Early detection and diversity of benthic alien species along the northeast Atlantic Ocean coastline

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Date: 2022-07-15
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Course code: MAE-79336

Keywords: ARMS, metabarcoding, benthic, marine, alien species

Abstract

Marine ecosystems are under severe pressure of biodiversity loss and deterioration, despite their key functions in nature and society. Monitoring biodiversity is key to address the status and change of marine coastal systems. One indicator of ecosystem health is the presence of alien species, as these can have adverse effects on the ecosystem such as introduction of diseases or competition with native species. Autonomous Reef Monitoring Systems (ARMS) can provide assessment of alien species through continuous deployment and the use of highly sensitive molecular tools.

This study tests if continuous and comparable genetic sampling across a larger coastal range can be used to detect potential alien species and understand their origin and introduction path. To this end we processed a large amount of sequence data provided by the ARMS-MBON program to detect three types of potential aliens: cryptogenic, hitchhiking and range expanding species. By processing sequence data from 72 ARMS deployed along the European coastline, from the Bay of Biscay to the Eastern Baltic, across a variety of habitats and cross-referencing the genetic observations against GBIF occurrence data, we show that 1) marine protected areas (MPAs) had a much higher biodiversity than non-protected and industrial environments, 2) the average number of alien species was not different between protected, non-protected, and industrial environments, and 3) hitchhiker species were most common in industrial ports and marinas, while being absent in MPAs. Results suggest that industrial ports are hotspots for the introduction of species native to the USA and east-Asia due to shipping traffic, while simultaneously representing a species-poor vulnerable ecosystem in which aliens could become invasive. These findings show the efficiency of metabarcoding, while also emphasizing the continuous need for coastal marine monitoring, especially in regions with considerable shipping traffic.

Introduction

Marine ecosystems are under severe pressure of biodiversity loss and deterioration, despite their key functions in nature and society (Lotze et al., 2006; Rockström et al., 2009). The decay of marine

ecosystems is especially notable in coastal areas, where anthropological effects, such as habitat destruction and increased aquaculture, are evident (Lotze et al., 2006; Worm et al., 2006). Monitoring biodiversity is key to address the overall state and resilience of marine coastal systems, but this can be a challenge on subtidal hard-bottoms substrates, which consist of complex three-dimensional structures. Monitoring programs generally make use of scientific diving and video recordings to index species abundance and diversity which, despite their extensive use, yield a low taxonomic resolution as well as low number of species, thereby leading to difficulty in (diversity) assessments (Beisiegel et al., 2017)

Autonomous Reef Monitoring Systems (ARMS) can provide a passive, low-cost and long-term monitoring system of marine benthic diversity, facilitated by stacked plates which imitate hard-bottom substrate to promote settlement of sessile and mobile benthic organisms (David et al., 2019; Obst, Exter, et al., 2020). The presence, abundance and biodiversity of benthic organisms, and thereby the status of the ecosystem, can be assessed through photography of individual ARMS plates (David et al., 2019) or using molecular tools (Leray & Knowlton, 2015; Obst, Exter, et al., 2020). One of these molecular tools, DNA-metabarcoding, has developed extensively over the last two decades to the extend where it is currently considered an effective tool for monitoring purposes, including the assessment of artificial hard-bottom substrate such as ARMS (Bourlat et al., 2013; Keck et al., 2022).

The prolonged deployment of ARMS and relatively broad variety of environmental conditions, in combination with the use of highly sensitive molecular tools, allow for detection of species which are otherwise hard to identify due to their low abundance, small appearance or resemblance to closely related species (Duarte et al., 2021). This is especially useful in the case of alien species, which usually arrive in low numbers and in case of small benthic organisms, are hard to distinguish from native species using traditional monitoring techniques, thereby going unnoticed for months or even years (Couton et al., 2019). Early detection of alien species, also known as "aliens", is essential, as they might harm the ecosystem by introducing diseases or through their competition advantage over native species (Bax et al., 2003).

Even though ARMS and subsequent metabarcoding are an effective tool for monitoring native and alien species, detecting newly introduced aliens (i.e. previously unreported) requires knowledge on the already present community, which includes native species as well as known aliens. The approach to finding new aliens is to filter any native or known alien species from the observed community, rather than using a predetermined species list. To understand the dispersal mechanisms of newly introduced aliens, it is crucial to identify the origin and potential dispersal pathway. Some species are dispersed through shipping traffic over long distances, whereas others disperse by physical movement or larval dispersal (Bax et al., 2003). In addition, cryptogenic species can be categorized as alien, due to the unknown origin and poorly understood abundance.

Introductions of alien species have become more common in the last decades due to increased boat traffic, with most marine taxonomic groups showing an exponential increase of introduction rate since the start of this century (Sardain et al., 2019; Seebens et al., 2017). Individual ARMS can record the presence and abundance of aliens over time, but to track the dispersal of aliens across continents, a widespread system of observatories is necessary. Detection and analysis of alien species is one of the reasons that the ARMS-MBON project established a broad ARMS network throughout Europe, deploying ARMS in a wide variety of seas, countries and habitats (Obst, Exter, et al., 2020). The wide spatial range of these ARMS and the long-term scope of the project allows for early detection of new aliens as well as

tracking dispersal and abundance over space and time. The ARMS-MBON project has, at the moment of publication by Obst et al. (2020), yielded sequence data of 134 ARMS, deployed at 20 observatories, including sampling locations in the Mediterranean Sea, Bay of Biscay, Greater North Sea, Baltic Sea and other sections of northeast Atlantic.

The northeast Atlantic is an essential region of the Atlantic Ocean for a vast number of European countries in terms of fisheries, energy production, boat traffic, aquaculture and simultaneously a significant region for numerous marine mammals, sea birds and other marine life (OSPAR Commission, 2000). Due to the high rate of shipping in the Northeast Atlantic, introduction of alien species is not uncommon. To detect, identify and track alien species at an early stage of introduction, a standardized approach is necessary, which is able to collect comparable genetic data and process these.

This study focusses on testing such an approach, which standardizes the methods to detect new aliens, rather than identifying them for each separate sampling location. Subsequently, presence of and diversity of new aliens in a wide variety of observatories in the northeast Atlantic are assessed. In this study, three indicators are assessed to test the impact of human activities in the coastal zone: 1) the biodiversity of native species, 2) the relative fraction of new alien species and 3) the type/origin of new alien species. Habitats are categorized according to the amount of human activities in the sample sites, including: 1) MPA (nature protected under (inter)national law), 2) Low Human Interference areas (LHI) (nature not protected, low level of human activities), 3) Marinas (recreational harbour) and 4) Industrial port (commercial harbour). The most pristine sites are expected to harbour the highest level of biodiversity, whereas sites with intense human imprint through shipping or boat traffic, are expected to be highly disturbed and therefore show lower levels of biodiversity, followed by higher levels of new aliens. In addition, due to the high levels of transportation in marinas and industrial ports and the corresponding possibility of alien introduction, it is likely that the origin of aliens differs among monitoring areas, with industrial ports being responsible for most of the long-distance introductions.

