to the world’s animal protein intake and that about 400 million people get more than 50% of their animal protein from fish (FAO, 2014; United Nations, 2014). There is concern that the availability of seafood may not be able to keep up with demand, considering that worldwide demand for seafood products has increased from about 10 kg per person each year during the 1960s to 19.0 kg per person in 2010–2012, and that the world’s population is expected to grow by 20% from 2010 to 2030 (FAO, 2014). Given a current fisheries and aquaculture production for a human consumption estimated to be about 136 million tonnes (animals from capture fisheries and aquaculture), with annual per capita fish consumption remaining at about 19 kg per person, a similar proportion of fish going into fishmeal, fish oil and other non-food uses as today, and a world population of 9.6 billion people, approximately 47.5 million additional tonnes of food fish will be needed in 2050 (FAO, 2014).

Although fish plays an extremely important role in the supply of protein in many developing coastal countries, it has sometimes been considered more important as a source of micronutrients as more than 2 billion people, especially in developing countries, are undernourished due to a lack of essential vitamins and minerals, often contained in seafood (FAO, 2014). Important micronutrients provided by fish consumption includes certain vitamins, the essential amino acid lysine (Médale et al., 2003) together with minerals such as selenium, an essential dietary trace element that plays an important role in antioxidant defence systems and may protect against cardiovascular disease and the toxic effects of mercury (Mozaffarian, 2009). Furthermore, seafood species are particularly rich in iron, and its deficiency is considered the most common single-nutrient deficiency syndrome in the world affecting haemoglobin counts most seriously in women, children and adolescents from developing countries (Trowbridge & Martorell, 2002; Bagchi, 2004).
The importance of seafood in the developed world

In the developed world (i.e. Europe and the USA), seafood does not play such an important role in food security, since people usually rely on animal protein from other sources (e.g. livestock). Most people in developed countries get sufficient protein in their diet and therefore much attention has been given to the contribution of seafood to a healthy diet because of the health benefits provided by the long-chain omega-3 (or n-3) fatty acids contained in seafood (reviewed by Brunner et al., 2009; Lloret, 2010). First, omega-3 fatty acids from fish oil help to improve cardiovascular health by decreasing risk factors such as triglyceride concentrations, blood pressure, platelet aggregation and heart arrhythmias, thus reducing coronary heart disease mortality (see e.g. He et al., 2004; Mozaffarian & Rimm, 2006). Second, fish-derived omega-3 fatty acids consumption protects against the development of certain cancers, e.g. breast and prostate cancers (see e.g. Fernandez et al., 1999). Cardiovascular diseases and cancer are amongst the most important causes of ill-health in developed countries according to the World Health Organization (2009a, b) and in this regard, the specific roles of α-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) in preventing cardiovascular disease and cancer are subjects of active research (see e.g. He et al., 2004; Mozaffarian & Rimm, 2006).

In addition to the cardiovascular and cancer protective effects of fish consumption, fish intake has been also associated with other health outcomes, such as reduced depression symptoms in adults and fewer asthmatic and respiratory allergies in children (reviewed by Lloret, 2010). Omega-3 fatty acids also mediate the inflammatory process and influence the general health status of the skeletal system (reviewed by Brunner et al., 2009). The omega-3 fatty acids found in seafood are an important component of the diet in some regions where people display better than average health outcomes. Thus for example, in Mediterranean countries, the traditional diet (so-called ‘Mediterranean diet’), rich in seafood, has consistently been shown to be associated with favourable health outcomes and a better quality of life (reviewed by Sofi et al., 2008; Sofi, 2009). Several epidemiological and observational studies suggest that the type of diet traditionally followed by Mediterranean people may protect against chronic diseases and mortality, with Mediterranean nations presenting lower rates of cardiovascular disease and cancer in comparison with other nations (Trichopoulou, 2001; Benetou et al., 2008). Greater adherence to this diet has also been associated with longevity (Trichopoulou, 2004), a reduction in depressive disorders (Sánchez-Villegas et al., 2009) and alleviation of iron deficiency (Mesias et al., 2009).

In particular, pelagic fish such as sardines (Sardina spp.), anchovies (Engraulis spp.), mackerels (Scomber spp.) and tunas (Thunnus spp.) represent a good dietary source of omega-3 fatty acids (reviewed by Lloret et al., 2014). The edible muscle of these pelagic fishes can contain up to 10 times more total lipids and omega-3 fatty acids than that of demersal fish species. This is because pelagic species tend to concentrate the lipid reserves in their muscle whereas demersal species tend to concentrate lipids in their liver or mesenteries, which usually are not consumed (Lloret et al., 2014). The key role of pelagic species in global omega-3 supply is more apparent if we consider that they comprise the largest proportion of the global marine catches: small pelagics (herrings, sardines, anchovies, etc.) contributed about 22% (19.9 million tonnes) of the total catch in 2009 (this share is down from 29% in the 1950s and 27% in 1970s), whereas the large pelagics (e.g. tuna, bonito, billfish etc.) accounted for 19% (16.6 million tonnes) of total catches in 2009 (FAO, 2011). Although marine capture fisheries have always been the largest contributor to world fish production, in the last two decades marine and inland aquaculture has expanded rapidly, and the relative contribution of marine capture fisheries to the growing total world fish production has fallen (FAO, 2014): in 1950, marine capture was 16.7 million tonnes and accounted for 86% of total world fish production whereas in 2009 marine capture contributed 49% of the world’s fish production in comparison with mariculture (21%), freshwater aquaculture (23%) and inland capture fishery (6%).

Balancing benefits and risks linked to eating seafood

Despite the potential human benefits derived from seafood consumption, pathogens, biotoxins and chemical contamination are threatening seafood quality and quantity throughout the world, thus seriously impacting the nutritional value of seafood and posing risks to human health and well-being (reviewed by Ross & Birnbaum, 2003; Fleming et al., 2006; Lloret, 2010). Pathogens including parasites, pollutants such as heavy metals, persistent organic pollutants (POPs) and biotoxins are affecting the safety of seafood. Although seafood safety concerns both the developing and the developed world, it is in the latter where most discussion and studies are focused. In Europe and the USA, the health benefits and risks associated with seafood consumption are leading to controversy. For example, concerns regarding potential health risks of polychlorinated biphenyls/dioxins and mercury, which are present in some fish species (particularly in large pelagic fish, which are also the richest in omega-3 fatty acids), have tempered the perception of fish as a healthy food (Mozaffarian & Rimm, 2006; Mozaffarian, 2009). Methylmercury, the predominant form of mercury in fish, can accumulate to high concentrations in predatory fish such as tunas and swordfish because once it enters the food web, it increases in concentration with each successive trophic level, posing a health risk to humans who eat these predatory fish (Drevnick et al., 2015). For developing foetuses, infants and children, the primary health impact of methylmercury is impaired neurological development (Rice et al., 2006) whereas for adults, methylmercury exposure has been associated with increased rates of cardiovascular disease (Guallar et al., 2002). It is also important to consider that, on average, seafood having greater ecological impact (i.e. captured species that show the highest vulnerability to fishing pressures) also present higher health risks (as indexed by mercury concentration) and do not necessarily provide higher health benefits (as indexed by omega-3 fatty acid concentrations; Gerber et al., 2012).

Another factor that is tempering the perception of fish as a healthy food is the presence of parasites because some fish parasites (e.g. Anisakids) can cause digestive disorders and/or allergies in humans as a consequence of accidental
ingestion of raw, undercooked or improperly processed fish and/or cephalopods parasitized by larvae of these parasites (Valero et al., 2003; Audicana & Kennedy, 2008). Parasites can also impinge fisheries, reducing the marketability of harvested products and reducing both abundance and yield by increasing mortality, reducing fish condition (lipids) as well as fecundity and egg quality (Dobson & May, 1987; Lloret et al., 2012; Gómez & Nichols, 2013; Ferrer-Maza et al., 2014, 2015).

Currently, the links between fish and human health are changing due to many factors, amongst which (over-)fishing, aquaculture and climate change are the most important. Because changes in fish condition and health will ultimately affect the health of consumers, it is important to consider how global change affects the condition and productivity of fish. The following sections attempt to summarize how the complex links between marine living resources and human health and well-being are being challenged by fisheries, aquaculture and climate change, and provides suggestions to promote fish security and safety in the context of global change.

FISHING AND AQUACULTURE: IMPACTS ON FISH AND HUMAN HEALTH

Capture fisheries

Fishing is threatening a number of fish stocks that have increasingly become over-exploited: the number of over-exploited marine fish stocks has increased during recent decades, from 10% in 1970 to 29% in 2011, while a further 61% of fish stocks are currently assessed as sustainably (fully) exploited (FAO, 2014). Over-exploitation, particularly of pelagic oily fish, is significantly reducing the overall supply of long chain omega-3 fats and proteins. For the developed world, the collapse of fisheries due to reduced fish productivity does not pose a significant risk to general food security, but more to health, because there is a danger that there will not be enough omega-3 from fish to assure optimum health support for all. The current recommendations of governmental health agencies to people in developed countries, to increase their intake of fatty fish by at least 2–3-fold, are inconsistent with the stagnation of global production of capture fisheries (Jenkins et al., 2009). In contrast, for many coastal countries of the developing world, the collapse of fish production poses a real risk for food security. Furthermore, overfishing is decreasing the biological and genetic diversity of fish worldwide, adversely impacting seafood production (Malin & Palumbi, 2014). Decreased diversity in marine ecosystems may also increase the risk of pathogen emergence that can pose a threat to consumer health, as it occurs in land, where decreased diversity in agroecosystems increases the risk of pest attack (Swift et al., 2004). Overfishing particularly affects the health of the poorest people because it undermines food security in the coastal regions of less economically developed countries, where overfishing reduces the supply of a vital source of dietary protein (Brunner et al., 2009). This inequality persists within rich economies, where the decreasing affordability of fish in the diet, likewise favours the better off (Brunner et al., 2009).

Fishing can further reduce the supply of omega-3 and proteins indirectly. First, by reducing the food supply of exploited fish where a reduced quality and quantity of prey translates into lower fish condition (Lloret et al., 2014). For example, trawling affects biomass and production of benthic communities (Jennings et al., 2001) that are the main food source for a number of exploited demersal species (Hoines & Bergstad, 1999). As a result, bottom trawling has the potential to affect the condition of exploited species, e.g. red mullet (Mullus barbatus) in the north-western Mediterranean (Lloret et al., 2007) and haddock (Melanogrammus aeglefinus) in the North Sea (Hiddink et al., 2005). Second, the effect of ‘selective fisheries’, i.e. fisheries that for commercial reasons mainly target certain fish sizes is also an issue. This problem is typical of some coastal small-scale (artisanal) and recreational fisheries which, through the selective removal of the largest individuals, affect the lipid reserves and viability of many coastal fish populations because larger individuals are usually better conditioned than the smaller ones (Lloret et al., 2008, 2012). Third, fishing-induced stress may also affect the condition of marine exploited species. For example, dredging chronically affects the condition of the clam Spisula solida in Portuguese waters, where dredging-induced stress provokes a decrease in lipid composition (Chicharo et al., 2002). Also, noise from fishing operations (e.g. from towed nets or from motors) may induce stress in fish and reduce condition (Anderson et al., 2011).

Fishing can also exert important effects on both marine community structure and ecosystem functioning via reductions in the abundance and/or diversity of parasites, many of which have a substantial ecological role in marine ecosystems (Wood et al., 2013). Parasites are extremely diverse, have key roles in ecological and evolutionary processes, and infection may paradoxically result in ecosystem services of direct human relevance (Gómez et al., 2012; Gómez & Nichols, 2013). Fishing can drive declines in overall parasite species richness and parasite abundance by reducing the availability of habitat and resources for parasites, probably because trophically transmitted parasites require multiple host species, some of which are the top predators most sensitive to fishing impacts (Wood et al., 2013). Nevertheless, the response of parasites to fishing is variable and context specific, with parasite responses being mediated by parasite traits and the host’s response to fishing in a particular area (Wood et al., 2014). This is an aspect of parasites that contrasts to the effects of fish parasites on human health and well-being noted earlier.

Marine protected areas

Marine protected areas, where fishing is restricted to varying degrees, have the potential to enhance not only fish abundance and biomass (Planes et al., 2000), but also the condition of fish species inhabiting these areas, and can be regarded therefore as a tool to enhance fish oil reserves and human health. For example, white seabream (Diplodus sargus) is better conditioned (higher lipid content in the muscle and higher hepatosomatic index, a proxy of fat reserves stored in the liver) within the rocky areas of two marine reserves in the northwestern Mediterranean than in adjacent unprotected rocky areas (Figure 1; Lloret & Planes, 2003). These examples show that marine reserves not only contribute to preserve biodiversity and enhance fish abundance and biomass, but also support increased lipid reserves in exploited species,
providing long-term benefits to local fisheries and consumer's health.

Furthermore, recent studies have shown that under the influence of certain parasites, fish stocks are less disposed to collapse if marine reserves are present (McCallum et al., 2005). These authors found that the presence of the Rickettsia-like prokaryote does not necessarily decrease yield of its host – the exploited invertebrate species abalone, Haliotis sp. – when a reserve is present. In contrast, when a reserve is absent, this highly transmissible pathogen causes a rapid decline in equilibrium yield for efforts beyond those that produce maximum sustainable yield, making the fishery more prone to collapse (McCallum et al., 2005). Notwithstanding these results, the links between parasites and marine reserves are still not well understood: several studies have found that areas where fishing is prohibited or strictly regulated can strengthen the life cycle of parasites and their trophic links and, consequently, facilitate parasite abundance and/or diversity among fished host species (e.g. Sasal et al., 1996; Bartoli et al., 2005, Loot et al., 2005, Hechinger et al., 2008; Wood et al., 2013; Aldana et al., 2014). Other studies have found lower parasite abundance in marine reserves compared with open access areas (Sonnenholzner et al., 2011); others have failed to find significant differences in abundance and diversity between reserve and open access areas (Ternengo et al., 2009), while still others have found negative effects of fishing on parasite diversity but variable effects on parasite abundance (Wood et al., 2014).

Aquaculture

Although aquaculture could also be regarded as a way to enhance fish proteins and lipids available to consumers and hence to promote consumer health, in fact the health benefits from reared fish are still controversial. Although farmed fish have a higher fat content than wild caught fish (because farmed fish feed abundantly; Hamilton et al., 2005), these cultured fish usually have a lower proportion of omega-3 fatty acids (the class of fish lipids that are most linked to human health) in their lipids compared with wild fish (reviewed by FAO, 2014; Lloret et al., 2014). Thus for example, despite the fat percentage in the muscle or farmed red porgy Pagrus pagrus (3.0%) being higher than that in wild fish (0.65%), wild red porgy shows higher levels of omega-3 fats than reared (Rueda et al., 1997). Similar to this, cultured Seriola dumerilii specimens present a lower proportion of DHA than wild specimens (Rodriguez-Barreto et al., 2012). The lower omega-3 fats in some farmed marine fish species, compared with their wild relatives, presumably is because of the lack of lipids originating from algae and marine phytoplankton (reviewed by Lloret et al., 2014).

Furthermore, although farmed fish can be a relatively good source of healthy n-3 fatty acids, they can contain high concentrations of organochlorine compounds such as PCBs, dioxins, chlorinated pesticides and other hazardous substances to human health such as organic contaminants and antibiotics (Hites et al., 2004; Hamilton et al., 2005). Sometimes reared fish have been associated with a higher presence of some of these hazardous substances (compared with wild fish), which reduces the net health benefits derived from the consumption of farmed fish compared with wild fish. For example, concentrations of organochlorine contaminants were found to be significantly higher in farmed Atlantic salmon (Salmo salar) than in wild, raising concerns that consumption of farmed fish may pose health risks that detract from the beneficial effects of fish consumption (Hites et al., 2004).

In addition, aquaculture activities are often related to environmental problems (Buschmann et al., 2006; Duarte et al., 2009) such as: (i) habitat loss, pollution and changes in benthic communities (alteration of seafloor fauna and flora communities) associated with the discharge of suspended solids, and nutrient and organic enrichment of waters resulting in build-up of anoxic sediments, (ii) impact of escaped fish on the native fish fauna; (iii) introduced species, pests and diseases (e.g. parasites), and (iv) use of fishmeal and fish oil to feed reared fish. From all these problems, the last two directly affect seafood quality and quantity. In marine ecosystems, most severe fish parasitic infections have been reported in aquaculture, probably related to artificial culture conditions, where fish densities are abnormally high (Rohde & Littlewood, 2005). Farm-origin parasites can spread and affect the survival of wild fish populations (Krkosek et al., 2006). Furthermore, current production of fishmeal for aquaculture appears to be unsustainable and raises social and environmental justice issues (Brunner et al., 2009). In order to ensure healthy fish and a final product comparable with their wild counterparts, farmed fish need to receive eicosapentaenoic and docosahexaenoic acids largely through their diets (FAO, 2014). Fish and crustacean mariculture currently depend on the use of feeds derived from wild fisheries to receive eicosapentaenoic and docosahexaenoic acids, taking 20–25 million metric tonnes of fishmeal to produce 30 million metric tonnes of fish and crustaceans (Duarte et al., 2009). It has been estimated that about 85% (~136 million tonnes) of the worldwide fisheries production are for direct human consumption (fresh, frozen and canned), whereas the 15% remaining (~22 million tonnes) is used for fishmeal and fish oil production as animal food including aquaculture (FAO, 2014). The aquaculture sector currently consumes about 75% of global fish-oil production, although this percentage seems to be declining owing to the increasing demand for fish oil for supplements and other food purposes (the demand for fish oil for direct human consumption is increasing at an annual growth rate of 15–20%; FAO, 2014).
POTENTIAL CONSEQUENCES OF CLIMATE CHANGE FOR FISH AND HUMAN HEALTH

Apart from the impacts of fishing and aquaculture, climate change is emerging as a key factor that has considerable implications for human-exploited natural resources worldwide. Climate change is leading to a warming in many of the world’s seas and oceans, producing spatial and temporal changes in the diversity and productivity of fish (see e.g. Hollwedel et al., 2013; Lloret et al., 2015), with consequences for fish security as well as on the availability of fish oils for consumers. Fish can respond to ocean warming by shifting their latitudinal range (e.g. Perry et al., 2005) and depth range (e.g. Durbuy et al., 2008). In the following sections, we discuss how changes in fish communities due to factors linked to climate change such as warming, changes in river runoff and ocean acidification may affect availability of fish oil for humans and seafood safety.

Direct impact of sea warming on fish oils available to consumers

Global warming can benefit warm-water species, allowing their expansion into areas they did not previously occupy (e.g. Sabatés et al., 2006; Petitgas et al., 2012; Lloret et al., 2015). The opening of the Suez Canal in the 19th century resulted in the migration of more than 600 tropical Indo-Pacific species into the Mediterranean Sea, and constitutes a sound example of how changes in fish communities may have large ecological and economic impacts (Galil, 2008; Lejeusne et al., 2009). The introduction or increase of warm-water (thermophilic) species in response to warming may provide new inputs of fish oil to consumers (local citizens will be able to access new fish oil sources). For example, the increase in abundance and expansion of thermophilic pelagic species such as Sardinella aurita, Trachinotus ovatus, Pomatomus saltatrix and Euthynnus alletteratus in the north-western Mediterranean (Lloret et al., 2015), is leading to new sources of oil for local communities.

On the other hand, sea warming threatens cold-water species in the colder places of the Mediterranean such as the Gulf of Lions. This leads to declining populations (e.g. Molva macrocephalma, Sardina pilchardus and Engraulis encrasicolus), range contractions (i.e. retraction to higher latitudes or deeper waters where waters are colder as with Sprattus sprattus), or even forcing local extinctions of certain boreal fish species such as Molva molva (Lloret et al., 2015). In the North Sea and Baltic Sea, this is leading to the replacement of cold-water assemblages, typically characterized by Atlantic herring and European sprat from the 1960s to 1980s, with warmer-water assemblages including Atlantic mackerel, Atlantic horse mackerel, European pilchard and European anchovy from the 1990s onwards (Montero-Serra et al., 2015). Nevertheless, the replacement of typical, cold-water fauna by warm-water species may result in suspicion of a non-traditional fish or low appreciation by consumers of the thermophilic species despite some possibly constituting a good source of fish oil. This is the case for Coryphaena hippurus in the northern Catalan Coast, where this relatively ‘new’ species has a lower market value (it sells for ≏€1.5 kg⁻¹ at auction in the fish markets of several ports of this area) compared with the southern Catalan Sea (≏€3.5 kg⁻¹) and the Balearic Islands (≏€6–9 kg⁻¹), where it has been historically more common and appreciated. This example shows that consumers often do not fully understand or appreciate the gastronomic value of marine exotic species, a fact that also occurs in freshwater ecosystems with the introduction of exotic species for aquaculture purposes (Bartley et al., 2005). Thus, we must take careful account of cultural histories and traditions. Such worries can be eased, as for example in the eastern Mediterranean with Lessepsian fish that originated from the Red Sea but now are accepted as a normal part of the diet following their migration through the Suez Canal to the Mediterranean (Öztürk, 2010).

Indirect impacts caused by sea warming to consumers

Predation of immigrated warm-water species may alter the local fauna, not only because they change the abundance of these fauna through competition but also because they may negatively affect fish condition. Where there is a high abundance of predators, prey must spend more time avoiding predators and can forage less often and over larger areas (Walsh et al., 2012). In addition, prey exposed to higher predation risk has higher mass-specific metabolic rates, resulting in more energy being required for maintenance (Walsh et al., 2012). Overall, behavioural or physiological changes in prey under high predator conditions may result in lower net energy intake, which may translate into lower mass gain or storage of energy in fat reserves (Garvey et al., 2004). This is an example of the complex links between climate change and fish condition, which needs further investigation in order to assess its ubiquity.

Sea warming is also exerting an indirect effect on fish lipid reserves, as rising seawater temperatures change primary and secondary production, e.g. the abundance and quality of the plankton fish feed upon. For example, climatic conditions partly determine feeding conditions for capelin (Mallotus villosus) and in this way influence both population biomass accumulation and fish fat content (Ellingsen et al., 2008; Orlova et al., 2010). Climate shifts could also change essential fatty acid production either by changing phytoplankton species composition or by changing essential fatty acid production within phytoplankton taxa. One of the best-studied examples of changing essential fatty acid production in response to environmental change comes from the Baltic Sea, where eutrophication has shifted phytoplankton dominance from diatoms to flagellates, apparently resulting in changes in essential fatty acid ratios throughout the food web, and possibly leading to a chronic reproductive disease in Atlantic salmon (Salmo salar; Ahlgren et al., 2005).

Another example of how sea warming negatively affects the condition of fish through changes in the plankton community is sprat from the Black Sea, where higher temperatures coincide with a decrease in the biomass of the cold-tolerant complex of phytoplankton, a decrease of mesozooplankton biomass, the main food of sprat, and a simultaneous decline of the abundance, biomass and the individual fat content of this small pelagic fish (reviewed by Lloret et al., 2014). The decline in the individual fat content of sprat in the Black Sea from the late 1990s (Figure 2; Nikolsky et al., 2011) provides a clear example of how sea warming can reduce the omega-3 supply available to consumers from local cold-water
species such as capelin, declined (Litzow, North Sea and Scotian shelf), while small pelagic lipid-rich abundance in four boreal zones (Bering Sea, Gulf of Alaska, gramma and other demersal lipid-poor species increased in Oscillation regime shift, walleye pollock of essential fatty acids. Following the 1970s Pacific Decadal were the result of climate-mediated changes in the availability in lipid content of different fish communities in the Pacific

fat content decreased from 20–30% to 10–15%, resulting in the collapse of the fishery (reviewed by Lloret et al. 2014). Another example of expanding biotoxin presence linked to sea warming is the case of lipid-soluble ciguatoxins produced by dinoflagellates (microalgae) of the genus Gambierdiscus that accumulate in some thermophilic fish, producing the so-called 'ciguatera fish poisoning', a seafood-borne illness that has become a hazard to consumers in non-endemic regions such as the USA and Germany (Dickey & Plakas, 2010). The expansion of ciguatera is due not only to the expanding international trade in seafood from tropical fisheries (Dickey & Plakas, 2010) but also because sea warming has contributed to the emergence of toxic dinoflagellate species and ciguatox fish in subtropical and even temperate regions that previously had been restricted to tropical areas (Mattei et al., 2014). Here, immigrating ciguatoxic, thermophile fish species, moving from their original subtropical and tropical habitats to temperate areas add risks to human health and well-being in these new localities. A growing number of ciguatera poisoning cases are being reported in Europe and the presence of Gambierdiscus spp. both in the Mediterranean Sea and Canary Islands has been linked to the spread of toxic dinoflagellates and ciguatoxic fish, suggesting that this problem is already affecting regions in more temperate latitudes (Otero et al., 2010; Alverca, 2011). In the Canary Islands, for example, at least three ciguatera outbreaks have been reported since 2010, which coincided with the detection of novel toxin-producing dinoflagellates (Boada et al., 2010; Nuñez et al., 2012). In the Mediterranean, reports suggest the presence of ciguatoxin-like substances in the Lessepsian rabbitfish Siganus rivulatus in the eastern Mediterranean (Bentur & Spanier, 2007). Additionally, during the last 15 years, reports of toxic episodes involving benthic dinoflagellates belonging to the ciguatera community, in particular Ostreopsis, have shown a marked increase in warm-temperate areas particularly the Mediterranean Sea, affecting several countries including Spain, Italy, France, Greece and more recently Portugal (Alverca, 2011). Another example of an expanding toxin linked to sea warming is tetrodotoxin (TTX), a potent neurotoxin produced by bacteria that is found in liver, gonads and gastrointestinal tract and skin of some puffer fish (Lagocephalus spp.) and some other marine organisms (Lee et al., 2000). Puffer fish are marine species that are distributed in tropical and subtropical areas of the Atlantic, Indian and Pacific Oceans that are spreading to temperate areas as sea temperatures rise, with the consequent spread of the tetrodotoxin that some species contain (see e.g. Golani, 2010). For example, a puffer fish (Lagocephalus sceleratus) invasion in the eastern Mediterranean is becoming a serious hazard to consumers due to tetrodotoxin (Sabrah et al., 2006; Katikou et al. 2009), with poisoning incidents following consumption of L. sceleratus being reported in some countries (see e.g. Bentur et al., 2008; Katikou et al., 2009).

Implications of sea warming for seafood safety Pathogens, contaminants and biotoxins are key to the safety of seafood as they pose health risks to consumers that are expected to increase due to sea warming, leading to illness in humans and other organisms (European Marine Board, 2013). Biotoxins provide a good example of the increasing threats arising from sea warming. Under favourable warmer conditions, some species of phytoplankton that produce potent biotoxins grow rapidly and multiply causing ‘blooms’ (so-called harmful algal blooms or HAB; see Berdalet et al., 2015), which can cause adverse health effects to wildlife through oxygen depletion leading to mass mortality of marine living resources, and to humans through the consumption of contaminated seafood leading to damage to the liver and nervous system (Moore et al., 2008; Berdalet et al. 2016). Harmful algal blooms are projected to increase in frequency and intensity, in part due to climate warming, together with increased microbial pollution from coastal populations and the resulting nutrient load (European Marine Board, 2013). Whereas warmer temperatures create a competitive advantage for certain types of harmful algae which favours their growth, other factors linked to climate change such as increases in dissolved carbon dioxide in marine ecosystems, coastal waters associated with sea level rises, and increased river runoff (and associated anthropogenic nutrients) may favour the growth of harmful algae (EPA, 2013). It is also relevant to note that this is often site-specific, with local hydrodynamic processes often determining whether or not blooms occur (Davidson et al., 2014).

An example of expanding biotoxin presence linked to sea warming is the case of lipid-soluble ciguatoxins produced by dinoflagellates (microalgae) of the genus Gambierdiscus that accumulate in some thermophilic fish, producing the so-called ‘ciguatera fish poisoning’, a seafood-borne illness that has become a hazard to consumers in non-endemic regions such as the USA and Germany (Dickey & Plakas, 2010). The expansion of ciguatera is due not only to the expanding international trade in seafood from tropical fisheries (Dickey & Plakas, 2010) but also because sea warming has contributed to the emergence of toxic dinoflagellate species and ciguatoxic fish in subtropical and even temperate regions that previously had been restricted to tropical areas (Mattei et al., 2014). Here, immigrating ciguatoxic, thermophile fish species, moving from their original subtropical and tropical habitats to temperate areas add risks to human health and well-being in these new localities. A growing number of ciguatera poisoning cases are being reported in Europe and the presence of Gambierdiscus spp. both in the Mediterranean Sea and Canary Islands has been linked to the spread of toxic dinoflagellates and ciguatoxic fish, suggesting that this problem is already affecting regions in more temperate latitudes (Otero et al., 2010; Alverca, 2011). In the Canary Islands, for example, at least three ciguatera outbreaks have been reported since 2010, which coincided with the detection of novel toxin-producing dinoflagellates (Boada et al., 2010; Nuñez et al., 2012). In the Mediterranean, reports suggest the presence of ciguatoxin-like substances in the Lessepsian rabbitfish Siganus rivulatus in the eastern Mediterranean (Bentur & Spanier, 2007). Additionally, during the last 15 years, reports of toxic episodes involving benthic dinoflagellates belonging to the ciguatera community, in particular Ostreopsis, have shown a marked increase in warm-temperate areas particularly the Mediterranean Sea, affecting several countries including Spain, Italy, France, Greece and more recently Portugal (Alverca, 2011). Another example of an expanding toxin linked to sea warming is tetrodotoxin (TTX), a potent neurotoxin produced by bacteria that is found in liver, gonads and gastrointestinal tract and skin of some puffer fish (Lagocephalus spp.) and some other marine organisms (Lee et al., 2000). Puffer fish are marine species that are distributed in tropical and subtropical areas of the Atlantic, Indian and Pacific Oceans that are spreading to temperate areas as sea temperatures rise, with the consequent spread of the tetrodotoxin that some species contain (see e.g. Golani, 2010). For example, a puffer fish (Lagocephalus sceleratus) invasion in the eastern Mediterranean is becoming a serious hazard to consumers due to tetrodotoxin (Sabrah et al., 2006; Katikou et al. 2009), with poisoning incidents following consumption of L. sceleratus being reported in some countries (see e.g. Bentur et al., 2008; Katikou et al., 2009).
Despite the alarming health hazards associated with the spread of ciguatera and tetrodotoxin-toxic thermophilic fish, it seems that the negative ecological and fishery effects of these fish once they enter the new habitat exceeds the health hazards. For example, an important economic impact of puffer fish in the eastern Mediterranean is to the small-scale local fishery sector, as the puffer fish feeds on commercial species entangled in nets, leading to significant losses of income and damage to fishing gear (Rousou et al., 2014). Similarly, rabbitfish in the eastern Mediterranean have profound negative effects on algal forests, which are among the most productive and diverse communities of temperate Mediterranean coasts, providing resources including food and habitat for large numbers of exploited fish and invertebrates (Vergès et al., 2014). These ecological/fishery effects have the potential to raise significant seafood security concerns in developing nations of the eastern Mediterranean.

Rising temperatures are also associated with changes in the distribution and occurrence of pathogens found in fish such as microbes and parasites that are harmful for consumers. During the last half century, ocean warming has favoured the spread of marine bacteria of the Vibrio genus, including *Vibrio cholera* (which can cause cholera in humans) and may be the cause of the globally increasing trend in their associated diseases (Vezzulli et al., 2012). There is also a clear effect of increased sea temperatures on the growth rates of parasites in fish hosts (Macnab & Barber, 2012). The potential ‘booster’ effect of sea warming on parasites can lead to additional worries for the consumer.

Finally, the bioavailability and toxicity of some contaminants found in fish such as mercury and persistent organic pollutants (POPs) is likely to increase in response to rising water temperatures, and hence the health risk for consumers (see e.g. Noyes et al., 2009; Dijkstra et al., 2013). Recent studies show that warmer sea temperatures may increase the ability of marine fish to accumulate mercury because fish in warmer water eat more and have higher methymercury levels in their tissues, suggesting that increases in their metabolic rate causes the increased mercury uptake (Dijkstra et al., 2013). Although still little is known about the interactions between temperature and other contaminants such as persistent organic pollutants that are found in exploited marine fish (Ross & Birnbaum, 2005), studies conducted in freshwater ecosystems demonstrated that the lethality of the persistent organic pollutant dieldrin and the toxicity of herbicide atrazine to freshwater fish increased with increasing temperatures (reviewed by Noyes et al., 2009), raising concerns that a similar effect may also occur in marine fisheries.

### Ocean acidification

Increased CO₂ from fossil fuel emissions enters the ocean and makes it more acidic (the pH decreases). When pH decreases, the carbon state, i.e. the balance between bicarbonate and carbonate in the ocean, changes so that there is less carbonate. This shift has important implications for plants and animals that build calcium carbonate (CaCO₃) structures (Haigh et al., 2015). Calcifying organisms such as molluscs and crustaceans comprise a large component of fisheries in many areas including the USA and Canada, where these animals can represent more than 65% of the total landed value (Cooley & Doney, 2009; Haigh et al., 2015). Furthermore, ocean acidification may affect the phytoplankton and zooplankton communities – particularly copepods and pteropods – that form a substantial biomass in the oceans and provide an important source of food for upper trophic levels in temperate marine food webs – and hence indirectly have a negative impact on fisheries (reviewed by Haigh et al., 2015).

Furthermore, with ocean acidification, some toxic phytoplankton species may gain a competitive advantage and threaten wild and cultured fish, e.g. *Heterosigma akashiwo* in the North-east Pacific, a raphidophyte that grows faster when dissolved CO₂ is higher, releasing peroxide free radicals that damage fish gills and cause significant mortality and monetary losses (Haigh et al., 2015). In a similar way, increasing partial pressure of carbon dioxide (P CO₂) has been shown to alter the mix of neurotoxins produced by toxic alga genera such as *Pseudo-nitzschia* and *Alexandrium* to favour more potent forms, posing a significant threat to higher trophic levels impacting the shellfish industry as well as overall food safety (Haigh et al., 2015). Furthermore, ocean acidification may also change the structure and composition of macroalgae and macroinvertebrate communities that provide food and essential habitats for commercially important fish species such as the calcified red algal deposits known as maerl beds (Biomar Team, 2003), the free-swimming echinoderms known as cninoids (Colloca et al., 2004), coralligenous assemblages (Ballesteros, 2006) and coral reefs (Kleypas & Yates, 2009).
However, there are still significant knowledge gaps on the nature and degree of these effects, particularly regarding the indirect effects of ocean acidification on particular life stages of exploited species and the prey they feed on, in particular the combined effects of multiple stressors such as climate change, chemical contamination and HABs (Denman et al., 2011; Haigh et al., 2015). In this sense, it is impossible to predict potential changes to exploited organisms due to a single factor alone (e.g. decreasing pH/increasing \( \text{PCO}_2 \)) when they are subjected to simultaneous changes in other variables that are expected to change with the climate such as increasing temperature and decreasing dissolved oxygen (Denman et al., 2011).

### Conclusions and Recommendations

Fishing activities, aquaculture and climate change are challenging the benefits and risks of marine resources to human health and well-being (Table 1; Figure 3). These challenges will have increasing global implications taking into account

<table>
<thead>
<tr>
<th>Challenges</th>
<th>Factors intervening</th>
<th>Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marine fisheries</td>
<td>Reductions in fish lipid supplies</td>
<td>- Decrease in abundance and biomass of living marine resources due to overfishing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Decrease in the abundance and diversity of preys available for fish</td>
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<tr>
<td></td>
<td></td>
<td>- Fishing-induced stress</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Environmental justice issues</td>
</tr>
<tr>
<td>Increase of health threats</td>
<td>Fish contaminated with parasites that pose a risk to consumers, such as Anisakids (although parasites can also provide ecological benefits)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Large pelagic fish contain high levels of contaminants</td>
</tr>
<tr>
<td>Aquaculture</td>
<td>Use of fishmeal and fish oil to feed reared fish</td>
<td>- Minimize the use of fishmeal and fish oil</td>
</tr>
<tr>
<td>Environmental and health</td>
<td>Other environmental issues: habitat loss, pollution, introduced species, pests and diseases, justice issues</td>
<td></td>
</tr>
<tr>
<td>issues</td>
<td>Reared fish often have a lower proportion of omega-3 fatty acids and a higher presence of hazardous substances to human health compared with wild fish</td>
<td></td>
</tr>
<tr>
<td>Climate change</td>
<td>Refusal or low appreciation by consumers of exotic fish</td>
<td>- For certain small and medium-sized pelagic fish, promote exotic warm-water and less-accepted species among consumers</td>
</tr>
<tr>
<td>Reduction in fish oil and</td>
<td>Ecological and fishery effects arising from exotic species</td>
<td>- Physical removal of exotic species that pose threats to the ecosystem and fisheries</td>
</tr>
<tr>
<td>proteins available to</td>
<td>Lower storage of fish lipids as rising seawater temperatures change the quantity and quality of the food items fish species feed upon</td>
<td></td>
</tr>
<tr>
<td>consumers</td>
<td>Negative effect of sea warming on condition of species inhabiting the warmest areas of their distribution range</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Riverine water inputs will affect fish oil reserves of pelagic fish inhabiting low latitude seas through a reduction in plankton productivity</td>
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<tr>
<td></td>
<td>Negative effect of ocean acidification on calcifying species that are important to fisheries</td>
<td></td>
</tr>
<tr>
<td>Emergence or increase of</td>
<td>Harmful algal blooms are projected to increase due to sea warming</td>
<td>- Physical removal of exotic fish species that pose threats to consumers</td>
</tr>
<tr>
<td>biotoxins, pathogens and</td>
<td>Expansion of exotic fish species containing biotoxins (e.g. ciguatera and tetrodotoxin) due to sea warming</td>
<td></td>
</tr>
<tr>
<td>contaminants</td>
<td>Spread of pathogens such as microbes and parasites in relation to sea warming</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In higher latitudes, riverine runoff increase could enhance pathogen pollution.</td>
<td>- Explore the use of the biotoxins for medical use</td>
</tr>
<tr>
<td></td>
<td>Bioavailability and toxicity of some contaminants found in fish will likely to increase in response to sea warming</td>
<td></td>
</tr>
</tbody>
</table>
Move towards better management of fisheries

Adoption of better management procedures will contribute to sustainable fishing practices that avoid overexploitation and support stock recovery. Although climate change (sea warming, ocean acidification, etc.) cannot be changed by fisheries management, the negative effects can be reduced if resilient (healthy) fish stocks are maintained through better management practices. These practices include: (a) the adaptation of fishing capacity to sustainable resource productivity; (b) the promotion of environmentally friendly fishing techniques that minimize discards and avoid degradation and loss of marine habitats and biodiversity; (c) the promotion of integrated ecosystem approaches (ecosystem-based fisheries management); (d) the establishment of new marine protected areas; (e) a solution would be to ban the discards of fish viscera over-board and their re-use promoted (under strict control rules to ensure their safety).

Promotion of sustainable aquaculture practices

In order to attain sustainable aquaculture, it is important to avoid practices that negatively impact the marine environment (habitat loss, pollution, introduced species, pests and diseases), and to minimize the unsustainable use of fishmeal and fish oil. Currently fish oil is the only economically viable source of long-chain omega-3 fatty acids for feed purposes in aquaculture (FAO, 2014). There are no good alternative sources of eicosapentaenoic and docosahexaenoic acids for feeding cultured fish at present because alternatives such as microalgae are too costly for feed purposes and not a viable option in the near future (FAO, 2014). Therefore, three possible approaches shall be considered in this context. First, the promotion of direct human consumption of the fish used to produce fishmeal (rather than the consumption of reared fish); this would be not only healthier but also ecologically sound. Second, mariculture must abandon its current dependence on fisheries catches for producing fish meal and fish oil and instead encourage the production of edible macroalgae, filter-feeder organisms and herbivorous or omnivorous fish, which use much less fishmeal than do carnivorous species per tonne of protein. Third, mariculture should abandon the use of agricultural products in marine animal feeds, which represents an indirect use of crop-land and water and leads to competition between fish and humans for food (Duarte et al., 2009).

Landing and use of fish-by-products

Livers and intestines, where a number of fish species store lipids (liver oil and perivisceral fat, respectively), are usually discarded as waste. Only the livers from some species (e.g. cod) are used to produce fish oil (omega-3) capsules for human consumption. If liver and perivisceral fats of more species could be used more effectively, sources of omega-3 would be greatly enhanced. However, since many fish accumulate pollutants and parasites in their viscera, or even bio-toxins such as TTX and ciguatera, the use of viscera should be carried out under strictly controlled product regulations. This procedure would also contribute to the management practices required in commercial fishing needed to ensure healthy fish stocks (McClelland, 2002; Lloret, 2010). For example, viscera of some Mediterranean groundfish species, such as the catshark *Scyliorhinus canicula* and *Lophius*, are often discarded at sea prior to reaching the fish market. This practice may result in heavier parasite loads in fish that feed on the discarded viscera (McClelland, 2002). Therefore, a solution would be to ban the discards of fish viscera over-board and their re-use promoted (under strict control rules to ensure their safety).

Promotion of warm-water species among consumers

For certain small and medium-sized pelagic fish, the promotion of warm-water (often under-exploited) and less accepted species among consumers could ease the pressure on cold-water (often over-exploited), traditional species. For example, in the north-western Mediterranean the consumption of warm-water species, such as *Sardinella aurita*, *Trachinotus ovatus* and *Sphyraena viridensis*, which are increasing due to sea warming (Lloret et al., 2015), could be...
promoted as a substitute for cold-water species that suffer from overfishing and sea warming, such as Engraulis encrasicolus and Sardina pilchardus (Martin et al., 2010). However, this suggestion must be cautiously considered and on a case-by-case basis. For example, it might not be suitable in the case of certain warm-water species such as thermophilic groupers or billfishes, which are also spreading into new areas because of sea warming but have life history traits that render them very vulnerable to fishing (see e.g. Lloret et al., 2015).

**Promotion of alternative/complementary sources of omega-3**

The overexploitation of certain fish stocks highlights the urgent need to seek alternative sources of omega-3 fatty acids such as marine algae, microorganisms and plants (Surette, 2008). Cultivated algal oils, for example, are free of contaminants and satisfy ethical considerations, but their value as a health asset remains to be demonstrated (Brunner et al., 2013). In fact, species deemed unsustainably fished have significantly higher levels of mercury but do not provide higher levels of long-chain omega-3 fatty acids (Gerber et al., 2012). Thus, the reduction in consumption of these species would have benefits for both human health and marine ecosystems. It is perhaps impossible to consume fish and have no risk of methylmercury exposure, but if high methylmercury containing species are substituted with low methylmercury containing species, methylmercury exposure could be minimized while retaining the beneficial health aspects associated with fish consumption (Johnston & Snow, 2007), and at the same time avoid pressures on unsustainable seafood (Gerber et al., 2012).

**The need for multidisciplinary research on oceans and human health**

More multidisciplinary research is needed to enhance our understanding of the ecological mechanisms behind seafood safety and security concerns. Additional multidisciplinary investigations should cover the impact of sea warming and fishing on fish oil reserves as well as the origin and dynamics of toxins in seafood (e.g. tetrodotoxin and ciguatera), health benefits of fish consumption, social and environmental equity issues arising from projected demands for caught and farmed fish, direct and indirect impacts of ocean acidification on fishery species, combined effects of multiple stressors (e.g. temperature–contaminant interactions), as well as effects of fishing on parasite abundance and diversity and the role of parasites in the efficacy of marine reserves.

**References**


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Mercury, selenium and fish oils in marine food webs and implications for human health

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Humans who eat fish are exposed to mixtures of healthful nutrients and harmful contaminants that are influenced by environmental and ecological factors. Marine fisheries are composed of a multitude of species with varying life histories, and harvested in oceans, coastal waters and estuaries where environmental and ecological conditions determine fish exposure to both nutrients and contaminants. Many of these nutrients and contaminants are thought to influence similar health outcomes (i.e., neurological, cardiovascular, immunological systems). Therefore, our understanding of the risks and benefits of consuming seafood require balanced assessments of contaminants and nutrients found in fish and shellfish. In this paper, we review some of the reported benefits of fish consumption with a focus on the potential hazards of mercury exposure, and compare the environmental variability of fish oils, selenium and mercury in fish. A major scientific gap identified is that fish tissue concentrations are rarely measured for both contaminants and nutrients across a range of species and geographic regions. Interpreting the implications of seafood for human health will require a better understanding of these multiple exposures, particularly as environmental conditions in the oceans change.

Keywords: Oceans and human health, OHH, mercury, selenium, fish oils, n-3 fatty acids, eicosapentaenoic acid, docosahexaenoic acid, ecotoxicology, public health

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The world’s oceans support marine fisheries for commercial, recreational and subsistence uses, and thus are directly linked to human health through fish consumption (i.e., Bergé & Barnathan, 2005; Kite-Powell et al., 2008; Halpern et al., 2012; Moore et al., 2013; Tacon & Metian, 2013). Fish comprise an important source of animal protein for much of the world’s human population, and in the next decade, total production from wild fisheries and aquaculture is expected to exceed production of beef, pork or poultry (FAO/WHO, 2011). In 2010, fish accounted for 16.7% of the world’s intake of animal protein, and the world fish food supply grew 3.2% per year from 1961–2012, nearly doubling from an average of 9.9 kg per capita to 19.2 kg per capita (FAO, 2014, pp. 3–4).

The ability of the global population to obtain healthful marine-derived food is dependent on well-managed ecosystems. A broad interdisciplinary approach is needed to understand the connections between the marine environment and human health (Kite-Powell et al., 2008; Moore et al., 2013), particularly for evaluating the risks and benefits of consuming seafood. This necessarily requires expertise from marine science as well as public health and biomedical science. This paper is authored by an interdisciplinary group comprising marine and human health scientists who have shared their expertise to synthesize current knowledge on the benefits and risks of consuming marine organisms as routes of human exposure to combinations of fish oils, selenium and the global contaminant mercury, particularly its highly bioavailable and toxic form, methylmercury. Other potential compounds of interest in marine organisms, including organohalogenics, natural toxins, arsenicals, trace essential elements and vitamins are beyond the scope of this review; however, introductions to such topics are available elsewhere (Jeandel & Minster, 1987; Edmonds & Francesconi, 1993; Neff, 1997; Garthwaite, 2000; Lail et al., 2007; Guglielmo et al., 2009; Shaw & Kannan, 2009; Yogui & Sericano, 2009; Dickey & Plakas, 2010; Buck et al., 2011; Cusick & Sayler, 2013; Prego-Faráldo et al., 2013; Skjanes et al., 2013; Ahrens & Bundschuh, 2014; Alonso et al., 2014; Šaňudo-Wilhelmy et al., 2014). This review provides a limited overview of

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select dimensions of marine seafood chemical content, and demonstrates the multidisciplinary issues at the interface of Oceans and Human Health (OHH). It does not set out to provide a comprehensive review of seafood content or the overall health implications of seafood consumption. The collaboration of the co-authors of this paper, hailing from diverse disciplinary backgrounds including veterinary medicine, toxicology, immunology, epidemiology, ecology, toxicology and geography, also exemplifies the goals of the OHH initiative which includes the sharing of insights and priorities across research communities (European Marine Board, 2013).

HEALTH BENEFITS OF FISH CONSUMPTION

Fish and shellfish contain protein, long-chain omega-3 fatty acids, vitamins, minerals and trace elements such as calcium and magnesium (Tacon & Metian, 2013). Seafood has the highest concentration of long-chain omega-3 polyunsaturated fatty acids (PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), of any foods (Tacon & Metian, 2013). EPA and DHA show beneficial associations with cardiovascular phenotypes including blood pressure (Campbell et al., 2013), vascular endothelial function (Xin et al., 2012), arterial stiffness (Pase et al., 2011) and heart rate variability (Xin et al., 2013). Fish or fish oil intake is also associated with decreased weight and waist circumference (Bender et al., 2014). Possible impacts of EPA and DHA on cholesterol in humans are unclear. Among persons with diabetes, fish oil supplementation may be associated with lower triglycerides and lower levels of very low density lipoprotein (VLDL) cholesterol, but with higher levels of low density lipoprotein (LDL) cholesterol (Hartweg et al., 2008). In dialysis patients, there are also associations of fish oil supplements with lower triglycerides, but also higher high density lipoprotein (HDL) cholesterol, and no association with LDL cholesterol (Zhu et al., 2014). However, the relationship of EPA and DHA to hard cardiovascular endpoints is less clear. A pooled meta-analysis of 68,680 fish oil supplement clinical trial participants, many of whom (more than half of the trials) had pre-existing cardiovascular disease and were being followed for a second event, did not show evidence for lower risk of mortality (from any cause), cardiac death, myocardial infarction or stroke (Rizos et al., 2012). In contrast, many observational studies report a decrease in cardiovascular disease and all-cause mortality with higher fish oil intake (Wang et al., 2006). The discrepancy between the clinical trial and the observational study results may reflect differences in study populations, or may suggest that another nutrient in fish (or an interacting cofactor in fish) is responsible for some of the cardiovascular benefits attributed to fish oils.

In addition to their possible relevance for cardiometabolic diseases, EPA and DHA fatty acids also may be associated with many other health outcomes. For example, observational studies suggest a lower risk of breast cancer with higher exposure (Zheng et al., 2013). DHA is essential for ophthalmological and neurological development (Uauy et al., 2001; Janssen & Kiliaan, 2014) and fish oil supplements may be associated with cognitive development among infants (Jiao et al., 2014). Among women who previously had delivered a pre-term baby, fish oil supplements appeared to be associated with longer latency and greater weight at birth of the child but did not appear to be associated with differences in risk of another pre-term birth (Saccone & Berghella, 2015).

Selenium, present in marine biota including fish and mussels (Ottzen et al., 2015), has biological effects that are dose-dependent: at low doses, selenium is an essential nutrient used in selenoproteins such as glutathione peroxidase (Barceloux, 1999), but at higher doses, selenium might be toxic to a range of animals including humans (Barceloux, 1999; Hoffman, 2002; Lemly, 2002; Adams et al., 2003; Ackerman & Eagles-Smith, 2009; Rigby et al., 2010; Hladun et al., 2013; Thomas & Janz, 2014), although the dose-response of selenium toxicity differs across animal species (Ackerman & Eagles-Smith, 2009). In humans, the health effects of selenium (total selenium and selenium species) are controversial, with ongoing research into possible elevations or decreases in risk of various health outcomes according to selenium intake (Sabino et al., 2013). A recent Cochrane review (a comprehensive review in medical sciences that aims to summarize published and unpublished data on a topic) of selenium and cancer prevention found heterogeneous studies furnishing no overall evidence that selenium reduces cancer risk (Vinceti et al., 2014).

HAZARDS OF MERCURY

Although seafood provides important nutritional benefits, there may also be hazards from contaminants such as mercury. Neurological impacts of high methylmercury exposure were described in mass poisoning events in Minamata Bay, Japan (Harada, 1995) from consumption of seafood contaminated by effluent from a chlor-alkali facility. 'Minamata disease' was characterized by deficits in sensation, vision, hearing, coordination (ataxia) and other problems associated with neurological function (Eto et al., 1999; Uchino et al., 2005). Children who had high in utero exposures suffered many neurotoxic effects including cerebral palsy, mental retardation, sensorimotor dysfunction and low birth weight (Chapman & Chan, 2000; Karagas et al., 2012). At lower doses, the neurological effects of methylmercury are less clear (Axelrad et al., 2007; Karagas et al., 2012).

Neurodevelopmental toxicity of mercury

Methylmercury neurotoxicity from consumption of seafood has been the focus of birth cohorts in the Faroe Islands, Seychelles and elsewhere (Table 1). In the Faroe Islands, where much of the mercury was acquired from consumption of marine mammals contaminated by organochlorines, there was an inverse association between mercury in cord blood and children’s performance on developmental tests (Grandjean et al., 2001, 2014). However, in the Seychelles, where much of the mercury was from fish, overall associations between foetal exposure to mercury and neurodevelopmental impairments were generally not observed (Carocci et al., 2014). However, at 9 years of age there appeared to be possible differences in fine motor function at higher levels of mercury exposure (Davidson et al., 2006; van Wijngaarden et al., 2006; Mergler et al., 2007), and evidence for interactions between fatty acids and mercury for cognitive processes (Strain et al., 2015). Emerging research suggests that genetic polymorphisms and epigenetic processes may account for some of the inter-individual variations of health effects given exposures (reviewed in Basu et al., 2014). A recent systematic review
<table>
<thead>
<tr>
<th>Population</th>
<th>Study sample</th>
<th>Measure of exposure</th>
<th>Average exposure (ppm)</th>
<th>Neurological associations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Faroe Islands (Grandjean et al., 1997; Grandjean et al., 2014)</td>
<td>1022 singleton births, 917 children at age 7</td>
<td>Hg concentrations in maternal hair at delivery, cord blood, child blood and hair at age 7 years</td>
<td>Geometric mean and IQR at 7 years; Hg 3.05 (1.68 - 6.33), maternal hair Hg in pregnancy: 4.35 (2.63 - 42.2)</td>
<td>Neurodevelopmental deficits (i.e. visuospatial memory) at birth and early school years when comparing high and low exposure groups. Children with high prenatal Hg exposure had lower verbal, scale and performance IQ than children with low prenatal Hg exposure, but this difference was not significant. In contrast, children’s fresh fish consumption was positively associated with scale and performance IQ.</td>
</tr>
<tr>
<td>Italy (Deronio et al., 2013)</td>
<td>149 children</td>
<td>Total Hg and MeHg in maternal hair and breast milk and child’s hair at 7–9 years</td>
<td>Median maternal hair Hg (total): 1.38</td>
<td>No evidence of prenatal Hg exposure linked to children’s neurodevelopment. Children’s fish intake, but not maternal PUFAs (EPA, DHA and other fatty acids), were positively associated with neurodevelopmental test scores.</td>
</tr>
<tr>
<td>Italy (Valent et al., 2013)</td>
<td>606 children at 18 months of age</td>
<td>Maternal and child fish intake; total Hg in maternal hair and blood during pregnancy, umbilical cord blood, and breast milk</td>
<td>Mean maternal hair Hg: 1.06</td>
<td>Increased maternal fish intake during pregnancy associated with increased infant cognition at 6 months of age. This association was stronger after adjusting for maternal hair Hg at delivery. Higher Hg levels were associated with lower infant cognition at 6 months of age.</td>
</tr>
<tr>
<td>United States – Massachusetts (Oken et al., 2005)</td>
<td>135 infant-mother pairs</td>
<td>Self reported Fish consumption during 2nd trimester of pregnancy, maternal total Hg in hair at delivery</td>
<td>Mean maternal hair Hg: 0.55 (range 0.02 – 2.38)</td>
<td>Hg not consistently associated with neurodevelopmental outcomes</td>
</tr>
<tr>
<td>Seychelles (Davidson et al., 1998; Myers et al., 2003; Myers et al., 2009)</td>
<td>Seychelles Child Development Study Main Cohort: 770 mother-child pairs (children through 107 months)</td>
<td>MeHg exposure (measured as total Hg in hair) from maternal hair, and children’s hair at 66 and 107 months</td>
<td>Mean maternal hair Hg: 6.8</td>
<td>No overall association of Hg with neurodevelopment, but evidence for possible interaction of Hg with fish oils for neurodevelopment: higher levels of Hg were negatively associated with psychomotor scores for children of mothers with higher ratio of n-6 to n-3 fatty acids; whereas higher Hg was positively associated with psychomotor development among children born to mothers with higher n-3 fatty acids.</td>
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<tr>
<td>Seychelles (Strain et al., 2015)</td>
<td>Seychelles Child Development Study Nutrition Cohort 2: 1265 mother-child pairs (children at age 20 months)</td>
<td>Total Hg in maternal hair at delivery and maternal weekly fish consumption</td>
<td>Mean child hair-Hg at 66 months: 6.3 (sd: 3.3); at 107 month: 6.1 (sd: 3.6)</td>
<td>No significant correlations between neurodevelopmental score and total mercury. Negative association of maternal hair Hg with academic attainment, language development, fine and gross motor coordination, and intelligence – after omitting one highly influential point from the analysis.</td>
</tr>
<tr>
<td>Seychelles (Davidson et al., 2008)</td>
<td>300 mothers and 229 children at ages 5, 9, 25 and 30 months</td>
<td>Number of fish meals per week of mother during pregnancy</td>
<td>Mean maternal hair MeHg: 5.9</td>
<td>Neurodevelopmental performance at 30 months decreased with increased MeHg, adjusted for nutritional factors.</td>
</tr>
<tr>
<td>Tohoku, Japan (Tatsuta et al., 2014)</td>
<td>387 42-month old children</td>
<td>Cord blood total Hg levels</td>
<td>Median cord blood Hg: 0.01</td>
<td>No significant correlations between neurodevelopmental score and total mercury.</td>
</tr>
<tr>
<td>New Zealand, North Island (Crump et al., 1998 re-analysis of Kjellstrom et al., 1986; Kjellstrom et al., 1989)</td>
<td>237 children ages 6–7 (paired with their mothers)</td>
<td>Average maternal hair Hg concentration during pregnancy</td>
<td>61 children with hair Hg &gt; 6 ppm matched to lower-Hg-exposed children. Crump et al. use continuous hair Hg measures, but do not report average Hg value</td>
<td>No overall association of Hg with neurodevelopment, but evidence for possible interaction of Hg with fish oils for neurodevelopment: higher levels of Hg were negatively associated with psychomotor scores for children of mothers with higher ratio of n-6 to n-3 fatty acids; whereas higher Hg was positively associated with psychomotor development among children born to mothers with higher n-3 fatty acids.</td>
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</tbody>
</table>

IQR, inter-quartile range (25th and 75th percentiles of distribution).
examined the associations between exposure to methylmercury from seafood consumption and developmental neurotoxicity from 164 studies in 43 countries and found that mercury might be impacting the health of Arctic and riverine populations near gold mining sites, and might also be relevant for public health in highly populated coastal regions (Sheehan et al., 2014).

**Immune toxicity of mercury**

Data are limited regarding whether mercury from fish affects the immune system, although studies have been conducted in human populations and in toxicological experiments. In cross-sectional studies in Amazonian Brazil, elevated mercury exposures were associated with increased levels of auto-antibodies in gold miners highly exposed to elemental mercury but also possibly exposed to some methylmercury (Silva et al., 2004; Gardner et al., 2010a). A cross-sectional, nationally representative survey of adults in the USA, showed that hair and blood mercury (thought to largely reflect methylmercury exposures) but not urine mercury (thought to largely reflect inorganic exposures) were associated with having anti-nuclear auto-antibodies (Somers et al., 2015). In *in vitro* toxicological experiments with sufficiently high doses of mercury (3.6 to 36 μM) to induce cell death within 24 h, exposure of human immune cells *in vitro* to methylmercury prevented B cell proliferation, and these suppressive effects were more severe if mercury exposure occurred prior to immune cell activation (Shenker et al., 1993). In T cells, proliferation was suppressed and apoptosis induced following mercury exposure *in vitro*, although these effects were examined in mixed culture systems (Shenker et al., 1992; Shenker et al., 1998). In mixed cultures of peripheral blood mononuclear cells stimulated with lipopolysaccharide, which stimulates macrophages, subcytotoxic concentrations of methylmercury increased production of pro-inflammatory cytokines TNF-α and IL-1β (Gardner et al., 2009, 2010b). Thus, stimulatory effects of methylmercury were observed at doses closer to the typical *in vivo* human exposure range, generally less than 200 nM (Mahaffey, 2004; Mahaffey et al., 2009), while higher doses were inhibitory. In the more environmentally relevant administered dose studies, effects were primarily observed when cells were stimulated, suggesting that immune activation state at least partially determines the sensitivity to toxic effects on the immune system. If mercury does affect inflammation, then inflammatory mechanisms could impact other organ systems including the cardiovascular system.

**Cardiovascular toxicity of mercury**

Mercury’s potential impacts on the cardiovascular system are a growing area of research (Roman et al., 2011). Mercury’s relationship to fatal heart attacks was recently cited as the potentially most expensive and therefore the most important uncertainty in the cost-benefit analysis for economic benefit of mercury pollution reductions to the USA (Rice et al., 2010). Myocardial infarction and mortality risks from mercury have been evaluated in several recent studies. A cross-sectional survey in a nationally representative sample of South Koreans found a higher odds of previous myocardial infarction with higher levels of blood mercury (Kim et al., 2014). A case-control study of 1408 men found that toenail mercury was associated with higher odds of myocardial infarction after accounting for levels of the heart-protective fatty acid DHA (Gualar et al., 2002). In contrast, a pooled convenience sample drawn from the Health Professionals Follow-up Study and Nurses’ Health Study in the USA (6045 adults) found non-significant, but potentially protective associations between toenail mercury and risk of myocardial infarction, stroke and coronary heart disease (Mozaffarian et al., 2011). This result is acknowledged by the authors to likely reflect the cardio-protective benefits of fish oils, rather than being an accurate portrait of mercury’s cardiovascular impact *per se*. A Swedish cohort also found lower risk of first myocardial infarction with higher erythrocyte mercury, even after controlling for a plasma biomarker of fish oils (Hallgren et al., 2001). In contrast, a large cohort study of 1871 elderly men in Finland found strong positive associations of hair mercury levels with acute coronary events, death, and with cause-specific mortality related to congestive heart failure and cardiovascular disease (Virtanen et al., 2005). Additional research is needed to clarify whether mercury is causally associated with fatal cardiovascular disease, and to tease apart the reasons for the apparently discrepant findings in the existing literature. It is likely that there are differing distributions of interacting and confounding variables (i.e. other dietary nutrients, or genetics) across these study populations. Data on geographic variation in joint distributions of nutrients and contaminants in seafood would provide important context for interpreting the human health literature.

**Concentrations of EPA + DHA**

Variability up to 128-fold has been documented in EPA and DHA levels across fish species (Gladyshev et al., 2013). EPA and DHA contents in aquatic animals depend on both taxonomic and ecological factors (Makhutova et al., 2011; Gladyshev et al., 2012b; Lau et al., 2012); other factors such as an anthropogenic pollution (Gladyshev et al., 2012a) may also be important. Research on the possible impacts of fish health status on fish fatty acid content is limited, but suggests the relationships may be complex and organism-specific. In a recent experiment with cultured puffer fish (*Fugu rubripes*) with or without *Trichodina* infection, flat fish (*Paralichthys olivaceus*) with or without streptococcus infection, yellowtail (*Seriola quinquergiata*) with or without jaundice, and amberjack (*Seriola purpurscens*) with or without *Photobacterium damselae* sp. *piscicida*, there was not a significant difference by fish disease status in the overall fish fatty acid composition in fish livers; however, liver DHA was significantly higher in the diseased fish than healthy fish for flat fish, yellowtail and amberjack (Tanaka et al., 2014). There is also growing interest in how oxidative stress in fish may affect fish lipids (Tanaka & Nakamura, 2012; Tanaka et al., 2014).

One objective for our review is to summarize data on EPA and DHA across fish populations. To identify EPA and DHA content of diverse marine fish species, including anadromous fish, we queried Web of Science, Core Collection on 2 October 2014 for ’fatty acid AND content AND fish AND marine’ (Table 2). Unfortunately, most studies report EPA and DHA as per cent of total fatty acids, and do not provide quantitative information on contents of these PUFA in mass units per fish portion (Gladyshev et al., 2007, 2012b; Huynh & Kitts, 2009).
Table 2. Content of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids (mg g\(^{-1}\), wet weight) in various wild fish species, their types of habitat (H1: p, pelagic; bp, benthopelagic; d, demersal; H2: c, cold waters; t, temperate waters; w, warm waters) and size (cm). Orders and species are ranked by EPA + DHA content values.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>EPA</th>
<th>DHA</th>
<th>EPA + DHA</th>
<th>H1</th>
<th>H2</th>
<th>Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order Clupeiformes</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Sardine (Sardinops sagax)</td>
<td>2.04</td>
<td>0.67</td>
<td>2.71</td>
<td>p</td>
<td>75</td>
<td>1.25</td>
<td>Huynh &amp; Kitts (2009)</td>
</tr>
<tr>
<td>Horse mackerel (Trachurus symmetricus)</td>
<td>1.39</td>
<td>0.95</td>
<td>2.34</td>
<td>d</td>
<td>100</td>
<td>1.01</td>
<td>Abd Aziz et al. (2013)</td>
</tr>
<tr>
<td>Order Perciformes</td>
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<tr>
<td>Horse mackerel (Trachurus symmetricus)</td>
<td>1.39</td>
<td>0.95</td>
<td>2.34</td>
<td>d</td>
<td>100</td>
<td>1.01</td>
<td>Abd Aziz et al. (2013)</td>
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<tr>
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<tr>
<td>Atlantic salmon (Salmo salar)</td>
<td>2.04</td>
<td>0.67</td>
<td>2.71</td>
<td>p</td>
<td>75</td>
<td>1.25</td>
<td>Huynh &amp; Kitts (2009)</td>
</tr>
<tr>
<td>Pink salmon (Oncorhynchus gorbuscha)</td>
<td>1.70</td>
<td>3.30</td>
<td>5.00</td>
<td>d</td>
<td>150</td>
<td>1.30</td>
<td>Castro-Gonzalez et al. (2013)</td>
</tr>
<tr>
<td>Order Salmoniformes</td>
<td></td>
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<tr>
<td>Rainbow trout (Oncorhynchus mykiss)</td>
<td>0.12</td>
<td>0.43</td>
<td>0.55</td>
<td>p</td>
<td>25</td>
<td>0.12</td>
<td>Chuang et al. (2012)</td>
</tr>
</tbody>
</table>

**Continued**
In this manuscript, we review data from 10 studies reporting direct measurements of EPA and DHA contents in wild fish biomass obtained using internal standards in chromatography (using capillary columns) over two recent decades. These had slightly different methodologies. For small fish, less than 35 cm (e.g. sardine or capelin), the fish were analysed whole (Huynh & Kitts, 2009). Larger fish species (e.g. salmon) were sampled by dissecting muscle tissue (filets without skin), usually under dorsal fin (e.g. Gladyshev et al., 2006, 2007, 2012b; Huynh & Kitts, 2009; Kitson et al., 2009; Abd Aziz et al., 2013; Sahari et al., 2014). In some studies (Chuang et al., 2012) ventral muscles were sampled. In other studies both small and large fish were taken whole, e.g. ground and homogenized (Castro-Gonzalez et al., 2013). Some authors did not report the sampling in detail (Garcia-Moreno et al., 2013).

