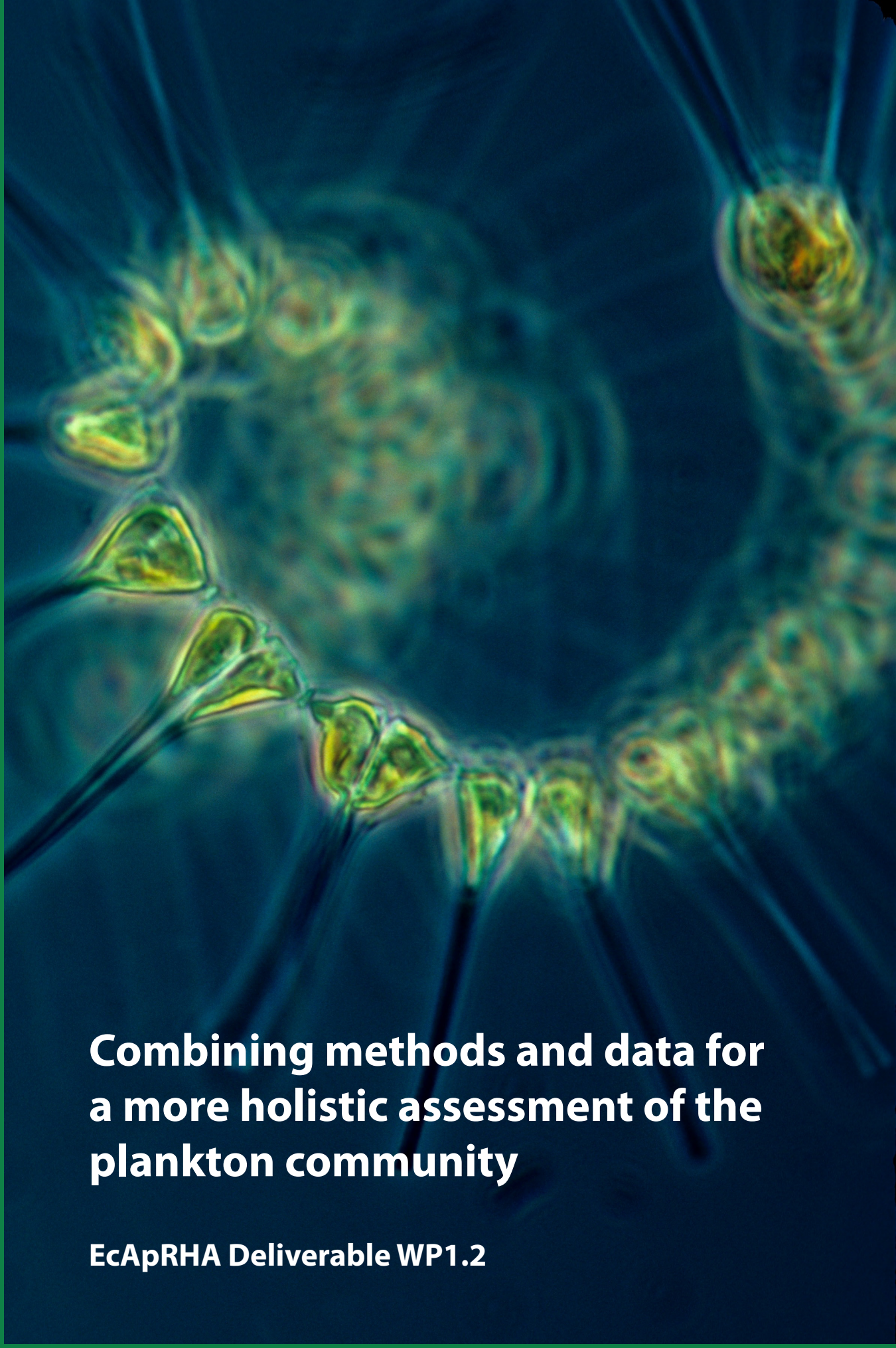

Combining methods and data for a more holistic assessment of the plankton community

EcApRHA Deliverable WP1.2

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EcApRHA an Ecosystem Approach to Regional Habitat Assessments

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Contents

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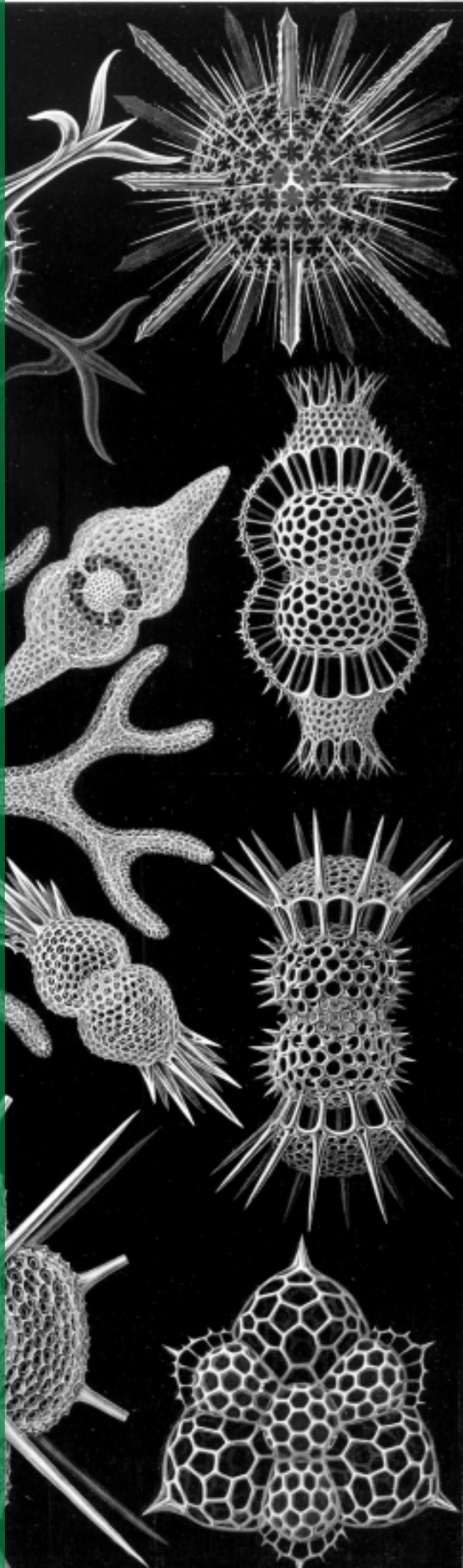

EcApRHA

The EcApRHA project (Applying an Ecosystem Approach to (sub) Regional Habitat Assessment) aims to address gaps in the development of biodiversity indicators for the OSPAR Regions. In particular, the project aims to overcome challenges in the development of indicators relating to the MSFD (Marine Strategy Framework Directive 56/2008/EU), such as Descriptor D1 (Biodiversity), D4 (Food webs) and D6 (Seafloor integrity), and to deliver an action plan to OSPAR that will enable monitoring and assessment at the (sub) regional scale, to contribute to OSPAR Intermediate Assessment 2017.

Indicators related to the benthic and pelagic habitats, as well as food webs, are investigated within the project at different levels (from data to indicator; from indicator to habitat assessment; from habitat to ecosystem assessment).

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Executive Summary

Plankton has a high potential in terms of their use as indicators of Good Environmental Status for marine management; this is notably due to their short life spans and thus rapid response. Three plankton indicators are currently being developed in OSPAR including to support the ongoing Marine Strategy Framework Directive policy mechanisms in Europe. These indicators are state indicators and have been based mostly on microscopic counts on samples gathered by classical collection methods (hydrographic bottles or plankton nets) or by the Continuous Plankton Recorder. These indicators are thus addressing the taxonomical diversity of only a defined size range of plankton organisms (microphytoplankton and mesozooplankton) as well as chlorophyll *a* concentrations or estimates. However complementary technologies and related types of data are existing, which would enable us to better understand plankton dynamics in relation to environmental changes and anthropogenic pressures. This report reviews the existing methods, which complement microscopic counts data and that could be used for informing the metrics necessary for the calculation of the plankton indicators developed through OSPAR. The usefulness of these methods and different types of data in the light of the most recent scientific findings for plankton is also underlined. Future lines of work and recommendations for the further development of the OSPAR plankton indicators are proposed.

Acronyms

Chl *a*: Chlorophyll *a*

CPR: Continuous Plankton Recorder

CTD: Conductivity, Temperature and Depth

FCM: Flow CytoMetry

FlowCam: Flow Camera

GES: Good Environmental Status

IFCB: ImagingFlowCytobot

LED: Light-Emitting Diode

LOPC: Laser Optical Plankton Counter

MSFD: EU Marine Strategy Framework Directive (2008/56/EC)

OCR: Optical Character Recognition Process

OPC: Optical Plankton Counter

OSPAR: The Convention for the Protection of the Marine Environment of the North-East Atlantic

PCI: Plankton Colour Index

PH: Pelagic Habitat (in PH1, PH2, PH3)

PSR: Polymerase Chain Reaction

UVP: Underwater Vision Profiler

VPR: Video Plankton Recorder

WFD: EU Water Framework Directive (2000/60/EC)

µ: micron

1 Introduction

Plankton organisms have short life-spans (from days to up to 5 years), which is a characteristic of interest in the frame of investigation of indicators of environmental status for marine management. Indeed, the short-life spans of most plankton give them the ability to respond more quickly than higher trophic levels to potential environmental or anthropogenic perturbations although higher trophic levels have been better considered in the marine management processes so far. Large scale studies focusing on copepods have shown for instance that they are the most evident example of a biogeographical shift toward the pole due to global warming, this shift being the largest and the fastest among marine and terrestrial biota (Beaugrand et al., 2002; Richardson, 2008, Poloczanska et al., 2013). This characteristic (response-time) should be prioritized in the goal of management measures (Lehtinen et al., 2012). Secondly, their small-size (except for some jellyfishes) and the fact that they are ubiquitous in the ocean are advantages for large-temporal scale monitoring programs.