Methods

Sequence data was available from a total of 73 ARMS deployments, consisting of 162 ARMS fractions (sessile, mobile 100 μm or mobile 500 μm), deployed in ten observatories throughout the northeast Atlantic Ocean between 2018 and 2020 (Figure 1, Supplementary table 1). DNA extraction, PCR and sequencing of ARMS were all performed to the protocol described by Obst (2020), targeting a 313 base pair (bp) region of the cytochrome oxidase I (COI) gene, which is a well-documented section of the mitochondrial DNA across many marine phyla. The resulting Illumina sequences were processed according to protocol described by Obst, Deneudt et al. (2020), using the *dada2* package in R (Callahan et al., 2016), where they were filtered on Q-score, de-noised and merged, followed by the removal of chimeras. Sequences with a length of 312 bp to 314 bp were then classified to species level using the BOLD reference library provided by the BOLDigger python package (Buchner & Leese, 2020; Ratnasingham & Hebert, 2007). Since the primer set is optimized for animals, any plant, fungi or protozoan, as well as common contaminants with no marine basis (*Homo sapiens* and *Canis lupus familiaris*) were removed from the dataset.

The analysis focussed on individual ARMS deployments, which was achieved by summing the read counts of (at most) three fractions. ARMS and fractions with less than 100 (summed) reads were removed from (alien) diversity analyses. In addition, read counts of amplicon sequence variants (ASVs) assigned to

the same species were summed. No cut-off was used for the number of reads per species. Based on location and applicable legislation, the ten sampling locations were divided into four so-called monitoring areas: Industrial port, marina, low human interference areas (LHI) and marine protected areas (MPAs). 'Confirmed presence' or 'new alien' status for each species in each sampling location was determined by finding the closest geographical observation over water, using the GBIF occurrence database and a R script to determine the shortest distance over sea. The script and parameters can be found on GitHub: https://github.com/DvBMolEc/rgbifanimals. Status of confirmed presence was assigned using a threshold of at most 250 km from the sampling location to the nearest occurrence on GBIF. If the distance over water from any sampling location to the nearest occurrence exceeded 250 km or no geographical occurrences were available, species were considered (potential) new alien and classified into either one of three classes: cryptogenic (less than 50 occurrences on GBIF), hitchhiker (more than 2000 km on average to the nearest GBIF observation, as the crow flies) or range expanding species (remaining observations). All following analysis and visualisation was performed using R (v. 4.2.0) and RStudio (v. 2022.02.3) (R Core Team, 2022; RStudio Team, 2022), using the packages *dplyr* and *tidyverse* for standard data manipulation (Wickham et al., 2019, 2022), the *phyloseq* package for initial analysis and creating a heatmap (McMurdie & Holmes, 2013), and the *ggpubr*, *ggplot2*, *rstatix* and *vegan* packages for additional visualisation and statistical analyses (Kassambara, 2022b, 2022a; Oksanen et al., 2022; Wickham, 2016).
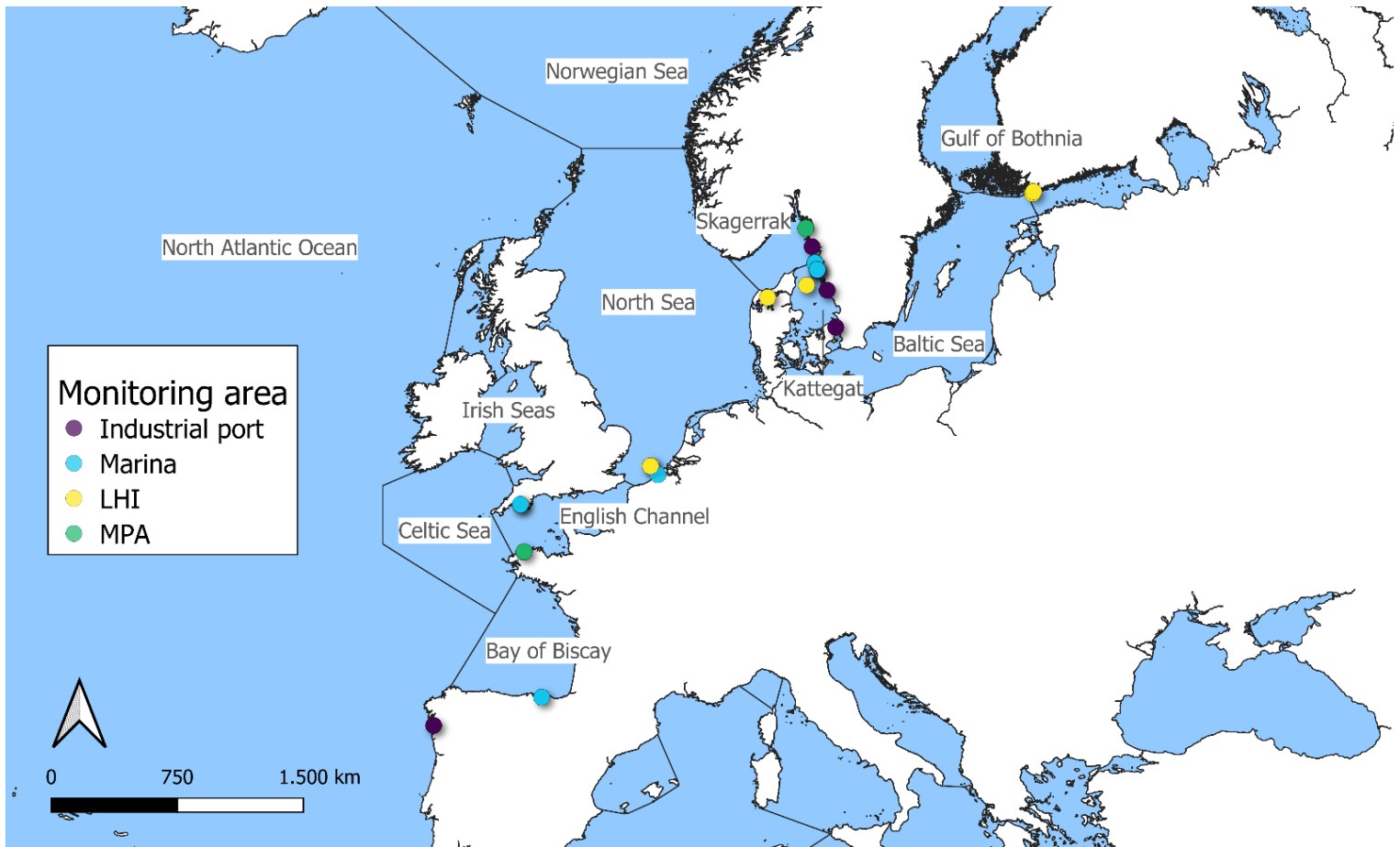

