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Molecular analysis of marine benthic biodiversity: methodological implementations and perspectives

Tesi di Dottorato di:
Laura Carugati

Tutor:
Prof. Roberto Danovaro

Co-Tutor:
Prof. Antonio Dell'Anno

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RIASSUNTO

La biodiversità marina regola il funzionamento ecosistemico, responsabile della produzione di beni e servizi importanti per la biosfera e il benessere umano. La stima della biodiversità è uno degli obiettivi fondamentali della scienza. I cambiamenti globali e le attività antropiche stanno avendo un impatto crescente sugli ecosistemi. Le valutazioni d'impatto e i programmi di monitoraggio si basano su specie di grandi dimensioni, trascurando gli organismi di piccola taglia come la meiofauna (20-500 μm), a causa delle difficoltà connesse con la tradizionale identificazione morfologica. L'obiettivo di questo lavoro è quello di implementare i metodi per lo studio della meiofauna, combinando l'approccio morfologico e molecolare. Al fine di rispondere alle esigenze della Direttiva Quadro sulla Strategia Marina sono stati testati, in diversi ecosistemi marini, uno strumento per la valutazione dello stato ambientale e varie metodologie innovative di campionamento. Gli strumenti molecolari, come il metabarcoding, insieme ai nuovi sistemi di campionamento, mostrano un grande potenziale per lo studio della biodiversità marina. Il protocollo molecolare qui messo a punto, permette di analizzare la diversità di organismi di piccola taglia in modo rapido ed economicamente efficiente, migliorando la capacità di studiare la biodiversità della meiofauna anche in ecosistemi marini profondi. Nonostante i numerosi vantaggi, l'applicazione del metabarcoding al monitoraggio richiede il superamento di alcuni limiti. L'incompletezza delle banche dati e la presenza di copie multiple di geni in individui della stessa specie ostacolano l'interpretazione dei dati e non consentono di effettuare stime quantitative di biodiversità. Per garantire che le analisi di metabarcoding possano essere ulteriormente convalidate, risulta cruciale conservare la competenza nell'identificazione morfologica. L'approccio tradizionale e quello molecolare forniscono informazioni diverse, quindi dovrebbero essere usati entrambi per ottenere stime accurate di biodiversità.

ABSTRACT

Marine biodiversity regulates ecosystem functions that are responsible for the production of goods and services for the entire biosphere and human wellbeing. Censusing species inhabit oceans is among the most fundamental questions in science. Global changes and human activities are having an increasing impact on ocean biodiversity and ecosystem functioning. Impact assessments and monitoring programmes are almost based on large and conspicuous species. Small and cryptic organisms including eukaryotic meiobenthic fauna (20-500 µm) remain overlooked due to the difficulties associated with the traditional, morphology-based identification. We here aim to implement methods for the study of meiofaunal biodiversity, combining classical morphological and molecular approaches. In order to answer to the needs of the Marine Strategy Framework Directive, an environmental status assessment tool and a variety of innovative sampling methodologies have been tested in different ecosystems. Along with new sampling approaches, molecular tools, as metabarcoding, have a great potential in innovating the analysis of marine biodiversity. Molecular protocol set up in this work allows to analyse the diversity of tiny organisms in a rapid and cost-effective way, thus increasing our ability to investigate meiofaunal biodiversity, also in the deep sea. Beyond its many advantages, the routine application of metabarcoding for monitoring requires overcoming some limitations. The incompleteness of public databases and the presence of multi-copy genes within meiofaunal species hamper the interpretation of metabarcoding data and do not allow to infer biodiversity quantitative estimates. It is thus critically important to maintain expertise in morphological identification to ensure that metabarcoding analyses can be further validated. Morphological- and molecular-based approaches provide different information, thus they should be combined to obtain better evaluations of the actual marine biodiversity.

CHAPTER 1. INTRODUCTION

Almost 20 years ago, Gray stated: "Complete loss of habitat is the most serious threat to marine biodiversity" (Gray, 1997). Although habitat loss would have dramatic effects on marine biodiversity, nowadays, it is recognized that pressures and threats due to anthropogenic activities and climate changes can erode biodiversity before habitat is completely lost. These stressors can act in synergy and cause changes in biodiversity that are more pervasive than those caused by single disturbances (Sala and Knowlton, 2006).

Human activities such as overfishing, agricultural expansion, dumping, mining, waste, pollution, species introductions can reduce marine biodiversity, by i) accelerating food webs (increasing the turnover of communities via fishing down food webs and enhancing microbial activity), ii) causing pollution-mediated mass mortalities of marine organisms (e.g., dead zones), and iii) facilitating the dominance of invasive species (Halpern et al., 2008). While anthropogenic activities are considered the main direct drivers of biodiversity loss during recent years (Hoffmann et al., 2010), climate change is likely to become the main cause of extinction over the coming century (Burrows et al., 2011; Doney et al., 2012). In addition, human-forced climate changes, such as those provoked by anthropogenic greenhouse gas emissions, have already caused increased temperatures, sea level rise, altered rainfall patterns and the frequency and severity of extreme events (IPCC, 2014). Climate changes induced by human activities may also have an indirect impact on marine ecosystems, altering how and where people interact with environment (Watson, 2014). The increasing attitude to build seawalls to protect against sea-level rise, has led to habitat loss (Dugan et al., 2008; Grantham et al., 2011) and changes in fish distribution (Pinsky and Fogarty, 2012). The impacts of relocation of urban areas and agricultural land due to future sea level rise may be more severe than the direct influence of sea-level rise (Wetzel et al., 2012).

Widespread impacts of human activities and climate changes on marine biodiversity can threaten ecosystem functions and thus ecosystem services, reduce ecosystem stability and resilience and hinder the recovery of biodiversity (Lotze et al., 2006; Sala and Knowlton, 2006; Worm et al., 2006; Danovaro et al., 2008; Halpern et al., 2008; Butchart et al., 2010). These effects have been reported for different geographic areas (Duffy, 2003; Springer et al., 2003; Sala and Knowlton, 2006). With the increasing awareness about the benefits that healthy marine ecosystems provide to people (MEA, 2005; McLeod and Leslie, 2009), the protection of biodiversity and the essential ecosystem services it supports has

become a priority for the scientific community, resource managers and national and international policy agreements.

Concerns about human impacts on marine life can be traced back in 1992 in Rio de Janeiro where Convention on Biological Diversity was signed. In 2002, during the Conference of the Parties (COP 6), the Strategic Plan for the Convention has been adopted and world leaders agreed "to achieve by 2010 a significant reduction of the current rate of biodiversity loss" (Secretariat of the Convention on Biological Diversity, 2005). The World Summit on Sustainable Development, held in Johannesburg in 2002, reviewed progress made during the last 10 years, confirming the "2010 target" and reaffirming that biodiversity plays a critical role in overall sustainable development. More recently, recommendations for marine biodiversity conservation were also risen by the United Nation Environmental Programme (Corrigan and Kershaw, 2008) and the Ocean Policy Task Force (Force, I.O.P. T. 2010). In September 2015 at an historic UN Summit in New York, the Sustainable Development Goals were signed. Goal 14 focuses on the conservation and sustainably use of the oceans, seas and marine resources for sustainable development (United Nations. General Assembly, 2015). For these reasons, regional and global assessments of the status, trends and future scenarios of biodiversity and ecosystem services need to be produced (Visconti et al., 2016).

Efforts to address the loss of biodiversity need to be strengthened, integrating biodiversity into broad-scale land-use planning and incorporating its economic value into decision making (Butchart et al., 2010). With the increasing appreciation of marine biodiversity and the importance of its conservation, directives that specifically address marine environmental protection and management began to appear in 1990, such as the EU Habitat Directive of 1992. More recently, in 2008, European Commission enacted the Marine Strategy Framework Directive (MSFD; 2008/56/EC), which aims to manage the European seas by using an ecosystem-based approach in order to achieve Good Environmental Status (GES; i.e. a healthy and productive state of marine ecosystems) by 2020 (Borja et al., 2013).

Despite the central role of marine biodiversity in conventions, regional and national legislations, our knowledge about how many species live in the ocean is still scant. Millennium Ecosystem Assessment (MEA, 2005) provided a state-of-the-art of the condition and trends of the world's ecosystems and the services they provide, making clear that obtaining realistic assessment of marine biodiversity is crucial to better understand the potential consequences of species loss on ecosystem functioning (Danovaro et al., 2008). Despite this, after 250 years of taxonomic classification only a small fraction of marine species (ca. 9%) have been indexed in central databases (Mora et al., 2011; Appeltans

et al., 2012). With extinction rates now exceeding natural background rates by a factor of 100 to 1,000 (Pimm et al., 1995; Payne et al., 2016), the slow advance in the description of species will lead to species becoming extinct before we know they even existed (Mora et al., 2011). Recent projections, based on genera containing IUCN-assessed species, predict an extinction rate of 24 to 40% of genera. The lower value is twice the background rate, and the higher is comparable to the End-Cretaceous mass extinction. If current biodiversity loss rates persist, the so called "sixth mass extinction" may exceed the magnitude of the five major extinctions occurred during the past 550 million years (Barnosky et al., 2011).