Plankton is considered through different aspects for the development of indicators in the goal of marine management. In Europe, the Marine Strategy Framework Directive (MSFD) considers plankton in four descriptors: Biodiversity (D1), Non-indigenous species (D2), Marine food webs (D4), and Eutrophication (D5) (European Commission, 2010). The biodiversity descriptor aims to describe the state and change in biological components and their habitats. Within the OSPAR Regional Sea Convention – the mechanism by which by which 15 Governments & the EU cooperate to protect the marine environment of the North-East Atlantic - plankton habitats are considered through three common indicators. These indicators consider plankton communities at different organizational levels: Changes in plankton abundance/biomass (PH2) at the broadest organizational level, changes in plankton lifeforms (PH1) at an intermediate level since it considers functional traits to group plankton taxa (McQuatters-Gollop et al., 2014; Tett et al., 2008), and changes in plankton diversity (PH3), at the finest level of organisation, if possible down to the species level (cf. OSPAR a, b and c(In Prep.)). A modified version of the PH2 indicator for zooplankton is also proposed as a candidate indicator for food-web (Zooplankton mean size and total abundance, FW6, cf. OSPAR d (In Prep)) and considers biomass of zooplankton (and aims to use zooplankton biomass per size spectra). The calculation and testing of these indicators have been based on microscopic counts only, performed on samples taken by hydrographic bottles as well as plankton nets or with the Continuous Plankton Recorder (CPR) meshes, except for the PH2 phytoplankton indicator, which uses total chlorophyll *a* (chl *a*) or the CPR Colour Index (PCI), a proxy for phytoplankton biomass (for details about these techniques and analysis, cf. OSPAR a, b, c, (In Prep); and Richardson et al. 2006). Indeed, long-term data sets on plankton are predominantly based on taxonomical microscopic work targeting only a size fraction of the plankton community (microphytoplankton, mesozooplankton), such as the long station plankton time-series of Helgoland Roads, Germany (Wiltshire et al., 2010); Marine Scotland Science, Stonehaven (Bresnan et al., 2015); Plymouth Marine Laboratory L4, UK (Harris, 2010); or Stazione Zoologica, Naples (Zingone et al., 2010); providing highly resolved taxonomic information for at least two decades. Other data-sets examples at a large spatio-temporal scale are the Continuous Plankton Recorder (CPR) and California Cooperative Oceanic Fisheries Investigations (CalCOFi), which have been monitoring plankton communities for 85 and 55 years, respectively (Edwards et al., 2010). Identifying and enumerating plankton using light microscopy is a long and precise taxonomical work carried out by experts. Historically, studies examining community composition have used data from datasets such as these comprising number and abundances of species.

In the last decades, the advancements in technologies offer the possibility to consider plankton data analysed through methods other than light microscopy. We are indeed in a new era of high-throughput

data linked with more advanced techniques such as 'omics' approaches and *in situ* single-cell optical characterisation and/or imaging technologies, together with new advances in automated classification, statistical and mechanistic modelling techniques (e.g. Follows et al. 2007). This has enabled advancement in our description and understanding of biodiversity and ecosystem processes and their responses to changing environmental conditions and anthropogenic pressures at unprecedented temporal and spatial scales (Root et al., 2003, Doney et al., 2012), and notably by considering other aspects of biodiversity than just taxonomic diversity. As mentioned, biodiversity has been so far assessed in the frame of the OSPAR plankton indicators development through taxonomical counts (except for chl *a*). Taxonomical diversity alone is thus considered and only for a fraction of the total plankton community. However, biodiversity, is a multifaceted concept that includes not only genera and species (taxonomical units), but also traits and evolutionary units so that organisms can also be classified according to their size, their morphology, genetics, functional/structural properties and/or phylogenetic relatedness. In relation to anthropogenic pressures and climate induced changes, which are the focus of any environmental and biological community management, responses of plankton communities will depend not only on their underlying taxonomic diversity, but also on their genetic and functional diversity. Genetic variation, for example, is assumed to reflect the ability of a population to adapt to changing environments (Fisher, 1930; Barrett and Schluter, 2008) where low genetic diversity would indicate that the species do not have sufficient adaptability and may not be able to survive an environmental hazard. Whereas losses in genetic diversity will weaken the capacity of a species to adapt, losses in functional diversity could affect the functioning of an entire ecosystem. This shows that if an only strictly taxonomic view of human impacts on ecological communities is adopted, important trait changes that are key to ecosystem functioning and stability could be concealed (Fisher et al., 2010). Hence, there is a current trend to integrate these "new" types of diversity and examine their different patterns of change together (Pavoine and Bonsall, 2010) since species, trait and phylogenetic diversity have been shown to be connected and complementary (Naeem et al., 2012). In conservation management, metrics of ecological function have been found to complement metrics of species diversity, including when identifying planning priorities and tracking changes to biodiversity values (Stuart-Smith 2013). Nowadays, studies of biodiversity and conservation objectives have thus shifted the emphasis away from key estimations of species diversity to trait- and phylogenetic approaches (Hardy and Senterre, 2007; Cavender-Bares et al., 2009; Pavoine et al., 2010). However, while such knowledge has expanded for many types of organisms or specific ecosystems, particularly for terrestrial ecosystems (Root et al., 2003), few studies have been applied to the marine plankton realm despite both methods and data being available.

Considering the potential of plankton as indicators for marine management and the on-going development of marine policies, trying to better understand plankton dynamics and response to environmental changes and anthropogenic pressures should be prioritised. High-throughput methods but also complementary information such as phylogeny, functionality and genetic information have a great potential to help this understanding. Since the ongoing development of European marine policies have not yet considered these methods and types of data other than light microscope taxonomy for plankton (and for only a part of the total size-range of plankton), this report will provide useful information for their future potential consideration. In the first section, the different existing complementary techniques available for plankton analysis, depicting the type of data they provide and their advantages and shortcomings in the frame of the development of regionally coherent plankton indicators for implementation of the MSFD through OSPAR, will be presented. In the second part, the usefulness to consider different facets of biodiversity such as genetic, functional and phylogenetic diversity, and most of all to combine this information in order to assess plankton community changes related to environmental changes and anthropogenic pressures, will be presented in light of the recent scientific findings.

Summary

- Plankton organisms present aspects of particular interest, notably their response time, in the frame of indicator development for assessing marine water good environmental state
- OSPAR plankton indicators undergoing development is based on taxonomic counts (except for phytoplankton biomass proxy such as chl *a*), thus assessing only the taxonomical diversity
- Other technologies (high-throughput techniques, genetic, phylogeny,…) have the potential to enlarge our understanding of the plankton communities in relation to environmental and anthropogenic pressures
- These techniques should be considered in the frame of OSPAR plankton indicator development
- This report firstly reviews these different techniques and then in a second time, shows how they can be useful to enlarge our understanding of plankton dynamic for the indicator development

2 Complementing light microscopy: methods for the acquisition and analysis of plankton

As mentioned earlier, we are in an era of high-throughput data thanks to the advancement in technologies (particularly semi-automated techniques) doubled with substantial improvements in data analysis tools, which have allowed the depiction of biodiversity at an unprecedented spatio-temporal scale. For plankton, the acquisition and analysis techniques have been particularly developed and improved in the past decade in order to 1) consider a wider size-range of the plankton community; 2) decrease analysis time compared to microscopic counts which require long hours of highly trained specialists using microscopes; 3) reduce the human bias linked to fatigue, inexperience and heterogeneity in taxonomical knowledge of operators that affect the quality of the analysis (Culverhouse et al., 2003); 4) improve the quantity of samples analysed by improving the speed of analysis; 5) to enlarge the spatio-temporal coverage of data thanks to the improved time and quantities that can be analysed; and 6) provide other levels of information in addition to standard taxonomic counts or chl *a* content, such as genetic or phylogenetic information.

In general, innovative methods complementary to microscopy are often constrained by the size of target organisms due to the technical properties of analysing devices. This is particularly obvious for plankton organisms since they can range from less than 1 µm to several meters size length. The following plankton size-groups are the most commonly considered: picoplankton (0.2–2 µm), which includes heterotrophic prokaryotes (Bacteria and Archaea) and the smallest phytoplankton (picoeukaryotes and cyanobacteria); nanoplankton (2–20 µm) which includes phytoplankton as well as nano-heterotrophic protists, microplankton (20–200 µm) which also includes phytoplankton and unicellular zooplankton (microzooplankton); mesoplankton (200 µm–2 mm) mainly including multicellular zooplankton, e.g. copepods, but can also include some large phytoplankton, and macro- (2-20 cm) and mega-plankton (20–>200cm) which includes large zooplankton such as fish larvae's and jellyfishes. We will use these size classes in the description of the different methods allowing acquiring and/or analysing plankton communities in this section.

Innovative complementary techniques to traditional microscopy have particularly flourished in the context of large spatio-temporal scale data acquisition. Due to local but parallel developments in different institutes, several names can exist for devices performing the same type of analysis, which can bring confusion. These semi-automated techniques as well as other techniques, which have the ability to complement light microscopy, are presented in this section. Techniques targeting more generally phytoplankton and smaller size classes are first presented before to present the ones targeting larger organisms, from large microplankton to macroplankton. The abilities of each technique to provide the metrics used in the frame of the OSPAR plankton indicators are presented for each main type of technique as a contoured paragraph within the section. These information and the main advantages and disadvantages of each technique presented are also synthetized in a table (Table I) at the end of this section 2.