Figure 1: Map of selected sample locations from the ARMS-MBON project, used for the present study. Base map supplied by Flanders Marine Institute (2018). IHO Sea Areas, version 3. Available online at https://www.marineregions.org/ https://doi.org/10.14284/323

Results

Description of dataset

After processing by the *dada2* pipeline and corresponding cleaning steps, 5,487,576 sequences used as input for the BOLDigger tool (ran on 04-02-2022), of which 1,882,886 sequences, approximately one-third, (34.3%) were identified to species level with at least 97% similarity. A small fraction of classified reads (2.36%) were removed, as these were assigned to plants, fungi, protozoans or non-marine life, leaving 1,838,434 sequences available for downstream analysis. Out of the initial 11,654 ASVs, 1,840 (15.8%) were classified by BOLDigger to species level, identifying, 426 species, of which 194 were identified as (potential) alien at one or more sampling locations, including eighteen cryptogenic species and five hitchhiker species. Similarity between ASVs and the classified species was found to be 99.41% ± 0.66% (mean ± SD). A total of 1788 ASVs had the target length of 313 bp, 47 had a length of 312 bp and the remaining 5 sequences had a length of 314 bp. In total, fourteen different phyla were found, with almost half (47.5%) of the species consisting of Arthropoda and Annelida, with 120 and 83 different species assigned to each phyla, respectively. After removal of ARMS with a summed read count less than 100, read count depth for each ARMS (N = 63) was 29,175 ± 32,641 reads (mean ± SD) and for ARMS fractions (N = 126) 14,583 ± 17,397 reads (mean ± SD).