The resulting data set includes 63 fish species across 11 orders (Table 2). Since PUFA contents in aquatic animals are known to depend on both phylogenetic and ecological factors (Makhutova et al., 2011; Gladyshev et al., 2012b; Lau et al., 2012), fish species were organized by their EPA and DHA values within taxonomic orders. Putative effects of ecological (habitat) factors were taken into account by dividing the fish species into pelagic, benthopelagic and demersal, as well as by category of water temperature of their habitat, i.e. cold-water, temperate and warm-water (tropical) species. Common size of the fish species was used as a proxy of logical (habitat) factors were taken into account by dividing the fish species into pelagic, benthopelagic and demersal, as well as by category of water temperature of their habitat, i.e. cold-water, temperate and warm-water (tropical) species. Common size of the fish species was used as a proxy of ecological (habitat) factors were taken into account by dividing the fish species into pelagic, benthopelagic and demersal, as well as by category of water temperature of their habitat, i.e. cold-water, temperate and warm-water (tropical) species. Common size of the fish species was used as a proxy of their trophic level, although this is an imperfect surrogate.

Values of EPA + DHA concentration in the 63 fish species varied from 25.60 mg g⁻¹ (sardine Sardinops sagax, order Clupeiformes) to 0.04 mg g⁻¹ (spotted weakfish Cynoscion nebulosus, order Perciformes) (Table 2). Clupeiformes had the highest median and maximum values of EPA + DHA contents, followed by Salmoniformes, while Perciformes, Scorpaeniformes and Gadiformes and miscellaneous had nearly similar median values (Figure 1). Nevertheless, ranges of values for EPA + DHA content of all orders overlapped in minimum values (Figure 1, Table 2). Thus, all orders, including Clupeiformes, have species with comparatively low content of EPA and DHA.

Interpretation of these results may be complicated by measurement error introduced by differing methods used for fish sampling and analysis, but some broad patterns in the data are interesting. Analysis of published EPA + DHA values found no statistically significant effect of type of habitat (pelagic, benthopelagic and demersal), or temperature of habitat, or their interaction on the PUFA content in fish. To visualize the results of ANOVA, a two-dimensional graph of the PUFA content in the groups of species was created (Figure 2). Since EPA + DHA contents in benthopelagic species overlapped completely with those of pelagic and demersal species, they were not included in the depicted groups. In addition, there were only six cold water species amongst pelagic, benthopelagic and demersal, which were joined in one group. The graph illustrates that EPA and DHA values of all the groups, pelagic temperate water, pelagic warm water, demersal temperate water, demersal warm water and cold water species, overlapped nearly completely.

This analysis of available data did not identify a strong predictor for EPA and DHA contents in fish. Temperature, for example, had limited impact: the contents of EPA + DHA in three pelagic planktivorous Clupeiformes with nearly identical sizes: sardine Sardinops sagax from temperate waters, shad Hilsa macrura from warm waters and herring Clupea

### Table 2. Continued

<table>
<thead>
<tr>
<th>Taxon</th>
<th>EPA (mg g⁻¹)</th>
<th>DHA (mg g⁻¹)</th>
<th>EPA + DHA (mg g⁻¹)</th>
<th>H₁</th>
<th>H₃</th>
<th>Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whiting (Gadus merlangus)</td>
<td>0.08</td>
<td>0.48</td>
<td>0.56</td>
<td>d</td>
<td>t</td>
<td>35</td>
<td>Chuang et al. (2012)</td>
</tr>
<tr>
<td>Order Pleuronectiformes</td>
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<tr>
<td>Rock sole (Lepidopsetta bilineata)</td>
<td>1.80</td>
<td>1.10</td>
<td>2.90</td>
<td>d</td>
<td>t</td>
<td>30</td>
<td>Gladyshev et al. (2007)</td>
</tr>
<tr>
<td>Largescale tongue sole (Cynoglossus arel)</td>
<td>0.08</td>
<td>1.13</td>
<td>1.21</td>
<td>d</td>
<td>w</td>
<td>30</td>
<td>Abd Aziz et al. (2013)</td>
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<tr>
<td>Order Siluriformes</td>
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<tr>
<td>Gray eel-catfish (Potosus spp.)</td>
<td>1.46</td>
<td>0.89</td>
<td>2.35</td>
<td>d</td>
<td>w</td>
<td>30</td>
<td>Abd Aziz et al. (2013)</td>
</tr>
<tr>
<td>Order Mugiliformes</td>
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<tr>
<td>Mullet (Mugil cephalus)</td>
<td>0.46</td>
<td>0.08</td>
<td>0.54</td>
<td>p</td>
<td>t</td>
<td>50</td>
<td>Chuang et al. (2012)</td>
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<tr>
<td>Order Beloniformes</td>
<td></td>
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<tr>
<td>Garfish (Belone belone)</td>
<td>0.01</td>
<td>0.15</td>
<td>0.16</td>
<td>p</td>
<td>t</td>
<td>70</td>
<td>Chuang et al. (2012)</td>
</tr>
<tr>
<td>Order Myliobatiformes</td>
<td></td>
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<tr>
<td>Long-tailed butterfly ray (Gymnura spp.)</td>
<td>0.03</td>
<td>0.09</td>
<td>0.12</td>
<td>d</td>
<td>w</td>
<td>30</td>
<td>Abd Aziz et al. (2013)</td>
</tr>
</tbody>
</table>

Fig. 1. Contents of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) in fish orders: minimum, maximum and median values and quartiles. Number of species, N: order Clupeiformes, N = 9; order Salmoniformes, N = 3; order Perciformes, N = 36; order Scorpaeniformes, N = 3; order Gadiformes, N = 4; miscellaneous (orders Osmeriformes, Pleuronectiformes, Siluriformes, Mugiliformes, Beloniformes and Myliobatiformes), N = 8.
harenus from cold waters were all similar (Table 2). Moths et al. (2013) analysed freshwater fish from the Great Lakes as well as 99 other species from freshwater and marine systems documented in seven other studies. As in this study, Moths et al. (2013) found that for marine systems, there was no relationship between latitude and omega-3 fatty acid composition of fish. However, in temperate climates, marine fish had higher omega-3/6 ratios than freshwater fish and for freshwater fish alone, there were higher omega-3 fatty acids in temperate fish as compared with tropical fish. While this study was based on relatively few datasets and many different species, it suggests some interesting patterns. For marine zooplankton, Kattner & Hagen (2009) did not find significant differences in latitudinal distribution of EPA and DHA levels. Since zooplankton are the main food of these three planktivorous fish species from different latitudes, Kattner & Hagen’s (2009) findings for zooplankton are consistent with those for the planktivorous fish. Thus, more specific characteristics of diverse aquatic ecosystems, such as levels of primary production of PUFA and the efficiency of their transfer through trophic chains (Gladyshhev et al., 2011), are likely to be contributing factors for EPA and DHA content of given fish species. In these large meta-analyses, many environmental and fish specific variables may obscure the potential effects of individual environmental factors such as temperature or trophic level, or pharmacokinetic compartment differences of lipids across fish tissues. More research directed to effects of fish phylogenetics, ecological niche, type of habitat, food quality and other possible determinants is needed to be able to predict EPA and DHA contents, particularly in marine fish.

Studies of fish from field sampling, particularly with heterogeneous methodology, are not conducive to investigating the mechanistic sources of difference between populations living in different environmental settings. In contrast to the analysis of metadata for fish fatty acids above, experimental laboratory studies suggest that fatty acid concentrations in plankton and fish may be influenced in part by the food and temperature environments to which they are exposed. Numerous studies have shown that EPA and PUFAs increase in cells grown at lower temperatures and saturated fatty acids decrease (Thompson et al., 1992; Jiang & Gao, 2004; Fuschino et al., 2011; Teoh et al., 2013). In addition, some fish either naturally occurring or cultured have higher concentrations of fatty acids when grown in colder temperatures. Fish need to adjust membrane fluidity for metabolic function in fluctuating temperatures (homeoviscous adaptation) and they do this by changing the concentrations and composition of individual fatty acids and sterols in cell membranes (Sinensky, 1974; Snyder et al., 2012). Several experimental studies show differences in fatty acid concentrations in fish exposed to different temperatures. Experiments with juvenile Atlantic salmon at two temperatures (14 and 19°C) found that n-3, n-5 and total fatty acids were higher in fish raised in colder water (Arts et al., 2012). Another study on cultured Atlantic salmon found that the temperature effect was dependent on the type of oil in their feed; temperature effects were more pronounced in fish fed copepod oil diets than fish oil diets (Bogevik et al., 2011). Another study found the digestibility of the lipids in Atlantic salmon to increase with increasing rearing temperatures suggesting that while colder temperatures may favour higher fatty acid concentrations, they may be less digestible than at warmer temperatures (Huguet et al., 2015). Laurel et al. (2012) found that lower temperatures also favoured increases in unsaturated fatty acids in newly hatched Pacific cod larvae but relative amounts of essential fatty acids did not change with temperature. Similarly, n-3 and n-6 fatty acids decreased with increased temperatures in eggs and larvae of the marine fish, Inimicus japonicus (Wen et al., 2013). Thus, there are a range of experimental studies supporting the role of temperature and potentially diet determining fatty acid composition in aquatic plankton and fish. They suggest that colder temperatures result in higher amounts and differing quality of fatty acids. However, the disparity between patterns observed in experimental and field-based studies should be further investigated.

**Variability in Fish Mercury Concentrations**

One of the major challenges in managing human exposure to mercury from fish consumption is that fish mercury concentrations are highly variable. Numerous studies have measured broad differences in mercury content across different finfish and shellfish taxa (Sunderland, 2007; Karimi et al., 2012). A recent review estimated that mercury content within a given taxon can also be highly variable, ranging from 0.3–2.4 orders of magnitude, depending on the taxon (Karimi et al., 2012). This variability poses a challenge to estimating mercury exposure from seafood consumption, and makes it difficult to quantify the risk associated with consuming specific fish taxa.

Numerous studies have shown that body size, age, trophic level and food source of fish are related to concentrations of methylmercury and the per cent of total mercury that is methylmercury (Chen et al., 2009; Piraino & Taylor, 2009). Across species, body size can be more strongly correlated with mercury concentration than trophic level (Karimi et al., 2013). In general, larger fish across and within species have
higher concentrations of methylmercury because larger fish eat higher trophic level prey and are older and have had a longer time to accumulate mercury (Cossa et al., 2012; Storelli and Barone, 2013). However, some studies have found that mercury concentration is more strongly correlated with age than length or weight (Braune, 1987; Burger & Gochfeld, 2011). For example, the size of Bluefin tuna is not related to mercury concentration (Burger & Gochfeld, 2011) and Atlantic herring in the Arctic show relationships at 3–5 years of age but a decrease at 1–2 years of age due to growth dilution (Braune, 1987). While there are clear positive relationships between total mercury and fish size and fish age, there is still variability in total mercury concentrations that is not explained by those two variables as well as the presence of interspecific and intraspecific variability (Tremain & Adams, 2012). Some of this unexplained variability likely comes from the food source and geographic range of the fish. Fish that have more pelagic than benthic food sources appear to bioaccumulate higher concentrations of mercury (Power et al., 2002; Chen et al., 2009; Karimi et al., 2013). Not surprisingly, fish that are exposed to higher water and sediment concentrations also have higher tissue concentrations of mercury (Lowery & Garrett, 2005; Chen et al., 2009; Gehrke et al., 2011; Taylor et al., 2012; Chen et al., 2014). However, levels of mercury may vary between similar species in a small geographic area and by tissue within a fish (Bank et al., 2007). A recent study also suggests increases in methylmercury bioaccumulation in fish experiencing warmer temperatures (Dijkstra et al., 2013). Overall, these studies show that fish size, age, trophic level, food source and geographic region each influence fish mercury content, with no strict rules for which of these factors explains the largest portion of mercury variability. While agencies such as the Food and Drug Administration (FDA) in the USA monitor mercury in marine fish consumed by humans, they do not report fish sizes or geographic location, both of which are extremely important when looking at mercury bioaccumulation.

**SELENIUM AND MERCURY CONCENTRATIONS IN FISH**

There is a long-running interest in nutrient-toxicant interactions between mercury and selenium (Ganther et al., 1972). Although recent evidence suggests possible synergistic interactions between mercury and selenium for fish development (Penglase et al., 2014), the weight of evidence suggests antagonistic interactions in which selenium mediates mercury toxicokinetics (reviewed in Peterson et al., 2009). Selenomethionine increases mercury elimination in zebrafish (Danio rerio) (Yamashita et al., 2013; Amlund et al., 2015), shrimp (Bjerregaard & Christensen, 2012) and goldfish (Carassius auratus) (Bjerregaard et al., 2012); selenite, and seleno-cysteine also increased mercury elimination in goldfish and shrimp. In humans, dietary organic selenium can increase mercury elimination (Li et al., 2012). Ralston and colleagues report that selenium not only ameliorates the toxic effects of methylmercury by sequestering methylmercury and reducing its bioavailability to organisms, but mercury and selenium may also have physiologically important interactions mediated by other mechanisms (Ralston et al., 2007; Ralston & Raymond, 2010). Based on rat data, Ralston (2008) suggests that where the selenium to mercury molar ratio exceeds 1:1, there is adequate selenium to counter mercury toxicity. However, this has not been clearly demonstrated in humans. In recent trout (Salmo trutta) studies in a Norwegian lake, the selenium to mercury molar ratio was a better predictor of trout metallothionein levels than was either selenium or mercury (Sørmo et al., 2011). However, human studies and clinical trials for selenium demonstrate mixed and inconclusive results for cardiovascular effects of methylmercury and selenium (Mozaffarian, 2009). It has been suggested that mercury cardiovascular toxicity may be modified by selenium intake (Cooper et al., 2007; Khan & Wang, 2009; Mozaffarian, 2009). This might arise through selenium impacts on mercury kinetics (Huang et al., 2013) or through impacts on oxidative stress mediators of mercury toxicity (Kaneko &Ralston, 2007; Ralston et al., 2007; Farina et al., 2011; Alkazemi et al., 2013; Drescher et al., 2014), although evidence for the oxidative stress mediation hypotheses is ambiguous (Belanger et al., 2008). Selenium-mercury interactions may also be relevant for neurodevelopmental outcomes (Choi et al., 2007).

In recent years due to the interest in selenium to mercury molar ratios, a number of studies have assessed mercury and selenium concentrations and the selenium to mercury molar ratios for a variety of fish species from field samples as well as fish purchased from supermarkets (Burger et al., 2005, 2013; Burger & Gochfeld, 2011, 2012; Gochfeld et al., 2012; Karimi et al., 2013, 2014). The relationship between body size and selenium to mercury molar ratios vary with species, tissues and geographic location. Selenium to mercury molar ratios decreased with size of fish for yellowfin tuna and windowpanewidener flounder in Delaware Bay and a wide variety of species in the Aleutians (Burger & Gochfeld, 2011, 2012). Some individuals of most of the 15 species studied in the Aleutians had ratios less than 1.0, where older, larger, higher trophic level fish had the lowest ratios. This was the result of mercury concentrations increasing with fish size but selenium concentrations not increasing with size. While selenium to mercury molar ratios were negatively correlated with fish length for bluefish, the ratios were lower for white muscle tissue, the portion of the fish that humans consume. In a study of 19 species off the coast of New Jersey (USA), (Burger & Gochfeld, 2011) mercury and selenium were positively related for five species and negatively related for two species, and across all species, selenium had no consistent relationship with length. However, for most species tested across all of these studies, the ratios were greater than 1.0, although 20% of the striped bass caught by trawling off the New Jersey coast had molar ratios of less than 1.0 (Gochfeld et al., 2012).

In general, studies of selenium to mercury molar ratios have found that mercury concentrations were positively related to fish length and trophic level but selenium concentrations were not, and selenium to mercury molar ratios are more strongly related to mercury content than selenium content (Karimi et al., 2013). This reflects the fact that mercury more strongly accumulates in the body, and biomagnifies through the food chain compared with selenium (Karimi et al., 2013). These findings are consistent with lower efflux (loss) rates of methylmercury than selenium, because lower efflux rates lead to greater bioaccumulation over time as body size increases (Karimi et al., 2010). However, bivalves (e.g. clams, mussels and oysters) are known to be relatively efficient selenium accumulators (Stewart et al., 2004; Presser & Luoma, 2010), and have higher selenium concentrations than finfish (Karimi et al.,
It also appears that the mean selenium to mercury molar ratio declines with mean size of fish species and with individual fish size within a species. Both suggest that larger, predatory fish as well as the largest individuals of many species have lower selenium to mercury molar ratios and may not provide selenium protection against mercury toxicity for human seafood consumers (although selenium may be available in their diet from other sources). Moreover, smaller fish of a given species may provide greater protective benefits suggesting that those age classes that reside in more estuarine and coastal environments may present lower human health hazards (Burger et al., 2013). However, the variability of selenium to mercury molar ratios found within and across species makes it difficult to use this ratio in risk assessment, risk management and risk communication at the present time. Most governmental organizations that develop fish consumption advisories do not have the data on both mercury and selenium levels in individual fish which are necessary to determine the selenium to mercury molar ratio variation within and across species. It is difficult for risk assessors to develop advisories that are protective without an estimate of this variability.

**FISH THAT OPTIMIZE POTENTIAL BENEFITS VS RISKS**

Recent research is beginning to address the need to quantify the overall nutritional and toxicological value of different types of fish and shellfish based on concentrations of multiple nutrients and contaminants in edible tissues. A recent study found unique, relative concentrations of mercury, omega-3 fatty acids, and selenium, or mercury-nutrient signatures, across seafood taxa (Figure 3, Karimi et al., 2014). Specifically, salmon and forage fish (herring, anchovies and sardines) are high in EPA and DHA compared with other seafood (Figure 3). In contrast, predatory fish have higher mercury concentrations than lower trophic level fish but nutrient concentrations do not appear to differ as strongly by trophic level. Karimi et al. (2014) found that these distinct mercury–nutrient signatures were reflected in the blood of seafood consumers based on their consumption habits. Most notably, consumers with a salmon-dominated diet had a high percentage of omega-3 fatty acids in their blood compared with other seafood consumers. Consumers who tended to eat top-predator fish had higher mercury, but similar nutrient concentrations in blood compared with consumers of lower trophic level seafood. These results suggest that consuming lower trophic level seafood can minimize the risk of mercury exposure without reducing the benefits of nutrient intake, and more broadly, demonstrate the value of examining nutrient and mercury exposure patterns simultaneously. Such research efforts are valuable in summarizing the largest signals among otherwise complex patterns of multiple nutrients and contaminants, but there is a need for a deeper understanding of these multivariate patterns at higher levels of taxonomic resolution. In some cases, the seafood categories used in this study include multiple

![Figure 3](https://example.com/image)(Karimi et al., 2014, reprinted with permission). Circles indicate 95% confidence limits for means of each seafood group and indicate the degree of difference among groups. Mercury and nutrient vectors (inset) represent the underlying structure of the axes. The position of circles relative to the direction of vectors indicates correlations between seafood groups and the concentration gradient of mercury or nutrients. Vector length indicates the overall contribution of mercury or nutrients in discriminating among seafood groups. Vector direction indicates the correlation of mercury or nutrient with each axis (vectors parallel to an axis are highly correlated with that axis). Angles between vectors represent correlations among mercury and nutrient concentrations. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; Hg, mercury; Se, selenium.
species that share market names in order to compare mercury–nutrient signatures between edible seafood and seafood consumers. For example, salmon includes Atlantic salmon and multiple species of Pacific salmon, and tuna steak includes bigeye and yellowfin tuna (Karimi et al., 2014). Future studies that examine the composition of individual fish of the same species would complement these broader analyses by examining nutrient-contaminant patterns at greater taxonomic resolution, and in relation to ecological and environmental factors. In addition, better information on the taxonomic identity of market fish and shellfish would improve estimates of co-exposure to nutrients and contaminants in seafood consumers.

Advice describing both the types and amounts of seafood consumption, while complex, is necessary to better manage risks and benefits of seafood consumption (Oken et al., 2008). Seafood risk communication also requires risks and benefits to be considered together for appropriate context (Kuntz et al., 2010; Turyk et al., 2013; Laird et al., 2013). Many fish advisories consider multiple chemical contaminants but provide minimal discussion of fish nutrients, focused on omega-3 fatty acids (Scherer et al., 2008). Compared with mercury concentrations, there are fewer studies quantifying fatty acids and selenium in seafood (Karimi et al., 2014). Therefore, to inform risk assessment more research is needed quantifying the risks and benefits associated with specific seafood consumption habits, such as considering the recommended daily consumption of seafood nutrients relative to reference doses (i.e. hazard quotients) of seafood contaminants (i.e. Gladyshev et al., 2009).

To conduct appropriate human health risk assessment for contaminants such as mercury requires an understanding of how mercury, fish oils and selenium co-exposures affect the human body. This work can be informed by studies from marine biology and fisheries science, coupled with epidemiological biomonitoring, anthropological and food science investigations into the role of culinary preparation and gut processing on mercury and nutrient bioavailability (Laird et al., 2009; Moses et al., 2009a, b; Costa et al., 2013). Acknowledging the concerns about contaminant exposure from seafood and its health benefits, the Joint FAO/WHO Expert Consultation on the Risks and Benefits of Fish Consumption (2010) recommended that government entities ‘Develop, maintain and improve existing databases on specific nutrients and contaminants, particularly methylmercury and dioxins, in fish consumed in their region’ and ‘Develop and evaluate risk management and communication strategies that both minimize risks and maximize benefits from eating fish’ (FAO/WHO, 2010, p. 33). Nevertheless, their general conclusions acknowledge fish as an important food source with clear benefits for reducing heart disease mortality and supporting optimal neurodevelopment in children.

CONCLUSIONS

Our current ability to properly estimate the risks and benefits to humans of seafood consumption are hampered by the common approaches of separately studying either contaminants or nutrients in fish. To date there are few studies in which fish tissue concentrations have been measured for both contaminants and nutrients across a range of species and geographic regions, even for the restricted set of chemicals considered in this review. There is tremendous variability between and within fish species in their mercury, EPA and DHA concentrations, leading to different versions of the ‘fish intake’ exposure across participants in epidemiological studies (Greenland & Robins, 2009), complicating the interpretation of studies on seafood health implications. Better characterizing the extent of interspecies and intraspecies variation of chemicals in fish may help inform future human exposure studies by allowing for more explicit accounting of measurement error (Spiegelman et al., 1997; Murad & Freedman, 2007; Guo et al., 2012; Pollack et al., 2013). Furthermore, statistical methods are improving for epidemiological studies to incorporate source (i.e. seafood) contaminant levels, intake frequencies, toxicokinetic processes and biomarkers for an integrated exposure assessment (Conti et al., 2003; Bartell & Johnson, 2011; Ian et al., 2012; Shin et al., 2014); or to consider complex interactions between multiple seafood contaminants (Lynch et al., 2011) Thus, additional research on the joint distribution of multiple chemicals in marine foods has potential to contribute directly to future epidemiological investigations. Bringing multiple stakeholders (i.e. fish consumers and marine scientists) together in a trans-disciplinary conversation with health scientists can also help target the science to relevant questions and improve on knowledge translation (Boote et al., 2002; Burger et al., 2013). Future assessments of the risks and benefits of fish consumption will require more detailed understanding of exposures to both fish contaminants and nutrients as well as the environmental and ecological drivers that control their chemical transformations, and flow through marine food webs. The processes affecting composition of marine fish may be altered by climate change impacts including but not limited to ocean warming and ocean acidification (Edwards & Richardson, 2004; Halpern et al., 2008; Kroeker et al., 2012); fishing (Micheli et al., 2014); emerging joint exposures such as pharmaceuticals and personal care products potentially changing xenobiotic kinetics for some other compounds (Smital et al., 2004; Epel et al., 2008; Bosnjak et al., 2009); and future changes in contaminant sources and inputs (UNEP, 2013). Together, these changes indicate a need for continued research on fish nutrients and contaminants in marine and medical science, as well as ongoing communication between these disciplines.

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Marine harmful algal blooms, human health and wellbeing: challenges and opportunities in the 21st century

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Microalgal blooms are a natural part of the seasonal cycle of photosynthetic organisms in marine ecosystems. They are key components of the structure and dynamics of the oceans and thus sustain the benefits that humans obtain from these aquatic environments. However, some microalgal blooms can cause harm to humans and other organisms. These harmful algal blooms (HABs) have direct impacts on human health and negative influences on human wellbeing, mainly through their consequences to coastal ecosystem services (fisheries, tourism and recreation) and other marine organisms and environments. HABs are natural phenomena, but these events can be favoured by anthropogenic pressures in coastal areas. Global warming and associated changes in the oceans could affect HAB occurrences and toxicity as well, although forecasting the possible trends is still speculative and requires intensive multidisciplinary research. At the beginning of the 21st century, with expanding human populations, particularly in coastal and developing countries, mitigating HABs impacts on human health and wellbeing is becoming a more pressing public health need. The available tools to address this global challenge include maintaining intensive, multidisciplinary and collaborative scientific research, and strengthening the coordination with stakeholders, policymakers and the general public. Here we provide an overview of different aspects of the HABs phenomena, an important element of the intrinsic links between oceans and human health and wellbeing.

Keywords: Harmful algal blooms, human health and wellbeing, marine biotoxins, ecosystem services

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OVERVIEW OF THE CHALLENGES

Aquatic ecosystems are supported by photosynthetic organisms (e.g. macrophytes, benthic and planktonic microalgae and cyanobacteria) that fix carbon, produce oxygen, and constitute the base of food webs. Under certain circumstances, however, the abundance of some taxa can reach levels that may cause harm to humans and other organisms. These proliferations often are referred to as ‘harmful algal blooms’ (HABs), a term that includes a variety of species and consequences that humans perceive as adverse. HABs occur in all aquatic environments (e.g. freshwater, brackish and marine) and at all latitudes. In this paper, we focus specifically on the threat that blooms of harmful microalgae pose to the benefits (food supplies, economic activities, tourism and recreation) that the oceans and seas provide to human health and wellbeing (Figure 1).

Of the many thousands of microalgal species described, about 300 are involved in harmful events (see e.g. http://www.marinespecies.org/hab/index.php). More than 100 of these species, with no apparent physiological, phylogenetic or structural commonalities, produce potent and persistent natural toxins that can be harmful or even lethal to humans and animals (Sournia, 1995; Moestrup et al., 2009). The chemically diverse compounds synthesized by toxic HABs species have been associated with different syndromes in humans (Box 1), and many may also adversely affect certain fish, seabirds, reptiles and marine mammals (Box 2).
In humans, toxicity is caused by the ingestion of contaminated seafood products (fish or shellfish), skin contact with toxin-contaminated water, or the inhalation of aerosolized toxins or noxious compounds. In the case of food-borne poisonings (Figure 2), HAB toxins are bio-concentrated, often without apparently harming the vector marine organism that ingested the toxin, and transferred up through the food web to humans. Toxic effects usually occur when the HAB species producing the toxin is present in high abundance, although seafood poisoning also can be caused by highly toxic microalgae at low abundances. In addition to the direct impacts on human health, these toxic outbreaks have associated consequences on other components of human well-being both in terms of their socio-economic impact and costs. Namely, HAB occurrences can lead to the closure of important shellfisheries (e.g. Jin et al., 2008) and increases in the costs of monitoring and management (Hoagland et al., 2002).

Another hazardous effect of some HABs taxa is the production of excess algal biomass, which can affect individual organisms and ecosystems in different ways (Box 2). When large blooms decay, the subsequent degradation by bacteria of accumulated biomass reduces oxygen concentrations in marine waters and can cause hypoxia, especially in bottom waters isolated by density gradients from surface waters. In addition to the benthic faunal mortalities related to oxygen depletion, the unpleasant appearance of surface scums and bad odours associated with some HABs can dissuade tourists from visiting coastal recreation areas (Scatasta et al., 2003). Phycotoxins also can cause morbidity and mortalities of wild and farmed fish (gill damage), birds (hypothermia), marine mammals, or certain invertebrates, resulting in economic losses in finfish aquaculture and tourism, and losses of the non-market, ‘passive’ values that humans may have for protected species or extraordinary ecosystems. These examples illustrate how HABs may also decrease the non-market, passive use values of marine ecosystems and their services, thereby limiting the way in which the marine environment can enhance the quality of life and wellbeing for humans (Hoagland & Scatasta, 2006).

Bloom of microalgae, including those deemed by humans to be harmful (see for instance, http://haedat.iode.org), are a natural phenomenon (e.g. Smayda, 1997; Margalef, 1998; Gowen et al., 2012 and references cited therein) and awareness of HAB events is embedded in the cultural heritage of many coastal human communities. Historically, this awareness has helped to mitigate some of the adverse effects of HABs. However, at present, increasing the information about public health risks and the strategies that scientists and policy makers, working together, can address to decrease the impacts of HABs on human health and wellbeing could still be beneficial. It is important to recognize that there is not a realistic way to prevent HABs occurrence as it results from complex interactions among physical, chemical, and biological processes operating at different spatio-temporal scales in the marine environment. Over the last 50 years, human modifications of the marine environment, particularly those occurring at the land-sea interface, may have influenced the incidence of HABs in certain locations. These modifications include the alteration of water circulation in harbours and artificial beach construction, the dispersal of species through ship ballast waters, and nutrient enrichment (Hallegraeff & Bolch, 1992; Anderson et al., 2002; Davidson et al., 2014).

Superimposed on these stresses, climate change is already leading to temperature increases in some areas of the earth’s oceans, and warmer waters could affect the occurrence of HABs (Moore et al., 2008; Backer & Moore, 2011; Hallegraeff, 2010 and references cited therein; Gowen et al., 2012). Specifically, changes in the frequency, intensity, and geographic extent of HABs may occur, but the possible responses are likely to be highly species-specific, given the diverse toxicity, physiology, biology, and ecology of HAB organisms. Thus, the forecasting of such changes is still quite speculative, requiring long time series of ecological processes, as well as more focused research (including modelling).

This review was stimulated by discussions at the ‘Oceans and Human Health at the beginning of the 21st century’ workshop held in Bedruthan (Cornwall, UK) in March 2014. This paper is not an exhaustive review of all the different factors concerning the occurrence of HAB events (see e.g. GEOHAB, 2001, 2005, 2006, 2008, 2010, 2012; Zingone & Wyatt, 2005; Gowen et al., 2012). Instead, we summarize the main direct impacts of HABs on human health. We describe briefly the influences of HABs on human wellbeing, mainly

Fig. 1. Conceptual links between the main drivers (natural dynamics, climate change and global warming and other anthropogenic forcings) involved in the occurrence of HABs, the main impacts of HABs on human health and wellbeing, and some of the tools to decrease these effects.
through the negative consequences to ecosystem services and other marine organisms and environments. Other aspects of the possible interconnections between human wellbeing and HABs have yet to be investigated. Based on this evidence, we highlight the main challenges posed by marine HABs, and we discuss the tools available to respond to HABs in the coming years, especially within the context of climate warming.

**DIRECT IMPACTS OF HABS ON HUMAN HEALTH**

As noted above, the direct impacts of HABs in marine waters on human health are linked to poisoning (Box 1) associated with eating contaminated seafood (Figure 2), skin contact with contaminated water, and/or inhaling aerosolized biotoxins. In addition to human health conditions associated with known toxins produced by microalgae, there exist emerging phycotoxins and risks of poisoning through biotoxin contaminated desalinated drinking water. In the remaining text, the terms ‘biotoxin’, ‘phycotoxin’ or ‘toxin’ will be used to refer to toxic compounds synthesized by the marine microalgae.

**Box 1. Main HAB toxic syndromes (in alphabetical order), Biotoxins (T), Causative Organisms (O), Symptoms (S), Route of exposure (E), Main Geographic Affected Areas (A), some References (R).**

*Amnesic Shellfish Poisoning (ASP):*

T: Domoic acid and isomers
O: *Pseudo-nitzschia* spp. and *Nitzschia*
S: Nausea, vomiting, diarrhoea, abdominal cramps, respiratory distress
E: Consumption of shellfish (possibly, fish)
A: Worldwide, affecting seafood and fisheries activities
R: Bates et al. (1989); Martin et al. (1993); Scholin et al. (2000); Fehling et al. (2004)

*Azaspiracid Shellfish Poisoning (AZP):*

T: Azaspiracid and its derivatives
O: *Amphidomataceae* (*Amphidoma languardia, Azadinium spinosum, Azadinium poporum, Azadinium dexteroporum*)
S: Nausea, vomiting, severe diarrhoea, abdominal cramps; effects on mice tests include severe damage to the intestine, spleen and liver tissues in animal tests
E: Consumption of shellfish
A: Seafood poisoning reported from shellfish in Europe and North America
R: Twiner et al. (2008, 2012a, b, 2014); Klontz et al. (2009); Tillmann et al. (2009, 2014); Hess et al. (2014)

*Ciguatera Fish Poisoning (CFP):*

T: Ciguatoxin
O: *Gambierdiscus* spp., *Fukuyo* spp.
S: Nausea, vomiting, diarrhoea, numbness of mouth and extremities. Neurological symptoms may persist for several months
E: Consumption of coral reef fish
A: Endemic in the tropics and subtropics, expanding to temperate latitudes
R: Friedman et al. (2008); Litaker et al. (2010); Chinain et al. (2010a, b); Tester et al. (2014)

*Diarrhetic Shellfish Poisoning (DSP):*

T: Okadaic acid and its derivatives (dinophysistoxins)
O: *Dinophysis* spp., *Proorocentrum lima*
S: Nausea, vomiting, severe diarrhoea, abdominal cramps, respiratory distress
E: Consumption of shellfish
A: Worldwide, affecting seafood and fisheries activities
R: Yasumoto et al. (1980); Kat (1983); Reguera & Pizarro (2008); Raine et al. (2010); Reguera et al. (2014)

*Neurotoxic Shellfish Poisoning (NSP) and respiratory irritation:

T: Brevetoxins
O: *Karenia brevis* (predominantly)
S: By seafood poisoning: nausea, temperature sensation reversals, muscle weakness, and vertigo. Exposure to aerosols related to respiratory and eye irritation particularly for asthmatics
E: Consumption of shellfish (and fish at least for marine mammals); inhalation of marine aerosols during active blooms
A: Particularly in the Gulf of Mexico and Japan, China, Korea, New Zealand
R: Watkins et al. (2008); Fleming et al. (2011)

*Palytoxicosis (foodborne poisoning) and other irritative symptoms:*

T: Palytoxin, Ostreocin, Ovatotoxin
O: *Ostreopsis* spp.
S: Associated to food-borne poisoning: nausea, vomiting, severe diarrhoea, abdominal cramps, lethargy, tingling of the lips, mouth, face and neck, lowered heart rate, skeletal muscle breakdown, muscle spasms and pain, lack of sensation, myalgia and weakness, hypersalivation, difficulty in breathing. Exposure to aerosols: eye and nose irritation, whinorrhoea, general malaise, fever. Cutaneous irritations in beach swimmers
E: Consumption of seafood; inhalation of marine aerosols; direct contact with water A: Food-borne poisoning in the tropics and subtropics; respiratory and cutaneous irritations in Mediterranean beaches
R: Deeds & Schwartz (2010) (references therein); Tubaro et al. (2011); Ciminiello et al. (2010, 2013); Vila et al. (2012)

*Paralytic Shellfish Poisoning (PSP):*

T: Saxitoxin and derivatives
O: *Alexandrium* spp., *Pyrodinium bahamense var. compressum* and other species, *Gymnodinium catenatum*, some calcareous red macroalgae
S: Nausea, vomiting, diarrhoea, numbness and tingling of
the lips, mouth, face and neck. Severe cases can result in paralysis of the muscles of the chest and abdomen leading to death.

E: Consumption of shellfish, crustaceans, fish
A: Worldwide, affecting shellfish activities
R: Ayres (1975); Gaines & Taylor (1985); Anderson et al. (1989, 2005a, b)

Food-borne diseases: overview

Poisoning through the ingestion of biotoxin-contaminated seafood is the best documented impact that HABs have on humans (Figure 2, Box 1). The poisoning process involves the bio-concentration of the biotoxins by filter feeding fauna (mostly bivalve molluscs, e.g. *Mytilus* spp.) which themselves are generally unaffected by these compounds. Other vectors include certain marine gastropods (e.g. whelks and moon snails), some crustaceans (e.g. crabs), echinoderms and fish (e.g. some planktivorous fishes or belonging to the tetraodontidae family) that acquire biotoxins through the food web (Deeds et al., 2008). Toxins accumulated in seafood tissues can remain for considerable lengths of time after the bloom has declined in the seawater. Further, these biotoxins are not destroyed by cooking or by the processing of seafood products, and because they do not have distinctive odours or taste, they can be detected only through specialized laboratory testing (Zaia et al., 2010).

Most algal toxins are primarily neurotoxins (e.g. brevetoxins affecting the Na$^+$ channels), although they are also known to affect human health through other routes (e.g. okadaic acid affecting phosphatase activity). The various toxic compounds can produce a wide range of symptoms and thus have been associated with several clinically described syndromes depending on the main symptomatic mode (Box 1): amnesic (ASP), azaspiracid (AZP), diarrhetic (DSP), neurotoxic (NSP) and paralytic (PSP) shellfish poisonings and ciguatera fish poisoning (CFP). The syndromes can present with symptoms from the nervous, digestive, respiratory, hepatic, dermatological or cardiac systems (e.g. Baden & Trainer, 1993). The effects can be acute (e.g. paralytic shellfish poisoning can occur within minutes to hours; Medcof, 1985) and can last for weeks to months (e.g. ciguatera fish poisoning; Friedman et al., 2008). Although there is considerable knowledge on the acute health effects caused by HAB biotoxins, many of the toxicological mechanisms are incompletely understood. In addition, we know little about the chronic effects of these biotoxins, either from acute exposure that produces long-lasting damage, or from chronic low-level exposures over long periods of time.

For the food-borne syndromes, the prevention of contaminated seafood reaching the markets (by monitoring the causative species and/or the presence of biotoxin in seafood in real time) is currently the only effective way to protect human health. In fact, well-structured monitoring programmes targeting the causative organisms and toxins in commercial seafood associated with the clinical syndromes (i.e. ASP, AZP, DSP, NSP and PSP) have proven effective in reducing the human exposure to biotoxins in many areas of the world. Information about operative HAB monitoring programmes is not compiled at a worldwide scale, only at regional as for the North Atlantic by the ICES (International Council for the Exploration of the Sea) – IOC UNESCO Working Group on Harmful Algal Blooms Dynamics (http://www.ices.dk/community/groups/Pages/WGHABD.aspx). However, monitoring could be enhanced by complementing it with additional actions such as informing the public in non-commercial areas affected by HABs (e.g. Reich et al., 2015) and increasing the understanding of the complex processes involved in harmful events (e.g. Whyte et al., 2014).

Unfortunately, due to increased human pressure on coastal marine ecosystems together with global warming, harmful blooms may occur in areas where they have not previously been reported (Trainer et al., 2013). Also, new biotoxins are continually being identified. For these cases, toxin detection and identification of the causative organism represent new challenges for monitoring and management procedures (Turner et al., 2015). It is also noteworthy that for ciguatera fish poisoning, the most frequent cause of HAB-associated poisoning in tropical waters, effective procedures to protect human populations are lacking (see Sections ‘Most common biotoxin syndromes in temperate latitudes’ and ‘Better quantification and prevention of the impacts on human health: HAB-related disease surveillance’). Increased international tourist travel and trade in

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**Fig. 2.** Biotxin transfer pathways through the marine food web to humans. A biotoxin-producing organism, such as the dinoflagellates *Dinophysis acuta* or *Alexandrium catenella*, is bioaccumulated by shellfish, which are apparently not affected by saxitoxin or lipophilic biotoxins. Consumption of the contaminated shellfish is a traditional way of diarrhetic or paralytic poisoning (DSP, PSP). Alternatively, some toxicogenic species attach to surfaces (macrophytes, corals) by an endogenous mucus (e.g. *Gambierdiscus, Ostreopsis, Proorocentrum lima*). Fragments of corals or macrophytes covered by the microalgae enter the food web through ingestion by herbivorous fish. This is the transmission mechanism of ciguatera fish poisoning (CFP). Certain fishes can also experience some sort of poisoning.
seafood can lead to poisonings in areas far from where the fish is caught (Mattei et al., 2014).

Most common biotoxin syndromes in temperate latitudes

In the temperate latitudes of Europe, South Africa, Asia, Australia, North America and South America, the most common HABs cause amnesic (ASP), azaspiracid (AZP), diarrhetic (DSP), neurotoxic (NSP) and paralytic (PSP) shellfish poisonings (Box 1).

Domoic acid, a neurotoxin produced by various species of *Pseudo-nitzschia* and *Nitzschia*, was identified as responsible for causing an outbreak of amnesic shellfish poisoning in humans (involving 107 illnesses and three deaths) after the consumption of blue mussels from Prince Edward Island (Canada) in 1987 (Bates et al., 1989; Todd, 1993). Since then, blooms of these penaean diatoms have resulted in a range of, often large-scale, shellfish toxicity events, affecting humans and other large vertebrates (see Section ‘Impacts of HABs on non-market, passive use values of marine ecosystems’ and Box 2). Symptoms of ASP poisoning in humans include short- and long-term memory loss.

The azaspiracids, first identified in mussels from Ireland in 1995 (Satake et al., 1998), belong to a novel group of polyether biotoxins produced by the small armoured dinoflagellate *Azadinium spinosum* (Tillmann et al., 2009; see also Section ‘Improving monitoring and research needs to forecast and predict HAB events’). This biotoxin causes symptoms similar to those displayed by DSP (Twiner et al., 2008), although slowly progressing paralyses have also been observed in mouse assays. Azaspiracids have now been found in a number of other European Union countries with ~20 different analogues of AZA identified.

Diarrhetic shellfish poisoning (DSP) was first linked to the presence of *Dinophysius fortii* in Japan (Yasumoto et al., 1980) and to *D. acuminata* in Dutch coastal waters (Kat, 1983), and it was recorded after consumption of mussels containing DSP biotoxins from the Northern Adriatic coast in 1989 (Boni et al., 1992). Diarrhetic shellfish poisoning is caused by okadaic acid or its derivative dinophysistoxins, produced by 10 species of the genus *Dinophysius*, two species of the genus *Phalacroma* (Reguera et al., 2012), and *Prorocentrum lima* (Koike et al., 1998). In humans, DSP biotoxins bind to phosphatase receptors, causing severe, but not usually fatal, gastrointestinal symptoms (with a rapid onset). DSP outbreaks are common in Europe, affecting shellfish consumers in at least 10 countries. On occasion, outbreaks have resulted in large numbers of people becoming ill, and shellfish harvest areas have been closed for up to 10 months (e.g. Fraga & Sánchez, 1985; Haamer et al., 1990; Lassus et al., 1985; Ramstad et al., 2001; Blanco et al., 2005, 2013; Vale et al., 2008; review by Reguera et al., 2014).

For regulatory purposes, the more recently discovered pectenotoxins and yessotoxins are classified within the DSP group. Pectenotoxins are produced by some of the *Dinophysis* species including *D. acuta* and *D. acuminata*. Yessotoxins induce similar symptoms but are produced by the dinoflagellates *Lingulodinium polyedrum* and *Protoceratium reticulatum* (Paz et al., 2004 and references cited there in). Recently, experts have recommended the deregulation of pectenotoxins and yessotoxins due to research results finding a non-toxic effect of the oral administration of these substances in mice (ICES, 2006).

Brevetoxin is the collective name given to a class of biotoxins that causes neurotoxic shellfish poisoning (NSP). Brevetoxins are produced primarily by the naked dinoflagellate *Karenia brevis*. In the Gulf of Mexico, and in isolated instances along the South-east Atlantic coast of the USA, blooms of *K. brevis* have caused water discoloration, large-scale fish mortality events, human poisonings due to the consumption of shellfish, and respiratory problems in asthmatics caused by inhalation of biotoxin in the form of an aerosol (Morris et al., 1991; Magaña et al., 2003; Kirkpatrick et al., 2004; Watkins et al., 2008; Fleming et al., 2011). Notably, NSP has not been linked to fatalities in humans (van Dolah, 2000). The occurrence of toxic *K. brevis* red tides was recorded as early as 1648 in the Western Gulf of Mexico and since the 1840s in Florida (Magaña et al., 2003; Kirkpatrick et al., 2004).

Paralytic shellfish poisoning (PSP) is caused by saxitoxin and its derivatives, potent neurotoxins that can cause headache, nausea, facial numbness, and, in severe cases, respiratory failure and death. The first likely cases in the UK were in 1872 in Leith and in 1888 in Liverpool (Ayres, 1975). In British Columbia (Gaines & Taylor, 1985) and Norway (Yndestad & Underdal, 1985), the first recorded outbreaks of PSP were in 1793 and 1901 respectively. Medcof (1985) reported toxic shellfish episodes from the 1930s and 1940s. In Europe and North America, PSP is mainly associated with blooms of the thecate (armoured) dinoflagellate genus *Alexandrium*, mainly *A. tamarense/funeyense/catenella* group and *A. minutum* (Medlin et al., 1998; Higman et al., 2001; Lilly et al., 2007; Touzet et al., 2007), although some *Alexandrium* species and strains are non-toxic. In Asia (the Philippines, Malaysia, Brunei and Indonesia, Papua New Guinea), PSP is mainly produced by outbreaks of the armoured dinoflagellate *Pyrodinium bahamense var. compressum*. In the Philippines, this species was responsible for 1995 cases with 117 deaths linked to PSP toxicity between 1983 and 1999 (Azanza, 1999; Azanza & Taylor, 2001).

Ciguatera fish poisoning (CFP)

Worldwide, ciguatera fish poisoning (CFP) is the most common food poisoning associated with a natural, non-bacterial chemical, and it occurs throughout the tropics, particularly in vulnerable island communities such as the Pacific Island Countries and Territories (PICT’s). Ciguatoxins, or their precursors, are produced by several species of the benthic dinoflagellate genus *Gambierdiscus* (mainly *G. toxicus*). The biotoxins are modified through metabolic pathways in the food web of coral reefs, and they tend to accumulate in fish, particularly larger carnivorous species such as barracuda (*Sphyraena* spp., Heymann, 2004), but they also have been found in more than 400 fish species. After consuming ciguatoxin-contaminated fish, a range of acute neurologic, gastrointestinal and cardiac symptoms have been reported, with some individuals experiencing chronic neurological symptoms lasting weeks to months (e.g. Freudenthal, 1990; Friedman et al., 2008; Skinner et al., 2011, and references cited therein). Repeated exposure to ciguatoxins can reportedly exacerbate the acute ciguatera symptoms (Bagnis et al., 1979; Pottier et al., 2001).

CFP is a threat to public health throughout tropical areas, and it is the most widespread, and hence best described, in the island...
nations of the Pacific and the Caribbean (for reviews, see Bruslé, 1997; Lehane & Lewis, 2000; Friedman et al., 2008; and references therein). Unfortunately, the real extent of illness is not well documented due to under-reporting and misdiagnosis (McKee et al., 2001; Radke et al., 2015). Thus, its impacts on human communities and ecosystem health are still poorly understood. Data from the Health and Fisheries Authorities of 17 PICTs (Skinner et al., 2011) estimated a mean annual incidence of 104 cases per 100,000 people across the region during 1973–83. Based on these estimates, the recorded CFP incidence in the South Pacific should be increased by 60%, i.e. up to 194 cases per 100,000 people between 1998–2008. CFP illness rates exhibit high variability, reaching particularly elevated levels in some areas. For instance, up to 497 cases per 10,000 population per year were recorded in French Polynesia including Raivavae Island (Austral archipelago) between 2000–08 (Chateau-Degat et al., 2009; Chinain et al., 2010b), and up to 440 cases per 10,000 population per year in the Caribbean between 1996–2006 (Tester et al., 2010). Despite uncertainty about the real incidence, data suggest that CFP constitutes an acute and chronic illness with major public health significance at both local and more widespread levels. In future, the health problem could be exacerbated due to anthropogenic pressures, such as the increased development of coastal zones, ocean warming (e.g. Villareal et al., 2007; Kibler et al., 2015), or natural disasters, such as hurricanes. These pressures can lead to the damage of coral reefs, favouring more resilient macroalgae that constitute new surfaces for the proliferation of the involved toxigenic benthic dinoflagellates (e.g. Chateau-Degat et al., 2005; Tester et al., 2010).

It is inherently difficult to cope with CFP as a public health problem. For example, the risks of CFP often have been traditionally 'managed' by native fishermen using their local, traditional knowledge, who may warn each other about areas where ciguatoxin-contaminated fish are known to occur. With increasing trade and coastal tourism, CFP cases could occur in non-tropical areas, where ciguatera often goes unrecognized or diagnosed only after expensive investigation (Epelboin et al., 2014; Mattei et al., 2014). There is neither a reliable, cost-effective method for detecting the biotoxin nor is there a biomarker to diagnose the illness in humans. A review by Friedman et al. (2008) describes the clinical course of CFP and its possible treatments, many of which may be unclear to other healthcare providers and public health practitioners, even in endemic areas.

**POISONING BY PALYTOXIN AND ANALOGUES**

*Ostreopsis* is a dinoflagellate genus co-occurring with *Gambierdiscus*. In tropical Indo-Pacific latitudes, *Ostreopsis* has been associated with poisoning after the ingestion of clupeid fishes, crabs or sea urchins contaminated with palytoxin (PLTX) (Noguchi et al., 1987; Taniyama et al. 2003; see review by Deeds & Schwartz, 2010, and references cited therein). This compound and its analogues (Ciminiello et al., 2011, 2015 and references therein; Brissard et al., 2015) are among the most potent biotoxins of marine origin. Nevertheless, in spite of the well-documented but very limited number of severe PLTX-related poisoning cases in these tropical areas, the true risk of PLTX poisoning through seafood consumption in humans is difficult to assess. This is due to the co-occurrence of PLTX with other seafood biotoxins (e.g. ciguatoxins, saxitoxins and tetrodotoxin), the distribution of PLTX mainly in the tropical areas of developing nations with little surveillance and reporting, and its prevalence in reef species that are not commercially harvested.

*Ostreopsis* seems to be expanding to temperate latitudes (Rhodes, 2011), and recurrent blooms have occurred in Mediterranean waters during the last two decades (e.g. Mangialajo et al., 2011; Illoul et al., 2012). In this region, *Ostreopsis* proliferations have been related to aerosol exposures at bathing beaches (see Section ‘Ostreopsis cf. ovata and respiratory irritations’) and macrofaunal mortalities (Box 2). Fortunately, to date, food poisonings related to the PLTX-like group have not been reported in the Mediterranean, although the biotoxin has been detected in certain marine fauna (e.g. Algizaki et al., 2011; Biré et al., 2013; Brissard et al., 2014; Ciminiello et al., 2015). The European regulation to monitor PLTX has not yet become established, although the Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA, 2009) assessed the risks to human health associated with the presence of PLTX-group biotoxins in shellfish, recommending a maximum concentration of 30 µg eqPLTX kg⁻¹ fresh weight. Overall, the few available data suggest a growing potential risk of seafood contamination (from commercial or recreational fishing or aquaculture) in Mediterranean coastal waters (especially in those affected by recurrent *Ostreopsis* blooms).

The possible transfer and accumulation of the PLTX-like group of biotoxins through the food web to humans should be investigated further. Because *Ostreopsis* exhibits both benthic and planktonic phases (Bravo et al., 2012), its dispersion is facilitated, increasing the number of seafood types that could become contaminated and complicating its monitoring. The planktonic stage can contaminate filter-feeding bivalves (e.g. mussels), while the benthic stage can enter the food chain through herbivores (e.g. sea urchins, crabs, gastropods, salps) that feed on macroalgae (Brissard et al., 2014). PLTX-like biotoxins can then be transferred to omnivores and carnivores, including humans.

**EMERGING BIOTOXINS**

*Cyclus imines*

Cyclic imines (gymnodimine, spirilides, pinnatoxins and others) have been produced reportedly by a number of dinoflagellates (*Karenia selliformis*, *Vulcanodinium rugosum*, *Alexandrium ostenfeldii* and *A. peruvianum*). They are classified typically as fast-acting biotoxins due to the rapid mortality occurring in the mouse bioassay for lipophilic biotoxins (Molgò et al., 2014, and references cited therein). Both gymnodimine and pinnatoxins had been associated initially with acute human food poisoning events (Seki et al., 1995; Uemura et al., 1995). Further studies indicated that other biotoxins (i.e. brevetoxins in the case of the New Zealand shellfish poisoning assumed to be caused by gymnodimine) or bacterial (Vibrio) contamination (in the case of the Chinese poisoning assumed to be caused by pinnatoxins) were in fact responsible. Pinnatoxin-G has been reported worldwide (e.g. Rhodes et al., 2010, 2011; Rundberget et al., 2011; McCarron et al., 2012), with the highest levels found in a Mediterranean lagoon in the south of France (Hess et al., 2013). It should be pointed out, however, that no food-poisoning-related events have yet been documented. The chemical stability of this biotoxin to both acids and bases (Jackson et al., 2012) and the high binding affinity to the nicotinic acetylcholine (nACh) receptor raises the spectre of potentially harmful effects to human health due to the chronic exposure of even low levels of pinnatoxins (Molgò et al., 2014).
BMAA
β-N-methylamino-L-alanine (BMAA), a non-protein amino acid initially reported to be produced by certain cyanobacteria proliferating in freshwater habitats (Cox et al., 2005), has been recently confirmed in marine diatoms (Jiang et al., 2014b) and in seafood from marine and coastal waters (Brand et al., 2010; Réveillon et al., 2014, 2015). Exposure to BMAA has been linked to an increased risk of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Parkinson’s and Alzheimer’s diseases (Bradley et al., 2013). Marine cyanotoxins in general are a potential cause of unexplained acute food poisoning (Golubic et al., 2010; Roué et al., 2013, 2014). Additional research is needed to confirm both these findings and the possible epidemiological associations between BMAA and neurodegenerative diseases.

Macroalgae
Food poisoning from macroalgae has been reported from Asian countries where such foodstuffs are traditionally consumed. Particularly important are the polycavernosides found in Gracilaria edulis (Louzao et al., 2014, and references cited therein). As postulated by Daigo (1959), domoic acid has now been confirmed as a metabolite in Chondria armata following the laboratory culture of these macroalgae (Jiang et al., 2014b). The occurrence of domoic acid and other glutamate receptor agonists in macroalgae may be of importance in studies on the effects of chronic exposures to subacute biotoxin concentration.

Effects of Chronic and Subacute Exposure
Marine biotoxins have traditionally been known for their acute effects. Consequently, regulation at a global scale (FAO, Codex alimentarius) has focused on preventing acute poisoning events (Lawrence et al., 2011). Only a few studies have investigated either chronic effects from an acute exposure episode or chronic exposure to sub-acute levels over time in humans. Some studies, using in vivo models, have shown that there may be some adverse health effects from low-level exposures (either single or repeated) to, for example, domoic acid. Levin et al. (2005) demonstrated that prenatal rats exposed to domoic acid exhibited postnatal effects, e.g. scopolamine susceptibility. In this study, rats showed persistent hypo-activity, and female offspring in particular showed poor performance in a maze, following a single low-dose early postnatal exposure to domoic acid. Baron et al. (2011) reported locomotor disorders in rats exposed to a low level of domoic acid. Finally, low-level repeated exposure over a 36-week period in zebra fish also led to gene alteration and impairment of mitochondrial function at cellular level (Lefebvre et al., 2012; Hiolski et al., 2014).

Sub-acute effects are also a potential problem for repeated or chronic ingestion of low doses of saxitoxins via drinking water produced from desalination of seawater. Until now, saxitoxins have been regulated only on the basis of their acute effects: a maximum permissible level of 3 μg L⁻¹ has been established for drinking water in Australia, Brazil and New Zealand. Recent studies suggest, however, that the effects of chronic ingestion of saxitoxins may include the alteration of antioxidant defences and the induction of oxidative stress in the brains and livers of mammals (Ramos et al., 2014; Silva et al., 2014). As mentioned above, the cyclic imine biotoxins also have the potential to interact with receptors involved in neurodegenerative diseases such as Parkinson’s and Alzheimer’s diseases (Molgó et al., 2014). Interestingly, in both in vitro and in vivo studies, gymnodimine and 13-desmethyl spirolide C showed some protective effects against Alzheimer’s disease, and hence these compounds should be considered for further research into their possible therapeutic applications (Alonso et al., 2011a,b, 2013).

Co-exposure and Exposure to Mixtures
Co-exposure to different agents can lead to confounding diagnoses in patients. For example, bacterial contamination in shellfish may lead to gastrointestinal illness (diarrhoea and vomiting) similar in degree to that caused by okadaic acid. Normally, the onset of illness following the consumption of shellfish contaminated with biotoxins is earlier (a few hours) than for those contaminated with bacterial pathogens (12–24 h), because bacteria typically require a period of incubation in the patient before causing the full effect.

Exposure to mixtures of different groups of biotoxins is another issue that has not been well studied. Many studies have reported the co-occurrence of biotoxins in single algal species or shellfish that were contaminated by different algal species simultaneously, so the issue should be considered seriously (Hess, 2002, 2010; Amzil et al., 2008; Twiner et al., 2008; Reguera et al., 2012; Suikkanen et al., 2013). In particular, there is a concern that some of the compounds that do not normally cause acute shellfish poisoning in humans (e.g. yessotoxins), may be absorbed following damage to the intestinal tract caused by other biotoxins known to cause harm to humans, (e.g. okadaic acid and azaspiracids). As pure biotoxin supplies are very limited for most of the HAB biotoxins, only a few studies have investigated this potential hazard. Aasen et al. (2011) investigated the combination of azaspiracids with yessotoxins and did not observe any potentiation when orally co-administered at sub-acute levels to mice. Similarly, even a combination of azaspiracids with okadaic acid, two biotoxins known for their potential to cause harm to the human digestive tract, did not cause synergistic effects when orally administered to mice at sub-acute levels (Aune et al., 2012). The combination of okadaic acid and yessotoxins did not cause any increased toxicity in the oral mouse model (Sosa et al., 2013). It should be noted that the digestive tract of mice differs significantly from that of humans (notably in pH), however, and further studies (possibly involving primates) may be necessary to rule out the likelihood of synergistic damage. Finally, some potentiation of domoic acid by peptibols, a fungal metabolite, was shown in a fly larval model (Ruiz et al., 2016). These varied findings are very much dependent on the biological model used, and they should be considered as very preliminary in their nature. Longitudinal studies of humans exposed naturally to mixtures of biotoxins at individually low levels over long periods of time are needed.

Water-borne diseases
In fresh waters, cyanobacteria blooms constitute the main hazard to the health of humans (and other animals), mainly through the contamination of drinking waters by cyanotoxins, such as microcystins (see e.g. Nishiwaki-Matsushima et al., 1992; Falconer, 1998; Stewart et al., 2008). Direct cutaneous contact, exposure to aerosols, or swallowing water during occupational or recreational activities conducted in freshwater...
environments affected by cyanobacteria blooms, also can cause health problems. In contrast, there is no published evidence, at present, of risks for adverse health effects in humans from swallowing seawater containing toxic marine microalgae, but this possibility cannot be discarded.

Toxic HABs have recently emerged as a potential risk for the contamination of drinking water supplied by desalination systems. Worldwide, desalination is rapidly growing to provide water for domestic consumption and industrial uses. In 2012, there were more than 14,000 desalination plants in 150 countries (Anderson & McCarthy, 2012). About 50% of this capacity was located in the West Asia Gulf region, 17% in North America, 10% in Asia (apart from the Gulf), 8% in North Africa and 7% in Europe. In 2008, the installed capacity was 52.3 million m³ per day. Based on a growth rate of 12% per year, the global production of freshwater by desalination will have reached a capacity of 94 million m³ per day by 2015.

Chemical and physical properties, such as the molecular weight of the common HAB biotoxins (saxitoxins, brevetoxins and domioc acid, i.e. 300–900 Da), suggest that they should be efficiently removed by reverse osmosis in the desalination process. Support for this assumption was provided by Seubert et al. (2012) in their study combining laboratory tests and a 5-year monitoring of an operational plant in California. It has been noticed, however, that some taste and odour compounds (e.g. geosmin) with low molecular weights similar to the biotoxins mentioned above can pass through pretreatment and reverse osmosis membranes (Reiss et al., 2006). Further, the complete removal of biotoxins is not guaranteed in membranes with micro-fissures caused by, for instance, high pressures within the desalination plant system. Excessive pressures can be caused by the obstruction of intake filters due to high-biomass blooms, such as the ones caused by Cochlodinium polykrkoides that occurred in the Arabian Gulf and Gulf of Oman (Richtlen et al., 2010). Still, the data are limited (e.g. Caron et al., 2010; Dixon et al., 2011a, b; Laycock et al., 2012), based mainly on laboratory studies (without the appropriate up-scaling), and there are only a few studies (Seubert et al., 2012) from plants operating during blooms of biotoxin-producing HAB species. Therefore, more research is needed to ascertain the fate of biotoxins during the desalination process. The potential risk of chronic exposure to biotoxins requires their monitoring in drinking water produced in desalination plants.

HABs and aerosolized biotoxins

KARENIA BREVIS AND BREVEOTOXINS

Over three decades, multi-institutional and multidisciplinary studies in the Gulf of Mexico and along the coast of Florida have explored how the inhalation of aerosols containing brevetoxins during high-biomass, toxic K. brevis blooms can cause respiratory symptoms (e.g. Fleming et al., 2005, 2006, 2011). Beach visitors and full-time lifeguards have reported respiratory disorders (Backer et al., 2003, 2005), and studies have shown that people with asthma (and possibly other lung diseases) were particularly at risk from more severe and longer-lasting symptoms (Fleming et al., 2005, 2006, 2011; Milian et al., 2007; Bean et al., 2011). More recent research has modelled the health costs of these effects (Hoagland et al., 2009, 2014).

The results of this research have been used to engage with stakeholders to develop local response management plans to help minimize societal impacts of Karenia blooms on human health (e.g. Kirkpatrick et al., 2010; Fleming et al., 2011; Zhao et al., 2013). Information about the likelihood of health impacts has been used to inform personal decisions as well. For example, the Gulf of Mexico HAB Forecast (see NOAA Harmful Algal Bloom Operational Forecast System (HAB-OFS), https://tidesandcurrents.noaa.gov/hab/, accessed 26 August 2015) in conjunction with a local Beach Condition Reporting System is used by windsurfers and beach visitors to decide, depending on the wind, tides and the presence of a Florida red tide, whether or not to visit a particular beach on a given day. Thus, current observations and forecasts of this HAB may help to protect public health by decreasing exposures to toxic aerosols.

OSTREOPSIS CF. OVATA AND RESPIRATORY IRRITATIONS

Over the past two decades, extensive blooms of Ostreopsis spp. have occurred in the Mediterranean (e.g. Algeria, France, Italy, Tunisia). During these blooms, beach users have experienced upper respiratory disorders (rhinorrhoa), eye and nose irritation, fever and general malaise, and sought medical care in hospital emergency departments and primary healthcare centres (Alvarez et al., 2005; Gallitelli et al., 2005; Brescianini et al., 2006; Durando et al., 2007; Barroso et al., 2008; Vila et al., 2008, 2012; Tichadou et al., 2010; Tubaro et al., 2011; Illoul et al., 2012). A similar situation could also be occurring in certain beaches in Brazil (Pronéca et al., 2010). The health effects could be caused by exposures to aerosols containing biotoxins or allergenic substances produced by Ostreopsis cell fragments or accompanying microbiota (Casabianca et al., 2013; Cimiello et al., 2014). Reported cases of cutaneous irritation were attributed (but not proven) to direct skin contact with seawater containing high concentrations of Ostreopsis cells. At present, studies (including epidemiological and ecological) are underway to improve the understanding of Ostreopsis bloom dynamics and the possible links to human health effects (Vila et al., 2014; Berdalet et al., 2015).

EFFECTS OF HABs ON HUMAN WELLBEING

In addition to the risks to human health posed by exposure to biotoxins, marine HABs can impact other aspects of human wellbeing, including human commercial and recreational uses of the coastal and marine environments, such as fishing, shellfish collection and growing, and tourism, and non-market, passive uses of the ocean, such as preferences for particular ecological states. Also, marine HABs may lead to complex societal responses in the affected human communities, such as the more general avoidance of coastal and ocean resources due to misconceptions and inadequate communications of health risks. In this section, we refer to the economic effects resulting both from biotoxin producing species (Box 1) and from algal taxa involved in high biomass events (Box 2). Difficulties in developing estimates of the economic costs associated with HABs are considered in detail in this section.
Box 2. Representative examples of HABs that affect marine organisms or ecosystems, with effects on well-being, and on human health in some cases.

Events presented following the alphabetical order of the causative organism (CO). The type (T) of bloom, their effect/mode of action in the marine organisms or ecosystem (E/m-a), effects on humans (E-h), main geographic affected areas (A) and some references (R) are indicated.

General information. Certain HABs affect marine organisms and ecosystems, due to the accumulation of biomass of particular taxa, which may or may not produce biotoxins. In most cases, fish-killing HAB species produce haemolytic or cytotoxic biotoxins, which cause necrotizing degeneration of the fish gills. Other, more rare or more recently discovered combinations of fish-killing algae and their biotoxins include goniiodomains (produced by G. pseudogonyaulax), amphidinols (synthesized by Amphidinium klesi彬 and A. carteri; reviewed by Louza et al., 2014) and karlotoxins from Karlodinium spp. (Bachvaroff et al., 2008; Place et al., 2014; Van Wagener et al., 2010). In many cases though, the toxic compound has not yet been clearly identified.

The breakdown of cells during the decline of a bloom, with the subsequent degradation and utilization of the organic matter by bacteria can result in low oxygen concentrations of isolated bottom water, causing subsequent macrofauna mortalities. Other impacts on the ecosystem include: the reduction of light penetration, production of excess ammonia, physical damage of fish gills, and hypothermia in marine birds due to the accumulation of surfactant-like proteins. Here we list examples of many microalgae and a macrophyte involved in HAB events. More details and examples of these blooms, mainly high biomass blooms, can be found, among others, in e.g. GEOHAB, 2001, 2006, 2010 (download free from http://www.geohab.info).