2.1 Techniques targeting phytoplankton and similar and/or smaller plankton size-classes

2.1.1 Inflow systems

The inflow techniques most developed for the small plankton part, in addition to traditional microscopy, uses the flow cytometry technology. In comparison with light microscopy which provides taxonomic detail, inflow systems allow to process larger sample volumes without the need for preservation (when applied *in*

vivo), at high speed with reduced reliance on the subjective visual inspection skills required in light microscopy. It thus reduces human intervention and the dependence on the operator for the acquisition and sample analytical steps (Alvarez, 2013). The total amount of data produced is even more important for devices incorporating the imaging technology, and particularly for *in situ* devices, allowing good resolution spatio-temporal datasets. Specialists are required for the maintenance of the machines and the data-analysis. Indeed, the complexity and large amount of data produced also requires large electronic storage capacities and strong analytical tools for their interpretation. If these techniques seem really promising, it is important to stress that the taxonomical level achieved is in general (depending on the magnification of the pictures taken) not often at the species level, meaning that these techniques have limitations providing robust information needed for diversity indicators (e.g. PH3 indicator). Nevertheless, by combining optical and image data, they could provide useful information needed for functional group-level indicators, as well as biomass and abundance. The main different types of flow cytometry techniques are depicted below.

Traditional flow cytometry

Flow cytometry (FCM) consists of generating a fluid stream containing the sample of interest which goes through a beam of light allowing analysis of the physical and chemical characteristics of each particle (cell or colony) it contains (Shapiro, 2003), generating a set of optical parameters defining each cell/colony analysed, in a "high-throughput" automated way. Indeed, flow cytometry is based on the optical characterization of cells/colonies based on their induced fluorescence (depending on their pigmentary composition or to the wavelength of the fluorochrome employed for non-pigmented cells), forward (size) and sideward scatter (composition), recorded as maximum response or as whole optical profiles (pulse shapes). Flow cytometry is thus able to analyse thousands of particles every second in "real time", with the ability to run particle separation based on specified properties. This technique was first developed in the 60's and started to be commercialized in the 70's but has been in widespread use since the 90's. Traditional flow cytometry can theoretically analyse any cell's particle ranging in size between 0.2 and 150 µm, thus enabling analysis of bacteria to large phytoplankton and some microzooplankton species (Shapiro, 2003). It is a useful technique for automated enumeration and size identification, producing precise estimates of the abundance and size of particles some of them, which are completely invisible to traditional microscopy (pico- and some nano-plankton cells). FCM has brought notifiable improvement to our understanding of marine plankton communities (Blanchot and Rodier, 1996; Gasol and del Giorgio, 2000, Veldhuis and Kraay, 2000; Li et al., 2009).

Pulse shape-recording flow systems

Improvements to flow cytometry have also become available, enlarging the size spectra of cells analysed and the type of results produced. This is particularly true with the dedicated pulse shape-recording automated flow cytometers from the CytoBuoy© company (CytoSense, CytoSub, Cytobuoy; Dubelaar et al., 2004) which are able to detect and characterize cells from 1 to 800 µm width and several mm length, then allowing to consider the whole size spectra of phytoplankton in "high-throughput" automated way and with the possibility of continuous recording from ships and fixed (mooring) stations. More details, as well as a proposed operational protocol for implementation, analytical tools and examples of combined techniques for phytoplankton monitoring in the field are provided in the deliverables of the DYMAPHY project (www.dymaphy.eu).

Imaging in flow systems

Another improvement of flow cytometry is notably the combination with the semi-autonomous imaging technology creating a new generation of flow cytometers, such as FlowCam (Alvares et al., 2011), CytoBuoy cytometers (Pereira et al., 2014; Dugenne et al., 2015), or Imaging FlowCytobot (IFCB) (Sosik and Olson 2007). These machines enable a better classification of plankton groups sometimes up to species level, based on image-analysing within up to a 10-800 µm size range, but also enable to analyse filamentous algae (up to 4mm length). They can also provide the chlorophyll fluorescence for each picture objects (Campbell et al. 2013). The FlowCam is a device which is used in the laboratory (Nour et al., 2014) while the CytoSub, CytoBuoy and IFCB provide similar type of analysis but *in situ*, generating optical profiles and/or images of particles (cells or colonies) in-flow taken from the aquatic environment. The IFCB is currently used for discriminating species from image analysis, whereas the CytoSub and CytoBuoy types base their analysis mainly on optical profiles and feature combinations. The great advantage of these *in situ* techniques is that they enable the production of a large quantity of data over long deployment duration, without any fixatives that could damage plankton cells.

Techniques usefulness for informing OSPAR Indicators metrics:
The use of flow cytometry (traditional, pulse shape-recording and/or imaging in flow systems) for building phytoplankton composition indicators for the Water Framework Directive (WFD) was recently explored in France through a contract between a national agency (ONEMA), IFREMER and CNRS. The first deliverables set the basis of further exploration of the determination of functional groups by this technique, combined to other techniques (Artigas et al., 2014 a, b; Artigas et al., 2015; Breton et al., 2017). Data gathered by pulse shape-recording FCM or by traditional flow cytometry could be used for calculating some of the lifeform pairs of the OSPAR PH1 indicator (completion of the large vs. small phytoplankton lifeform pair, proposition of new lifeform pairs), PH2 indicator (total red fluorescence of cells assimilated to total chlorophyll *a* fluorescence). FlowCam and IFCB can permit the calculation of diversity indices (PH3 indicators) based on different metrics than the ones provided by traditional microscopy.

In vivo fluorescence

In vivo fluorescence is one of the most common monitored parameters in oceanography, as being most often measured in combination to basic physical parameters (temperature and salinity), through various types of commercial benchtop and *in situ* sensors (the measurement is widely implemented in ferry boxes, moorings, research cruises, etc…). It has been introduced in the 60's in order to monitor the changes in phytoplankton biomass (Babin et al., 2008). One of the advantages of this method is that the often combined measurement with physical parameters allow to produce data for the water-column vertical profile, and thus not only for surface (compared to satellites outcomes for instance). The widely implementation of this method have produced large spatio-temporal coverage data sets. *In vivo* fluorescence can provide a proxy for phytoplankton biomass (assessment of the total chlorophyll a concentration) for the PH2 indicator and, when using a multi-spectral device (Beutler et al., 2002), can provide information on spectral pigmentary groups composition (which could be used for PH1; for more details see www.dympahy.eu and the JERICO-Next project (H2020; JERICO-Next, 2015-2019; www.jerico-ri.eu)."

Technique usefulness for informing OSPAR Indicators metrics:
Total *in vivo* fluorometry has the potential to inform PH2 indicator, and when using a multi-spectral device, can inform PH1 indicator.

2.1.2 Pigments analysis

Pigment analysis aims at detecting or analysing the pigment content of cells and thus targets photosynthetic organisms (phytoplankton) only. The most used pigment marker, chl *a*, has been recognized for a long time in marine oceanography as a strong indicator of biomass production for oceanic phytoplankton (Jeffrey et al., 1997).

The most accurate method for determining pigments is High Performance Liquid Chromatography (HPLC). HPLC allows for estimating the precise concentration of pigments in the whole size range of phytoplankton species, by collecting cells into filters and extracting the pigments on an organic solvent, that will be further analysed and chromatogrammed in order to identify the different pigments present in the sample. To further address the phytoplankton community biomass composition, in terms of pigmentary-class specific chl *a*, conversion of pigment data is required. This can be achieved using either multiple linear regression analysis or matrix factorisation methods. However, conversion of pigments into class apportioned chl *a* is not straightforward. Some pigments are indeed unambiguous markers of specific phytoplankton groups, whereas the pigment to chl *a* ratios varies for different species and even within a species, according to environmental factors including light intensity, nutrient concentrations and growth rate (Henriksen et al., 2002; Garibotti et al., 2003; Llewellyn et al., 2005). The most reliable and best developed of these methods is a matrix factorisation software programme, CHEMTAX (CHEMical TAXonomy), developed by Mackey et al. (1996), which seeks to accommodate for potential changes in class pigment:Chl *a* ratios by subsetting the data according to known environmental conditions (Llewellyn et al., 2005; Muylaert et al., 2006).

The fact that pigment analysis is easily performed makes it more relevant than microscopy in the scope of large scale monitoring (Sherrard et al., 2006). The method also allow a more homogenous calibration between devices (as less dependent on human operator) in comparison with microscopy making it more inter-comparable between institutions (Havskum et al., 2004) and is better adapted for fragile forms that are often not well preserved through fixative procedures (Ansotegui et al., 2001). However, since this technique does not provide a taxonomical identification, a complement with rapid light microscopy screening is recommended in general.

Technique usefulness for informing OSPAR Indicators metrics:
Indicator using HPLC definition of pigmentary groups was proposed in coastal waters for the French WFD by Lampert (2015). Data gathered by HPLC could be used for calculating PH1 (proposition of new lifeform pairs) and PH2 (total chlorophyll *a*). It is less likely that the technique could be used for the calculation of diversity indices, such as PH3 indicator. This will require further investigations as the technique alone discriminate main large plankton groups, some of them belonging to the same taxonomy group.