Comparison of environmental classes

Native diversity, measured by the total number of species with confirmed presence found on a single ARMS, was highest in MPAs with 57.4 ± 15.6 (mean ± SD) species found per ARMS, and lowest in LHI areas with 27.9 ± 17.0 (mean ± SD) species found per ARMS. All monitoring areas showed similar species diversity, except for MPAs, which had a higher species diversity compared to the other three monitoring areas (p = 0.038 for Industrial port - MPA, p = 0.038 for Marina - MPA, p = 0.014 for LHI - MPA, n = 12 for industrial ports, n = 14 for LHI, n = 19 for Marina, n = 8 for MPA) (Figure 2). The fraction of new alien species in the overall dataset was 9.63 % ± 11.2% (mean ± SD) and was found to be similar between all four monitoring areas. The lowest new alien fraction was found in MPAs with 4.89% ± 4.18% aliens (mean ± SD) and the highest new alien fraction was found in LHI areas with 21.3% ± 17.7% alien species (mean ± SD) (Figure 3). In addition, a higher native diversity seemed reduce the fraction of new alien species, as a negative linear relationship was found across the overall dataset (Figure 4). A strong relationship was found between the type of alien species and the type of monitoring area (X^2 = 37.865, df = 6, p < 0.001). Five hitchhiker species were found, which were most abundant in industrial ports with fourteen occurrences, while being completely absent in MPAs (Figure 5). Marinas had a total of seven hitchhiker occurrences, while only two were found in LHI areas.
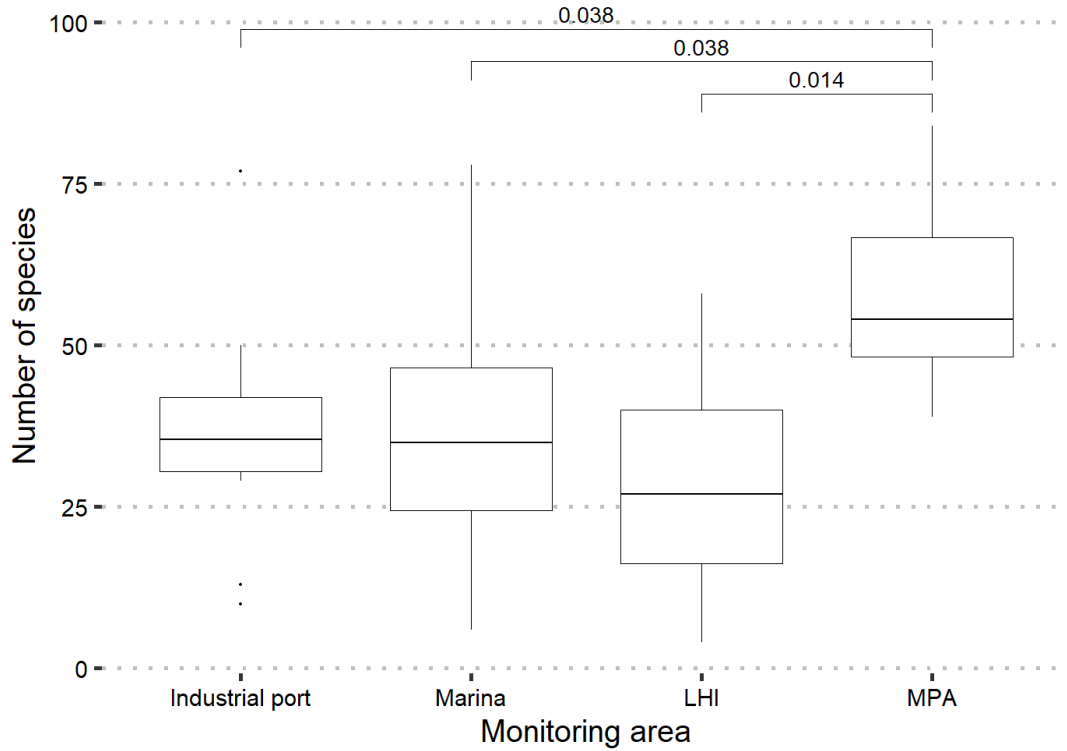
Figure 2: Boxplot of number of species, with confirmed presence at the sampling location, in each ARMS (summed fractions) for each of the four monitoring areas. All significant differences in diversity between groups determined using a Wilcoxon test are shown.
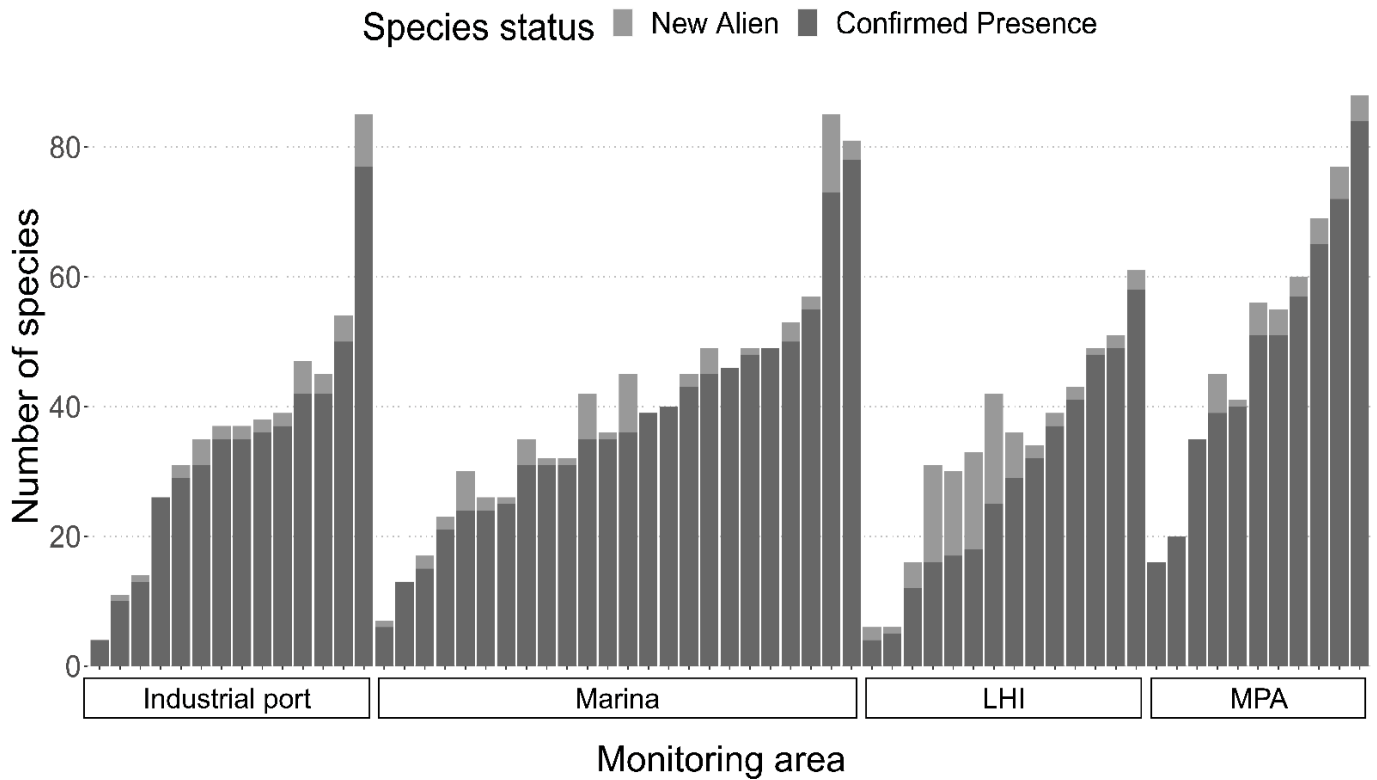
Figure 3: number of new alien species and species with confirmed presence found for each ARMS deployment, grouped by monitoring area.
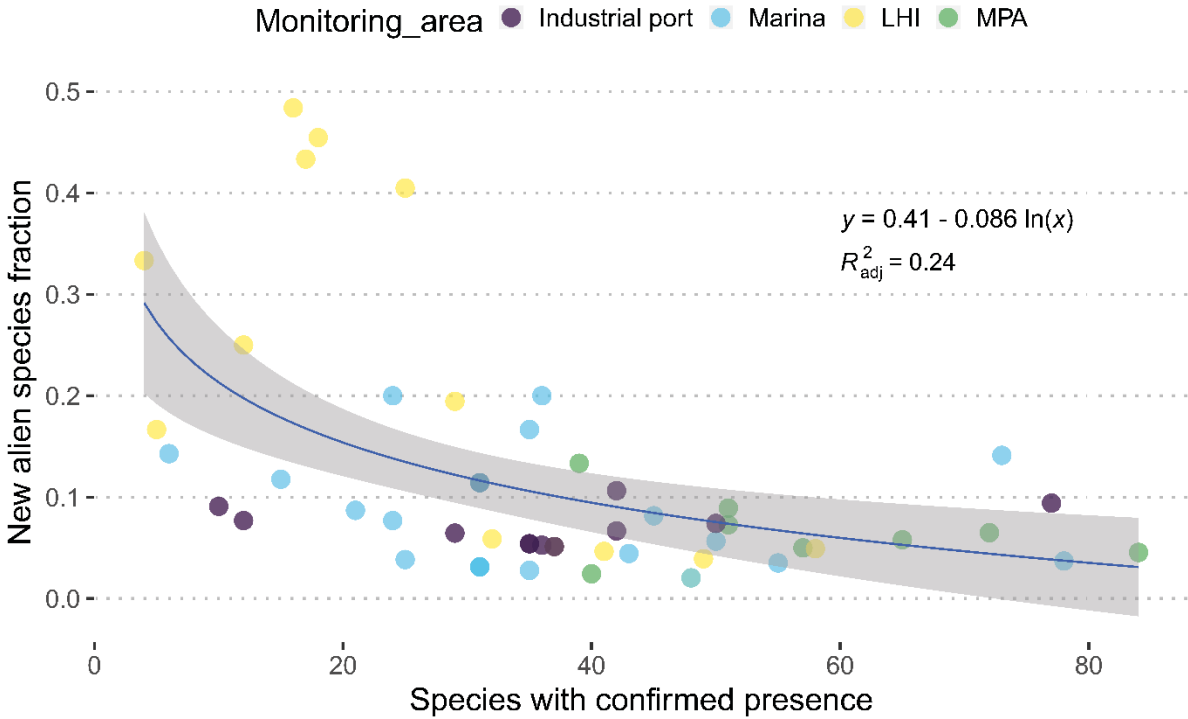
Figure 4: fraction of new alien species and species with confirmed presence found for each ARMS deployment, grouped by monitoring area. Trend line indicated in blue, with 95% confidence interval indicated in grey. Trend line formula and fit indicated in figure.