The biodiversity loss should incentive the study of marine living species and lead to a shift in the business-as-usual course for marine management (Payne et al., 2016). So far, impact assessments and monitoring programmes are based almost exclusively on large and conspicuous species that represent a small portion of marine biodiversity (Leray and Knowlton, 2016). Tiny organisms (with body size < 2 mm) including eukaryotic meiobenthic fauna, remain overlooked. Meiofauna are defined on the basis of body size as organisms passing through 500 μm mesh net and retained by a 20 μm mesh net (Mare, 1942). Meiofaunal organisms dominate benthic metazoan assemblages in all marine systems, from intertidal ecosystems down to the deep sea. Meiofauna play key roles in ecosystem functioning, contributing to nutrient cycling and being an important link between macrofauna and microbial assemblages (Danovaro and Fraschetti, 2002; Giere, 2009). All these characteristics make meiofaunal organisms excellent candidates to study biodiversity patterns (Snelgrove, 1999; Danovaro et al., 2001, 2008; Nascimento et al. 2011, 2012; Bonaglia et al. 2014). Meiofauna are also recently used as indicators of the status of marine environments, due to their high sensitivity to environmental changes and anthropogenic impacts (Moreno et al., 2011; Pusceddu et al., 2011; Bonaglia et al. 2014). The identification bottleneck associated with meiofaunal taxonomy is related to the classification of species, based on morphological characters, which is time-consuming, requires taxonomic expertise and does not allow to identify cryptic species (Bhadury et al., 2008; Fontaneto et al., 2009; Carugati et al., 2015). All these issues hamper the inclusion of meiofaunal diversity in marine monitoring programs. Molecular methods can open new perspective in the field of marine ecology, enhancing our ability to assess the biodiversity of tiny metazoans. Standard barcoding, based on Sanger sequencing, can allow us to study cryptic diversity within marine species, but it is not an ideal tool for investigating biodiversity at large spatial scales. At the beginning of XXI century, the advent of high-throughput sequencing platforms, capable of producing millions of sequences per run, may significantly increase our ability to assess meiofaunal biodiversity at larger spatial scale (Creer et al., 2010; Fonseca et al., 2010, 2014; Pawlowski

et al., 2011; Porazinska et al., 2010, 2012). “Metagenetics” or “Metabarcoding” refer to large-scale analyses of biodiversity through the amplification and sequencing of homologous genes (Creer et al., 2010).

So far, metabarcoding analyses carried out to assess marine metazoan biodiversity have been primarily based on protocols and methods implemented from prokaryotic studies. However, how such approaches could be transferred over to the metazoan assemblages has not been extensively explored. Morphological and molecular approaches have long been considered complementary, but doubts still exist regarding the accuracy and reliability of metabarcoding. Some attempts to compare morphological and metabarcoding-based taxonomic identification have been recently performed on macrofauna (Cowart et al., 2015). We still lack a pilot study aiming to analyse the reliability of metabarcoding to assess the biodiversity of meiofaunal organisms.

We here aim to implement methods for the study of marine benthic biodiversity, by using a combined approach based on classical morphological identification and molecular tools. In order to answer to the needs of the Marine Strategy Framework Directive, an environmental status assessment tool and a variety of innovative sampling methodologies, including metabarcoding, have been tested in different ecosystems. Then, we evaluated the reliability of metabarcoding for the study of meiofaunal biodiversity, through the following main steps:

i) the identification of the limits of high-throughput sequencing analyses for the assessment of marine biodiversity; such limitations need to be addressed in order to use metabarcoding in routine marine monitoring;

ii) the set up of a molecular method to analyze meiofaunal biodiversity by using high-throughput sequencing platforms (e.g., 454 pyrosequencing, Illumina MiSeq);

iii) the comparison of the morphology- and molecular-based approaches to assess the biodiversity of meiofaunal organisms, in different marine ecosystems, spanning from the deep sea to extreme environments, such as Antarctica.

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1.1 A comparison of the degree of implementation of marine biodiversity indicators by European countries in relation to the MSFD

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Abstract

The degree of development and operability of the indicators for the Marine Strategy Framework Directive (MSFD) using Descriptor 1 (D1) Biological Diversity was assessed. To this end, an overview of the relevance and degree of operability of the underlying parameters across 20 European countries was compiled by analysing national directives, legislation, regulations, and publicly available reports. Marked differences were found between countries in the degree of ecological relevance as well as in the degree of implementation and operability of the parameters chosen to indicate biological diversity. The best scoring EU countries were France, Germany, Greece and Spain, while the worst scoring countries were Italy and Slovenia. No country achieved maximum scores for the implementation of MSFD D1. The non-EU countries Norway and Turkey score as highly as the top-scoring EU countries. On the positive side, the chosen parameters for D1 indicators were generally identified as being an ecologically relevant reflection of Biological Diversity. On the negative side however, less than half of the chosen parameters are currently operational. It appears that at a pan-European level, no consistent and harmonized approach currently exists for the description and assessment of marine biological diversity. The implementation of the MSFD Descriptor 1 for Europe as a whole can therefore at best be marked as moderately successful.

Keywords: Indicators, MSFD, descriptor, marine biodiversity, operability

Introduction

From June 2008 the member countries of the European Union (EU) have been working towards the implementation of the Marine Strategy Framework Directive (MSFD). The MSDF aims to provide a holistic and effective mechanism for the protection of the marine environment with the ultimate aim being to achieve Good Environmental Status (GES) of the European marine water bodies by 2020. The timeline for MSFD implementation includes an initial status assessment (2012); an identification of environmental indicators and targets (2012); establishment of a monitoring programme (2014) and the implementation of a programme of measures towards achieving GES (2016). One of the most challenging aspects of the implementation has been the development of a consistent, scientifically-sound and harmonized approach for describing the marine environment utilising indicators of environmental status at a national and pan-European level. These indicators and their associated targets provide the information required against which the appropriate policy and adaptive management tools can be used to achieve the delicate balance between environmental protection and the sustainable use of the critical marine zone.

Among the positive aspects of implementing the MSFD are that it promotes cooperation among the involved countries and institutions, particularly through the Regional Sea Conventions i.e. the Barcelona Convention for the Mediterranean; Bucharest Convention for the Black Sea; the Oslo-Paris Convention (OSPAR) for the NE Atlantic, and the Helsinki Convention (HELCOM) for the Baltic. The MSFD also promotes the integration of approaches to inventory environmental issues at an international and national level, partly due to its robust legal and obligatory character (Milieu 2014b). It is also intended to provide a more thorough and complete "picture" of the marine environment as a whole by complementing earlier directives such as the Water Framework Directive (WFD) for transitional and coastal waters (up to 1nm or 3nm offshore).

Although the willingness to implement the MSFD may seem high in many countries, in practice there is a wide divergence in the degree to which new indicators and targets have been developed to operationalize the directive. Regarding the development of the indicators, there is a tendency among member states to extract parameters already used for OSPAR or HELCOM, Natura 2000, the Bird Directive or WFD (European Commission, 2012; OSPAR Commission, 2012b; BMUB, 2014c). Although this is necessary as a first step to ensure standardization across the various pieces of legislation, merely limiting the key parameters to those used in previous instruments would undermine the spirit and usefulness of the (new) MSFD directive.

A current key question therefore is to what extent are the GES descriptors and their underlying indicators developed in the different European countries. Moreover, since many countries are relying heavily on indicators and associated parameters from earlier directives, the question arises to what extent these parameters are relevant to the overlying descriptor. Both these questions are important as reported legal compliance with the directive presented as progress (against the MSFD milestones) may mask underlying issues with the basic science needed to report on progress towards GES.

The aim of the current study therefore was to assess the degree of development and operability of the indicators for MSFD using Descriptor 1 (D1) Biological Diversity as a test case. Descriptor D1 is a key descriptor focusing on whether Biological Diversity is maintained, and should be able to show whether the quality and occurrence of habitats and the distribution and abundance of species are in line with prevailing physiographic, geographic and climatic conditions (Directive 2008/56 EC[1]; European Commission, 2011). Good Environmental Status for Descriptor 1 should be achieved by ensuring on the one hand no further loss of the diversity of genes, species and habitats/communities at ecological relevant scales and, on the other hand, that deteriorated components, where intrinsic environmental conditions allow, are restored to target levels.

The assessment of MSFD D1 Biological Diversity parameters was carried out by compiling an overview of the relevance and degree of operability of the parameters across different European countries.

The difficulty of accessing all the relevant information means the study is not exhaustive but the large number of countries involved means it should be comprehensive enough to provide a unique assessment of the relative progress across member states.

Material and methods

Information Collation

A survey of 20 European countries was conducted to determine the actual status and degree of development of Descriptor 1 of the MSFD. The survey was undertaken by compiling and analyzing national directives, legislation, regulations, and publically available reports. Where feasible, the available information was supplemented with interviews of experts from ministries and research institutes.

[1] Directive 2008/56/EC Establishing a framework for community action in the field of marine environmental policy (Marine Strategy Framework Directive) http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32008L0056&from=EN

As some countries voluntarily have adopted the MSFD Descriptor system or installed homologous systems, the survey was extended with some neighbour-countries of the EU to assess the degree of concurrence or differentiation of those countries with the EU.