CO: Akashiwo sanguinea (Dinophyceae)
T: High biomass, non-toxic
E/m-a: Extensive marine birds mortality caused by hypothermia. The microalgae produced high amounts of organic matter that accumulated at the sea surface. The foam contained surfactant-like proteins, which destroyed the waterproof and insulation characteristics of the bird feathers
E-h: Unknown
R: Jessup et al. (2009)
A: Pacific North-west (Monterey Bay, California)

CO: Chaetoceros wighami, C. debile (Bacillariophyceae);
Dicyocha speculum (Silicoflagellate);
T: High biomass, non-toxic
Physical damage (silicic cell cover) of the fills gills
E/m-a: Farmed fish kills
E-h: Economic losses (several million pounds)
R: Bruno et al. (1989); Treasurer et al. (2003)
A: Europe (Loch Torridon, Scotland; Shetland Isles)

CO: Chattonella antiqua and C. marina (Raphidophyceae);
T: High biomass, toxic (haemolytic, haemagglutinating and neurotoxic effects, molecule similar to brevetoxin)
E/m-a: Aquaculture fish and natural fauna kills
E-h: Economic losses (US$0.5 billion in 1972; Australia
AU$15.0 million loss of caged bluefin tuna in April 1996)
R: Endo et al. (1985), Marshall & Hallegraeff (1999), and references cited therein
A: Japan, Australia, India, Florida and China

CO: Cocchlidinium polykriloides, Cocchlidinium spp. (Dinophyceae)
T: High biomass, toxic. The toxic compound and mechanism have not been identified yet, with controversial results (Tang & Gobler, 2008, and refs therein). Toxicity could be caused by non-hydrogen peroxide, highly reactive, labile biotoxins such as ROS-like chemicals
E/m-a: Wild and farmed fish kills; coral and shellfish
E-h: Economic losses. Hundreds of millions of US$ in fisheries losses in Korea alone; more than US$3.0 million in Vancouver (in 1999); in the Arabian Gulf and Gulf of Oman, the long-lasting Cocchlidinium bloom of 2010 killed thousands of tonnes of fish, limited traditional fishery operations, damaged coral reefs, impacting coastal tourism, and forcing the closure of desalination systems
R: Yuki & Yoshimatsu (1989); Kim et al. (1999); Whyte et al. (2001); Gobler et al. (2008); Richlen et al. (2010); Kudela & Gobler (2012), and references cited therein
A: North America, Asia, Australia and Europe

CO: Heterosigma akashiwo (Raphidophyceae)
T: High biomass, toxic. No identified toxic nor toxin mechanism, which may affect other aquatic organisms (zooplankton, copepods, benthic larvae
E/m-a: Aquaculture fish kills
E-h: Economic losses
A: Atlantic and Pacific coast: Canada, Chile, Japan and New Zealand

CO: Karenia brevis (Dinophyceae)
T: High biomass, toxic (brevetoxin, haemolytic)
E/m-a: Toxicity to humans and marine fauna by direct ingestion or aerosolization; manatee mortality during the active bloom phase; dolphin and manatee poisoning and mortality through the ingestion of contaminated fish and aquatic plants during the senescence phase of the bloom. Fish kills
E-h: Health costs; tourism losses, passive use losses; costs of beach cleanups
R: Tangen (1977); Steidinger et al. (1998); Bossart et al. (2002); Magaña et al. (2003); Flevelling et al. (2005); Naar et al. (2007); Campbell et al. (2013)
A: Gulf of Mexico, Florida, North Carolina

CO: Karenia mikimotoi (Dinophyceae)
T: High biomass, toxic (gimmocin, haemolytic)
E/m-a: Fish kills. Mortalities of marine fauna
E-h: Economic losses; passive use losses
R: Dahl & Tangen (1990, 1993); Nakamura et al. (1995); Gentien (1998); Raine et al. (2001); Satake et al. (2002); Silke et al. (2005), Vanhoutte et al. (2008); Davidson et al. (2009)
A: Asian and Australian, Northern European waters

CO: Noctiluca (heterotrophic Dinophyceae)
T: High biomass, non-toxic
E/m-a: Hypoxia and excess ammonia in the environment; unpleasant odours; water discolouration
E-h: Economic losses (on tourism, not estimated)
R: Bricelj & Lonsdale (1997); Elbrächter & Qi (1998); Murray & Suthers (1999); Fonda-Umani et al. (2004)
A: Worldwide

CO: Ostreopsis cf. ovata, O. siamensis (benthic Dinophyceae)
T: High biomass, toxic
E/m-a: Cells attach to corals, macrophytes or macrofauna by mucous substances. Toxicity (Palytoxin and analogues) to marine fauna by direct ingestion or by anoxia. The particular mechanisms have not been identified yet
E-h: Respiratory irritation, possible gastrointestinal illness
R: Shears & Ross (2009); Mangialajo et al. (2011) and references cited therein
A: Mediterranean, New Zealand, Brazil, Japan

CO: Phaeocystis (Prymnesiophyceae)
T: High biomass, non-toxic
E/m-a: Production of foam or mucilage (alteration of gas diffusion and rheologic properties) causing farmed fish kills; discolouration and repellent odour can impact tourism
E-h: From ~US$8.7 million in Vietnam to ~US$7.5 million in Asian aquaculture (1997, Quanzhan Bay, Fujian province, China)
R: Lancelot et al. (1987); Schoemann et al. (2005); Hai et al. (2010) and references cited therein; Arin et al. (2013)
A: Asian coastlines, North Sea, Antarctica

CO: Prymnesium parvum (Prymnesiophyta)
T: High biomass, toxic. A variety of toxic compounds with lytic effects (allelopathy) that would affect other organisms in the food webs, as well as haemolytic and cytotoxic effects on fishes (Blossom et al., 2014)
E/m-a: Aquaculture fish kills
E-h: Economic losses (US$5.0 million)
R: Kaartvedt et al. (1991)
A: Norwegian fjords

CO: Pseudochantonella verruculosa (Dictyochophyceae)
T: High biomass, toxic
E/m-a: Aquaculture fish kills
E-h: Economic losses
R: Mackenzie et al. (2011)
A: New Zealand

CO: Pseudo-nitzschia spp. (Bacillariophyceae)
T: High biomass, toxic (domoic acid, neurotoxic)
E/m-a: Morbidity and mortality of large vertebrates, including seabirds, sea lions, sea otters, seals, and possibly whales
E-h: Possible passive use losses
R: Sierra-Beltrán et al. (1997); Scholin et al. (2000); Hall & Frame (2010)
A: Especially, Pacific coast of North America

CO: Ulva (Ulvaceae), macroscopic benthic macroalgae
T: High biomass, non toxic
E/m-a: Hypoxia in the environment; unpleasant odours; impacts on tourism

E-h: Beach cleanup costs: US$87.3 million in China; US$10.3 – 165.9 million in France, depending on the extent of affected areas (approximate period 1989 – 2006)
R: Hu & He (2008); Zhou et al. 2015 and references cited therein.
A: China (Qingdao) during the Olympics, Atlantic French coast
In China, disrupted activities during the Olympic games in 2008

Economic effects of HABs linked to food security and seafood-related activities

Certain HABs constitute a threat to aquaculture food production, which has become a major source of protein (linked to decreasing wild fish stocks) for expanding human populations, particularly in coastal communities in developing countries. Like farming, and sometimes in conjunction with it, aquaculture helps to support many local and regional economies (FAO, 2006). Currently, and with respect to the cultivation (and wild-harvest) of shellfish, the most effective way to protect humans from HAB-related seafood poisoning is to monitor for the presence of HAB species or biotoxins and to enforce periodic closures of commercial and recreational harvesting or growing areas (Figure 3). Contamination of seafood products can result in economic losses in shellfish collection and growing, and in the aquaculture of certain finfish. There may also be losses in ancillary industries, including processing, distribution, wholesaling and retailing of seafood (Larkin & Adams, 2007; Morgan et al., 2010).

Estimating the overall economic costs associated with the occurrence of HABs is complex. These costs vary markedly, depending on geographic region, seafood product, the frequency and intensity of HAB-related events, the duration of fishery closures and the costs of healthcare provision. As an example, Hoagland et al. (2002) estimated the annual economic effects of HABs in the USA between 1987 and 1992. At that time, the economic effects were valued in the order of US$50.0 million per year; this calculation has been subsequently adjusted for inflation to ~US$100.0 million per year (Hoagland & Scatasta, 2006). It is important to note that there is considerable variation in estimated impacts from year to year. This estimate was based on a compilation of the assessments of economic effects in public health, commercial fisheries, recreation and tourism, and monitoring and management. Notably, public health effects are the largest component, representing about 42% of nationwide average effects during that time period. Although the actual incidence of ciguatera fish poisoning (CFP) is very uncertain in many tropical locations, estimates of CFP illnesses comprise 95% of the total public health costs.

With regards to the specific economic costs of HAB-induced illnesses, there are very few studies available. The UNEP Global Environmental Outlook lists the worldwide economic impacts of algal biotoxins on human health from seafood alone as ~US$4.0 billion a year (GESAMP, 2001). As a comparison, the impact of infectious hepatitis from seafood consumption was estimated to be ~US$7.2 billion per year. The direct medical care costs associated with the respiratory and digestive illnesses caused by K. brevis blooms along the Florida coast of
the Gulf of Mexico ranged from US$0.1–0.7 million annually, depending on the intensity of the event (Hoagland et al., 2009, 2014). Illness costs might exceed US$1.0 million per year for large, long-lasting blooms, however, such as one that occurred during 2005–06. Assuming that the average annual illness costs of *K. brevis* blooms would persist into the future, using a discount rate of 3%, the capitalized costs of future illnesses would range between US$2.0–24.0 million.

In the particular case of ciguatera fish poisoning, the average medical costs of diagnosis and treatment in traditionally non-endemic areas, as for instance in Canada, was estimated at about CA$2470 per case, with about 1000 cases per year related to tourism and food importation in 1990 (Lange et al., 1992; Todd, 1995; Fleming et al., 2002). Accurate estimates of the human costs of these diseases necessitate an adequate knowledge of their prevalence and incidence, as well as an understanding of their acute and chronic human health effects (Pennotti et al., 2013).

Any estimation of the economic effects of HABs also should include an evaluation of the costs of monitoring and management, which would include, for instance, sampling programmes or strategies to decrease the risk of HAB occurrences (e.g. reducing coastal eutrophication, sustainable use of the coastal zone, and other human-related activities) or their impacts (Hoagland et al., 2002). For instance, limiting shellfish harvesting closures or varying the timing of shellfish or finfish harvesting could be useful strategies to reduce the impacts of HABs, but these actions also may have associated costs in terms of product marketability. Wessells et al. (1995) found that the occurrence of paralytic shellfish poisoning in blue mussels from the Canadian maritimes, leading to illnesses and subsequent harvest closures, adversely affected the market for mussels from Maine. Whitehead et al. (2003) found that, after consumer demand for seafood contracted upon learning of a HAB-induced fish kill, mandatory seafood inspection programmes were more effective in restoring consumer confidence and expanding demand than broad public assurances of seafood safety.

Experience suggests that closure strategies in shellfish production areas could be beneficial over the long term, despite leading to losses in the short term. For example, this approach has been applied recently in the northern areas of Hokkaido and Tohoku (Japan) with some benefits. The region provides and Tohoku (Japan) with some benefits. The region provides

**Impacts of HABs on non-market, passive use values of marine ecosystems**

Certain HABs have noxious, even lethal, consequences on marine organisms and ecosystems (Box 2), thereby potentially leading to passive value losses or the degradation of ecosystem services.

Some taxa produce particular toxic compounds that, given their high concentrations in the water when the species bloom, have been involved in mass mortalities of wild fauna. According to the US National Oceanic and Atmospheric Administration (NOAA), more than 50% of all Unusual Mortality Events (UMEs) of wild animals (turtles, dolphins, manatees, whales, birds; e.g. Scholin et al., 2000), could have been due to microalgal toxins, although it is evident that this is not a new phenomenon and is also a part of the natural ecosystem functioning. There are reports of mortalities of wild fish associated with HABs that date back to the latter part of the 19th century (Whitelegge, 1891). In 1968, mortalities of sand eels (*Ammodites* spp.) and an estimated 80% of the breeding population of shag (*Phalacrocorax aristotelis*) coincided with a bloom of *Alexandrium tamarense* off the north-east coast of the UK (Adams et al., 1968; Coulson et al., 1968). White (1984) documented four cases of fish kills associated with saxitoxins. Other historical examples include the deaths of 19 humpback whales off Cape Cod.
(USA), and probably the deaths of pygmy, dwarf sperm and North Atlantic right whales in the same region, as well as Southern right whale mortalities in Peninsula Valdés (Argentina). More recently, Scholin et al. (2000) reported the deaths of over 400 California sea lions (Zalophus californianus) along the central Californian coast during May and June 1998. Coincident with these mortalities, a bloom of *Pseudo-nitzschia australis* was reported and domoic acid was detected in planktivorous fish and in sea lion body fluids.

Jessup et al. (2009) reported extensive marine bird mortality in the Pacific North-west (Monterey Bay, California) in winter 2007 coinciding with a bloom of the non-toxic dinoflagellate *Akashiwo sanguinea*. Affected birds had a slimy yellow-green material on their feathers, and they were diagnosed as severely hypothermic. This dinoflagellate excretes high amounts of organic matter that accumulate at the sea surface. In this case, the sea foam contained surfactant-like proteins, which destroyed the waterproofing and insulative characteristics of the bird feathers.

*Ostreopsis* blooms constitute a particular case of an emerging harmful event that is a growing problem in coastal environments. Since the end of the 20th century in temperate waters (e.g. in the Mediterranean, Brazilian and New Zealand coasts), blooms of this benthic dinoflagellate genus have been linked to damage to marine fauna (i.e. mussel mortalities, loss of spines and death of sea urchins, loss of one or more arms in sea stars, and coral bleaching) and subsequent alterations of the coastal ecosystems (Sansoni et al., 2003; Simoni et al., 2003; Shears & Ross, 2009). In some cases, the invertebrate community of the affected area has not recovered to date (Vila et al., 2012). Coinciding with these effects, high cell concentrations of *O. cf. ovata* (mainly in the Mediterranean) or *O. siamensis* (mainly in New Zealand) were recorded in the water column. Also, highly visible distinctive rusty-brown coloured mucilaginous films covered the reef, macroalgae and other sedentary organisms, or floated in the water column or on the surface. Oxygen depletion seems to be the most likely cause of the observed effects. Direct toxicity to the fauna (via ingestion of *Ostreopsis* through the food web) cannot be ruled out, given that palytoxin analogues (i.e. ostreocin and ovatoxin) have been isolated from certain macrofauna at other Mediterranean sites (Aligizaki et al., 2008, 2011; Amzil et al., 2012; Biré et al., 2013).

High-biomass *Phaeocystis* blooms also have been linked to the deterioration of water quality in some coastal regions of the North Atlantic and the Mediterranean (e.g. Lanceot & Mathot, 1987; Arin et al., 2013). This species produces macroscopic colonies embedded into a mucilaginous matrix. The decay of the blooms results in brownish foams containing 3-dimethylsulphoniopropionate (DMSP), which is responsible for bad odours (Liss et al., 1994). Although this may result indirectly in the loss of recreational activities, no economic assessment has been undertaken yet, and no direct harm to humans has been reported.

Finally, some studies have suggested that certain HAB species also could have subtle impacts on ecosystems, by altering fundamental physiological and biological processes (e.g. feeding behaviour, life stages, survival strategies, reproductive capacity) in other organisms of food webs, likely through allelopathic effects (e.g. Fistarol et al., 2003; Tillmann, 2003; Granelli & Hansen, 2006). These particular effects are not easy to detect, however, and the underlying mechanisms of damage to other components of the food webs remain difficult to understand (Landsberg, 2002; Shumway et al., 2003). This is due, in part, to the spatial and temporal variability of HAB events and also to the difficulty of isolating their impacts from those associated with other environmental and/or anthropogenic factors (e.g. increased temperature, wave action, habitat destruction). Both long-term field studies and experimental approaches in the laboratory are required to ascertain the direct and indirect impacts of HABs in the different components of the ecosystems.

### The challenges of estimating the economic costs of HABs

As explained, HABs can lead to economic losses associated with the costs of treating human illnesses, closures of commercial and recreational shellfisheries, mortalities of fish in commercial finfish aquaculture operations, declines in coastal recreation and tourism, and additional investments in environmental monitoring and the prevention, control or mitigation of blooms or their consequences (Hoagland et al., 2002). Hoagland & Scatasta (2006) estimated economic effects (adjusted for inflation into 2015 US$) in the order of US$1.0 billion per year in Europe and US$100.0 million per year in the United States. The European estimate was influenced largely by estimated losses to coastal tourism, which is affected mostly by high biomass, noxious (blooms causing high foam volumes, discolouration of the ocean, noxious odours, or beach closings), but not necessarily toxic, HABs. Any estimates of the scale of the economic effects of HABs should be considered very rough approximations, even in areas where they have been relatively well studied, including the USA and Europe. Estimates in other parts of the world, particularly in developing countries, are much more speculative and uncertain. The development of comprehensive and consistent estimates of losses in economic welfare as a consequence of HABs remains a clear priority for future research.

The reasons why economic estimates often are uncertain include the wide variety of methodologies used to compile the assessments, some of which produce estimates of economic measures that may not be strictly comparable (e.g. sales, indirect or induced impacts, consumer or producer surpluses, agency budgets); the wide variety of physical and economic effects; the episodic and often unpredictable nature of blooms of different types in many areas; and the wide ranges of responses of humans to mitigate the adverse impacts, including, most importantly, switching to next-best alternative activities or foods. Moreover, these reasons tend to be context-dependent, because of differences in the capabilities of human communities for responding to HABs as a natural hazard. For example, shellfish management measures are well-practiced in many parts of the developed world, such as in Maine or Florida in the USA, but often non-existent or difficult to enforce in parts of the developing world, such as in the coastal bays of the Bohol and Visayan Seas in the Philippines. Morbidities and mortalities from shellfish poisonings, particularly among the underprivileged, are common in the latter but now exceedingly rare in the former (but see Reich et al. (2015) for a modern counter-example of neurotoxic shellfish poisonings from Florida).

Other so-called non-market, ‘passive’ uses of the coastal and marine ecosystems, such as for the conservation of protected species (including marine mammals and sea turtles),
can in theory be affected by HABs. Even if favoured species or unique ecosystems are not used directly, humans may experience a sense of loss that might be evaluated in economic terms. Studies of passive value losses due to the adverse effects of HABs on protected species or ecosystems are almost non-existent. One reason for the lack of estimates of effects on passive use values is that, although individuals may become ill or die as a consequence of HABs, such as the deaths of endangered West Indian manatees (Trichechus manatus) due to Karenia brevis blooms in south-western Florida, the populations often are largely unaffected by the mortalities (in essence, K. brevis blooms could be viewed as one of many natural sources of mortality). Similarly, the media often report on ‘large-scale’ finfish kills resulting from HABs, but the impacts on fish biomass and the growth rates of fish stocks in the wild often are minor or imperceptible.

Of greater concern to users of coastal and marine systems is the loss of access to commercial or recreational fisheries, especially to high-valued shellfish stocks, such as oysters, scallops, clams, mussels, crabs, or marine snails, or to areas used to grow shellfish. Even in such cases, commercial fishermen (a market-based, direct use) tend to relocate or to switch fisheries or occupations, thereby mitigating substantial economic losses. Recreational fishermen (a non-market, direct use) choose another target or another pastime. Further, seafood consumers also can switch among food options. When switching occurs, other fisheries, recreational destinations, or food providers may benefit. Because the latter typically are not ‘first-best’ choices, economic losses are ipso facto incurred in all cases, but the human behavioural switching response clearly serves to blunt the impacts. Here, policies incurred in all cases, but the human behavioural switching response clearly serve to blunt the impacts. Here, policies aimed at reducing passive use values may become widespread. Given that, researchers and managers may wish to prioritize characterizing the extent of this immunity to biotoxins is that areas closed to shellfish harvesting may become de facto fishery reserves, helping to conserve stocks, provided that the biotoxin is naturally metabolized or lost after the bloom disappears.

Social impacts of the health risks caused by toxic HABs. The case of ciguatera fish poisoning

Within the human communities whose revenue is based on aquaculture and shellfish activities, HABs may have adverse social impacts including damaged reputations, decreased incomes, employment losses, and long-term changes in seafood consumption patterns (Magnien, 2001). There has been very little study of these other human dimensions, and many social impacts remain mostly conjectural and unexplored (Bauer et al., 2009).

In the case of the ciguatera fish poisoning (CFP), it has been associated with the loss of a traditional food source, losses in commercial fishing businesses, losses of tourism, increased costs of medical care, and high costs of monitoring and management (Epstein & Rapport, 1996). Fear of CFP can lead to reduced fishing in coral lagoons (Dewailly et al., 2008), and to a corresponding increased reliance on pelagic fish or on a less healthy diet of lower quality, imported, canned fish or red meat. This new diet, combined with reduced levels of physical exercise, may be one factor contributing to the trend of increasing obesity among Pacific Islanders, accompanied by a rising prevalence of chronic diseases (e.g. diabetes, hypertension and cardiovascular diseases) in indigenous Pacific populations (Lewis & Ruff, 1993).

Ciguatera fish poisoning outbreaks could harm the economies of tropical islands, many of which are highly dependent on local reef fisheries for subsistence, export products, and tourism. Reduced fishing effort due to endemic ciguatera has been blamed for losses of traditional fishing knowledge, as new generations abandon artisanal or commercial fishing. One example concerns Rarotonga, an island located in the southern Cook Islands that exhibits the world’s highest incidence of ciguatera poisoning. On Rarotonga, the per-capita fresh fish consumption decreased from 149 g d$^{-1}$ in 1989 to 75 g d$^{-1}$ in 2006, due to concerns about CFP (Rongo & van Woessik, 2012). Consequently, over the same period, the consumption of alternative proteins, particularly imported meats, increased. The direct loss in value of marketable goods from commercial fisheries amounted to NZ$60.8 million (about US$0.5 million) per year, and the approximate costs associated with dietary shifts amounted to NZ$1.0 million (about US$0.7 million) per year. With a decline in cases of ciguatera poisoning in recent years, fresh fish has returned to the menus of Rarotongans, and per-capita fresh fish consumption increased to 104 g d$^{-1}$ in 2011.

In a discussion of the effects of HABs on public health, it should be highlighted that the management of tropical waters for ciguatera fish poisoning is not well-developed. In these regions, fishermen usually are able to continue to access areas where finfish may have bioaccumulated ciguatoxin, leading to circumstances in which CFP illnesses may become widespread. Given that, researchers and managers may wish to prioritize characterizing the extent of the CFP problem in tropical regions and developing ways to alleviate it.

Present and future challenges, strategies and opportunities

HABs are natural phenomena that have almost certainly occurred for thousands of years before recorded history (Mudie et al., 2002). To humans, HABs constitute a problem worldwide, but the idea that HABs might be prevented from occurring is not a viable option. However, improvements in our understanding of HAB dynamics during the last 40 years have led to insights for designing strategies to mitigate their impacts. Successful strategies consider the present situation and the future prospects of the potential spread and increase in HAB occurrence as a result of climate and other environmental changes. Common strategies can be adapted to address specific, local problems in different parts of our world. Despite some limitations, new advances in technology and coordinated international research efforts may help to improve the management of the impacts of HABs.

Improving monitoring and research needs to forecast and predict HAB events

Monitoring programmes for HAB species and their toxins implemented in different areas of the world have clearly
reduced fatal poisonings in humans (e.g. Davidson & Bresnan, 2009). For example, current monitoring programmes of the European Union are driven by its Food Hygiene Regulations (EC) No. 853/2004 and (EC) No. 854/2004, which require Member States to monitor both for biotoxin concentrations in shellfish tissues and the presence of marine biotoxin-producing phytoplankton in coastal waters. Due to different capacities for undertaking local risk assessments, there are local differences in the implementation of these monitoring activities. In general, these programmes have been successful in safeguarding human health, but continued refinement will be needed in the future as environmental conditions shift (particularly climate warming) and human uses of the coastal ocean change.

Currently missing are programmes of robust monitoring in developing countries where they are often at best rudimentary, despite increasing exports of seafood products to other regions (Reich et al., 2015). Despite their success, established monitoring programmes are not without technical, logistical or cost-related limitations (DeGrasse & Martinez-Diaz, 2012; Hess, 2012; Suzuki & Watanabe, 2012). Since their inception, improvements have been linked to advances in technologies related to sampling, taxonomic and biotoxin analyses, and observing systems.

Light microscopy constitutes the most widespread monitoring tool for harmful algal species, but it is time consuming to employ, and it requires specialized training and expertise. Development of quantitative biomolecular tools to facilitate monitoring could improve monitoring. Given the number of different organisms that have to be tracked, and the lack of fully quantitative molecular tools for their enumeration, monitoring agencies cannot currently replace light microscopy as the primary tool. Maintaining and supporting the training of microscopists in the identification and quantification of HABs species is necessary for successful monitoring.

While the ability to detect the presence of harmful micro-algal species in coastal waters can provide an excellent early warning of the likely occurrence of biotoxins in, for example, shellfish (e.g. Trainer & Suddleson, 2005), many uncertainties remain. These include the fact that the presence of potentially toxic organisms is not always linked to toxicity in shellfish. For example, in Scottish waters, morphologically indistinguishable toxic and non-toxic Alexandrium tamarense cells have been known to occur simultaneously (Touzet et al., 2010). Furthermore, known-toxic organisms are not always detected efficiently due to their benthic character, fragility when preserved, or small size. For example, the azaspiracid producer Azadinium sp. measures only 7 × 12 μm. The link with its produced biotoxin was established recently in 2007 (Tillmann et al., 2009), only after standards for biotoxin analysis were isolated (Satake et al., 2002; Jauffrais et al., 2012; Kilcoyne et al., 2012) and biomolecular tools for tracking the organism in water were developed (Kilcoyne et al., 2014). Further, toxicity itself is not always well described. For example, not all of the biotoxins of many Gambierdiscus species have been described, and they may be present in complex mixtures in different individual organisms and blooms. Finally, toxicity may be associated with new, unexpected vectors or species. For example, in 2002, the consumption of puffer fish from Florida caused neurological disease in 21 people (Centers for Disease Control and Prevention (CDCP), 2002). Initially, based on patient symptoms and reported puffer fish ingestion, the outbreak was attributed to tetrodotoxin. A mouse bioassay confirmed the presence of a Na+ channel blocking toxin, tentatively identified as tetrodotoxin (usually associated with puffer fish), in unconsumed portions of fish. Direct measurement analyses (LC/MS, immunoassay, and receptor binding assay) confirmed the unexpected presence of saxitoxin in the fish samples, however, but not tetrodotoxin. Analysis of urine samples from the victims of the outbreak confirmed these findings. This was the first confirmed report of saxitoxin poisoning associated with puffer fish ingestion in North America (Landsberg et al., 2006).

Concerning biotoxin detection, bioassays (i.e. the mouse bioassay) had been used traditionally as a fast way to detect the presence of toxins in potentially contaminated seafood, thus raising warnings and preventing the harvest of contaminated shellfish. Bioassays are associated with a number of technical and ethical issues (Hess et al., 2006), however, and led to their recent replacement with non-animal alternatives (i.e. cell tissue cultures; e.g. Van Dolah et al., 2012) in many countries, particularly in Europe (European Commission, 2011). Research efforts are now focused on the development of multi-toxin methods for the detection of multiple groups of compounds in biosensor systems (Campbell et al., 2014, and references cited therein). The large number of compounds that need to be detected and an inability to validate new tools for detection mean that many monitoring agencies will need to continue to rely on either insensitive (and ethically questionable) mouse bioassays or on comparatively complex and expensive LC-MS/MS detection methods, neither of which can be implemented in situ.

A potentially useful sampling technique based on the passive capture of biotoxins by resins was developed by MacKenzie et al. (2004). This method has been used successfully to trace biotoxins when the producing organisms are difficult to monitor due to their small size (e.g. Alexandrium spp.) or their benthic habit (e.g. Prorocentrum lima and Vulcanodinium rugosum; Fux et al., 2009; Zeddong et al., 2014). The technique has not been standardized, however, and it will need further development for hydrophilic biotoxins. At present, the passive capture of biotoxins cannot easily be automated or adapted for in situ detection. Furthermore, ongoing refinement of its analytical methods will be required to meet the challenges of emerging biotoxins (Turner et al., 2015).

Issues arise with the existing technologies with the frequency of representative sampling of in situ biotoxins and the toxin-producing microalgae, which rarely takes place more than weekly, although higher frequency sampling may be desirable in some coastal waters. In the particular case of the benthic harmful species (e.g. Gambierdiscus, Ostreopsis, Prorocentrum lima), sampling techniques require refinements and standardization (e.g. Tester et al., 2014). As yet automated, reliable, and affordable systems to detect harmful species or their biotoxins for routine monitoring in situ are lacking. While recently some promising techniques have been developed to detect biotoxin-producing organisms (e.g. Hess et al., 2012 and references cited therein; ICES, 2015), they are still viewed mainly as research tools and have seldom been used in high frequency in situ monitoring. This is the case of the Environmental Sample Processor (ESP; Greenfield et al., 2008; Scholin et al., 2009) and the Imaging Flow Cytobot (IFCB; Olson & Sosik, 2007), two automated
in situ monitoring platforms deployed in Monterey Bay, the Gulf of Maine, Puget Sound and the Gulf of Mexico. The ESP collects and processes water samples in situ, using sensitive and specific molecular assays to detect target HAB cells and toxins, and transmitting the data to the laboratory in near real time. The IFCB is an imaging flow cytometer that captures the high-resolution images and fluorescence characteristics of all plankton cells (in the 5–150 μm size range) at a high frequency (3 samples per hour); the data can also be sent to a laboratory in near real time (e.g. Campbell et al., 2010, 2013). At present, however, they have very high costs and cannot be deployed to monitor individual shellfish production areas. Although they cannot replace more traditional forms of monitoring at present efforts are being made to facilitate their use for this purpose. Fortunately, these sophisticated systems are useful tools for advancing an understanding of the links between HABs and ocean dynamics.

The adoption of monitoring technologies are affected strongly by economic costs (Frolov et al., 2013). Some systems are relatively simple, comprising microscopic analysis of water samples for particular HAB species and analysis of shellfish tissues for the presence of biotoxins. Other programmes involve in situ monitoring of phytoplankton and model-based forecasts of bloom occurrence, such those carried out in the Gulf of Maine (e.g. Anderson et al., 2005a, b; McGillicuddy et al., 2005; http://www.whoi.edu/main/topic/harmful-algae-red-tides) or in California Bay (e.g. Ryan et al., 2005, 2011). According to Bernard et al. (2014), the costs of monitoring could be on the order of 1 billion USD annually, accounting for 10% of the overall costs of HABs worldwide, estimated to be at 10 billion USD annually for marine and fresh waters. A monitoring system including satellite observations could be made more efficient by improving international cooperation in the collection, interpretation and sharing of Earth observation information, as conceived by the Global Earth Observation System of Systems (GEOSS, Fritz et al., 2008).

Monitoring, combined with operational oceanography and modelling, offers the hope of providing forecasts and early warnings and ultimately predictions of HAB events (e.g. GEOHAB, 2011; Davidson, 2014). Achieving a predictive capability would require an adequate understanding of the eco-physiology of HAB species and the physical and chemical processes that influence the occurrence of the blooms, however. One problem is that, in many contexts, such as remote sensing from satellites, HAB species do not exhibit characteristics that distinguish them from other phytoplankters. Our understanding of the many different processes (biological, ecological, physical, chemical, meteorological) that control HAB dynamics and the dynamics of microalgae in general is incomplete, making their parameterization in numerical models extremely difficult. To date, progress has been constrained by the complexities of biological interactions and the resulting difficulties in developing ecological models. One example is the HAB operational forecast (HAB-OFS) developed by NOAA for Florida and Texas (Stumpf et al., 2008; http://tidesandcurrents.noaa.gov/hab/). HAB-OFS combines satellite imagery, field observations, models, public health reports and buoy data to help forecast K. brevis blooms. Another NOAA effort for forecasting Pseudo-nitzschia has incorporated input from stakeholder communities in the state of Washington (Brown et al., 2012). In the Gulf of Maine, a coupled physical/biological model (e.g. He et al., 2008) predicts the transport of Alexandrium fundyense cells, controlled by plume advection and wind forcing. Statistically based models (Anderson et al., 2009; Lane et al., 2009) have successfully hindcast Pseudo-nitzschia blooms in Monterey Bay and Chesapeake Bay. Finally, conceptual models, such as for Phaeocystis blooms in Vietnam (Hai et al., 2010) and Dinophysis acuminate (Velo-Suárez et al., 2014) in the Rías of north-western Spain, have provided new insights into understanding the dynamics of blooms and designing preventive strategies.

Sustained fundamental research could help generate a better understanding of HAB dynamics, to inform and improve monitoring programmes, and to design methods to mitigate the impacts of HABs on human health and well-being.

**Better quantification and prevention of the impacts on human health: HAB-related disease surveillance**

Determining the true incidence of HAB-related diseases remains a very significant challenge. The lack of experience of professionals in the public health and medical communities with patients exposed to marine biotoxins (e.g. Pennotti et al., 2013) has led to incorrect diagnoses and failures to report illness. Inexperience, in turn, likely results in the under-reporting of HAB-related disorders, even in areas where such diseases are endemic. Furthermore, in general, there is a lack of coordination between marine biotoxin monitoring and public health surveillance activities.

Under-reporting due to erroneous diagnosis is common and widespread, and is related, in part, to the lack of diagnostic tools. For instance, the symptoms of diarrhetic shellfish poisoning (DSP) are non-specific, and, except during exceptional outbreaks, neither seafood consumers nor their clinicians may recognize the incidence of DSP illness (e.g. Davidson et al., 2011; Taylor et al., 2013). Taylor & Harrison (2002) query that “[n]o diarrhoeic shellfish poisoning has been diagnosed in humans in British Columbia, but, given its resemblance to diarrhoea caused by bacterial contamination (Vibrio haemolyticus, in particular), would DSP be detected without testing specifically for okadaic acid or dinophysistoxin? Indeed, routine methods for the detection of either biotoxins or biomarkers in human tissues or fluids are virtually absent. [In 2011, nine years after Taylor had queried whether diarrhetic shellfish poisoning would be detected in the absence of testing for okadaic acid or dinophysistoxin, 62 illnesses of diarrhetic shellfish poisoning were reported in British Columbia. Changes were made to the shellfish monitoring programme following this outbreak to include more stringent testing for DSP toxins (Taylor et al., 2013).]

Diagnostic tools for saxitoxins in human urine have recently been developed (Johnson et al., 2009). A biomarker for low-level domoic acid exposure of zebra-fish and sea lions (Lefebvre et al., 2012), is a promising technology for identifying such biomarkers for humans. The verification of ciguatoxins in contaminated seafood has been particularly challenging. Recent advances suggest that an effective and reliable method will soon be available. Under the auspices of the International Atomic Energy Agency (IAEA), a radio-labelled receptor binding assay (RBA) was developed for ciguatoxin, and this
assay has been tested successfully in the Caribbean and the Pacific at IAEA-UNESCO-IOC sponsored training workshops (Tester, personal communication). A more recent advance is a fluorescent RBA method that compares favourably with the radio-labelled RBA approach. The fluorescent method is analogous to a technique implemented for brevetoxins (e.g. McCall et al., 2012), in that no radioactivity is involved, making it less expensive to use, and avoiding hazardous waste disposal costs that can be significant, for example, in the Pacific islands.

HAB-associated illnesses are a public health issue, and, accordingly, public health surveillance could be coordinated with environmental monitoring. Until now, epidemiological studies typically have been conducted after clusters of disease outbreaks have occurred (although unfortunately usually without long-term follow-up to explore the chronic health effects from acute exposures). New multidisciplinary approaches could help to identify relevant bloom events, measure biotoxins in seawater (and in some cases air) and seafood, and identify symptoms in humans and other animals associated with exposures. In addition, communications with potentially affected communities could be conducted to promote the understanding that a particular symptom may be the result of exposure to a HAB biotoxin.

An example of such an approach can be found in the Gulf of Mexico, where the link between Karenia brevis blooms and human exposures and health effects, particularly to contaminated aerosols, was established. These efforts facilitated the successful understanding and mitigation of the human health risks associated with these events. This large and costly initiative included coordination among physicians, toxicologists, ecologists, stakeholders (including businesses, public health agencies, Poison Information Centres) and end-users (Fleming et al., 2005, 2011).

Another initiative with the same aim was the Harmful Algal Bloom-related Illness Surveillance System (HABISS) (http://www.cdc.gov/hab/surveillance.htm) that the US Centers for Disease Control and Prevention (CDC) and other public health and environmental organizations established to create a coordinated human, animal and environmental health surveillance network. The simultaneous collection of environmental and health data over time was considered to have helped public health practitioners identify long-term trends in HAB-related diseases in humans and animals. The CDC also created a module within the National Outbreak Reporting System (NORS) to capture HAB-related illnesses. Beta-testing of the system began in the summer of 2015.

In Europe, similar initiatives, although at a smaller scale, have been established in the Mediterranean region to develop tools and strategies to manage the impacts of Ostreopsis blooms on human health before they can have a wider impact. Examples conducted at local scale include France (Tichadou et al., 2010; Lemée et al., 2012) and the Spanish Catalan coast (Vila et al., 2012), and the Accord RAMOGE (http://www.ramoge.org) at Mediterranean region level (mainly on the coasts of Italy, Monaco, France and Spain).

**HABs dynamics in the context of global climate change**

Climate plays a fundamental role in the physical dynamics of the water masses and energy pathways in the ocean, which in turn modulate the biogeochemical fluxes and thus nutrient supplies to microorganisms at the bases of food webs (Beaugrand et al., 2010). Climate variability modulates ecological events (phenology) and ecosystem characteristics at different spatio-temporal scales (Longhurst, 2007). As a result, the dynamics of HABs, as a natural phenomenon, are also influenced by climate variability.

The Earth’s climate has changed continually over scales of millions of years, as evidenced by glacial and interglacial periods. Its climate also exhibits much shorter-term cyclic changes at multiannual to decadal and multi-decadal scales, such as those reflected in the El Niño Southern Oscillation (ENSO) and the North Atlantic Oscillation (NAO). Photosynthetic organisms have evolved and adapted with changes occurring at these different temporal scales (e.g. Beardall & Raven, 2004). In the last 200 years, however, since the industrial revolution, human activities have impacted the Earth dramatically, mainly as a consequence of the increasing release of carbon dioxide (CO₂), methane and nitrous oxides into the atmosphere. In particular, atmospheric CO₂ concentration has increased from 280 ppm to >380 ppm at present, with values of 570–1000 ppm predicted by 2100 (IPCC, 2008), in comparison to fluctuations between 180 and 300 ppm during the previous 800,000 years.

This rapid and uncontrolled rate of increase in so-called greenhouse gases has been responsible for an augmentation in about 1°C of global temperature in the past 20–30 years (data from the Hadley Centre for Climate Prediction and Research, shown in figure 1 of Hallegraeff, 2010), with a further rise of 2–4°C predicted over the next 100 years. Climate warming is already directly and indirectly impacting terrestrial and marine ecosystems. In the oceans, increased temperature in the upper layers can lead to changes in density which in turn affect the seasonal patterns of mixing, stratification and circulation, acidification, weakening or reinforcement of upwelling winds, and modification of the freshwater inflows to coastal regions (e.g. Bindoff et al., 2007). These changes may influence many ecological processes, including the occurrences of HABs. Given the impacts that HABs have on humans, there is now an urgent need to investigate the potential effects of climate warming on the occurrence of HABs. In addition, it will be important to develop tools for tracking and evaluating recent and future trends in HAB dynamics at both local and global scales.

The possible impacts of climate change on toxic marine HABs include: (1) range-changes in both warm- and cold-water species; (2) changes in abundance and toxicity; and (3) changes in the timing of the seasonal window of growth (e.g. Hays et al., 2005; Moore et al., 2008; Hallegraeff, 2010; Anderson et al., 2012; Fu et al., 2012). On the other hand, the effects of climate change on other components of the food webs (e.g. predators, competitors, parasites) could modulate the impact of climate change on the occurrence, magnitude and duration of HABs. Some HAB species may benefit such that their impact on human health and well-being becomes more severe, whereas others may diminish in areas that are currently impacted (Hallegraeff, 2010). Our ability to forecast the directions of change for toxic marine HABs is constrained by inadequate understanding of the interactions among multiple climate change variables and non-climate stressors in conjunction with inadequately
designed experiments for investigating decadal- or century-scale trends (Hallegraeff, 2010; Fu et al., 2012).

Few studies have investigated the effects of projected future climate change conditions on individual species or genera of toxin-producing marine microalgae. Of the few studies to date that have directly investigated future climate change effects on certain HAB species or genera, most have focused on the effects of warming. For example, the 2.5–3.5°C projected increase in sea surface temperature in the Caribbean over the coming century has been estimated to increase the incidence of CFP by 200–400% (Gingold et al., 2014). In Puget Sound, warming has been projected to increase the seasonal window of growth for Alexandrium by ~30 days by 2040, allowing blooms to begin earlier in the year and to persist for longer periods (Moore et al., 2015). It is important to remember that these projections are primarily based on projected changes to water temperature. Other aspects of bloom ecology and oceanography, such as the location of nutrient availability, competition with other phytoplankton species, grazing, and infection by parasites, were not considered. Therefore, while these initial assessments of greenhouse gas-driven changes to HAB risk provide insight into potentially important climate pathways that are relevant for bloom development, more work is needed to understand better the interactive effects of drivers other than temperature on HAB occurrence.

An emerging body of work is also focusing on the effects of ocean acidification on marine HABs. Some experiments performed on *Pseudo-nitzschia fraudulenta* found significantly high production of the domoic acid neurotoxin in response to a combination of low pH and some other factor that limits growth (Sun et al., 2011; Fu et al., 2012; Tatters et al., 2012a, b, 2013), provided that the limiting resource was not needed for toxin synthesis. Saxitoxin (paralytic shellfish toxin) production increased with elevated temperature and increased CO₂ supply (Kremp et al., 2012), although the response was highly strain specific. Much more work is needed to understand the effect of increasing ocean acidification on HAB species, and thus the potential effect on human health and the ecosystems under this scenario.

In order to evaluate future trends of HAB events, there is a need to establish the present baseline. Several studies report that the occurrence and geographic distribution of toxic marine HABs has increased in certain areas during the past few decades (Hallegraeff, 1993, 2010; Van Dolah, 2000; Lewitus et al., 2012). Increased awareness, expanded and better monitoring and detection of toxic HABs, and the multiple effects and interactions of human activities (including, but not limited to, increased nutrient loading to coastal waters, aquaculture development and transport of seed stock, ballast water transport) and climate warming may have contributed to this putative increase (Smayda, 1990; Hallegraeff, 1993, 2010; Sellner et al., 2003; Gowen et al., 2012). Direct observations of microalgae in coastal marine waters on timescales sufficient to evaluate climate change effects and to discriminate the role of other natural or anthropogenic forcings (e.g. nutrient loads) are typically lacking. This makes it difficult to establish whether the observed trends are global or local (Davidson et al., 2012). While a few isolated time series do exist (e.g. Borkman & Smayda, 2009; Kim et al., 2009), they have not yet been fully utilized to describe changes in the abundances of HAB species. In some cases, the available HAB data are from offshore, such as that generated by the Continuous Plankton Recorder (CPR) in the North Atlantic (Edwards et al., 2001, 2006; Hinder et al., 2012). Even though humans typically encounter HABs and their toxins at the coastal margins, these offshore time series provide valuable insights into the ecological responses of HAB species on timescales relevant to climate change. A small number of toxic HAB species leave a long-term record of their abundance in bottom sediments as microfossils (Dale et al., 2006) which could be used to explore time-series of bloom events. On the basis of similarities in the sediment cyst records in the Pacific and Atlantic regions of Canada, Mudie et al. (2002) concluded that climate change (including surface temperature and storminess) was a main factor stimulating blooms.

Reported illnesses are also an indicator of toxic HABs (Van Dolah, 2000); however, seafood is often consumed at long distances from where harvested. Unless robust information on the source of the seafood consumed is reported, it is difficult to identify potential relationships between food-borne HAB-related diseases and climate. Because most of the HAB-related diseases are associated with the consumption of contaminated shellfish, time series of HAB toxins in shellfish tissues provide valuable data for evaluating long-term trends and relationships to climate and in some cases date back to the 1950s (Trainer et al., 2003). The interactions of weather and climate are important for understanding patterns of disease, with climate determining the range of species and weather determining the timing and intensity of outbreaks (Dobson & Carper, 1993; Epstein, 2001).

At present, it is clear that we lack sufficient scientific understanding of climate-driven changes to nearshore marine environments, as well as HAB responses to these potential changes, to provide accurate predictions of future HAB occurrences in space and time. One option to improve understanding of these changes would be to facilitate multidisciplinary research and coordination including stakeholders and policy makers with the objective to mitigate the impacts of HABs on human health and well-being. The concern about this need motivates discussions and meetings in different fora, such as the Symposium on ‘HABs and Climate Change’ held in Göteborg, Sweden, 19–22 May 2015 (https://pices.int/meetings/international_symposia/2015/2015-HAB/scope.aspx).

**Mitigation strategies for aquaculture**

In Japan, several strategies have been adopted to minimize economic losses to aquaculture (Imai et al., 2014). In southwest Hokkaido’s Funka Bay, for example, the scallop culturing industry has adapted to frequent occurrences of dinoflagellate blooms that produce the dinophysis toxin and paralytic shellfish poisoning (PSP), which typically occur in May and continue into the summer. Modified approaches to harvesting were necessitated, in part, by high PSP contamination in the 1980s that stopped shipments of all bivalves. Timing the scallop harvest every year from December to April avoids the impacts associated with the toxic bloom season. Scallops are harvested both for immediate fresh consumption, and they can be inventoried by boiling and freezing in order to provide a source of supply during the closed season.

In the Okhotsk Sea, recent occurrences of paralytic shellfish poisoning contamination of scallops have been predicted on the basis of movements of the Soya Warm Current.
The strength of this current prevents the transport of *Alexandrium tamarense*, which typically resides in the offshore water masses of the Okhotsk Sea, to locations such as Monbetsu and Abashiri on the northern coast of Hokkaido. Adaptive harvesting in response to these forecasts is an effective way to avoid the impacts of toxic blooms on the bivalve aquaculture industry. This strategy is effective because of the fundamental understanding of local HAB dynamics.

Some strategies to mitigate the impacts of HABs on aquaculture may have been discounted prematurely and are worth revisiting. The use of clay to flocculate and remove toxic cells at some aquaculture sites in Asia (Sengco & Anderson, 2004) is one example. This approach could be studied further to explore tradeoffs between the economic losses associated with fish kills from HABs, the environmental consequences of clay dispersal, the costs of implementing the mitigation technology, and toxin accumulation in the benthos.

**Communication with the public and improving literacy about oceans and HABs**

Throughout history, humans have learned how to cope with natural phenomena, including HABs. In some areas, such as in the Pacific Islands (Chinain et al., 2010b), local traditional knowledge about ciguatera is functionally correct, albeit scientifically incomplete.

The Spanish explorer Alvar Nuñez Cabeza de Vaca (1490–1558) recorded a place along the northern coast of Mexico in the Gulf of Mexico, probably not far from Apalache Bay, where indigenous peoples were unable to relate the passage of time to the movements of the sun and the moon, but instead marked the seasons by fishkills (Ferrando, 1984), which could have been caused then, as now, by blooms of *Karenia brevis*.

To people making a living around the Galician Rías, red-tides (‘purgas de mar’) are familiar events, traditionally compared with menstruation, through which local waters are cleansed, usually in the autumn. It was general knowledge that it was unsafe to eat shellfish gathered when the ocean exhibited a reddish-brownish hue. When mass cultivation of mussels in the Galician Rías was undertaken by developers, it was general knowledge about ciguatera is functionally correct, albeit scientifically incomplete.

Fukuyo et al. (2002) note that in Northern Japan, local folklore advises not to eat shellfish during runoff of snow-melt into the sea occurring in the early spring. In the UK and the USA, there is a similar folklore (O’Connor 2008; Whyte 2011): one should eat shellfish only during months that are spelled with the letter ‘r’, i.e. avoid shellfish consumption the summer months (May to August) when HABs are more likely to occur (although bacterial contamination of the food cannot be discounted). Perhaps, as Fukuyo et al. (2002) suggest, such folklore has arisen because:

- this indicates that toxin contamination of shellfish has repeatedly occurred almost every year over a long time, leading to many tragedies among the local people. Community education resulted in self-regulating behaviour to avoid high-risk fish species and certain fishing locations.

Successful communication about HABs and their effects can be problematic for many different reasons. In part, this is due to the complexity of HAB phenomena, which are currently difficult to predict and to prevent or control. Most public health and medical personnel do not have in-depth knowledge about HABs and their potential effects on human health and wellbeing. Nowadays, many coastal communities are not fully informed of the public health risks of HABs, however, which can result in a phenomenon known as the ‘halo effect’, where human activities unrelated to the HAB hazards are scaled back or discontinued (Wessells et al., 1995; Whitehead et al., 2003; Parsons et al., 2006). A common form of the halo effect is a reduction in the consumption of all types of seafood, regardless of the actual risks of consuming phyco-toxins. Other forms of the halo effect can influence commercial and recreational fishing behaviour and coastal tourism. Consequently, it would be beneficial to improve the scientific messages about HABs to the many and diverse end users and stakeholders.

Fortunately, there are now examples of interesting and potentially successful dissemination and early warning activities related to safeguarding human health from HAB-related toxic episodes. For example, the US National Oceanographic and Atmospheric Administration (NOAA) produces HAB Bulletins which provide forecasting of HABs for managers in the Gulf of Mexico (for *Karenia brevis*), in the Pacific North-west (for toxic *Pseudo-nitzschia*) and the Great Lakes (for cyanobacteria). The bulletins incorporate oceanographic modelling, satellite imagery and on-the-ground monitoring, as well as other information (e.g. real-time reports from lifeguards and managers). These materials were developed with stakeholder engagement and input, so that the outputs are actually useful for and used by the people (such as beach managers) who need timely information. Another example of appropriate communication was the implementation of a toll-free, 24/7 telephone number at the Miami Poison Information Centre staffed by poison specialists trained to discuss HAB exposure and illnesses in Florida. This resource was signposted on information panels and brochures placed in beaches, hotels and other tourist venues. Another useful aspect of this centralized information resource was that the poison control phone calls also could be incorporated into HAB human health surveillance activities to increase case reporting (Fleming et al., 2011).

Examples in Europe include HAB bulletins that are produced weekly in both Ireland and Scotland for the aquaculture industries in these countries (https://www.marine.ie/Home/site-area/data-services/interactive-maps/weekly-hab-bulletin; http://www.somuchtosea.co.uk/news/ bulletin_for_shellfish_farmers.aspx). In the Baltic, the Swedish Meteorological and Hydrological Institute Service offers reports and almost real-time information about the algae situation in the Baltic obtained from satellite data (http://www.smhi.se/en/weather/sweden-weather/1.11631). In the NW Mediterranean, the RAMOGE Accord is working to communicate with the public and stakeholders, communicating through brochures and web pages, with dissemination activities about the occurrence of the *Ostreopsis* blooms and their impacts on human health and the environment, and searches to provide tools to manage them (http://www.ramoge.org/fr/ostreopsis_ovata.aspx). RAMOGE also fosters the coordination of international research around the understanding and management of these events in the affected countries.
International coordination: the GEOHAB and GlobalHAB programmes

HABs are a global challenge which needs to be addressed at local levels by implementing a broad global vision. During the last few decades, much work has been accomplished to understand HAB dynamics with the ultimate aim of predicting their occurrences and mitigating their impacts. Studies and monitoring of toxic phytoplankton and biotoxins have been conducted at local, national and regional levels, each with their own particular resources. In addition, international and interdisciplinary cooperation has been invaluable in advancing the science of HABs. As an international exemplar, this cooperative research has been fostered from 2000 to 2014, by the GEOHAB programme, ‘Global Ecology and Oceanography of Harmful Algal Blooms’ (http://www.geohab.info), with the financial support of SCOR (Scientific Committee on Oceanic Research) and IOC/UNESCO (Intergovernmental Oceanographic Commission of UNESCO). As stated in its Science Plan (GEOHAB, 2001): ‘[t]he scientific goal of GEOHAB was to improve the ability to predict HABs by determining the ecological and oceanographic mechanisms underlying their population dynamics, and integrating biological, chemical, and physical studies supported by enhanced observation and modelling systems.’

GEOHAB acted as an umbrella and catalyst for organizing scientific research on HABs. Moreover, GEOHAB provided a common and interconnecting aim for individual needs and efforts. The initiatives sponsored by GEOHAB, including open science meetings, specific workshops, and training activities, were publicized as reports, books and special issues in international journals (http://www.geohab.info).

At the termination of GEOHAB in 2014, the international scientific community agreed that a coordinated research approach to HABs was beneficial and still necessary (see GEOHAB, 2014). Starting in 2015, a new programme, GlobalHAB, will build on the GEOHAB Science Plan, incorporating the present challenges and opportunities that international research on HABs requires (Figure 3). Taking multidisciplinary and international perspectives, GlobalHAB will integrate key aspects of climate warming and global change on HABs within the context of the field of ‘oceans and human health.’

![Fig. 3. Main elements of the international GEOHAB (2000–2014) and GlobalHAB (2015–2018) programmes, that aim to contribute to the advance of the research on HABs.](image)

CONCLUSIONS

Microalgal blooms are a natural part of the seasonal cycle of the marine ecosystems around the world. They are key components of the structure and dynamics of the oceans and thus sustain the benefits (food supply, recreation, commerce, livelihood,) that humans obtain from this aquatic environment.

However, some microalgal blooms can be harmful to human health, impact valuable fisheries, and degrade other marine and coastal ecosystem services. The impacts of these HABs comprise acute and chronic health effects in humans, financial losses from contaminated seafood, mortalities of farmed fish, reduced coastal tourism and altered socio-cultural aspects. The losses due to HABs of passive values that humans place on protected species and extraordinary marine ecosystems are more difficult to quantify. There is evidence in some littoral areas that human pressures may have increased the occurrence of HABs. Further, global warming and changes in the climate regime also could affect HAB occurrences and toxicity, although forecasting the possible trends is still speculative.

At the beginning of the 21st century, with expanding human populations, particularly in coastal and developing countries, there is an urgent need to prevent and mitigate the impacts that HABs pose on human health and wellbeing. Because HABs are natural phenomena, it is not possible to prevent their occurrence. However, the scientific research conducted over the last four decades, with the support of stakeholders, policymakers and the general public, has improved the understanding of HAB dynamics.

The achieved insights allow defining key priorities and designing strategies to mitigate the HABs impacts. In this paper, we have summarized part of the present knowledge and available tools to address this general objective. Around the world, at varying levels of government, certain promising efforts are being attempted to mitigate HAB impacts. These efforts include:

- Integrating both ecosystem and human health monitoring for HAB impacts. This involves maintaining or expanding existing HAB and biotoxin monitoring, and implementing new monitoring programmes where necessary. It also includes the monitoring of physico-chemical and meteorological variables to help ascertain the real effects of climate and other environmental changes on HAB occurrences and their impacts.
- Maintaining and reinforcing initiatives and local and international policies to reduce human pressures on the marine environment that may increase the occurrence of HABs and the severity of associated events.
- Developing an increased public “ocean literacy” and expanded engagement with coastal and ocean stakeholders.

We have also identified key research needs that, if addressed, could greatly enhance our understanding over HABs and their impacts on health. These questions include:

- Ascertaining real trends in ciguatera fish poisoning incidence, and provide informed projections of potential future trends.
- Ascertaining the risks of new emerging HABs and biotoxins (e.g., azaspiracid food-borne poisonings, BMAA and
neuromuscular diseases, and respiratory irritation in new areas impacted by *Ostreopsis* blooms).

- Investigating the responses of HABs (especially regarding toxin production) to multifactorial physico-chemical climate drivers and the potential of marine microalgae to adapt genetically and phenotypically to the unprecedented rapidity of current climate and other environmental changes. This will require multidisciplinary collaboration and appropriately scaled experimental designs.

- Investigating possible interconnections between sociocultural aspects of human wellbeing and HAB events.

- Advancing in the estimation of the economic costs of HAB events.

Overall, we emphasize that collaborative research across natural and social scientific disciplines, as for example, the Florida Red Tide Research Group and GEOHAB, can lead to significant advances in our understanding of HABs, helping to develop approaches at local and global levels to lessen their impacts on public health and human wellbeing.

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The association between marine bathing and infectious diseases – a review

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Worldwide, infectious diseases represent a leading cause of death and disability. Exposure to the ocean, whether through recreation or occupation, represents a potentially significant, but poorly understood, source of infectious diseases in man. This review describes the potential mechanisms whereby marine bathing could lead to infectious diseases in man. Sources of pathogens in the marine environment are described, including human sewage, animal sources, fellow bathers and indigenous marine organisms. The epidemiological evidence for the association between marine bathing and infectious disease is presented, including a consideration of the differing relationship between faecal indicator bacteria levels and illness at point source compared with non-point source settings. Estimating the burden of infectious disease is reliant on public health surveillance, both formal and informal, which is described from a UK perspective in this review. Potential emerging threats at the marine–human interface are discussed, including infections caused by Shewanella and Vibrio bacteria, and the presence of human pathogens in the marine environment that are resistant to antimicrobials.

Keywords: infectious disease, marine pollution, faecal indicator bacteria, emerging infections

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INTRODUCTION

Infectious diseases in man result from invasion by microbes and subsequent injury, or, less commonly, through contact with non-invasive microbial toxins (Spicer, 2008). Pathogenic organisms, i.e. those capable of causing disease, range from microscopic agents such as prions and viruses – incapable of independent reproduction or prolonged survival – through to bacteria, eukaryotic organisms like moulds, fungi and larger protozoa, and then multicellular animals such as helminths.

Worldwide, infectious diseases represent a leading cause of death and disability, with over 3 million deaths from lower respiratory infections and 1.5 million from diarrhoeal illnesses in 2012 (World Health Organization, 2014); food and waterborne illnesses are estimated to kill 2.2 million people per year, most of whom are children (World Health Organization, 2012). Human exposure to marine micro-organisms can occur as a result of bathing, recreation, the food chain and occupation. Nonetheless, any potential risks from infectious disease need to be balanced against the benefits of exposure to the marine environment, including as a source of food and the demonstrable effects on physical and mental well-being (Depledge & Bird, 2009).

With estimates of more than a million bacteria present per mL of seawater, and 10–100 times as many viral particles, the ocean represents a potentially significant, but poorly understood, source of infectious diseases in man; and of the estimated 1 billion marine bacterial species only a small proportion have been characterized (Thomas & Bowers, 2012). Human exposure to marine micro-organisms can occur as a result of bathing, recreation, the food chain and occupation. Nonetheless, any potential risks from infectious disease need to be balanced against the benefits of exposure to the marine environment, including as a source of food and the demonstrable effects on physical and mental well-being (Depledge & Bird, 2009).

This review will present the theoretical basis of infectious disease transmission, and how it relates to marine bathing; describe sources of marine pathogens; and present evidence to support associations with disease. Additionally, infectious disease surveillance and the risks of emerging infections related to marine bathing will be considered. Finally public health measures informed by evidence and research priorities to reduce the burden of disease will be presented.

INFECTIOUS DISEASE TRANSMISSION

For a host to become infected with an infectious disease, a sequence of events – referred to as the Chain of Infection – needs to occur (United States Centers for Disease Control & Prevention, 2015). A pathogenic organism leaves its reservoir through a portal of exit, reaches a host via a mode of transmission, entering through a portal of entry to cause infection and disease in a susceptible host. Infection control strategies are focused on breaking one or more links in the chain.

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Potential reservoirs for marine bathing-related infection include seawater – with or without anthropogenic input (Kay et al., 1994; Wade et al., 2003; Tolba et al., 2008; Vignier et al., 2013), beach sand and sediment (Solo-Gabriele et al., 2000; Beversdorf et al., 2007; Yamahara et al., 2007), zooplankton (Huq et al., 1990), fellow bathers (Plano et al., 2011) and animals (Wang et al., 2010). Gastrointestinal pathogens, for example norovirus and E. coli, leave human and animal hosts respectively through faeces (Ihekweazu et al., 2005), whereas skin shedding of methicillin-resistant Staphylococcus aureus may potentially increase the risk to fellow bathers (Tolba et al., 2008; Plano et al., 2011, 2013). The mode of transmission for gastrointestinal pathogens is faeco-oral – once organisms leave an infected host subsequent ingestion can lead to infection. In addition to faeco-oral transmission, marine bathers may become ill through inhalation of pathogens in marine aerosols – leading to respiratory tract disease – and through direct contact with skin, ears and eyes.

Numerous host factors determine susceptibility to marine-derived pathogens and the severity of any subsequent illnesses. Breaches of innate defences (e.g. skin ulcers and penetrating trauma) increase the risk of bathing-related infection (Vignier et al., 2013; Janda & Abbott, 2014); subsequent disease risk depends on age (Lepesteur et al., 2006; Wade et al., 2008); co-morbid conditions such as diabetes mellitus and impaired immunity (Frank et al., 2006; Vignier et al., 2013); and the presence of immunity from previous infection (Prietó et al., 2001) or vaccination. The ability of an organism to cause disease, and the subsequent severity, is termed ‘virulence’; some pathogens, such as noroviruses, generally cause mild self-limiting illness, whereas ingestion of a limited number of Verotoxin-producing E. coli can lead to severe illness and even death.

The development of infectious disease in man is determined by complex interactions between the host, pathogens and the environment (Scholthof, 2007). This interaction is apparent in marine bathing; host behaviour is associated with the illness, with activities that increase duration of exposure, and swallowing of water increasing risk (Wade et al., 2003; Papastergiou et al., 2012; Harder-Lauridsen et al., 2013); increased risk is suggested with surfing activities, diving and athletic events (Bradley & Hancock, 2003; Wade et al., 2003; Schijven & de Roda Husman, 2006; Harder-Lauridsen et al., 2013). Environmental conditions such as eutrophication, sunlight and rainfall will all determine the growth and fate of micro-organisms and so alter the probability of disease (Yau et al., 2014). In a single study, swallowing water was a risk factor for gastrointestinal illness only in the presence of high groundwater discharge and low solar radiation (Yau et al., 2014).

Bradley & Hancock (2003) elegantly demonstrated an example of the complex host-pathogen-environment relationship in marine bathing. During meteorological conditions with greater wave height; this is associated with higher numbers of bathers participating in high risk activity (surfing) and where levels of faecal indicator bacteria were significantly higher than during calm weather (Bradley & Hancock, 2003).

**SOURCES AND SURVIVAL OF PATHOGENS IN THE MARINE ENVIRONMENT**

The predominant sources of human pathogens in the marine environment in the UK are considered to be from: human sewage, whether through poorly connected domestic sewers or combined sewage outflows or treated waste; agricultural run-off; urban area run-off; and animal and bird faeces (Environment Agency, UK, 2014).

Faecal indicator bacteria (FIB) are a limited number of organisms (typically Coliforms, E. coli, faecal Streptococci and Enterococci) used as markers for the presence of sewage containing human faeces. Not necessarily pathogenic per se, FIB are used as a proxy measure for the vast number of pathogens potentially present in human waste (Wade et al., 2008). Measurement of these organisms is undertaken widely and their association with disease described in areas with point sources of microbial pollution, but the discharge of pathogenic organisms into the sea and their persistence, survival and subsequent virulence are poorly understood. Microbial source tracking has demonstrated potential in identifying the sources of pathogen in the marine environment (Rusinol et al., 2014).

Current established methods for measuring FIB are based on culturing organisms; therefore requiring a minimum of 24 – 36 h to provide results. Novel molecular methods, specifically quantitative Polymerase Chain Reaction (qPCR), can provide measures of microbial contamination in bathing waters within a few hours and therefore have the potential to inform short-term public health interventions such as beach closures (Oliver et al., 2014). There is some epidemiological evidence linking qPCR measures of FIB and illness (Wade et al., 2010), but further studies would be required if slower, culture-based measures are to be replaced (Oliver et al., 2014).

Many human pathogens have been identified in seawater and the marine environment, including: viruses – adenovirus (Pina et al., 1998; Jiang & Chu, 2004; Colford et al., 2007), noroviruses (Victoria et al., 2010), enteroviruses (Pina et al., 1998; Jiang & Chu, 2004) and hepatitis A (Jiang & Chu, 2004); bacteria – S. aureus (including methicillin-resistant Staphylococcus aureus (MRSA)) (Plano et al., 2013), Bacteroides spp. (Colford et al., 2007), Clostridia (Wade et al., 2010), also demonstrated in sand (Heaney et al., 2012; Plano et al., 2013), Pseudomonas spp. (Khan et al., 2010); protozoa – Cryptosporidia in seawater (Fayer et al., 1998) and giardia in the faeces of marine mammals (Measures & Olson, 1999).

*Salmonella* bacteria have been found in small numbers of samples of bathing water classified using FIB measurements as excellent by European bathing water standards; *Salmonella* levels were positively correlated with FIB levels (Efstatiou & Tsirtsis, 2006).

In addition to pathogens from sewage, run-off and animal sources, there is potential for colonized individuals to infect others with potentially pathogenic organisms. Under experimental conditions mimicking marine bathing, individuals can release $10^7 - 10^6$ S. aureus per 15 min immersion; with 15–20% of isolates being resistant (MRSA), capable of causing serious soft-tissue infections (Plano et al., 2011). Plano et al. (2013) demonstrated a correlation between bather density and concentrations of S. aureus in sub-tropical recreational waters, with levels of 2–780 colony forming units (CFUs) per mL (Plano et al., 2013). Experimental evidence suggests that S. aureus may be able to persist in seawater for several days (Fujikawa & Umuta, 2006). Further experimental work suggests that individuals shed in the region of 1 million *enterococci* per bathing episode (Elmir et al., 2007, 2009) and potentially up to the same number of

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- Measures & Olson, 1999
- Pina et al., 1998
- Jiang & Chu, 2004
- Colford et al., 2007
Bacteroidales – a potential indicator of human contamination (Elmir et al., 2009).

In addition to faecal pathogens from external sources, indigenous marine bacteria are capable of causing disease in humans; examples include Vibrio spp. (Huq et al., 1990; Andersson & Ekdahl, 2006; Frank et al., 2006; Kushawaha et al., 2010), Shewanella (Dominguez et al., 1998; Vignier et al., 2013; Srinivas et al., 2015) and mycobacteria (Cheung et al., 2010). In a review of 171 patients from a coastal area of South Korea diagnosed with necrotizing fasciitis – a potentially fatal soft-tissue infection – marine bacteria were considered to be the causal pathogen in two-thirds of cases (Park et al., 2009).

**SURVEILLANCE OF INFECTIOUS DISEASES**

Public health surveillance is defined as “the continuous, systematic collection, analysis and interpretation of health-related data needed for the planning, implementation, and evaluation of public health practice” (World Health Organization, 2015b); robust systems are fundamental to public health practice. Surveillance data are used to identify trends, both short- and long-term; identify disease outbreaks and emerging threats; provide hypotheses of causal associations; and provide policymakers with evidence to prioritize resources. Surveillance can take many forms – both formal and informal – and recent advances in genomics have the potential to provide public health authorities with additional information with which to identify previously elusive shared exposures among cases of infectious disease (Dallman et al., 2015).

Many European countries have well-developed infectious disease surveillance systems; as in many countries, in the UK registered medical practitioners are legally required to notify certain infectious diseases – whether suspected or confirmed. Notifiable diseases listed in the UK Public Health (Control of Disease) Act 1984 include several relevant to marine exposure, including Verotoxin-producing E. coli (Ihekweazu et al., 2005), Vibrio cholerae (Huq et al., 1990) and Campylobacter spp. (Harder-Lauridsen et al., 2013). Despite robust systems being in place, cases reported represent only a small proportion of persons infected, particularly if their illness is mild and self-limiting; based on data from UK primary care, it is estimated that for every case of gastrointestinal infection reported to national surveillance, there are 10 primary care consultations and 147 cases in the community (Tam et al., 2012). In addition to physician reporting, there is a reporting system which collects laboratory confirmed infections to public health; as described previously, this will only detect the tip of the ‘clinical iceberg’ in milder disease, but would be expected to detect a larger proportion of more severe infection such as Verotoxin-producing E. coli that can cause bloody diarrhoea and haemolytic-uraemic syndrome (Heymann, 2008).