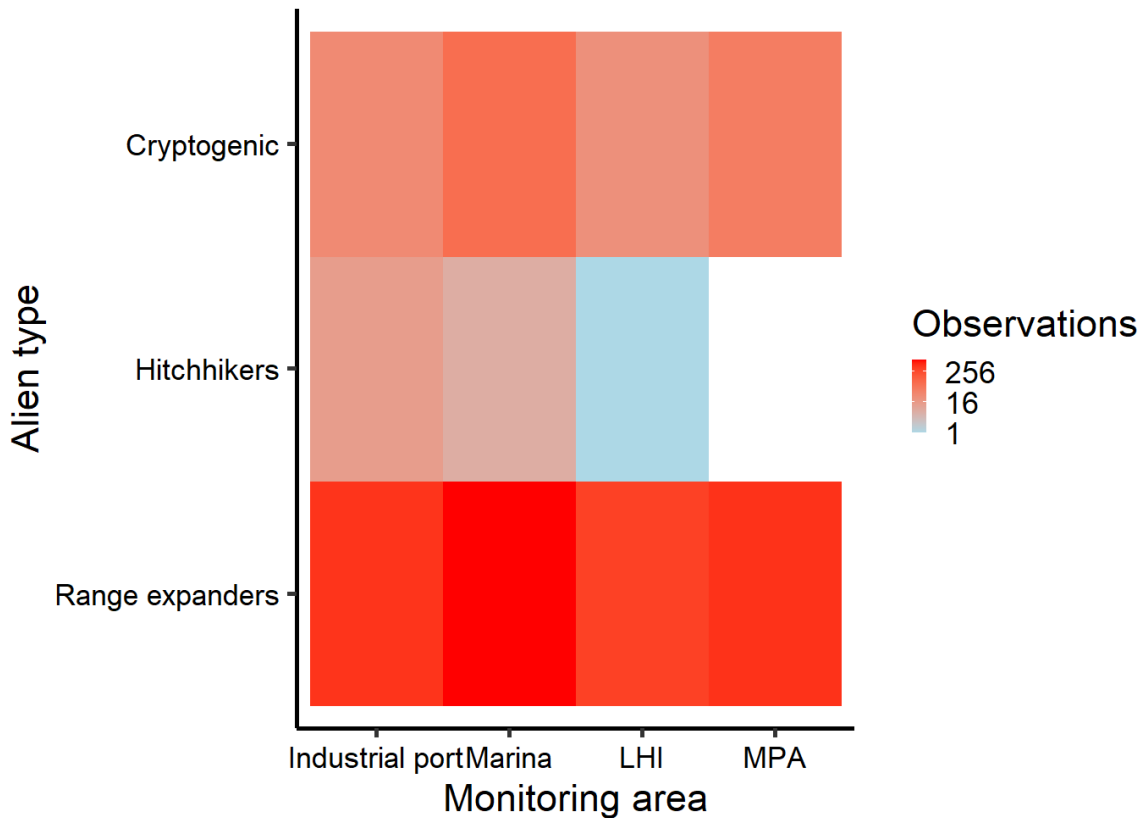$$y = 0.41 - 0.086 \ln(x)$$
$$R^2_{adj} = 0.24$$

Figure 5: heatmap of abundance of three different type of aliens, found in four types of monitoring areas. All ARMS and ARMS fraction read counts are summed within monitoring areas, as well as the read count of separate alien species.

Discussion

ARMS-MBON monitoring

This study presents the first case in which the extensive ARMS-MBON COI sequence database is explored on a large spatial scale, focusing on the early detection of alien species. Using the GBIF database, it was possible to detect five aliens which had no prior documented presence in Europe. Extensive monitoring in a broad range of environmental factors has provided insight in the abundance and type of alien species in relation to the level of human activities, in particular shipping, which allows for more specific monitoring and prevention of alien or invasive species.

Biodiversity of monitoring areas

The first main finding was that the biodiversity of each of the four monitoring areas was highest in the MPAs, which follows expectations. MPAs are least affected by human activities, leading to little to no disturbance of settlement or growth. A higher diversity of species is generally associated with a healthier, more resilient ecosystem (Chapin III et al., 2000). The low diversity found in LHI areas was unexpected, since LHI area relatively pristine compared to industrial ports and marinas. However, this result could explained by the fact that the sampling locations in Finland are included in the LHI monitoring area. The Finnish locations are located in the eastern part of the Baltic Sea, which is characterized by brackish water, rather than the saline water present at all other sampling locations. As a result of the brackish water, the native species diversity at the Finnish observatory is relatively low, with under 300 species, compared to over 1500 in e.g. Skagerrak (HELCOM, 2018, p. 13). The other LHI sampling locations in Belgium showed a much higher species diversity, which was more comparable to that of the MPAs.

New alien species fraction

Secondly, the relative number of new alien species was observed to be equal across all four monitoring areas, which did not correspond with the hypothesis. The unexpected even distribution between monitoring areas is likely caused by the fact that the majority of alien species were the so-called range expanders, of which the dispersal is extremely hard to prevent. Relative new alien species presence was relatively high in the LHI areas, which could be another effect of the sampling locations in Finland. The low diversity of the native benthic community means that 1) the pool of alien species is vastly bigger, as there are simply more species considered alien, and 2) any occurrence of alien species has a bigger influence on the relative abundance of alien species (Furman et al., 2014, p. 58). Again, the other LHI areas, in Denmark and Belgium, had a much more comparable alien species fraction to other monitoring areas, supporting the statement that the level of human activities does not influence the number of alien species. The large overlap of alien fraction and type of level of human activities was not found by Pearman et al. (2021), who found significantly more alien species in an international port, compared to nearby marinas. However, it is believed that their study presents a different alien introduction scenario, as the study was conducted on the island of Tahiti, which is extremely secluded compared to the coastline of West- and North Europe. Due to the isolation of their sampling sites, arguments can be made that these aliens would classify as hitchhikers, which would place their results in line with the results found in this study, presented below. In addition, a negative linear relationship was found between the biodiversity of present species and the fraction of new alien species. While the loss of native biodiversity is often

mentioned as a result of alien introductions (Bax et al., 2003; Katsanevakis et al., 2014), our results suggest that a species-rich ecosystems with a high biodiversity are more resilient against alien introductions. These results imply that species-rich ecosystems might not only be considered healthier and more productive (Chapin III et al., 2000), they are also more capable of mitigating alien introductions.