2.1.3 Epifluorescence

Epifluorescence microscopy works the same way as a light microscope but instead of using light reflection and absorption, it uses fluorescence and phosphorescence in order to study properties of organic and inorganic compounds. It allows the discrimination of plankton types in term of functional groups, autotrophic-myxotrophic (pigmented cells) versus heterotrophic and allows a finer size resolution than inverted microscopy (epifluorescence microscope is able to reveal the presence of a single molecule). The results obtained are fluorescence-emitting cells in different wavelengths according to the excitation wavelength chosen, the pigments content or the fluorochrome applied. For instance, applying the

fluorescence in situ hybridization (FISH) technique would enable in situ phylogenetic identification and enumeration of individual microbial cells by whole cell hybridization with oligonucleotide probes (Amann et al. 1995). Most of the time, epifluorescence is performed in fixed samples (as for pico- and nanoplankton counts, benefiting from auto-fluorescence properties of phytoplankton pigments) but it is also applied in vivo for microplankton (dinoflagellates, ciliates). The fact that it does not necessarily require preservation of the sample in fixative products is one of the great advantages of this technique since fixative products have been shown to degrade the chl *a* pigments (Havskum et al., 2004). Abundances of pico- and nano-phytoplankton derived from this technique have the potentiality to be transformed into biomass, which could be used as a proxy of phytoplankton biomass (PH2 indicator) with the same confidence level as PCI (Plankton Colour Index, from the CPR) or remote sensing."

Technique usefulness for informing OSPAR Indicators metrics:
Epifluorescence could, as for Flow cytometry, be used to complement light microscopy in improving the confidence of PH1 lifeform pairs, as small vs. big phytoplankton, heterotrophic vs. autotrophic and mixotrophic dinoflagellates.

2.1.4 Remote sensing

Remote sensing consists of the acquisition of data without physical contact with the object of study. As such, sonar technology (already described in section 2.1.4) can be considered as remote sensing. Earth observing satellites are notably able to measure the characteristics of light, or radiance, coming from the Earth's surface. This information is used as a proxy for chl *a* concentration, which is applied as a proxy for phytoplankton biomass and which allows a quasi-permanent monitoring of chl *a* at the earth's surface. This is possible using the radiance calibration between water samples analysed for chlorophyll content linked with the output of ocean colour sensors fixed on the satellites. Chl *a* measurements are then back calculated from water-leaving radiances (nLw) provided by the satellites with specific algorithms. This approach allows also, when combined with other algorithms and combined to *in situ* optical measurements (PhySat method), identifying the main taxonomical groups of phytoplankton (diatoms, haptophytes, cyanobacteria) (Alvain et al., 2005; Thyssen et al., 2015). The near global spatio-temporal coverage allowed by such devices shows the usefulness of such data sets in calculating the phytoplankton annual mean biomass, in order to produce ecological indicators, with a low error for 95 % of the global oceans, for instance in the study of Racault et al. (2014). The geographical localisation of these types of data should be further investigated to see if the spatial resolution of current satellites could be pertinent in the frame of the OSPAR indicator testing at the eco-hydrodynamic scale.

Technique usefulness for informing OSPAR Indicators metrics:
These types of data would be useful for the calculation of the PH2 indicator for phytoplankton, since the technique provides chl *a* proxy at the large spatio-temporal scale. Some lifeform pairs of the PH1 indicator could also be potentially addressed with this technique if combined with *in situ* optical measurements. The technique cannot however address the metrics required for PH3 indicators as it discriminates plankton groups based on their pigment composition, as such that no taxonomic resolution is achievable.

2.2. Techniques targeting zooplankton and large phytoplankton cells

The development towards depicting organisms larger than phytoplankton (but also particulate matter), more generally meso- and macro-zooplankton, have been focused on semi-automated techniques for obvious reasons (cf. first paragraph of section 2). All of the following techniques presented in this section are thus semi-automated ones.

2.2.1 Optical Plankton Counter (OPC) and Laser Optical Plankton Counter (LOPC)

Within the Optical Plankton Counter (OPC)(Herman, 1988) and Laser Optical Plankton Counter (LOPC)(Herman, 2004), the flow of water with plankton organisms and particles pass through a tunnel equipped with the light emitting diodes-LEDs (OPC) or with a laser reading system (LOPC). This device has been developed for large particles with a target size spectra of 100 µm to <3 cm, thus allowing analysis from micro-plankton to meso-zooplanton. They allow measuring the optical density and cross-sectional area of each particle while passing through the sampling device, and thus allow estimation of the biomass of each individual as well as the total biomass of the sample (Suthers et al. 2006; Basedow et al., 2010). Particle (plankton) counters are instruments that are generally used *in situ*, mounted on a towed undulating vehicle that is used underwater, but they are compact enough to also be mounted on autonomous underwater vehicles or even on an oceanographic float, such as the Sounding Oceanographic Lagrangian Observer (SOLO, Checkley et al., 2008), so reducing the ship time required. They can also be used in the laboratory for processing of plankton samples that have been obtained using a net. The LOPC can be towed at up to 12 knots, which allows surveys of very large areas. Data are recorded continuously but the internal processor collates data every half second. At a 12 knot towing speed this equates to a spatial resolution of 3 m. These devices have been in use for more than 20 years and have been shown to produce useful data for the understanding of marine plankton ecology (Marcolin et al., 2013).

Technique usefulness for informing OSPAR Indicators metrics:
These devices only produce size spectra results and as such are unable to identify plankton taxonomically. The only way to relate these size particle results to taxonomy is to run plankton nets in parallel at the same locations, which will require taxonomical identification analysis by light microscopy (Rissik et al., 1997; Gaardsted et al., 2010). Thus, OPC and LOPC have limited abilities in complementing light microscopy and to inform the OSPAR common plankton indicators. They are interesting tools in providing quick results on biomass per size spectra, thus the metrics required for the FW6 plankton indicator, without the possibility to relate these size spectra to taxonomical groups unless long time-consuming work is added.

2.2.2 Imaging systems

All of the semi-automated imaging devices, despite different functioning modes, provide pictures of every single organism or entity (whether organic or not) in the sample by isolating each object passing in front of the analyser. The enormous amount of images produced needs to be automatically pre-grouped (manually grouping would require an enormous amount of time). Automated pre-grouping into large taxonomical groups is then possible, as well as size-spectra analysis of the whole sample community (as done with the previous devices presented, CytoSense, IFCB, FlowCAM, OPC and LOPC). Software running this pre-grouping have been particularly improved (and are in constant improvement), using specific statistical methods, and have shown to be able to produce high success of automatic classification for the most common taxa, comparable to what can be achieved by traditional microscopy by a trained biologist (considering only

these taxa of course) (Tang and al., 1998; Gorsky et al., 2010). The pictures are also associated with morphological data (i.e. size) allowing getting total- or group-specific biovolumes and/or size spectra of the zooplanktonic communities. However, these devices are called semi-automated since the production of these taxonomic and associated size results require first the definition of the training set, pictures checking and then re-grouping by a trained expert after the initial autonomous analysis. Zoo/PhytoImage (developed by Ifremer France, cf. Nour et al., 2014) is an example of such software which is free on request. Online collaborative tools allowing helping the recognition of plankton pictures produced by imaging systems became also available such as Ecotaxa (developed by Villefranche-sur-Mer laboratory in France, http://ecotaxa.obs-vlfr.fr/). Even after these steps, the taxonomic resolution attained is rarely possible down to the species level, but good identification to the family or genus level is achievable (Davies et al., 1992, Benfield et al., 2007).

Similarly to Flow Cytometry, IFCB and FlowCAM, some of these devices allow *in situ* deployment (UVP, VPR and ZooCAm), and thus a high geographical coverage, and as well the possibility to analyse the samples directly on board for the ZooCam (cf. following paragraphs).

Concerning their shortcomings, it has to be mentioned that operator-dependent sample preparation is also often required before the imaging analysis and the deployment of some techniques, such as the UVP and VPR systems, requires on-board trained technicians. Their technological development is however moving toward autonomous systems. The large amount of data produced by these techniques requires large electronic storage capacities and strong analytical tools for their interpretation. For the plankton scanners (used in laboratory conditions), the samples are first collected with a plankton net, rendering the sampling process similar to the reference one, analysed by light microscopy.

We present four main types of semi-automated imaging analysis tools, which come in addition to imaging flow cytometry and in-flow image acquisition, presented previously in the “Flow cytometry” section.

UVP (Underwater Vision Profiler)

The UVP5 (last version of the UVP) is an instrument used *in situ* as a sensor embedded to the CTD system on research vessels. It can be used down to 6000 m depth. It uses an intelligent camera and a powerful lighting system to record images in a fixed volume of water making the results independent from descending speed. The UVP images zooplankton and large phytoplankton above 700µm, as well as suspended particles above 100µm. Pressure and angle sensors are included in the system to label the images. As all *in situ* imaging systems, it provides detailed vertical plankton information (in contrast with instruments dedicated to net collected samples which gives water-column integrated plankton information). The device can also be used incorporated into autonomous underwater vehicles and drifting (but also geostationary) moorings, thus allowing large spatio-temporal data sets acquisition. In addition, the UVP has been developed since the 90's, providing already a large inter-calibrating data base over the global ocean (6500 inter-calibrated profiles). For more info, see the specific section in chapter 2 of Le Galliard et al. (2012) (but not updated for technological advancements made after 2012).

Video Plankton Recorder (VPR)

The Video Plankton Recorder (VPR) can be thought of as an ‘underwater microscope’ which can detect plankton and/or particles, from 100 µm and up to few centimetres in size, in real time (Davies et al., 1992). The instrument is composed of two parallel arms, with a camera and a strobe light mounted facing each

other on each arm. This instrument is used generally in situ and towed underwater (but it can be used in the laboratory as well). The latest version of the instrument can be towed at a speed of up to 12 knots and takes pictures at a rate of 30 per second. At a speed of 12 knots, for example, a picture is taken every 20 cm. The principal shortcoming of this technique is related to the focal length of the camera. In order to have clear image, the focal length needs to be short which implies that only a small volume of sample can be analysed. Increasing the analysis volume involves increasing the focal length, which most often leads to the production of images too blurry for a proper analysis. This type of device thus cannot be used in highly eutrophic areas such as part of the Celtic Seas, the Bay of Biscay, the North Sea or English Channel during the productive season and is better adapted to oligotrophic waters, such as the Mediterranean Sea (personal communications). In addition, the images provided by the VRP device are three-dimensional objects in arbitrary positions and orientations rendering the picture analysis complex.