Formal notification provides only part of the information used to monitor infectious diseases in the UK; many other sources of data are available. In England a syndromic surveillance team analyses health data from several sources in real-time, allowing rapid dissemination of trend data to identify emerging threats (Public Health England, 2014b); syndromes pertinent to marine exposure include gastroenteritis and acute respiratory infection. Syndromic surveillance has demonstrated research utility in the field of environmental and human health (Elliot et al., 2013).

In addition to formal surveillance by public health authorities, additional sources of data can provide insights into the emergence of infectious disease outbreaks, particularly in areas where robust systems are absent. In 2003, the World Health Organization (WHO) was alerted to the emergence of the SARS epidemic in Guangdong Province, China by an early warning tool searching global media reports; this method detected the emergence of SARS significantly in advance of formal surveillance (Heymann & Rodier, 2004; Keller et al., 2009). In addition to public health bodies, stakeholders (including charities), have set up surveillance systems. For example, Surfers Against Sewage (SAS), a UK environmental charity, collects reports of illness among bathers/surfers, and has conducted a self-reported beach user health survey in collaboration with the European Centre for Environment and Human Health (ECEHH; http://www.ecehh.org) in the UK; there is an antecedent to the use of online surveys to record water-associated illness (Turbow et al., 2008).

Identifying infectious disease outbreaks related to recreational use of the marine environment poses challenges as exposed populations quickly disperse, particularly tourists. In response to notified cases of infectious diseases in the UK, questionnaires are administered in an attempt to identify shared exposures and therefore potential sources of disease; for example, persons diagnosed with Verotoxin-producing E. coli in England are specifically asked about any site and type of exposure to seawater. In 2006, an outbreak of seven cases of E. coli was identified associated with playing in the same beach stream (Ihekweazu et al., 2005). By their nature, diseases with longer and less precise incubation periods, such as some types of viral hepatitis, often identify a greater number of potential causal exposures; apparent clustering of cases related to a single exposure therefore becomes harder to identify.

The use of questionnaire data to identify epidemiological links requires considerable resource and cooperation from cases, often introducing additional delay in the process of identifying common exposures. Novel molecular techniques, such as whole-genomic sequencing, are beginning to provide opportunities to rapidly identify common exposures among populations that are temporally and geographically dispersed (Dallman et al., 2015). Nevertheless this approach may be negated by outbreaks caused by several pathogens released following a particular environmental or anthropogenic event (Harder-Lauridsen et al., 2013).

**EVIDENCE FOR THE ASSOCIATION BETWEEN MARINE BATHING AND INFECTIOUS DISEASE**

Numerous studies have demonstrated an increased risk of gastrointestinal illness (Corbett et al., 1993; Kay et al., 1994; Colford et al., 2007; Wade et al., 2008; Fleisher et al., 2010; Papastergiou et al., 2012; Yau et al., 2014), ear infections (Corbett et al., 1993; Fleisher et al., 1996; Papastergiou et al., 2012; Wade et al., 2013), eye infections (Corbett et al., 1993; Fleisher et al., 1996; Papastergiou et al., 2012), respiratory illness (Corbett et al., 1993; Prieto et al., 2001; Fleisher et al., 2010).
The association between levels of FIB and illness among swimmers has been studied. Kay et al. (1994) demonstrated an association between any illness and faecal streptococci counts measured at chest level, suggesting a threshold level of 32 CFUs per 100 mL below which there is no increased risk (Kay et al., 1994). In a systematic review and meta-analysis, Wade et al. (2003) estimated that for a log$_{10}$ unit increase in enterococci, the risk of gastrointestinal illness was increased by 1.34 times; additionally noting an association between enterovirus and illness in a small number of studies (Wade et al., 2003). The presence of any illness following marine bathing has been shown in a single study to be more closely associated with mean coliform counts than faecal streptococci levels (Corbett et al., 1993). Furthermore, levels of enterococci in sand are positively associated with gastrointestinal illness among beachgoers digging or buried in sand (Heaney et al., 2012).

Despite evidence of association between FIB levels and illness, this is not a consistent finding. Colford et al. (2007) demonstrated higher rates of gastrointestinal and skin symptoms among a cohort of swimmers compared with non-swimmers, but no association between enterococci or faecal coliform levels and reported illness. Likewise, the BEACHES study found elevated incidence of gastrointestinal, acute febrile and skin illness in bathers compared with those non-bathing bathers under sub-tropical conditions, the only association with FIB levels was between enterococci levels and skin illness (Fleisher et al., 2010). Both studies were carried out at beaches with a non-point source run-off, suggesting that where high exposure to an output of human waste is not the predominant source of pathogens traditional FIB may not be predictive of human health impacts (Colford et al., 2007). Microbiological testing conducted in areas of California urban run-off have demonstrated a lack of association between adenovirus, a potential viral pathogen, and FIB levels (Jiang et al., 2001).

Under the European Union revised Bathing Water Directive (2006/7/EC), coastal water is classified as 'excellent' and awarded 'blue flag status' if E. coli are predominately below 250 per 100 mL and intestinal enterococci below 100 per 100 mL. Among bathers at sites with demonstrable excellent water quality by EU standards, there was still evidence of increased gastrointestinal, respiratory, eye and ear infections compared with non-bathers; with 3% of bathers having gastroenteritis compared with 0.7% of non-bathers in the 10 days following exposure (Papastergiou et al., 2012). Furthermore, it is estimated that at the threshold for excellent bathing water quality for enterococci by European standards, it would be expected that 2.5–4.0% of bathers would develop gastrointestinal illness (Saliba & Helmer, 1990). Estimates suggest that 71% of gastrointestinal episodes in Southern California occur when the water quality is considered safe for bathing (Brinks et al., 2008), indicating that a 'prevention paradox' may exist – most cases of illness associated with marine bathing arise from the lowest risk exposure, but higher numbers of people exposed; this observation has implications for preventative strategies aimed at reducing the burden of disease.

The majority of epidemiological studies investigating the associations between marine bathing and illness have relied on self-reporting to define cases; however, there are clearly limitations to this approach. Bathers may be more likely to report symptoms if they believe they have had an exposure, such as head immersion, and in retrospective studies 'recall bias' may lead to increased reporting of events – such as swallowing water – in persons who have become unwell. A limited number of studies have attempted to obtain microbiological confirmation of the cause of illness; an association between exposure to seawater and confirmed staphylococcal skin infections has been shown (Charoenca & Fujioka, 1995). Following heavy rainfall in Copenhagen, 42% of competitors in a sea-swimming event reported gastrointestinal illness; competitors who submitted stool samples had confirmed illness as a result of various pathogens including Campylobacter jejuni, Giardia lambia and diarrheagenic E. coli. However, microbiological testing of the vast majority of stool samples submitted by competitors did not reveal any pathogens, suggesting a viral cause for illness in these competitors as very few specimens were tested for viruses (Harder-Lauridsen et al., 2013).

Simulation models incorporating bathing water quality have estimated a considerable burden of disease related to bathing in coastal waters in Southern California, with 689,000 to over 4 million episodes of gastrointestinal disease and 693,000 episodes of respiratory illness among the 100 million visitors per year (Brinks et al., 2008). Marine-bathing associated earache in the United States has the potential to result in 260,000 visits to the doctor, and direct health costs of nearly US$60 million (Wade et al., 2013).
and *Shewanella algae* as potential emerging human pathogens (Goyal et al., 2011; Vignier et al., 2013; Janda & Abbott, 2014). *Shewanella* infection, of which 80% are caused by *S. algae*, can lead to skin and soft tissue infection, septicaemia, ear infections and death (Goyal et al., 2011; Vignier et al., 2013; Janda & Abbott, 2014; Srinivas et al., 2015). Case series of *Shewanella* infection, with case-fatality rates of 20–25%, have been reported in warmer climates, including Martinique and India (Vignier et al., 2013; Srinivas et al., 2015); although a systematic review by Vignier et al. (2013) identified cases from northern Europe, including France, Denmark and the UK (Vignier et al., 2013). The majority (Vignier et al., 2013) or all (Srinivas et al., 2015) of *Shewanella* cases in reported series had skin breaks or penetrating injury providing a portal of entry; a risk factor often identified along with chronic health conditions with potential immunosuppression such as diabetes mellitus in people infected with marine indigenous bacteria (Gomez et al., 2003; Frank et al., 2006; Aighibivulu & Maraqa, 2009; Cheung et al., 2010). Multidrug resistance has been demonstrated in some isolates of *Shewanella* (Holt et al., 2005). During a notable warm summer in 1994 in Denmark, *S. algae* was isolated from ear swabs from 67 patients mostly aged between 3–15 years; most of the patients had symptoms of acute or chronic otitis (52%) or a non-specific aural discharge; *S. algae* was isolated from bathing sites in the same area where cases were identified (Holt et al., 1997).

Vibrios are bacteria found in estuarine and salt water environments; species that cause illness in man include *V. cholerae* – causing gastrointestinal illness (including cholera with toxin-producing forms); *V. parahaemolyticus* – gastroenteritis; *V. vulnificus* – skin infections, septicaemia and death; and *V. alginolyticus* – wound, ear and eye infections (Spicer, 2008). In 2006, a notably warm summer in Europe when sea temperatures across much of the Baltic Sea were >20°C, cases of *V. vulnificus* and non-toxin-producing *V. cholerae* were reported in Germany and Sweden, respectively; cases of vibrio infection were also reported in Denmark in the same year including a fatality associated with *V. vulnificus* – all cases identified in the three countries had bathed in the Baltic Sea (Frank et al., 2006). *Vibrio* spp. were identified widely in the Baltic Sea in July and August 2006 (Frank et al., 2006) and have since also been found to be common in Dutch waters (Schets et al., 2011); cases of *V. alginolyticus* were detected in people bathing in Dutch Waters of the North Sea in 2006 (Schets et al., 2006). The pandemic strain of *V. parahaemolyticus* has recently been identified in UK waters for the first time (Powell et al., 2013); it has been suggested that, as patterns of infection with *Vibrio* spp. are associated with climate trends, increasing sea temperatures have the potential to increase the range and burden of related disease. Furthermore, following Hurricane Katrina, an extreme weather event in 2005, there were five reported deaths from vibrio-related wound infections in persons from affected states (United States Centers for Disease Control & Prevention, 2005). Robust surveillance systems will be required to detect emerging trends (Baker-Austin et al., 2010).

Antimicrobial resistance (AMR) is a major emerging global public health threat that challenges our ability to treat common infectious diseases; resistant strains can emerge and disseminate worldwide resulting in a highly significant disease burden (World Health Organization, 2015c). *Escherichia coli* resistant to antibiotics, notably third-generation cephalosporins, have been demonstrated in coastal waters across England and Wales, posing a potential risk to recreational users through ingestion; although the clinical significance of this exposure is uncertain (Leonard et al., 2015). The ‘Beach Bums’ study, conducted by the ECEHH (http://www.ecehh.org), will use rectal swabs from regular surfers to investigate gut colonization by AMR bacteria in this group highly exposed to seawater.

**CONCLUSIONS**

Current evidence suggests that the burden of infectious disease from marine bathing is significant at a societal level, although, with notable exceptions, less so individually. Priorities for further research include better understanding of sources, reservoirs and the burden of disease from pathogens in the marine environment. Additional studies are required to identify appropriate markers for risk at non-point source beaches. The use of novel, rapid, molecular techniques can potentially inform rapid interventions to protect the health of bathers. Nonetheless, despite limitations, practical advice can be given to bathers based on current evidence to minimize their risk of illness; the public should be advised to avoid bathing following heavy rainfall, not ingest seawater, cover all skin lesions, shower with fresh water before and after bathing, wear protective equipment if necessary, and be aware of the additional risks in the presence of impaired immunity and open wounds. Bathers who become unwell should seek medical attention, with medical practitioners encouraged to seek microbiological confirmation of the cause of illness.

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Beach sand and the potential for infectious disease transmission: observations and recommendations

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Recent studies suggest that sand can serve as a vehicle for exposure of humans to pathogens at beach sites, resulting in increased health risks. Sampling for microorganisms in sand should therefore be considered for inclusion in regulatory programmes aimed at protecting recreational beach users from infectious disease. Here, we review the literature on pathogen levels in beach sand, and their potential for affecting human health. In an effort to provide specific recommendations for sand sampling programmes, we outline published guidelines for beach monitoring programmes, which are currently focused exclusively on measuring microbial levels in water. We also provide background on spatial distribution and temporal characteristics of microbes in sand, as these factors influence sampling programmes. First steps toward establishing a sand sampling programme include identifying appropriate beach sites and use of initial sanitary assessments to refine site selection. A tiered approach is recommended for monitoring. This approach would include the analysis of samples from many sites for faecal indicator organisms and other conventional analytes, while testing for specific pathogens and unconventional indicators is reserved for high-risk sites. Given the diversity of microbes found in sand, studies are urgently needed to identify the most significant aetiological agent of disease and to relate microbial measurements in sand to human health risk.

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INTRODUCTION

Transmission of infectious diseases in terrestrial beach environments can occur via direct exposure to microbes found in sand or through the flux of microbes from water to sand within the swash or intertidal zone. Exposure to pathogens can include routes such as dermal contact, contact with eyes and ears, inhalation and ingestion. Recent studies suggest that direct exposure to beach sands is a risk factor for infectious disease, particularly in children. An epidemiological study found that gastrointestinal (GI) illness in beach users was associated with exposure to water and intertidal sand (Bonilla et al., 2007; Pinto et al., 2012a, b; Sabino et al., 2014a). A separate epidemiological study (Heaney et al., 2009) found that digging in the sand was positively associated with GI illness and was associated with levels of faecal indicators (FIO), enterococci (Heaney et al., 2012). While correlations between beach sand exposure and infectious disease exist, the specific causative mechanisms of infection are yet to be shown including identification of the aetiological agent.

In addition to direct exposure, sand can also serve as a vehicle for transferring pathogenic microbes to and from the adjacent water. Studies on southern Lake Michigan found that densities of *Escherichia coli* were highest in core samples taken from foreshore sands, often by several logs, but rapidly decreased from that maximum both landwards and lakewards (Whitman & Nevers, 2003; Kinzelman et al., 2004; Whitman et al., 2006a). Studies at marine beaches in Florida found that the intertidal zone (Figure 1), in particular the upper fringe of this zone, was a hot spot for the accumulation of microbes (Shibata et al., 2004; Wright et al., 2011). These microbes can be mobilized from this reservoir by mechanisms such as wave action (Phillips et al., 2014), pore water transport (Phillips et al., 2011b), and then impact adjacent water quality (Phillips et al., 2011a). Microbes may also be re-deposited in sand by incoming current and waves (Ge et al., 2010). Maximal FIO levels in both marine and freshwater beaches generally occur just behind the highest wave up-rush along the beach. Of particular significance is that the foreshore or intertidal zones are areas where beach goers congregate, and where children tend to play with sand. Thus, the swash zone and foreshore is a dynamic area of the shoreline in terms of microbe accumulation and potential exposures. It is also a zone where the interactions between the water and sand are strongest allowing for an interchange of microbes to and from this zone.

To evaluate the current state of knowledge and most pressing research needs in the area of beach sand microbiology, a panel session was convened as part of the TEMP/H2014 (Trends in Environmental Microbiology and Public Health, 2014) conference held in Lisbon, Portugal during September 2014. The purpose of the panel was to discuss the potential inclusion of sand quality assessments in monitoring programmes for recreational beaches. The interdisciplinary group of participants held particular expertise in one of two primary categories, recreational beach water quality and environmental mycology. The ideas presented in this article provide an interesting meld of concepts that would benefit beach sand monitoring programmes. Specifically, this work begins by reviewing documents that provide recommendations for changes to existing recreational water quality monitoring guidelines (‘Call-to-Action’ documents), and expands upon these documents by emphasizing the merits of including measures of sand. The manuscript then focuses on describing the spatial distribution and temporal characteristics of microbes in sand, which is necessary for developing general recommendations for sampling programmes. Recommendations for sampling programmes begin by identifying appropriate beach sites and inclusion of beach sanitary assessments. Strategies for sampling and analysis follow, including an emphasis on recommending which microbes to measure and on advances in microbe measurement techniques. We conclude with an identification of research needs and a call for the inclusion of microbial monitoring in sand as an integral part of routine beach health assessments.

OVERVIEW OF ‘CALL-TO-ACTION’ DOCUMENTS

Microbial contamination in recreational waters is monitored through measures of FIOs, including ‘generic’ (non-pathogenic) *E. coli* and enterococci (EU, 1976, 2006; USEPA, 1986). The enterococci are a group of bacterial species belonging to the genus *Enterococcus*. FIOs are seldom in themselves pathogenic, but since they are found in faeces of both human and animals they are useful indicators for faecal contamination of water. The ubiquitous distribution of FIOs in faeces stands in contrast to the relatively infrequent occurrence of pathogens, whose detection is complicated by their great diversity. Reliance on FIOs for water quality assessment is thus a matter of practicality, as a set of general targets that are highly concentrated and widely distributed in faeces provides an economical metric, while testing large volumes of water for innumerable pathogens is impractical for monitoring.

The sole reliance on FIO levels in water as a mechanism for classifying recreational waters was challenged by the Annapolis Protocol, a document prepared for the World Health Organization and authored by many of the acknowledged world experts in the field (WHO, 1999), well over a decade ago. The Annapolis Protocol first identified the value of a comprehensive sanitary inspection of recreational waters to identify all sources of potential pathogens, a concept further developed over the past decade (Boehm et al., 2009a; Gooch-Moore et al., 2011; Abdelzaher et al., 2013). The Annapolis Protocol introduced the concept of a risk-based approach, and acknowledged that common FIO standards across all waters did not account for the unequal probability of pathogen presence in faecal contamination from different sources. In particular, it identified the probable...
reduced health risk when FIOs were primarily from non-human sources. Many of the recommendations in the Annapolis Protocol were incorporated into the World Health Organization (WHO) guidelines for recreational waters (WHO, 2003, 2009). Implicit in the WHO approach is the notion that health risks are unacceptable when they exceed a set FIO threshold level.

As understanding of the differential risk inherent in faeces from different sources was accumulating, microbial source tracking (MST) emerged as a discipline. The goal of MST is to determine the host animals responsible for faecal contamination of water. It is accomplished by analysis of host-associated microorganisms (or host genes in the case of mitochondrial DNA) in the faeces of humans and various animals (reviewed in Stoeckel & Harwood, 2007; Harwood et al., 2014). In 2005 the US Environmental Protection Agency (EPA) produced a document that outlined the uses of MST to aid in total maximum daily load (TMDL) and risk assessment for recreational waters (USEPA, 2005). Since that time, the ability to discriminate among different sources of contamination in water has steadily improved, and MST has been used to explore FIO sources in beach sand (Russell et al., 2013).

The 2003 WHO report has an extensive review of the risk from microbes in sand, but concluded that there was insufficient evidence to support the establishment of a guideline value for indicator or pathogenic microorganisms in beach sand. Neither the subsequent European Union (2006) Bathing Water directive, nor the USEPA (2012) ‘NEEAR’ criteria consider sand contamination, other than acknowledging that it may be a source of FIOs in the adjacent water (EU, 2006; USEPA, 2012). Health Canada recreational water quality guidelines indicate that testing of sand may be warranted in circumstances such as support for sanitary surveys or disease outbreak investigations, though stated that more research was needed before guideline values for sand could be established (Health Canada, 2012). Thus, no regulatory criteria exist currently for microbial levels in sand.

**MICROBIAL CHARACTERISTICS OF BEACH SAND**

**Microbes found in beach sand**

Numerous studies have been conducted that document the existence of pathogenic microbes in beach sands, providing evidence for sand as a potential reservoir for aetiological agents of disease (Whitman et al., 2014; Sabino et al., 2014a). The pathogenic organisms found in sand come from many groups, including bacteria, viruses, protozoa, helminths (worms) and fungi. For example, pathogenic bacteria detected in beach sands include *Vibrio vulnificus* (Abdelzaher et al., 2010; Shah et al., 2011), *Salmonella* (Yamahara et al., 2012), *Campylobacter* (Yamahara et al., 2012), *Pseudomonas aeruginosa* (Esiobu et al., 2004) and *Staphylococcus aureus* including methicillin resistant strains (Plano et al., 2013). Viruses found in sand have included enterovirus by culture (Shah et al., 2011). Protozoans have included *Giardia* spp. and *Cryptosporidium* spp. in nearshore sands (Abdelzaher et al., 2010). Nematode larvae and eggs have also been readily detected in beach sands (Shah et al., 2011). Many species of potentially pathogenic yeasts and fungi have been found including *Aspergillus* spp., *Chrysosporium* spp., *Fusarium* sp., *Scedosporium* sp., *Scytalidium* sp., *Scopulariopsis* sp. (Sabino et al., 2011), *Candida* sp. (Shah et al., 2011), *Penicillium* sp., *Rhodotorula mucilaginosa* (Vogel et al., 2007), *Cladosporium* sp., *Mucor* sp. and *Stachybotrys* sp. (Gonzales et al., 2000; Migahed, 2003; Gomes et al., 2008; Bik et al., 2012). Fungi with propensity
to infect skin and nails include Trichophyton sp. and Microsporum sp. (Sabino et al., 2011). The presence of black yeasts of the genera Aureobasidium and Exophiala, causing allergies, subcutaneous phaeohyphomycoses and neurotropic infections (de Hoog et al., 2009), have been reported once (Efstratiou & Velegraki, 2009), probably reflecting their slow growth and consequential difficulty of detection.

Spatial distribution and temporal characteristics

The spatial and temporal distribution of FIOs and fungi is highly variable in sand over distances as small as a few centimetres (WHO, 2003; Bonilla et al., 2007; Whitman et al., 2006b). The heterogeneity at this scale may be due to limited transport and mixing of microbes in sand such that once the sand is inoculated under conditions suitable for growth, microbial distribution becomes very patchy. Extreme patchiness can be due to discrete inputs from dog droppings, seabirds and onshore drift, followed by growth. Different zones of the beach may be more or less conducive to microbe growth. Research suggests that the swash zone, and in particular the area just above the maximum up-rush, may also be conducive for regrowth of FIOs through distinct wetting and drying actions and unique characteristics afforded by wrack, which is defined to include seaweed, algae, Sargassum, kelp, Cladophora, macrophytes and other vegetation-like accumulations (Alm et al., 2003; Whitman et al., 2003; Olapade et al., 2006; Ishii et al., 2007; Yamahara et al., 2009). Although sand moisture content of approximately 8% is sufficient to permit the survival of bacteria, yeasts and nematodes (Whitman et al., 2014), there is a lack of consistency in the literature over the distribution of organisms in sand and their relation to moisture content. Generally, a greater density of FIOs in wet foreshore sand has been observed compared with either subemerged, backshore, sand at depth, or dry sand (Whitman et al., 2014). Conversely, studies at a Florida marine beach found higher concentrations of E. coli and enterococci in supratidal sand (above the high water mark) than in intertidal sand (Abdelzaher et al., 2010).

The accumulation of wrack in the swash zone also serves to maintain FIO populations by serving as a source of nutrients (Byappanahalli et al., 2003; Imamura et al., 2011), providing protection from UV light (Feng et al., 2013), and regulating the temperature and moisture conditions in sands located immediately below them. Studies have shown that E. coli and enterococci can survive for over 6 months in sun-dried algal mats (Cladophora) stored at 4°C, and the residual bacteria in the dried alga readily grew upon rehydration (Whitman et al., 2003). Experimental work in the UK found that FIOs are liable to persist, and possibly proliferate, in supra-littoral wrack piles on a beach (Ward, 2009; Dunhill et al., 2013). In addition to the unique hydrodynamics of this area, which is conducive to the accumulation of wrack, the swash zone also attracts shorebirds that feed and roost in this area and may contribute to the microbial load through their faeces (Lévesque et al., 1993; Fogarty et al., 2003; Wright et al., 2009; Edge & Hill, 2007; Lu et al., 2011). Bird faeces may contribute directly to beach water contamination, although microbial source tracking techniques have found that beach sand (with bird-derived E. coli) can be a more significant secondary source of contamination to adjacent beach water than directly from the bird droppings themselves (Edge & Hill, 2007).

The growth of microbes in sand is not limited to bacteria. It is well recognized that fungi survive, and even grow in sand (Anderson, 1979). This has been demonstrated using both culture and microscopic analyses. For example, Khiyama & Makemson (1973) reported that culturable fungi in 42 Mediterranean beaches can reach as high as $\sim 7 \times 10^6$ CFU g$^{-1}$ (Larrondo & Calvo, 1989). Fungi levels at beaches have been observed to vary temporally with extreme events. In the volcanic islands of Madeira and Porto Santo, an archipelago of Portugal, pathogens in the beach sands have been associated with intense rainfall events, flash floods and debris flow (Fereira et al., 2013; Marzol et al., 2006a, b). In a study of 15 Portuguese Atlantic Coast beaches, the highest number of viable fungal colony forming units in sand was in supratidal sand, at around 500 CFU g$^{-1}$ (Brandão et al., 2002).

Antimicrobial resistance

Environmental reservoirs of both antibiotic resistant bacteria (Francino, 2012; Wellington et al., 2013) and antifungal resistant fungi have been emerging. The causes may be associated with the release of antibiotic and antifungal residues, from agriculture, animal feeding, aquaculture and also hospital wastewater (Jiang et al., 2011; Suzuki & Hoa, 2012; Diwan et al., 2013). A variety of antibiotic resistant bacteria have been isolated from sand and beach water, which can be in contact with humans (Velonakis et al., 2014). Examples include MRSA (mecillinam-resistant Staphylococcus aureus) which has been detected in correlation with the quality of water and sand, showing a relationship with beach-user overcrowding, the concentration of other microorganisms, the presence of yeasts from human origin, as well as water temperature (Papadakis et al., 1997; Plano et al., 2011; Roberts et al., 2013). Mudryk et al. (2013) showed that Vibrio species inhabiting sand were more resistant to antibiotics than those isolated from seawater; in addition, more than 90% of planktonic and benthic Vibrio-like bacteria could present multiple antibiotic resistance. Also multidrug resistant Enterococcus faecium from beach sand were identified with similar features to those from clinical human isolates (Heikens et al., 2008) indicating that enterococci can be included in the monitoring of sand, with the respective characterization of antibiotic resistance and virulence factors (Pinto et al., 2012a, b).

Fungi can have an intrinsic antifungal resistance to certain antifungal substances (primary resistance) but initially susceptible microorganisms can also develop resistance (secondary resistance). In the first case, we have examples such as Candida krusei, resistant to fluconazole (Orozco et al., 1998) or specific Fusarium species (Carneiro et al., 2011), resistant to the majority of antifungals used in clinical practice (Alastuey-Izquierdo et al., 2008). These species have coded in their genome molecular mechanisms that enable them to survive in presence of those antifungals. Candida spp. and Fusarium spp. are frequently found in sand samples and are considered as parameters to evaluate the microbiological quality of a given sample (Sabino et al., 2011). Nevertheless, the number of fungi showing antifungal resistance has been rising over the years. Aspergillus is one of the major fungal threats showing high rates of resistance to azoles, especially in Europe (Sabino et al., 2011) with the environment serving as one of the possible sources of resistant strains.
Nevertheless, in to azoles, especially due to antifungal prophylaxis. Candida
Most alarming in recent years, resistant strains of certain
mechanisms underlying their high rates of resistance to
class-specific resistance mechanisms (Pfaller
ently less sensitive species and/or acquisition of drug
use of antifungal drugs has been associated with increasing
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Verweij
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The European Union under the 1976 directive has a specific
two-part definition for bathing waters. It defines bathing
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competent authorities of each member State, or where bathing
is not prohibited and is traditionally practiced by a large
number of bathers; The second criteria means that the public
largely self select what will become a bathing area and it is
then up to the authorities to ensure suitable microbial quality.
For purposes of this review, we consider a recreational
beach to serve as a bathing water. We also consider a recrea-
tional beach to be a designated shore and water complex
largely used for recreation. Waters may be marine or fresh
water (fluvial or lentic), and sands may be calcareous, basaltic
or siliceous in origin. Indeed, some very well-known beaches
are made up of cobble or even bedrock, but here we restrict
our discussion to sands. Biologically, beaches like all shore-
lines are ecotonal, where the terrestrial and nearshore ecosys-
tems interact and overlap (Pennak, 1951). Here organisms
interact at many different trophic levels including the
microbes, which are found at the lowest levels. Little is
known about obligate or specific bacteria, fungi or viruses in
sand but we describe in this paper a diverse array of
common indicators or pathogenic species known to occur in
marine and freshwater sands.

Beach characteristics
At the local level, the beach itself is divided into zones (Figure 1) largely influenced by hydrology: the swash –
where wave run-up and return occurs; intertidal – the hori-
zontal extent of tides; the berm – a raised sand ridge of
sand deposited by maximum wave run up; foreshore – area
under influence of waves and tides; and backshore – landward
side of the beach generally not affected by water except during
storms. The berm tends to have the highest concentration of
microbes due to filtering waves as they infiltrate the sand.
The berm may also be a significant source of bacteria to
bathing waters when waves or tides re-suspend stored material
and return it to the nearshore (Whitman et al., 2014). The back-
and foreshore may also have significant input from animals
especially seabirds. More research is needed on the transport
of bacteria or fungi to or from the beach via groundwater but
it is presumed that some microbes, especially the smaller
ones (<5 microns) (Solo-Gabriele et al., 1998), can pass
through shallow groundwater relatively easily. Boehm et al.
(2004) found that microbes could be potentially transported
to the surf zone through tidally driven exchange of ground-
water. de Sieyes et al. (2008) hypothesized that the transport
of nutrients via groundwater promotes the persistence and
population replication of bacteria within the surf zone.

Sanitary assessments and sand remediation
methods
A sanitary survey is the first step in evaluating pollution
sources of a beach. This requires that the beach be viewed
within the context of its beachedness. A 'beachedness' is 'a
defined stretch of shoreline and the biogeochemical factors
that influence it' (Whitman et al., 2014). The extent of the
beachedness and its potential influence on a beach should be
considered before conducting a sanitary survey, developing a
monitoring programme, estimating risk or conducting a
microbial source tracking exercise. This would include ground-
water, runoff, incoming streams, anthropogenic and natural
faecal input, surface water dynamics, offshore influences and
general water quality. A common mistake is to seek a single
cause of poor sand quality or to finalize a survey after discover-
ning obvious or superficial factors. Multiple sources of contam-
ination are illustrated by the 63rd Street Beach, Chicago
(Whitman et al., 2001). Investigation has shown that FIOs

RECOMMENDATIONS FOR SAND
MONITORING PROGRAMMES

Criteria for selecting designated recreational
beaches
The European Union under the 1976 directive has a specific
two-part definition for bathing waters. It defines bathing
waters as areas where bathing is explicitly authorized by the
competent authorities of each member State, or where bathing
is not prohibited and is traditionally practiced by a large
number of bathers; The second criteria means that the public
largely self select what will become a bathing area and it is
then up to the authorities to ensure suitable microbial quality.

(Snelders et al., 2008; Verweij et al., 2009; Mortensen et al.,
2016; Chowdhary et al., 2013). In a recent study (Sabino
et al., 2014b), the cryptic species of A. fumigatus, A. lentulus
and Neosartorya pseudofischeri were detected in samples
from beaches. These species have been reported to be resistant
in vitro to the azole antifungals (Balajee et al., 2005; Yaguchi
et al., 2007; Alcazar-Fuoli et al., 2008).
The rapid increase of antibiotic and antifungal resistance
compromises the treatment of several bacterial (ECDC,
Antibiotic and antifungal resistance is considered a serious
threat to human health because of the limited treatments
available resulting in greater costs (Finley et al., 2013) and
increased morbidity and mortality (Ashbolt et al., 2013;
World Health Organization, 2014). MRSA is particularly
notorious among the antibiotic resistant bacteria as it causes
life-threatening skin ailments that are difficult to treat.
Escherichia coli, although a natural inhabitant of the human
intestine, has several pathogenic forms causing extreme
gastrointestinal infection, some of which exhibit cephalo-
sporin resistance (de Kraker et al., 2011). Antibiotic resistant
bacteria are found in bathing waters and studies have shown
that risks are related to the type of water activity (Leonard
et al., 2015). Regarding fungi, invasive Candida infections
are the fourth leading cause of hospital-acquired bloodstream
infections, and they are associated with a high mortality
(>40%) (Sipsas et al., 2009). Candida and Aspergillus
species cause a majority of serious infections in non-HIV
patients. Because of the high risk of fungal infections in
immunocompromised individuals, antifungal prophylaxis is
often used to treat these patients. However, the expanding
use of antifungal drugs has been associated with increasing
incidence of antifungal drug resistance resulting from inher-
ently less sensitive species and/or acquisition of drug
class-specific resistance mechanisms (Pfaller et al., 2011).
Most alarming in recent years, resistant strains of certain
Candida or Aspergillus species have emerged that are resistant
to azoles, especially due to antifungal prophylaxis. Neverthe-
less, in Aspergillus, and considering the molecular
mechanisms underlying their high rates of resistance to
azoles, especially in Europe (Chowdhary et al., 2013) (TR34/
L98H and TR46/Y121F/T289A mutations of the CYP51A
gene), it was hypothesized that one of the possible sources
of resistant strains is the environment (Snelders et al., 2008;
Verweij et al., 2009; Mortensen et al., 2016; Chowdhary
et al., 2013).
may be introduced into the foreshore from (1) direct defecation from birds, (2) accumulation of sand wave infiltration, (3) shoreward drift from re-suspended bacteria, (4) growth in stranded green algae Cladophora and (5) in situ growth of bacteria in moist sand (Halliday & Gast, 2011; Whitman et al., 2014). Human faecal sources should be given high priority because they not only pose the greatest health risk, but may lend themselves to engineering and management solutions.

One of the obvious benefits of conducting a sanitary survey is discovering the source or factors contributing to sand contamination. Practical beach management and visitor education are good first steps. An adequate number of animal-proof rubbish receptacles will reduce disease-carrying wildlife, the spread of spoiled, discarded food and waste, and the associated vectors on the beach. Wrack management may help in minimizing the persistence of indicator microbes and some pathogens. Removal of nearby air pollution sources (in the case of airborne fungal spores) could help in the management of fungi in beach sands. As mentioned above, humans are sources of pathogenic microbes and limiting the number of people at a beach to prevent overcrowding will also avoid excessive microbial contributions within a congested environment (Brandão et al., 2002). The number of beach visitors could possibly be controlled by limiting access and, in some cases, through the availability of parking. Encouraging visitors to shower before and after returning from the beach and hand washing before eating may reduce illnesses. One study showed that even just rinsing hands in the beach water greatly reduces bacterial eating may reduce illnesses. One study showed that even just rinsing hands in the beach water greatly reduces bacterial adherence on hands (Whitman et al., 2009). If birds contribute to poor sand quality an assessment would be necessary to determine whether deterrence is in line with local ecosystem preservation efforts. If so, there are techniques such as landscaping, sand grooming, and even the use of dogs and other tactics that can be used to deter birds. Nearby streams and margins may also contain high bacteria levels and swimmers should be encouraged avoid these areas. Often break-walls direct contaminants shoreward and managers may wish to have visitors avoid these areas (Byappanahalli et al., 2015). Seepage or runoff on to the beach might also increase contamination. Thus, comprehensive sanitary surveys coupled with visitor education, adaptive management, and well-designed monitoring, will go far in providing safer enjoyment of recreational beaches.

In cases where a sanitary assessment and prevention is not enough, remediation may be needed. Remediation technologies include sand grooming (Kinzelman et al., 2003; Kinzelman & McLellan, 2009), sand re-nourishment (Hernandez et al., 2014) and treatment through chemical disinfectants and physical sterilization. Iodine spraying is one of the options currently employed in Portugal (Costa et al., 2009) but theoretically other non-hazardous options exist such as sonication and high-energy light bathing (such as UV and infra-red radiation, ozone). As harmless for beach users as all these possibilities may be, the downstream pollution cleanup procedures inevitably will act both upon harmful contaminants and normal innocuous flora. Care should be taken that these methods are used only in extreme pollution events rather than as routine procedures.

General considerations for developing a monitoring programme

Designing a sampling programme begins with several preliminary questions: where should we sample, how do we sample, and how often should we sample? Deciding where to monitor can be difficult: experience shows that contaminants arising from the water accumulate along the foreshore but substantial contamination may be occurring from surrounding areas, wildlife, pets and humans themselves higher on the beach. A programme that encompasses areas where visitors might encounter pathogens, ranging from backshore sand to the swash zone, may be appropriate at many beaches. Longitudinal transects along the beach at pre-selected intervals are preferred from a statistical standpoint, but known ‘hotspots’ should not be excluded from these studies.

Achieving representative sampling at beaches is difficult due to diverse inputs of microorganisms that create a heterogeneous community landscape. Sand is arguably a more problematic matrix than water, as it is relatively less prone to mixing than water. Sample replication is essential since microbrial distribution in sand is patchy. An alternative or companion strategy to replication is to collect many individual samples and mix them to create a composite sample, keeping in mind that pseudo-replicate sampling of composite samples should also be carried out to avoid placing undue weight on data based on a very small fraction of the sample. For example, Phillips et al. (2011a) collected 60 shallow core samples (each 2.5 cm diameter and 2.5 cm deep) along target transects. They combined these core samples, mixed them thoroughly, and utilized an aliquot for analysis.

Temperature and irradiation of exposed sand can vastly reduce levels of surface microbes. While deep within the sand, communities change due to more negative redox potential and lack of oxygen. Core samples are essential in order to provide an integrated survey of potential microbial pathogens. Sampling depths to 20–30 cm are the most practical and protective for the casual beach visitor. Once collected samples require extraction or elution prior to analysis (Boehm et al., 2009b).

Decisions on the frequency of monitoring should be based on the amount of beach use, susceptibility to contamination, and also on cost. In temperate climates, beach use is very limited during cold weather, and sampling programmes may be minimized. It is known that sand microbial quality changes much less rapidly than the frequent temporal variability observed in water (Boehm et al., 2002; Enns et al., 2012). On the other hand, studies have shown correlations between water and sand FIO content, especially at the foreshore (Whitman & Nevers, 2003; Phillips et al., 2011a, b). Generally, any monitoring programme should account for tradeoffs between visitor risk, budget, effectiveness, and accounting for expected variations in targeted microbes. Beach sand should be routinely sampled at least annually and whenever there has been an event such as a sewage release, major storm events or known seasonal events. Pre- and post-event monitoring where large crowds are anticipated might be considered. High-risk beaches, such as those potentially impacted by human sewage, require more frequent monitoring.

Sampling strategies based upon desired outcomes and integrating traditional approaches

Development of a monitoring programme for beach sand requires first that the desired outcomes of such a programme are clearly defined. An example of a two-part outcome is
Many cases are difficult to assess in terms of human health outcomes. Ultimately, each individual has its exposure limit to fungi, which is dependent in part upon immune status. As a result, the environmental mycology community recognizes the need to simplify its approach for assessing the potential for transmission of infectious disease within beach settings. The ‘tiered’ approach described below represents a compromise between the recreational water quality community and those whose primary focus area is in environmental mycology.

A tiered approach for beach sand monitoring programmes

The ‘first pass’ of most monitoring programmes is culturable microbes. From the recreational monitoring community these microbes are normally FIOs. But from the environmental mycology perspective these may include total culturable fungi. The methods for analysing culturable microbes are relatively inexpensive, can be performed in laboratories with minimal specialized equipment and expertise, and in many cases have extensive historical use, providing context to new measurements. However, enumerating traditional FIOs, in particular, provides no information about contamination source in sand or water (recently reviewed in Harwood et al., 2014), and the dearth of sand-related epidemiology studies leaves a substantial knowledge gap about human health risks (Whitman et al., 2014).

In contrast to traditional regulatory approaches, the classic approach in environmental mycology has been to measure all fungi and FIO present in sand together with total counts of viable colony-forming units, as described in Brandão et al. (2002). More recently, however, environmental mycologists have started to focus on measures of specific microorganisms, more relevant in terms of public health (i.e. black moulds and keratinophilic fungi) (Sabino et al., 2014). This change is due to the lengthy and expensive practice of identifying all possible organisms. Thus, Environmental Mycology is gradually migrating towards the concept of measuring representative microbes or a fungal indicator microbe. As such the first tiered approach for monitoring the microbiological quality of water and/or beach sand should focus on measures of indicator microbes. This is consistent with current regulatory approaches used to assess recreational water for faecal contamination and is also consistent with the more recent evolving approaches in environmental mycology. In the case of fungi, the first tier analysis approach could include measures of total culturable fungi.

A second-tier approach using source-specific testing (microbial source tracking: MST) may be undertaken if information on the source of faecal contamination or fungi is required, either for mitigation or for risk assessment. Recent studies have estimated very different human health risks from faecal contamination originating from different host animals (Soller et al., 2010, 2014) and so acceptable levels of indicator microbes should consider the potential source of the FIO. For fungi, species identification may help in identifying sources. For example, some fungi are associated with superficial infection, like the contagious Tinea corporis (ringworm). The Trichophyton and Microsporum genera include species of human, animal or soil origin (Badillet, 1973). Thus, identification of species may point to the contamination source for fungi in particular.
A third-tier approach would generally be most useful where many people are likely to be exposed (e.g. a crowded beach with human densities greater than 1 per square metre of beach sand area), and where MST has indicated the likely presence of high-risk faecal or fungal sources. The information provided by MST could be used to target particular pathogens that are known to be shed by the indicated host types. It can also require a great number of separate tests if several host types contribute to contamination. Alternatively, one can use a microarray approach, where hundreds of nucleic acid sequences representing FIOs, pathogens and MST markers can be simultaneously queried (Weidhaas et al., 2014). An issue with microarray is that sample sizes are very small, so very efficient concentration methods that do not result in interference with nucleic acid hybridization are necessary, and these are still under development.

The second tier and third tier of analyses may be left for reference laboratories, laboratories with the technical expertise to analyse MST markers and pathogens from environmental samples, including capabilities for molecular analyses. These reference laboratories should assess regional specificities of indicator microorganisms and support or validate laboratories capable of basic analysis. For this level of reference, accreditation by ISO 17025 (ISO/IEC 17025, 2005) will ensure the technical proficiency of a laboratory and technical personnel. Inter-laboratory quality assessment schemes will capacitate the laboratories at this level to the point where they can validate first tier analytical approaches and provide expertise for non-standardized analytical methods. In this case, reproducibility and repeatability will ensure results independent of laboratories and technicians.

What should be measured?

The rapid pace of technological advances in the environmental detection and quantification of microbial targets has created what might be considered an embarrassment of riches. It engenders questions such as, should we test for a representative pathogen or two, or a broad suite of pathogens? Does testing need to be quantitative, or do binary results (plus/minus) (presence/absence) suffice? Should the focus be on one microbial type, such as viruses, or should the group of targets be broadened? Ideally, monitoring methods for beach sand monitoring should be inexpensive, provide instant, or at least same-day results, and be directly connected with human health outcomes (Figure 2). The current reality of monitoring methods is that there is no ready protocol that leads precisely to such an elegant outcome. Instead, compromise on one or more aspects of the ideal indicator is necessary and for this reason a tiered approach is recommended by the authors of this review as described above.

Over the past 60 years FIOs have proved a useful surrogate for measuring pathogens. The FIOs most commonly used for regulatory purposes are enterococci for marine waters (WHO, 2003) and E. coli for fresh water (USEPA, 2012). Other alternative indicator microbes that have been recommended include Clostridium perfringens (Fujioka & Shizumura, 1985; Roll & Fujioka, 1997; Boehm et al., 2009a), Bacteroidales (Boehm et al., 2009a) and coliphage (Havelaar et al., 1993; Luther & Fujioka, 2004; Boehm et al., 2009a). Although FIOs are utilized extensively worldwide, their limitations have been recognized (EU, 2006). Limitations include the fact that commonly used FIOs are invariably bacterial species, whereas the majority of the reported illnesses are believed to be caused by viruses (particularly norovirus), and analytical techniques suitable for routine use are poor at distinguishing between human and animal sources of bacterial FIOs. Data from the USA identified the following eight faecal pathogens as dominating waterborne illness: norovirus, rotavirus, adenovirus, Cryptosporidium spp., Giardia lamblia, Campylobacter jejuni, Salmonella enterica and E. coli O157:H7 (Mead et al., 1999; Vital et al., 2008). Investigations at beaches in Miami, FL, USA support the hypothesis that existing indicator microbes indirectly monitor several pathogens through common factors, at least in sand. However, for pathogens such as Cryptosporidium spp., Giardia spp. and enterovirus, generalizations about the predictive ability of indicator microbes must be treated with caution owing to the sparseness of data.

Soller et al. (2010) concluded that in fresh water, enteric viruses and Giardia appear to account for the vast majority of the observed swimming-associated GI illnesses, and when treated sewage effluent predominates, norovirus alone may represent the primary concern. The pre-eminence of noroviruses is supported by work by Public Health England, where norovirus dominated the identified cause of illness from consuming sewage-contaminated shellfish (Figure 3). Evaluating trends from 1991 through 2011, the aetiological agent most frequently identified as the cause of an outbreak was norovirus. This is particularly apparent for more recent years where detection technologies have been capable to identify the aetiological agent responsible for the outbreaks (David Lees, personal communication, CEFAS UK).

The European Union has sponsored an investigation (Virobathe) into analytical methods for viruses for possible incorporation into the 2020 revision of the European Union’s (2006) Bathing Water Directive. The report of this work concluded that whilst adenoviruses were a possible control parameter, noroviruses were encountered too infrequently to be considered (EU, 2009; Wyr et al., 2012). However the authors of Virobathe have since recommended to the European Commission that a viral pathogen standard should not be adopted on both analytical and public health grounds. They recommend instead that future risk continues to be managed through demonstrating connectivity to faecal sources, rather than proving that a pathogen is being excreted by the contributing population (Kay, 2015). Overall, because of their link to gastrointestinal disease and/or detection in recreational waters, viruses that should be considered when evaluating potential aetiological agents in sand include norovirus, adenovirus, rotavirus, enterovirus and hepatitis.

Fig. 2. Conceptual triangle for ideal characteristics of an indicator organism used for the first tier of screening sand quality at beaches.
and Fonsecaea are causative agents of chromoblastomycosis in subtropic and tropic regions and since they have been detected on wood, soil, plant material and in environments polluted with oil or creosote, their presence should also be evaluated in sand (Vicente et al., 2008), especially on inland beaches. Since they are often detected in beach sand, species like F. pedrosoi, F. monophora, C. bantiana and E. dermatitidis should be included in future legislations of beach sand quality. Organisms of biosafety level 3 like Cladophialophora bantiana should also be considered. Figure 4 brings together data on fungi found in water and sand environments and those in clinical experiments to identify those most of concern in studies of recreational waters.

Technological advances in microbe measurement techniques

Improving technology has provided several new, but relatively expensive, methodologies for determining the safety of beach sand, e.g. quantitative PCR for specific pathogens or host-specific gene markers, or multi-target methods such as microarray or next-generation nucleic acid sequencing. However, it is important to note that molecular analysis will only reveal the presence of microbial genetic material, which does not always represent viable microbes. Culturable organisms must be capable of replication in order to be detected, a condition that is closer to an infective state that simply possessing genetic material. An example of this discrepancy is the 2012 US Environmental Protection Agency criteria for recreational water quality, which estimates 36 cases of gastroenteritis per 1000 exposed individuals in waters containing a geometric mean level of 35 culturable enterococci per 100 mL, but 470 'cell equivalents' per 100 mL by qPCR (USEPA, 2012). Conversely, some researchers have reported that FIO can enter a viable but non-culturable (VBNC) state in water from which they may be infective (Heim et al., 2002; Lleo et al., 2005; Boehm & Sassoubre, 2014), and these forms can be detected by molecular methods. Thus although molecular methods can detect specific microbes that are associated with human health outcomes, what they detect is different from culture-dependent methods, which adds to the complexity of adopting new methods based upon knowledge gained from older technologies. Few epidemiology studies have been conducted to determine whether new methods to detect specific pathogens are better assessments of human health outcomes than the century-old FIO paradigm. So a knowledge void exists that fosters indecision about which methods(s) of assessment should be used. Although more information through epidemiology studies may be deemed better, economic constraints become quite important to regulatory agencies and the citizens who must ultimately pay for the testing.

Given the advent of new genomics approaches, measurements of the entire microbial community represent a potential new approach (see Application of Metagenomics to Assess Microbial Communities in Water and Other Environmental Matrices by Staley and Sadowsky in this issue). Many of the microbes living in sand have not been cultured and may not be culturable; thus a complete understanding of the microbial ecology of sand communities has not been possible. Furthermore, the lack of detection of unculturable and potentially infectious microorganisms has confounded monitoring efforts to protect public health. Consequently, metagenomic and 16S-amplicon-based studies to characterize microbial
communities in sand, water and sediment habitats will offer great insight into the ecology of these systems (Lozupone & Knight, 2007; Staley et al., 2015). In addition, characterization of these communities will provide a context for the role and relative abundance of potential pathogens, which has previously only been assessed using a relatively small number of ephemeral molecular targets (Aw & Rose, 2012). While in its infancy, this type of approach has been taken by Cui et al. (2013) who used 454 sequencing to find backshore sands in Hawaii had a more diverse community and contained different populations than other beach zones. Piggot et al. (2012) found that the Proteobacteria and Bacteroidetes dominate biofilm communities in South Florida beach sand, with microbial communities that vary by location within the tidal zones and in relation to water activity. More recently, Halliday et al. (2014) reported that sand at the high tide line, intertidal sand and adjacent water samples contained different overall bacterial communities, that there was some similarity in community composition between coastal water samples from two distant sites, and there was dissimilarity between bacterial communities from high tide and intertidal sands.

Recently, 16S rDNA amplicon analysis, using the Illumina HiSeq and MiSeq platforms was used to examine microbial communities in sands obtained from an estuarine beach and a marine site in Tampa, FL; a freshwater lake in Saint Paul, MN; and Lake Michigan, near Chicago, IL (Whitman et al., 2014). Among all three sites, Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria were the most abundant phyla, with families at all sites including Rhodobacteraceae, Flavobacteriaceae, Flavimicrobiaceae and Campylobacteraceae. Sand from the marine sites had greater richness and higher non-parametric diversity indices than the other sites examined (Figure 5). More recently a programme ‘sands of the world’ has been initiated which utilizes, 16S amplicon sequencing and Illiumina HiSeq to examine spatial and temporal diversity of bacterial as well as fungal communities in beach sands collected from fresh (Great Lakes) and salt water beaches around the world. More specifically, this project will characterize microbial diversity in sands from four beaches along the Great Lakes as well as marine beaches on both US coasts, the Gulf of Mexico, Hawaii, Japan and Korea. This information will be useful to determine what environmental factors control beach microbial communities and whether sands harbour unique or similar bacteria, archael and fungal microbial communities that vary in some predictable manner. In addition, these data may give us insight into which microbial taxa are related to specific sand habitats.

Fig. 4. Presence of fungal genera in environmental and clinical studies. Blue circle includes fungal genera reported from seawater and ocean studies, orange circle presents fungi isolated from sand. In red circle there are genera reported as causative agents for human disease. The intersection of the circles includes fungi, isolated from two (seawater – beach sand, beach sand – clinical samples, clinical samples – seawater) or all three sampled sites (seawater – beach sand – clinical samples).
SCIENTIFIC RESEARCH NEEDS

The research community recognizes that the traditional FIO paradigm is limited. This paradigm has served the public health community well for more than a century, especially in areas directly impacted by sewage. In developed countries, direct sewage impacts on beaches are the exception rather than the rule. In beaches impacted by non-point source contamination, the relationships between FIOs and human health outcomes are not well understood; therefore it is unclear whether continued monitoring for FIOs in these cases is relevant. Regardless of the source of contamination, the incidence of illness is higher for beach bathers relative to non-bathers (Colford et al., 2007; Fleisher et al., 2010; Sinigalliano et al., 2010) and also for beach users who play in the sand relative to those who do not play in the sand (Heaney et al., 2012).

It is also recognized that sand can serve as a reservoir of pathogenic microbes, including faecal pathogens of human or animal source, as well as fungi, which are generally considered to be associated with environmental sources. Thus, pathogens may be present and transmitted in the beach environment, even in the absence of sewage contamination. We recommend research to establish a cause and effect relationship for infectious disease contracted within beach environments. First and foremost is the identification of the aetiological agent of disease followed by establishing stronger links between environmental monitoring parameters and human health risk. Specific recommendations include:

1. Identification of the aetiological agent(s) of disease. A better understanding is needed of the aetiologic agents responsible for the majority of disease attributed to recreational water and sand contact. Identifying the aetiological agent will provide a stronger mechanistic understanding for disease transmission in beach settings. With this understanding, effective control and monitoring programmes can be implemented. To narrow the list of possible aetiological agents, public health data should be examined. A preliminary assessment of reportable diseases in the EU and USA (Table 1) suggests that significant pathogens that have potential sand reservoirs include GI pathogens Salmonella, Shigella, verotoxin-producing E. coli, Campylobacter, Cryptosporidium, Cyclospora, Vibrio, Giardia, hepatitis A and Listeria. Those associated with sediment and water reservoirs include Yersinia, Leptospira and Tularaemia. Although a list of reportable diseases is available, low-level self-limiting diseases (e.g. GI illness, mild skin infections and mild respiratory infections) are usually not reported and the aetiological agents are typically not measured. Wheeler et al. (1999) found that the incidence rate for mild gastroenteritis was under-reported by a factor of 31. Thus numbers listed in Table 1 may significantly underestimate the incidence of reportable diseases. Concerted efforts are needed to encourage clinical practitioners to more often request an evaluation of etiological agents of disease to better track them. Because of unique clinical manifestations, the tracking of fungal infections may be more easily accomplished as opposed to GI infections.

2. Quantitative microbial risk assessment (QMRA). QMRA methods should be utilized to specifically estimate public health risks from various pathogens (bacteria, fungi, viruses, protozoa, helminths) in beach sand, which can transmit diseases by various exposure routes (contact, ingestion, inhalation). QMRA methods are generally less expensive and less time consuming than epidemiological studies; however, in some cases relationships needed in calculating risks and disease rates are not available (e.g. dose-response relationships for some microbes). An assessment must be made as to which pathogens in beach sand can and cannot be evaluated by QMRA. Preliminary assessments (Shibata & Solo-Gabriele, 2012) have identified the need for dose-response estimates for fungi and helminths. Moreover, to obtain a better estimate of skin-related ailments, the impacts of wounds should be evaluated on the dose-response of various aetiological agents known to cause skin disease. In some cases, such as for helminths,
(3) Epidemiological studies. Epidemiological studies measure disease in the exposed population and are the method of choice for establishing the link between human health (GI illness, acute febrile respiratory illness, skin ailments, ear and eye infections) and environmental factors. Since this method is expensive, work intensive and time consuming, it should be used at selected beach sites, bearing in mind the slow onset of infections caused by fungi and parasites. Epidemiologists should determine if study designs can specifically measure pathogen disease rate and measure exposure to the sand by three separate routes (contact, ingestion, inhalation). Controlled cohort or randomized trial studies – similar in aim to those undertaken in water – are needed to better quantify disease risk from exposure to sand.

(4) Evaluate alternatives to FIO for beach monitoring programmes. Although measurements of FIOs should not be discontinued because many beaches are susceptible to sewage contamination, their usefulness needs to be reassessed. The most common FIOs (E. coli, enterococci) fail to fulfil the following three scientifically based ideal criteria or cellular properties of the indicator bacteria, which are required to ensure that the numbers of FIO will correspond to the numbers of sewage-borne contaminants.

Table 1. Number of cases of reportable diseases and incidence rates in the EU and US. Data from ECDC (2011a, b, 2012, 2014a, b) and CDC (2013, 2014). Incidence rates based upon population estimates of 506–503 million and 309–314 million for the EU and US, respectively.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Year</th>
<th>European Union</th>
<th>United States</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total no. of cases</td>
<td>Incidence rate (per 100,000 habitants)</td>
</tr>
<tr>
<td>Salmonellosis</td>
<td>2010</td>
<td>90,764</td>
<td>17.9</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>88,577</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>87,719</td>
<td>17.4</td>
</tr>
<tr>
<td>Shigellosis</td>
<td>2010</td>
<td>68,39</td>
<td>1.35</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>66,55</td>
<td>1.32</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>66,43</td>
<td>1.32</td>
</tr>
<tr>
<td>VTEC infection*</td>
<td>2010</td>
<td>37,48</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>9661</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>5954</td>
<td>1.18</td>
</tr>
<tr>
<td>Listeriosis</td>
<td>2010</td>
<td>1686</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>1538</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>1642</td>
<td>0.34</td>
</tr>
<tr>
<td>Legionellosis</td>
<td>2010</td>
<td>5854</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>4449</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>5856</td>
<td>1.16</td>
</tr>
<tr>
<td>Vibriosis, non-cholera</td>
<td>2010</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Campylobacteriosis</td>
<td>2010</td>
<td>218,957</td>
<td>43.28</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>227,803</td>
<td>45.02</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>218,153</td>
<td>43.37</td>
</tr>
<tr>
<td>Yersiniosis</td>
<td>2010</td>
<td>6614</td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>6810</td>
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<tr>
<td></td>
<td>2012</td>
<td>6102</td>
<td>1.21</td>
</tr>
<tr>
<td>Leptospirosis</td>
<td>2010</td>
<td>822</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>685</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>778</td>
<td>0.15</td>
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<tr>
<td>Tularaemia</td>
<td>2010</td>
<td>888</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>755</td>
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</tr>
<tr>
<td></td>
<td>2012</td>
<td>1003</td>
<td>0.20</td>
</tr>
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<td>Cryptosporidiosis</td>
<td>2010</td>
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</tr>
<tr>
<td></td>
<td>2011</td>
<td>685</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>778</td>
<td>0.15</td>
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<tr>
<td>Cyclosporiasis</td>
<td>2010</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Giardiasis</td>
<td>2010</td>
<td>17,130</td>
<td>3.39</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>16,473</td>
<td>3.26</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>16,424</td>
<td>3.27</td>
</tr>
<tr>
<td>Hepatitis A</td>
<td>2010</td>
<td>13,471</td>
<td>2.66</td>
</tr>
<tr>
<td></td>
<td>2011</td>
<td>12,706</td>
<td>2.51</td>
</tr>
<tr>
<td></td>
<td>2012</td>
<td>13,156</td>
<td>2.62</td>
</tr>
</tbody>
</table>

*VTEC, verotoxin-producing Escherichia coli, the Shigella-like toxin (includes toxin producing O157). Also known as shiga toxin producing E. coli (STEC).
Develop techniques for detection and quantification of pathogens in the water samples tested (Bonde, 1966; Yates, 2007): (a) The indicator should be consistently and exclusively associated with a source of human pathogens (e.g. human faeces/sewage); (b) FIO should not be able to multiply under environmental conditions because they would no longer track some sewage-borne pathogens (viruses, protozoa), which presumably do not multiply in the environment; (c) their resistance or survival characteristics to environmental conditions and to wastewater treatment processes should be similar to that of pathogens. Without these cellular characteristics and multiplication in human intestinal sources, the FIO would not track pathogens and subsequently human health. Research is needed to evaluate the reliability and feasibility of monitoring sand for alternative faecal indicators that meet the above three ideal criteria and thus appropriately track pathogens. Since noroviruses are one of the more likely aetiological agents for water-borne transmission of diseases during recreational uses of water, there is a pressing need to develop appropriate viral indicator(s). Viral indicators could include bacteriophages (bacterial viruses), which have similar size, chemical composition and survival characteristics as human enteric viruses. Phages, which are considered promising indicators of human enteric viruses, are F-specific RNA bacteriophages (Havelaar et al, 1993; Luther & Fujioka, 2004), phages of enterococci bacteria (Santiago-Rodriguez et al, 2013) or phages of Bacteroides (Ebdon et al., 2007; McMinn et al., 2014). In addition, it will be important to evaluate beach sand for the alternative FIO (C. perfringens) because it is a conservative indicator of sewage contamination (Fujioka & Shizumura, 1985; Roll & Fujioka, 1997). Because of their persistence as spores, the use of C. perfringens may be most useful in areas where currents dilute and remove existing contamination.

Develop techniques for detection and quantification of microbe levels. There is a need for improved cultivation methods for detection of fungi, viruses, helminths, protozoa and bacteria in environmental samples. There is a need to determine if the method to detect a specific pathogen or class of pathogens is feasible and reliable for monitoring purposes. If a pathogen detection method is not feasible, then a feasible and reliable indicator monitoring method should be implemented, which should provide data on the quantity and infectivity for that pathogen or that class of pathogens. In this regard, cultivable methods provide information on the theoretical infectivity of the pathogen and this kind of data can be used to determine public health risks. Currently, many molecular methods have been developed to rapidly and reliably detect specific pathogens. The limitation of this method is that it does not differentiate between dead and living pathogens. As a result, public health assessments must be based on some assumptions. The value of molecular methods is that they can be used to confirm the presence or absence of specific pathogens in beach sand, regardless of their viability. Improved molecular techniques for the detection of medically important fungi in sand are needed. Also, the ecological role of fungi in coastal reservoirs such as beach sand is little understood (Migahed, 2003) and needs to be investigated. In securing better protection, unnecessary complexity in monitoring is to be avoided. Concern has been expressed on the cost burden of monitoring – particularly in developing regions – of even the existing criteria (WHO, 2003). Against this must be balanced a better cost-benefit balance of targeted improvements and the avoidance of expenditure on ineffective measures undertaken simply to meet flawed criteria (Kay et al., 1999).

Pathogen levels and survival in sands. A more complete picture of the types and levels of pathogens in sand is needed, including a focus on evaluating their geographic, spatial and temporal distribution. Multiple studies have documented E. coli and enterococci reservoirs in sand, but few studies have concurrently measured pathogens to determine if E. coli and enterococci are indicative of faecal pollution that carries pathogens, or uncoupled from their original source through prolonged survival or growth. The general consensus among researchers is that pathogens do not multiply in the environment. So prolonged survival or growth of E. coli and enterococci would result in their presence in the absence of pathogens. New research is needed that can provide tools to determine whether FIO are indicative of pathogens within sand environments. The sources of faecal pollution will largely determine the types of pathogens that may be present in sand. Faecal pollution can be deposited directly in sand through outfall runoff or wildlife, or may be delivered through contaminated water. Understanding how faecal pollution and its co-occurring pathogens are modulated in both the sand and water environment can guide the types of indicators or pathogens chosen. Further, gaining a more complete picture of the pathogens that persist in the beach sand and the causative agents for disease, will guide choices of indicators for monitoring and improve assessments of risk.

Develop tools to identify sources. Although considerable advances have been made through MST, more work is needed to identify and approve/agree methods that distinguish between human and non-human sources. There is a need to understand differences in risk among these sources. An improved understanding of the relative risk of faecal contamination from human and other sources is necessary to establish acceptable levels of FIOs in the environment.

Regulations are to reflect microbial sources. Through application of the Annapolis Protocol the WHO has confirmed the need to consider all potential sources of pathogens, not only those from faecal point sources, an approach endorsed by the Rotorua declaration of 2011 (IWA, 2011). The beach environment is an important contributor to water, both through the retention, and possible regrowth, of FIOs within the sand matrix and beach wrack, but also for the presence in sand of non-faecal pathogens including fungi, protozoa and parasites. Forthcoming developments of regulatory standards need to reflect this evolving understanding of microbial sources, the pathogens they contain, and the associated health risks.

Develop reliable sand collection methods designed to recover average pathogen loads for a given beach site or at a specific site where pathogens are suspected. Since pathogen contamination at sandy beaches is expected to be patchy, sand samples from multiple sites should be collected and pooled to determine average concentrations of pathogens in sandy areas. However, targeted sampling
(within decaying algae, bird roosts, swash zone, public showers, land-based discharges onto beach sand) should also be conducted where pathogen contamination is suspected. These contaminated patches of sand are good sources for microbial source tracking analysis.

(10) Determine beach sand quality at freshwater vs. marine beaches. Fresh and marine beach sands have been reported to be contaminated by different sources of FIO and pathogens (Whitman et al., 2014). For example, different species of algae have been reported to contaminate shorelines of freshwater beaches (Byappanahalli et al., 2003) vs. marine beaches (Imamura et al., 2011). In this regard, decay of Cladophora in freshwater beaches has been reported to allow for the growth of FIO and other pathogenic bacteria (Ishii et al., 2006). Therefore, sand at freshwater beaches and sand at marine beaches can be expected to differ with respect to sources of contamination, types of pathogens and their survival characteristics.

(11) Assess beach sand quality based on contamination by land- and air-based discharges, which are known to be major factors that determine the sources as well as persistence of microorganisms in beach sand. High rainfall patterns cause land-based discharges such as streams and storm drains and may include discharges from agricultural and animal raising facilities. Even beaches with low rainfall can receive substantial urban discharges (storm drains) that include effluents from sewage and industrial facilities as well as discharges of human faeces directly into storm drains. The impacts of these land-based discharges affect the quality of beach sand differently at different beaches and must be assessed as site-specific factors. Air transportation plays the same role for sporulating microorganisms.

(12) Assess standardized methods to recover and disinfect FIO and pathogens from silica-based vs. calcium carbonate-based sands. Both silica sand and calcium carbonate sand are chemically stable sand particles. However, calcium carbonate sand is more reactive and dissolves in dilute acid more readily compared with silica sand. As a result, each may not react similarly to all reagents and may have different influences on survival of microorganisms. There is a need to determine the impact of silica-based and calcium carbonate-based sands on survival characteristics of microbes, on the use of reagents to recover microbes from sand and in the use of chemical reagents to disinfect these two types of beach sand. As a corollary to disinfection, efforts are needed to evaluate the impacts of sand disinfection on the microbial ecosystems and at upper trophic levels.

SUMMARY AND CONCLUSIONS

There is compelling scientific evidence that beaches, through their sands, are a significant contributor to the pathogen load to which beach users are exposed. Many beach epidemiological studies have focused on the impacts of bathing. At beaches that are not impacted by sewage effluent, the source of pathogens originates from the local beach site itself and includes human visitors at the beach, animals, local runoff and the release of microbes from sand. The microbes released from sand can include native microbes (autochthonous) or those that have been deposited from outside sources (allochthonous). Studies have identified the presence of pathogenic microbes in beach sand and have identified factors other than point source pollution that contribute to their presence (e.g. moisture, wrack, wildlife, domestic animals, beach morphology, currents). More recent epidemiological studies have shown that children who play in sand are subject to higher rates of illness relative to those who do not play in the sand. Thus beach sand can serve as a vehicle for disease transmission, either through direct sand contact containing microbes or indirectly through contact with water containing microbes washed off from sand. Given the ability of sand to harbour microbes, we recommend the inclusion of sand measurements in all beach monitoring programmes.