Descriptor 1 'Biological Diversity' is comprised of 7 criteria each including a range of associated indicators (or 'types' of indicators) for which parameters have been developed. Most countries have developed the indicators by breaking down the ecosystem into several components or features (i.e. functional groups and categories of taxa or habitats) (Cochrane, 2010; European Commission, 2011) (see Table 1) the key ones being Marine Mammals; Fish; Birds; Benthos; Pelagic habitats; Rock and biogenic reef habitats; Sediment habitats; and Other habitats. This results in about 40 "State Variables", also called Parameters or Metrics (OSPAR Commission, 2012a; henceforth referred to as 'parameters')[2], to be classified for D1. Since not every country develops parameters for the same component set, the number of Parameters may differ slightly between countries. The assessment omitted components for which few (less than 6) countries have developed parameters such as jellyfish, turtles and cephalopods.

[2] OSPAR Commission 2012a, p. 113: A parameter or metric is a measureable single characteristic of a species or habitat (e.g. number of individuals, biomass in g dry weight, sediment particle size diameter in mm). Parameters of this nature can be used as simple indicators, and indeed several such metrics are included in the list of indicators provided in the Commission Decision on criteria and indicators (e.g. indicator 1.2.1, population biomass).

Table 1. Overview of the criteria, indicators and most common groups for which Parameters are described with regard to Descriptor D1 Biological Diversity (codes are used in Table 2).

Criteria	Indicator	Group
1.1 Species distribution	Distributional range (1.1.1)	a. Mammals
		b. Fish
		c. Benthos
		d. Birds
	Distributional pattern within the latter (1.1.2)	a. Mammals
		b. Fish
		c. Benthos
		d. Birds
	Area covered by the species (1.1.3)	Benthos
1.2 Population size	Population abundance and/or biomass (1.2.1)	a. Mammals
		b. Fish
		c. Benthos
		d. Birds
1.3 Population condition	Population demographic characteristics (e.g. body size or age class structure, sex ratio, fecundity rates, survival/mortality rates) (1.3.1)	a. Mammals
		b. Fish
		c. Benthos
		d. Birds
	Population genetic structure (1.3.2)	Benthos
1.4 Habitat distribution	Habitat distributional range (1.4.1)	a. Pelagic habitats
		b. Rock and biogenic reef habitats
		c. Sediment habitats
		d. Other habitats
	Habitat distributional pattern (1.4.2)	a. Pelagic habitats
		b. Rock and biogenic reef habitats
		c. Sediment habitats
1.5 Habitat extent	Habitat area (1.5.1)	a. Rock and biogenic reef habitats
		b. Sediment habitat
		c. Other habitats
	Habitat volume where relevant (1.5.2)	
1.6 Habitat condition	Condition of the typical species and communities (1.6.1)	a. Pelagic habitats
		b. Rock and biogenic reef habitats
		c. Sediment habitat
		d. Benthos
	Relative abundance and/or biomass (1.6.2)	a. Pelagic habitats
		b. Rock and biogenic reef habitats
		c. Other Habitats
	Physical, hydrological and chemical conditions (1.6.3)	Sediment habitat
1.7 Ecosystem structure	Composition and relative proportions of ecosystem components (habitats and species) (1.7.1)	a. Fish
		b. Pelagic habitats
		c. Foodweb

Analysis of information

Two criteria were used to assess the indicators as described below. The evaluation was firstly carried out in May 2013, and updated from May 2014 onwards by scientists assembled in two meetings as part of the EMBOS network (COST Action ES1003 on the European Marine Biodiversity Observatory System).

CRITERION 1

Firstly, the ecological relevance of a parameter proposed by each country was assessed as to its ecological relevance, i.e. how realistically it was likely to represent the impacts of the state of the natural species or community diversity or the natural habitats in an area. The two key determinants when considering this were firstly, whether a parameter is easy to measure or not and secondly how representative a parameter is of the structural and functional state of diversity in a coastal system. For the latter determinant, an additional consideration is how sensitive the parameter is to stressors and other impacts so state change can be identified. The indicators were classified on a scale representing not relevant (0); somewhat relevant (1); definitely relevant (2).

For example, the presence of a specific rare seabird species (as in Italy; MATTM, 2009), which is difficult to observe, would not be deemed as being a proper ecological measure of the diversity of a coastal system, both due to the problems with measuring the parameter and in being a poor link to the state of the coastal biodiversity it is supposed to be an indicator for. Thus the parameter would be judged as not relevant (0) or at most somewhat relevant (1). In contrast, parameters based on multivariate measures of diversity are more likely to be representative of the biological diversity of the habitat and can be linked to pressures through known effects on biodiversity.

In most cases, the ecological relevance of the indicator was based on the expert judgment of the scientists undertaking the assessment supplemented with information from the literature on the importance given to the different parameters (e.g. for Spain: Borja *et al.*, 2011; Velasco *et al.*, 2012).

CRITERION 2

The second criterion for the assessment was the degree of operability of a parameter. Parameters were considered as not being operational when they were either still under discussion by member states or not being taken forward at all at the present time, in which case they were assigned a score of 0. If a parameter is in development (e.g. R&D is being carried out to operationalize it) then it was assigned a score of 1. Finally, if an indicator is already operational then it was assigned a score of 2.

The sum of the scores was calculated with the score for each parameter ranging from 0 to 4. A combined score of 0 means that the parameter is currently not deemed to be implemented or is poorly implemented at best; a score of 4 means the parameter is being well implemented by that member state.

Results and discussion

Marked differences were found between countries in the degree of ecological relevance as well as in the degree of implementation and operability of the parameters chosen to indicate biological diversity (Figure 1, Table 2).

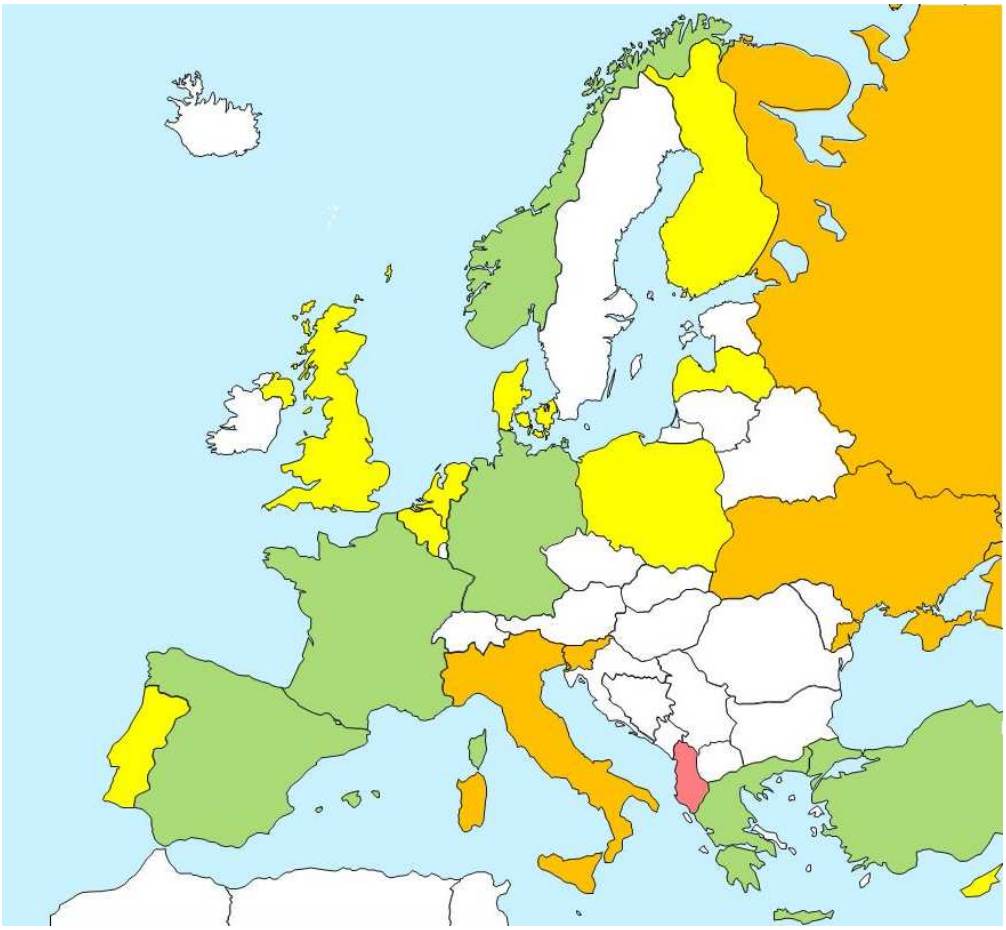

Figure 1. Average score on relevance and operability of Parameters for Descriptor D1 on Biological Diversity proposed by the different European countries. Color codes mean that the MSFD D1 has been implemented: poorly (red), inadequately (orange), moderately (yellow), good (light green) and fully (dark green) (see for color codes also the legend in Table 2; data were checked for blank countries; not any country falls in the 'fully implemented' category).

The first thing to notice in the results of the evaluation is the wide disparity between countries in the implementation of D1 via its parameters. There is also variation within countries with some functional components and some indicator classes are better developed than other. For example, in the Netherlands, Norway and Poland some clusters of related parameters are considered relevant and operational while other clusters are neither relevant nor operational. It is also noticeable that in certain countries such as Turkey, relevant parameters have been developed for almost all groups but with a relatively low degree of operationalization. This variation illustrates strong differences between countries in their strategies for developing this descriptor, some developing parameters that are relevant and largely operational, whereas other focusing on the development of a wider range of (theoretical) parameters but which are far from being made operational.