ZooCam

The ZooCam is a benchtop in-flux imaging prototype developed by Ifremer (France) enabling quasi real-time imaging of living zooplankton samples collected by nets or pumps directly on board, as well as laboratory analysis of preserved zooplankton samples (ICES, 2014). After collection, the zooplankton sample is aliquoted and then poured into a gently stirred tank. A peristaltic pump drives the fluid from the tank through a cell where the fluid and its content are imaged in a continuous flow. The sample is finally recovered on a sieve of appropriate mesh and concentrated for long-term storage in a preservative fluid. The system saves sequences of raw images of zooplankton ('films') that are analysed with classical and robust image analysis methods, as described in the introduction for semi-automated imaging analysis. The great advantage of this technique is that it allowed direct analysis on board.

Plankton scanner

Plankton scanners are similar to basic office scanners, allowing the production of a digital image of what has been placed on the glass window of the scanner. Instead of using an optical character recognition process (OCR), the plankton scanning technology allows the automatic detection of particles according to size and shape producing raw images of zooplankton. Samples are scanned as a whole. Plankton scanning requires a careful deposition of the sample on the window glass and preparation of the sample, and thus can only be used in a laboratory (not on board). Indeed, particles need to be carefully separated from each other in order to perform analysis correctly. While some specific devices have been developed through different institutes, such as the ZooScan (Grosjean, 2004), simple office scanners can be turned into plankton scanners as shown in the study of Uusitalo et al. (2016). However, the use of the same device and software allow procedures to be standardized and to ensure better inter-comparability of data, which is required in the frame of potential metrics monitoring used to inform the regional OSPAR indicators. In general, a scan contains between 1500 and 2000 individuals <0.5 mm (Grosjean, 2004). Large numbers of images are produced at a two-dimensional scale which lowers the occurrence of arbitrary positions and orientations of the objects compared to the three others semi-automated imaging systems presented above.

Technique usefulness for informing OSPAR Indicators metrics:

These methods, as flow cytometers and imaging inflow devices, have to be seen as complementary to light microscopy as they allow size-spectra analysis, processing a higher amount of samples, or grouping into large taxonomical groups. In some cases, they allow a taxonomical resolution down to genus or even species (but for a restricted number of species compared to light microscopy, and mostly after image

validation by expert). These techniques can provide quick useful biomass information per size spectra required for the FW6 indicator and for some lifeforms of the PH1 zooplankton indicator, with a relatively good precision and at large spatio-temporal coverage. They can also provide total abundance counts of mesozooplankton or copepods necessary for the PH2 indicator. An important advantage is that they, most of the time, can provide these information for the whole water-column (the CPR device only samples the 10 first meters of the water column). To date, they cannot provide metrics of sufficient resolution for the diversity indices of the PH3 indicator. The interpretation of the data produced requires strong analytical tools and a minimum of taxonomic expertise.

2.2.3 Acoustic technology

Acoustic technology has been widely used and developed notably in order to target fish for biomass estimation for fisheries (Misund, 1997). Echo sounders send waves through the water which, when encountering objects (e.g. fish), are reflected and this reflected sound is sent back toward the sound source. Depending on the density of the object, the reflected sound or "echo" will have different characteristics giving information on the size, location and abundances of the encountered objects. In addition to emitting and receiving sounds, echo sounders filter, amplify and analyse the echoes. The sounds are specifically calibrated according to the characteristic of the objects targeted, mostly related to their size. While the technology has been clearly applied to fish, echo-sounder calibration is in constant development, allowing the targeting of plankton. However, the organisms that can be currently detected by acoustic devices are restricted in size, the lower size-limit detection being the mesozooplankton size-class, as such that the technology does not allow the consideration of phytoplankton and small zooplankton. Abundances of plankton organisms can be derived from acoustic information using both size-related info (mean size of zooplankton groups or species) and target strength (taking into account the density of organisms). Greater is the density of organisms, greater is their target strengths and thus ability to be detected by acoustic devices. Thus low-density plankton organisms such as gelatinous ones are less adequately detected by acoustic devices despite of their potential large sizes for some jellyfishes (Brierley et al., 2004, 2005). The data provided by acoustic devices are size spectra data, which do not provide taxa identification. Attribution to large taxonomical group (genus) is however possible if net sampling is done concomitantly (Berge et al., 2014). However, it means that additional work is necessary (taxonomy through light microscopy) if the taxonomical identification is required. The inter-comparison with net samples makes the abundances data per taxa acquired associated with a substantial bias. There is still a lot of advancement needed before acoustic data can be used for potential plankton taxonomic grouping in the future.

<u>Technique usefulness for informing OSPAR Indicators metrics:</u>

Despite the need for further advancement of this technique for the plankton study, acoustic devices can be already considered for estimation of mean abundance of total zooplankton for instance, as shown in the study of Cisewski and Strass (2016), and can provide information for the PH2 indicator for zooplankton. Acoustic devices should therefore not be excluded from marine management consideration in the future despite their inability to inform diversity indices. They have the great advantage to have the potential to give abundance and biomass derived estimation over the whole water-depth for zooplankton (thus estimating in the 3-D dimension, which is not possible through the CPR device for instance), and have the ability to be run at a large spatio-temporal scale (not allowed by plankton mesh sampling due to deployment time and ship access).

2.3. Genetic, metagenomics and phylogeny

A large range of different genetic methods exist to identify and elucidate functional capacity of plankton: DNA barcoding and metabarcoding, metagenomics, microarrays, quantitative real-time polymerase chain-reaction or transcriptomics. For the identification of planktonic organisms, molecular methods such as DNA barcoding and metagenomics have evolved significantly in the last decades beyond the scope of this review. For more details, Bourlat et al. (2013) has provided a full review of these methods in the context of marine management for assessing the marine health status. An advantage of these methods is that they produce more objective results, from an analytical point of view, compared to methods where identification of an organism is dependent on the skill of a person (ICES, 2015). DNA barcoding and metabarcoding have the potential to increase speed, accuracy and resolution of the identification of operational taxonomical units (OTUs), while decreasing its cost in biodiversity monitoring (Ji et al. 2013). However, the analyses of the resulting data can be time consuming since molecular techniques may generate large quantities of data. As such, their handling and analysis needs to be considered when being used to inform the design of management plans. However, there are two essential prerequisites for DNA barcoding: the creation of a reference library of species names for which an expert taxonomist is required to identify each species correctly (Bourlat et al., 2013) and the need for specialists of bio-informatics in order to analyse the data obtained and to refer to the library name (assuming the species found had been previously barcoded by extracting and characterizing its DNA, which is something not yet accomplished for most plankton species).

A molecular-based phylogenetic pattern supports the use of DNA barcoding programs for biodiversity conservation planning since it can provide information on species-level "complementarity" values – measures of biodiversity gains or losses (Smith and Fisher, 2009). Therefore, it is important to provide links between species diversity and genetic diversity by making species level indicators relevant to genetic diversity (Graudal et al. 2014). However, before the results of molecular methods can be used alongside those of microscope-based methods, a comprehensive comparison of the two techniques is still required for the use on phytoplankton data (ICES, 2015, Pawlowski et al. 2012). Additionally, genomic methods such as single nucleotide polymorphism (SNP) array, genome analysis and transcriptomics yield novel information on the biogeography, population structure and the potential and actual physiological capacity of plankton. This is especially useful for organisms < 5 µm, that are highly abundant, yet hard or impossible to identify by other means (ICES, 2015, Vargas et al., 2015).

Technique usefulness for informing OSPAR Indicators metrics:

In the future, genetic and phylogenetic data for plankton have the potential to be used for the three OSPAR plankton indicators, but most likely for PH3 (Sommerville et al., 2008). Actually, some phylogenetic data are already available and their potential for informing the PH3 indicator for the phytoplankton could be already explored. The potential of these techniques or approaches to enlarge our understanding of the plankton communities in relation to environmental changes and anthropogenic pressures is further discussed in the part 3 of this report.

2.4. Synthesis: abilities of the presented techniques to inform the metrics of the OSPAR plankton indicators

A table (Table I) is used to synthetize the abilities of each of the techniques presented in informing the metrics for the plankton indicators currently in development within OSPAR and aiming to be used for the MSFD implementation.

For further details about the advantages and shortcomings of the presented techniques in a more general context than the one of the OSPAR indicator development, see the work of Broutin et al. (2011, for MSFD work on innovative techniques) or the DYMAPHY project (www.dymaphy.eu), devoted to the study of phytoplankton. The JERICO-NEXT project (http://www.jerico-ri.eu/) will also provide deliverables (in 2019) on technical aspects but also on good practices, operational procedures and implementation advises, as well as examples of their application in targeted studies focusing on phytoplankton blooms and HABs, related to the different presented techniques.