We provide a series of recommendations for beach monitoring programmes that begin by identifying designated recreational beach areas, beach sanitary surveys inclusive of remediation methods, general considerations for monitoring programmes, and a sampling strategy based upon desired outcomes. Given the large number of potential aetiologic agents of disease, a tiered approach is recommended for beach sand monitoring. The approach should begin with measures of FIOs and/or total culturable fungi followed by microbes with potential for source tracking. For microbes transmitted via faecal-oral routes, sources should be identified through microbial source tracking. For fungi, specific species can be used to help identify sources. For the third tier, the specific aetiologic agent responsible for disease should be measured. No beach epidemiological study to date (whether focused on water or sand) has directly measured pathogens in human subjects to confirm the aetiologic agent of disease. According to QMRA methods, the most likely aetiologic agents for faecal-oral beach illnesses include norovirus and Giardia. So far, measurements of fungi have not been included in beach epidemiological studies. However, given their propensity in beach sands, agents that we recommend for inclusion in the third tier of measurements are pathogenic Aspergillus sp., Candida sp., Microsporum sp., and Trichophyton sp. Measurement techniques include culture-based methods and quantitative PCR. An alternative approach can include the measure of the beach metagenome as a means of assessing the microbial ecological factors that may facilitate the presence of pathogens.

Considerable evidence exists that sand can serve as a reservoir of enteric microorganisms and fungi, which can be vehicles of disease transmission at beach sites. Current policies worldwide, at both national and international levels, give scant regard to the impact of sands on the health of users of beaches. We recommend that sand quality measures should be considered with some urgency for inclusion in regulatory programmes aimed at protecting recreational beach user health. Contaminated sands present health and economic costs that can and should be known by decision makers, communities and by individuals. Available evidence should be evaluated by both scientists and regulators with a view to filling the data gaps outlined here, which should be followed by sound policy development for safeguarding public health.

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Application of metagenomics to assess microbial communities in water and other environmental matrices

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The emergence of metagenomics-based approaches in biology has overcome historical culture-based biases in microbiological studies. This has also enabled a more comprehensive assessment of the microbial ecology of environmental samples. The subsequent development of next-generation sequencing technologies, able to produce hundreds of millions of sequences at improved cost and speed, necessitated a computational shift from user-supervised alignment and analysis pipelines, that were used previously for vector-based metagenomic studies that relied on Sanger sequencing. Current computational advances have expanded the scope of microbial biogeography studies and offered novel insights into microbial responses to environmental variation and anthropogenic inputs into ecosystems. However, new biostatistical and computational approaches are required to handle the large volume and complexity of these new multivariate datasets. While this has allowed more complete characterization of taxonomic, phylogenetic and functional microbial diversity, these tools are still limited by methodological biases, incomplete databases, and the high cost of fully characterizing environmental biodiversity. This review addresses the evolution of methods to monitor surface waters and characterize environmental samples through the recent computational advances in metagenomics, with an emphasis on the study of surface waters. These new methods have provided an abundance of opportunities to expand our understanding of the interaction between microbial communities and public health. Specifically, they have allowed for comprehensive monitoring of bacterial communities in surface waters for changes in community structure associated with faecal contamination and the presence of human pathogens, rather than relying on only a few indicator bacteria to direct public health concerns.

Keywords: environmental samples, metagenomics, next-generation sequencing, 16S rDNA

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ADVANTAGES OF METAGENOMICS TO STUDY ENVIRONMENTAL SAMPLES

Historically, investigation of microbial communities has been performed using culture-based methodologies. However, less than 1% of bacterial species in environmental communities are thought to be culturable on standard laboratory growth media (Amann et al., 1995). To overcome these limitations, a metagenomic approach was suggested to characterize total microbial community DNA (including viruses, prokaryotes and eukaryotes) (Handelsman et al., 1998). Such techniques have revealed unprecedented taxonomic and functional diversity in aquatic and terrestrial habitats (Rondon et al., 2000; Venter et al., 2004; Sogin et al., 2006). Metagenomics encompasses two types of study – whole genome shotgun (WGS) sequencing of all the genes in the microbial community or those targeting a single, taxonomically important gene (e.g. 16S rDNA for bacteria) (Gilbert & Dupont, 2011). Whole genome shotgun sequencing studies generally fall into one of three categories: (1) vector cloning and sequencing studies in which community DNA is cloned into a fosmid, cosmid, or bacterial artificial chromosome (BAC) and the library is screened for particular genes of interest, usually for a particular species or group; (2) Sanger sequencing-based shotgun metagenomic studies in which community DNA is cloned into a vector and randomly sequenced for assembly and/or annotation; and (3) next-generation shotgun sequencing studies that utilize sequencing-by-synthesis technology to generate millions of sequence reads without the need for cloning (Gilbert & Dupont, 2011). In contrast, amplicon sequencing does not encompass the sequencing of total environmental DNA, and is not viewed by many as a metagenomic method in the strict sense. However, the ability to exploit millions of sequence reads to characterize thousands of species has revealed unprecedented diversity in marine samples and may represent a more promising alternative to assess ecosystem health and public health risks than previous culture-based or molecular methods (Unno et al., 2010; Gibbons et al., 2013; Staley et al., 2014a).

Early applications of metagenomics to assess marine biodiversity

The study of marine biodiversity and biogeography was among one of the first applications of a metagenomics-based approach, utilizing the traditional Sanger method to sequence millions of small inserts from clone libraries (Venter et al.,...
An early study conducted in the Sargasso Sea identified 1800 genomes, including 48 unknown bacterial taxa and ~70,000 novel genes, using novel bioinformatics techniques for metagenomic assembly (Venter et al., 2004). This study was followed by the Global Ocean Sampling (GOS) expedition to the north-west Atlantic to the eastern Pacific Oceans (Rusch et al., 2007), which revealed previously unprecedented diversity and heterogeneity within and between marine ecosystems using >7 million sequence reads. The GOS dataset was subsequently used to demonstrate a relatively ubiquitous and consistent distribution of aerobic, anoxygenic, photosynthetic bacteria among marine habitats, and these results suggested that environmental conditions may explain geographic variations in the relative abundance of this group (Yutin et al., 2007). Similarly, a comparison of the genome sequence of a SAR11 marine alphaproteobacterium to the Sargasso Sea dataset revealed a high degree of conservation among core functional genes within this group, despite several hypervariable genome regions potentially associated with biogeographic variation (Wilhelm et al., 2007). These classical studies revealed the power of metagenomics-based approaches to reveal previously unknown organisms and to greatly expand the scope of microbial biodiversity and biogeography studies.

Here, we review the applications of metagenomics-based approaches, with a focus on emerging next-generation sequencing strategies, to the study of aquatic ecosystems, the computational advances associated with increasingly larger datasets, and current limitations to this rapidly expanding field of research.

**NEXT-GENERATION SEQUENCING INVESTIGATION OF AQUATIC ECOSYSTEMS**

In response to the success of early metagenomics studies, several next-generation sequencing (NGS) platforms have been developed that are able to produce $10^5 – 10^7$ sequence reads of short-to-intermediate length (approximately 30–500 nt) using massively parallel sequencing approaches (Margulies et al., 2005). The most popular of these platforms are the 454-FLX (Roche), Genome Analyzer (Illumina) and SOLiD (Applied Biosystems) systems. The primary differences among these platforms are the sequence length and number of sequence reads achieved, although more detailed comparisons of these systems, including reaction chemistries and costs per sample, have been reviewed (Mardis, 2008). These approaches have greatly reduced the cost and increased the speed at which metagenomics-based approaches can be applied, without the need for the construction of extensive clone libraries, as can be seen by the exponential increase in the amount of sequence data uploaded to public repositories, such as the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (Figure 1).

**Exploration of the ‘rare biosphere’**

Among the first applications of NGS was an amplicon-sequencing study of deep sea water masses in the North Atlantic Ocean (Sogin et al., 2006). This study, which targeted the V6 region of the 16S rDNA, revealed that bacterial communities in the deep sea were comprised of a small number of dominant taxa, but that much of the phylogenetic diversity was contained in a high number of taxa at low abundance, which was termed the ‘rare biosphere’. This finding was supported by a study published 3 years later that assessed the variation in bacterial community structure over a 1-year period in the English Channel (Gilbert et al., 2009). Among 12 samples collected, a small fraction (0.5% of >17,000 unique sequences) of sequence reads represented 50% of the total sequence reads from each sample, and 78% of the operational taxonomic units (OTUs) identified were only found in a single sample. Furthermore, this study demonstrated seasonal variation, primarily among dominant OTUs, that were associated with changes in temperature as well as phosphate and silica concentrations (Gilbert et al., 2009).

Study of the English Channel over a 6-year period further confirmed that variation in seasonal parameters, especially day length, better explained variation in bacterial community structure than did trophic interactions, measured as protozoan and metazoan biomass (Gilbert et al., 2012). Furthermore, these seasonally driven shifts resulted in strongly reproducible patterns in variation of community structure. Similar NGS studies targeting the 16S rDNA in riverine systems have similarly revealed seasonally recurrent patterns in bacterial community structure (Crump & Hobbie, 2005; Staley et al., 2015a). Conversely, a recent study of the viral biogeography using the Pacific Ocean Virome dataset concluded that seasonal parameters were less important in shaping viral assemblages than were parameters such as depth and proximity to shore (Hurvitz et al., 2014). However, despite apparent variation both among dominant bacterial taxa as well as within the rare biosphere, deeper sequencing – increasing sequencing depth from a few thousand reads to several million – has revealed that there may potentially be a globally conserved marine microbial seed bank (Gibbons et al., 2013).

Results of these studies are highly suggestive that, even given the wealth of new information regarding the biodiversity and biogeography already obtained using NGS methods, future improvements in sequencing technologies may yield...
even more valuable insights into the microbial ecology of aquatic and other diverse ecosystems.

**Evaluation of water quality and public health risk**

Recently, NGS metagenomics methods have been employed to assess potential public health risks related to anthropogenic impacts on surface waters as well as to evaluate how these practices are influencing microbial community structure in these ecosystems and other recreational biomes such as beach sands (see the article by Solo-Gabriele et al., in this issue). The scope of these studies has ranged from the identification and characterization of previously unidentified viruses and bacteria, which may pose a health risk to humans or livestock, to evaluating the effects of eutrophication from agricultural runoff on total microbial community structure. In addition, these methods have been used to determine sources of faecal pollution to surface waters. While these metagenomic techniques have allowed for more thorough characterization of previously unknown species in these studies, they remain limited by a lack of genomic data among these, often uncultured, groups as well as a paucity of metadata to explain community variation and allow meaningful comparisons between datasets.

**IDENTIFICATION OF POTENTIAL PATHOGENS**

Catfish farming in the Mississippi Delta accounts for more than 50% of farmed catfish in the USA (Tucker, 1996). However, freshwater bodies such as catfish ponds represent important vectors for interspecies disease transmission due to the wide variety of interactions between humans, wildlife and the surface water. A metagenomic study of four catfish ponds utilized 454 pyrosequencing to identify 48 sequences that were found to belong to the viral family Asfarviridae (Wan et al., 2013). The only known member of this family prior to this study was the African swine fever virus. While the authors did not conclude that this represented a definite health risk associated with these novel viruses, the study was the first to identify members of this virus family in North America. They suggested that further study was necessary to evaluate the pathogenic potential of these viruses.

**APPLICATION TO SUSTAINABILITY EFFORTS**

Due to the scarcity of water in many regions of the world, the use of alternative water supplies to support rapid population growth remains a key component of sustainable agricultural practices (Levine & Asano, 2004). Reclaimed water has been proposed as an alternative source of non-potable water for purposes including agricultural irrigation. However, because it is an end-product of wastewater treatment, there are concerns regarding the possibility of pathogen transmission. While the concentration of virus-like particles in reclaimed water is ~1000-fold higher than in potable water (Rosario et al., 2009), metagenomic analysis revealed that most of these particles in both water types (46% in potable water and >50% in reclaimed water) did not have matches in existing databases, suggesting that they were novel. In addition, no viruses that did match database entries matched viruses known to be pathogenic to humans, but members of Siphoviridae were proposed as markers for faecal pollution. Similar to the catfish pond study, however, further study will be necessary to evaluate the host-specificity and pathogenic potential of the novel viruses identified.

Differences in the bacterial community of surface vs ground water have been investigated to determine the effects of different water sources on the bacterial community associated with the surfaces of tomatoes (Telias et al., 2011). Surface waters are exposed to a number of human, animal, and climate impacts that may result in the spread of pathogens when surface waters are applied directly to crops. Communities in groundwater had significantly higher relative abundances of Betaproteobacteria than did more diverse surface waters. However, no differences were observed in bacterial communities in the phyllosphere, the total above ground portion of plants, of tomatoes treated with different water types, and it was found that these communities were dominated by members of the Gammaproteobacteria (Telias et al., 2011). Furthermore, >90% of sequence reads were shared among all phyllosphere samples. Despite these results, the authors were unable to conclude that fruits treated with surface waters were completely safe due to the possibility of sequencing errors and an inability to identify OTUs at the species level.

**APPLICATIONS FOR WATER QUALITY MONITORING**

Runoff from agricultural practices is known to increase concentrations of nitrogen, phosphorus and other nutrients in surface waters, including rivers, lakes and coastal marine waters. Evaluation of the total microbial community of a freshwater Mediterranean lagoon that was eutrophic as a result of primarily agricultural impacts revealed that it was distinctly different from previously characterized freshwater systems (Ghai et al., 2012). Notably, ultramicrobacteria, specifically lineages of Actinobacteria and Alphaproteobacteria, that comprise well-known, ubiquitous freshwater lineages were minority members in this system. Furthermore, the genus Polynucleobacter, a member of the Betaproteobacteria, which is cosmopolitan in freshwater systems, was also absent. Instead, the community was dominated by cyanobacteria, in particular Synechococcus spp. Prevalence of cyanobacteria among eutrophic freshwater bodies was expected, yet the near absence of other major freshwater groups in this system was unusual. Results of this study highlight the potential detrimental effects of high levels of agricultural runoff on bacterial communities and water quality.

Similarly, the role of specific types of anthropogenic impacts in contributing nutrients (e.g. nitrogen and phosphorus) and chemicals (e.g. pharmaceuticals and agrochemicals), as well as in altering the bacterial community structure, have been investigated in the Mississippi River in Minnesota (Staley et al., 2013, 2014a, b). Initial results revealed that, despite various land coverage types throughout the study area, a core microbiome persisted over a reach of >400 km, such that ~90% of sequence reads were shared among the 10 sites sampled (Staley et al., 2013). Furthermore, bacterial communities at sampling sites could be grouped based on major surrounding land cover type (i.e. developed, forested or agricultural), suggesting that runoff from specific types of anthropogenic activities resulted in specific shifts in bacterial community structure. Investigation of local and regional microbial community dynamics in the Mississippi River, in Minnesota, revealed that local variations were primarily linked to within-community dynamics, but regional changes could be
associated with variations in specific nutrient concentrations, specifically total dissolved carbon and dissolved solids (Staley et al., 2014). Furthermore, increases in the relative abundances of specific orders were associated with broadly characterized land cover types. In addition, while the distributions of the majority of functional genes were conserved throughout the Mississippi River in Minnesota, there was slight variation in functional traits of bacterial communities between two basins that were surrounded by primarily agricultural vs primarily urban land cover (Staley et al., 2014). Results of these studies highlight the utility of metagenomic-based approaches to investigate taxonomic and functional variation as it relates to water quality, potential public health risk, and ecosystem health and sustainability at both local and regional scales.

**MICROBIAL SOURCE TRACKING**

Traditional indicators of water quality, in terms of risk to public health, have relied on culture-based enumeration of indicator bacteria (e.g., *Escherichia coli* and enterococci), which have failed to serve as a robust marker for the presence of pathogens due, in part, to their ubiquity among non-human sources (Harwood et al., 2014). In light of this obstacle, metagenomic approaches have proven useful in microbial source tracking (MST) studies, offering more specific characterization of sources of faecal contamination by comparing faecal microbial communities to those in the water column (Unno et al., 2010, 2012; Newton et al., 2013). The first implementation of metagenomic-based source tracking, PyroMiST (Unno et al., 2012), employed existing subroutines and available software (i.e. cd-hit) as well as Perl script automation in a web-based interface to identify sources of faecal contamination from 16S rDNA sequence data. However, in order to accommodate advances in the recent expansion of NGS technologies, including longer read lengths and variation between platforms (e.g. 454 and Illumina), it was necessary to develop a more flexible pipeline to identify sources.

SourceTracker, a subroutine implementable in the R software package [http://www.r-project.org](http://www.r-project.org), has since been developed to offer more flexibility in determining the contribution of known sources to an environmental community using taxonomic marker genes (Knights et al., 2011). While the use of this subroutine is not limited to MST studies in recreational water quality, it has been successfully utilized to identify, and to some extent quantify, sewage contamination in surface waters (Newton et al., 2013; Shanks et al., 2013). Moreover, this technology has been an adjunct to determining potential health risks associated with recreational water.

**Metagenomic characterization of functional diversity**

Taxonomic marker genes do not provide information regarding the distribution of functional traits. However, based on the known distributions of core genes among prokaryotic lineages, phylogenetic trees can be constructed from functional genes that closely resemble those built from taxonomically relevant sequences (Segata & Huttenhower, 2011). This suggests that taxonomic information alone may also be used to infer the distribution of functional genes on the basis of phylogenetic relationships. A recently developed subroutine, PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states), has been developed to infer functional traits for prokaryotes using 16S rDNA sequence data and the GreenGenes reference database (Langille et al., 2013). Functional inferences from PICRUSt were significantly correlated with shotgun metagenomic data from the Human Microbiome Project as well as from soils and a hypersaline microbial mat (Langille et al., 2013). However, the accuracy of these inferences in diverse environmental habitats, such as soils and waterways, requires further validation as these environments contain structurally and functionally diverse microbiota (Staley et al., 2014).

Due to the complexity of microbial communities, novel genes are unlikely to be successfully detected and characterized using WGS NGS data. However, functional metagenomic studies to characterize patterns in functional trait distribution as well as novel functional genes for traits (e.g. antibiotic resistance and heavy metals) remain promising areas of study (Torres-Cortés et al., 2011; Staley et al., 2014, 2015). To this end, the study of marine metagenomics has allowed for the discovery of novel enzymes that catalyse the formation of potentially useful metabolites (Barone et al., 2014). Traditionally, marine metagenomic studies aimed at discovery of novel bioactive compounds have relied on the functional or sequence-based screening of large clone libraries (see reviews: Kennedy et al., 2008; Barone et al., 2014; Reen et al., 2015), with few exploiting NGS approaches. However, Woodhouse et al. (2013) recently applied tag-encoded pyrosequencing as well as whole genome shotgun sequencing of the microbiome of Australian sponges to assess the diversity of non-ribosomal peptide synthetase and polyketide synthase genes. Using the tag-encoded approach, this group demonstrated the utility of using conserved domains in conjunction with NGS to identify genes involved in natural biosynthesis.

**COMPUTATIONAL ADVANCES FOR METAGENOMIC ANALYSIS**

The emergence of NGS technologies brought about a requirement to develop computational approaches to process and analyse the massive volumes of data generated. Advantages and limitations of these tools are outlined in Table 1. Preliminary computational tools, originally designed to handle <10⁶ sequence reads, such as LIBSHUFF (Singleton et al., 2001) and ARB (Ludwig et al., 2004) were encumbered by the computational demands of the emerging datasets, and their limited functionality. Moreover, performing one or a few functions, made them difficult to integrate. As a result, software programs such as mothur and QIIME (Quantitative

<table>
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<th>Advantages</th>
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<td>Identify unculturable microbes</td>
<td>Primer bias</td>
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<tr>
<td>Automated annotation of taxa/function</td>
<td>Sequencing errors</td>
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<tr>
<td>Integrated analysis pipeline</td>
<td>Incomplete databases</td>
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<td>Robust statistical analysis</td>
<td>Limited taxonomic resolution</td>
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<td>Functional prediction from marker genes</td>
<td>Prohibitive cost for sequence depth</td>
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Insights into Microbial Ecology) have become popular applications to process and analyse taxonomic marker genes (Schloss et al., 2009; Caporaso et al., 2010). These programs employ a pipeline of subroutines – a series of computational steps to perform multiple functions – for joining paired-end sequence reads (i.e. forward and reverse sequences), quality control procedures (e.g. quality trimming and chimera removal), sequence alignment, OTU clustering, and taxonomic assignment using standard reference databases such as GreenGenes, SILVA or the Ribosomal Database Project (RDP) (DeSantis et al., 2006; Pruesse et al., 2007; Cole et al., 2009). Statistical tools for analysis of complex datasets have also been incorporated into these programs allowing more thorough and complex analyses of ecological datasets.

Similar to NGS of taxonomic marker genes, results of WGS sequencing studies were also initially difficult to interpret due to the increased computational requirements. Metagenomic Analyzer (MEGAN) software was developed specifically to deal with the computation of NGS shotgun sequences, bypassing the limitations of extensive sequence assembly from environmental sequence data and the lower abundance of phylogenetically relevant marker genes (Huson et al., 2007). The Community Cyberinfrastructure for Advanced Marine Microbial Research and Analysis (CAMERA) was developed as an online repository for sequence and metadata, and integrates existing and emerging bioinformatics tools for the analysis of metagenomic data originally incorporating analytical packages and workflows used for the Global Ocean Sampling (GOS) expedition (Seshadri et al., 2007). The program compares sequence reads against a reference database and outputs the results for exploration using a graphical interface. Similarly, web servers like the Metagenomics RAST (MG-RAST) have also been developed where data can be uploaded and stored, and taxonomic and functional annotations are performed automatically (Meyer et al., 2008). While these tools have facilitated analysis of datasets of previously unprecedented depth and coverage, they each have their unique advantages and disadvantages.

Metagenomic studies to date have been carried out primarily from a descriptive, data discovery perspective, with a focus on what is in an environmental sample and how diversity, as well as the presence and abundance of community members or functional genes, change between samples and habitats. Ordination techniques such as principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) have enabled visualization of these complex datasets to facilitate these studies. In fact, one of the main drawbacks of these studies is the enormous datasets generated and the inability to view all the data in an easily interpretable format. Network-based approaches have also been developed to allow visual comparisons from complex metagenomic shotgun and taxonomic marker datasets (Mitra et al., 2010; Larsen et al., 2012). Statistical software packages such as STAMP (Statistical Analysis of Metagenomic Profiles) have been developed to clearly summarize statistical trends allowing meaningful biological inferences to be made from these complex data (Parks & Beiko, 2010). The recent and continuing decline in the cost of NGS will soon allow for better sample replication, enabling more powerful statistical comparisons, and the generation of terabyte datasets that will allow for a better quantitative assessment of taxa and genes present in the environment (Gilbert & Dupont, 2011).

**Computational Limitations to Metagenomic Analyses**

Prior to 2006, metagenomics studies were limited to clone-based studies and Sanger sequencing, and the relatively high cost of sequencing (∼$500 per Mb) restricted the size of these early datasets, as well as replication efforts for robust statistical comparisons (Kircher & Kelso, 2010; Temperton & Giovannoni, 2012). These methods were subject to PCR amplification and cloning biases. Next-generation sequencing methods have greatly reduced the costs associated with generating larger volumes of sequence data, and have, to some extent, alleviated bias associated with cloning (Wooley et al., 2010). However, PCR primer bias remains an intrinsic limitation and this issue, coupled with shorter sequence read lengths, can significantly affect the diversity inferred from NGS data (Youssef et al., 2009). In addition, next-generation sequences are subject to error due to DNA polymerases, chimera formation and sequencing errors (Kircher & Kelso, 2010; Patin et al., 2013). Bias and error can be reduced by improving reaction chemistries, reducing PCR cycle numbers, using well-designed primer sets, and refining the quality of reagents. However, computational approaches must also be considered to account for these errors as well as difficulties arising from the massive volumes of data generated.

The intrinsic problem of sequencing error is mitigated in single organism genomic sequencing by sequence assembly and high coverage depth (Goldberg et al., 2006); however, taxonomic marker surveys using NGS methods are prone to overestimation of diversity resulting from sequence error (Kunin et al., 2010). Early analysis of sequence errors in a NGS dataset has shown that exclusion of sequences containing ambiguous bases (Ns) and primer or barcode mismatches reduces the sequence error rate in the dataset to less than that of Sanger sequencing while retaining >90% of sequence reads (Huse et al., 2007). Subsequent studies have shown that a 2% pre-clustering step and OTU binning at ≤97% similarity greatly reduced inflated diversity estimates associated with sequencing error (Huse et al., 2010; Kunin et al., 2010). In addition, several computational approaches, such as UCHIME (Edgar et al., 2011), have been developed to identify and remove chimeric sequence reads (Wooley & Ye, 2009).

Despite these processing steps to improve sequence quality, inherent bias and limitations still exist when using small subunit rRNA genes for taxonomic surveys. Due to fundamental differences in rDNA sequences, prokaryotes and eukaryotes must be sequenced separately. Among prokaryotes, utilization of the 16S rDNA presents unique challenges in that species resolution can be difficult as a result of the highly conserved nature of this gene (Gürtler & Stanisich, 1996). Furthermore, a single cell can contain up to 15 copies of rDNA and copies may be heterogeneous within the same genome, further complicating species identification and accurate quantification of taxonomic abundances (Klappenbach et al., 2001). Finally, even when universal primers are used for amplification, it is unlikely that all members of a certain group (e.g. bacteria or archaea) will be amplified due to the higher diversity of these domains (Davenport & Tümmler, 2013).

For WGS studies of environmental samples, sequencing coverage is often extremely low, thus it is difficult to discern individual genomes within a community due to differences in relative abundances of highly diverse organisms. The first,
Sanger-sequencing-based, metagenomic studies had some success using single genome sequence assemblers to build contigs, contiguous sequence fragments, from metagenomic data, owing to relatively small numbers of sequence reads and longer read lengths compared with NGS methods (Venter et al., 2004; Rusch et al., 2007). However, the large number and small read length of NGS reads limit the effectiveness of sequence assembly by these methods (Wooley et al., 2010). Large amounts of nearly identical sequences and the possibility of assembling sequences from different OTUs require a different assembly strategy that has been met by several new programs such as IDBA-UD and meta-VELVET (Namiki et al., 2012; Peng et al., 2012), which take into account short sequence reads and uneven coverage due to differences in OTU abundance. Nevertheless, there is still a trade-off between assembly, which can reveal novel genes or species not previously described, and read mapping without assembly, which allows semi-quantitative inferences (Davenport & Tümmler, 2013).

While NGS costs have declined compared with Sanger sequencing, complete metagenomic characterization of highly diverse environmental samples remains limited by the prohibitive cost required (Gilbert & Dupont, 2011; Knight et al., 2012). Even with the recent advances in sequencing technology, <0.000001% of the metagenome in seawater is estimated to have been sequenced based on average genome sizes and bacterial density in a one litre sample (Gilbert & Dupont, 2011). Furthermore, 4–5 × 10^14 bp and 3 × 10^15 bp of sequence data are estimated as the requirement for 1× coverage of a one litre seawater and one gram soil sample, respectively (Gilbert & Dupont, 2011; Knight et al., 2012). To accomplish this sequencing depth, >800 and approximately 5000 full runs would be required on an Illumina HiSeq2000 platform, at a cost of tens of millions of dollars currently (Caporaso et al., 2012). However, 6× to 8× coverage is considered the standard to ensure adequate representation of all of the genomes in the community (Akondi & Lakshmi, 2013), further increasing costs and effort. Thus, analyses of complex environments at the degree of coverage needed may need to wait for the development of new, even less expensive, sequencing technology.

Regardless of the type of sequencing study performed, the taxonomic and functional annotations that result are dependent on the databases used. Databases are known to have a compositional bias, favouring sequences from easily cultivable and accessible organisms (Pignatelli et al., 2008). The quality of the assemblies and the application used for gene or taxonomic annotation can have significant effects on the percentage of reads annotated as well as the accuracy of the prediction (Mavromatis et al., 2007). Furthermore, the completeness of the database dramatically influences the classification of sequences, and taxonomic and functional composition of samples may change depending on the version of the database used, even among recently updated databases (Pignatelli et al., 2008). These changes primarily result from the assignment of previously unclassified reads to newly sequenced taxa. However, sequencing of closely related species can also result in shifts in assignment (Pignatelli et al., 2008).

**PERSPECTIVES**

The studies reviewed here, highlight the efficacy of employing metagenomics approaches in the study of environmental samples, specifically water samples, to better characterize biodiversity, biogeography, the effects of anthropogenic impacts, and potential public health risk. Development of new computational tools to process and analyse NGS data has facilitated the identification of previously unidentified microorganisms, some of which may have important public health implications. Furthermore, more thorough characterization of microbial communities is facilitating better interpretations regarding which practices are important in shaping bacterial community structure. Despite recent advances, drawbacks to these methods still exist, including sequencing error as well as biases and gaps in reference databases favouring easily culturable microorganisms. Further advances in technology and metagenomics studies will allow microbial ecologists and physiologists to fill these knowledge gaps and thus provide a more complete understanding of the interaction between anthropogenic practices, the environment and microbial communities.

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**CONFLICTS OF INTEREST**

The authors declare that they have no conflicts of interest.

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and


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The preliminary assessment of abundance and composition of marine beach debris in the northern Persian Gulf, Bandar Abbas City, Iran

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Marine debris is a major challenge threatening ocean and coastal environment with no easy solution in coming years. The problem is totally manmade and extends to coastal areas around the world. The accumulation of marine debris is largely due to lack of awareness and environmental education among the public reinforced with mismanagement of municipal litter in coastal cities. Iran has about 2415 km of coastlines in the north and south of the country that suffer severely from a marine debris problem. Despite the great scale of the problem that leads to the shutdown of some beaches to beachgoers, there are no documented data on the abundance, composition and sources of marine debris dispersed on beaches. With the cooperation of marine biology student volunteers, we surveyed beaches at two scales, 100 m and 1 km searching for litter in varying ranges. The results revealed that the most common items are made of plastic/polystyrene. Tourism and recreational activities are responsible for more than 90% of litter production on the study beach. Fisheries and aquaculture are the second source of most abundant marine litter found on the beach with considerable distance from tourism activities. We conclude that lack of education about the consequences of marine litter is the main reason for marine debris at the study beach. Another reason is related to rapid construction of coastal areas and development of coastal cities with no proper infrastructure to manage municipal littering.

Keywords: marine beach debris, assessment, the Persian Gulf, Bandar Abbas city

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INTRODUCTION

One of the most ubiquitous and major problems threatening our marine environment is accumulation and fragmentation of solid debris particularly plastics (Barnes et al., 2009; Thompson et al., 2009). This versatile, inexpensive, light weight, durable and transparent material has many applications in our daily life with the annual global production at 245 million tonnes (Andrady, 2011). Since the mass production of plastics commencing around the 1950s, plastic items have accumulated around the world from pole to pole and are even reported from remote islands (Moore et al., 2001a, b; Edyvane et al., 2004; Barnes, 2005; Thompson et al., 2009; Eriksson et al., 2011; Thiel et al., 2013). Despite the widespread recognition of the problem, evidence suggests that plastic debris accumulation is still increasing (Moore, 2008; Barnes et al., 2009; Ryan et al., 2009). The sources of the marine debris are from both land-based and marine-based activities, although it is estimated that half of the marine debris is produced from land-based activities (Shevly & Register, 2007). On shorelines, accumulation of marine debris, especially plastics, is greater in the northern than in the southern hemisphere and there is evidence that plastic fragments are buried in the sediment (Barnes et al., 2009; Ryan et al., 2009). Marine debris is not only an eyesore but the potential hazard to marine biodiversity from tiny filter feeders to giant mammals in all oceans (Laist, 1997; Thompson et al., 2009; Van Franeker et al., 2011). Entanglement in plastic items, particularly discarded fishing gear is a serious threat to marine animals (see Laist, 1997; Moore et al., 2009). Ingestion of stranded and fragmented plastic items can have fatal consequences for marine species (Browne et al., 2008; Van Franeker et al., 2011, Ramos et al., 2012, Cole et al., 2014). Many comprehensive studies have been done to quantify and monitor coastal debris around the world (OSPAR, 2007; Cheshire et al., 2009; information is also available at marinedebris.noaa.gov) but no such data have been reported for the coastline of the Northern Persian Gulf. The data collected in this research have been derived from volunteer beach debris monitoring efforts conducted during May 2014. Here we present the first quantitative assessment of marine debris found along Bandar Abbas beach. We aimed to understand the amount and composition of marine debris along the coastline of Bandar Abbas to determine their origins. The data
are valuable for decision makers in the initiation of national projects to deal with the marine debris problem.

MATERIALS AND METHODS

This study was conducted as a pilot project to assess marine litter on the Bandar Abbas coastline with a quantitative approach. Marine beach litter was distinguished in the field following the definition of marine debris of UNEP (2009): ‘Marine debris is any manufactured or processed solid waste material that has been accidentally or deliberately released to the sea’, available at http://www.unep.org/regionalseas/marine-litter/about/default.asp. Beach debris was collected based on the standard method introduced by OSPAR commission entitled ‘OSPAR Pilot Project on Monitoring Marine Beach Litter, Monitoring of Marine Litter in the OSPAR region’ (OSPAR, 2007b). This survey was completed by student volunteers from Bandar Abbas University. The survey site is a stretch of 6 km upper limit of a beach located in Bandar Abbas coastal park (27°11′7.22″N 56°20′10.89″E).

The selection of sites was made according to OSPAR (2007b) guidelines. The criteria include sediments of sand or gravel, exposed to open water, according to visual observation frequently littered, good accessibility to the surveyors, over 1 km in length, and not located near input sources of wastewater (OSPAR, 2007b).

The selected area is used heavily as an intertidal fishing ground and known as a recreational site for youth and families. Beach litter was collected and sorted at two different scales (100 and 1000 m). Materials used for this survey were simple and inexpensive. We used gloves, pencils, printed paper forms and rubbish bags prepared by Bandar Abbas University. Ten categories of debris were determined based on OSPAR monitoring beach litter guidelines (2007b). Each debris item was identified, photographed and documented in the printed survey forms. Data analysis was done using Excel.

RESULTS

An average of 456 items of varying sizes were found per 100 m stretch of Bandar Abbas beach. Following the global tendency
(Derraik, 2002) the largest proportion of marine litter found in coastlines was made of plastics. In this survey at the scale of 100 m, more than 80% of total items were plastic debris. Paper and wooden materials ranked second and third (Figure 1).

The most common items found in the highest number were made of plastic/polystyrene including caps/lids, drinks (bottles, containers, drums) and crisp/sweet packets and lolly sticks (Figure 2). Our results indicate an average density of 4.5 items per metre in this 6 km beach survey.

To estimate the sources of marine litter in this area, we defined some marine litter items as indicators for five categories of potential origins of marine debris (Table 1). The possible sources of marine coastal litter includes fisheries and aquaculture, shipping activities, sanitary and sewage-related waste, shipping including offshore activities, tourism and recreational based waste.

Analysis shows that the marine debris left on the beach is mainly produced by beach users and recreational activities (Figure 3). About 94% of all marine items originated from beach users. Fisheries and aquaculture in the area is responsible for about 2% of observed marine litter on the beach.

For the 1000 m scale of the survey, the 4 km of the beach were investigated to determine the quantity and composition of all marine litter

<table>
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<tr>
<th>Sources</th>
<th>Indicators</th>
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<tbody>
<tr>
<td>Fisheries including aquaculture</td>
<td>Jerry cans, fish boxes, fishing line, fishing weights, rubber gloves, floats/buoy, ropes/cords/nets, crane/lobster pot, oyster nets and mussel bags,</td>
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<tr>
<td></td>
<td>plastic sticks, plastic sheeting from mussel culture ('Tahitians')</td>
</tr>
<tr>
<td>Galley waste from shipping, fisheries and</td>
<td>Cartons/tetrapaks, cleaner bottles, spray cans, metal food cans, plastic gloves, plastic crates, sanitary towels/panty liners/backs strips,</td>
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<tr>
<td>offshore activities</td>
<td>tampons/tampon applicators, other items</td>
</tr>
<tr>
<td>Sanitary and sewage-related waste</td>
<td>Condoms, cotton bud sticks, sanitary towels/panty liners/backs strips, tampons/tampon applicators, other items</td>
</tr>
<tr>
<td>Shipping, including offshore activities</td>
<td>Strapping bands, Industrial packaging, Hard hats, Wooden pallets, oil drums (new and old), light bulbs/tubes, injection gun, containers</td>
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<tr>
<td>(operational waste)</td>
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<tr>
<td>Tourism and recreational activities</td>
<td>4–6-pack yokes, plastic shopping bags, plastic bottles/containers for drinks, metal bottles/containers for drinks, plastic food containers,</td>
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<tr>
<td></td>
<td>glass bottles, crisp/sweet packets and lolly sticks, caps/lids, cutlery/trays/straws, cigarette butts, cups, small plastic bags, freezer bags, cloth</td>
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Table 1. Sources and indicators selected for data analysis in the survey area on Bandar Abbas beach.

Fig. 3. Sources of all marine litter found on on 100 m stretches of beach in Bandar Abbas, northern part of the Persian Gulf, Iran.

Fig. 4. Proportion of marine debris >50 cm recorded in 1000 m stretch of beaches during the project on May 2014 on the coastline of Bandar Abbas, northern part of the Persian Gulf, Iran.
The most common items were plastic/polystyrene materials and clothing was the second-most common item observed on the reference beach (Figure 5). Of the other 10 categories found, plastic rope from the plastic/polystyrene category was third, which mostly indicates residuals of fisheries activities on the beach.

**DISCUSSION**

Marine debris is a multidimensional problem of global scale that particularly challenges coastal communities (Cheshire et al., 2009). Despite the large-scale effort from different commissions and institutes (OSPAR, 2007b; Cheshire et al., 2009; NOAA available at marinedebris.noaa.gov) the problem remains unsolved till today. Iran has an extended sea coast border which is estimated as about 644 km lying along the southern Caspian shore and about 1770 km on the northern part of the Gulf of Oman and Persian Gulf (Fisher, 1968). Globally, the main source of marine litter is from land-based and oceanic-based activities, with 50% originated from land (Sheavly & Register, 2007). The Persian Gulf is under tremendous pressure from many levels such as hydrocarbon pollution, heavy construction, offshore activities, severe drought caused by construction of dams, and changes associated with global warming (Sheppard et al., 2010). The rapid pace of heavy construction in coastal areas and mismanagement of municipal litter results in beaches and intertidal zones being dumping places of litter. As our results revealed (Figure 3) the origin of marine debris found on these beaches is mainly due to beach users’ behaviour, in that they accidentally or intentionally left their litter on the beach. A volunteer-based study conducted on Chilean beaches showed that the average of small plastic items per m² is 27 and that these originated from coastal urban centres and their economic activities (Hidalgo-Ruz & Thiel, 2013). Another similar study on a Caribbean coast in mangrove beach sites revealed that plastic and polystyrene items were the most abundant debris (Debrot et al., 2013). The second source of marine litter is from fisheries activities (Figure 3) although it is not considerable compared with tourism and recreational activities. The traditional fishing method that is known as ‘Moshta’ is frequently observed at the intertidal zone. This efficient and environmentally friendly method of fishing is a cage of wooden bars that is embedded on the intertidal areas with a coverage of nets around it that simply traps fish and other marine animals. Fishermen collect animals at low tide by walking on the dry beach. It is estimated that some wood and nets are lost in storms or destroyed.

As well as the health and aesthetic problem, marine debris has fatal consequences for marine biota (Laist, 1997; Browne et al., 2008; Cole et al., 2014). Our great concern is about the amount of plastic items found on the beach (Figures 1 & 4). Following the global tendency (Ryan et al., 2009; Thompson et al., 2009) this non-degradable, long durable and inexpensive polymer makes up the highest percentage of total items recorded in the area. Recent studies show that fragmentation of plastic debris produces small fragments called ‘microplastics’ with harmful impacts on all trophic levels either through direct consumption or by release of chemicals (Rios et al., 2007; Teuten et al., 2009; Thompson et al., 2009; Ivar do Sul & Costa, 2014).

We suggest expanding the environmental educational systems in coastal borders along with establishing anti-littering policies, to raise awareness about the consequences of litter on the marine environment. Littering behaviour is the one that needs to be changed. Due to the fatal consequences of marine debris on marine biota, immediate concern is vital in Iran. We recommend to marine biologists and policymakers to open up serious and immediate discussion in this field of research in Iran.

**ACKNOWLEDGEMENTS**

We would like to thank marine biology and fisheries students of Bandar Abbas University for their enthusiastic volunteer participation in this project. We appreciate the anonymous reviewers for the valuable suggestions.
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and


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email: zhalehsarafraz@gmail.com
Exposure of bivalve shellfish to titania nanoparticles under an environmental-spill scenario: Encounter, ingestion and egestion

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Nanoparticles have applications in a diverse range of products including medications, detergents, cosmetics, paint, sunscreen and electronics, with an economic worth projected to reach $2.5 trillion dollars in 2015. Research into the effects of manufactured nanomaterials on the environment, however, has failed to keep pace with the high volume of commercial production. Whereas a number of studies have examined the effects of nanoparticles on aquatic species, little work has focused on the way in which benthic marine species encounter, ingest and depurate these materials. The purpose of this study was to examine the ingestion and depuration of titania nanoparticles (anatase) by the blue mussel (Mytilus edulis) and the eastern oyster (Crassostrea virginica) during a spill scenario (an acute exposure to elevated concentrations). Bivalves were exposed to nanoparticles either incorporated into marine snow, an environmentally relevant medium for pollutants, or added directly to seawater at a concentration of 4.5 mg L\(^{-1}\) for 2 h. After feeding, the animals were transferred to filtered seawater and allowed to depurate. Faeces and tissues were collected at 0, 6, 24, 72 and 120 h, post-exposure, and analysed for concentrations of titanium by inductively coupled plasma-mass spectrometry. Results indicated that the capture and ingestion of titania nanoparticles by both species was not dependent on the method of delivery (incorporated into marine snow or freely suspended). Additionally, greater than 90% of the titania nanoparticles, on average, were eliminated from the tissues after 6 h, and only trace amounts remained after 72 h. These data demonstrate that mussels and oysters readily ingest titania nanoparticles, but rapidly depurate the material within hours of an acute exposure suggesting that little would be transferred to secondary consumers including humans. Further research is required to determine if other species of suspension-feeders handle titania nanoparticles in a manner similar to bivalves.

Keywords: nanoparticles, environmental spill, bivalves, ingestion, bioaccumulation, depuration

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INTRODUCTION

Emerging contaminants such as manufactured nanomaterials are of growing concern in the marine environment, not only because they can directly impact resident organisms, but also because they can potentially be transferred to humans via the food chain. One group of nanomaterials that has received scrutiny is the particles composed of titania. At the nanoscale, titania possesses unique physicochemical and photocatalytic properties that have been implemented in a range of industrial and commercial applications. For example, titania nanoparticles (TiO\(_2\) NPs) are used in paint as pigments and opaquers (Carp et al. 2004), in cosmetics and sunscreens to absorb UV radiation (Jaroenworaluck et al. 2006; Siddiquey et al. 2007; Labille et al. 2010), as antimicrobials (Ame´zaga-Madrid et al. 2003; Kim et al. 2003; Kühn et al. 2003; Robertson et al. 2005; Adams et al. 2006; Li et al. 2006; Foster et al. 2011) and in the degradation of organic pollutants (Chatterjee & Mahata 2002). TiO\(_2\) NPs can exist in either the rutile, anatase, or brookite crystalline phases (Markowska-Szczupak et al. 2011), of which rutile and anatase are the most common (EPA 2010). Both the rutile and anatase crystalline phases demonstrate a tetragonal structure, however, the anatase form tends to be less dense and more photocatalytically active than the rutile structure (Serpone et al. 2007; Dankovic & Kuempel 2011).

Although the precise production rates of manufactured nanomaterials are not typically released, estimates of TiO\(_2\) NPs in the USA alone are projected to reach 2.5 million tonnes annually by the year 2025 (Robichaud et al. 2009). Mueller & Nowack (2008) estimated that the concentration of TiO\(_2\) NPs in aquatic environments were between 0.7–16 µg L\(^{-1}\) for realistic and high exposure scenarios, respectively, however, the estimates were made in 2008, and represent end-use concentrations of TiO\(_2\) NPs in Switzerland only (Robichaud et al. 2009). Naturally derived titanium weathered from the earth’s crust (Schroeder et al. 1963; Fishbein et al. 1982; Orians et al. 1990) is estimated to be 4–8 picomolar (~0.3–0.6 ng L\(^{-1}\); Orians et al. 1990) at the ocean’s surface, with lower nanomolar concentrations present in estuarine environments (Yokoi & van den Berg 1991; Skrabal et al. 1992; Skrabal 1995). Thus, conservative estimates show that anthropogenic loads of TiO\(_2\) NPs in aquatic environments could be three orders of magnitude higher than the
concentration of naturally derived titanium. As populations in coastal and estuarine regions continue to rise (National Research Council, 2007), the prevalence of novel contaminants such as NPs entering aquatic systems in sewage effluents, industrial waste, and surface runoff have also increased (Kolpin et al., 2002; Farré et al., 2008).

Nanomaterials entering the marine environment are exposed to dissolved, colloidal and particulate organic matter that will increase their potential for homo- and hetero-aggregation (Brant et al., 2005; Xie et al., 2008; Sharma, 2009; Doyle et al., 2014). Additionally, physical and biological processes aggregate particulate matter suspended in the water (including nanoparticles) into larger masses known as marine snow (Alldredge & Silver, 1988; Jackson, 1990; Doyle et al., 2014). In fact at certain times of the year, large proportions (>70%) of natural particulates are incorporated in marine snow (Alldredge et al., 1993; Crocker & Passow, 1995). Marine snow has a complex three-dimensional structure that is physically and chemically distinct from the surrounding water (Silver et al., 1978; Alldredge, 2000; Ploug, 2001), is important for the vertical transport of material to the benthos (Kierboe et al., 1990; Passow & Wassmann, 1994; Crocker & Passow, 1995; Waite et al., 2000), and can facilitate the trophic transfer of dissolved and particulate matter to benthic suspension-feeders (Alber & Valiela, 1994, 1996; Kach & Ward, 2008). Certain types of metal-oxide NPs have a high agglomeration potential in seawater (Sillanpää et al., 2011; Shih et al., 2012; Doyle et al., 2014).

Formation of larger-diameter NP agglomerates can cause an increased collision rate with other particles suspended in the water column (Jackson, 1990), and a higher incorporation rate in marine snow. Nanoparticles that are incorporated in marine snow will sink faster than the same particles that are freely suspended (Stokes Law; Hill, 1998; Waite et al., 2000). Higher sinking rates would increase deposition rates to the bottom, exposing benthic organisms to a higher concentration of NPs over a shorter time period. Additionally, tides and bottom, exposing benthic organisms to a higher concentration of naturally derived titanium. As populations in coastal and estuarine regions continue to rise (National Research Council, 2007), the prevalence of novel contaminants such as NPs entering aquatic systems in sewage effluents, industrial waste, and surface runoff have also increased (Kolpin et al., 2002; Farré et al., 2008).

Dense populations interact strongly with near-shore water columns, removing phytoplankton, depositing faeces and pseudofaeces (material rejected prior to ingestion), cycling dissolved nutrients (Dame, 1993, 1996; Prins et al., 1998; Newell, 2004), and contributing to the concentration of transparent exopolymer particles (TEP; McKenzie et al., 2005; Heinonen et al., 2007; Li et al., 2008). Additionally, bivalves process large amounts of organic matter, converting some of it into body tissues that can be used by higher trophic levels including humans. Many bivalve species are commercially important, providing a source of jobs and food to people worldwide. For example, the global catch of bivalves as of 2010 was approximately 1.7 million tonnes, whereas world-wide aquaculture production of bivalves was about 13 million tonnes (FAO, 2012). These characteristics make suspension-feeding bivalves an important group of organisms to study. Defining how bivalves interact with, and are affected by, manufactured nanomaterials is critical to an understanding of the potential broad-scale impacts of these materials on water quality and productivity of coastal ecosystems. Such data are also important to define which types of nanomaterials are bioaccumulated and could be transferred up the food chain to higher-level consumers including humans.

Several studies have demonstrated the cytotoxic and genotoxic effects of TiO2 NPs on suspension-feeding bivalves (see Table 1 for a more complete review). For example, the haemocytes of Crassostrea virginica (eastern oyster) were found to have reduced phagocytosis after exposure to TiO2 NPs (Abbott-Chalew et al., 2012). Mytilus galloprovincialis (Mediterranean mussel) demonstrated oxidative stress, reduced transcription of immune-function genes, decreased lysosomal membrane stability, reduced haemocyte phagocytosis, activation of MAPK stress genes, larval malformations, and decreased numbers of mitochondria following exposure to TiO2 NPs (Canesi et al., 2010a, b, 2014; Giacci et al., 2012; Barmo et al., 2013; Libralato et al., 2013). A reduction in haemocyte viability and phagocytosis as well as the production of reactive oxygen species (ROS) were observed in Perna viridis (Asian green mussel; Wang et al., 2014). Dreissena polymorpha (zebra mussel) showed reduced haemocyte phagocytosis and activation of the MAPK stress genes after exposure to TiO2 NPs (Couleau et al., 2012). Despite the observed cellular and genotoxic effects, no studies have addressed the ways in which suspension-feeding bivalves encounter TiO2 NPs in the environment. Furthermore, no data exist concerning the ingestion and depuration rates of TiO2 NPs in exposed bivalves.

The goal of the research presented here was to understand the ingestion and depuration rates of Mytilus edulis (blue mussel) and Crassostrea virginica (eastern oyster) exposed to a high concentration of TiO2 NPs over an acute time interval. Dosing the animals at a high concentration for a short period of time was used as a proxy for an environmental spill...
Table 1. A summary of the cytotoxic and genotoxic effects of nanoparticles on various marine species of bivalves.

<table>
<thead>
<tr>
<th>Species</th>
<th>Nanoparticle</th>
<th>Concentration</th>
<th>Exposure</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oyster</td>
<td>Crassostrea virginica</td>
<td>Ag, TiO$_2$</td>
<td>1–400 µg L$^{-1}$</td>
<td>15–120 min</td>
<td>Reduced phagocytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag</td>
<td>0.02–20 µg L$^{-1}$</td>
<td>48 h</td>
<td>Decreased LMS, ROS production, reduction in total proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_{60}$ Fullerene</td>
<td>1–500 µg L$^{-1}$</td>
<td>4 days</td>
<td>Decreased LMS, accumulation in lysosomes, impaired development</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag</td>
<td>1.6–16 µg L$^{-1}$</td>
<td>48 h</td>
<td>Impaired development, Decreased LMS, increased metallothionein gene expression</td>
</tr>
<tr>
<td>Mussel</td>
<td>Mytilus edulis</td>
<td>Fe</td>
<td>1 mg L$^{-1}$</td>
<td>1–12 h</td>
<td>Bioaccumulation, lipid peroxidation, decreased LMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SiO$_2$</td>
<td>Not reported</td>
<td>12 h–16 days</td>
<td>Decreased LMS, oxidative stress, increased phagocytosis, apoptosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Au</td>
<td>750 µg L$^{-1}$</td>
<td>24 h</td>
<td>Oxidative stress</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polystyrene</td>
<td>0.1–0.3 g L$^{-1}$</td>
<td>8 h</td>
<td>Increased pseudofaeces, reduced filtration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag</td>
<td>0.7 µg L$^{-1}$</td>
<td>3.5 h</td>
<td>Bioaccumulation, presence in extrapallial fluid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ag</td>
<td>0.7 µg L$^{-1}$</td>
<td>3.5 h</td>
<td>Alterations of shell nacre</td>
</tr>
<tr>
<td>Mussel</td>
<td>Mytilus galloprovincialis</td>
<td>C$_{60}$ Fullerene</td>
<td>100–1 mg L$^{-1}$</td>
<td>3 days</td>
<td>DNA damage, histological abnormalities, bioaccumulation, decreased clearance rates</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>1–100 µg L$^{-1}$</td>
<td>96 h</td>
<td>Oxidative stress, reduced transcription of immune-function genes, decreased LMS, reduced phagocytosis, pre-apoptotic effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCB, TiO$_2$, SiO$_2$</td>
<td>1–10 mg L$^{-1}$</td>
<td>60 min</td>
<td>Increased ROS production, decreased mitochondria, activation of MAPK stress genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_{60}$ Fullerene, TiO$_2$, SiO$_2$</td>
<td>1–10 mg L$^{-1}$</td>
<td>30 min–4 h</td>
<td>Increased oxidative stress, activation of MAPK stress genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NCB, C$_{60}$ Fullerene, TiO$_2$, SiO$_2$</td>
<td>50 µg L$^{-1}$–5 mg L$^{-1}$</td>
<td>24 h</td>
<td>Decreased LMS, oxidative stress</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>10 mg L$^{-1}$</td>
<td>30–60 min; 96 h</td>
<td>Decreased LMS, reduced phagocytosis, genotoxicity, bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$, SiO$_2$, ZnO, CrO$_2$</td>
<td>1–10 mg L$^{-1}$</td>
<td>30 min–4 h</td>
<td>Decreased LMS, hormetic effects on phagocytosis, increased ROS production, decreased mitochondria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CuO</td>
<td>10 µg L$^{-1}$</td>
<td>15 days</td>
<td>Bioaccumulation, oxidative stress, neurotoxicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CuO, Ag</td>
<td>10 µg L$^{-1}$</td>
<td>15 days</td>
<td>DNA damage</td>
</tr>
<tr>
<td>Mussel</td>
<td>Mytilus galloprovincialis</td>
<td>Ag</td>
<td>10 µg L$^{-1}$</td>
<td>15 days</td>
<td>Bioaccumulation, changes in protein expression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnO Quantum Dots, Fe</td>
<td>0.1–2 mg L$^{-1}$</td>
<td>84 days</td>
<td>Bioaccumulation, reduced growth, mortality</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CdSe Quantum Dots, Fe</td>
<td>92–2 mg L$^{-1}$</td>
<td>18 &amp; 24 h</td>
<td>Bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>0.5–64 mg L$^{-1}$</td>
<td>48 h</td>
<td>Larval malformations</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CeO$_2$, ZnO</td>
<td>1–10 mg L$^{-1}$</td>
<td>4 days</td>
<td>Bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_{60}$ Fullerene</td>
<td>1–10 mg L$^{-1}$</td>
<td>60 min</td>
<td>Decreased LMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SWCNH</td>
<td>1–10 mg L$^{-1}$</td>
<td>24 &amp; 48 h</td>
<td>Reduced ROS production, decreased LMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>2.5–10 mg L$^{-1}$</td>
<td>24–216 h</td>
<td>Decreased hemocyte viability, decreased phagocytosis, reduced ROS production</td>
</tr>
<tr>
<td>Clam</td>
<td>Macoma balthica</td>
<td>Ag, CuO</td>
<td>200 µg g$^{-1}$</td>
<td>35 days</td>
<td>Bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnO</td>
<td>0.1–2 mg L$^{-1}$</td>
<td>84 days</td>
<td>Bioaccumulation, reduced growth, mortality</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CdSe Quantum Dots, Fe</td>
<td>92–2 mg L$^{-1}$</td>
<td>18 &amp; 24 h</td>
<td>Bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>0.5–64 mg L$^{-1}$</td>
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<td>Larval malformations</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CeO$_2$, ZnO</td>
<td>1–10 mg L$^{-1}$</td>
<td>4 days</td>
<td>Bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_{60}$ Fullerene</td>
<td>1–10 mg L$^{-1}$</td>
<td>60 min</td>
<td>Decreased LMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SWCNH</td>
<td>1–10 mg L$^{-1}$</td>
<td>24 &amp; 48 h</td>
<td>Reduced ROS production, decreased LMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>2.5–10 mg L$^{-1}$</td>
<td>24–216 h</td>
<td>Decreased hemocyte viability, decreased phagocytosis, reduced ROS production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZnO Quantum Dots, Fe</td>
<td>92–2 mg L$^{-1}$</td>
<td>18 &amp; 24 h</td>
<td>Bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>0.5–64 mg L$^{-1}$</td>
<td>48 h</td>
<td>Larval malformations</td>
</tr>
<tr>
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<td>CeO$_2$, ZnO</td>
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<td>4 days</td>
<td>Bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_{60}$ Fullerene</td>
<td>1–10 mg L$^{-1}$</td>
<td>60 min</td>
<td>Decreased LMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SWCNH</td>
<td>1–10 mg L$^{-1}$</td>
<td>24 &amp; 48 h</td>
<td>Reduced ROS production, decreased LMS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TiO$_2$</td>
<td>2.5–10 mg L$^{-1}$</td>
<td>24–216 h</td>
<td>Decreased hemocyte viability, decreased phagocytosis, reduced ROS production</td>
</tr>
</tbody>
</table>

Continued
scenario. Under such conditions, animals would be subjected to a large amount of material, but natural processes such as tidal flushing, currents, and dilution would render the exposure time relatively short. This study is unique in that it also tests how several ecologically relevant modes of delivery (i.e. marine snow, aged suspended, un-aged suspended) affect encounter and ingestion of nano-titania by bivalves.

**MATERIALS AND METHODS**

### Production of marine snow

A stock solution was prepared by suspending TiO$_2$ NPs (Meliorum Technologies, 99.9% pure anatase) in MQ-water at a concentration of 250 mg L$^{-1}$. X-ray diffraction (XRD) analysis of the TiO$_2$ NPs showed the characteristic anatase crystalline phase and a mean particle size of 7.4 nm$.^{+2.5}$ $(Doyle et al., 2014)$. The stock suspension was placed on a stir plate and subjected to ultrasonication (Fisher Scientific FB-505; calibrated according to Taurozzi et al., 2012) at 13.8 Watts for 30 min (modified from Wang et al., 2009). Following ultrasonication, TiO$_2$ NPs from the stock suspension were added to filtered-seawater (210-μm mesh) to achieve a final concentration of $\approx$4.5 mg L$^{-1}$ (4.4–4.7 mg L$^{-1}$). The working solution was mixed on a stir plate and then poured into 1-L Nalgene rolling bottles in quarter-litre aliquots. The solution was stirred and agitated after dispensing each aliquot to ensure that the NPs remained well mixed. This process was repeated until all the rolling bottles were full. Bottles designated as rolled samples (hereafter referred to as marine snow samples) were placed on a roller table for 72 h at 15 rpm (Shanks & Edmondson, 1989). Unrolled bottles consisted of the same solutions as described above, but instead of rolling, the bottles were placed next to the roller table for 72 h. A second treatment was prepared as described above to calculate the per cent incorporation, which was determined by the concentration of TiO$_2$ NPs in the marine snow when compared with the initial concentration of TiO$_2$ NPs (4.5 mg L$^{-1}$) added to the water. The TiO$_2$ NPs and marine snow were characterized using a suite of analytical techniques (dynamic light scattering, zeta potential, field-emission scanning electron microscopy and inductively coupled plasma-mass spectrometry) as previously described (Doyle et al., 2014).

### Feeding experiments

Mussels (5.0–6.5 cm in shell length) were collected from a local population at Avery Point (Groton, CT, USA), and oysters (5.0–6.5 cm in shell height) were obtained from the Noank Shellfish Cooperative (Noank, CT, USA). Prior to the experiments, bivalves were cleaned of all fouling organisms. A Velcro$^\text{TM}$ strip was attached to one of the animals’ shells using a two-part marine epoxy (Ward & Kach, 2009). Animals were held in an environmental chamber and fed *Tetraselmis* sp. for several days in order to acclimate to a temperature between 18$^\circ$ to 20$^\circ$C. Before the commencement of the feeding experiments, the bivalves were secured to craft sticks with Velcro$^\text{TM}$ and transferred to a large holding tray filled with aerated seawater, fed *Tetraselmis* sp., and allowed to acclimate at least 1 h prior to the beginning of the experiments.

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### Table 1. Continued

<table>
<thead>
<tr>
<th>Species</th>
<th>Nanoparticle</th>
<th>Concentration</th>
<th>Exposure</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mercenaria mercenaria</td>
<td>Au</td>
<td>5.6 μg/kg$^{-1}$</td>
<td>11 days</td>
<td>Bioaccumulation</td>
<td>Burns et al. (2013)</td>
</tr>
<tr>
<td>Ruditapes philippinarum</td>
<td>Au</td>
<td>6 &amp; 30 μg L$^{-1}$</td>
<td>18 days</td>
<td>Oxidative stress, changes in burrowing</td>
<td>Garcia-Negrete et al. (2013)</td>
</tr>
<tr>
<td>Scrobicularia plana</td>
<td>CuO</td>
<td>10 μg L$^{-1}$</td>
<td>16 days</td>
<td>Oxidative stress, changes in burrowing</td>
<td>Buffet et al. (2011)</td>
</tr>
<tr>
<td>Scallop</td>
<td>CuO</td>
<td>100 μg L$^{-1}$</td>
<td>16 days</td>
<td>Bioaccumulation</td>
<td>Pan et al. (2012)</td>
</tr>
<tr>
<td>Chlamys islandica</td>
<td>Ag</td>
<td>110 &amp; 151 ng L$^{-1}$</td>
<td>12 h</td>
<td>Bioaccumulation followed by depuration</td>
<td>Al-Sid-Cheikh et al. (2011)</td>
</tr>
</tbody>
</table>
Each animal was exposed to one of four treatments. Two of the four treatments (marine snow and unrolled) were described above. The third treatment consisted of TiO₂ NPs spiked directly into 1-L Nalgene bottles containing filtered seawater (210-μm mesh) just prior to the start of the feeding assay (hereafter referred to as freely suspended). The last treatment, which served as blanks, consisted of 1-L Nalgene bottles containing filtered-seawater devoid of NPs to account for background concentrations of titanium.

Bottles containing these four treatments were arranged on multi-position stir plates. Each bottle was supplied with gentle aeration using glass Pasteur pipettes and a stir bar. The stir plates were programmed to agitate the water for 10 s every 15 min to prevent the marine snow from settling on the bottom for too long. The water was then spiked with 10-μm polystyrene beads at a concentration of 2000 beads L⁻¹. The 10-μm polystyrene beads have a diameter large enough to ensure a capture efficiency of approximately 100% in both species, and were used as a means of determining feeding activity (Ward & Kach, 2009).

Animals with their shells open and mantles extended were transferred from the holding tray into the bottles. One bivalve was placed into each bottle and its craft stick secured to the rim by means of a wooden clip so that the animal was in the centre of the bottle (Ward & Kach, 2009). Animals were allowed to feed for 2 h with time commencing after they showed signs of suspension feeding (i.e. shells open, mantles extended). After 2 h, the animals were transferred from the bottles to clean 1-L beakers containing filtered seawater (0.22-μm membrane) at 18°C–20°C. Faeces were collected immediately from the 1-L Nalgene bottles and labelled as 0 h (immediately following exposure to NPs) were considered as 0.91 ± 0.06 mg L⁻¹ (mean ± standard deviation of six solutions containing TiO₂ at a concentration of 1.0 mg L⁻¹). The limits of detection of the ICP-MS were calculated as 3.75 × 10⁻⁴ μg g⁻¹ (three times the mean of the standard deviation of three replicate solutions containing TiO₂ at a concentration of 1.0 mg L⁻¹, and converted to μg g⁻¹ assuming the density of MQ-water is 1000 g L⁻¹).

Sample analysis

Animals were euthanized after feeding at 0, 6, 24, 72 and 120 h, post-exposure. The visceral mass, mantle and gills were removed by dissection and placed in 20-mL scintillation vials. Tissues were stored at −20°C overnight and then lyophilized for 48 h to remove any remaining moisture. A dry mass was obtained, and the organs were digested in 2 mL of 18 M H₂SO₄ and 16 M HNO₃ in a 3:7 v/v ratio for 24 h. The acid digest was then diluted to a 1% solution using MQ-water. A sub-sample of the dilution was collected and the average number of 10-μm polystyrene beads in the faeces was determined using a haemocytometer. Animals that had an average of less than one bead in their faeces (equivalent to the ingestion of <1% of available beads), or no TiO₂ present in the visceral mass or faeces at T = 0 (immediately following exposure to NPs) were considered not to have fed during the experimental period and were removed from the analyses (one mussel and eight oysters). Background concentrations of TiO₂ detected in the faeces and tissues of blank animals (not exposed to NPs) were averaged and subtracted from the concentrations of TiO₂ measured in exposed animals. This step was taken to ensure that only the titanium from the NPs was being measured in the exposed animals. Concentrations of TiO₂ were then standardized to the dry mass of the tissue and faecal material to account for differences in the size of experimental animals (see Supplementary Table S1).

Tissue and faeces samples were analysed for titanium using an ELAN DRC II inductively coupled plasma-mass spectrometer (ICP-MS; Perkin Elmer) to examine the concentration of TiO₂ present. The ICP-MS was tuned to detect the titanium-47 isotope in the tissue and faeces samples to avoid interference from the high levels of the titanium-48 isotope found in natural seawater. The analytical error of the ICP-MS was calculated as 0.91 ± 0.06 mg L⁻¹ (mean ± standard deviation of six solutions containing TiO₂ at a concentration of 1.0 mg L⁻¹). The limits of detection of the ICP-MS were calculated as 3.75 × 10⁻⁴ μg g⁻¹ (three times the mean of the standard deviation of three replicate solutions containing TiO₂ at a concentration of 1.0 mg L⁻¹, and converted to μg g⁻¹ assuming the density of MQ-water is 1000 g L⁻¹).

Statistical analysis

Two-way analysis of variance (ANOVA) tests were used to compare the effects of treatment and time on the concentration of TiO₂ NPs measured in the gills, mantles, visceral masses and faeces of the mussels and oysters. Effects of the two independent variables (time, treatment) within a given tissue/faecal sample and bivalve species were of primary interest, so two-way procedures were applied. If no differences were found between treatments at each time period, data were pooled and reanalysed to examine effects of time and species on TiO₂ concentrations within tissue/faecal samples. Following ANOVA analyses, a Tukey’s HSD post hoc test was applied to examine differences between levels of the independent variables. Prior to statistical analyses, data were assessed for homoscedasticity and normality using an Equality-of-Variance test and Kurtosis test, respectively. Data sets that did not meet the underlying assumptions were transformed by means of a square-root or natural-log transformation. In all tests, an alpha level of 0.05 was used.

RESULTS

Incorporation into marine snow

The marine snow produced in the laboratory ranged in size from approximately 1–10 mm. Incorporation efficiency of TiO₂ NPs in laboratory-made marine snow was ~52% ± 5.7% (standard error; N = 9) after 72 h (~2.3 mg of the
4.5 mg in each 1-L bottle). This efficiency is similar to that obtained in previous studies examining the incorporation of nano-titania (anatase form) into marine snow (Doyle et al., 2014). Analysis using field emission scanning electron microscopy with energy dispersive X-ray spectroscopy (FESEM-EDX) revealed that agglomerates of TiO$_2$ NPs were distributed throughout the organic matrix of the marine snow (Doyle et al., 2014).