These differences between countries are corroborated by the German National Measuring Programme (BLMP, 2014) stating that the listed German indicators are at different stages of development, some being operational, while others are lacking evaluation criteria and/or monitoring.

Some differences have been identified across sub-regions within the same country. The same way, indicators and descriptors have not been implemented/assessed in all the regions of a country (e.g. Spain).

Table 2: Country scores for the ecological relevance and operability of Parameters for Descriptor D1 on Biological Diversity proposed by the different countries (group-codes are according Table 1).

References used are for Belgium: Belgische Staat 2012, État Belge 2012; Cyprus: DFMR 2012a,b,c, 2014a,b; Denmark: DME 2012, 2014; Finland: SYKE 2012, 2014; France: Guérin et al 2013, Dupont et al 2014, SRMC 2014, SRMG 2014, SRMM 2014; Germany: BLMP 2014, BMUB 2014a,b,c; Greece: MEECC 2014, Italy: MATTM 2009, 2010, Munari & Mistri 2010, COREM 2012, Cima & Ballarin 2013, ISPRA 2013, Tunesi et al 2013, Milieu 2014a; Latvia: LHEI 2012, 2014, MARMONI 2014; Netherlands: MIE 2012, 2014; Norway: Certain et al 2011, Nybø et al 2011, 2012, Aslaksen & Garnåsjordet 2012, Aslaksen et al 2012; Poland: Krzymiński 2013, 2014; Portugal: MAMAOT 2012, 2014; Russia: Guide to hydrological work in the oceans and seas 1977, Anonymous 1985, VNIRO 2004; Slovenia: Peterlin et al 2013; Spain: Anonymous 2010, Borja et al 2011, Arcos et al 2012, Gil et al 2012, Gil de Sola et al 2012, Hernández et al 2012, MAGRAMA 2014, Ruiz et al 2012, Santos Vázquez et al 2012, Velasco et al 2012, Milieu 2014b, Palialexis et al 2014; Turkey: UNEP/MAP 2007, MFWM 2008, Şekeroğlu et al 2011, Sumer & Muluk 2011; Ukraine: Losovskaya 2005, Zamora et al 2005, Anistratenko et al 2007, Mikhalev 2008, Gladilina 2010, MENRU 2010, Petrenko 2013, Vishnyakova & Gol'din 2014, United Kingdom: Cook et al 2012, ICES 2013, OSPAR Commission 2013, Burrows et al 2014a,b, DEFRA 2014, Fariñas-Franco et al 2014, Haynes et al 2014.

Legend to Table 2:

Relevance of Parameter chosen by country	Operational status	Combined score	Color code for combined score
2: Parameter is definitely ecological relevant			
1: Parameter is somewhat ecological relevant			
0: No Parameter available or not relevant			
	2: Operational		
	1: Under Development		
	0: Not operational		
0	0	Sum count 0	0.0 – 0.8: Implementation of MSFD D1 is Poor
0 / 1	1 / 0	Sum count 1	0.8 – 1.6: Implementation of MSFD D1 is Inadequate
1	1	Sum count 2	1.6 – 2.4: Implementation of MSFD D1 is Moderate
1 / 2	2 / 1	Sum count 3	2.4 – 3.2: Implementation of MSFD D1 is Good
2	2	Sum count 4	3.2 – 4.0: Implementation of MSFD D1 is Full

Group	Albania	Albania	Albania	Belgium	Belgium	Belgium	Cyprus	Cyprus	Cyprus	Denmark	Denmark	Denmark	Finland	Finland	Finland
Code	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined
1.1.1.a	0	0	0	1	2	3	1	1	2	1	2	3	2	2	4
1.1.1.b	0	0	0	1	2	3	2	2	4	0	0	0	1	2	3
1.1.1.c	0	0	0	2	0	2	2	2	4	0	0	0	0	0	0
1.1.1.d	0	0	0	1	2	3	1	1	2	1	2	3	2	1	3
1.1.2.a	0	0	0	1	0	1	1	1	2	1	2	3	2	2	4
1.1.2.b	0	0	0	1	0	1	2	2	4	0	0	0	0	0	0
1.1.2.c	0	0	0	2	0	2	1	2	3	0	0	0	0	0	0
1.1.2.d	0	0	0	1	0	1	1	1	2	1	2	3	2	1	3
1.1.3	0	0	0	2	1	3	1	2	3	2	0	2	1	1	2
1.2.1.a	0	0	0	1	2	3	1	1	2	1	2	3	0	0	0
1.2.1.b	0	0	0	1	2	3	2	2	4	0	0	0	2	1	3
1.2.1.c	0	0	0	2	1	3	1	2	3	0	0	0	0	0	0
1.2.1.d	0	0	0	1	2	3	1	1	2	1	1	2	2	1	3
1.3.1.a	0	0	0	1	2	3	1	1	2	1	2	3	2	2	4
1.3.1.b	0	0	0	1	2	3	2	2	4	0	0	0	2	2	4
1.3.1.c	0	0	0	2	0	2	0	0	0	0	0	0	2	2	4
1.3.1.d	0	0	0	1	1	2	1	1	2	2	2	4	2	2	4
1.3.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.4.1.a	0	0	0	1	0	1	1	0	1	0	0	0	2	1	3
1.4.1.b	0	0	0	1	0	1	1	0	1	2	1	3	2	1	3
1.4.1.c	0	0	0	2	1	3	1	0	1	1	1	2	2	1	3
1.4.1.d	0	0	0	1	1	2	1	1	2	1	1	2	2	1	3
1.4.2.a	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.4.2.b	0	0	0	1	1	2	0	0	0	1	1	2	0	0	0
1.4.2.c	0	0	0	2	1	3	0	0	0	1	1	2	0	0	0
1.5.1.a	0	0	0	2	0	2	0	0	0	2	1	3	2	1	3
1.5.1.b	0	0	0	1	0	1	0	0	0	1	1	2	2	1	3
1.5.1.c	0	0	0	1	0	1	1	1	2	1	1	2	2	1	3
1.5.2	0	0	0	0	0	0	0	0	0	1	1	2	2	1	3
1.6.1.a	0	0	0	1	0	1	0	0	0	0	0	0	2	1	3
1.6.1.b	0	0	0	0	0	0	1	2	3	1	2	3	2	1	3
1.6.1.c	0	0	0	2	2	4	1	2	3	1	2	3	2	1	3
1.6.1.d	0	0	0	1	1	2	2	2	4	1	2	3	2	1	3
1.6.2.a	0	0	0	1	0	1	1	0	1	0	0	0	0	0	0
1.6.2.b	0	0	0	2	0	2	1	2	3	2	1	3	0	0	0
1.6.2.c	0	0	0	2	0	2	1	2	3	1	2	3	1	1	2
1.6.3	0	0	0	2	0	2	1	1	2	2	2	4	2	2	4
1.7.1.a	0	0	0	2	0	2	1	1	2	1	1	2	2	1	3
1.7.1.b	0	0	0	1	0	1	0	0	0	1	1	2	2	1	3
1.7.1.c	0	0	0	1	0	1	0	0	0	1	1	2	2	1	3
Average score			0			1,875			1,825			1,775			2,3

Group	France	France	France	Germany	Germany	Germany	Greece	Greece	Greece	Italy	Italy	Italy	Latvia	Latvia	Latvia
Code	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined
1.1.1.a	1	2	3	2	2	4	1	1	2	0	0	0	0	0	0
1.1.1.b	2	1	3	2	2	4	2	2	4	0	0	0	2	2	4
1.1.1.c	0	0	0	2	1	3	1	2	3	0	0	0	2	2	4
1.1.1.d	2	2	4	2	2	4	0	2	2	0	0	0	2	1	3
1.1.2.a	1	2	3	2	2	4	1	1	2	0	0	0	0	0	0
1.1.2.b	2	1	3	2	2	4	2	2	4	0	0	0	0	0	0
1.1.2.c	0	0	0	2	1	3	0	1	1	0	0	0	0	0	0
1.1.2.d	2	2	4	2	2	4	0	2	2	0	0	0	2	1	3
1.1.3	0	0	0	2	1	3	2	2	4	0	0	0	0	0	0
1.2.1.a	2	2	4	2	2	4	1	1	2	1	2	3	0	0	0
1.2.1.b	2	1	3	1	1	2	2	2	4	1	2	3	2	1	3
1.2.1.c	0	0	0	2	2	4	2	1	3	1	2	3	0	0	0
1.2.1.d	2	2	4	2	2	4	1	1	2	1	1	2	2	2	4
1.3.1.a	1	2	3	2	2	4	1	1	2	0	0	0	0	0	0
1.3.1.b	2	1	3	1	1	2	1	2	3	1	2	3	0	0	0
1.3.1.c	0	0	0	2	1	3	0	0	0	1	2	3	2	2	4
1.3.1.d	2	2	4	2	2	4	1	1	2	1	1	2	2	1	3
1.3.2	1	0	1	0	0	0	0	1	1	1	1	2	0	0	0
1.4.1.a	2	1	3	0	0	0	2	2	4	0	0	0	0	0	0
1.4.1.b	2	1	3	2	1	3	1	1	2	0	0	0	2	2	4
1.4.1.c	2	1	3	2	1	3	1	1	2	0	0	0	0	0	0
1.4.1.d	1	0	1	0	0	0	2	2	4	0	0	0	0	0	0
1.4.2.a	2	1	3	1	1	2	1	1	2	0	0	0	0	0	0
1.4.2.b	2	1	3	2	1	3	1	1	2	0	0	0	0	0	0
1.4.2.c	2	1	3	2	1	3	1	1	2	0	0	0	0	0	0
1.5.1.a	2	1	3	2	1	3	2	1	3	1	2	3	2	1	3
1.5.1.b	2	1	3	2	1	3	2	1	3	0	0	0	0	0	0
1.5.1.c	1	0	1	0	0	0	2	2	4	1	2	3	0	0	0
1.5.2	1	1	2	0	0	0	1	0	1	0	0	0	0	0	0
1.6.1.a	2	1	3	1	1	2	1	1	2	0	0	0	2	1	3
1.6.1.b	2	1	3	0	0	0	1	1	2	1	2	3	2	2	4
1.6.1.c	2	1	3	0	0	0	2	2	4	0	0	0	2	2	4
1.6.1.d	1	0	1	2	1	3	2	2	4	1	2	3	2	2	4
1.6.2.a	2	1	3	1	1	2	1	1	2	1	2	3	2	1	3
1.6.2.b	2	1	3	2	1	3	1	1	2	0	0	0	2	2	4
1.6.2.c	1	0	1	2	1	3	1	1	2	1	2	3	1	1	2
1.6.3	2	1	3	1	1	2	2	1	3	0	0	0	1	1	2
1.7.1.a	2	1	3	2	1	3	1	2	3	1	1	2	2	2	4
1.7.1.b	2	1	3	1	1	2	1	1	2	1	1	2	2	1	3
1.7.1.c	2	1	3	2	1	3	0	0	0	1	1	2	0	0	0
Average score			2,475			2,575			2,45			1,125			1,7