Origin of new aliens

Lastly, as expected, the relative abundance of range expanders and cryptogenic species was very similar amongst all monitoring areas, but the abundance of the hitchhiker species seems highly influenced by the amount of human activity and corresponding boat traffic. As mentioned, the dispersal over relatively short distances by range expanders is hard to prevent, as it is mostly driven by larval dispersal through sea currents, or physical movement of specimens (Kinlan & Gaines, 2003). The status and impact of cryptogenic species are hard to determine, as little is known regarding their spatial occurrence and reproduction patterns. Until more is spatial information is published, it is advised to remain vigilant and treat these species as potential aliens. As for the hitchhiker species, these introductions are assisted by long distance boat trafficking. The most probable transport mechanisms are either by attachment to the hull of a ship, or through ballast water (Hewitt et al., 2009). Since the dispersal techniques are so specific, prevention of introduction is relatively easy compared to range expanders, which is why already several measures are enforced to reduce this type of alien introduction (Vidas & Kostelac, 2011).

Expansion of pipeline

Two major public databases were used for taxonomic assignment of ASVs and spatial occurrence data (BOLD and GBIF, respectively). Both are amongst the most complete in their respective field, which allows for a fast data output, while keeping accuracy of results high. Even though these two databases provide a significant amount of data, additional resources could be utilized in future research to improve the classification of ASVs as well as supplement geographical occurrences. As for classification of sequences, additional resources consist of, but are not limited to, the MIDORI reference library, whereas the OBIS databank could increase the number of georeferenced occurrences (Leray et al., 2018; *OBIS*, 2022). In addition, the mechanism of the tool presented in this study is to find new, undiscovered aliens, and lacks the ability to detect settled alien species. Once an alien species has been observed and is included in the GBIF occurrence database, it does not meet the 250 km threshold for aliens and is therefore not classified as new alien anymore. In case future research wish to include known, settled aliens, it is advised to include the recently published R package *dasco* in the analysis. Addition of this package to the tool will severely increase running time, but will provide more insight in the process of alien introduction, as it is able to identify settled aliens by using historical occurrence data of local databases, GBIF and OBIS (Seebens & Kaplan, 2022). Moreover, to increase the quality of the data and rule out contamination and PCR or sequencing artefacts, a stricter analysis of potential contamination is essential. The *decontam* package is able to qualify and quantify contamination, and works seamlessly with the *phyloseq* package, which is currently used in the data analysis (Davis et al., 2018).

Comprehensive reference library

DNA metabarcoding has been used successfully in dozens of monitoring studies to identify species, but the classification of ASVs and operational taxonomic units (OTUs) to species level has been a

topic of discussion (Weigand et al., 2019). Using BOLDigger as database, this study was able to classify 15.8% of all ASVs to species level, which is comparable to the 15.1% Cahill et al. (2018) were able to classify with BOLD as only reference library. Ip et al (2021) were able to increase the fraction of classified molecular OTUs (MOTUs) to 24.7% by using multiple reference libraries, including local databases, demonstrating the benefit of multi-library classification. Other studies found much lower classification rates, such as Pearman et al (2021), who were only able to classify only 3.6% of COI ASVs to species level using MIDORI, but had higher efficiency with the 18S gene (31.4%). Besides, their study was conducted in a secluded region, of which the reference library is likely incomprehensive.

Despite the relatively high fraction of reads classified (34.3%), almost 85% of ASVs could not be classified to species level, leading to a vast amount of missing data. A complete reference library, and therefore aiming for 100% accurate classification of all ASVs or (M)OTUs is key in gaining insight in species diversity and identification of NIS. Use of additional reference libraries such as MIDORI is highly recommended for future studies to decrease the number of unclassified ASVs, but even then it is unlikely that all ASVs will be classified. The need for an extensive and complete library has been expressed in prior studies, and the results of the current study emphasize the value of such a database.

The ratio of alien species found in this study, with on average 1 in 10 species considered alien at a single sampling location, is in line with other studies. Ibabe et al. (2021) reported similar results, as they detected 141 OTUs marine OTUs in sediment and water samples in one of the main sea ports of Spain, and identified 14 of those as NIS. Out of these 14 NIS, 12 species could be classified as hitchhiker species, as they originated from the Northern Pacific and the Southwest Atlantic, emphasizing the relationship between shipping and hitchhiker aliens. In the study by Tamburini et al. (2021), a different type of ASU, with smaller plates and a shorter deployment time was used. However, 10% of the identified species was still considered as alien, despite using traditional identification methods and no genetic tools.

Expansion of ARMS-MBON data

This study is one of the first to explore the vast amounts of data produced by the ARMS-MBON project and has demonstrated one of many possible implications, by detecting alien species patterns across a broad range of observatories. Alien introductions have been extensively studied, but most often within a single sampling site. While this is still possible with the vast amounts of sequence data provided by the ARMS-MBON project, the current study has demonstrated the possible implications of using a wide variety of sampling locations. Despite the current findings, the alien identification tool is built to be future-proof, in order to process the increasing amount of sampling locations. On top of the continued sampling by existing observatories, ARMS are now being deployed in Ireland, Germany, Norway and other locations across and outside Europe, providing an even wider range of environmental factors to explore.

Conclusions

In summary, the present study presents a tool which is able to identify alien species on a large scale, which was applied on a large dataset of benthic communities. The corresponding results suggest that MPAs show a high degree of biodiversity, while sampling sites with more and intense human activity showed lower biodiversity. Results imply that the amount and type of human activities does not influence the number or fraction of alien species. However, species rich ecosystems were found to contain less undiscovered aliens. In addition, a strong relationship was found between the type of alien species and

shipping traffic, with hitchhiker species, often coming from the USA or east Asia, being more abundant in industrial ports and marinas, while being absent in MPAs.

Acknowledgements

Data availability

All sequence data used in the current study is readily available and can be found on https://plutof.ut.ee/#/study/view/81139.