**Encounter, ingestion and egestion**

No significant difference was found in the number of 10-µm polystyrene beads removed from suspension by animals delivered TiO$_2$ in the marine snow, unrolled or freely suspended treatments (ANOVA, data not presented). This finding indicates that the animals all fed at the same rate regardless of the treatment to which they were exposed. The mean concentration of background TiO$_2$ in the tissues and faeces of the blank animals (not exposed to NPs) was 1.6% ± 0.7 (mean ± SE) of the mean concentration of TiO$_2$ measured in the same samples from animals exposed to NPs. Overall, data analyses of the concentration of TiO$_2$ NPs on the gills, in the visceral mass, and in the faeces of both mussels and oysters yielded similar results. These included significant time effects, no significant treatment effects (marine snow vs unrolled vs free), and no significant interaction effects between the two independent variables (two-way ANOVA; Table 2; Figure 1). The only exceptions to this general trend were for the gills of oysters, which demonstrated no significant effect of time on the concentration of TiO$_2$, and the faeces of mussels, which demonstrated a significant interaction effect between time and treatment (Table 2). In general, pairwise comparisons indicated that the concentration of TiO$_2$ in tissues and faeces immediately after the 2-h feeding exposure (0-h) was significantly greater than that after 6, 24, 72, and 120 h of exposure (Tukey’s, $P < 0.05$; Figure 1, Supplementary Figures S1, and S2). Concentrations were typically lowest or not detectable after 72 h. As mentioned above, treatment had no significant effect on the concentration of TiO$_2$ measured in the tissues or faeces. The only exception being a slight but significant difference in the egested concentrations of TiO$_2$ in the marine snow and freely suspended treatments of mussels at 24 h (Supplementary Figure S2A). Therefore treatment data for each sampling period were pooled in order to more easily compare elimination of NPs over time between the two species (Figure 2, Supplementary Table S1).

Analyses of the pooled TiO$_2$-concentration data for the gills, the visceral mass, and the faeces also demonstrated common trends. In all cases, significant time effects were found (two-way ANOVA; Table 3, Figure 2), with significantly higher concentrations immediately after the 2-h feeding exposure (0-h; Tukey’s, $P < 0.05$). A significant species effect was also found for the faecal samples, and significant interaction effects found for both visceral mass and faecal samples (Table 3). The concentration of TiO$_2$ on the gills was more than an order of magnitude lower than in the visceral mass (Figure 2A). At 0-h post exposure, a significant difference in the concentration of TiO$_2$ in the visceral mass was found between mussels and oysters (Tukey’s, $P < 0.01$; Figure 2B). A similar difference was found in the faeces at

### Table 2. Results of two-way analysis of variance tests for (A) mussel and (B) oyster data. The concentration of TiO$_2$ nanoparticles in each tissue type (gill, visceral mass) and faeces was compared over five time periods (0, 6, 24, 72, 120 h) and between three treatments (marine snow, unrolled and freely suspended)

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Mussel Gill</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>0.083</td>
<td>4.115</td>
<td>0.005</td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.002</td>
<td>0.115</td>
<td>0.891</td>
</tr>
<tr>
<td>Time × treatment</td>
<td>8</td>
<td>0.003</td>
<td>0.125</td>
<td>0.998</td>
</tr>
<tr>
<td>Error visceral mass</td>
<td>75</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>3.586</td>
<td>24.63</td>
<td>0.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.037</td>
<td>0.257</td>
<td>0.774</td>
</tr>
<tr>
<td>Time × treatment</td>
<td>8</td>
<td>0.168</td>
<td>1.157</td>
<td>0.337</td>
</tr>
<tr>
<td>Error faeces</td>
<td>75</td>
<td>0.146</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>331.3</td>
<td>199.6</td>
<td>0.000</td>
</tr>
<tr>
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<td>2</td>
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<td>0.823</td>
</tr>
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<td>2.851</td>
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</tr>
<tr>
<td>Error</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>(B) Oyster Gill</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>0.009</td>
<td>0.686</td>
<td>0.604</td>
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<tr>
<td>Treatment</td>
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<td>0.011</td>
<td>0.989</td>
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<td>Time × treatment</td>
<td>8</td>
<td>0.009</td>
<td>0.644</td>
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</tr>
<tr>
<td>Error visceral mass</td>
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<td>0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>0.907</td>
<td>7.615</td>
<td>0.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>0.016</td>
<td>0.132</td>
<td>0.876</td>
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<tr>
<td>Time × treatment</td>
<td>8</td>
<td>0.041</td>
<td>0.344</td>
<td>0.946</td>
</tr>
<tr>
<td>Error faeces</td>
<td>67</td>
<td>0.119</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>70.06</td>
<td>75.54</td>
<td>0.000</td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>1.260</td>
<td>1.358</td>
<td>0.259</td>
</tr>
<tr>
<td>Time × treatment</td>
<td>8</td>
<td>0.618</td>
<td>0.666</td>
<td>0.721</td>
</tr>
<tr>
<td>Error</td>
<td>247</td>
<td>0.947</td>
<td></td>
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</tr>
</tbody>
</table>
both 0- and 6-h post exposure (Tukey’s, $P < 0.01$; Figure 2C). These data suggest that mussels ingested more NPs than oysters over the 2-h exposure period. The concentration of TiO$_2$ in the faeces of both mussels and oysters diminished more gradually over time compared with that on the gills and in the visceral mass (Figure 2). Visual observation showed a colour transition in the faeces, likely due to the presence of TiO$_2$ NPs, over the course of the 120-h depuration period. For example, the faeces produced at both the 0- and 6-h time intervals were white in colour, while a mix of both white and greenish-brown faeces was observed at 24 h. These observations correspond to the significantly higher concentrations of TiO$_2$ found at 6 h, post exposure, compared with >6 h, and at 24 h, post exposure, compared with >24 h. The faeces produced during the 72- and 120-h time intervals were the typical greenish-brown hue.

Subsamples of mantle from the mussels and oysters (16 of each species) were examined at 0, 6, 24, 72 and 120 h, and measurable concentrations of TiO$_2$ NPs were detected only in the 0-h samples. Data analyses of the concentration of TiO$_2$ in the mantle of mussels and oysters at 0 h revealed no significant treatment effects ($P > 0.1$; one-way ANOVA).

**DISCUSSION**

The results of this study demonstrate that mussels and oysters are able to capture and ingest TiO$_2$ NPs regardless of how they encounter the material (incorporated in marine snow or freely suspended). Mussels are able to ingest significantly more NPs than oysters over a 2-h exposure period. Once the TiO$_2$ NPs are ingested, both species of bivalves are able to eliminate the majority of NPs within the first 6 h from their gills, mantles and visceral masses. Additionally, the majority of TiO$_2$ NPs are depurated in the faeces over the course of 72 h, with only trace amounts remaining after this time. Data demonstrate that after an acute exposure to a high concentration of TiO$_2$ NPs, accumulation in the tissues of mussels and oysters does not occur.

Counter to our main alternative hypothesis, we found no increase in ingestion when NPs were incorporated in marine snow. This finding was likely a result of the agglomeration potential of TiO$_2$ NPs in seawater (Christian et al., 2008; Handy et al., 2008; Tiede et al., 2009; Sillanpää et al., 2011). When TiO$_2$ NPs are immersed in seawater, dissolved organic matter (DOM) begins to coat the particles, creating a uniform negative charge at the surface of the particle.
Thus, the agglomeration of TiO$_2$ NPs in natural seawater is likely that the TiO$_2$ NPs will be encountered by the gills of the bivalve and ingested. Our data examining the physicochemical behaviour of TiO$_2$ NPs immersed in natural seawater show the formation of agglomerates ranging in size from $\sim0.5$–$3\ \mu$m (Doyle et al., 2014). Particles $>1.5\ \mu$m can be captured by both mussels and oysters at an efficiency of between 50 and 75% (see Ward & Shumway, 2004 for review). Thus, the agglomeration of TiO$_2$ NPs in natural seawater is as effective as marine snow at increasing the particle diameter, and enabling capture on the gills of mussels and oysters. Over the course of 2 h, mussels can filter a litre of water approximately 3 times, whereas oysters can filter a litre of water approximately 10 times (assuming a dry tissue mass of $\sim0.5\ \text{g}$ for mussels and $1.0\ \text{g}$ for oysters; see Newell, 1988; Newell et al., 2005). Considering these clearance rates and the intermittent stirring of water to counter the effects of particle settling, we conclude that bivalves were exposed to all of the NPs added to each bottle ($\sim4.5\ \text{mg}$). They did not, however, ingest all of the material, likely because of the production of pseudofaeces and the lower capture efficiency of agglomerates $<1.0\ \mu$m in size. Our results support the findings of other research which report that bivalves can effectively capture a variety of NP types including Au, ZnO, CeO$_2$, TiO$_2$, SiO$_2$, carbon black and C$_{60}$ fullerene (Koehler et al., 2008; Canesi et al., 2010b; Tedesco et al., 2010; Montes et al., 2012). It is likely that these particles were captured and ingested by the bivalves because they were in an agglomerated form. In contrast, NPs that remain more monodispersed in seawater would be captured at very low efficiencies unless they were incorporated in marine snow (Ward & Kach, 2009).

The bulk of the TiO$_2$ NPs were removed from the gills and visceral masses of both mussels and oysters between 0 and 6 h, post-exposure. On the gills, the average residence time of particles is on the order of minutes, as material is rapidly transported to the labial palps or mantle (Milke & Ward, 2003). In the gut, food particles with high nutritive value are retained for extracellular digestion in the stomach, followed by intracellular digestion in the cells of the digestive gland. Conversely, particles with little or no nutritive value are subjected to minimal extracellular digestion in the stomach and transported to the intestine for egestion (Bricelj et al., 1984; Brilliant & MacDonald, 2002, 2003; Ward & Shumway, 2004). Furthermore, bivalves retain larger, less dense particles longer than smaller, denser particles because organic matter tends to be larger and lighter than inorganic particles that contain little nutritive value. Thus, larger, less dense material remains suspended in the stomach for more thorough processing, whereas smaller, denser particles settle into ciliary selection tracts where they are transported rapidly to the intestine for egestion (Reid, 1965; Brilliant & MacDonald, 2000). The separation of particles in the gut of bivalves based on size and density increases digestive efficiency and reduces digestive investment in material with little to no nutritive value (Brilliant & MacDonald, 2000). Previous studies regarding gut-retention time (GRT) report that M. edulis retain natural food particles for a period of approximately 2.5 h (Bayne et al., 1989), whereas C. virginica was found to retain natural food particles for approximately 9 h (Owen, 1966, 1974; Morton, 1977) depending on feeding rate and tidal cycle. Therefore, in the current study, bivalves handled the bulk of TiO$_2$ NPs as small, dense particles with little nutritive value, moving the material quickly to the intestine for egestion. Mass-balance analysis demonstrates the rapid depuration process that occurred with both mussels and oysters (Figure 3). Immediately following exposure (0 h), $>70\%$ of the ingested TiO$_2$ NPs had been eliminated in the faeces, and at 6 h, $>90\%$ of the ingested TiO$_2$ NPs had been egested. Only trace amounts of TiO$_2$ NPs were associated with the gills, visceral masses, and faeces 24 h after exposure.

The concentration of NPs to which bivalves were exposed in this study was greater than those deemed environmentally relevant (low $\mu\text{g}\ \text{L}^{-1}$; see Mueller & Nowack, 2008). Such conditions are possible, however, in a scenario where NPs are released into the near-shore environment during a spill (e.g. nanoparticle manufacturers located close to rivers or estuaries experiencing a failure). The effluent would be dispersed through the action of currents and tides, exposing coastal organisms to a high concentration of NPs over a short time interval. The impacts of spill-scenario concentrations of NPs on aquatic and terrestrial organisms have been examined in previous studies with effects ranging from the production of reactive oxygen species and activation of stress genes to delays in moulting and reduced fecundity (Oberdörster et al., 2008). The negatively charged surface then begins to attract cations dissolved in solution promoting Columbic attraction and enhanced agglomeration (Handy et al., 2008; Lead & Smith, 2009). As agglomeration of the NPs increases so does the particle diameter making it more likely that the TiO$_2$ NPs will be encountered by the gills of the bivalve and ingested. The impacts of spill-scenario concentrations of NPs on aquatic and terrestrial organisms have been examined in previous studies with effects ranging from the production of reactive oxygen species and activation of stress genes to delays in moulting and reduced fecundity (Oberdörster et al., 2008).

### Table 3

Results of two-way analysis of variance tests for mussel and oyster data (treatments pooled). In each tissue type (gill, visceral mass) and faeces, the concentration of TiO$_2$ NPs was compared between the two species (mussels, oysters) over five time intervals (0, 6, 24, 72, 120 h).

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>MS</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gill</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>0.071</td>
<td>4.615</td>
<td>0.002</td>
</tr>
<tr>
<td>Species</td>
<td>1</td>
<td>0.015</td>
<td>0.829</td>
<td>0.364</td>
</tr>
<tr>
<td>Time × species</td>
<td>4</td>
<td>0.019</td>
<td>1.256</td>
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</tr>
<tr>
<td>Error visceral mass</td>
<td>162</td>
<td>0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>4.070</td>
<td>31.94</td>
<td>0.000</td>
</tr>
<tr>
<td>Species</td>
<td>1</td>
<td>0.367</td>
<td>2.881</td>
<td>0.092</td>
</tr>
<tr>
<td>Time × species</td>
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<td>0.371</td>
<td>2.910</td>
<td>0.023</td>
</tr>
<tr>
<td>Error Faeces</td>
<td>162</td>
<td>0.127</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
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<td>180.6</td>
<td>280.6</td>
<td>0.000</td>
</tr>
<tr>
<td>Species</td>
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<td>4.304</td>
<td>6.687</td>
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</tr>
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<td>Time × species</td>
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<td>4.564</td>
<td>7.092</td>
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</tr>
<tr>
<td>Error</td>
<td>502</td>
<td>0.644</td>
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</tbody>
</table>
suggests the need for more long-term studies that consider: (1) the routes of entry and fate of nanomaterials in the marine environment, and (2) the way in which marine animals encounter, handle, ingest and egest the nanomaterials. Such research would provide valuable information regarding bioaccumulation and potential for food-chain transfer, and would be essential for commercially important marine species that are consumed by humans.

**SUPPLEMENTARY MATERIAL**

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0025315415001174

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**REFERENCES**


The marine biodiscovery pipeline and ocean medicines of tomorrow

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Marine organisms possess the capacity to produce a variety of unique and biologically potent natural products for treating human diseases, many of which are currently commercially available or are in advanced clinical trials. Here we provide a short review on progress in the field and discuss a case study of an EU-funded project, PharmaSea, which aims to discover novel products for the treatment of infections, inflammation and neurodegenerative diseases. Research in this sector is opening new doors for harnessing the potential of marine natural products with pharmaceutical properties.

Keywords: drug discovery, ocean medicines, PharmaSea project

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INTRODUCTION

A significant portion of the Earth’s biodiversity (an estimated 25% of the total number of species on Earth) is comprised of marine species (Mora et al., 2011). These have evolved mechanisms to survive in an extremely different and hostile environment compared with land in terms of light, salinity and pressure. This is reflected by the myriad of secondary metabolites (or natural products) that they produce to defend themselves against predators, to locate mates and to out-compete competitors for limited resources. Many of these compounds have no terrestrial analogues and are unique in terms of chemical structure and biological activity. What makes these products interesting for humans are their potential applications as pharmaceuticals for the treatment of numerous diseases or as templates for medicinal chemistry. Humans have been trying to understand and use ocean resources for medicinal purposes since ancient times. The Chinese and Japanese were eating a variety of iodine-rich seaweeds already in 1400 BC that accounted for their low incidence of goitre (Leoutsakos, 2004). In Ireland, the red algae Chondrus crispus and Mastocarpus stellatus were used as a folk cure for colds, sore throats, chest infections and bronchitis for several centuries (Dias et al., 2012). In the early 20th century cod liver oil was an important nutritional supplement in many northern European countries. However it was only after the 1950s, with the advent of scuba diving and new sampling technologies that scientists began to systematically probe the oceans for useful therapeutics. The number of potential compounds isolated from marine organisms now exceeds 28,000 with hundreds of new compounds being discovered every year (Blunt et al., 2015). However, despite the number of compounds isolated from marine organisms and the biological activities attributed to many of these, those that have either been marketed or are under development are relatively few.

There are several reasons for this including the time and cost it takes to reach the market, difficulties in harvesting the organism, low titres of natural product in producing organisms, difficulties in isolation and purification procedures, problems in obtaining a sustainable supply of the compound, high toxicity of the active compound, ecological impact on natural populations, and insufficient investment by pharmaceutical companies (Torjesen, 2015). However, notwithstanding these difficulties there has been a ‘renaissance’ in marine drug discovery in the last decade due to technological developments that have accelerated structural elucidation and screening, and the use of marine microbial genomics to provide biosynthetic pathways for the production of marine natural products (Glaser and Mayer, 2009). The development of emerging ‘omics’ tools such direct sequencing of eDNA, next generation sequencing technologies, metaproteomic and synthetic biology, heterologous expression and bioinformatic tools will improve the discovery and production of these compounds and facilitate the study of biosynthetic pathways of organisms previously inaccessible by traditional methods (see review by Rocha-Martín et al., 2014). This is coupled with the fact that alternative technologies such as combinatorial chemistry have failed to provide the pharmaceutical industry with the chemical diversity necessary to significantly increase the number of new drug-like leads.

Here we discuss the current state of art of marine compounds approved, developed or in clinical trials for treating various diseases, or marketed as nutraceuticals and cosmeceuticals. Several excellent reviews already exist on marine drug...
discovery so this paper does not attempt to provide a comprehensive overview, but rather to illustrate some examples of the recent advances in this field. We also discuss the ambitions and efforts of an on-going EU project to find new molecules from microorganisms for the treatment of bacterial and viral infections, and inflammatory and neurodegenerative diseases. With a focus on under-exploited marine phyla of cultivable microorganisms, essentially photo- and chemosynthetic bacteria together with fungi and microalgae, this project aims to achieve optimized and sustainable production of relevant biomass and high added-value compounds for pharmaceutical, nutraceutical and cosmeceutical applications, and to overcome some of the major bottlenecks in the drug discovery pipeline.

MARINE DRUGS IN CLINICAL USE

Marine-derived compounds are more bioactive than those of terrestrial origin, especially in terms of cytotoxicity. It is no surprise, therefore, that marine natural products have their stronghold in the area of anticancer chemotherapy, as indicated by the list of compounds that have already made it to the market or are currently under clinical investigation. To date, the global marine pharmaceutical pipeline consists of seven approved pharmaceuticals in clinical use, four of which are anticancer drugs (Figure 1). The first marine-derived anticancer agent to be developed for clinical use, cytarabine or Ara-C, is a synthetic analogue of a C-nucleoside from the Caribbean sponge, Cryptothelya crypta, approved in 1969 in still in use today to treat acute myelocytic leukaemia and non-Hodgkin’s lymphoma (Sagar et al., 2010). Almost 20 years later in 2007 the next anticancer agent from the tunicate Ecteinascidia turbirata would be approved, trabectedin (Yondelis), for the treatment of soft tissue sarcomas and ovarian cancer (Schoffski et al., 2008). A third marine anticancer success story was the discovery of the polyether metabolite halichondrin A from the sponge Halichondria okadai (Hirata & Uemura, 1986). A simpler structure containing the pharmacophore obtained synthetically gave rise to Eribulin (Halaven), the most complex synthetic derivate of the potent antitubulin agent dolastatin 10 (Katz et al., 2013) used for the treatment of breast cancer in 2010 (Menis & Twelves, 2011). The most recently approved anticancer compound from the marine environment is brentuximab vedotin (Adcetris), a chimeric antibody attached through a protease-cleavable linker to a derivative of the potent antitubulin agent dolastatin 10 (Katz et al., 2013) used for the treatment of Hodgkin’s lymphoma and anaplastic large cell lymphoma.

Marine natural products have also found applications as antiviral drugs used to treat herpes simplex infections. Vidarabine or Ara-A is a synthetic analogue of spongouridine with improved antiviral activity, originally isolated in 1950 from the sponge Tethya crypta (Sagar et al., 2010), but currently obtained from fermentation cultures of the bacterium Streptomyces antibioticus. Another important marine natural product, ziconotide (commercial name Prialt; Klotz, 2006), is a synthetic calcium-channel binding conotoxin from Conus magus, the Magician’s cone snail, which has proved effective in patients with intractable severe pain who either do not respond to or cannot tolerate other drugs. Additional conotoxins are in clinical development with potential applications in pain management and are widely employed tool compounds in neurotoxin research (Daly & Craik, 2011). Finally, marine products obtained from fish oils, typically from oily fish such as mackerel and anchovy, have found applications as a lipid-regulating agent (commercial name Lovaza) to reduce severe elevations of triglycerides associated with conditions such as obesity, insulin resistance, diabetes mellitus and other factors that contribute to the risk of atherosclerosis or hardening of the arteries responsible for coronary artery disease. According to Gerwick & Moore (2012) the current success rate of discovery from the marine world, namely seven clinically useful and approved drugs from 28,175 discovered molecular entities (e.g. one drug per 4025 natural products described) is approximately 1.2- to 2.5-fold better than the industry average (1 in 5000–10,000 tested compounds) (http://www.pharma.org/sites/default/files/159/rd_brochure_022307.pdf).

NEW DRUGS IN DEVELOPMENT

Currently there are about 26 natural products in Phase I to Phase III clinical trials, 23 as anticancer cancer agents, two for schizophrenia and Alzheimer’s, and one for chronic pain (http://marinepharmacology.midwestern.edu/clinPipeline.htm). Thus, the pipeline of promising marine derived compounds is very strong, and several of these agents are likely to reach the market in the coming years (Mayer et al., 2010). Some of these new marine drugs are discussed briefly in the next section.

Anticancer

Aplidine (dehydrodidemnin B), a depsipeptide dehydrodiddemnin isolated from the Mediterranean tunicate Aplidium albicans has antiproliferative activity by blocking the cell cycle and inducing apoptosis, with strong activity against multiple myeloma cells. PharmaMar is currently developing Aplidin for the treatment of multiple myeloma (phase III of clinical trials), and for solid and haematological malignant neoplasias, like T-cell lymphoma (phase II of clinical trials) (http://www.pharmamar.com/aplidin.aspx).

Plinabulin is a synthetic analogue of a natural product isolated from a marine fungus (Aspergillus sp.) that inhibits tubulin polymerization, leading to the disruption of the vascular endothelial architecture of the tumour. Beyondspring Pharmaceuticals is developing plinabulin and announced the start of phase III clinical trials in patients with non-small cell lung cancer in 2015 (http://www.beyondspringpharma.com/press-release-plinabulin-phase-3-trial/).

Salinosporamide A (Marizomib; Potts et al., 2011) is a novel, potent proteasome inhibitor from the marine actinomycete, Salinispora tropica, that induces apoptosis by a caspase-8 dependent mechanism in multiple myeloma and leukaemia cells. Currently, combination therapies of salinosporamide A with other drugs are under investigation in phase I clinical trials.

Further examples of anticancer drugs of marine origin in clinical development are discussed by Newman and Cragg (Newman & Cragg, 2014).

Alzheimer’s disease

Bryostatin 1, a macrolide lactone isolated from the bryozoan species, Bugula neritina, is a potent modulator of protein kinase C that is currently in phase II clinical trials for the
treatment of Alzheimer’s disease by Neurotrope Bioscience (http://www.neurotropebioscience.com/). The drug has shown pre-clinical efficacy to not only treat Alzheimer’s disease symptoms, but also its underlying causes. Bryostatin was originally intended for anti-cancer chemotherapy, but was then discovered to potentially arrest Alzheimer’s disease (Lorente et al., 2014). Pre-clinical testing revealed that it reduced the toxic Alzheimer’s disease protein amyloid-β and the deposits of amyloid-β called amyloid plaques, restored lost synapses, and protected against the loss of memory functions.

DMXBA, a synthetic analogue of the toxic alkaloid produced by several nemertean worm species, such as Paranemertes peregrine and Amphiporus lactiflorus, improves cognition and sensory deficit in several animal models, and has shown neuroprotective effects in vitro and in vivo (Rangel & Falkenberg, 2015). Phase I and II clinical trials showed a significant cognitive improvement in healthy young adults and in schizophrenic patients (Rangel & Falkenberg, 2015). Comentis Inc. is developing the drug for treatment for Alzheimer’s disease and schizophrenia (http://comentis.com/).

**Analgesics**

The guanidine alkaloid tetrodotoxin (TTX), a blocker of voltage dependent sodium channels isolated from fish, algae and bacteria, has shown therapeutic efficacy as an analgesic in cancer patients. Two formulations are currently under evaluation in phases II and III of clinical trials by the Canadian WEX Pharmaceuticals Inc.: the first formulation is in phase III, indicated for the treatment of neuropathic pain in cancer patients; the second one is in phase II of clinical trials, for peripheral pain and cancer-related pain (http://www.wextech.ca/clinical_trials.asp?m=1&s=0&p=0; http://www.clinicaltrials.gov).

**Antibacterials**

Despite the urgent need for new antibiotics, particularly to tackle the rise of antibiotic-resistant bacteria, new antibiotic development has moved slowly and there are few compounds in the antibiotic development pipeline. This lack of activity reflects market failure as the risk–reward ratio has been considered unattractive for pharmaceutical companies. Mayer et al. (2013) lists 23 antibacterial compounds in preclinical pharmacological research. An interesting example is Anthracimycin, a polyketide antibiotic discovered in 2013, derived from marine actinobacteria that has shown significant activity against Bacillus anthracis, the bacteria that causes anthrax (Jang et al., 2013).

**MARINE NUTRACEUTICALS AND COSMECEUTICALS**

Currently there is also great interest in marine-derived products as nutraceuticals and cosmeceuticals because of their beneficial effects on human health. These often have drug-like properties (hence the term -ceutical) and contain active ingredients such as vitamins, phytochemicals, enzymes, antioxidants and essential oils which are finding uses as natural additives in foods, as nutritional supplements including colour additives and antioxidants, and as vitamins, oils and cofactors which enhance general well-being. Compared with...
high-value pharmaceuticals, these medium-value products have a rapid route to market and many companies have chosen to go along the functional product route as it offers lower risk and a quicker potential return on investment than the high-risk high-reward pharmaceutical market.

The main products of primary interest for marine nutraceuticals include omega-3 fish/algal oil, phospholipids (bound omega 3-fatty acids), micro/macro algal nutrition supplements, fish proteins and peptides, hydrolysates, shellfish chitin, fish collagen and mineral supplements (Figure 2). Polyunsaturated ω−3 fatty acids are purported to have a range of beneficial effects including improved heart health and reduced inflammation. Several population studies report that dietary ω−3 fatty acids or fish oil may also reduce the risk of developing breast, colon or prostate cancer. In addition to the traditional sources of the ω−3-fish oils, krill oil has also become very popular. This oil is different from the traditional fish oils because it contains three active components: ω−3 fatty acids, phospholipids and the carotenoid astaxanthin, a potent antioxidant. Marine microalgae are also rich in ω−3 oils. Made from various species of microalgae, this new omega-3 oil is reported to contain the same lipid type as fish oil, triglycerides. The advantage in this case is that production from this source is more eco-sustainable and less damaging to the environment than production of fish and krill oils.

Current nutraceutical markets from marine organisms are also focused on chemicals such as carotenoids due to their high market value, projected to reach €1.27 billion by 2019 (http://www.marketsandmarkets.com/PressReleases/carotenoid.asp). Carotenoids have much potential as food colourants, feed supplements, nutraceuticals, and for cosmetic and pharmaceutical purposes. There are well over 600 known carotenoids, with beta-carotene, alpha-carotene, lutein, zeaxanthin, lycopene, fucoxanthin and astaxanthin being the most common. Astaxanthin is one of the best known in terms of its status as a ‘supernutrient,’ as it is the only one to easily cross the blood–brain barrier and other bio-membranes which makes it more easily absorbed and transported to all tissues and organs in the body. Research suggests that astaxanthin is an optimum ingredient choice for eye protection, skin health, anti-ageing, anti-fatigue or any condition where protection from free radicals is needed (Fiedor & Burda, 2014).

Another carotenoid, fucoxanthin, may play a role in reducing obesity (Gammone & D’Orazio, 2015). So far, only animal studies have been conducted but these show that fucoxanthin, found in edible brown seaweed and microalgae such as the diatoms Phaeodactylum tricornutum or Cylindrotheca closterium, promotes the loss of abdominal fat in obese mice and rats. Although it is not fully understood how fucoxanthin works, it appears to target protein UCP1 that increases the rate at which abdominal fat is burned. Fucoxanthin has also been found in animal studies to decrease insulin and blood glucose levels. Researchers hypothesize that fucoxanthin may have anti-diabetes effects because it stimulates the formation of ω−3 fatty acids which are thought to increase insulin sensitivity, improve triglycerides and reduce LDL (‘bad’) cholesterol.
Together with the pharmaceutical and nutraceutical industries, the cosmeceutical industry is increasingly turning to the sea in the search for new molecules. One of the most common molecular classes of compounds used in the personal care bioactive ingredients sector is the exopolysaccharides (EPS). Various microorganisms produce EPS, including proteobacteria, cyanobacteria and archaea. Abyssine by Lucas Meyers is an Alteromonas fermentation extract containing the EPS HYD657, named deepsane, produced and secreted by the strain Alteromonas macleodii subsp. fijienis biovar deepsane (Cambon-Bonavita et al., 2002). DeepSANe is commercially available under the name of Abyssine for soothing and reducing irritation of sensitive skin against chemical, mechanical and ultraviolet B aggression (Martins et al., 2014). A ferment of the deep sea hydrothermal vent bacterium Thermus thermophilus (Venecana) produced by Sederma Cosmetics is a potent antioxidant that inhibits damage from reactive oxygen species, protects natural defense enzymes, shields from UV damage, and restores barrier lipids.

Resilience is a line of skincare products from the Estée Lauder Company that contains a special extracellular extract from the Caribbean Sea whip (gorgonian) Pseudopterogorgia elisabethae. This extract is mainly composed by pseudopterosins, which are potent anti-inflammatory and analgesic agents that inhibit eicosanoids biosynthesis by inhibition of both phospholipase A2 and 5-lipoxygenase. Additionally, a derivative of a natural pseudopterosin, methopterosin, has completed phase I and II clinical trials as a wound-healing agent.

Microalgae are the source of some of the most innovative skincare products today. Some examples include Dermochlorella DG, XCELL-30, Alguronic Acid and Alguard. Dermochlorella DG from CODIF Recherche et Nature (Brittany, France) is a Chlorella sp. extract containing oligosaccharides that increases firmness and skin tone. XCELL-30 from Greenssea (Meze, France) is developed from microalgae endemic to Madagascar, and specifically acts on cellular turnover in the basal layer of the epidermis, thus allowing the preservation of the youthful characteristics of the skin. Alguronic Acid from Algenist (San Francisco, CA, USA) is an undetermined mix of polysaccharides produced by microalgae with significant anti-ageing properties, helping to rejuvenate the skin for a more youthful appearance. Alguard is a natural sulphated polysaccharide compound isolated from a single red microalga (Porphyridium sp.) that protects against photo damage, ageing and micro-abrasion of the skin.

**PHARMASEA: A CASE STUDY OF A DRUG DISCOVERY PROJECT**

Marine natural products constitute a strategic research area with potentially enormous economic and social revenues. According to EuroOcean (http://www.eurocean.org/npa/2502.html) there are over 590 European marine projects that have been funded under both FP6 and FP7 programmes (see also Martins et al., 2014), which shows the engagement of academia and industry in bringing more marine bioactives into the market.

An example is the EU PharmaSea project, an SME-academia-driven project initiated by 24 partners in 2012 to discover novel products for the treatment of bacterial infections, inflammation and neurodegenerative conditions such as Alzheimer’s disease. The novelty of the project is that it searches for new microbial biodiversity to treat these diseases in some of the deepest, coldest and hottest places on the planet. By choosing deep and cold marine environments scientists hope to tap novel diversity not seen before. Deep ocean trenches are islands of diversity in which evolution may have progressed differently. The work on microorganisms from deep trenches shows they are indeed different. The chemistry derived from these, though limited in scope, shows high novelty.

Why study these diseases? In the case of antibiotics, the pipeline for new antibiotics has paradoxically experienced a long-term decline. Antibiotics are being developed, but not ones targeting the most urgent needs, and not in the diverse portfolio required to combat the rise of bacterial resistance. No new class of antibiotics has been discovered since 1987 but a new infection emerges on an almost yearly basis (Bérdy, 2012). Diseases such as MRSA have become major problems in hospitals, evolving to become resistant to the limited number of antibiotics available. Hence the urgent need to discover new antimicrobials in many EU drug discovery projects (e.g. MicoB3, MACUMBA and others).

PharmaSea is also looking for (a) anti-inflammatory compounds targeting diseases such as atherosclerosis and Alzheimer’s, and (b) neuroactive compounds targeting CNS disorders such as epilepsy. An estimated 44 million people worldwide suffer from dementia and Alzheimer’s disease is the biggest cause, but the available treatments are inadequate and only improve some of the symptoms. There are also an estimated 70 million epilepsy patients worldwide, and 30% of these are pharmacoresistant, creating an urgent need for novel antiepileptic drugs. With the growing and ageing population worldwide, and without the effective cures, it is anticipated that the number of patients with these diseases will increase dramatically in the coming decades. To avert the worst of this crisis, and to secure a sustainable source of more effective drugs, bottle-necks in the drug discovery process must be overcome.

Having its main focus on the discovery of new compounds bioactive in the above-mentioned therapeutic areas, PharmaSea also aims to overcome some of the bottlenecks currently found in the biodiscovery process, leading to (1) improvements in the quality of marine resources available for biotechnological exploitation, (2) to shorten time to market, and (3) develop sustainable modes of supply of raw materials for industry. The first challenge is being targeted by exploring the biotechnological potential of microorganisms such as bacteria, fungi and microalgae to overcome problems associated with supply of bioactives since microorganisms are generally more amenable to mass cultivation in large-scale enclosed bioreactors, as already demonstrated for the biofuel and nutritional supplement industries. The two last challenges centre on enabling activities to clarify legal aspects to facilitate access to marine resources, their sustainable use, and their secure exploitation; and second, to create an improved framework for accessing marine biotechnology data and research materials. To achieve these goals, PharmaSea brings together complementary and world-leading experts, integrating biology, genomics, natural product chemistry, bioactivity testing, industrial bioprocessing, legal aspects, market analysis and knowledge exchange.

Furthermore, to address the issue of inaccessible biodiversity, innovative technologies are being developed to allow more frequent and cost efficient retrieval of, for example, deep-sea microbes. Selection of new habitats such as the deep-sea has increased the number of novel species, which
are obtained using selective isolation techniques. Phylogenetic and genomic strategies have further guided selection of strains based on biosynthetic capacity. These high-quality strains are being cultured in varying conditions to elicit their biosynthetic repertoire while those recalcitrant to culture are being exploited using a metagenomic approach.

To date the PharmaSea project has cultivated and extracted more than 1,400 microbial strains from extreme marine environments, with the majority being fungi and actinobacteria (Figure 3A). These have been cultivated in a number of different media with different stresses applied to elicit production of secondary metabolites. The strains investigated so far have yielded a total of over 15,000 extracts and fractions which have been subjected to a broad range of antimicrobial and central nervous system disease assays (Figure 3B). The pathogenic bacteria and fungi used in this assay panel include Gram-positive (methicillin resistant *Staphylococcus aureus*, MRSA) and Gram-negative bacteria (*Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Escherichia coli*), fungi (*Candida albicans* and *Aspergillus fumigatus*) (Audoin et al., 2013), and two surrogate (avirulent) strains of *Mycobacterium*, namely *Mycobacterium tuberculosis* H37Ra and *Mycobacterium bovis* BCG. Additionally, all samples are also tested in an assay to detect compounds potentiating the effect of the β-lactam antibiotic imipenem against *P. aeruginosa*, with extracts and fractions tested against the pathogen in the presence or absence of a sub-lethal concentration of the antibiotic. Assay incubation times range from overnight in the antibacterial and antifungal assays to 7-day incubations in assays against *Mycobacterium* strains. In order to reduce the high number of hits in anti-infective screens (Figure 3C) we are using a state of the art LC/MS based dereplication approach. This immediately excludes extracts containing known antibiotics and identifies those containing chemical novelty (Lacet et al., 2015). These extracts are being subjected to further work to identify and chemically characterize the active components.

Neuroactive extracts and fractions, for potential applications to treat epilepsy and other CNS disorders, are tested using high-throughput behavioural fingerprinting and seizure assays in zebrafish larvae (e.g. VanHook, 2010; Cunliffe, 2015). Zebrafish-based behavioural fingerprinting provides initial data as to the putative mechanism of action of neuroactive molecules, and provides preliminary mechanism of action information for isolated compounds, thereby helping to rapidly prioritize those with novel mechanisms of action. In addition to anti-infective and neuroactivity screens, anti-inflammatory and toxicity screens are also carried out (Figure 3C). Figure 3D further shows the different assays used to evaluate the neuroactivity and anti-inflammatory activity in the primary screen. It also shows the percentage of screening events performed to evaluate toxicity.

Simultaneous with the screening process, extracts are being dereplicated at an early stage through the use of innovative chemometric methods. Extracts identified as validated actives in a selected assay and which are non-toxic have also been evaluated for their likelihood of containing novel compounds using chemometrics. Extracts identified using this protocol are purified.
using targeted chromatography followed by pioneering compound dereplication strategies leading directly into an accelerated workflow for full structure determination. This is only possible through the involvement of one of the world’s largest chemical databases and a chemical software company, which has enabled the prediction of NMR and MS spectra of most known marine and microbial natural products.

The global aim of PharmaSea is to produce two compounds at a larger scale and advance them to pre-clinical evaluation. Assay cascades are used as the first step in a protocol to decide which extracts to progress. Those showing good in vitro potency, low toxicity and high probability of containing novel compounds are taken forward to isolate the active compounds as potential pharmaceutical candidates. Scale-up fermentation in saline media is being used to generate adequate amounts of target compounds. In addition, identified novel gene clusters are being expressed in heterologous hosts. Extracts that show interesting bioactivity e.g. low cytotoxicity and/or dual activity from cell based screens e.g. anti-inflammatory and antioxidant, will be subjected to additional screens to identify their potential value as novel applications of marine natural products in personal care and nutrition as part of follow-on projects.

Bioactive compounds will be developed by SME partners and with larger pharmaceutical companies, if appropriate. To address relevant challenges in marine biodiscovery related to policy and legal issues, PharmaSea has brought together practitioners, legal experts, policy advisors/makers and other stakeholders, focusing on the feasibility of harmonizing, aligning and complementing current legal frameworks with recommendations and ready to use solutions tailored to marine biodiscovery (Lallier et al., 2014). This work will also generate a toolkit to enable users to explore the existing difficulties of access and benefit sharing under different legal regimes and find practical guidance to secure access to marine genetic resources. Uptake of products derived from marine bioresources will be improved via a number of mechanisms making PharmaSea data/IP available under licence to interested end-users.

CONCLUDING REMARKS

Discovery of natural bioactive molecules is, of course, only the first step in the biodiscovery pipeline. The hardest challenge is turning these products into useful medicines because the costs of bringing a new drug to market from discovery through clinical trials to approval is currently $2.6 billion, according to a new study by the Tufts Center for the Study of Drug Development (http://csdd.tufts.edu/news/complete_story/pr_tufts_csdd_2014_cost_study), costs that have more than doubled in the last decade. The same Center also reports that only 11.8% of drug compounds entering clinical testing are eventually approved. Another major challenge is to reduce the time to market, which is currently ~10 years. Both factors are discouraging pharmaceutical industries which also have to cope with the fact that the marine environment is largely unexplored and does not offer certainty on the successful outcome of investment. This is complicated by the large investment required to organize oceanographic cruises and to sample in extreme environments where chances of finding greater biodiversity are higher. A further complication regards the legal aspects to access and utilization of marine natural products (or genetic resources) that have been formalized in a series of agreements such as the Convention on Biological Diversity (CBD) and its Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (Nagoya Protocol, as well as the United Nations Convention on the Law of the Sea (UNCLOS)). The Nagoya protocol, expected to enter into force in 2015, will enable stakeholders to better understand and comply with national access and benefit-sharing procedures. For example, when the genetic material is accessed from an ‘ex situ’ collection such as a biorepository then the access provision of the CBD and the Nagoya protocol apply. If the genetic material is collected ‘in situ’ during an expedition then UNCLOS provisions will also apply (for further details on legal framework to access and benefit sharing see Lallier et al., 2014 and references therein).

Notwithstanding these difficulties, the discovery of new ocean medicines is one of the most promising new directions of marine science today. Novel initiatives including partnering between governmental organizations and industry are being developed to meet the difficulties incurred in the drug discovery pipeline. Projects such as PharmaSea represent an example of such a partnership, as will future programmes being organized within the framework of Horizon 2020.

The oceans can therefore provide us with many invaluable benefits and services, including some of the medicines we use to cure human disease. However, the oceans are under serious threat due to pollution and global change. We even contaminate the ocean and its inhabitants by dumping chemicals from drug manufacturing into the world’s waterways, polluting villages, cities and aquatic ecosystems around the world, eventually contaminating the fish we eat (Larsson, 2014) and the very organisms that could provide us with new cures for human diseases in the future. With the global population projected to increase from 7 to 9 billion people in the next few decades, there is an urgent need to secure a healthy and productive global ocean. A healthier ocean that is better managed could provide more food and employment. It could also ensure that the benefits from the exploitation of ocean resources can be sustainably managed and equitably shared. Knowledge on the effects of pollution and global change on marine organisms are limited and still relatively unexplored, leaving critical knowledge gaps for those seeking to develop effective policies for sustainable use of marine resources and environmental and human health protection (European Marine Board, 2013). A better understanding of the potential health benefits from marine organisms, the compounds they produce, and the environmental conditions affecting their production will allow for the better management and sustainable development of these valuable marine resources in the future. By pursuing the medical promises hidden within the ocean, while also managing the dangers to human health found in this new frontier, ocean scientists can make a major contribution to improving human health in the 21st century and beyond.

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Rates of warming and the global sensitivity of shallow water marine invertebrates to elevated temperature

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Assessing the sensitivity of ectotherms to variability in their environment is a key challenge, especially in the face of rapid warming of the Earth’s surface. Comparing the upper temperature limits of species from different regions, at different rates of warming, has recently been developed as a method to estimate the long term sensitivity of shallow marine fauna. This paper presents the first preliminary data from four tropical Ascension Island, five temperate New Zealand and six Antarctic McMurdo Sound species. The slopes and intercepts of these three assemblages fitted within the overall pattern for previously measured assemblages from high temperature tolerance in tropical fauna and a shallow slope, to low temperature tolerance and a steep slope in Antarctic fauna. Despite the tropical oceanic Ascension Island being subject to upwelling events, the fit of the intercept and slope within the overall assemblage pattern suggests that the upwelling is sufficiently predictable for the fauna to have evolved the plasticity to respond. This contrasts with previously analysed species from the Peruvian upwelling region, which had a steeper slope than other temperate fauna. The speed and capacity of faunal assemblages to acclimatize their physiology is likely to determine the shape of the rates of warming relationship, and will be a key mechanism underpinning vulnerability to climate warming.

Keywords: climate change, assemblage, vulnerability, warming, rates of warming, Ascension Island, New Zealand, McMurdo Sound, Antarctica

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INTRODUCTION

Understanding how animals respond to climate variation, and the mechanisms underlying changes in species distributions, are key scientific challenges (Pennisi, 2005). One of the most common ways to address these challenges is to compare geographical differences in physiological tolerance and plasticity between populations, species and communities across latitudes (see Gaston et al., 2009 for recent review). Estimates of physiological limits can then be combined with climate envelope models to improve predictions of how patterns of biodiversity will change in the future (Helmuth, 2009; Peck et al., 2009). Studies have, however, highlighted the importance of comparing physiological traits at an environmental scale that is relevant to the variability of the organisms experienced microhabitat, otherwise there may be a poor match between species range (realized niche) and the range predicted by physiological tolerance (physiological niche, Helmuth, 2002; Helmuth et al., 2002; Sanchez-Fernandez et al., 2012).

Whilst many physical and biological factors are known to affect the distribution of marine ectotherms, temperature is pervasive, as it affects the rates of all biochemical reactions (Hochachka & Somero, 2002). When testing temperature limits, the rate of warming markedly affects the measured temperature limit, which is set by a combination of the magnitude and duration of temperature exposure; limits are expected to be lower at slower rates of warming (Mora & Moya 2006; Terblanche et al., 2007; Chown et al., 2009; Peck et al., 2009). However, if the rate of warming is slow enough, then animals may be able to adjust their physiology to a new stable state, with this acclimation is expected to lead to an increase in temperature limits (Beneficial Acclimation Hypothesis; e.g. Leroi et al., 1994). Different life history stages may have different degrees of physiological plasticity, potentially altering their vulnerability. Acclimatory capacity may, therefore, be the component of phenotypic plasticity that confers most resistance to environmental change (Somero, 2010; Peck et al., 2014). Modelling relationships of species’ thermal limits at different rates of warming has recently been developed as a technique for extrapolating the results of short term laboratory experiments, run over hours to months, towards annual and decadal scales, which are more closely related to the scales over which climate is
changing (Peck et al., 2009; Barnes et al., 2010). The upper temperature limits of ectotherms are set by a combination of both the magnitude of temperature exposure and the duration of that exposure. This results in ectotherms having higher lethal limits at faster rates of warming and lower limits at slower rates. If the lethal limits of species within an assemblage are tested at different rates of warming in the laboratory, then long term limits can be estimated from the resultant relationship (Peck et al., 2009). The shape of the relationship also allows assessment of the relative vulnerability of different assemblages to the rate at which climate is warming. The intercept indicates the maximum CTmax of the assemblage which is expected to be correlated with habitat temperature. A steeper relationship between CTmax and the rate of warming will indicate a reduced ability to cope with long term warming. The slope will also be shallower if species within an assemblage have the capacity to acclimate their physiology at slower rates of warming.

Assemblage level studies have shown that marine species from the Antarctic (Peck et al., 2009) and coastal tropics (Nguyen et al., 2011), which experience stable year round water temperatures, are more sensitive to acute elevated temperature than temperate species, which experience a wider annual range (Richard et al., 2012). A similar pattern was found for the ability of the same marine ectotherms to acclimate their physiology; tropical and polar species had reduced physiological capacity compared to temperate species (Peck et al., 2014). This differs from findings for a range of terrestrial species, which are more sensitive in the tropics than temperate regions, but also have increased tolerance capacities to cope with the hugely variable air temperature range in high latitudes (Deutsch et al., 2008; Tewksbury et al., 2008; Sunday et al., 2012).

To further test the global patterns of thermal sensitivity in shallow subtidal marine ectotherms, this study presents the first, preliminary, data on the effect of different rates of temperature change on the upper temperature limit of common shallow subtidal marine ectotherms, collected from additional tropical (the oceanic Island of Ascension), temperate (Dunedin New Zealand) and Antarctic (McMurdo Sound) locations. Methodologies were standardized so that data sets could be directly compared with published data for a coastal tropical site in Singapore (Nguyen et al., 2011), temperate regions (Richard et al., 2012) and the Antarctic Peninsula (Peck et al., 2009).

MATERIALS AND METHODS

Shallow water subtidal species were collected by divers from less than 20 m depth; four species were studied from Ascension Island, five from Dunedin, New Zealand and six from McMurdo Sound, Antarctica (Table 1). Apart from Ascension, which is tropical, all samples were collected in summer. For these trials, the site-size of each species was deliberately limited to reduce the variability in thermal limits attributable to size (e.g. Peck et al., 2009). Ascension Island is an isolated volcanic Island on the Mid-Atlantic Ridge (7.89°S 14.38°W) and is subject to an annual seawater temperature range of 22–28°C (Irving, 1989; Weber et al., 2011). The Island has a narrow shelf and is surrounded by deep water, which leads to a cooler and more variable sea surface temperature than many coastal tropical sites (e.g. Singapore, 27–31°C (Chou & Lee, 1997)). Species were collected from Dunedin Harbour on the south-east coast of New Zealand (45°49.6′S 170°38.4′E) and Doubtful Sound, on the south-west Fiordland coast (45°25′S 167°6′E). Dunedin Harbour has a narrow entrance with an annual temperature range of 7.1–16.1°C (Shaw et al., 1999). Doubtful Sound is a narrow fjord, surrounded by mountains, and has a reduced annual temperature range, at 15 m, of 12–18°C (Goodwin & Cornelsen, 2012). Species were also collected from McMurdo Sound, by US Antarctic Program divers (77°51′S 166°40′E). McMurdo Sound has the coldest and one of the most stable marine temperature regimes (−1.9 to −0.5°C (Hunt et al., 2003)). In each location specimens were transported in insulated containers to marine aquarium systems, in the nearest laboratory facility. During trials carnivores were fed to satiation twice a week on pieces of fish, whilst herbivores were fed on recently collected macroalgae, algal cultures or algae in the water column, which were replaced through daily water changes or a flow-through system in longer incubations at Portobello Marine Laboratory. Daily water exchange, with water pre-heated to the experimental temperature, also ensured water quality never deteriorated. Mortality in controls (at ambient temperature) was monitored to check for any adverse effects of culture conditions. Any species that suffered significant mortality of control animals was removed from the analysis.

In Ascension Island, specimens were transported to the Ascension Island Conservation Office where they were placed in 100 l aerated water baths with 500 W titanium heaters (Aqua Medic) controlled by a 0.1°C digital thermostat (MJA Heat trace Ltd). The species were separated, with two compatible species in each water bath, and then temperature was either kept at ambient (control 26.1 ± 0.1°C; mean ± 1 standard error), or raised at one of three rates 0.04°C, 1°C and 1.3°C (Table 1; Supplementary Figure S1). Acute temperature tolerance tests were conducted in insulated jacketed tanks attached to thermocirculators (Grant Instruments, LTDG20) which were able to maintain seawater temperature to a precision of ±0.1°C. Temperatures were raised at 0.04°C, 1°C and 1.3°C; temperatures between 0.04°C and 1°C were maintained at a constant 1.0°C, while temperatures between 1°C and 1.3°C were maintained at a constant 1.0°C, and temperatures between 1°C and 1.3°C were maintained at a constant 1.0°C. Lower term temperature tolerance was estimated following the methodology of Peck et al. (2009). Animals used in longer term experiments were housed in insulated flow-through aquaria (~10 l). Briefly, seawater was heated in a header tank (50 l) with 50 W aquarium tank heaters and inflow was via gravity into aquaria at a rate of 60 ml min−1 (controlled by the diameter of the outflow valves). Flow of cold seawater into the header tank was maintained with a float valve so that the header pressure remained constant. Temperature was raised at 5°C until target temperatures of 22.9 ± 0.3°C and 26.2 ± 0.6°C were reached. These temperatures were then maintained until more than 50% mortality had occurred. The number of days that 50% of individuals survived at elevated temperature was converted into a rate of temperature change by dividing the number of degrees that temperature was elevated by the number of days (following Peck et al., 2009).
At Scott Base (Antarctica New Zealand) animals were transferred to a flow-through aquarium at ambient temperature (−1.7°C). Acute temperature tolerance tests were conducted in insulated jacketed tanks attached to thermo-circulators which were able to maintain seawater temperature to a precision of ±0.1°C. Temperatures were raised at 0.04 °C h⁻¹ and 1 °C h⁻¹. Trials at slower rates of warming were not possible.

Mortality was assessed every hour in 0.04 °C h⁻¹ experiments or two to three times a day at slower rates of temperature change (after the method of Peck et al., 2009). For species that normally live attached to the substratum, e.g. starfish, sea urchins and gastropod molluscs, the first sign that competence had been compromised was when they lost their ability to remain attached. Unattached individuals were stimulated with a blunt seeker to illicit a response; non-response (loss of equilibrium) was recorded as CTmax and the size of each individual was measured. Temperature limits for individual species in the current study were not normally distributed even after transformation, and so non-parametric Mann–Whitney multiple comparisons were used with a Bonferroni corrected acceptance probability. The global comparison of temperature limits was conducted using a linear mixed effects model with species as a random factor and natural logarithm of rate of change as a covariate. The model fit and residual behaviour were visually validated independently using the function ‘varIdent’ and the transformation, and so non-parametric Mann–Whitney multiple comparisons were used with a Bonferroni corrected acceptance probability. The global comparison of temperature limits was conducted using a linear mixed effects model with species as a random factor and natural logarithm of rate of change as a covariate. The model fit and residual behaviour were visually validated independently using the function ‘varIdent’ and the transformation, and so non-parametric Mann–Whitney multiple comparisons were used with a Bonferroni corrected acceptance probability. The global comparison of temperature limits was conducted using a linear mixed effects model with species as a random factor and natural logarithm of rate of change as a covariate. The model fit and residual behaviour were visually validated independently using the function ‘varIdent’ and the transformation, and so non-parametric Mann–Whitney multiple comparisons were used with a Bonferroni corrected acceptance probability. The global comparison of temperature limits was conducted using a linear mixed effects model with species as a random factor and natural logarithm of rate of change as a covariate. The model fit and residual behaviour were visually validated independently using the function ‘varIdent’ and the

**RESULTS**

**Ascension Island**

There was no mortality of control animals. All species had a significantly higher CTmax at the fastest rate of change (Tables 1, 2). At warming rates of 0.04 °C h⁻¹ and 1 °C h⁻¹,
the bivalve, *Acar domingensis* was the most temperature tolerant (medians of 39.0 and 37.1°C, respectively; Tables 1, 2) and the brittlestar, *O. abnorme*, was the least tolerant at both these rates of change (35.6 and 31.1°C respectively; Tables 1, 2). At the slowest rate of change, 1.2 or 1.3 d°C⁻¹, there was no difference in the temperature limits of *Acar domingensis* or *Echinometra lucunter*, but *Eucidaris tribuloides* had a significantly higher temperature limit at 1.3 d°C⁻¹ than at 1.0 d°C⁻¹.

**New Zealand**

After 45 d there were mortalities in controls of *Pseudechinus huttoni* (8 out of 12) and *Liothyrella neozelanica* (9 out of 13). Data from beyond 30 d were, therefore, not used for these species. Mortalities in controls of *Cnemidocarpa bicornata* (N = 2), *Cookia sulcata* (N = 1) and *Ophiioneries fasiata* (N = 1) were low, and so mortality could be estimated from long term trials. This was with the exception of *C. sulcata*, which did not suffer 50% mortality during the incubation period at 22.9°C. Long term mortality could, therefore, not be estimated at 22.9°C for this species.

In all cases upper temperature CT max was lower at 1 d°C⁻¹ than at the fastest rate of warming, 0.04 d°C⁻¹ (Tables 1, 2). 50% of *Cnemidocarpa bicornata* survived for a median of 17 and 37.5 d at 26.2°C and 22.9°C, for which calculated rates of change were 2.3 d°C⁻¹ and 8.9 d°C⁻¹ respectively. 50% of *Cookia sulcata* survived for a median of 23 d at 26.2°C which gave a rate of change of 3.2 d°C⁻¹. 50% of *Ophiioneries fasiata* survived for a median of 20 and 44 d at 26.2°C and 22.9°C for which calculated rates of change were 2.7 d°C⁻¹ and 10.5 d°C⁻¹, respectively.

**McMurdo Sound**

In all but *Limatula hodgsoni* upper temperature limits were lower at 1 d°C⁻¹ than at the fastest rate of warming, 0.04 d°C⁻¹ (Tables 1, 2).

**Global comparisons between assemblages**

In the global comparison of CT max at 1 d°C⁻¹, each assemblage had a tolerance that was positively correlated with their mean environmental temperature, except McMurdo Sound, which although a colder environment than the Western Antarctic Peninsula, had a higher tolerance than

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**Table 2. Mann-Whitney comparison tests of thermal limits of 4 species of invertebrate at the fastest rates of temperature change.**

<table>
<thead>
<tr>
<th>Location</th>
<th>Species</th>
<th>Mann-Whitney values for quoted rate of warming d°C⁻¹</th>
<th>Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.04 VS 1.0</td>
<td>0.04 VS 1.2</td>
</tr>
<tr>
<td>Ascension</td>
<td><em>Acar domingensis</em></td>
<td>W = 532</td>
<td>W = 434</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td><em>Echinometra lucunter</em></td>
<td>W = 408</td>
<td>W = 472</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td><em>Eucidaris tribuloides</em></td>
<td>W = 493</td>
<td>W = 340</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td><em>Ophiostigma abnorme</em></td>
<td>W = 350</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>–</td>
</tr>
<tr>
<td>New Zealand</td>
<td><em>Cnemidocarpa bicornata</em></td>
<td>W = 134</td>
<td>W = 133</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td><em>Cookia sulcata</em></td>
<td>W = 456</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><em>Liothyrella neozelanica</em></td>
<td>W = 190</td>
<td>W = 120</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td></td>
<td><em>Ophiioneries fasiata</em></td>
<td>W = 36</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>–</td>
</tr>
<tr>
<td>McMurdo Sound</td>
<td><em>Pseudechinus huttoni</em></td>
<td>W = 120</td>
<td>W = 196</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P &lt; 0.01</td>
<td>P &lt; 0.01</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Temperature limits of different faunal assemblages from the tropics to the Antarctic. Tropical Singapore data from Nguyen et al. (2012), Antarctic Peninsula data from Peck et al. (2009), and new data from Ascension Island, New Zealand and McMurdo Sound. Fitted lines from linear mixed effect regression fits (Table 3).
As expected, CT\text{max} values were generally lower at slower rates of warming. The only species that had a temperature limit that was significantly higher at a slower rate of warming was the urchin Euchidaris tribuloides from Ascension Island. The first that a shallow water Ascension Island species has the capacity to rapidly adjust its physiology. Tropical species are expected to have limited acclimatory capacity as they live in thermally stable environments, with little variation in temperature through the year (Spicer & Gaston, 1999; Deutsch et al., 2008; Tewksbury et al., 2008; Peck et al., 2014). However, the shelf seas around Ascension Island are surrounded by deep oceanic water and so the water temperature is cooler and more variable than in coastal tropical locations. The near-shore water temperature rose gradually between September 2012 and June 2013, from 24 to 28°C (Brickle, unpublished data). If this is a predictable seasonal change in seawater temperature, then Ascension Island marine species may have evolved the plasticity to adjust their physiology in response to this variation. When warm water species have the capacity to acclimate their physiology, acclimation is expected to be rapid, occurring within a few hours to days (Spicer & Gaston, 1999; Verberk & Calosi, 2012; Peck et al., 2014). In polar waters, which are also thermally stable through the year, species are similarly expected to have limited acclimatory capacity (Peck et al., 2014). When acclimation of invertebrates does take place at the poles, it may take many months. For example, the acclimation of the Antarctic limpet, Nacella concinna, took between 2 and 5 months (Peck et al., 2014). It is possible that longer duration experiments will find that McMurdo Sound fauna will also have limited acclimation capacity but the preliminary experiments conducted here do not allow this to be tested.

Assemblages from warmer environments had higher intercepts, indicating their higher CT\text{max} (Deutsch et al., 2008; Tewksbury et al., 2008; Sunday et al., 2011). The overall relationship shows that the CT\text{max} of each assemblage was negatively correlated to the slope of the relationship between CT\text{max} and the rate of warming; the slope was also shallower in assemblages from warmer environments (Figure 3). Tropical species are often thought to be living at temperatures close to the upper limit that can be sustained through acclimatisation and, therefore, have limited ability to further adjust their temperature limits (Stillman & Somero, 2000; Stillman, 2003; Deutsch et al., 2008; Richard et al., 2012). This is thought to result in tropical species having temperature limits that are only slightly above their experienced environmental temperatures and a rates-of-warming relationship with a reduced slope. Temperate marine assemblages, such as the New Zealand fauna tested here, generally live in cooler, but more variable environments, and their intercepts and slopes are closer to those of tropical species than polar ones. To cope with the variability of their environments they have evolved higher thermal tolerances with higher buffers above their experienced environmental maxima (Deutsch et al., 2008; Tewksbury et al., 2008; Sunday et al., 2011). The increased acclimatory capacity of temperate species would also tend to reduce the slope of the rates-of-warming relationship closer to those of tropical rather than polar species (Figure 3). Whilst the maintenance of physiological heat tolerance is expensive and is generally expected to be lost in species from colder latitudes (Stillman

\begin{table}
\centering
\caption{Summary table for the linear mixed effects model estimates fitted using restricted maximum likelihood for the global relationship of upper temperature limit and rate of warming (as in Figure 1) is plotted against the intercept. Singapore data from Peck et al. (2012), temperate data from Richard et al. (2012) with the Peruvian upwelling fauna indicated with an open diamond, Antarctic Peninsula data from Peck et al. (2009). The regression line was fitted excluding this outlying Peruvian fauna. \textit{Slope} = 0.07, \textit{intercept} = -0.27, \textit{R}^2 = 0.97; F = 252, P < 0.01.} 
\begin{tabular}{|l|l|l|l|l|l|l|}
\hline
\textbf{Assemblage} & \textbf{Slope Estimate} & \textbf{SE} & \textbf{d.f.} & \textbf{t-value} & \textbf{P-value} & \textbf{Intercept Estimate} & \textbf{SE} & \textbf{d.f.} & \textbf{t-value} & \textbf{P-value} \\
\hline
Singapore & -0.0288 & 0.0021 & 66 & 13.3 & <0.01 & 3.61 & 0.01 & 66 & 280.9 & <0.01 \\
Ascension & -0.0258 & 0.006 & 66 & 0.37 & 0.71 & 3.5388 & 0.03 & 43 & 2.66 & 0.01 \\
McMurdo & -0.104 & 0.027 & 66 & 2.87 & <0.01 & 2.549 & 0.057 & 66 & 18.6 & <0.01 \\
New Zealand & -0.047 & 0.01 & 66 & 1.83 & 0.07 & 3.2385 & 0.031 & 43 & 12.09 & <0.01 \\
WAP & -0.1949 & 0.02 & 66 & 6.71 & <0.01 & 2.099 & 0.071 & 66 & 21.23 & <0.01 \\
\hline
\end{tabular}
\end{table}
Fig. 3. Theoretical representation of the effect of the rate of warming on the lethal limits of marine ecospheres across latitudes. The dashed line represents a tropical assemblage with a shallow slope, due to rapid but limited acclimation capacity. The solid lines represent a temperate assemblage with an intermediate slope, a slower rate of acclimation, but with greater acclimation capacity. Two examples are shown with different rates of acclimation. The dotted line represents a polar assemblage with a steep slope of the relationship for this assemblage (Figure 3), indicating their increased sensitivity to long term elevated temperature. The compression of upper temperature limits, and therefore shallower slopes, at higher temperatures was also apparent when the slopes and intercepts of temperate species were compared between summer and winter (Richard et al., 2012).

Isolated oceanic islands are often regions of upwelling, as they have narrow shelves and are surrounded by deep water (Pinheiro et al., 2010). Rapid changes in temperature, of up to 2.5°C in less than 24 h, were recorded at Ascension Island, particularly during March and April 2013 (Brickle, unpublished data). Whilst the occurrence of upwelling events at Ascension Island may be predictable enough that faunal species have sufficiently regular cues to acclimate their physiology, the assemblage living in the Peruvian upwelling region, studied by Richard et al. (2012), did not have the ability to respond to the episodic warming associated with El Niño events. When the slopes of regression fits were plotted against the intercept for each assemblage (following Richard et al., 2012), the relationship was consistent across assemblages, except for the Peruvian upwelling fauna. This indicates that the Peruvian fauna would have a reduced long term temperature tolerance, compared to other temperate assemblages, and are less likely to be able to adjust their thermal tolerance to cope with the warmer temperatures experienced during unpredictable El Niño warming events (Figure 1). This is supported by evidence that species experience large scale mortality during these periods. Although further data are required, the fact that Ascension Island fauna fit with the general pattern provides preliminary evidence that the annual variation in temperature at Ascension (6°C) is sufficiently variable yet predictable, that some of the marine fauna have the ability to acclimate their physiology. The Ascension experiments were conducted in August, when the water temperature was close to its annual minimum of 22°C. If Ascension Island invertebrates can acclimate their physiology in response to seasonal changes in temperature, they might be expected to have higher temperature limits when water is at its annual maximum (28°C). Experiments testing more species, and at different times of year, are required to confirm the presence of seasonal plasticity. Comparisons of thermal tolerance across environments are often, out of necessity, conducted in different years and whilst temperate and polar experiments were conducted in summer, there can be inter-annual differences in thermal limits (e.g. Morley et al., 2012). Assessing long term thermal limits through projections based on different rates of warming are likely to reduce the impact of differences between years and give a better assessment of the differences between assemblages.

The relationship between CT max and the rate at which temperature is increased measures a combination of acute thermal tolerance and acclimatory capacity. These relationships between CT max and the rate of warming help explain why tropical species, which are already living close to their upper limits, have limited capacity for acclimation to warmer temperatures (Stillman & Somero, 2000; Stillman, 2003), whilst temperate latitude freshwater beetles with higher CT max’s had higher acclimation capacity (Calosi et al., 2008). The global relationship between these parameters is consistent across assemblages allowing long term thermal sensitivities to be predicted for other assemblages. This pattern, however, only holds in environments where predictable temporal cues have lead to the development of the ability to respond to environmental variability.

ACKNOWLEDGEMENTS

Bev Dickson and the staff at both Portobello and Doubtful Sound provided vital technical support in New Zealand. Jess Ericson, Tom and Linnaea Bird helped maintain the long term incubations in Portobello Marine Laboratory. The United States Antarctic Program divers, under the leadership of Rob Robbins, collected specimens at McMurdo. We are grateful to the Shallow Marine Surveys Group and the South Atlantic Environmental Research Institute for organizing the expedition. We are also very grateful to the Ascension Island Government, the members of staff at the Conservation Centre and Ascension Island Dive Club for their cooperation, accommodation and hospitality.