Group	Nether lands	Nether lands	Nether lands	Norway	Norway	Norway	Poland	Poland	Poland	Portugal	Portugal	Portugal	Russia	Russia	Russia
Code	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined
1.1.1.a	1	2	3	2	2	4	0	0	0	0	0	0	1	1	2
1.1.1.b	2	1	3	2	2	4	1	0	1	0	0	0	2	2	4
1.1.1.c	2	1	3	2	2	4	1	0	1	0	0	0	2	2	4
1.1.1.d	1	2	3	2	2	4	0	0	0	0	0	0	0	0	0
1.1.2.a	1	2	3	2	2	4	0	0	0	0	0	0	1	1	2
1.1.2.b	2	1	3	2	2	4	1	0	1	2	1	3	1	1	2
1.1.2.c	2	1	3	2	2	4	1	0	1	2	1	3	2	2	4
1.1.2.d	1	2	3	2	2	4	0	0	0	2	1	3	0	0	0
1.1.3	2	2	4	1	1	2	0	0	0	0	0	0	2	1	3
1.2.1.a	1	2	3	2	2	4	2	2	4	0	0	0	0	0	0
1.2.1.b	2	1	3	2	2	4	2	1	3	2	1	3	1	2	3
1.2.1.c	2	2	4	2	2	4	1	0	1	2	1	3	2	2	4
1.2.1.d	1	2	3	2	2	4	0	0	0	2	1	3	0	0	0
1.3.1.a	0	2	2	1	1	2	2	2	4	0	0	0	0	0	0
1.3.1.b	1	1	2	1	1	2	2	2	4	0	0	0	0	0	0
1.3.1.c	1	2	3	1	1	2	2	1	3	0	0	0	1	0	1
1.3.1.d	1	2	3	1	1	2	2	2	4	0	0	0	0	0	0
1.3.2	0	0	0	1	1	2	1	0	1	1	0	1	2	0	2
1.4.1.a	0	0	0	2	2	4	0	0	0	2	1	3	0	0	0
1.4.1.b	0	0	0	2	2	4	0	0	0	2	1	3	0	0	0
1.4.1.c	0	0	0	2	2	4	0	0	0	2	1	3	1	0	1
1.4.1.d	1	2	3	1	1	2	0	0	0	2	1	3	0	0	0
1.4.2.a	0	0	0	0	0	0	0	0	0	2	1	3	0	0	0
1.4.2.b	0	0	0	0	0	0	0	0	0	2	1	3	0	0	0
1.4.2.c	0	0	0	0	0	0	0	0	0	2	1	3	0	0	0
1.5.1.a	0	0	0	2	2	4	1	2	3	2	1	3	0	0	0
1.5.1.b	0	0	0	2	2	4	1	2	3	2	1	3	1	0	1
1.5.1.c	1	2	3	1	1	2	1	2	3	2	1	3	0	0	0
1.5.2	0	0	0	0	0	0	1	2	3	0	0	0	0	0	0
1.6.1.a	0	0	0	2	2	4	2	2	4	2	1	3	1	0	1
1.6.1.b	0	0	0	2	2	4	0	0	0	2	1	3	0	1	1
1.6.1.c	0	0	0	2	2	4	2	2	4	2	1	3	1	2	3
1.6.1.d	2	2	4	2	2	4	2	2	4	2	1	3	1	2	3
1.6.2.a	0	0	0	2	2	4	2	2	4	2	1	3	1	2	3
1.6.2.b	0	0	0	2	2	4	0	0	0	2	1	3	0	1	1
1.6.2.c	2	2	4	0	0	0	2	2	4	2	1	3	0	1	1
1.6.3	2	1	3	0	0	0	2	2	4	0	0	0	2	2	4
1.7.1.a	0	1	1	1	1	2	2	2	4	0	0	0	1	0	1
1.7.1.b	0	1	1	1	1	2	1	0	1	0	0	0	0	1	1
1.7.1.c	1	2	3	0	0	0	0	0	0	0	0	0	1	0	1
Average score			1,825			2,8			1,725			1,75			1,325

Group	Slovenia	Slovenia	Slovenia	Spain	Spain	Spain	Turkey	Turkey	Turkey	UK	UK	UK	Ukraine	Ukraine	Ukraine
Code	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined	Relevance	Operational status	Combined
1.1.1.a	2	2	4	2	1	3	2	1	3	1	2	3	1	2	3
1.1.1.b	0	0	0	2	2	4	2	1	3	2	2	4	2	0	2
1.1.1.c	0	0	0	2	0	2	2	1	3	0	0	0	0	0	0
1.1.1.d	2	2	4	1	2	3	2	1	3	0	0	0	1	2	3
1.1.2.a	0	0	0	1	1	2	2	1	3	1	2	3	2	0	2
1.1.2.b	0	0	0	1	2	3	2	1	3	2	2	4	1	0	1
1.1.2.c	0	0	0	1	0	1	2	1	3	0	0	0	0	0	0
1.1.2.d	0	0	0	1	2	3	2	1	3	1	2	3	0	0	0
1.1.3	2	1	3	2	0	2	2	1	3	0	0	0	0	0	0
1.2.1.a	2	2	4	1	2	3	2	1	3	1	2	3	2	2	4
1.2.1.b	0	0	0	2	2	4	2	1	3	2	2	4	2	0	2
1.2.1.c	0	0	0	2	0	2	2	1	3	0	0	0	2	1	3
1.2.1.d	2	2	4	1	2	3	2	1	3	1	2	3	2	0	2
1.3.1.a	0	0	0	1	2	3	2	1	3	1	2	3	2	2	4
1.3.1.b	0	0	0	2	2	4	2	1	3	1	2	3	2	2	4
1.3.1.c	0	0	0	1	0	1	2	0	2	0	0	0	2	1	3
1.3.1.d	0	0	0	0	2	2	2	1	3	1	2	3	2	1	3
1.3.2	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0
1.4.1.a	2	1	3	1	1	2	2	1	3	2	2	4	0	0	0
1.4.1.b	2	1	3	2	2	4	2	1	3	1	1	2	0	0	0
1.4.1.c	2	1	3	2	2	4	2	1	3	1	1	2	0	0	0
1.4.1.d	0	0	0	0	0	0	2	1	3	0	0	0	0	0	0
1.4.2.a	0	0	0	1	1	2	2	1	3	2	2	4	0	0	0
1.4.2.b	0	0	0	1	0	1	2	1	3	1	1	2	0	0	0
1.4.2.c	0	0	0	1	0	1	2	1	3	1	1	2	0	0	0
1.5.1.a	2	1	3	1	2	3	2	1	3	1	1	2	0	0	0
1.5.1.b	2	1	3	1	2	3	2	1	3	1	1	2	0	0	0
1.5.1.c	2	1	3	0	0	0	2	0	2	0	0	0	0	0	0
1.5.2	0	0	0	0	0	0	2	0	2	0	0	0	0	0	0
1.6.1.a	2	1	3	2	1	3	2	1	3	2	2	4	0	0	0
1.6.1.b	2	1	3	2	2	4	2	1	3	2	2	4	0	0	0
1.6.1.c	2	1	3	2	2	4	2	1	3	2	2	4	0	0	0
1.6.1.d	2	1	3	2	2	4	2	1	3	0	0	0	0	0	0
1.6.2.a	0	0	0	2	0	2	2	1	3	1	2	3	0	0	0
1.6.2.b	0	0	0	2	2	4	2	1	3	1	1	2	0	0	0
1.6.2.c	0	0	0	1	0	1	2	1	3	0	0	0	0	0	0
1.6.3	0	0	0	1	1	2	2	2	4	2	1	3	2	2	4
1.7.1.a	0	0	0	1	2	3	2	1	3	1	2	3	2	1	3
1.7.1.b	0	0	0	1	1	2	2	1	3	2	2	4	2	1	3
1.7.1.c	0	0	0	2	1	3	2	0	2	0	0	0	2	1	3
Average scores			1,225			2,425			2,9			2,075			1,225

The EU countries that scored highest in the evaluation were France, Germany, Greece and Spain, those scoring lowest were Italy and Slovenia (Figure 1, Table 2). No country achieved maximum scores by demonstrating that its parameters were all ecologically relevant (thus scientifically robust) and operational.