References

Bax, N., Williamson, A., Aguero, M., Gonzalez, E., & Geeves, W. (2003). Marine invasive alien species: A threat to global biodiversity. *Marine Policy*, *27*(4), 313–323. https://doi.org/10.1016/S0308-597X(03)00041-1

Beisiegel, K., Darr, A., Gogina, M., & Zettler, M. L. (2017). Benefits and shortcomings of non-destructive benthic imagery for monitoring hard-bottom habitats. *Marine Pollution Bulletin*, *121*, 5–15. https://doi.org/10.1016/j.marpolbul.2017.04.009

Bourlat, S. J., Borja, A., Gilbert, J., Taylor, M. I., Davies, N., Weisberg, S. B., Griffith, J. F., Lettieri, T., Field, D., Benzie, J., Glöckner, F. O., Rodríguez-Ezpeleta, N., Faith, D. P., Bean, T. P., & Obst, M. (2013). Genomics in marine monitoring: New opportunities for assessing marine health status. *Marine Pollution Bulletin*, *74*(1), 19–31. https://doi.org/10.1016/j.marpolbul.2013.05.042

Buchner, D., & Leese, F. (2020). BOLDigger – a Python package to identify and organise sequences with the Barcode of Life Data systems. *Metabarcoding and Metagenomics*, *4*, e53535. https://doi.org/10.3897/mbmg.4.53535

Cahill, A. E., Pearman, J. K., Borja, A., Carugati, L., Carvalho, S., Danovaro, R., Dashfield, S., David, R., Féral, J. P., Olenin, S., Šiaulys, A., Somerfield, P. J., Trayanova, A., Uyarra, M. C., & Chenuil, A. (2018). A comparative analysis of metabarcoding and morphology-based identification of benthic communities across different regional seas. *Ecology and Evolution*, *8*(17), 8908–8920. https://doi.org/10.1002/ECE3.4283

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods*, *13*(7), 581–583. https://doi.org/10.1038/nmeth.3869

Chapin III, F. S., Zavaleta, E. S., Eviner, V. T., Naylor, R. L., Vitousek, P. M., Reynolds, H. L., Hooper, D. U., Lavorel, S., Sala, O. E., Hobbie, S. E., Mack, M. C., & Díaz, S. (2000). Consequences of changing biodiversity. *Nature*, *405*, 234–242. https://doi.org/10.1038/35012241

Couton, M., Comtet, T., Le Cam, S., Corre, E., & Viard, F. (2019). Metabarcoding on planktonic larval stages: An efficient approach for detecting and investigating life cycle dynamics of benthic

aliens. *Management of Biological Invasions*, *10*(4), 657–689.
https://doi.org/10.3391/MBI.2019.10.4.06

David, R., Uyarra, M. C., Carvalho, S., Anlauf, H., Borja, A., Cahill, A. E., Carugati, L., Danovaro, R., De
Jode, A., Feral, J. P., Guillemain, D., Martire, M. L., D'Avray, L. T. D. V., Pearman, J. K., & Chenuil,
A. (2019). Lessons from photo analyses of Autonomous Reef Monitoring Structures as tools to
detect (bio-)geographical, spatial, and environmental effects. *Marine Pollution Bulletin*, *141*,
420–429. https://doi.org/10.1016/j.marpolbul.2019.02.066

Davis, N. M., Proctor, D. M., Holmes, S. P., Relman, D. A., & Callahan, B. J. (2018). Simple statistical
identification and removal of contaminant sequences in marker-gene and metagenomics data.
Microbiome, *6*(1), 226. https://doi.org/10.1186/s40168-018-0605-2

Duarte, S., Vieira, P. E., Lavrador, A. S., & Costa, F. O. (2021). Status and prospects of marine NIS
detection and monitoring through (e)DNA metabarcoding. *Science of The Total Environment*,
751, 141729. https://doi.org/10.1016/j.scitotenv.2020.141729

Furman, E., Pihlajamäki, M., Välipakka, P., & Myrberg, K. (Eds.). (2014). *The Baltic Sea Environment and
Ecology*.

HELCOM. (2018). *State of the Baltic Sea – Second HELCOM holistic assessment 2011-2016. Baltic Sea
Environment Proceedings 155.*

Hewitt, C. L., Gollasch, S., & Minchin, D. (2009). The Vessel as a Vector – Biofouling, Ballast Water and
Sediments. In G. Rilov & J. A. Crooks (Eds.), *Biological Invasions in Marine Ecosystems:
Ecological, Management, and Geographic Perspectives* (pp. 117–131). Springer.
https://doi.org/10.1007/978-3-540-79236-9_6

Ibabe, A., Miralles, L., Carleos, C. E., Soto-López, V., Menéndez-Teleña, D., Bartolomé, M., Montes, H. J.,
González, M., Dopico, E., Garcia-Vazquez, E., & Borrell, Y. J. (2021). Building on gAMBI in ports
for a challenging biological invasions scenario: Blue-gNIS as a proof of concept. *Marine
Environmental Research*, *169*. https://doi.org/10.1016/j.marenvres.2021.105340

Ip, Y. C. A., Tay, Y. C., Chang, J. J. M., Ang, H. P., Tun, K. P. P., Chou, L. M., Huang, D., & Meier, R. (2021).
Seeking life in sedimented waters: Environmental DNA from diverse habitat types reveals
ecologically significant species in a tropical marine environment. *Environmental DNA*, *3*(3), 654–
668. https://doi.org/10.1002/edn3.162

Kassambara, A. (2022a). *ggpubr: 'ggplot2' Based Publication Ready Plots*. https://cran.r-
project.org/package=ggpubr

Kassambara, A. (2022b). *rstatix: Pipe-Friendly Framework for Basic Statistical Tests*. https://cran.r-
project.org/package=rstatix

Katsanevakis, S., Wallentinus, I., Zenetos, A., Leppäkoski, E., Çinar, M., Oztürk, B., Grabowski, M., Golani,
D., & Cardoso, A. (2014). Impacts of invasive alien marine species on ecosystem services and
biodiversity: A pan-European review. *Aquatic Invasions*, *9*, 391–423.
https://doi.org/10.3391/ai.2014.9.4.01

Keck, F., Blackman, R. C., Bossart, R., Brantschen, J., Couton, M., Hürlemann, S., Kirschner, D., Locher, N.,
Zhang, H., & Altermatt, F. (2022). Meta-analysis shows both congruence and complementarity of
DNA and eDNA metabarcoding to traditional methods for biological community assessment.
Molecular Ecology, *31*(6), 1820–1835. https://doi.org/10.1111/mec.16364

Kinlan, B. P., & Gaines, S. D. (2003). Propagule Dispersal in Marine and Terrestrial Environments: A Community Perspective. *Ecology*, *84*(8), 2007–2020. https://doi.org/10.1890/01-0622

Leray, M., Ho, S.-L., Lin, I.-J., & Machida, R. J. (2018). MIDORI server: A webserver for taxonomic assignment of unknown metazoan mitochondrial-encoded sequences using a curated database. *Bioinformatics*, *34*(21), 3753–3754. https://doi.org/10.1093/bioinformatics/bty454

Leray, M., & Knowlton, N. (2015). DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. *Proceedings of the National Academy of Sciences*, *112*(7), 2076–2081. https://doi.org/10.1073/pnas.1424997112

Lotze, H. K., Lenihan, H. S., Bourque, B. J., Bradbury, R. H., Cooke, R. G., Kay, M. C., Kidwell, S. M., Kirby, M. X., Peterson, C. H., & Jackson, J. B. C. (2006). Depletion degradation, and recovery potential of estuaries and coastal seas. *Science*, *312*(5781), 1806–1809. https://doi.org/10.1126/science.1128035

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE*, *8*(4). https://doi.org/10.1371/journal.pone.0061217

OBIS. (2022). Ocean Biodiversity Information System. https://obis.org/data/access/#

Obst, M. (2020). *Molecular Standard Operating Procedure (MSOP) for Marine Biodiversity Observation network for genetic monitoring of hard-bottom communities (ARMS-MBON)*. https://www.assembleplus.eu/sites/assembleplus.eu/files/public/ARMS/ARMS_MSOPs.pdf

Obst, M., Deneudt, K., & Exter, K. (2020). *Data Management Plan (DMP) for Marine Biodiversity Observation Network for genetic monitoring of hard-bottom communities (ARMS-MBON)*.