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SUPPLEMENTARY MATERIALS AND METHODS

The supplementary material referred to in this article can be found online at journals.cambridge.org/mbi.
REFERENCES


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Stock structure of the English Channel common cuttlefish *Sepia officinalis* (Linnaeus, 1758) during the reproduction period

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Within the English Channel, the common cuttlefish *Sepia officinalis* is a semelparous species for which a 2-year life cycle was exclusively described in the 1980s. In the 1990s, new research indicated that whilst a 2-year life cycle was still evident for females and the large majority of males, a small proportion of males were actually maturing at only 1 year of age. Since 1980, the interest of French and UK fishers for this resource has increased and it is nowadays one of the most important demersal species of the area and is considered to be fully exploited. From the start of the 20th century, fishing effort and sea surface temperatures have increased in the English Channel and have probably impacted the life history traits of *S. officinalis*. A 2-year sampling programme was undertaken at French landing sites of the English Channel during the reproduction season in 2010 and 2011 to estimate if the proportion of 1-year-old mature animals has changed. Age determination was carried out by coupling polymodal decomposition and lipofuscin measurement. Size-at-maturity for each year and each sex was estimated by fitting a binomial error GLM. Results highlight that a variable percentage of males and females belonging to the first cohort are mature and that size-at-maturity was lower than that observed in the 1990s. Finally, different parameters, such as temperature and fishing pressure are explored to discuss changes in life history traits suggesting that cuttlefish could be an indicator of the temperature regime shift in the English Channel.

Keywords: *Sepia officinalis*, English Channel, life history traits, maturity, warming, fishing pressure

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INTRODUCTION

The common cuttlefish, *Sepia officinalis* (Linnaeus, 1758), is a short lived and semelparous cephalopod distributed from the West African coast (Senegal and Mauritania) to the English Channel and in the Mediterranean Sea (Mangold, 1987). It has a life cycle lasting between 1 and 2 years depending on the latitude at which it lives (Domingues et al., 2006; Guerra, 2006). From West African to the Portuguese coasts, the entire population has a 1-year life cycle (Mangold-Wirz, 1963; Mangold, 1966; Guerra & Castro, 1988; Coelho & Martins, 1991). The Bay of Biscay is a transition zone where two different groups were identified: Group I Breeding (GIB), can breed at 1 year of age (45% of male specimens and 20% of female specimens), Group II Breeding (GIIB), breeds at 2 years of age (Le Goff & Daguzan, 1991; Gauvrit et al., 1997). In the English Channel, the life cycle of *S. officinalis* was originally described as lasting exclusively 2 years (Boucaud-Camou & Boismery, 1991; Boucaud-Camou et al., 1991). A decade later, Dunn (1999a) found that the entire female population and 96% of male specimens had a 2-year life cycle (GIIB). The remaining males (4%) were found to be mature at the age of 1 year old, constituting a GIB.

The English Channel population of *S. officinalis* was mainly considered as a non-valuable by-catch before the 1980s (Dunn, 1999a). Since then, the increasing price of this species and the depletion of finfish stocks (Royer et al., 2006; Molfese et al., 2014) led French and UK fishers to exploit this stock. Landings increased from 4350 tonnes in 1992 to a maximum of 17,400 tonnes in 2004 (Gras et al., 2014). During the 2000–2010 decade, an average of 11,000 tonnes was landed annually for an average turnover of €20 M (Pierce et al., 2010; Portail CHARM III–Interreg IV, ©, 2012). The English Channel stock of *S. officinalis* was assessed in trials using a depletion model (Dunn, 1999b), a Virtual Population Analysis (Royer et al., 2006) and a two-stage biomass model (Gras et al., 2014). Whilst Dunn (1999b) does not give a clear indication of the stock status, Royer et al. (2006) and Gras et al. (2014) concluded that *Sepia officinalis* has been fully exploited since the beginning of the 1990s. Moreover, Gras et al. (2014) showed that *S. officinalis* abundance decreased during the period 2001–2008 and consequences of the fishing pressure on life history traits should be investigated.
High fishing pressure directly and indirectly affects fish communities and populations. Among the direct effects of high fishing pressure on populations are increased mortality and size selectivity that tend to reduce the proportion of old and large specimens in the population (Smith, 1994). After several years of high exploitation, smaller specimens maturing faster are favoured, leading to decrease in mean size and length (or age) at maturity by favouring slow growth and early maturing specimens (Bianchi et al., 2000; Shin et al., 2005; Kantoussan et al., 2009). Observations of such processes have been carried out in various stocks of the N-E Atlantic such as the North Sea herring (Shin & Rochet, 1998; Engelhard & Heino, 2004), the North Sea plaice (Grift 2003) and the northern cod (Olsen et al., 2000; Shin et al., 2003). Early maturing specimens (Bianchi et al., 2000) suggest that fish assemblages become dominated by species maturing faster and reproducing at a shorter length (or age) at maturity in demersal communities has been observed, a decline in community level, average length and length-at-maturity in demersal communities has been observed, suggesting that fish assemblages become dominated by species maturing faster and reproducing at a shorter length (Hawkins et al., 2003). Due to their short life cycle and their high metabolic rate, cephalopods are assumed to be very sensitive to environmental warming (Pierce et al., 2010).

In the context of high fishing pressure and climate warming (including global warming which is made of natural and anthropogenic components) are also known to influence the abundance and spatial distribution of marine species (Beaugrand & Reid, 2003; Hawkins et al., 2003; Beaugrand, 2009). According to the data collected by the Marine Biological Association of the United Kingdom (MBA), significant warming of the English Channel occurred throughout the 20th century (Southward & Roberts, 1987). Between 1986 and 2006, Saulquin & Gohin (2010) estimated this warming to be between 0.3 °C in the southern part of the Channel and 1.5 °C in its northern part. This warming has led to modifications in phytoplankton assemblages, fish larval abundances and pelagic fish abundances (Southward et al., 1988). Moreover, a decline in community level, average length and length-at-maturity in demersal communities has been observed, suggesting that fish assemblages become dominated by species maturing faster and reproducing at a shorter length (Hawkins et al., 2003). Due to their short life cycle and their high metabolic rate, cephalopods are assumed to be very sensitive to environmental warming (Pierce et al., 2010).

Sample collection

Samples of S. officinalis were collected from three French landing sites along the English Channel coastline in spring 2010 and 2011. Specimens were caught by trawlers or trap fishers in the Normano-Breton Gulf (ICES rectangles 26E8 and 27E8) and in the Bay of Seine (ICES rectangles 27E9 and 28E9). In 2010, from 12 April to 9 July, 734 specimens (395 males, 339 females) were collected in Erquy, Cherbourg and Port-en-Bessin landing sites. In 2011, from 5 April to 30 June, 655 specimens (444 males and 211 females) were collected in Port-en-Bessin and Cherbourg landing sites. The sex ratio was tested using a chi-square test.
Biometry dissection and sex determination

The Dorsal Mantle Length (DML) of each specimen was measured to the lowest cm and length frequencies were represented on four histograms, one per sex and per year. Length classes are defined every cm, immature specimens are represented in white; mature animals are represented in grey. An attempt was made to fit two Gaussian models associated with a mixing effect to the two cohorts. If the model fitted the first cohort well, due to its shape, it was not possible to fit it to the second cohort. A truncation point between the first cohort well, due to its shape, it was not possible to fit it to the second cohort. A truncation point between the first and second cohort is then defined by the least numerous fit it to the second cohort. A truncation point between the first and second cohort is then defined by the least numerous growth rate in *S. officinalis* after the first mode. However, due to the high inter-individual variability of the growth rate in *S. officinalis* (Challier et al., 2002, 2006), this criteria cannot be used alone to determine which cohort a particular specimen belongs to. The measurement of lipofuscin (age pigment accumulated in tissues; Zielinski & Portner, 2000; Doubleday & Semmens, 2011) was used in this work to give a second indicator of age estimation. Fresh total Body Weight (BW) and Gonad Weight (GW) were also measured for each specimen.

The mantle of each specimen was opened by a ventral incision to determine the sex and maturity stage using the macroscopic scale developed in the framework of the international Workshop on Sexual Maturity Staging of Cephalopods (WKMSCEPH; ICES, 2010). Six maturity stages are described from undetermined to spent (post-reproduction) for each sex. In female specimens, maturity stages are mainly defined using oocyte size and development of the Nidamental Glands (NG). In male specimens, maturity stages are defined using testis size and spermatophore position in the Spermatophoric Complex (SC). This international scale is based on the same number of stages and the same criteria as the one developed by Dunn (1999a). Mantle tissue samples were collected from a subsample of specimens to measure lipofuscin concentrations. Tissue samples were kept frozen in liquid nitrogen to avoid tissue degradation and transported to the laboratory to be analysed.

Lipofuscin measurement

Experiments carried out in the past to study English Channel *S. officinalis* based age estimation only on polymodal decomposition of length frequencies (Boucaud-Camou & Boismery, 1991; Boucaud-Camou et al., 1991; Dunn, 1999a). In cephalopods, growth rate is influenced by environmental conditions and presents a high inter-individual variability making this method inaccurate. Various hard structures have been tested for age determination in *S. officinalis* (Le Goff et al., 1998; Neige, 2006) but the only consistent results were obtained from statolith daily rings during the juvenile phase (Challier et al., 2002, 2006). However, statolith rings are very difficult to read in *S. officinalis* and are not usable after 240 days of age (Bettencourt & Guerra, 2001). Above this threshold, some studies proposed to use the concentration of lipofuscin as a proxy of age: lipofuscin is a pigment that accumulates in tissues through the lifetime of some invertebrates (Sheehy et al., 1996; Ju et al., 1999; Bluhm & Brey, 2001). This method has been successfully used in cephalopods to provide a rough estimation of *S. officinalis* age (Zielinski & Portner, 2000; Doubleday & Semmens, 2011).

Lipofuscin was measured according to Zielinski & Portner (2000). Mantles of *S. officinalis* were ground under liquid nitrogen and homogenized in a chloroform-methanol mixture (1:2, v/v). After centrifugation for 10 min at 2000 g, lipofuscin was found in the chloroform phase. In this phase an emission spectrum between 350 and 550 nm was obtained at an excitation wavelength of 350 nm using a Mithras LB940 fluorimeter (Berthold, Thoiry, France). The luminescence of the sample was determined at the maximum emission at 420 nm. Lipofuscin concentrations were expressed as relative fluorescence intensity according to Hill & Womersley (1991), using 0.1 μg quinine per mL 1N H₂SO₄ as a standard.

In a first step, we tested the applicability of lipofuscin concentrations to fish market samples of *S. officinalis* by measuring post-mortem variability over the 3 days following the death. A mantle was sampled on one living animal and lipofuscin was measured on the fresh tissue. The tissue was then kept on ice during the following 3 days. A large piece of mantle was taken every 24 h randomly on the mantle and used to perform replicated measures of lipofuscin. Differences were tested using an ANOVA. In a second step, lipofuscin concentrations were measured on two subsamples. A subsample of 11 immature specimens was taken from the first cohort (DML ranging 10–13 cm and assumed to be 1 year olds as they are short and immature) and a subsample of

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**Fig. 1.** Life cycle of the English Channel population of *S. officinalis* L. as described by Boucaud-Camou & Boismery (1991), Boucaud-Camou et al. (1991) and Dunn (1999a).
17 mature specimens was taken from the group which length is highly over the boundary between the two cohorts (DML ranging 20–31 cm and therefore assumed to be 2-year-olds). This experiment describes inter-cohort differences in lipofuscin concentrations. Finally, results of lipofuscin concentration measured from the smallest mature specimens (two animals of 11 cm DML and one of 13 cm DML) were used in a Student t-test to estimate the probability of each small mature specimen to belong to each cohort.

Size-at-maturity estimation

The maturity stage of each specimen, observed in each length class, was used to fit a binomial error Generalized Linear Model (GLM) to estimate size-at-maturity. Specimens were considered as mature when they reached the 'Maturing stage' (2b), assuming that if they are maturing during the spring, they will become mature and able to breed and spawn before the end of the reproduction season in late summer.

Gonado-Somatic Index

An index of sexual development was computed with the Gonado-Somatic Index (GSI):

\[
GSI = \frac{GW}{BW - GW}
\]

GSI is a proxy of the energy allocated by the specimen to reproduction to the detriment of somatic growth. This index is a quantitative measure of sexual maturity and the relationship between GSI and DML complements the estimation of size-at-maturity. Student t-tests were performed to look for differences in GSI between the two sampled years, between mature and immature animals and between males and females. An F-test was used to highlight differences between GSI variances of mature males and females. An F-test was used to highlight differences between GSI variances of mature males and females. Finally, a linear model was fitted to explore the correlation between mature male DML and GSI.

RESULTS

Length frequency of samples

Female specimens ranged from 6 to 27 cm DML in 2010 and from 7 to 26 cm DML in 2011. Male specimens ranged from 6 to 36 cm DML in 2010 and from 7 to 32 cm DML in 2011 (Figure 2). Length frequencies highlighted that a first cohort was identifiable and the maximum size of this first cohort could be determined at 16 cm for both sexes in 2010 and at 14 and 13 cm for females and males respectively in 2011. In 2010, the smallest mature females were 7 cm long while the smallest mature males were 8 cm long. In 2011, the smallest mature females were 11 cm long and the smallest mature males were 10 cm. In 2010, 17% of females and 30% of males assigned to the first cohort were found to be mature and in 2011, 3% of females and 13% of males of the first cohort were found to be mature. For both years, sex ratios were significantly different from 1:1 with males more numerous than females, particularly in 2011.

Size-at-maturity determined using a binomial error GLM

For both sexes, the size-at-maturity (Figure 3) was higher in 2010 than in 2011 and for both years, size-at-maturity in females was larger than in males. For male specimens, the smallest length class within which all individuals were found to be mature was 16 cm DML in 2010 and 14 cm DML in 2011; above these length classes all male specimens were found to be mature. In contrast, for female specimens, the smallest length within which all individuals were found to be mature was smaller at 14 cm in 2010 and 13 cm in 2011; however, above these length classes, immature individuals were still observed in larger length classes.

Variability in lipofuscin measurement and age estimation

Lipofuscin measurement from the first experiment (Figure 4) showed that, over a period of 3 days, no significant trend was observed in lipofuscin concentration; however, the standard deviation of the measure did increase with time. In the second experiment, lipofuscin accumulation measured in specimens belonging to the first and second cohorts were significantly different (Figure 5). Results of the lipofuscin measurement performed on the three small mature males (Figure 5, Table 1) showed that lipofuscin accumulation of these three specimens was not significantly different from that of the first cohort, but was significantly different from lipofuscin accumulation measured in GIB. These three specimens could thus be considered as part of the GIB group.

Gonado-Somatic Index

The GSI (Figure 6) for each sex and each year, does not reveal any significant inter-annual differences. In both sexes, the GSI during the reproduction period was significantly higher for mature specimens than for immatures. Mature female specimens were characterized by a significantly higher GSI and a higher inter-individual variability than mature males. Finally, the GSI of mature male specimens was found to be significantly correlated with DML.

DISCUSSION

The life cycle of the Sepia officinalis population in the English Channel was described in the past as lasting 2 years for females and for the large majority of males (Boucaud-Camou et al., 1991; Dunn, 1999a). The present study highlights that, during the 2010 and 2011 reproduction seasons, a variable percentage of mature male and female S. officinalis belongs to the first cohort and could thus be considered as a GIB. Age determination was based on polymodal decomposition and adaption of lipofuscin measurement protocol (Zielinski & Portner, 2000; Doubleday & Semmens, 2011). The association of both methods is more accurate than the polymodal decomposition used alone and enabled to disentangle the two sampled cohorts. It has corroborated that the smallest mature animals observed are most likely 1-year-old S. officinalis and not small 2-year-old specimens. Finally, the GSI illustrates the amount of energy dedicated by each sex
to the development of the gonad to the detriment of the somatic growth.

Lipofuscin measurement has helped in determining the cohort to which each specimen belongs, but this method is not accurate enough for age estimation (Zielinski & Portner, 2000; Doubleday & Semmens, 2011). Moreover, accuracy decreases with increasing time between death and sampling; as the sampling process was based on commercial fishery landings, this parameter was not under control. Finally, lipofuscin measurement is expensive and time consuming and for this reason experiments cannot be carried out on a large number of specimens. An alternative method for rough age estimation could be set up using statoliths. Although statolith daily rings are not accurately readable in 1-year-old *S. officinalis* (Bettencourt & Guerra, 2001; Challier et al., 2002, 2006), studies of several fish species have found that otolith length, thickness and weight continued to grow with age even when body growth rate was null (Reznick et al., 1989; Campana, 1990; Newman, 2002). Age frequency distribution derived from otolith weights were found to be consistent with those derived from increment counts (Pilling et al., 2003; Mc Dougall, 2004). Moreover, statolith diameter has also been successfully used as a proxy of age in the planktonic tunicate *Oikopleura vanhoeffeni* (Choe & Deibel, 2009). If *S. officinalis* statolith experiences daily increments throughout its life, measuring dome diameters could be another way to separate the two annual cohorts.

In cephalopod species, rearing experiments have shown that maturation is driven by different factors including photoperiod, food availability and temperature (Mangold, 1987). Cephalopods are known not to store ingested energy in their tissues and as such individuals, particularly females, need a regular food availability in order to reach maturity as observed for *Sepioteuthis australis* (Ho et al., 2004) and *Loligo vulgaris* (Moreno et al., 2005). Thus, the variability observed in the female GSI could be explained by food availability experienced by each specimen. In male specimens, the lower amount of energy dedicated to reproduction allows more energy for somatic growth and therefore explains both the low variability of the GSI and a larger DML than in females. It is also worth noting that, within this study, the size-at-maturity plots for males are quite homogeneous and do not reveal subgroups maturing at different sizes as previously described for other cephalopod species (Castro et al., 1991; Coelho et al., 1994; Moreno et al., 1994; Boyle & Rodhouse, 2005). Finally, the English Channel warming (Hawkins et al., 2003; Saulquin & Gohin, 2010) leads to the modification of species communities by increasing abundance of warm water species and decreasing abundance of cold water species. Such modifications could also influence the English Channel population of *S. officinalis* by modifying the competition and the predator-prey relationships.

Temperature is a key parameter influencing cephalopod life cycles, from egg development to reproduction (Rodhouse et al., 1992; Boyle & Pierce, 1994; Robin et al., 2014). An increase in environmental temperature generally accelerates the somatic growth of cephalopods, shortening the life cycle duration by accelerating maturation (Moreno et al., 2005).

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**Fig. 2.** Length frequencies of the female and male specimens collected during spring 2010 and 2011 in French English Channel landing sites. Immature specimens are displayed in white and mature specimens are displayed in grey. The vertical line drawn indicates the boundary between the first and the second cohorts.
et al., 2007). This is a well-known feature in the life cycle of *S. officinalis* which shortens from the north of its distribution area (English Channel) to the south (West African coasts; Mangold-Wirz, 1963; Mangold, 1966; Guerra & Castro, 1988; Coelho & Martins, 1991). The warming of the English Channel (Hawkins *et al.*, 2003; Saulquin & Gohin, 2010) could be the cause of appearance of a GIB composed of males and females and the observation of a lower size-at-maturity (on average 12.16 cm for males and 12.41 cm for females in 2010 and 2011 respectively) than that observed by Dunn (1999a) (14.6 cm for males and 16.4 cm for females). Further investigations are required to investigate if this size-at-maturity variability could be correlated with environmental indices (such as NAO index) and

**Fig. 3.** Size-at-maturity determined using a binomial error GLM for female and male samples collected during spring 2010 and 2011 in French English Channel landing sites. Number of specimens measured per DML class is shown in Figure 2.

**Fig. 4.** Trend in lipofuscin concentration measured in the mantle sampled on one living *S. officinalis* from the death and every 24 h until 72 h after the death. Error bars are the standard deviations of each measure.

**Fig. 5.** Lipofuscin concentration measured in *S. officinalis* mantles for juveniles (Juv, 11 specimens, ranging 10–13 cm DML), adults (Ad, 17 specimens, ranging 20–31 cm DML) with the inter-individual standard deviation and lipofuscin concentration measured in three small mature specimens (I1, 11 cm DML; I2 11 cm DML; I3, 13 cm DML).
whether GIB is actually involved in reproduction by analysing the sperm reservoirs of females to understand whether cross-mating between generations exists (Gauvrit et al., 1998).

Warming of the English Channel could also lead to an extension of the reproduction season allowing the more precocious hatchlings sufficient time to mature prior to the following spring.

In the *S. officinalis* population of the English Channel, Challier et al. (2006) showed that recruitment in the trawling fishery occurs throughout the year at a constant age leading to the conclusion that *S. officinalis* hatches throughout the year. GIB observed in this study could thus be individuals which had hatched early in the reproduction season and which had had enough time before offshore migration to start maturation and finish it before the end of next spring.

The English Channel has long been exploited by both French and UK fishers. Trawling started 200 years ago and fishing pressure has regularly increased since the beginning of the 20th century (Hawkins et al., 2003; Guenette & Gascuel, 2012; Gras, 2013). High fishing pressure greatly impacts demersal communities and can modify life history traits by favouring specimens which mature faster or reach maturity at a smaller size or lower age (Shin et al., 2005; Kantoussan et al., 2009). This phenomenon has been observed in a range of N-E Atlantic stocks (Shin & Rochet, 1998; Griff et al., 2003; Engelhard & Heino, 2004; Olsen et al., 2004) and could also have influenced the English Channel population of *S. officinalis*, which has been fully exploited since the beginning of the 1990s (Royer et al., 2006; Gras et al., 2014), leading to the appearance of a GIB and a potential decreasing trend in size-at-maturity. Modifications in demersal community assemblages caused by high fishing pressure could have modified the prey availability for *S. officinalis*. Maturation efficiency in cephalopods, which could be unable to store energy in their tissue (Mangold, 1987; Moreno et al., 2005) could therefore be impacted.

*Sepia officinalis* is one of the top predators of the English Channel ecosystem (Araújo et al., 2005). In contrast to other top predators, *S. officinalis* has a short lifespan and as such, the consequences of any regime shift can be easily observed in the short term and could thus provide a useful indicator of climate change. According to Jackson et al.

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**Table 1.** Probabilities of three small male mature specimens to be different from the GIB and GIIB cohorts.

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Length (cm)</th>
<th>P, GIB</th>
<th>P, GIIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cohort (immature; 11 specimens) 10 &lt; L &lt; 13</td>
<td>0.12</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Second cohort (mature; 17 specimens) 20 &lt; L &lt; 31</td>
<td>0.08</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Individual 1</td>
<td>11</td>
<td>0.12</td>
<td>0.02</td>
</tr>
<tr>
<td>Individual 2</td>
<td>11</td>
<td>0.08</td>
<td>0.03</td>
</tr>
<tr>
<td>Individual 3</td>
<td>13</td>
<td>0.22</td>
<td>0.02</td>
</tr>
</tbody>
</table>

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**Fig. 6.** Gonado Somatic Index (GSI) vs Dorsal Mantle Length (DML) for female and male samples collected during spring 2010 and 2011 in French English Channel landing sites. Immature specimens are displayed with circles while mature specimens are displayed with crosses.
(2000), an indicator of climate change must meet four criteria: (i) relevance to assessment and ecological functions, (ii) feasibility of data collection, (iii) response to variation and (iv) interpretation and utility of data to highlight the changing environment. Although S. officinalis is a non quota species (Pierce et al., 2010), two scientific bottom trawl surveys sample it each year (Carpentier et al., 2009; Gras et al., 2014) and maturity stages can be easily determined using the international WKMScEPH scale (ICES, 2010). This could be complemented by data collected at landing sites throughout the year according to the Data Collection Framework (DCF) programme. Sepia officinalis therefore meets the four criteria listed above and should be considered as an indicator for environmental shift.

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Stock assessment, spatial distribution and biological parameters of the clam *Venerupis decussata* along the Sfax coasts (Tunisia, Central Mediterranean)

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The grooved carpet shell *Venerupis decussata* (=Ruditapes decussatus) (Linnaeus, 1758) is a Mediterranean autochthonous species that in Tunisia is heavily harvested from natural populations. It represents one of the important economic issues, especially in terms of employment and the trade balances of the country via its exportation. Despite its economic value, no studies have been carried out about the spread of this mollusc. Thus the status of this species is still poorly known and there is a knowledge gap about its stocks assessment. This study aimed to investigate the distribution, abundance and population structure of the carpet shell clam in Sfax coastal areas. In terms of geographic occupation, maps of the population distribution were drawn. The results showed a scattered distribution pattern of the species according to location ranging between 0 and 152 ind m$^{-2}$. The consequence was a remarkable biomass which represented 891 tonnes of total fresh weight and high abundance levels reaching over 261.7 ± 84.2 million individuals estimated in the area of 4182 hectares. The distribution of the species has also been investigated in consideration with the size which varied markedly according to location, the shell length ranged between 2 and 41 mm. The overall sex ratio (F:M) was 1:1.17, which was significantly different from parity (1:1). The size at which 50% of the population reached maturity was 24.08 and 24.76 mm shell length for males and females, respectively. Findings of the current study suggest that *V. decussata* proliferates along southern Tunisian coasts.

**Keywords:** Bivalve, clam, *Venerupis decussata*, distribution, stock assessment, population structure, sexuality, size at first maturity, Tunisian waters

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**INTRODUCTION**

The carpet shell clam *Venerupis decussata* (Linnaeus, 1758), is a commercially valuable bivalve mollusc. It is widely distributed in the Mediterranean and its adjacent Atlantic waters from the North Sea to the coast of Senegal (Breber, 1985). In Tunisia, *V. decussata* is widely distributed along the southern coasts. It represents an important economic endpoint being mostly exported to Europe, and is exclusively collected in the Gulf of Gabes. Natural populations of *V. decussata* can be found in sandy and muddy-sand sediments in bays, estuaries coastal lagoons and other sheltered environments, living on tidal flats and below tidal marks. The target species also has an important role in ecosystems. They are filter-feeders pumping water through their siphons at sediment level, and they can bury in the sediment to a depth of 10–12 cm (Vilela, 1950).

In Tunisia, extensive studies have been conducted on *V. decussata* occurrence (Medhioub, 1983, 1993; Trigui, 1995; Trigui-El Menif et al., 1995), ecotoxicological studies (Smaoui-Damak et al., 2003; Gharbi et al., 2010), reproductive biology (Zamouri-Langar, 1991; Trigui-El Menif et al., 1995; Zamouri-Langar & Tritar, 1995; Hamida, 2004; Medhioub et al., 2006) and ecotoxicology (Trigui-El Menif, 1995; Dellali et al., 2001; Hamza-Chaffai et al., 2003; Dellali et al., 2004; Banni et al., 2009; Fathallah et al., 2010). Nevertheless, available information on stock size assessment and spatial distribution of this target species is insufficient. What is known about this species is limited to the contributions of Haouas-Gharsallah et al. (2008) and Charef et al. (2011) who conducted research in coastal areas of northern Tunisia. The literature review clearly indicates the lack of studies aimed at characterizing the target species stocks size and its spatial distribution in the Gulf of Gabes.

*Venerupis decussata* is one of the most important components of benthic fauna. Studies concerning its stock distribution and densities underpin basic knowledge in different fields of environmental research. These studies constitute the first step for any future work and can be useful as a reference for studies in marine invertebrates and cultivation. Accordingly, the aim of this study was to assess the current status of *V. decussata* along the Sfax coasts depending upon the species distribution, abundance and for the first time the sex ratio and size at first sexual maturity. The purpose was to give an outline of the status of this target clam along the
coastal areas as a background of southern Tunisia waters poorly known from this point of view.

**MATERIALS AND METHODS**

**Study area**

The study area, located in southern Tunisia and in the northern part of the Gulf of Gabes, extends along 235 km (Figure 1). Both wide and shallow continental shelves are topographically regular. The bottom slightly declines towards the sea and 60 m depth occurs at 110 km from the coast (Ben Othman, 1973). The main characteristic of substrates in the inshore area is muddy sand, being covered in same area by the seagrass *Cymodocea nodosa* (Ucria) Ascherson and *Zostera noltii* Hornemann.

**Sampling protocol and operations**

In the present study, systematic surveys were carried out from May 2012 to August 2014 within the 100 km Sfax coastal part located in south Tunisia. *Venerupis decussata* was well known in five sites of production: Site 2 (Gargour), Site 3 (Nakta-El Gtifa), Site 4 (Sidi Hmed-Oued Malaine), Site 5 (Maouma-Zabboussa) and Site 6 (Skhira-Bousaid) (Figure 1). Transects were systematically performed along the Sfax coast during low tides. Clams were collected every 50 m along transect lines from extreme high water tide to the extreme low water tide. Along transects, 4–10 stations were sampled. In each one, two replicates were taken from quadrats (0.25 m²) using a shovel. Large specimens were collected by hand and small ones were taken using a 2 mm mesh size sieve. During each sampling period, seawater temperature and salinity were recorded at the same time as the clam collections.

The materials were put in labelled plastic bags, subsequently preserved in a 7% formaldehyde solution and then transported to the laboratory. In the laboratory, samples were sorted and washed to remove all adhering organisms and other debris. Specimens were identified, counted and measured for shell length (SL) with a digital caliper to the nearest 0.01 mm and weighed for total weight (TW) on a toploading digital balance (precision of 0.001 g). The obtained data set was registered and maps of population distribution were drawn.

After identification to species level, data were pooled within each station to obtain a mean density (inds m⁻²) and mean biomass (g m⁻²) per site, and subsequently pooled across stations to assess stocks. Obtained data were exploited also for the cartography based on the method of kriging using Arc View v. 3.2 software and to evaluate the population densities and biomass according to the following equation (Gulland, 1969):  

\[ B_i = N_i \times A_i/a_i \times 1/X_i \]

where \( B_i \) – represents the total biomass of cockles; \( N_i \) – the mean abundance in the sample; \( A_i \) – the whole study area; \( a_i \) – the swept area and \( X_i \) – is the proportion retained.

For statistical analysis, data were tested for homogeneity of variance and normality using Leven’s and Kolmorogov–Smirnov tests, respectively. The Kruskal–Wallis median test was used to compare densities and sizes in all depth ranges. The effect of site on SL and on abundance was investigated using one-way ANOVA. Similarities between sites in terms of abundances and biomasses were investigated using Cluster analysis (group average). In addition, the harmonic Spearman correlation coefficient was also applied to identify any significant correlation between density and biomass in each site. The results are presented as a mean ± 95% CI and the significance level used for the tests was \( P < 0.05 \).

**Fig. 1.** Geographic position of sampling transects in the littoral zone of Sfax (Gulf of Gabes).
The relationship between SL and TW was described using the exponential regression (TW = aSL^b).

For biological study, in order to study the first sexual maturity and sexuality of the clam *V. decussata* in the Gulf of Gabes, a monthly sampling was carried out during its first period of reproduction (from May to July 2009). A total of 862 clams with sizes ranging from 19 to 40 mm were collected and analysed. Initially, *V. decussata* specimens were measured for shell length (SL, mm) and weighed for total weight (TW, g). The sexuality of *V. decussata* was determined by examination of (1) macroscopic appearance of the gonad and (2) microscopic examination of gametes. The visceral mass was then teased apart and smears of the visceral wall with attached gonad were examined at 100× magnification and sex of specimens was determined.

For both sexes, the shell length at first maturity, defined as the length at which 50% of clams mature, was estimated by means of a logistic function fitted to the proportion of the mature specimens pooled in 1 mm shell length classes (L). The quasi-Newton algorithm for non-linear least square estimation of function parameters was applied to data according to the following equation: 

\[ P = \frac{1}{(1 + e^{-a \times (L - L_{50})})} \]

where *P* is proportion of mature individuals; *a* is estimated parameter (slope of the curve); *L* is shell length corresponding to the proportion (*P*); *L_{50}* is total length of 50% mature clams. The sex ratio (expressed as number of females per males; F:M) was determined. Statistically significant deviations from a balanced sexual proportion of 1:1 were assessed by the *x^2* test, with statistical significance considered at *P* < 0.05 (Zar, 1996). Statistical package used was STATISTICA v. 6.0.

### RESULTS

#### Occurrence and abundance

*Venerupis decussata* was encountered throughout the areas surveyed and was found in various substrates. This includes muddy-sand substrate covered in some areas by the marine seagrass *C. nodosa* or *Z. noltii* or by mixed vegetation consisting of these two seagrasses. In some localities, samples of *V. decussata* were also encountered loose on the sandy bottom. Overall, a total of 143 transects were made from extreme high water tide to the extreme low water tide, corresponding to 966 samples in total (Table 1).

Changes in water temperature in the study area ranged from 12 to 13.9 °C in winter and from 21.7 to 27 °C in summer. Highest values were recorded in July (27 °C), whereas the lowest values were recorded in February (12 °C). Salinity measurements showed an annual fluctuation between 36 in winter and 48 in summer.

Data on clam densities were not normally distributed (Kolmogorov–Smirnov test, *P* < 0.05) and were not homogeneous (Leven’s test, *P* < 0.05). Therefore, the Kruskal–Wallis median test was adapted to compare densities in all depth ranges because it is suitable when many cases in the data are at either extreme of the scale. The overall clam densities in the study areas were significantly different (χ^2 = 14.08, *P* < 0.05). The results showed a scattered distribution pattern of the target species according to location ranging between 0 and 152 ind m^-2 for densities and from 0 to 292 g m^-2 for biomass (Figures 2 & 3). Pairwise comparisons of densities showed that overall data in the five sites were significantly different (*P* < 0.05).

The total stock was estimated to be 891 tons (total fresh weight), with a mean biomass around 25.9 g m^-2 and a total abundance reaching over 261.7 million individuals. The population was limited to mean densities of 7.6 ind m^-2 (Table 1).

Overall, 1290 specimens were harvested from the six aforementioned sites (4182 hectares). Significant variations in abundance and biomass values were recorded among sites (Figure 4). In term of abundance, there is a significant difference between all prospecting sites (*P* < 0.05) except site 3 with respect to sites 4 and 6 and among site 2 with respect to site 6 (*P* > 0.05). As for abundance, mean biomass showed significant variations among sampling sites (*P* < 0.05) except ones among site 3 with respect to sites 4 and 6 (*P* > 0.05). A Spearman correlation coefficient was performed to compare between densities and biomass values in each site. Obtained values exceed 0.95 so there is a strong positive correlation between densities and biomass. Moreover, by means of cluster analysis of sites applied to sampling period two groups were defined; the first composed of site 2 whereas the second grouped the remaining sites.

#### Population structure

The size frequency distribution of *V. decussata* was determined in all sites (Table 2). The broad size range of specimens varied between 2 and 41 mm SL. The mean size of the length distribution was 21.47 ± 0.38 mm SL. The majority of species populations were attributed to size classes (15–31 mm) which represented 79.15% of total samples collected in the present study. Large-size individuals (>30 mm) represent only 6.2%. The populations of clams are not distributed in an even manner: larger individuals are distributed in a relatively heterogeneous way over the whole study area, however smaller specimens were more geographically restricted to the southern part (sites 4, 5 and 6).

The mean sizes ranged from 19.82 ± 0.47 mm in site 6 to 24.24 ± 0.69 mm in site 2. The coefficient of variation of the

---

**Table 1.** Surface of sites, number of transects and replicates, and stock assessment of *Venerupis decussata* in littoral zone of Sfax.

<table>
<thead>
<tr>
<th>Sites</th>
<th>Surface (ha)</th>
<th>% of all surface</th>
<th>Number of transects</th>
<th>Number of replicates</th>
<th>Stock size (millions)</th>
<th>Stock size (tonnes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 2</td>
<td>505</td>
<td>12.08</td>
<td>18</td>
<td>130</td>
<td>74.1</td>
<td>264.0</td>
</tr>
<tr>
<td>Site 3</td>
<td>790</td>
<td>18.89</td>
<td>39</td>
<td>194</td>
<td>52.9</td>
<td>242.7</td>
</tr>
<tr>
<td>Site 4</td>
<td>845</td>
<td>20.21</td>
<td>33</td>
<td>210</td>
<td>51.0</td>
<td>162.9</td>
</tr>
<tr>
<td>Site 5</td>
<td>1560</td>
<td>37.5</td>
<td>17</td>
<td>114</td>
<td>13.2</td>
<td>73.2</td>
</tr>
<tr>
<td>Site 6</td>
<td>482</td>
<td>11.52</td>
<td>36</td>
<td>286</td>
<td>68.5</td>
<td>146.2</td>
</tr>
<tr>
<td>Total</td>
<td>4182</td>
<td>100</td>
<td>143</td>
<td>966</td>
<td>261.7</td>
<td>891.0</td>
</tr>
</tbody>
</table>
size data in the current study area was relatively higher in sites 4 and 5. In addition, individuals’ sizes appeared greatly influenced by location. Significant variations were recorded among sites 2 and 3 when compared with sites 4 and 6 ($P < 0.001$) (Table 2). However, no significant differences were detected between sites 4, 5 and 6. On other hand, the shell length-weight relationship for overall data (1290 individuals) is shown in Figure 5. The calculated $R^2$ was positively correlated
to total weight ($0.924; P < 0.001$). The slope $b$ and condition factor were 2.783 and 0.0004 respectively. The relationship was: $W = 0.0004L^{2.783}$ ($R^2 = 0.9242$, N = 1290).

**Size at first maturity**

The changes in the proportion of the mature individuals showed that at a size (SL) $< 22$ mm, no individual was mature and that in a size (SL) $> 27$ mm, all observed individuals were mature. The results obtained by the application of the logistical function showed that size at first maturity ($L_{50}$) was 24.08 mm for males, 24.76 mm for females and 24.37 mm for the two sexes combined (Figure 6). The $\chi^2$-test did not show any significant difference between the theoretical proportions and the observed ones of mature individuals (Table 3).

**Sexuality**

From all samples examined during the study period, we counted 459 males (53.2%), 392 females (45.5%) and 11 indeterminate sex (1.3%). For both sexes, specimens presented a broad size range, both in term of shell length (19–39.8 mm) and total weight (1.26–11.65 g). The overall sex ratio (F:M = 1:1.17) was significantly divergent from parity (F:M = 1:1; $\chi^2$ test, $P < 0.05$).

**DISCUSSION**

The present paper describes for the first time the stocks assessment, spatial distribution and first maturity of the clam *Venerupis decussata* in the Gulf of Gabes, an important bivalve habitat. Findings of recent research indicate an extraordinary stock of this species. The consequence was a remarkable biomass which represented 891 tonnes of total fresh weight and high abundance levels reaching over 261 million individuals. This stock was smaller than that reported from the Gabes coastline area (2405 tonnes of fresh weight and high abundance levels reaching over 908 million individuals) and higher than that recorded from the Medenine littoral zone located in the southern Tunisian part (730 tonnes; unpublished data).

Mapping stocks revealed the fluctuation of *V. decussata* habitat in the prospected area. It seems that stocks varied substantially from one locality to another. Elsewhere, obtained densities was considered much higher than that recorded in the Arcachon Basin (128 g m$^{-2}$; Bertignac *et al.*, 2001) and...
that focused in later works in the same area (0.4 ind m$^{-2}$; Caill-Milly et al., 2006) but much smaller than that signalled for other populations for Mellah lagoon in Algeria (528 inds m$^{-2}$; Draredja & Beldi, 2004). The same authors indicated also that densities varied substantially according to strata.

It appears that clam populations has been influenced by strong impacts of various levels (e.g. physicochemical, edaphic and hydrological factors of the study area). Due to high water temperature levels (12–27°C) and shallow waters, the salinity remains fairly stable throughout the year. It was recorded with high concentrations in summer (48) and often in winter (36) (Derbali, 2011). The temperature and the food potentials are the most important factors affecting phenotypic differences in growth patterns and maximum sizes in a variety of marine organisms (Stergiou et al., 1997; Derbali et al., 2011). Elevated water temperatures and phytoplankton levels may promote rapid growth rates in many local bivalve species. On the opposite side, high temperatures (above 27°C) can be stressful to V. decussata as shown by Sobral & Widdows (2014) for clam populations in southern Portugal.

Other factors structuring the clam populations include soft bottoms and organic matter content. The high diversity of shellfish species is particularly interesting when the depth of the organic matter layer (<1 m) and the muddy-sand bottom are considered. In fact, these factors can provide ecological conditions that are able to maintain highly diverse reef communities in southern Tunisian waters. Indeed, the prospect areas maintain high diversity if compared with other areas in southern Tunisian waters where only a few species (Hexaplex trunculus (Linnaeus, 1758), Conus mediterraneus (Bruguieres, 1792), Gibbula ardens (Von Salis, 1793), Cerithium vulgatum (Bruguieres, 1792), Cerithium scabridum (Philippi, 1848) and Bittium reticulatum (Da Costa, 1778)) were found on rock and hard substrates. It seems that this low diversity is primarily attributed to bottom characteristics.
This hypothesis can be supported by the relationship between the sedentary nature of some shellfish species such as \textit{V. decussata} and substrates.

With respect to faunal diversity and substrate types, this species was harvested from areas sheltered by seagrasses \textit{C. nodosa} and \textit{Z. noltii}, covering more than 50% of the muddy-sand bottoms. It seems that the heterogeneity of geographic distribution of \textit{V. decussata} was not significantly correlated to the distribution of the two seagrasses. \textit{Venerupis decussata} prefers feeding on planktonic and benthic microalgae, especially diatoms. The same conclusion was pointed out by Vilela (1950) from natural populations of clams in Portugal. Several studies have confirmed the correlation between patterns of community structure with the primary production. In particular, the local abundance and biomass of filter-feeders was correlated with both intertidal productivity and nearshore primary productivity (Menge & Olson, 1990; Bustamante et al., 1995).

These assumptions of spatial distribution patterns may explain the prevalence of the clam species in these particular grounds. In the same order, it shows the importance of environmental factors in controlling the density and then the biomass of shellfish aggregations. The broad size range of specimens varied between 2 and 41 mm SL. The mean size of the length distribution was 21.47 mm SL. The majority of specimens belonged to size classes (15–31 mm) which represented 79.15% of total samples collected in the study area. The size range of populations varied subsequently between localities. Variability in \textit{V. decussata} size could be related to environmental factors that vary from one habitat to another. In fact, the growth, development and survival of bivalves are generally mental factors that vary from one habitat to another. In fact, the appearance and colour of foot tissue (Lucas, 1965) can be determined from macroscopic properties, i.e. from and the degree of their development, though sometimes it can be determined from macroscopic properties, i.e. from the appearance and colour of foot tissue (Lucas, 1965). Similar trends were found in other bivalve families, such as in Pectenidae, Mytilidae, Limidae and Cardiidae, both the sex and degree of gonad development can be determined from the colour of gonads (Lubet, 1959; Derbali et al., 2009a, b, 2012, 2014). Accordingly, investigations on the size at sexual maturity revealed that males and females matured at 24.08 and 24.76 mm respectively, and at 24.37 mm for combined sexes. In comparison, present outcomes were not in agreement with those of findings made in other geographic areas differing by their bottom characteristics, vegetation cover and physicochemical factors. Indeed, for French coasts, the first maturity recorded was at about 20 mm (Conti et al., 2014) which is slightly smaller than in our data.

Finally, the present paper revealed the localization of the main concentrations of the clam \textit{V. decussata} with potential commercial value along the Sfax coastal areas. Furthermore, this contribution was the first attempt to estimate its stock. It gives more information about its stocks in the colonization area, but it was not possible to reveal the influence of physical processes (tide), intraspecific interactions and spatio-temporal change of the target species. Future studies could be done in two ways: firstly, stock assessments should be carried out each year in order to show the variations of \textit{V. decussata} stocks and to check changes in population size structure. Secondly, the relationship between the spatial distribution of populations and hydrodynamic surveys, combined with topography could allow the assessment and quantification of the effect of transport processes in the regulation of the population.

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Effects of temperature and feeding regime on food consumption, growth, gonad production and quality of the sea urchin *Strongylocentrotus intermedius*

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*These authors contributed equally to this work.

Water temperature is one of the most important factors greatly affecting the aquaculture of sea urchins. However, no information is available on how to improve commercial traits of sea urchins reared at high water temperature. Here, we investigated the effects of water temperature and feeding regime on food consumption, growth, gonad production, gametogenesis and gonad quality of the sea urchin *Strongylocentrotus intermedius*. We found that high water temperature (22°C) significantly decreased dried food consumption and gonad production of *S. intermedius*, but not the somatic growth. The feeding regime of formulated feed and kelp has direct application potential in *S. intermedius* aquaculture, especially at field temperature. Feeding kelp alone is not effective in supporting growth and gonad production for *S. intermedius* cultured at high water temperature. This finding greatly challenges the current commonly used feeding regime (feeding macroalgae only) for *S. intermedius* cultured at high water temperature. Based on the current results, we suggest the feeding regimes of formulated feed and kelp or formulated feed alone for *S. intermedius* aquaculture at high water temperature. The present study provides new information for aquaculture of *S. intermedius* at high temperature and for production out of season.

Keywords: Sea urchin, *Strongylocentrotus intermedius*, high temperature, feeding regime, gonad, aquaculture

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INTRODUCTION

Water temperature is one of the most important environmental factors significantly affecting the fitness of commercially important marine invertebrates (Lemoine & Burkepile, 2012), and consequently impacting their aquaculture industries (Watts et al., 2011). The sea urchin is a good example because its aquaculture industry is adversely affected by high water temperature (e.g. Lawrence et al., 2009; Watts et al., 2011). It has been well documented that food consumption (Siikavuopio et al., 2008; Lawrence et al., 2009), growth (Lawrence et al., 2009; Azad et al., 2011), gametogenesis (Gibbs et al., 2007; Uthicke et al., 2014) and reproduction (Garrido & Barber, 2001) are significantly affected by high water temperature in sea urchins. Three major methods for sea urchin aquaculture include culturing them offshore at shallow depths in suspended cages and feeding them macroalgae or formulated feeds; releasing them into managed areas of sea floor; and industrial on-land indoor aquaculture (Chang et al., 2004). Water temperature cannot be controlled in the sea when sea urchins are cultured in cages or on the shallow sea floor, where they are greatly affected in areas with seasonal high water temperature (Brothers & McClintock, 2015). Even in industrial aquaculture, it is very expensive to keep the seawater cool in these areas during seasonal high temperatures. This highlights the importance of the evaluation of potential negative impacts on commercially important traits of sea urchins and of approaches to improving sea urchin aquaculture in the expanding areas with seasonal high temperature.

The sea urchin *Strongylocentrotus intermedius*, which was introduced into China from Japan in 1989 for its commercial value (Chang et al., 2004), is endemic to intertidal and subtidal bottoms in northern Pacific coastal waters of Hokkaido off Japan, Korea and Far East Russia (Agatsuma, 2013). The annual production of sea urchins from fisheries and aquaculture in China was 6791 tonnes in 2014 (Zhao, 2015). As a cold-water, commercially important species, *S. intermedius* is a good model to investigate the effects of high water temperature on commercially important traits of sea urchins in aquaculture and to contribute a cost-effective approach to improving these traits of urchins cultured at high water temperature. The lethal temperature for *S. intermedius* is 23°C (Chang et al., 2004; Agatsuma, 2013). In Japan, over half of a population of juvenile *S. intermedius* in shallow water died at temperatures >23°C (Hokkaido Central Fisheries Experimental Station et al., 1984). Food consumption and gonad production of *S. intermedius* were significantly affected at 22°C (Lawrence et al., 2009). Aside from production, gonad

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quality of sea urchins is very important to determine their market values, which includes gonad moisture, colour, flavour and nutrient components (e.g. protein). However, we know of no information on the effects of high water temperature (for example, 22°C) on gametogenesis and gonad quality of S. intermedius. This lack greatly hampers the development and promotion of aquaculture of S. intermedius, especially in areas with seasonal high water temperature.

Formulated feeds have been well documented to be effective in supporting growth and gonad production in a number of sea urchin species (e.g. Pearce et al., 2002; Watts et al., 2011), including S. intermedius (Chang et al., 2005; Lawrence et al., 2011), although their effects on gonad quality are still debatable (e.g. Pearce et al., 2002; Azad et al., 2011). Shpigel et al. (2005) reported that the feeding regime of formulated feed and macroalgae produced the optimal combination of desired gonad colour and production. Their explanation was that the formulated feed is effective in increasing gonad production, while macroalgae help improve gonad quality (Shpigel et al., 2005). However, they did not evaluate the effectiveness of this method in unfavourable conditions (for example, at high water temperature). In S. intermedius, formulated diet has been well documented to effectively support gonad production (Chang et al., 2005; Lawrence et al., 2011). Kelp Saccharina japonica is the natural optimal feed for S. intermedius (Chang et al., 2004). Thus, we hypothesized that a feeding regime of formulated feed and kelp would probably improve gonad production as well as achieving the desired gonad quality and that it might be an effective feeding regime for S. intermedius at a high water temperature of 22°C.

The purposes of the present study are to investigate (1) whether high water temperature (22°C) significantly affects food consumption, growth, gonad production, gametogenesis and gonad quality of S. intermedius; (2) whether a feeding regime of formulated feed and kelp is more effective for gonad production in S. intermedius than formulated feed or kelp alone; (3) whether a feeding regime of formulated feed and kelp is a cost-effective method to improve gonad production and quality of S. intermedius cultured at high water temperature.

MATERIALS AND METHODS

Sea urchins

Sea urchins with a test diameter about 5 cm (5.2 ± 0.3 cm for 54 haphazardly chosen individuals) were transported from Dalian Haibao Fishery Company to the Key Laboratory of Mariculture & Stock Enhancement in the North China’s Sea, Ministry of Agriculture at Dalian Ocean University on 2 April 2014. Sea urchins were maintained in the laboratory at 10°C, pH 8.05 and 31.6‰ salinity for a week and fed kelp S. japonica. They then were held without feeding for a week before the experiment began on 15 April 2014.

Experimental design

Water temperature and feeding regime were the two experimental factors in the study. 22°C was set as the high water temperature group. Water temperature was gradually increased from 10.6°C (the initial laboratory in situ water temperature) to 22°C by 1°C every 2 days and kept at 22°C using a seawater temperature control system (Huixin Co., China). The laboratory in situ water temperature (10.6–18.8°C during the experiment) was set as the control temperature (designated as field temperature group). Filtered seawater was used in this study.

Three feeding regimes comprised formulated feed alone, formulated feed and kelp (S. japonica) and kelp alone. Formulated feed was prepared at Texas AgriLife Research Mariculture Laboratory at Port Aransas, USA. The composition of the formulated feed was analysed and provided by Dr A. Lawrence (Table 1). Wild fresh kelp, which was bought from a Dalian local market, was collected in Dalian Bay (120°37’E 38°56’N). The organic composition and ash were measured during the experiment (Table 2). Kelp alone and formulated feed alone refer to feeding S. intermedius only kelp and only formulated feed during the 6 weeks of the experiment, respectively. Formulated feed and kelp refers to feeding S. intermedius kelp for 3 weeks and formulated feed for the following 3 weeks. Nine replicates were used in each experimental treatment.

According to the experimental design described above, sea urchins were put into 27 individual cylindrical cages (11 cm in diameter, 15 cm high) in each of two large tanks (180 × 100 × 80 cm). Water temperature was controlled in one tank. Water temperature in the second tank was environmental temperature.

Food consumption

Strongylocentrotus intermedius were fed according to the feeding regimes described above. Uneaten kelp was collected, cleaned to remove the seawater on the surface and then weighed every day before re-feeding. Uneaten formulated feed was collected every day before re-feeding, dried at 72°C for 4 days and weighed. Seawater was changed every 2 days. Dried food consumption of kelp was calculated by dried amount of kelp provided minus the dried amount of uneaten kelp. Food consumption of formulated feed was

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squid/soybean meal</td>
<td>22.00</td>
</tr>
<tr>
<td>Other marine ingredients</td>
<td>3.00</td>
</tr>
<tr>
<td>Other non-marine ingredients</td>
<td>10.10</td>
</tr>
<tr>
<td>Mineral premix</td>
<td>23.43</td>
</tr>
<tr>
<td>Carotenoid premix</td>
<td>1.70</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.70</td>
</tr>
<tr>
<td>Lipid premix</td>
<td>5.20</td>
</tr>
<tr>
<td>Soybean meal (isolated soy protein)</td>
<td>5.30</td>
</tr>
<tr>
<td>Wheat starch, purified</td>
<td>28.57</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Crude protein</th>
<th>Crude fat</th>
<th>Crude fibre</th>
<th>Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8.23</td>
<td>1.00</td>
<td>9.82</td>
<td>0.20</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>0.066</td>
<td>0.040</td>
<td>0.047</td>
<td>0.008</td>
</tr>
</tbody>
</table>
calculated as follows (according to Zhu, 2005 with some revisions):

\[
\text{FCR} = \frac{\text{FI} \times (1 - R_1) - R_2 \times W_{\text{seawater}} - (W_{\text{R}} - R_3 \times W_{\text{n}})}{(W_{\text{f}} - 0.037 \times W_{\text{n}})}
\]

where

\[
\text{FI} = W \times (1 - R) - R_2 \times W \times (1 - R_1) - (W_{\text{R}} - R_3 \times W_{\text{n}})
\]

\[
= 1.5 \times (1 - 0.09) - 0.17 \times 1.5 \times (1 - 0.09)
\]

\[
- (W_{\text{f}} - 0.037 \times W_{\text{n}})
\]

\[
W_{\text{f}}, \text{ weight of residual feed after drying; } W_{\text{seawater}}, \text{ weight of seawater in residual feed; } W, \text{ amount of feed provided; } 1.5, \text{ g; } R, \text{ water content rate of the formulated feed; } 0.09, \text{ lost rate of the formulated feed; } 0.17, \text{ rate of residual elements of seawater after drying; } 0.037.
\]

Food consumption of sea urchins in the group of formulated feed and kelp was calculated as the arithmetic mean of the dried food consumption of kelp in the first 3 weeks and that of dried formulated feed in the following 3 weeks.

**Body size, gonad weight, index and moisture**

Test diameter, height and body weight of *S. intermedius* were measured using digital vernier calipers (Mahr Co., Germany) and an electric balance (G&G Co., USA). After dissection, test, lantern and gonads were weighed, dried for 4 days at 72°C and an electric balance (G&G Co., USA) under the standard light of D65. One gonad was placed on dishes for subjective colour and sweetness assessment.

**Feed conversion ratio**

Feed conversion ratio (FCR) was calculated according to the method used by Siikavuopio et al. (2012) as follows:

\[
\text{FCR} = \frac{\text{FI} / (W_2 - W_1)}{100}
\]

where

\[
\text{FI}, \text{ food consumption (g); } W_2, \text{ final weight; } W_1, \text{ initial weight; } FI, \text{ food consumption.}
\]

**Gonad colour and sweetness**

One gonad of each urchin was used for objective colour measuring of \(L^*, a^*\) and \(b^*\) readings (\(L^*\) = lightness, \(a^*\) = redness, \(b^*\) = yellowness) using PANTONE Color Cue® 2 (Carlstadt, NJ, USA) under the standard light of D65. One gonad was placed on dishes for subjective colour and sweetness assessments under standard light of D65. The sensory panel consisted of six individuals who were familiar with colour and flavour analysis of sea urchin gonads. Before the experiment, they were carefully trained to distinguish among the subjective ratings of gonad colour and sweetness of *S. intermedius* according to the training method of Phillips et al. (2009). Gonad colour and sweetness of sea urchins were evaluated according to the ranking standard of Pearce et al. (2002):

Gonad colour (rating 1 - 4):

1 = bright yellow or orange; 2 = pale yellow or orange, mustard; 3 = yellow - brown, orange - brown, red - brown, cream; 4 = any other colour (e.g. dark brown, grey).

Gonad sweetness (rating 1 - 5):

1 = sweet; 2 = a bit sweet; 3 = not sweet, not bitter; 4 = a bit bitter; 5 = bitter.

**Gametogenesis**

One gonad from each individual was collected and fixed in the Bouin’s solution (saturated picric acid solution: formaldehyde: glacial acetic acid = 15: 5: 1) for 24 h, followed by dehydration, transparency, wax dip, sectioning, HE staining and microscopic examination. The method was fully described in Ren et al. (2007).

The state of the gonad was divided into four stages according to the description of James & Siikavuopio (2011).

**Stage I: Inter-gametogenesis and NP phagocytosis**

This stage occurs after spawning. The reproductive cells start to appear around the gonads and the number of NP cells increases.

**Stage II: Pre-gametogenesis and NP renewal**

In this stage the size of the gonads and the number of NP cells increase continuously. The reproductive cells exist in the periphery of the follicle in the gonad.

**Stage III: Gametogenesis and NP utilization**

The reproductive cells develop continuously with a decrease in size and number of NP cells. Reproductive cells migrate into the centre of follicle in the gonad.

**Stage IV: End of gametogenesis and spawning**

Differentiated reproductive cells exist in the lumen, stored and ready for spawning. At the end of this stage all or some of the mature reproductive cells will be released.

**Crude protein content of gonads**

Semi-micro Kjeldahl nitrogen was used to determine crude protein content of 0.5 g dried gonad in each individual. This method was fully described in Chen et al. (2008). The procedure includes digestion, distillation, absorption and titration. Crude protein content of gonads was calculated as follows:

\[
\text{Crude protein content(%) = } \frac{[V_2 - V_1] \times C \times 0.014 \times 6.25 \times 100}{[M \times V']/V}
\]

where

\[
V_1, \text{ consumption of hydrochloric acid titration (ml); } V_2, \text{ consumption of hydrochloric acid titration for the blank (ml); } C, \text{ concentration of hydrochloric acid standard solution (mol L}^{-1}); M, \text{ sample weight (g); } V', \text{ the total dilution volume of the collected liquid (ml); } V, \text{ the distillation volume of the collected liquid (ml).}
\]

**Statistical analysis**

The data were tested for homogeneity of variance and normal distribution before statistical analysis. Two-way ANOVA was used to analyse the effects of water temperature and feeding regime on all experimental traits apart from gametogenesis. Because no significant interaction was found in all experimental traits except for feed conversion ratio, one-way ANOVA was carried out to separately analyse the effects of the two factors.
Duncan multiple comparisons were then performed when significant differences were found with the ANOVA analysis. Independent-samples t-test was used to compare the differences of all experimental traits between the final and initial conditions apart from gametogenesis. Kruskal–Wallis H test was used to analyse the stage frequency of gametogenesis. All data analysis was performed using SPSS 13.0 statistical software. A probability level of $P < 0.05$ was considered as significant.

## RESULTS

### Test diameter, height, weight, lantern weight and body weight

Compared with the initial condition, test diameter increased significantly only in *S. intermedius* fed formulated feed at the field temperature ($P = 0.006$, Table 3). Body weight
significantly increased in *S. intermedius* fed formulated feed and kelp at both temperatures (*P* < 0.05), and individuals fed kelp at high water temperature (*P* = 0.046, Table 3). Test height was not significantly affected in *S. intermedius* fed formulated feed and kelp at both temperatures (*P* > 0.05, Table 3).

Water temperature significantly affected test diameter of *S. intermedius* (*P* = 0.003, Figure 1) but did not significantly impact test height, weight, lantern weight and body weight (*P* > 0.05, Figures 1 & 2). Feeding regime, on the other hand, significantly affected body weight (*P* = 0.006), test diameter (*P* = 0.021), test height (*P* = 0.002), test weight (*P* = 0.007) and dried test weight (*P* = 0.045) of *S. intermedius* (Figures 1 & 2), but did not significantly affect the wet and dried lantern weight (*P* > 0.05, Figure 2). There was no significant interaction on all these traits between water temperature and feeding regime (*P* > 0.05).

At the field temperature (10.6–18.8°C), *S. intermedius* fed formulated feed and kelp had the significantly highest test diameter (*P* = 0.004), test weight (*P* = 0.002), dried test weight (*P* = 0.007), test height (*P* = 0.002) and dried lantern weight (*P* = 0.045).
weight ($P = 0.003$) and body weight ($P = 0.003$). Significant differences of these traits were not found in individuals at high water temperature ($P > 0.05$, Figure 1).

Dried food consumption and feed conversion ratio

Dried food consumption by $S$. intermedius was significantly affected by both water temperature ($P < 0.001$) and feeding regime ($P < 0.001$, Table 4, Figure 3). Food consumption of $S$. intermedius held at the high water temperature was significantly less than those at the field temperature ($10.6–18.8^\circ$C) with all feeding regimes ($P < 0.001$).

$S$. intermedius fed formulated feed consumed significantly less food at both water temperatures ($P < 0.001$). Individuals fed formulated feed and kelp did not consume significantly more dried food than those fed kelp alone at both water temperatures ($P > 0.05$).

Feed conversion ratio was significantly affected by water temperature ($P < 0.001$), but not feeding regime ($P > 0.051$, Table 4, Figure 3). Feed conversion ratio was significantly higher at high water temperature ($22^\circ$C) than at field temperature ($10.6–18.8^\circ$C) regardless of feed ($P < 0.001$).

Gonad weight, index and moisture

Compared with the initial condition, gonad weight significantly increased in $S$. intermedius in all experimental groups except the individuals fed kelp at high water temperature (Table 3). Gonad moisture significantly decreased in $S$. intermedius in all experimental groups ($P < 0.001$, Table 3). Gonad index significantly increased in $S$. intermedius cultured at field temperature with all feeding regimes ($P < 0.05$) and in the individuals fed formulated feed at high water temperature ($P < 0.001$).

$S$. intermedius at high water temperature ($22^\circ$C) had significantly lower gonad weight and index than those at field temperature ($10.6–18.8^\circ$C) ($P < 0.001$). Gonad weight and index of $S$. intermedius fed formulated feed alone and formulated feed and kelp were significantly higher than those fed kelp alone at both water temperatures ($P < 0.001$). However, there was no significant difference of

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**Table 5.** Statistical results of two-way ANOVA to compare the differences of gonad weight, gonad index, gonad moisture, $L^\ast$, $a^\ast$, $b^\ast$, gonad colour rating, gonad sweetness rating and crude protein of gonads (water temperature and feeding regime as the two factors).

<table>
<thead>
<tr>
<th></th>
<th>GW</th>
<th>GI</th>
<th>GM</th>
<th>$L^\ast$</th>
<th>$a^\ast$</th>
<th>$b^\ast$</th>
<th>GC</th>
<th>GS</th>
<th>GP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temp</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Field</td>
<td>28.10</td>
<td>&lt;0.001</td>
<td>42.77</td>
<td>&lt;0.001</td>
<td>9.55</td>
<td>0.003</td>
<td>0.76</td>
<td>0.389</td>
<td>4.42</td>
</tr>
<tr>
<td>High</td>
<td>19.59</td>
<td>&lt;0.001</td>
<td>24.72</td>
<td>&lt;0.001</td>
<td>0.37</td>
<td>0.694</td>
<td>4.64</td>
<td>0.015</td>
<td>6.81</td>
</tr>
<tr>
<td><strong>Feeding</strong></td>
<td>2.37</td>
<td>0.105</td>
<td>0.99</td>
<td>0.378</td>
<td>0.43</td>
<td>0.653</td>
<td>0.28</td>
<td>0.757</td>
<td>1.15</td>
</tr>
<tr>
<td><strong>Temp × feeding</strong></td>
<td>2.37</td>
<td>0.105</td>
<td>0.99</td>
<td>0.378</td>
<td>0.43</td>
<td>0.653</td>
<td>0.28</td>
<td>0.757</td>
<td>1.15</td>
</tr>
</tbody>
</table>

Note: Temp refers to water temperature, GW refers to gonad weight, GI refers to gonad index, GM refers to gonad moisture, GC refers to gonad colour rating, GS refers to gonad sweetness rating, GP refers to crude protein of gonads. Bold data indicate significant at $P < 0.05$. Significant differences of these traits were not found in individuals at high water temperature ($P > 0.05$, Figure 1).
gonad weight and index between *S. intermedius* fed formulated feed alone and formulated feed and kelp (*P* < 0.003, Figure 4). Feeding regime, however, did not significantly affect gonad moisture (*P* > 0.05).

**Gonad colour and sweetness**

Compared with the initial condition, only *a*’ was significantly reduced in *S. intermedius* in all experimental groups except the individuals fed kelp at high water temperature (Table 3). Subjective gonad colour rating was significantly impacted only in *S. intermedius* fed formulated feed and kelp at high water temperature (*P* = 0.0002, Table 3). Subjective gonad colour rating was significantly affected in *S. intermedius* cultured at high water temperature in all feeding regimes (*P* < 0.05) and in individuals fed kelp at field temperature (*P* < 0.001, Table 3).

Water temperature did not significantly affect either objective (*L*, *a*’ and *b*’ readings) or subjective colour ratings (*P* > 0.05, Table 5, Figures 5 & 6). Feeding regime, on the other hand, significantly affected *L*’ (*P* = 0.015) and *a*’ (*P* = 0.003) readings, but did not significantly affect *b*’ and subjective colour ratings (*P* > 0.05). There was no significant difference of both objective and subjective colour ratings between *S. intermedius* fed formulated feed alone and those fed formulated feed and kelp (*P* > 0.05).

Gonad sweetness of *S. intermedius* at the high water temperature (22°C) was significantly better than those at the field temperature (10.6–18.8°C) (*P* = 0.042). However, feeding regime had no significant effect on gonad sweetness of *S. intermedius* (*P* > 0.05, Table 5, Figure 6).

**Gametogenesis**

At the beginning of the experiment, gametogenesis of sea urchins were at stage 1 (*N* = 9). At the end of the experiment, the stage of the gonads was variable and not significantly different among all experimental groups (χ² = 5.331, *P* = 0.377, Figure 7).

**Crude protein content of gonads**

Feeding regime significantly affected crude protein content of gonads (Table 5, *P* = 0.015). However, water temperature did not significantly affect the crude protein of content of gonads (*P* > 0.05, Table 5). At the field temperature (10.6–18.8°C), *S. intermedius* fed formulated feed alone had the significantly highest gonad crude protein content (*P* < 0.05, Figure 8).

**DISCUSSION**

The effects of water temperature on feed conversion, gonad quality and gametogenesis have never been investigated in...
Fig. 5. $L^*$, $a^*$ and $b^*$ readings of *Strongylocentrotus intermedius* cultured at different temperatures and feeding regimes. Different letters above the bars refer to significant difference in each temperature group.

Fig. 6. Subjective gonad colour and sweetness ratings of *Strongylocentrotus intermedius* cultured at different temperatures and feeding regimes. Different letters above the bars refer to significant difference in each temperature group.
S. intermedius, although the effects of temperature on food consumption, growth and gonad production have been reported by Lawrence et al. (2009). In the present study, we found that high water temperature significantly impacted gonad production of S. intermedius after 6 weeks, but not the body, test and lantern weights. This is partly consistent with the finding by Lawrence et al. (2009) that high water temperature for 3 months significantly impacted both gonad weight and body weight of S. intermedius. The different results of the two studies clearly indicate that gonad production of S. intermedius is more vulnerable than test and lantern growth under stress (for example, high water temperature) even in a relatively short duration. This conclusion can be supported by our previous finding that diel intermittent feeding/fasting significantly affected gonad weight, but not the body weight (Zhao et al., 2013). In the present study, dried food consumption of S. intermedius held at high water temperature was significantly lower than at the field temperature, which agrees with the results of Lawrence et al. (2009). Moreover, we found that feed conversion ratio (FCR) significantly increased in S. intermedius reared at the high water temperature (22°C), clearly indicating that S. intermedius not only consumes less, but utilizes food less effectively. This can be explained by the significantly reduced absorption rate and assimilation efficiency of sea urchins held at suboptimal water temperatures (Lawrence et al., 2009; Azad et al., 2011). Thus, a reasonable explanation of the reduced gonad production is that sea urchins probably use the nutrients stored in gonads for maintenance when they are under stress (Lares & Pomory, 1998), although energy budget was not analysed in the present study. However, high water temperature did not significantly affect the crude protein of gonads in the present study. This indicates that the reduced gonad production is probably due to other nutrient elements except crude protein. The present finding increases our understanding of how high water temperature significantly negatively impacts gonad production of sea urchins. Together with previous studies (e.g. Lawrence et al., 2009; Watts et al., 2011), the present study suggests the importance of avoiding various stresses to achieve optimal gonad production and quality in sea urchin aquaculture. In addition, S. intermedius cultured at the high water temperature showed significantly reduced gonad moisture compared with those at the field temperature. This agrees with the study by Spirlet et al. (2000) on the sea urchin Paracentrotus lividus. High water temperature did not significantly affect gonad colour, according to both objective and subjective methods. However, the subjective sweetness rating was significantly better in S. intermedius held at the high water temperature than at the field temperature. This can be partly explained by the significantly reduced gonad moisture, which might result in the improvement of gonad flavour (McBride et al., 2004). To be noted, we did not measure the potential effects of the natural slow increase at temperature (10.6–18.8°C) on the traits of S. intermedius, although it was not great, only 8.2°C in the experimental duration of 6 weeks.