Among countries not belonging to the EU the results also vary. In Ukraine and Albania, for example, the implementation of instruments homologous to the MSFD seems to be rather lacking as they are not obliged to follow the EC regulation. In contrast, Turkey and Norway although also under no obligation, have chosen to implement the MSFD. Both these countries are among the highest scoring in the evaluation meaning that they have outperformed many EU countries that were legally required to transpose the directive.

Although the MSFD descriptor for biodiversity appears to be well-developed with at least 40 parameters, the evaluation as undertaken for this study reveals serious weaknesses in many of the parameters in terms of ecological relevance. Many of the parameters are no more than general qualitative statements with little quantitative underpinning or information on species groups (e.g. changes of plankton form-types, or presence of monk seal). Moreover, as the development of the indicators and underlying parameters is often based on previously available data, the degree of development is strongly biased in favor of species of commercial interest (e.g. proportion of large fish) or endangered species or habitats: these may not necessarily reflect wider marine biodiversity. Such a biased approach, often top-down (politically) driven, resulted in low numbers of relevant parameters for biological diversity (as in the Netherlands), yielding a low average score. On the other hand, in countries where in-depth consultations with experts have taken place, such a bottom-up process can result in the selection of relevant biodiversity parameters. Yet, a low degree of operationalization, as e.g. for Turkey having a very high score for relevance, may yield again a somewhat lower average score.

Other reasons for low performance of parameters and the wide disparity in performance deduced from the country reports to the EU or similarly relevant documentation include the lack of clear and shared homologous definitions on the criteria whereby the choice of parameters relied more on (expert) opinion than on true data. As Palialexis *et al.* (2014) stated, although some indicators of D1 are very clear and specific (e.g. 1.2.1 Population abundance and/or biomass) having a straight-forward implementation, many other are more sophisticated and general (as e.g. 1.7.1 Composition and relative proportions of ecosystem components); more open to interpretation and reliant on a suite of methods and models to be implemented. Even for established parameters there may be major knowledge gaps and a lack of quantification of the targets (e.g. threshold level values) for what is, or is not, GES (Milieu, 2014b). The

lack of shared definitions can be overcome by collaboration between member states. In certain cases, however, lack of collaboration and coordination between governmental and research institutions, or lack of communication among regions within each country, and even conflicts between scientists and policy makers were also mentioned in interviews as a reason for a delayed development of GES indicators. A complex reporting structure and lack of funding for reaching more detailed and advanced results was often pointed out too in the interviews.

As a consequence of all these flaws in the establishment and implementation of the GES indicators and underlying Parameters, for Europe as a whole, the average combined score (for all 40 Parameters of all 20 countries) is only 1.9 (out of a score of maximally 4). This means that the performance of most European countries with regard to the implementation of the MSFD is still far below that necessary if GES is truly to be achieved. There is also an urgent need for harmonised monitoring networks and standardized sampling strategies, for a full implementation of the MSFD to all European countries, as advocated by e.g. the COST Action EMBOS (Heip & McDonough, 2012, p. 19). This would facilitate the establishment of a proper internationally integrated set of parameters, and allow a full gaps and weakness analysis to be undertaken.

In conclusion, even though it is clear a lot of effort has gone in to ensuring parameters are ecologically relevant reflections of Biological Diversity, the real weakness is in the lack of operational indicators: less than half of the established Parameters at this point in the process are operational. There also needs to be more effort for coordination at the Pan-European level so a consistent and harmonized approach to describing marine biological diversity with comparable parameters can be developed. Although a couple of countries are on track in implementing the MSFD, our results suggest that several European countries are not properly prepared to introduce the MSFD, partly because in those countries most parameters are neither bottom-up science driven, nor well-described. Therefore, the implementation of the MSFD Descriptor 1 can for Europe as a whole only be marked as moderate. Ultimately, the need to summarize the large environmental variability and assess impacts using a relatively small group of parameters is a hugely ambitious task. From a scientific point of view, several parameters are still under development because of the need to better understand the functional relationships between biological and abiotic factors, or on how to discriminate between the natural variability of the ecological systems in space and time and the shifts caused by human pressures. Official reports may contain parameters and monitoring as required for legal obligations but the need to scrutinize the scientific robustness of the MSFD work is more crucial than ever.

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1.2 Indicator-based assessment of marine biological diversity – lessons from 10 case studies across the European Seas

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Abstract

The Marine Strategy Framework Directive requires the environmental status of European marine waters to be assessed using biodiversity as 1 out of 11 descriptors, but the complexity of marine biodiversity and its large span across latitudinal and salinity gradients have been a challenge to the scientific community aiming to produce approaches for integrating information from a broad range of indicators. The Nested Environmental status Assessment Tool (NEAT), developed for the integrated assessment of the status of marine waters, was applied to 10 marine ecosystems to test its applicability and compare biodiversity assessments across the four European regional seas. We evaluate the assessment results as well as the assessment designs of the 10 cases, and how the assessment design, particularly the choices made regarding the area and indicator selection, affected the results. The results show that only 2 out of the 10 case study areas show more than 50% probability of being in good status in respect of biodiversity. No strong pattern among the ecosystem components across the case study areas could be detected, but marine mammals, birds, and benthic vegetation indicators tended to indicate poor status while zooplankton indicators indicated good status when included into the assessment. The analysis shows that the assessment design, including the selection of indicators, their target values, geographical resolution and habitats to be assessed, has potentially a high impact on the result, and the assessment structure needs to be understood in order to make an informed assessment. Moreover, recommendations are provided for the best practice of using NEAT for marine status assessments.

Keywords: biodiversity, assessment tool, MSFD, environmental status, spatial aggregation, integration, indicator sensitivity

Introduction

Biological diversity is widely recognized as one of the cornerstones of healthy ecosystems (e.g., Worm et al., 2006). Diversity may safeguard ecosystems against undesired regime shifts (Folke et al., 2004) and guarantee the continued delivery of ecosystem goods and services (Duarte, 2000; Beaumont et al., 2007). The need to maintain biodiversity is also recognized by international legislation (e.g., Convention of Biological Diversity; UNEP, 1992); to European Union (EU) level, the Marine Strategy Framework Directive (MSFD; European Union, 2008) requires its member states to assess the status of marine biodiversity and take action to guarantee that it remains at, or is restored to, Good Environmental Status (GES). A definition of what can be interpreted as good status can be consulted in Borja et al. (2013).

In order to conduct an assessment of status, and to determine the effectiveness of any implemented remedial measures, we need a clear definition of biodiversity and a unified approach for its assessment. In the marine assessments like MSFD, biodiversity is defined on the level of species, communities, habitats, and ecosystems, as well as in the genetic level (Cochrane et al., 2010). Indicators that show the ecosystem response to human pressures form the basis of the tool kit with which we can describe environmental status (Borja et al., 2016). Based on qualitative environmental objectives, targets are set for each indicator which allow policy makers to implement management measures should these not be reached (Borja et al., 2012).

One of the challenges faced during the first round of MSFD initial assessments is the diverging data availability for biodiversity across highly variable systems, but yet an overarching need to conduct compatible assessments across European regional seas (Hummel et al., 2015). European marine ecosystems comprise a complexity and variability both in space and time, ranging from fully saline systems such as in Mediterranean and Atlantic waters to the brackish Baltic Sea, and exposed open water systems such as in the northern Norwegian and Barents seas to fully enclosed systems such as the Black Sea. The levels of available knowledge and data within these systems vary, as well as the biological parameters and indicators used for assessments (Hummel et al., 2015).

The conclusions of the European Commission, in their evaluation of the EU member states' reports on the initial assessment carried out in 2010–2012 was that there is an apparent lack of coherence and comparability in the indicators used and in the final evaluation of the overall status, between the countries and within all regional seas (Palialexis et al., 2014). Therefore, there is an urgent need for coherent frameworks and methodologies to allow consistent approach in biodiversity status assessment across the European Regional Seas. This would also be needed in

order to allow coherence in the biodiversity assessments for the EU Birds and Habitats directives and the EU Biodiversity Strategy 2020.