Obst, M., Exter, K., Allcock, A. L., Arvanitidis, C., Axberg, A., Bustamante, M., Cancio, I., Carreira-Flores, D., Chatzinikolaou, E., Chatzigeorgiou, G., Chrismas, N., Clark, M. S., Comtet, T., Dailianis, T., Davies, N., Deneudt, K., de Cerio, O. D., Fortič, A., Gerovasileiou, V., … Pavloudi, C. (2020). A Marine Biodiversity Observation Network for Genetic Monitoring of Hard-Bottom Communities (ARMS-MBON). *Frontiers in Marine Science*, *7*. https://doi.org/10.3389/fmars.2020.572680

Oksanen, J., Simpson, G. L., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., Solymos, P., Stevens, M. H. H., Szoecs, E., Wagner, H., Barbour, M., Bedward, M., Bolker, B., Borcard, D., Carvalho, G., Chirico, M., Caceres, M. D., Durand, S., … Weedon, J. (2022). *vegan: Community Ecology Package*. https://CRAN.R-project.org/package=vegan

OSPAR Commission. (2000). *Quality status report 2000*. OSPAR Comission.

Pearman, J. K., von Ammon, U., Laroche, O., Zaiko, A., Wood, S. A., Zubia, M., Planes, S., & Pochon, X. (2021). Metabarcoding as a tool to enhance marine surveillance of nonindigenous species in tropical harbors: A case study in Tahiti. *Environmental DNA*, *3*(1), 173–189. https://doi.org/10.1002/edn3.154

R Core Team. (2022). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.R-project.org/

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Notes*, *7*(3), 355–364. https://doi.org/10.1111/j.1471-8286.2007.01678.x

Rockström, J., Steffen, W., Noone, K., Persson, Å., Chapin, F. S., Lambin, E. F., Lenton, T. M., Scheffer, M., Folke, C., Schellnhuber, H. J., Nykvist, B., De Wit, C. A., Hughes, T., Van Der Leeuw, S., Rodhe, H.,

Sörlin, S., Snyder, P. K., Costanza, R., Svedin, U., … Foley, J. A. (2009). A safe operating space for humanity. *Nature*, *461*(7263), 472–475. https://doi.org/10.1038/461472a

RStudio Team. (2022). *RStudio: Integrated Development Environment for R*. RStudio, PBC. http://www.rstudio.com/

Sardain, A., Sardain, E., & Leung, B. (2019). Global forecasts of shipping traffic and biological invasions to 2050. *Nature Sustainability*, *2*(4), 274–282. https://doi.org/10.1038/s41893-019-0245-y

Seebens, H., Blackburn, T. M., Dyer, E. E., Genovesi, P., Hulme, P. E., Jeschke, J. M., Pagad, S., Pyšek, P., Winter, M., Arianoutsou, M., Bacher, S., Blasius, B., Brundu, G., Capinha, C., Celesti-Grapow, L., Dawson, W., Dullinger, S., Fuentes, N., Jäger, H., … Essl, F. (2017). No saturation in the accumulation of alien species worldwide. *Nature Communications*, *8*(1), 1–9. https://doi.org/10.1038/ncomms14435

Seebens, H., & Kaplan, E. (2022). DASCO: A workflow to downscale alien species checklists using occurrence records and to re-allocate species distributions across realms. *NeoBiota*, *74*, 75–91. https://doi.org/10.3897/neobiota.74.81082

Tamburini, M., Keppel, E., Marchini, A., Repetto, M. F., Ruiz, G. M., Ferrario, J., & Occhipinti-Ambrogi, A. (2021). Monitoring Non-indigenous Species in Port Habitats: First Application of a Standardized North American Protocol in the Mediterranean Sea. *Frontiers in Marine Science*, *8*. https://doi.org/10.3389/fmars.2021.700730

Vidas, D., & Kostelac, M. M. (2011). *Ballast Water and Alien Species: Regulating Global Transfers and Regional Consequences*. 371–392. https://doi.org/10.1163/9789004204225_022

Weigand, H., Beermann, A. J., Čiampor, F., Costa, F. O., Csabai, Z., Duarte, S., Geiger, M. F., Grabowski, M., Rimet, F., Rulik, B., Strand, M., Szucsich, N., Weigand, A. M., Willassen, E., Wyler, S. A., Bouchez, A., Borja, A., Čiamporová-Zaťovičová, Z., Ferreira, S., … Ekrem, T. (2019). DNA barcode reference libraries for the monitoring of aquatic biota in Europe: Gap-analysis and recommendations for future work. *Science of The Total Environment*, *678*, 499–524. https://doi.org/10.1016/j.scitotenv.2019.04.247

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag. https://ggplot2.tidyverse.org

Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T. L., Miller, E., Bache, S. M., Müller, K., Ooms, J., Robinson, D., Seidel, D. P., Spinu, V., … Yutani, H. (2019). Welcome to the Tidyverse. *Journal of Open Source Software*, *4*(43), 1686. https://doi.org/10.21105/joss.01686

Wickham, H., François, R., Henry, L., Müller, K., & RStudio. (2022). *dplyr: A Grammar of Data Manipulation* (1.0.9) [Computer software]. https://CRAN.R-project.org/package=dplyr

Worm, B., Barbier, E. B., Beaumont, N., Duffy, J. E., Folke, C., Halpern, B. S., Jackson, J. B. C., Lotze, H. K., Micheli, F., Palumbi, S. R., Sala, E., Selkoe, K. A., Stachowicz, J. J., & Watson, R. (2006). Impacts of biodiversity loss on ocean ecosystem services. *Science*, *314*, 787–790. https://doi.org/10.1126/science.1132294