Main plankton targetted	Methods		Target size-range organisms	OSPAR plankton common Indicator						OSPAR FW6	Main advantages	Main shortcomings
				PH2		PH1		PH3				
				Phyto	Zoo	Phyto	Zoo	Phyto	Zoo			
Phyto	**Inflow systems**	**Traditional flow cytometry (FCM)**	Pico- and nano-plankton	No	No	Partly	No	No	No	No	Pico- and nano-plankton precise counts and estimation of some groups. Single-cell analysis. Adding fluorochromes allows to count pico- and nano-heterotrophs	No species level possible
		Pulse shape-recording flow cytometer	±1 - 800 µm	Potential (using total red fluo. Which is often correlated to total chl *a*)	No	Partly	No	Potential (biodiversity index can be calculated)	No	No	Whole phytoplankton size-range considered. Single-cell analysis and determination of the size-structure of the phytoplankton community.	Large groups determined but species level not always possible (except if coupled with Image analysis) Strong analytical tool required for automated data analysis.
		Imaging in flow systems (FlowCAM, IFCB)	±10 - 800 µm	Potential (depending on the type of devices used)	No	Partly	No	Potential (if representative training set and good classification tool = possible discrimination at the genus	No	No	Can be used *in situ:* high spatio-temporal resolution possible (large amount of samples at high-speed	Strong analytical tool required for automated data analysis.

								at least)			analysis), allowing large geographical coverage.	
	***In vivo* fluorescence (total or multi-spectral)**		Phytoplankton	Yes	No	Partly (if spectral pigmentary groups discriminated)	No	No	No	No	Can be used *in situ:* high spatio-temporal resolution. Vertical profiles provided most often. Continuous recording too.	Sensitivity of the fluorescence assessment depending on pigmentary composition, physiology and environmental light conditions.
	Pigments analysis (HPLC)		All phytoplankton	Yes	No	Partly	No	Potential (to investigate)	No	No	Enable large spatio-temporal scale. Considers the whole phytoplankton size-range. Functional (pigmentary) groups are addressed	No identification of groups or species without light microscopy complement
	Epifluorescence		Pico-nano plankton Microphyto-plankton	No	No	Partly	No	No	No	No	Considers the whole phytoplankton size-range. Allows to discriminate pigmented and non-pigmented plankton	Time consuming

											organisms	
	Remote sensing		Phytoplankton	Yes	No	Partly (if combined with *in situ* optical measurements)	No	No	No	No	Enable really large geographical scale	Depends on the strength of the algorithm applied (limitations in highly turbid waters). Not sure the spatial resolution of current satellites is pertinent for OSPAR indicators (to investigate)
Zoo- and large phyto	**OPC LOPC**		100 µm to <3 cm	No	Yes	No	No	No	No	Potentially	Can be used *in situ*: large temporal geographical scale. Measure of total biomass	No identification of any taxonomical species or groups unless light microscopy is complementing
	Imaging systems	**UVP**	From 100 µm and up to few centimeters	Potential (for large phyto. cells and during bloom)	Yes		Partly		Potential	Yes	Used *in situ* and great potential for: large temporal geographical scale. Enable fast analysis in coarse taxonomical groups. Provide size spectra data	Strong analytical tools required for data interpretation

		VPR	From 100 µm and up to few centimeters	Potential (for large phyto. cells and during bloom)	Yes	No	Potential (to investigate)	No	No	Yes	Used *in situ*, enable large spatio-temporal scale. Enable fast analysis in coarse taxonomical groups.	Strong analytical tools required for data interpretation. Few organisms identifiable at the specie level
		ZooCam	From 100 µm and up to few centimeters	Potential (for large phyto. cells and during bloom)	Yes	No	Partly (for some life-forms)	No	Potential (to investigate)	Yes	Used *in situ*, enable direct analysis on board. Enable fast analysis in coarse taxonomical groups. Measure of size-spectra.	Requires net sampling. Strong analytical tools required for data interpretation. Few organisms identifiable at the specie level
		Plankton Scanners	300 µm Ø (spherical) to few centimeters	No	Yes	No	Partly (for some life-forms)	No	Potential (to investigate)	Yes	Enable fast analysis in coarse taxonomical groups. Measure of size-spectra.	Requires net sampling. Only usable in laboratory conditions. Strong analytical tools required for data interpretation. Few organisms identifiable at the specie level
	Acoustic technology		Mesoplankton	No	Yes	No	No	No	No	Not at the current level of development	Enable really large spatio-temporal resolution	No identification of any taxonomical species or groups unless light microscopy is complementing
Genetic, metagenomics and			All plankton	No	No	Future	Future	Future	Future	No	Future	Not enough

phylogeny				potential	potential	potential	potential		potential of measurements of biodiversity much more precisely than traditional methods (and also through determination of phylogenetic relatedness)	developed yet for measuring biodiversity Not a quantitative method.

Table I: Abilities of each of the different presented techniques (complementing light microscopy) to inform the metrics of the OSPAR plankton indicators currently under development (the common indicators PH1, PH2 and PH3, and the candidate indicator FW6). The main advantages and shortcomings of these techniques are also presented. The green colour indicates that the technique can inform the metric, the yellow colour indicates that the technique partly or could potentially (with development or with investigation) inform the metric, and the red colour indicates that the technique cannot inform the metric.

3 Towards combining different types of methods and data for assessing OSPAR plankton indicators

Each of the techniques presented above has advantages and shortcomings and can complement data acquired through conventional or CPR sampling, and analysis performed by light microscopy, spectrometry/fluorometry (for chl *a*) or PCI index, in different ways. Case studies from the scientific literature showing the usefulness of each of these techniques separately in enlarging our knowledge of the plankton community's dynamics are numerous and there is not an attempt to review them here. In addition, combining innovative and reference techniques for phytoplankton monitoring are being explored and improved in the frame of cross-border (DYMAPHY INTERREG IV A project) or European (JERICo-Next H2020) projects or networks. The focus is thus given on how these techniques could complement or enlarge the potential of plankton indicators in informing the good environmental status of marine waters, which is the goal of MSFD indicators. Examples are selected from the most recent findings in the scientific literature.

3.1. Complementary techniques to traditional microscopy: which plankton aspects, not yet considered, can they add to the current PH indicators development?

Compared to reference microscopy and *in vitro* spectrometry/fluorometry, a number of techniques previously presented, have the ability to treat autonomously a greater amount of samples. Some of them can provide data at a wide spatio-geographical scale, particularly for the *in situ* ones (pumping or used as probes, and/or towed) as does the CPR, which is not possible through conventional plankton acquisition techniques (such as net and hydrological bottle sampling). To have large spatio-temporal coverage allows having a much better estimation of plankton dynamics and changes than looking at data acquired at specific stations. It is then also possible to make the links with environmental changes and/or anthropogenic pressures at a finest scale. For instance, if only data for specific locations are provided (but also to some extent, if only CPR data are used, since their transects are fixed, they don't cover coastal areas in general and each sample represents 10 nautical miles of tow), it may happen that the pressure data available have not been acquired for the same zone, thus implying a geographical mismatch of data. It has to be also remembered as well that the only data used at the large spatio-temporal scale for the PH indicators come from the CPR which provides plankton counts estimates for the only 10 first integrated-meters of the water column. Still only few techniques allow having vertical profiles or depth-integrated plankton counts over the major part of the water column. This is a major point to address for the indicators development, particularly for the ones considering plankton biomass or production (so for FW6 for instance or for some of the PH1/FW5 lifeforms). Some of the techniques presented (some of the inflow or semi-automated techniques) can allow to overcome some of these problems in the future (geographical mismatch of data, true measurement of biomass). They will also permit to elaborate indicators usable for multi-metric index, considering other biological component of the food-web for instance, which is the goal of the MSFD food-web indicators (multi-indices food-web indicator including plankton have not been tested yet through in the OSPAR process). In addition, the CPR device does not provide taxonomic counts for plankton organisms smaller than the microphytoplankton and mesozooplankton size class. This aspect has not been considered yet, for the OSPAR PH indicators and is discussed separately below.

One shortcoming of the current indicators developed within OSPAR is the lack of consideration of the small size-classes smaller than microphytoplankton and mesozooplankton (but also bigger than mesozooplankton concerning gelatinous organisms) in order to inform PH1 and PH3 indicators at the

large geographical scale. Whilst microscopic counts consider only a fraction of the community and are subject to biases due to differences in taxonomic expertise, the application of state-of-the-art (semi-automated methods, such as automated pulse shape-recording flow cytometry (Thyssen et al. 2015; Bonato et al., 2015) and image analysis, such as FlowCAM (Álvarez et al. 2012), could enlarge the range of organisms considered and allow a higher spatial and temporal resolution, as mentioned before. Several studies have combined information from traditional microscopy with high-throughput data such as flow cytometry. On the other hand, Bosak et al. (2012) have defined phytoplankton indicators of trophic status using traditional flow cytometry to analyse the picophytoplankton while using, in parallel, traditional microscopy to determine nano- and micro-phytoplankton. High-throughput techniques combining flow cytometry with imaging systems, such as Cytobuoy FCMs and the Imaging FlowCytobot, have been shown to be efficient for monitoring HAB (Harmful Algal Bloom) in coastal areas (Sosik et al., 2011; Dugenne et al, 2015) and notably for detecting early warnings of these blooms, as shown by the study of Campbell et al. (2013) for the highly polluted Gulf of Mexico.

On the other hand, the work of Seoane et al. (2011) investigated the combination of pigments analysis with epifluorescence microscopy, as well as the use of HPLC, in order to identify indicators of good ecological status for the Water Framework Directive, as did also Lampert (2015) for the composition index in France. In their study, Seoane et al. (2011) clearly showed the effectiveness of epifluorescence microscopy in complementing traditional microscopy, since it allowed better evaluation and quantification of plankton groups, notably investigating the link between nanoplankton abundances and anthropogenic nutrients (ammonium/phosphate). A clear link between large phytoplankton cells and the higher nutrient concentrations, in areas highly impacted by humans, was also shown by Hlaili et al. (2008) in the Mediterranean. However, the very time consuming aspect of the epifluorescence method was highlighted, in addition to the fact that it necessitates a high level of taxonomic skills by the operator (Havskum et al., 2004; Seoane et al., 2011). The use of HPLC, notably the HPLC-CHEMTAX approach, which has been shown to be more cost-effective than microscopy and has a better potential to link functional groups with environmental conditions, was recommended by Seoane et al. (2011) despite that they did not find clear pattern between anthropogenic pressures and phytoplankton communities in their study. The advantages of the HPLC-CHEMTAX technique were highlighted notably in the frame of a large-scale monitoring programme when no identification to the species or genus level is required, however such level of identification is required for the OSPAR PH3 indicators.