While we could argue that we cannot compare studies if we do not have directly comparable datasets, in practice this is rarely possible, and certainly not at large spatial scales, or involving multiple research institutes and member states. Since there is no single way of describing biodiversity that fits all purposes, and since regional seas have intrinsic differences, we need a pragmatic selection of indicators which are appropriate to the specific questions asked, as well as a flexible and transparent indicator-based tool for assessment of biodiversity status. There is a large number of operational indicators, which have been used to describe the status in different types of aquatic systems (Birk et al., 2012; Borja et al., 2016). As biological diversity is multifaceted, including different taxonomic and functional groups, it cannot be expressed with a single indicator. Consequently, sets of different indicators are needed to cover the broad aspects of biological diversity and it is their combination into a single assessment that becomes a challenge (Borja et al., 2014; Probst and Lynam, 2016). In order to obtain a single overall assessment value, or conclusion, the results of the multiple indicators used in the assessment need to be aggregated, depending on the purpose of the assessment; e.g., if the aim is to inform different stakeholders and to set overall targets for the improvement of the marine environment, or depending on the assessment scale (Borja et al., 2014). Clear and transparent aggregation and integration rules are needed to interpret indicator information onto an environmental status assessment (see Borja et al., 2014 for a review on integration methods).

A variety of assessment tools enabling the integration of indicators already exists (see e.g., HELCOM, 2009a; Andersen et al., 2014; Borja et al., 2016). However, only few of them have treated biological diversity in a comprehensive way, have been tested broadly (i.e., outside the region in which they have been developed), or consider the complexity at an adequate level of detail for the spatial scale for which they are applied. To overcome these issues, in the context of the EU funded project DEVOTES (DEVelopment Of innovative Tools for understanding marine biodiversity and assessing GES), the Nested Environmental status Assessment Tool (NEAT; Berg et al., 2016; Borja et al., 2016) has been developed to assess biodiversity status of marine waters under the MSFD. NEAT uses a combination of high-level integration of habitats and spatial units, and averaging approach (Borja et al., 2014), allowing for specification on structural and spatial levels, applicable to any geographical scale.

In this contribution NEAT is applied to the assessment of marine biological diversity in 10 different case studies distributed across the European regional seas (Figure 1). The assessment results are discussed, but the main focus of the paper is on:

(i) analyzing the outcome of these assessments in light of the practical choices that have to be made to apply this tool, and

(ii) proposing best practices for marine biological diversity assessment using this tool.

Materials and methods

Case study areas

The case study areas were selected to represent a wide range of marine systems (Figure 1), with different climatic and hydrographic characteristics as well as exposure to different human activities and management challenges (Table 1).

These areas represent a wide range of marine biogeographical areas from subtropical waters to temperate and Arctic, covering the four European regional seas (i.e., Mediterranean, Atlantic, Black, and Baltic Seas). The surface areas of these case studies varied from <3000 km2 in Saronikos Gulf (Greece) to >820,000 km2 in the Barents Sea (Norway; Table 1). Detailed descriptions of the case study areas, with relevant references, can be found in Supplementary Material (S1–S10).

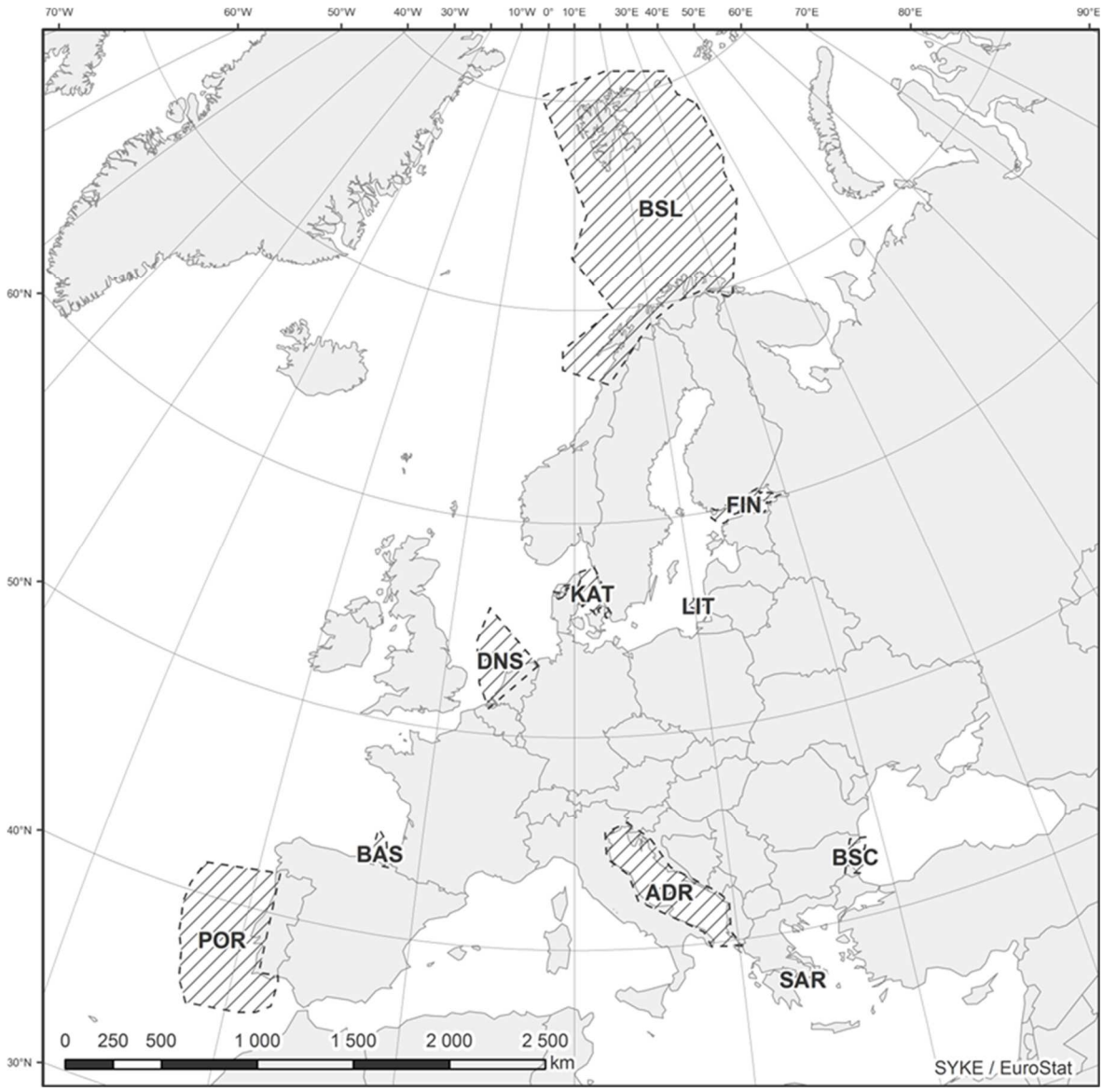

Figure 1. The case study areas. For the area codes, see Table 1. More detailed case study maps can be found in Supplementary Material (S1-S10).

Table 1. Characteristics of the case study areas.

Case study name	Code	Regional Sea, subregion	Salinity range	Seas Surface temperature range (°C)	Mean/max depth (m)	Special features	Main pressures	Biodiversity components in poorest status	Biodiversity components in best status	Observed gaps in biodiversity components	Observed gaps in habitats	Key references
Dutch North Sea	DNS	North Sea	27.6–34.8	4–18	6/54	Shallow and highly productive area, soft bottom sediments, contrasted benthic habitats (stressed in the southern part, rarely disturbed in the northern one)	Beam-trawl fishing, organic loading, harbor infrastructures	Fish	Birds, mammals, benthic fauna	Benthic vegetation		Herman et al., 2014
Basque Coast	BAS	North-East Atlantic	32.5–35.5	11–23	Range 0–5000	High wave exposure, long fetch	Fishing/ shipping	Mammals	Phytoplankton, zooplankton, benthic habitat, water column habitat	Microbes	Deep and abyssal habitats	Borja and Collins, 2004
Portuguese continental subdivision	POR	North-East Atlantic	35–36	13–23	Range 0–6000	High wave exposure and seasonal upwelling at north-west coast, smoother conditions at south and south-west coast	Coastal trawling (also dredging, sediment disposal, harbors)	Fish, birds, pelagic fauna	Zooplankton	Reptiles, mammals, microbes. Species distribution and coverage area. The current status of all stocks has been assessed, although using secondary indicators	Deep and abyssal habitats and open-sea areas (>20 nm)	MAMAOT, 2012 (https://dl.dropboxusercontent.com/u/103729442/EstrategiaMarinha_subdv_Continente.pdf) Dupont et al., 2014
Black Sea coast (Large Varna Bay)	BSC	Black Sea	11–18	0–28	30	Hydrologically dynamic area	Tourism industry, urbanization, port activity, aquaculture	Fish, phytoplankton, zooplankton	Water column habitat, benthic habitat	Microbes, alien species, food-web indicators; insufficient fish monitoring	No monitoring in the open sea areas	National Initial Assessment GES Report, 2013

Case study name	Code	Regional Sea, subregion	Salinity range	Seas Surface temperature range (°C)	Mean/max depth (m)	Special features	Main pressures	Biodiversity components in poorest status	Biodiversity components in best status	Observed gaps in biodiversity components	Observed gaps in habitats	Key references
Saronikos Gulf	SAR	Mediterranean (Eastern Mediterranean/ Aegean Sea)	38–39	12–28	100/450	It is the natural marine gateway of the city of Athens and Piraeus harbor. It receives the effluents of the central sewage outfall of Athens through a deep underwater outlet.	Activities: Waste Water Treatment Plant (WWTP), Shipping, tourism, fishing. Pressures: input of organics/nutrients, contaminants, habitat loss, resource exploitation, alien species, litter	Mammals, fish	Reptiles	Birds, zooplankton		Simboura et al., 2014, 2015; SoHelME, 2005; SoHelFI, 2007
Adriatic Sea	ADR	Mediterranean	37–39	18–25	35/1200	Cyclonic circulation; input from the Po river	Organic loading; overexploitation; bottom trawling	Mammals, phytoplankton		Fish, birds, zooplankton	Reef and mud habitats	Artegiani et al., 1997a; Artegiani et al., 1997b; (UNEP(DEPI)/MED WG.408/Inf.14, 2015).