Dietary intervention successfully reduced the mortality of cultured Australian greenlip abalone, Haliotis laevigata, at high water temperature (Stone et al., 2014). Like abalones, a number of sea urchin species (for example, S. intermedius) are greatly affected by high water temperature in aquaculture, which greatly hampers the development of the industry. However, no information is available on how to improve commercial traits of sea urchins reared at high water temperature, although a number of impacts have been reported on food intake (Siikavuopio et al., 2006, 2008; Lawrence et al., 2009; Watts et al., 2011), somatic growth (Lawrence et al., 2009), gametogenesis (Garriro & Barber, 2001; James & Heath, 2008), gonad production (McBride et al., 1997; Siikavuopio et al., 2006, 2008; Gibbs et al., 2007; James et al., 2007; Azad et al., 2011; Watts et al., 2011) and quality (Azad et al., 2011) in sea urchins. In the present study, we found that the feeding regime of formulated feed and kelp has obvious advantages in test size, body weight and gonad production at the field temperature and does not have significant disadvantages on these traits at high water temperature as well as gonad quality at both water temperatures, compared with feeding kelp or formulated feed alone. Thus, this feeding regime has direct application potential for S. intermedius aquaculture, especially at field temperature. The present result enriches the finding of Shpigel et al. (2005) that
mixed feeding of formulated feed and macroalgae is applicable not only at the field temperature, but also at high water temperature. In the present study, *S. intermedius* fed formulated feed showed significantly highest crude protein of gonads and lowest food consumption and feed conversion ratio, which greatly contributes to the advantages of formulated feed at both field and high water temperatures. Macroalgae (for example, kelp) is commonly used in sea urchin aquaculture. In the present study, however, *S. intermedius* fed formulated feed and kelp and formulated feed alone showed significant growth at high water temperature but not in the individuals fed kelp alone. This result clearly indicates that kelp alone is not effective in supporting growth and gonad production by *S. intermedius* held at a high water temperature. This novel finding greatly challenges the current commonly used feeding regime for *S. intermedius* in aquaculture with high water temperature (Chang et al., 2004). According to the present results, we suggest the feeding regimes of formulated feed and kelp and formulated feed alone, which have advantages of growth and feed conversion for *S. intermedius* at high water temperature.

In conclusion, high water temperature significantly reduced food consumption and gonad production of *S. intermedius*, but not the somatic growth. The feeding regime of formulated feed and kelp has direct application potential in *S. intermedius* aquaculture, especially at field temperature. Feeding kelp alone can be poorly effective to support growth and gonad production for *S. intermedius* cultured at high water temperature. Based on the current results, we suggest the feeding regimes of formulated feed and kelp or formulated feed alone for *S. intermedius* aquaculture with high water temperature. The present study provides new information into the aquaculture of sea urchins at high temperature and for production out of season.

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Fatty acid component in sea cucumber 
*Apostichopus japonicus* from different tissues and habitats

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Fatty acids (FA) are a non-protein energy source and can act as trophic biomarkers in benthic food webs. We analysed the FA profiles of sea cucumber *Apostichopus japonicus*, comparing tissues of body wall, gut sediment and ovaries in two habitats. Rongcheng Bay: kelp raft cultivation area with high organic matter in sediment; Laoshan Bay: strong current with low sediment organic matter. The results showed that body wall and ovary tissues were rich in long chain polyunsaturated FA (LC-PUFA), which contributed ≏31% to the FA dissimilarity between the two tissues. SIMPER (similarity percentages routine) results showed that C20:5 (EPA), C18:1 (AA), C16:0, C14:1 and C20:1 contributed to dissimilarity between the body wall and ovary tissues, while 16:1, 20:5, C16:0, C18:1 and C14:1 contributed more to the dissimilarity of body wall tissues between the two habitats. FA biomarkers showed that sea cucumbers from the two habitats had different food sources, with brown kelp and vascular plants being the main food for sea cucumbers in Rongcheng and diatoms for those in Laoshan. To better understand differences in FA composition in sea cucumbers, more research is needed examining a wider diversity of tissue types and habitats.

**Keywords:** fatty acid, sea cucumber, *Apostichopus japonicus*, food resource

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**INTRODUCTION**

Sea cucumbers *Apostichopus japonicus* (Selenka) are harvested as a dietary delicacy and medicinal cure with high nutritional value (Chen, 2004). Moreover, *A. japonicus* play an ecological role as suspension feeders, detritivores and prey for different marine species. Through their trophic activities, deposit feeding holothurians can change the size of benthic particles and turn over sediment (Uthicke, 1999; Anderson et al., 2010). As an obligate deposit-feeding species, the sea cucumber *A. japonicus* plays an important trophic role in marine ecosystems (Slater & Carton, 2009; Ren et al., 2010). The feeding activities of this species could effectively remove nutrient matter deposited in benthic habitats and consequently may reduce the nutrient loadings in coastal ecosystems (Zheng et al., 2009; Sun et al., 2012).

Lipids are a non-protein energy source, which provide energy and essential fatty acids for normal growth and survival of aquatic animals (Seo & Lee, 2011). Fatty acids (FA) are considered to serve a dietary function in benthic food webs (Kelly & Scheibling, 2012). FA and FA ratios are used as biomarkers to confirm and trace food resources and trophic relationships in different aquatic habitats (Penha-Lopes et al., 2009; Coelho et al., 2011).

*Apostichopus japonicus* is the main cultured species in northern China, and there are three main cultured ways: in ponds, at the coast and in cages. There has been much research on FA of *A. japonicus*, and many have shown that *A. japonicus* has different FA profiles in different sampling times and areas (Kasai, 2003; Dantong et al., 2009; Gao et al., 2011; Han, 2011; Lee et al., 2012). The fatty acid values varied depending on the sampling time and collection region, however, an FA biomarker was not used as a biomarker for many studies.

In the present study we compare FA contents in the body wall and ovary of *A. japonicus* and also study FA biomarkers in sea cucumbers from two different habitats with organic matter in sediment (Rongcheng Bay: kelp raft cultivation area, high organic matter in sediment; Laoshan Bay: strong current area with low sediment organic matter) to determine habitat effects on FA content. Our hypotheses were that (1) fatty acid value especially polyunsaturated FA in ovary was higher than that in body wall and gut sediment; (2) FA composition were different and FA biomarkers may reveal main food resources for *A. japonicus* in the two areas.

**MATERIALS AND METHODS**

**Sampling sites**

Samples were taken at two different period in two different habitats. The first samples were taken in Laoshan Bay,
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Qingdao in April 2011. Organic matter in sediment in this bay was about 1–2%, because of strong currents (0.6–0.8 m s⁻¹) and low organic matter input. The second samples were taken in Rongcheng Bay (37°15.29′N 122°35.25′E), Weihai in June 2012, and where the organic content in sediment was high and can reach 7% (Xu et al., 2014) (Figure 1).

Sea cucumbers were taken in Laoshan Bay (36°15.07′N 122°40.80′E), while sea cucumber, sediment in the stomach as well as gonads from females were sampled in Rongcheng Bay. The sampled sea cucumber weight from both habitats was 200–300 g.

The sea cucumber samplings (12 individuals in each area) were dissected in the field after being washed with distilled water. The body wall, female gonad tissues and in situ sediment in the stomach were frozen and stored in the laboratory in a freezer at −20 °C.

**Fatty acid analysis**

Tissue, sediment and gonad samples were ground after being lyophilized. The same amount of three samples was chosen randomly to create a mixture sample. Fatty acid analyses of the mixture sample were carried out as in Liu et al. (2011). Samples were placed in DCM: MeOH (dichloromethane: methanol) in a proportion of 2:1 (v/v) with 0.01% BHT (butylhydroxytoluene) as an antioxidant. After sonication, the mixture was separated by centrifugation. The organic extracts hydroxytoluene) as an antioxidant. After sonication, the mixture was separated by centrifugation. The organic extracts were combined after three extractions and then evaporated under high purity nitrogen. Two millilitres of 6% KOH/Methanol was added to hydrolyse the samples at 80 °C for 2 h under the protection of high purity nitrogen. After acidification to pH 4 with aqueous hydrochloric acid (1:1), 1 mL of 14% boron trifluoride-methanol was added and the samples were esterified at 80 °C for an hour under the protection of high purity nitrogen. Fatty acid methyl esters (FAMEs) were recovered from the mixture by extraction with 3 × 2 ml of hexane.

Quantification of FA was carried out using an Agilent 7890A instrument with FID (Agilent Technologies, Wilmington, DE, USA) equipped with a DB-FFAP capillary column (30 m × 0.25 mm ID coated with 0.25 μm film thickness). The program of temperature was as follows: 70 °C for 5 min, held at 220 °C for 33 min after increasing at 3 °C min⁻¹ rate. Temperatures of the injector and detector were maintained at 220 and 280 °C, respectively. FAME identification was performed by comparing relative retention times with those of known standard (see Supplementary material Figure S1) and mixture standard (37-FAME Mix, 46-FAME Mix; Sigma, USA). Equivalent chain lengths were used as an aid in peak localization and identification. Each FAME area was corrected from the corresponding FID response factor and from the difference in weight between the FAME and its corresponding free fatty acid (Dubois et al., 2014). We then used the quantitative method from Bai et al. (2010) to calculate the FA content.

**FA biomarkers**

Biomarkers (also expressed as ratios) indicate the relative importance of one food source over another. We used Alfaro et al. (2006) and other articles to calculate FA biomarkers. The odd and branch-chain FA (odd&br FA), the ratio of C18:1ω7/C18:1ω9 was used to show bacterial food sources (Budge et al., 2001; Li et al., 2007). The sum percentage content of C18:2ω6 and C18:3ω3 was used as a continental food source marker (above 2.5 implies a continental source) (Budge & Parrish, 1998; Cui et al., 2012). C16/ΣC18, C16:1/C16:0 and C20:5ω3/C22:6ω3 (EPA/DHA) ratios were used as markers for diatom food resources (Budge et al., 2001). C18:1ω9 indicates brown kelp food resources (Alfaro et al., 2006).

**The statistics analysis**

FA values were presented as means ± SD. FA and FA biomarkers mentioned above were subjected to a one-way ANOVA with post hoc Tukey test using SPSS 15.0 statistical software package. Significance was accepted at P < 0.05. One-way analysis of similarities (ANOSIM) were calculated on the Bray–Curtis similarity of body wall in the two habitats, sediment, body wall and gonad. Differences in FA (using percentage content) in the sediment, body wall and ovary in Rongcheng, body wall in Rongcheng and Laoshan were explored using the similarity percentages routine (SIMPER) based on the Bray–Curtis similarity (Hughes et al., 2005; Budge et al., 2007). All multivariate analysis was carried out using the PRIMER-E V6.0 (Clarke & Warwick, 2001; Clarke & Gorley, 2006).

**RESULTS**

**Differences in FA among sediment, body wall and gonads of sea cucumbers in Rongcheng Bay**

Total FA content was the lowest in gut sediment (700.96 ± 538.92 μg g⁻¹) and highest in the ovary (89164.13 ± 8363.35 μg g⁻¹) in Rongcheng Bay. FA in the body wall of sea cucumbers from Rongcheng Bay was 46746.99 ± 4196.47 μg g⁻¹. Absolute FA content is shown in supplementary material. FA profiles were dominated by polyunsaturated FA (PUFA) in body wall and ovary tissues, and by saturated FA (SFA) in the sediment (Figure 2).

Most FA, especially long chain PUFA (LC-PUFA, including 20 or more carbon atoms in polyunsaturated FA) were significantly different between body wall and gonad tissues, except for some monounsaturated FA (MUFA) and non PUFA (containing two or more double bonds, C14:1ω5, C16:2ω4, C16:4ω3, C20:1ω9, C22:6ω3). In gut sediment, C16:0 was the dominant FA, accounting for 20.46% of the total FA, followed by C18:0 (15.03%), C16:1ω7 (8.93%), C18:1ω7 (6.43%) and EPA (6.34%). EPA accounted for 15.24% of total FA in the ovaries, followed by C16:1ω7 (8.60%), C18:1ω7 (7.94%), C16:0 (7.92) and AA (7.35%).

The ANOSIM results show that percentage content of FA in the sediment, body wall and gonad in Rongcheng Bay were significantly different (Table 1). The dissimilarity between sediment and body wall was 33% (Table 2). The SIMPER results showed that C16:0, C18:0, EPA, C22:6ω3, C18:1ω7, AA (arachidonic acid, C20:4ω6), C16:0, C14:1 and C20:1ω11 contribute most to the dissimilarity between sediment and body tissue of A. japonicus in Rongcheng Bay (Table 2). However, EPA, C18:1ω7, AA, C16:0, C14:1 and C20:1ω11 contributed most to differences between the body
wall and ovary of *A. japonicus* in Rongcheng Bay (Table 3). LC-PUFAs contributed $\approx 30.95\%$ to the difference between the two tissues.

**Differences in body wall FA**

EPA was the most abundant FA in the body wall of sea cucumbers both in Rongcheng (11.51%, 5380.4 ± 607.11 µg g$^{-1}$) and Laoshan (15.29%, 2978.3 ± 1113.68 µg g$^{-1}$). C16:1ω7, C16:0, C18:0 and DHA were most abundant in Rongcheng sea cucumbers, accounting for 10.41, 8.92, 8.13 and 8.02% of total FA, respectively. However, C18:0, DHA, C16:0 and AA were most abundant in Laoshan sea cucumbers, accounting for 8.82, 8.61, 8.56 and 8.18% of total FA, respectively.

Results show that total FA, SFA, MUFA and PUFA in the body wall of sea cucumbers from Rongcheng were significantly higher than in Laoshan (see supplementary material). However, the percentage content of PUFA in Laoshan (44.67%) was higher than that in Rongcheng (35.83%, see Figure 2).

**Food resources of sea cucumbers from different habitats**

The odd&br FA content in Rongcheng was higher than that in Laoshan, while C18:1ω9/C18:1ω7 ratio (bacterial food sources) in the body wall of sea cucumbers from Laoshan (3.05 ± 0.54) was higher than that in Rongcheng (2.25 ± 0.13), but was not significantly different (Figure 3).

In Rongcheng sediment, continental food source biomarker was 4.26, which implies FA was mainly from a continental source. But this biomarker in the body wall of sea cucumbers from the two habitats was below 2.5, which showed continental organic matter took little percentage.

The diatom food resource biomarkers had different trends in the two areas. The ratio of total C16 and C18 in Laoshan was slightly higher (1.18 ± 0.07), but not significantly different, than that in Rongcheng (1.07 ± 0.09). The ratio of C16:1/C16:0 was also higher in Laoshan (1.49 ± 0.39) than in Rongcheng (1.43 ± 0.13). The ratio of EPA/DHA

---

**Table 1.** Pairwise ANOSIM results of FA profiles in sediment in gut, body wall and gonad of *Apostichopus japonicus* from Rongcheng Bay.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Global R</th>
<th>Significance level</th>
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<tbody>
<tr>
<td>Sediment, Body wall</td>
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<td>2.9</td>
</tr>
<tr>
<td>Sediment, Gonad</td>
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<tr>
<td>Body wall, Gonad</td>
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<td>2.9</td>
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</table>

---

**Fig. 1.** The two sample areas for the sea cucumber *Apostichopus japonicus* in Shandong peninsula (▲ indicates Rongcheng Bay and Laoshan Bay).
had the same trend (Laoshan: 1.73 ± 0.30, Rongcheng: 1.43 ± 0.08). This series of ratios indicates higher variability in diatoms as a food resource of sea cucumbers from Laoshan than that of Rongcheng.

The percentage of brown kelp food resources biomarker (C18:1\textit{\textit{\alpha}9}) in body wall in Rongcheng (3.24 ± 0.17) was significantly higher than in Laoshan (2.12 ± 0.35, Figure 3).

**Table 2.** SIMPER results of sediment in gut (S) and the body wall (C) of *Apostichopus japonicus* from Rongcheng Bay (the dissimilarity of the two groups was 33.32%).

<table>
<thead>
<tr>
<th>FA</th>
<th>Av. Abund of S</th>
<th>Av. Abund of C</th>
<th>Average dissimilarity</th>
<th>Dissimilarity/SD</th>
<th>Contribution %</th>
<th>Cumulative %</th>
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</tr>
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<td>1.76</td>
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<td>57.94</td>
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<td>0.95</td>
<td>90.70</td>
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</table>

had the same trend (Laoshan: 1.73 ± 0.30, Rongcheng: 1.43 ± 0.08). This series of ratios indicates higher variability in diatoms as a food resource of sea cucumbers from Laoshan than that of Rongcheng.

The percentage of brown kelp food resources biomarker (C18:1\textit{\textit{\alpha}9}) in body wall in Rongcheng (3.24 ± 0.17) was significantly higher than in Laoshan (2.12 ± 0.35, Figure 3).

**DISCUSSION**

**FA differences between sediment, body wall, ovary and role of sea cucumbers**

Sea cucumbers are suspension and deposit feeders (Massin, 1982). Deposit feeders have an important role in FA transformation from sediment and benthic creatures to the upper water ecosystem through eggs and larvae (Mileikovsky, 1974). Some deposit feeding sea cucumbers, such as *Holothuria (Halodeima) atra* and *Stichopus chloronotus* have bioturbate effects and can disturb the entire upper 5 mm of sediment once a year (4600 kg (dry wet) year$^{-1}$ km$^{-2}$), significantly reducing the microalgal biomass in the sediment and playing a substantial role in the recycling of nutrients in oligotrophic environments where nutrients would otherwise remain trapped in the surface sediment (Uthicke, 1999). Uthicke (2001) also showed that a small sediment area may be fertilized by nutrients released via the body wall of sea cucumber, approximately 25% of which is in direct contact with the sediment.

Species of other feeding types also have the same ecological role. Sea urchin can deliver more LC-PUFA to the pelagic and benthic food webs through their gonads and subsequently in

<table>
<thead>
<tr>
<th>FA</th>
<th>Av. Abund of B</th>
<th>Av. Abund of G</th>
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<td>1.59</td>
<td>0.78</td>
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</table>
producing larvae, than they can through production of faecal pellets (Hughes et al., 2011).

Most sea cucumbers have the ability to select the organic food. Some study revealed that Mediterranean holothuroids show different selectivity for organic matter (Plotieau et al., 2014). In our study, the continental food source marker (sum of C18:2\(^{\Delta 6}\) and C18:3\(^{\Delta 3}\)) in the body wall was lower than that in sediment, which indicates that A. japonicus have the ability to feed selectively.

In a benthic ecosystem, A. japonicus may select the much more organic sediment matter and then use some methods to transfer the nutrients. That is the same as sea urchins, related above. Sea cucumber may use their gonad to transfer nutrients. This study revealed that sea cucumbers can store more LC-PUFA in the ovary. The eggs released by sea cucumbers may be a suitable food source for other marine organisms especially carnivores living in upper water and can also provide abundant nutrients to the planktonic ecosystem. However, the ecological role of holothuroids is often neglected by researchers. Seagrass habitat research using carbon and nitrogen stable isotopes showed holothurians enhanced the sediment uptake of organic carbon and total nitrogen by up to 30× and 3×, respectively (Costa et al., 2014). Another study in a reef area revealed that sea cucumbers play an important ecological role in the coral reef CaCO\(_3\) cycle (Schneider et al., 2013). So further laboratory and field simulation experiments are needed to fully understand the ecological effects of this species, which is the main cultured species in northern China coastal area.

FA in different habitats

FA composition, especially LC-MUFA, was different depending on sampling times, regions and age of species. Previous studies have shown differences in FA of A. japonicus. EPA, AA and DHA in the body wall of pond-cultured A. japonicus was between 7.24–14.45%, 4.54–8.16% and 2.41–4.45%, respectively over 1 year (Gao et al., 2011), while higher EPA levels (9.94–11.24%) and lower DHA levels (5.83–7.02%) have been shown for A. japonicus caught in Zhangzi Island coast (Dantong et al., 2009). The percentage of EPA and AA in the present study are in agreement with the ranges reported above, but the DHA was a little higher. DHA content had a larger difference.

![Fig. 3. Fatty acid biomarkers of the body wall of Apostichopus japonicus from Rongcheng Bay and Laoshan Bay and sediment in gut in Laoshan Bay odd&br FA: odd and branch-chain FA, EAP/ DHA: C20:5\(^{\Delta 3}\)/C22:6\(^{\Delta 3}\).](image)

Table 4. SIMPER results of the body wall of Apostichopus japonicus from Rongcheng Bay (RC) and Laoshan Bay (LC, the dissimilarity was 21.26%).

<table>
<thead>
<tr>
<th>FA</th>
<th>Av. Abund of RC</th>
<th>Av. Abund of LC</th>
<th>Average dissimilarity</th>
<th>Dissimilarity/SD</th>
<th>Contribution %</th>
<th>Cumulative %</th>
</tr>
</thead>
<tbody>
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<td>2.79</td>
<td>1.55</td>
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could be partially due to the sampling time. *Apostichopus japonicus* will reduce feeding activities in cold water and enter aetivation in summer (Gao et al., 2011). The sampling of the two areas was done in spring, which is the optimal season for feeding in *A. japonicus*. Another reason is the habitats. Sea cucumber living in open areas may feed on more detritus from kelp and other materials, so those individual may have higher MUFA from food than that inhabited in pond and cage.

FA is known to be a useful biomarker, with FA profiles indicating different food resources in different habitats (Braeckman et al., 2012; Kelly & Scheibling, 2012). Some studies have used FA to indicate food supply in different habitats. Coelho et al. (2011) studied FA of mud snails *Hydrobia vulvae* from mudflats and seagrass meadows in the same estuary and found significant differences of food resources. SFA and MUFA were found in greater abundances in the eggs of crabs *Uca annulipes* inhabiting a peri-urban mangrove subjected to domestic sewage discharges compared with those inhabiting pristine mangroves (Penha-Lopes et al., 2009). Further, FA can be used to distinguish different geographic populations. Dong et al. (2013) showed that five kinds of FA, C18:1ω9, C22:1ω9, C20:4ω6 and C20:5ω3, were effective in identifying six populations of crabs *Portunus trituberculatus* from the China Sea with a total discriminant accuracy of 88.46%.

Food supply has a significant effect on the FA profile of sea cucumbers (Alfaro et al., 2006). Some studies have shown that FA composition may be influenced by the feeding and environmental conditions of the habitat (Iverson et al., 1997). Neto et al. (2006) studied FA of three types of deep sea holothurians and suggested that the FA profile response to variations in food supply appears to depend on feeding mode. Lipid content of *Psychropotes longicauda* showed a strong positive correlation with the contents of lipids in the surficial sediments (Neto et al., 2006). This may be because *P. longicauda* uses peltate tentacles to sweep sediments into the mouth to obtain food from the top ~5 mm of the sediment.

Similarly, the holothuria *A. japonicus* in the present study is also a surface sediment feeding species. Our results showed that some FA contents differed markedly between the body walls of *A. japonicus* from the two studied coastal areas. In this cultured environment, sea cucumber is often released and scatter-fed in the seabed. Rongcheng Bay is famous for this cultured environment, sea cucumber is often released top sweep sediments into the mouth to obtain food from the tops of the sediment.

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CONCLUSION

This study reveals significant differences among FA from sediment, the body wall and the female ovary of sea cucumbers. LC-PUFA contributed the greatest differences in FA between the body wall and ovary. FA biomarkers show that sea cucumbers inhabiting Rongcheng and Laoshan have different food resources. Diatoms may contribute more to the diets of sea cucumbers in Laoshan, while brown kelp and vascular plants may play a more significant role in the diets of those in Rongcheng. It is necessary to do further research over longer time periods to better understand differences in FA composition in different tissues and habitats.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S002531541500168X

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Trace metal bioavailabilities in the Thames estuary: continuing decline in the 21st century

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Levels of pollution, including contamination by toxic metals, in the Thames estuary reduced over the last four decades of the 20th century. This 2014 study investigates whether the declines in the bioavailabilities of trace metals (Ag, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V, Zn) have continued in the 21st century, using a suite of littoral biomonitors also employed in 2001 – the brown seaweed Fucus vesiculosus, the strandline, talitrid amphipod Orchestia gammarellus and the estuarine barnacle Amphibalanus improvisus. Bioaccumulated concentrations represent relative measures of the total bioavailabilities of each metal to the biomonitor over a previous time period, and can be compared over space and over time. Trace metal bioavailabilities varied along the estuary, and, in general, fell between 2001 and 2014, a reflection of the continuing remediation of the Thames estuary from its severely polluted state in the middle of the 20th century.

Keywords: trace metals, biomonitoring, bioavailability, Thames estuary, Fucus vesiculosus, Orchestia gammarellus, Amphibalanus improvisus

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INTRODUCTION

In the middle of the 20th century, the estuary of the River Thames was heavily polluted with severe ecotoxicological effects on the fauna and flora (Wheeler, 1979; Andrews, 1984). The forms of pollution were manifold, and included high concentrations of suspended solids originating from sewage, leading to high biological oxygen demand (BOD), reduced dissolved oxygen levels and high total ammonia concentrations (Andrews, 1984; Power et al., 1999). Additionally industrial and domestic effluents contributed very high concentrations of toxic metals, of organic contaminants such as organochlorines and PCBs, and of synthetic detergents into the estuary (Andrews, 1984). Fortunately, between 1960 and the late 1970s, the water quality of the Thames estuary was greatly improved as a result of the completion or extension of major sewage treatment plants discharging into the estuary (e.g. at Beckton and Crossness), and greater control of industrial effluents (Andrews, 1984; Power et al., 1999). By 1980, the Thames estuary had changed from an ‘open sewer’ to a waterway allowing the free passage of salmon (Wheeler, 1979; Andrews, 1984).

The clean-up of the Thames estuary did not stop in the early 1980s. For example, dissolved concentrations of cadmium, copper, mercury, nickel and zinc showed further exponential declines between 1980 and 1997 (Power et al., 1999). Dissolved lead concentrations also showed a decline over the initial years of this period, but this decline was reversed in the early 1990s by the onset of drought and by the then continuing effect of the use of leaded petrol by Londoners before its eventual phase out (Power et al., 1999).

This study is concerned with the status of trace metals in the Thames estuary in the second decade of the 21st century, particularly examining whether or not toxic metal bioavailabilities have continued to fall over time since the late 1990s, and whether or not these metal bioavailabilities can still be considered to be high and of potential ecotoxicological significance.

The study by Power et al. (1999) reported on changes in dissolved metal concentrations in the Thames estuary over time. An alternative method of assessing metal contamination, or more specifically the bioavailabilities of trace metals to local biota, is to use biomonitors. Biomonitor must be net accumulators of contaminants (in this case trace metals) in proportion to the total ambient bioavailability of the contaminant, and the resulting bioaccumulated concentrations represent relative measures of the local bioavailabilities of the trace metals at a site to that particular biomonitor, integrated over a preceding time period (Bryan et al., 1980, 1985; Luoma & Rainbow, 2008). The use of biomonitors has advantages over the measurement of trace metal concentrations in local water or sediment, because the measured bioaccumulated concentrations concern only the bioavailable fraction of the local metal, the specific fraction that has the potential to be of ecotoxicological significance (Bryan et al., 1980, 1985; Luoma & Rainbow, 2008). The use of trace metal biomonitors is now routine in the comparative assessment of potential toxic metal
bioavailabilities, whether between locations at any one time or between sampling occasions at the same site. Given that the accumulated metal concentrations in a biomonitor strictly represent the integrated total bioavailability of a metal to that biomonitor at a site, it is preferable to use a suite of biomonitors to reflect different possible sources of bioavailable metals to the local biota (Bryan et al., 1980, 1985; Bryan & Gibbs, 1983; Rainbow et al., 2002, 2011; Luoma & Rainbow, 2008).

In the context of this study, biomonitoring data are available for trace metals in the estuary of the Thames. McEvoy et al. (2000) and Langston et al. (2004) measured the bioaccumulated trace metal concentrations in suites of biomonitors along the Thames estuary in 1997 and 2001 respectively. McEvoy et al. (2000) confirmed that bioaccumulated concentrations of most trace metals analysed had fallen between 1980 and 1997, in agreement with the dissolved metal data of Power et al. (1999). Comparisons of bioaccumulated concentrations between 1999 and 2001 data were more equivocal, to be expected given the short time period concerned (Langston et al., 2004).

Of particular relevance here is the biomonitoring study of the Thames estuary carried out in 2001 by Rainbow et al. (2002). These authors used a suite of three littoral biomonitors – the brown seaweed Fucus vesiculosus (bladder wrack), the talitrid amphipod Orchestia gammarellus and the estuarine barnacle Amphibalanus improvisus. Accumulated metal concentrations in the seaweed reflect dissolved trace metal bioavailability (Bryan et al., 1980). Orchestia gammarellus feeds on cast up seaweed in the strandline, a trophic source of trace metals that, to a degree, indirectly reflects local dissolved metal bioavailability (Weeks & Rainbow, 1993). The barnacle takes up metals from solution and from suspended material in the water column, including re-suspended sediment, their bioaccumulated metal concentrations reflecting metal bioavailabilities from these sources (Luoma & Rainbow, 2008). Some invertebrates (for example decapod crustaceans) are able to regulate body concentrations of essential trace metals such as zinc to approximately constant levels under different bioavailable zinc exposures, and are, therefore, unsuitable as biomonitors of these metals (Luoma & Rainbow, 2008). Neither amphipods nor barnacles are regulators of body concentrations of essential metals, and are, therefore, suitable for use as biomonitors (Luoma & Rainbow, 2008).

The present study repeated the 2001 biomonitoring survey of Rainbow et al. (2002) in order to address directly the question whether toxic metal bioavailabilities in the Thames estuary have continued to fall over time since the turn of the century. The study employed the same biomonitor from essentially the same locations, and was carried out at the same time of the year to avoid any seasonal effects. As in the 2001 study, the data collected (a more extensive data set involving additional metals) will also serve as a new baseline for future studies of the ongoing remediation of this significant estuary.

**MATERIALS AND METHODS**

Three littoral species Fucus vesiculosus, Orchestia gammarellus and Amphibalanus improvisus were collected at low tide from up to five sites along the Thames estuary on 16 and 17 July 2014: Woolwich (51°29.684′N 0°2.186′E), Erith (O. gammarellus and F. vesiculosus at 51°28.767′N 0°11.600′E and A. improvisus at 51°28.927′N 0°10.855′E), Greenhithe (51°27.222′N 0°17.063′E), Gravesend (51°26.659′N 0°22.260′E) and Canvey Island (51°30.810′N 0°35.232′E) (Figure 1). Fucus vesiculosus was not found at Woolwich, and A. improvisus was not found at Woolwich and Canvey Island.

Samples of 10 F. vesiculosus fronds were collected from pier piles or their equivalent at each site, in order to ensure that the individual fronds selected were not in contact with any sediment. At least 10 'large' (>0.5 mm) O. gammarellus were collected from the strandline in the littoral fringe at each site. Barnacles were scraped from the rock face or pier on which they were found straight into sample collection bags, ensuring that more than 100 individuals were collected from each site. All samples were transported to the laboratory in a chilled cool box on the day of collection, and then transferred to a −20°C freezer for storage for between 1 and 4 weeks before analysis.

In the laboratory, epiphyte-free regions, about 2 cm long and located 10 cm from the distal tip, of each F. vesiculosus frond were cut with stainless steel scissors, rinsed in double distilled water, and dried to constant weight at 60°C in individual acid-washed test tubes. This region of the frond was chosen because it is reproducible for each sample and represents tissue in which the accumulated metal concentrations have been integrated over several months (Bryan et al., 1985; Rainbow et al., 2002). Ten O. gammarellus from each site were rinsed individually in double distilled water, and also dried to constant weight at 60°C in individual acid-washed test tubes. The bodies of the barnacles were removed with clean stainless steel forceps from the surrounding calcareous plates with associated mantle tissue. Ten bodies were pooled in each of 10 acid-washed test tubes to provide 10 replicate pooled samples per site (in total 100 individual bodies from each site), before drying to constant weight at 60°C. The body (strictly the thorax with six pairs of filtering thoracic limbs (cirri), the reduced abdomen and part of the head, the oral cone) of a barnacle is easily recognized and

**Fig. 1.** Locations of the five sampling sites (Woolwich, Erith, Greenhithe, Gravesend and Canvey Island).
the most reproducible tissue to use for analysis (Rainbow et al., 2002, 2004). Remaining tissues, including the rest of the head forming the mantle tissue, base, shell plates and opercular plates, and any egg masses in the mantle cavity were ignored. Neither amphipods nor barnacles were maintained in the laboratory in any effort to depurate the gut contents, in order to avoid laboratory contamination. All the amphipods and barnacle bodies, therefore, contain gut contents, but these are considered to represent only a small (and location-dependent) proportion of total body metal content (Rainbow & Moore, 1986; Moore & Rainbow, 1987; Rainbow et al., 1989; Moore et al., 1991), particularly in the case of barnacles which are well known to be particularly strong trace metal accumulators (Rainbow, 1987, 2007; Rainbow & Blackmore, 2003; Luoma & Rainbow, 2008). Laboratory depuration of biomonitorors, on the other hand, is to be recommended in the case of deposit feeders with gut contents consisting of metal-rich sediment (Bryan et al., 1985; Casado-Martínez et al., 2009; Rainbow et al., 2009; Kalman et al., 2014).

The seaweed sections, amphipods and barnacle bodies were digested on a heating block in concentrated nitric acid (HNO₃, Aristar grade, BDH Ltd, Poole, UK) at 100 °C, and diluted to 10 mL with double distilled water. Samples were analysed for the trace metals Ag, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Zn and V by Inductively Coupled Plasma Spectroscopy (ICPS) (Thermo iCap 6500 Duo ICP-AES), together with blanks and samples of two Standard Reference Materials (Mussel Tissue ERM-CE278 from the Institute for Reference Materials and Measurements, 2440 Geel, Belgium, and Oyster Tissue 1566b from the US Department of Commerce, Technology Administration, National Institute of Standards and Technology, Gaithersburg, MD). Agreement with the certified concentrations of the reference material, where available, is considered acceptable (Supplementary Table 1). All metal concentrations are quoted in terms of µg g⁻¹ dry weight.

All accumulated metal concentration data were transformed logarithmically (to the base 10) to reduce deviations of data sets from normal distributions before the application of parametric statistical tests. Concentrations of metals in the seaweed F. vesiculosus were compared by analysis of variance (ANOVA) using logged data. For the amphipods and barnacle bodies, it is important to take into account any effect of size (in this case body dry weight) on accumulated trace metal concentrations before making comparisons between sites, or indeed between times at the same site. The power function $y = ax^b$, where $y$ is the metal concentration (µg g⁻¹), $x$ is body dry weight (g), and $a$ and $b$ are constants, is an appropriate model for the relationship between accumulated trace metal concentrations and individual body weights in both amphipods and barnacles (Rainbow et al., 1989, 2002, 2004; Moore et al., 1991). Here $x$ is the dry body weight of an individual amphipod, or the mean body dry weight of the 10 pooled barnacle bodies in each replicate sample. Amphipod and barnacle body data were, therefore, first analysed for significant regression coefficients (slopes) in the best-fit linear regressions of log individual body weight $(\log x)$ against log metal concentration $(\log y)$ in the data set for each crustacean for each metal at each site, and in the whole species data set for each metal.

For many, but not all, metals in each species, there was, in fact, a significant effect of body weight on accumulated body metal concentration in the whole data set and/or in at least one of the data sets for an individual site. In these cases, it is, therefore, not meaningful to quote means or use ANOVA to make comparisons between the amphipod or barnacle body accumulated metal concentrations at the different sites. In contrast, in the absence of any such size effect for a metal in a particular species, ANOVA can be, and was, used to make statistical comparisons between sites, still using logged data.

If a size effect was detected, Analysis of Covariance (ANCOVA) was used to make allowance for this size effect when comparing between sites. In the ANCOVA comparisons, the data for each metal in each crustacean were first analysed for significant regression coefficient significantly different from those of the other sites for that species. When this occurred, that data set was removed from any further statistical comparison against the remaining sites. Analysis of the data for those sites for which the slopes of best-fit regressions did not differ significantly, was continued to identify any differences in elevation of metal concentration/body dry weight double log regressions between sites. If a priori analysis showed significant differences in accumulated metal concentrations between sites, Tukey’s Honest Significant Difference (HSD) test was then applied a posteriori, to identify which sites differed significantly from each other in accumulated metal concentrations. Linear regression analyses, ANOVA and ANCOVA were carried out using STATISTICA (Statsoft).

Because mean accumulated metal concentrations in the bodies of amphipods or barnacles from different sites have no comparative meaning in the presence of size effects, comparative accumulated metal concentrations are then quoted as the estimated accumulated concentrations (with 95% confidence limits) in amphipod or barnacle bodies of a standardized dry weight, as calculated from each best-fit double log regression. The standardized dry weight chosen for O. gammarellus was 0.01 g. Mean dry weights of the amphipods at the five sites ranged from 0.0045 g (Woolwich) to 0.0181 g (Greenhithe), with a grand mean across sites of 0.0143 g, so a standardized dry weight of 0.01 g is appropriate for this data set. Furthermore, comparisons were to be made (see below) against the equivalent 2001 Thames estuary data set for O. gammarellus of Rainbow et al. (2002), in which study the grand mean dry weight of amphipods from the five sites was 0.0099 g (range 0.0053–0.0155 g). Rainbow et al. (2002) also quoted accumulated metal concentrations of O. gammarellus for a 0.01 g dry weight amphipod, as indeed did Rainbow et al. (1989) and Moore et al. (1991) for the same amphipod from wider ranges of sites across the UK. For the bodies of A. improvisus, the mean individual body dry weight ranged from 0.00106 to 0.00195 g (grand mean 0.00153 g) across the three sites in 2014, while ranging from 0.00165 to 0.00227 g (grand mean 0.00209 g) in 2001 (from data of Rainbow et al., 2002). For ease of comparisons, the standardized individual barnacle body dry weight chosen was 0.002 g.

Finally, comparisons were made between the 2014 data set and that of 2001 (Rainbow et al., 2002), to investigate whether trace metal bioavailabilities to each of the three chosen biomonitor species had changed over the intervening years. Samples were taken at the same time of year (mid July in 2014, early August in 2001) to eliminate any seasonal effects. In 2001, Rainbow et al. (2002) employed atomic absorption spectrophotometry for metal analysis, while in 2014 we used the more sensitive analytical technique of ICPS, giving a wider range of accumulated metal concentration data for the later year. Data are, therefore, here presented for the metals cobalt, chromium, nickel and vanadium, which cannot be
Table 1. *Fucus vesiculosus* mean concentrations (µg g⁻¹ with 95% confidence limits interval, unlogged data) of trace metals in seaweed samples from four sites in the Thames estuary in 2014, and results of Tukey post hoc ANOVA comparisons of logged metal concentration data between sites. 2014 sites sharing a common letter do not differ significantly in seaweed concentrations of that metal (*P > 0.05*). Also shown are equivalent concentrations (mean, 95% CL, unlogged data) of samples collected from approximately the same sites in 2001 (after Rainbow *et al.*, 2002), and results of ANOVA comparisons of logged data for each site between 2001 and 2014 (*P < 0.05*, **P < 0.01**, ***P < 0.001, NS P > 0.05*).

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Compared against any equivalent 2001 data, but are now available for any future comparisons. Furthermore, the summary 2001 metal concentration data presented here may appear to differ slightly from those in Rainbow *et al.* (2002) who, for example, presented mean metal concentrations in ANOVA comparisons in the form of anti-logged means of logged concentrations. While logged data were still used for statistical comparisons here, mean data are presented for unlogged concentrations, showing very slightly different values from those of Rainbow *et al.* (2002). In the case of the barnacle body data used for ANCOVA, Rainbow *et al.* (2002) quoted estimated metal concentrations for a barnacle of the mean dry weight for each site, while we have used the standardized 0.002 g dry weight for all sites.

RESULTS

2014 *Fucus vesiculosus*

Table 1 presents the mean accumulated concentrations of 11 trace metals in *F. vesiculosus* from four sites in the Thames estuary (Figure 1) in July 2014. Seaweed samples from sites sharing the same letter do not differ significantly (ANOVA, Tukey’s post hoc, *P > 0.05*) in concentration of the trace metal concerned. For all 11 metals there was a significant difference (*P < 0.05*) in the bioaccumulated concentrations in the seaweed across the four sites.

2014 *Orchestia gammarellus*

Table 2 gives accumulated trace metal concentrations in the amphipod *O. gammarellus* from all five sites in the Thames estuary sampled in July 2014. If there was no size effect on the concentration of a particular metal in the amphipods at any one site or in the whole data set for that metal across all five sites, then accumulated concentrations are quoted as means with 95% confidence limits. For each such metal, sites are presented in descending order of mean bioaccumulated concentrations (Table 2). As in Table 1, sites sharing the same letter do not differ significantly (ANOVA, Tukey’s...
Table 2. *Orchestia gammarellus*: Concentrations (µg g⁻¹) of trace metals in amphipods from up to five sites in the Thames estuary in 2014, expressed as the mean with 95% confidence limits interval (unlogged data), or as the concentration ([M] with 95% CL) in a standardized amphipod of 0.01 g dry weight as estimated from best fit double log regressions of body dry weight (g) against metal concentration (µg g⁻¹) when the accumulated concentration has been shown to be significantly related to weight. Also shown are 2014 results of statistical comparisons between sites using Tukey post hoc ANOVA (logged metal concentration data) or ANCOVA (double log regressions). 2014 sites sharing a common letter do not differ significantly in amphipod concentrations of that metal (P > 0.05). Also shown are equivalent concentrations (means with 95% CL or concentrations in 0.01 g amphipods [M] as estimated from double log regressions) of samples collected from approximately the same sites in 2001 (after Rainbow et al., 2002), and the results of ANOVA or ANCOVA comparisons of logged data for each site between 2001 and 2014 (∗P < 0.05, ∗∗P < 0.01, ∗∗∗P < 0.001, NS P > 0.05).

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Continued
In the presence of a size effect for a particular metal, data were compared by ANCOVA, and the concentrations presented ([M] in Table 2) are those in a standardized 0.01 g dry weight amphipod, as estimated from best fit double log regressions of body dry weight (g) against metal concentration (µg g⁻¹). 95% confidence limits are asymmetrical about the estimated concentrations [M] after anti-logging of estimates made using logged data. Again, amphipods from any sites sharing a common letter in the post hoc column for a particular metal do not differ significantly in body concentrations of that metal. In Table 2, sites listed under each metal showing a size effect have been ordered firstly in terms of the post hoc ANCOVA groupings to which they belong (as denoted by a single letter), and secondly within those groups in terms of the estimated body metal concentration of a body of 0.01 g dry weight amphipod. In ANCOVA, it is best-fit regression lines that are being compared. These lines will inevitably cross, even when there are no significant differences between slopes, and estimated metal concentrations for different standardized body weights chosen are likely to give different site orders. Furthermore, according to how the chosen standardized weight falls inside or outside the range of body weights in the data set analysed for a given site, the 95% confidence limits of the estimated concentrations might be narrow or broad. Finally, when the number of measurable replicates is small, confidence limits about estimated concentrations in a standard sized amphipod will be large, again affecting placement in a descending order of sites for a particular metal. Therefore, post hoc ANCOVA site groupings will not always follow the strict rank order of the weight standardized metal concentrations quoted (e.g. for Mn in Table 2). Occasionally, and perhaps inevitably by chance at the 5% level given the number of statistical comparisons made, significant differences in regression coefficients (slopes of best fit lines) ruled out follow-up comparisons of the elevations of those lines (in practice the logged accumulated metal concentrations). Three such examples are apparent in the 2014 data set for O. gammarellus (Table 2).

In the 2014 data set, nine of the 11 trace metals investigated showed significant differences (P < 0.05) in the bioaccumulated concentrations in the amphipods across the five sites (Table 2). The two remaining trace metals, copper and silver, showed no significant differences in accumulated metal concentration in O. gammarellus across the four sites that could be compared in each case, the fifth site being eliminated from the ANCOVA comparison because of a significantly differing regression coefficient (Table 2).

### 2014 Amphibalanus improvisus

Table 3 presents accumulated concentrations of the 11 trace metals in bodies of the barnacle A. improvisus from the three sites at which it occurred. Arrangement of the 2014 barnacle data in Table 3 follows the same pattern as that for the amphipod data in Table 2, with use of ANOVA or ANCOVA in the absence or presence respectively of any size effect. The concentrations presented ([M] in Table 3) are those in a standardized 0.002 g dry weight barnacle body.

In the 2014 data set for A. improvisus, nine of the 11 trace metals investigated showed significant differences (P < 0.05) in the bioaccumulated concentrations in the barnacle bodies across the three sites (Table 3). The two remaining trace metals, lead and nickel, showed no significant differences in accumulated metal concentration in the barnacles across the three sites (Table 3).

### 2014 Bioavailabilities

The bioaccumulated trace metal concentrations in each of the three biomonitor sites represent relative measures of the local bioavailabilities of the trace metals at a site to that particular biomonitor integrated over a preceding time period.

The dissolved bioavailability of zinc, as reflected by the seaweed F. vesiculosus, showed a fall between Erith and both Greenhithe and Canvey Island downstream, but Gravesend, between Greenhithe and Canvey Island (Figure 1), showed matching high zinc bioavailability to that at Erith (Table 1). Erith, joined now by Woolwich, showed the highest bioavailability of zinc to O. gammarellus, while Gravesend was at the bottom of this rank order of sites (Table 2). The barnacle A. improvisus showed a downstream pattern of decreasing zinc bioavailability between sites, zinc bioavailability at each of Greenhithe and Erith being greater than that at Gravesend (Table 3).

Copper bioavailability to F. vesiculosus did not differ between Erith, Gravesend and Canvey Island, but was lower at Greenhithe (Table 1). There was no geographic variation in copper bioavailability to O. gammarellus along the Thames estuary in 2014 (Table 2). Copper bioavailability to the barnacle A. improvisus did show geographic variation, but Greenhithe now topped the list of sites (Table 3). Cadmium bioavailabilities to all three biomonitors varied geographically (Tables 1 – 3). There was consistency of site pattern
between *F. vesiculosus* and *O. gammarellus* with bioavailabilities at Erith and Gravesend being higher than at Greenhithe and Canvey Island (Tables 2 & 3). As for copper, cadmium bioavailability to the barnacle was highest at Greenhithe and lowest at Erith (Table 3).

Lead bioavailabilities to the seaweed and amphipod did vary geographically but not consistently between the two biomonitors (Tables 1 & 2), but lead bioavailability to the barnacle showed no regional variation (Table 3).

Iron bioavailabilities to *F. vesiculosus* showed limited variation between sites (Table 1), while that to *O. gammarellus* was strikingly raised at Gravesend (Table 2). Iron bioavailability to *A. improvisus*, on the other hand, was not particularly raised at Gravesend, and was highest at Greenhithe (Table 3). The site patterns of manganese bioavailabilities to each of the biomonitors reflected that of iron (Tables 1–3).

Iron bioavailabilities to *F. vesiculosus* showed limited variation between sites (Table 1), while that to *O. gammarellus* was strikingly raised at Gravesend (Table 2). Iron bioavailability to *A. improvisus*, on the other hand, was not particularly raised at Gravesend, and was highest at Greenhithe (Table 3). The site patterns of manganese bioavailabilities to each of the biomonitors reflected that of iron (Tables 1–3).

**Table 3.** *Amphibalanus improvisus*: Concentrations (µg g⁻¹) of trace metals in barnacle bodies from three sites in the Thames estuary in 2014, expressed as either the mean with 95% confidence limits interval (unlogged data), or as the concentration ([M] with 95% CL) in a standardized body of 0.002 g dry weight as estimated from best fit double log regressions of body dry weight (g) against metal concentration (µg g⁻¹) when the accumulated concentration has been shown to be significantly related to the body weight. Also shown are 2014 results of statistical comparisons between sites using Tukey post hoc ANOVA (logged metal concentration data) or ANCOVA (double log regressions). 2014 sites sharing a common letter do not differ significantly in barnacle body concentrations of that metal (P > 0.05). Also shown are equivalent concentrations (means with 95% CL or concentrations in 0.002 g bodies [M] as estimated from double log regressions) of samples collected from approximately the same sites in 2001 (after Rainbow et al., 2002), and the results of ANOVA or ANCOVA comparisons of logged data for each site between 2001 and 2014 (*P < 0.05, **P < 0.01, ***P < 0.001, NS P > 0.05).

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</tr>
<tr>
<td></td>
<td>Erith</td>
<td>4680</td>
<td>418</td>
<td>6940</td>
<td>2880</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td></td>
<td>Gravesend</td>
<td>3790</td>
<td>458</td>
<td>4960</td>
<td>820</td>
<td>**</td>
<td>**</td>
<td></td>
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<tr>
<td>Manganese</td>
<td>Greenhithe</td>
<td>107</td>
<td>9.4</td>
<td>108</td>
<td>98.6, 116</td>
<td>75.4</td>
<td>58.6, 97.0</td>
<td>a</td>
</tr>
<tr>
<td></td>
<td>Erith</td>
<td>107</td>
<td>12.4</td>
<td>88.8</td>
<td>16.9</td>
<td>NS</td>
<td>NS</td>
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<td></td>
<td>Gravesend</td>
<td>78.8</td>
<td>9.9</td>
<td>83.7</td>
<td>34.6</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Silver</td>
<td>Greenhithe</td>
<td>19.4</td>
<td>2.9</td>
<td>18.3</td>
<td>16.1, 20.9</td>
<td>10.6</td>
<td>8.42, 16.7</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Erith</td>
<td>17.8</td>
<td>2.1</td>
<td>10.5</td>
<td>2.1</td>
<td>***</td>
<td>***</td>
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<tr>
<td></td>
<td>Gravesend</td>
<td>8.63</td>
<td>1.53</td>
<td>7.31</td>
<td>1.51</td>
<td>NS</td>
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<tr>
<td>Cobalt</td>
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<td>14.8</td>
<td>12.8, 17.0</td>
<td>14.4</td>
<td>12.9, 16.1</td>
<td>NS</td>
<td>NS</td>
<td></td>
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<tr>
<td></td>
<td>Erith</td>
<td>14.4</td>
<td>12.9, 16.1</td>
<td>11.1</td>
<td>10.3, 11.9</td>
<td>NS</td>
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<td>Chromium</td>
<td>Greenhithe</td>
<td>21.1</td>
<td>13.7, 32.4</td>
<td>10.5</td>
<td>8.8, 12.5</td>
<td>NS</td>
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<td>Erith</td>
<td>10.5</td>
<td>8.8, 12.5</td>
<td>11.1</td>
<td>10.3, 11.9</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Nickel</td>
<td>Erith</td>
<td>33.5</td>
<td>18.2, 61.5</td>
<td>32.5</td>
<td>22.6, 46.7</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>Greenhithe</td>
<td>32.5</td>
<td>22.6, 46.7</td>
<td>22.4</td>
<td>3.8, 132</td>
<td>NS</td>
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<tr>
<td>Vanadium</td>
<td>Gravesend</td>
<td>13.4</td>
<td>6.65, 27.0</td>
<td>13.4</td>
<td>6.65, 27.0</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td></td>
<td>Greenhithe</td>
<td>8.57</td>
<td>7.56, 9.71</td>
<td>8.16</td>
<td>7.07, 9.41</td>
<td>NS</td>
<td>NS</td>
<td></td>
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</tbody>
</table>

*Size effect prevents comparison by ANOVA, and significant difference in regression coefficients prevents comparison by ANCOVA.
O. gammarellus did not vary between sites (Table 2). In contrast to the rank order of sites for the seaweed (Table 1), silver bioavailability to A. improvisus was highest at Greenhithe (Table 3). In the case of cobalt, bioavailability at Gravesend was relatively high to the seaweed and the amphipod (Tables 1 & 2), but not to the barnacle (Table 3). Chromium bioavailability was particularly high to the amphipod (Table 2) and the barnacle (Table 3) at Gravesend, but not to the seaweed (Table 1). Nickel bioavailability to O. gammarellus was also relatively high at Gravesend (Table 2), but showed little or no geographic variation for F. vesiculosus (Table 1) and A. improvisus (Table 3). Vanadium bioavailability to O. gammarellus was significantly raised at Gravesend (Table 2), where it was also relatively high for A. improvisus (Table 3), but not for F. vesiculosus (Table 1).

2001 vs 2014 Bioavailabilities

Tables 1–3 also show comparisons (whether by ANOVA or ANCOVA) of accumulated metal concentrations in the three biomonitors between 2001 (from the raw data of Rainbow et al., 2002) and 2014. On occasion, the between-year comparisons needed the use of ANOVA or ANCOVA, differently from the inter-site comparison used for the 2014 data. Thus metal concentration data for some sites in Tables 2 & 3 show both mean concentrations and concentrations estimated for standard-sized amphipods or barnacle bodies. Also included in Tables 1–3, are indications of the direction of any significant change in accumulated metal concentration between 2001 and 2014. As above, intraspecific changes in bioaccumulated trace metal concentrations are interpreted as changes in total metal bioavailabilities to the particular biomonitor at the particular site, in this case between 2001 and 2014.

The bioavailabilities of several trace metals in the Thames estuary have fallen significantly, and to different extents, between 2001 and 2014 (Tables 1–3). More specifically, the bioavailability of zinc to F. vesiculosus fell significantly at three out of four sites (Table 1), and zinc bioavailability to O. gammarellus fell at three from five sites (Table 2). Zinc bioavailabilities to the barnacle A. improvisus, however, did not change significantly at any of three sites. Copper bioavailabilities to the barnacle were similarly unchanged over time (Table 3), as was the case also for the amphipod (Table 2). Bioavailabilities of copper to the seaweed did, however, fall at Greenhithe and Canvey Island (Table 1). Both cadmium and lead bioavailabilities to all three biomonitors fell significantly at all sites (with one exception of no change) where comparisons could be made (Tables 1–3). The bioavailabilities of silver to F. vesiculosus fell at all four sites examined (Table 1), but rose in the case of A. improvisus at two out of three sites (Table 3). Comparative silver data are not available for O. gammarellus.

Iron bioavailabilities are in effect unchanged to the three biomonitors. In the case of the seaweed, bioavailabilities have increased at one site, decreased at another, and remained unchanged at the other two (Table 1). A similar situation exists for iron bioavailability to the amphipod, with a decrease at one site, an increase at a second, and no change at three sites (Table 2). The increase in iron bioavailability to O. gammarellus at Gravesend is, however, considerable (Table 2), suggesting some relatively local source. For the barnacle, iron bioavailability is unchanged at two sites, and has fallen at one (Table 3). Comparative data for manganese are only available for the seaweed and the barnacle (Tables 1 & 3). Manganese bioavailability to F. vesiculosus fell at two sites, and remained unchanged at two others (Table 1). Manganese bioavailability to the barnacle was unchanged (Table 3).

Discussion

The bioaccumulated trace metal concentrations in a biomonitor represent relative measures of the total bioavailabilities of the metals to that particular biomonitor at a particular site integrated over a preceding time period. This time period may be of the order of a few months in the case of F. vesiculosus, and, so long as the frond is not in contact with sediment, the seaweed will take up and accumulate metals only from solution (Bryan et al., 1985). Amphipods store accumulated trace metals in detoxified form in the ventral caeca of the alimentary tract, the cells of which turn over in a cell cycle over a period of between 15 and 30 days (Galay Burgos & Rainbow, 1998; Luoma & Rainbow, 2008). Thus the preceding time period of trace metal bioavailabilities represented by the accumulated trace metal concentrations in O. gammarellus would be of this order (15–30 days). Talitrid amphipods, such as O. gammarellus, take up and accumulate trace metals from both solution and from their food, typically decaying seaweed in the strandline (Weeks & Rainbow, 1991, 1993). The metal concentrations in this food source essentially depend on metal dissolved in the local water, and so this talitrid might be considered as an indirect biomonitor of local dissolved metal bioavailabilities. Being a detritivore, however, O. gammarellus may also consume other material from the strandline, for example sediment particles on decaying seaweed fronds, making any reflection of dissolved bioavailabilities less straightforward.

Barnacles take up trace metals from solution through the large permeable surface area of their bodies with their extensive cirri, and from the suspended food filtered by these cirri. In the case of balanid barnacles, including A. improvisus, the filtered food consists of fine detritus particles (including re-suspended fine sediments), phytoplankton and zooplankton (Anderson, 1994). Although barnacles have high uptake rates of trace metals from solution in comparison to other crustaceans (Luoma & Rainbow, 2008), the high trophic input of metals from filtered particles, coupled with high assimilation efficiencies, mean that barnacles take up most of their accumulated metals from the diet (Wang et al., 1999a, b; Rainbow et al., 2003; Rainbow, 2007). Barnacles also have extremely low excretion rates of accumulated trace metals, which they store in the body in detoxified forms (Rainbow, 2007; Rainbow & Luoma, 2011), leading to accumulated concentrations that are extremely high amongst invertebrates (Rainbow, 2007; Luoma & Rainbow, 2008). The low excretion rates also mean that the period of metal exposure represented by an accumulated concentration in the body of a barnacle is very long, of the order of months or even more than a year (Rainbow et al., 2003; Rainbow & Luoma, 2011). For these reasons A. improvisus can, therefore, be regarded as a biomonitor of the bioavailable trace metals mostly in the suspended matter on which it has been feeding for an extended period, approximating to the lifetime of the barnacles which were probably mostly 1 year old at the time of collection (Southward, 2008).
When considering geographic changes in trace metal bioavailability along the Thames estuary, it is to be expected that the total dissolved concentrations of any metals of domestic or industrial origin upstream would be sequentially diluted with seawater down the estuary to Canvey Island. Furthermore the dissolved bioavailability of the many trace metals that exist in solution as the cation (M⁺ or M²⁺) can be modelled by the availability of the free uncomplexed metal ion (Campbell, 1995; Luoma & Rainbow, 2008). This is the case for nine of the 11 trace metals investigated here, the remaining two, chromium and vanadium, being present as oxy-anions, based on chromat and vanadate respectively (Luoma & Rainbow, 2008). The dissolved cations of Ag, Cd, Co, Cu, Fe, Mn, Ni, Pb and Zn in seawater are complexed by inorganic anions, particularly chloride ions in the cases of Ag, Cd and, to a lesser but significant extent, Zn (Luoma & Rainbow, 2008). This inorganic complexation is progressively reduced with decreasing salinity upstream, and the correlated dissolved bioavailabilities of these trace metals can be expected to decrease downstream with increased salinity, simply as a physicochemical effect of salinity change (Luoma & Rainbow, 2008). In the Thames estuary, salinity varies from 1 to 12 at Woolwich, 13 to 27 at Gravesend, to above 30 at Canvey Island (Anon, 1998). These interdisc differences in salinity are certainly strong enough to be correlated with changes in dissolved trace metal bioavailabilities (Luoma & Rainbow, 2008), as shown for zinc and cadmium in the specific case of *O. gammarellus* (Rainbow et al, 1993). This physico-chemical effect affecting the dissolved bioavailabilities of many of the trace metals would enhance any expected effect of sequential dilution of upstream metal contamination, leading to decreased dissolved trace metal bioavailabilities downstream. There are, however, significant potential sources of bioavailable trace metals into the estuary downstream of the top site Woolwich, particularly effluents from the two major sewage works at Beckton and Crossness, between Woolwich and Erith (Figure 1). It is also likely that there are other potentially significant sources of trace metals along the stretch of estuary examined. It is not surprising, then, that the descending rank orders of trace metal bioavailabilities at each site in 2014 do not systematically follow from upstream to downstream (Tables 1–3).

The apparent anomalies in the downstream changes in the bioavailabilities of many of the trace metals (for example copper, cadmium, iron, manganese and silver) between the barnacles and the other two biomonitors may be, at least partially, caused by regional differences in the bioavailabilities of the metals in the sediments. The barnacles are able to filter re-suspended sediments, and, therefore, access a source of metals not bioavailable to the other two biomonitors. Trace metals associated with sediments are held there for very long periods of years or decades without being eluted, even into uncontaminated flowing water, and yet may still be trophically bioavailable to organisms ingesting these sediments, whether by deposit feeding or by filtration after sediment re-suspension (Bryan et al., 1980, 1985; Rainbow et al., 2011). If the concentrations, and potentially the trophic bioavailabilities, of trace metals in the sediments in the Thames estuary have reduced over time (as in the case of dissolved metals – Power et al., 1999), and if Thames estuary sediments do have an expected net movement downstream by mass transport, then it is indeed possible that barnacles at sites downstream of historic sources of metal contamination are filtering locally re-suspended older sediments with relatively high metal loadings. Thus the seaweed and amphipods are sampling the present day dissolved bioavailabilities of trace metals at particular sites in the Thames, while the barnacles at these sites may be accessing a bioavailable metal source of older origin.

In the 2014 data set, the geographic patterns of manganese bioavailabilities to each of the biomonitors reflected that of iron. This should not be surprising because both trace metals often co-occur in sediments (Luoma & Rainbow, 2008).

Raised bioavailabilities of both nickel and vanadium can indicate the presence of spilled oil or refined fuel in a coastal system, for both trace metals are commonly associated with oil (Pearson & Green, 1993). There was some similarity between rank orders of sites for nickel and vanadium bioavailabilities for *O. gammarellus* (Table 2), but not a significant correlation (Spearman’s Rank). Nor was there any similarity of rank orders of sites for nickel and vanadium bioavailabilities for the other two biomonitors.

To turn to the comparison between bioavailabilities in 2001 and 2014, it is clear that the bioavailabilities of several trace metals in the Thames estuary have fallen significantly, to different extents, over this time period.

There is generally good agreement between the three biomonitors on this point, with the slight exception of the bioavailabilities of zinc and silver to the barnacles. Zinc bioavailabilities to *A. improvisus* did not change significantly at any of three sites (Table 3), perhaps partly because the barnacles are accessing zinc in re-suspended sediments, a source that does not decrease in strength over time without mass export (Rainbow et al., 2011). Similarly, the bioavailabilities of silver to *F. vesiculosus* fell at all four sites examined (Table 1), but rose in the case of *A. improvisus* at two out of three sites (Table 3). Comparative silver data are not available for *O. gammarellus*. Perhaps again the barnacles at Greenhithe and Gravesend are accessing older silver-rich sediments, transported downstream since an expected reduction in upstream silver-rich effluents originating in the photographic industry prior to the huge rise in popularity of digital photography.

As relative measures of bioavailabilities, the bioaccumulated concentrations of the trace metals, and any changes observed over time, need to be put into an international context of what is typical of estuaries today and what might be considered as atypically high. Table 4 makes such an attempt. Furthermore, Table 4 calls on historical literature data for the Thames estuary to extend the temporal comparison back in time.

The comparisons made here between 2001 and 2014 trace metal bioavailabilities in the Thames estuary follow on from existing literature data showing considerable reductions in dissolved metal concentrations (Power et al., 1999), sediment metal concentrations (Attrill & Thomas, 1995; McEvoy et al., 2000; Langston et al., 2004) and trace metal bioavailabilities to a number of biomonitors (McEvoy et al., 2000; Langston et al., 2004) from 1980 to the turn of the century. For example, there were significant decreases in accumulated concentrations of As, Cd, Hg, Ni and Zn in *Fucus vesiculosus* in the Thames estuary between 1980 and 1997 (McEvoy et al., 2000). Comparisons between 1999 and 2001 accumulated concentrations in this seaweed showed further decreases for Co and Hg, while accumulated concentrations of Cd and Pb rose.
somewhat (Langston et al., 2004). The comparison made here over the longer period of 2001 to 2014 has shown that the decrease in cadmium and lead concentrations in F. vesiculosus has been re-established. Table 4 confirms that the bioavailabilities of many trace metals, for example Cd, Cu and Zn to A. improvisus in the Thames estuary in the early 1980s were extraordinarily high. By 2014, trace metal bioavailabilities have become more typical of estuaries that are either uncontaminated or with some moderate, perhaps expected, contamination (Table 4). To generalize across the suite of biomonitor, bioavailabilities of Cd, Cr, Cu, Ni (probably) and Pb are now typical (Table 4), in the case of Pb presumably in correlation to the removal of lead from petrol at the end of the 20th century. Bioavailabilities of Ag, Cu and Zn are typical in the cases of F. vesiculosus and O. gammarellus, but are still high for A. improvisus (Table 4), perhaps in reflection of re-suspended older sediments still being accessed trophically by the barnacles. Bioavailabilities of Fe and (to some extent) Mn in 2014 are high (Table 4), perhaps reflecting high sediment loadings in the estuary. More comparative data, however, for the trace metals less often analysed, for example Co, Cr, Ni and V, are needed to strengthen these conclusions.

The general conclusion to be drawn from this study is that the bioavailabilities of several trace metals in the Thames estuary have continued to fall significantly, to different degrees, between 2001 and 2014, in reflection of the continuing remediation of the Thames from its severely polluted state in the middle of the 20th century. Encouragingly, the biodiversity of the Thames has continued to recover from its previous desperate condition, but there is still room for further improvement (Henderson & Rainbow, 2012). Further falls in the bioavailabilities of trace metals in the Thames estuary can be expected as local environmental management proceeds, and in the longer term historically contaminated sediments (Attrill & Thomas, 1995) are transported downstream and out of the estuary.

The continuing reductions in the bioavailabilities of toxic metals in the Thames estuary do also have some significance for human health. The outer Thames estuary is a source of shellfish such as mussels, oysters, cockles, winkles and prawns for the human seafood market. Samples of such shellfish in 2001 contained accumulated concentrations of toxic metals within public health safety guidelines, although the margins of safety were not always very large (Langston et al., 2004). It is reassuring that falling toxic metal bioavailabilities will continue to increase such safety margins over time.

**SUPPLEMENTARY MATERIAL**

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0025315415001952

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CONFLICT OF INTEREST

None.

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