Semi-automated and inflow imaging systems presented in this report can complement the taxonomic information by providing biomass per plankton size-spectra. This information is indeed required for informing the FW6 candidate indicator for instance (OSPAR d, (In Prep))). The techniques currently considered for the OSPAR plankton indicators do not provide direct measure of biomass for either phytoplankton nor for zooplankton. The use of semi-automated imaging systems seems to be the best techniques to provide the metrics for the FW6 food-web indicator, but would also allow to better quantify at the large spatio-temporal scale phytoplankton biomass (for the moment chl *a* is used as a proxy) and zooplankton abundance for the PH2 indicator. In addition, further consideration of the whole plankton size spectra and (on routine use of *in situ* devices) could allow developing indicators which would include the small plankton size groups currently not considered. In general, plankton size based indicators could be developed in the future. The size distribution of an assemblage is indeed an important factor that determines the direction and magnitude of energy and carbon fluxes in marine pelagic food webs (Riegman et al. 1993, Legendre and Rassoulzadegan

1995, Bosak et al. 2014), consequently affecting ecosystem productivity (for which measurements are also necessary to calculate another food-web candidate indicator, FW2).

3.2. Why to increase the focus on plankton functionality?

The consideration of functional diversity, through the choice of specific functional traits, is an alternative and/or complementary approach to taxonomy diversity, known to better reflect the effect of environmental changes and to be more responsive to disturbances in an ecosystem (McGill et al., 2006; Mouillot et al., 2013). Lately, there has been an increasing effort to depict traits for both phytoplankton and zooplankton (Benedetti et al., 2016; Edwards, 2016), although considerable work remains. Attempts at using the functionality of plankton organisms with other techniques have been made in order to increase our predictability of plankton dynamics and diversity at the large geographical scale. Example of links between functionality and size-spectra with ocean colour satellite outputs are reviewed in Nair et al. (2008) showing the high potential of these combinations for better modelling and predicting of the phytoplankton role in carbon storage, for instance, but also to develop chl *a* based indicators at the high spatio-temporal scale. In a recent paper, Breton et al. (2017) applied a functional approach to the analyses of FCM data in a three-year time series from the English Channel. In the study of Sarmento and Descy (2008), they complemented the use of the HPLC-CHEMTAX technique with the consideration of functional traits of phytoplankton and enabled the assessment of the status of the phytoplankton assemblages in lakes for the WFD. Such combination has been also considered in Lampert (2015) and Seoane et al. (2011). The trait approach linked with indices based on taxonomy and describing the distribution of species and of their abundance allows the measurement of diversity in a multidimensional space (Mouillot et al., 2013). Other indices have been also developed to measure the functional dissimilarity between assemblages (Villéger et al., 2013). These tools, if applied to different sets of data generated by complementary techniques, could allow the testing of theoretical approaches such as spatial congruence between taxonomy and functionality within plankton groups or congruence between the functionality of different plankton groups having interaction, such as phytoplankton and grazing zooplankton. These approaches have the potential to increase our understanding of which factors shape planktonic assemblages (Mouchet et al., 2010), and thus to better understand their relation with environmental changes and anthropogenic pressures. It will notably enable the selection of new lifeforms, which are potentially more sensitive to environmental changes and/or anthropogenic pressures, thus enabling to improve the PH1 OSPAR indicator for instance (modifying the chosen lifeforms and to propose potential new ones) and to relate them better to GES (Good Environmental Status).

3.3. Genomics and phylogenetic diversity: what is their potential for complementing the plankton indicators under development?

Knowledge of plankton traits (functionality) is gradually building but is a difficult process since it requires the measurement of several functional traits, most of them only achievable through experimentation, at the species/genus level. Phylogeny (based on morphological and molecular tools and applied to microscopy counts) and genomics (especially metabarcoding and metagenomics) are tools, which can be used in complementarity or as alternatives to trait-based approaches, but also to taxonomic diversity. Phylogeny considers the phylogenetic tree, which is based on phylogenetic distances between species, for which the DNA sequences are needed. Hinchliff at al. (2015) have

created an automated and efficient process for assembling published trees into a complete tree of life. They have also highlighted the gaps in biodiversity component and sampling, in addition to the difficulty to have digital formats of some scientific articles. Indices already exist that measure the different facets of phylogenetic diversity for biological assemblages, as well as phylogenetic dissimilarity (Chao et al., 2014; Tucker et al., 2016). Somerfield et al. (2008) notably indicated the usefulness to use indices of distinctiveness on functionality as an indicator of ecosystem functioning that can be used for marine management. A number of studies have proposed several diversity indicators for copepods at the large- geographical scale, which could be complemented by phylogenetic aspects. There hasn't been application of any of these indices to marine management at the present time. There is now an opportunity to complement these facets of biodiversity with the genetic one. Indeed, the knowledge of plankton genomics has increased in particular; one notable example is the metagenomics data set acquired through the Tara Oceans expeditions, which has made it possible to disentangle the main environmental drivers of diversity for marine prokaryotes in the ocean (de Vargas et al., 2015) or the study of Lima-Mendez et al., (2015) which described the effects of abiotic and biotic factors on plankton species interaction networks, using phylogeny and genomic information.

Santoferrara (2016) showed for microbial communities that the use of organism abundance versus gene abundance showed different inferences about community diversity. The different facets generally agreed, but the study showed that both genotypes and phenotypes are important and should be used in combination (using multidisciplinary analyses and reliable database). Genomic approaches allow depicting the composition of the community in detail but this is not yet possible for most plankton organisms. Bulk diversity alone is most often available for plankton at the moment (and only for small plankton organisms). As a complement to the bulk genomics, single-cell approaches have also developed (pioneered in the medical sector) which are increasingly applied to plankton organisms. For the moment this work has focused on marine bacteria, mostly obtained through laboratory cultures (e.g. Swan et al., 2013; Rinke et al., 2013; Kashtan et al., 2014). This type of information allows comparison of the phenotype with the genotype such that the genomic architecture of free-living bacterioplankton has been depicted by Swan et al., (2013) for instance, or that niche partitioning within lineages of marine microbes globally distributed have been uncovered (Kashtan et al., 2014). We can imagine that it will be possible one day in the future to apply this type of approach to larger plankton organisms. Combining the bulk and single-cell approaches together can also help to reveal the biological functions that organisms perform (Thrash et al., 2014; Rinke et al., 2013). However, these techniques often result in large quantities of data that cannot be used due to the lack of a species-attributed DNA genomics database (e.g. Sunagawa et al., 2015). There are few marine phytoplankton species that have been actually completely sequenced, and even fewer for mesozooplankton. The study of Asai et al. (2015) is one of the few which has performed RNA extraction on three common large copepods species, *Calanus helgolandicus*, *Centropages typicus,* and *Temora stylifera,* for instance. This highlights that there are important gaps in our ability to describe the plankton diversity through the genetic facet, but this is also true for the phylogenetic and functional facets. While these knowledge gaps remain, there is at the same time an increasing quantity of vast new data streams, presenting clear challenges to be quickly overcome, especially in the light of the need for marine management and conservation.

4. Knowledge gaps and recommendations

4.1. Further testing of the PH Indicators

Methods and data that could complement those currently being used for the calculation and testing of OSPAR Pelagic Habitat indicators have been presented in this report. The techniques presented have already been considered in the context of specific research studies or projects that have produced operational procedures, analytical tools and examples of the production of data which could be used and tested for the further development of the OSPAR PH indicators. These data (if they respect the monitoring OSPAR guidelines in term of standardized procedures of course) could have both the ability to complement microscopic counts in term of plankton indicator interpretation and understanding toward defining GES, and increase the spatio-temporal scale considered, thus increasing the confidence of plankton indicators. The main data gaps that exist for the local and large geographical scale are identified in Table II, and potentially known existing data sets are proposed for filling these gaps (as examples only). This shows that some extra data sets allowing the calculation of the different PH indicators exist, even for the most-innovative semi-automated techniques. However, the major problems lie in the support of these actions that would need to be sustained in a regular and long-term basis. It is also necessary to ensure access to the data sets and ensure the human resources for their management and analysis. The problem of data access has been notably highlighted through the data-call made in the frame of this project through OSPAR, for which only few local stations data sets have been provided (cf. deliverable 1.2 EcApRHA WP1).

Indicators	Plankton type	Local scale (coastal stations)	Large Geographical scale	Potential data-sets Local scale	Potential data sets large scale
PH1/FW5	**Phyto**	Restricted and only microscopic data used	Good but mainly CPR and light microscopy data used. Some gaps are important in areas without regular CPR sampling (nor FerryBox implementation)	More local stations from Contracting Parties should be included, for instance PhytObs data (France)	For instance, data from fisheries cruises (France, other?), research cruises, ships of opportunity (this implies that indicators could be developed only for specific season, for instance focusing on the productive season). Some semi-automated data are currently under a plan to be put in an online global data-bases (JERICO-Next and SeaData net projects for automated FCM data)
	Zoo	Restricted and only microscopic data used	Good for some life forms/weak for other life-forms, only CPR data used	More local stations from CPs should be included. For instance, Spanish stations, Swedish stations, French stations (ongoing building of a French zooplankton observation network), etc…. (and through COPEPOD data basis potentially)	For instance, data from fisheries cruises (France, other?), COPEPOD data basis for some life-forms (http://www.st.nmfs.noaa.gov/copepod/)
PH2	**Phyto**	Restricted and only chl *a* data used	Good but only PCI data used up to now.	More local stations from CPs should be included, for instance, PhytObs data set in France, monitoring stations in other CPs	For instance, fisheries cruises data (France, other?), remote sensing (potential example: Global SeaWiFS chlorophyll Sep97 - Dec04 database). Semi-automated data are currently under a plan to be put in online global data-bases (JERICO-Next and SeaData net projects for automated FCM data)
	Zoo	Restricted and only microscopic data used	Good but only CPR data used	More local stations from CPs should be included, for instance, Spanish stations, Swedish stations, etc…. (and through COPEPOD data basis potentially)	For instance: fisheries data (France), COPEPOD data basis, data from semi-automated devices (for instance UVP data are currently under a plan to be put in an online global data-base)
PH3	**Phyto**	Restricted and only microscopic	Very weak, no available data	More local stations from CPs should be included	For instance: fisheries data (France), TARA-OCEANS plankton data set. Semi-automated data are currently under a plan to be put in online

		data used	through OSPAR		global data-bases (JERICO-Next and SeaData net projects for automated FCM data, other potential data sets more complete with Image Inflow or Image analysis data (FlowCAM, IFCB)
	Zoo	Really weak and only microscopic data used	Good but only CPR data available through OSPAR	More local stations from CPs should be included, for instance, Spanish stations, Swedish stations, etc…. (and through COPEPOD data base potentially?)	For instance: fisheries cruises data (France), through COPEPOD data basis potentially, TARA-OCEANS plankton data set, …
FW6	**Zoo**	Not yet tested but no local data available in OSPAR	Not yet tested but only CPR data available potentially through OSPAR	More local stations from CPs should be included where total biomass is measured and/or zooplankton size-spectra (as example in France: local station sampled by Parc Marin Iroise in the Celtic Seas sub-region)	Data from semi-automated devices (for instance UVP data are currently under a plan to be put in an online global data-basis), fisheries cruises data using the ZooCam (France), …

Table II: Main data gaps for the local and large geographical scales for the different OSPAR plankton indicators in development and potential known existing data sets identified to fill these gaps (considering both reference methods and the complementing techniques presented in this report).

One of the biggest bottlenecks for further testing relies on the access of data, which sometimes (for semi-automated approaches mainly but not exclusively) have not yet been gathered together and referenced in national or European data bases (only in local ones). A clear policy mechanisms should be implemented in the frame of marine monitoring for helping all the spread data to be concentrated at least into national data bases, and to provide real data access. There is also a clear need to raise the necessity to have the data provided in an adequate format for their analysis and their inter-comparability, and the human resources to do so (at national and/or local levels). OSPAR could raise this main bottleneck to the European Commission. The establishment of a common data-base, where the real data are actually stored, at the European scale would overcome this problem in the future and is further discussed in the following section on monitoring.

Summary

- Data for further testing of the OSPAR PH indicators exist for both reference techniques and for some of the techniques presented in this report
- The main problem lies in their access (→ need for clear policy mechanisms for data access) and also in the sustainable funding of these monitoring (following precise standardized guidelines)
- Also, there is a need of a strong message to support both the acquisition of new types of data or of data filling the spatial or temporal gaps, as well as to support the inclusion of these data on solid and reliable data bases
- There is also a need to further explore PH indicators in order to being able to cope with this new types of data, by adapting the existing indicators to different types of metrics (considered separately or combined)

4.2. Monitoring programs

In complement to the microscopic counts used for the current development of the pelagic indicators, we showed that inflow and semi-automated methods, such as automated or traditional flow cytometry (Bonato et al., 2015; Morán et al. 2015; Thyssen et al. 2015) and imaging systems, such as FlowCAM and IFCB (Álvarez et al. 2012) or UVP for instance, could enlarge the size-range of organisms considered and/or allow a higher spatial and temporal resolution. Microscopy has been indeed found to underestimate the species richness of marine phytoplankton communities (Rodriguez-Ramos, 2014), especially for the less abundant as well as for the smaller cells in the assemblage. Semi-automated methods could thus fill this gap for the plankton community. In addition, molecular approaches, DNA barcoding and metabarcoding, have the potential to increase speed, accuracy and resolution of species identification, considering the whole community, while decreasing its cost in biodiversity monitoring (Ji et al. 2013). They can also resolve cryptic species that cannot be discriminated by microscopy. Hence, combining methods may fill the gaps in microscopic examinations as well as in temporal and spatial resolution, and the complementary methods will allow for monitoring the whole size range of the plankton community.

Previous or current projects also propose recommendations in relation to innovative sensors and methods, at least for phytoplankton monitoring, which should be considered by OSPAR. As such, the recent INTERREG IV A "2 Seas" programme DYMAPHY (2010-2014; www.dymaphy.eu), a cross--border partnership for the North Sea – English Channel, was established for the assessment of marine water quality based on phytoplankton analysis using innovative methods. It has provided a first set of inter comparisons and recommendations for the use of some techniques as automated

flow cytometry and multi-spectral fluorometry. It has also allowed the implementation of different techniques in targeted cruises in spring in the North Sea (Thyssen et al., 2015) and E. Channel (Artigas et al., 2015; Bonato et al., 2015) as well as in autumn in Dutch Estuaries (Créach et al., 2015). Moreover, within the current Joint European Research Infrastructure for Coastal Observatories – New Expertise (H2020; JERICO-Next, 2015-2019; www.jerico-ri.eu), experts from all Europe are working to define the best practices of using automated optical sensors, proposing some technical and analytical improvements and applying them in combined international case studies of phytoplankton blooms from eutrophic to oligotrophic systems.

Another important point concerning the monitoring is the need for high quality data which follow standardised procedures. Indeed, quality and standard procedures have to be ensured in order to have inter-comparable data at a large geographical scale and to make datasets compatible for the extraction and calculation of PH indicators. Efforts have been made through OSPAR to ensure the delivery of consistent, high-quality data that can be used to evaluate the state of each of the plankton indicators, e.g. CEMP monitoring guidelines (OSPAR, 2016). Since these indicators belong to several descriptors, they require the same or similar data so that integration of data collection and analysis can be sought. In these guidelines, the most routinely used analytical methods to characterize the taxonomic structure and biomass of phytoplankton are being considered but additional methods are also proposed. Whilst the CEMP monitoring guidelines have been drafted for microphytoplankton species composition data, there is currently no equivalent document for zooplankton, neither for other types of plankton data provided by semi-automated approaches.

In general, a further revision of the CEMP monitoring guidelines for phytoplankton (OSPAR Agreement 2016-06) is advised, which would take into account at least some of the techniques presented in this report, and would include all components of the phytoplankton community, such as pico- and nanoplankton, which are currently understudied or not studied at all, and which are not subject of standard monitoring procedures. In this respect, ICES has advised OSPAR to encourage its Contracting Parties to invest in both the equipment and personnel necessary to properly monitor picoplankton (and also nanoplankton). Autotrophic picoplankton, such as the *Synechococcus* genus, is probably the most abundant phytoplankton in European coastal waters in winter (Bonato et al., 2015; Thyssen et al., 2015) and also in summer and plays an important role in the marine food web.

It is also a recommendation that a CEMP monitoring guideline for zooplankton should be created to support the work of OSPAR Contracting Parties, and included in the context of the MSFD implementation. It has to be noted that the large macro- and mega-plankton, notably jellyfishes, have not been treated here, but they should be considered as well in these future guidelines.

The revision/creation of these guidelines should clearly consider at least some of the different techniques presented in this report.
The outcomes of these projects should be considered and articulated in the revision/creation of the new CEMP OSPAR guidelines for plankton.

Summary

- Quality and standard procedures of monitoring data should be ensured by OSPAR for the whole plankton realm (considering the whole size classes of plankton including the small size-classes such as pico- and nanophytoplankton)
- CEMP monitoring guidelines should be revised and amended for phytoplankton and smaller size-range organisms and created for zooplankton taking into account the techniques presented in this report

4.3. Towards a better understanding of plankton dynamics in relation to environmental changes and anthropogenic pressures and thus GES

One of the main scientific challenges in relating plankton indicators to environmental changes and anthropogenic pressures remains the disentanglement of climate change effects from other pressure drivers that affect plankton assemblages (environmental, anthropogenic pressures such as pollution trophic cascades due to fishery pressure, etc...). As we have shown in this report, studies on biodiversity should include not only species, traits, phylogenetic diversity and abundance, but also aspects of richness, regularity and divergence (different types of diversity indicators) associated with the various diversity aspects. Combining diversity indices into two-dimensional plots or by using multidimensional statistical techniques could help to tease out the mechanisms that shape communities. In order to allow a better understanding of plankton communities' change in relation to environmental changes and anthropogenic pressures, we thus propose to consider the other types of methods and data which are complementary to light microscopy taxonomy. From the short review carried out here, it appears that data addressing different aspects of plankton diversity do exist but with limitations in their application and/or that they are not yet really included in current and sustained monitoring networks. General theoretical approaches but also applied approaches have been developed, allowing the depiction of facets of plankton biodiversity determining key ecosystem processes, separately. However, studies addressing response to multi-pressures and environmental conditions for plankton should be the focus in the near future, in the context of ongoing policy mechanisms. This is essential since it is their aim to assess marine health status and to find relevant indicators in order to monitor the effects of environmental changes and anthropogenic pressures at a relevant temporal and spatial scale.

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