NEAT

NEAT is a structured, hierarchical tool for making marine status assessments (Berg et al., 2016; Borja et al., 2016), and freely available at www.devotes-project.eu/neat. In NEAT, the study area can be divided into hierarchical spatial assessment units (SAU) and habitat types (HBT); e.g., SAU "archipelago zone" could include "inner archipelago" and "outer archipelago" as lower-level SAUs, and they, in turn, could include, e.g., water bodies as yet lower-level SAUs. Similarly, the HBT "seafloor" could include HBTs "soft bottom" and "hard bottom," which again could be further sub-divided (Figure 2). NEAT classifies the status of each SAU based on indicators that have been defined for that SAU; if one SAU has indicators describing different HBTs, the status of each HBT within a SAU is assessed first, and each HBT is then given equal weight in assessing the status of the SAU. The overall assessment is an average of the SAUs, weighted by their surface areas (km2). Other weighting schemes can be applied, if desired.

Each indicator must be explicitly linked to a SAU and a HBT— the same indicator, e.g., "the maximum depth of seaweed," can be included multiple times for multiple SAUs and HBTs if it has been assessed for multiple areas. These instances of indicators are called "indicator values" in this paper, while the indicators describing a certain ecological concept, e.g., the growth depth of a macrophyte species, or the reproduction rate of a bird species, are called "unique indicators."

In order to aggregate indicators by weighted average, it is necessary to transform all indicators to a common scale. In NEAT, indicators are transformed into values that range from 0 to 1 using a continuous piecewise linear function. On this scale, the value of 0.6 corresponds to the boundary between good (>0.6) and not good (<0.6) status. Transformation to this scale is defined by specifying the values of the indicator in the original measurement scale, which corresponds to the transformed values of 0, 0.2, 0.4, 0.6, 0.8, and 1.0. Though the transformation function is piecewise linear, the definition of 5 segments allows a reasonable approximation to non-linear functions. These five segments are also used here for illustrative purposes, and they are called bad/poor/moderate/good/high classes, although it is recognized that the boundary between GES and non-GES lies between the "moderate" and "good" classes.

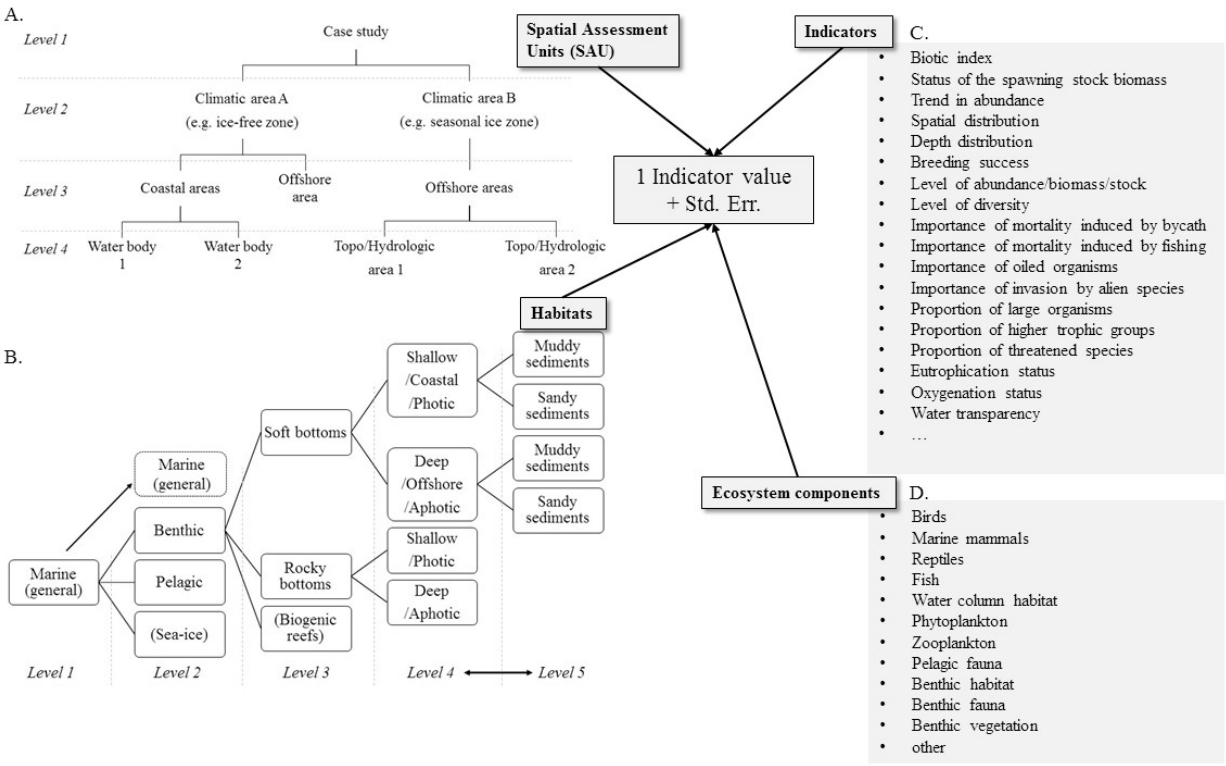

Figure 2. The indicator values are specific for (A) a Spatial Assessment Unit (SAU) at any level of the hierarchy, (B) a habitat (HBT) at any level of the hierarchy, (C) an indicator which is definer for (D) an ecosystem component.

Indicator selection and specification

The indicators used for this assessment represent the best available data and expertise for the six biological descriptors of the MSFD [i.e., D1 (biodiversity), D2 (non-indigenous species), D3 (commercially important species), D4 (food webs), D5 (eutrophication), and D6 (sea floor integrity)] in each case study area. These indicators include the national and regional indicators used for the MSFD assessment, and indicators derived from scientific literature and expertise. They have been selected to be representative of various biodiversity components, habitats, and geographical areas relevant for each case study area; however, it is possible that no indicators exist to be used for some relevant components. The list of indicators included in each case study is available in Supplementary Material S11.

Each indicator is associated to an ecosystem component class that describes the ecosystem component that the indicator describes. In this study, 12 ecosystem components were defined in order to accommodate all indicators used in all of the case studies. These components were phytoplankton, zooplankton, fish, reptiles, marine mammals, birds, benthic fauna, benthic vegetation, pelagic fauna (composite indicators consisting of data from multiple pelagic fauna groups), all taxa (composite indicators consisting of data from multiple taxa), benthic habitat, and

water column habitat. The latter two components gathered indicators related to physico-chemical conditions of the habitat, necessary to maintain life (e.g., oxygen or nutrients), whilst the "all taxa," benthic fauna, and pelagic fauna groups included composite indicators encompassing many species groups; the other nine ecosystem components were taxonomic groups.

Biodiversity status

The status of the biological diversity was assessed for each case study area using NEAT. The analysis provides an overall assessment for each case study area and a separate assessment for each of the ecosystem components included in the assessment. The final value has an associated uncertainty value, which is the probability of being in a determinate class status (GES/non-GES). This uncertainty was determined by the standard error linked to the indicator values (Carstensen and Lindegarth, 2016).

Evaluation of assessment design and its effects on the status assessment

The application of NEAT to a broad range of marine regions provides an opportunity to test and compare the NEAT assessment approaches and evaluate the consequences of design choice for the general environmental status assessment. How the available data are combined within the tool might have consequences on the results of the status assessment of biodiversity (Borja et al., 2014; Probst and Lynam, 2016). Therefore, one of our aims is to evaluate the consequences of the way the assessment was designed on the general assessment result.

NEAT gives a framework to organize the assessment, but it does not prescribe the number of assessment components, i.e., indicators, SAUs, HBTs, or ecosystem components to be used in an assessment. The user has the option to organize the different components of NEAT depending on the case, e.g., the morphological characteristics of the area, availability and resolution of data, and how the selected local indicators are defined.

In order to describe the assessment design, the following key components were summarized for each case study: (i) the total number of SAUs and how many hierarchical SAU levels there are, (ii) the total number of HBTs and their hierarchical levels, (iii) the number of ecosystem components covered by the indicators, (iv) the number of unique indicators (i.e., not repetition of the same indicator on a different spatial unit), as well as (v) the quantity of data, defined as the number of different indicator values (e.g., if the same indicator is defined separately for five different SAUs, they would comprise five indicator values).

NEAT assigns weights to the indicators based on the SAU and HBT that they represent (see Section Evaluation of the Assessment Results). The SAUs are weighted according to their surface area and the HBTs are weighed equally within a SAU. Therefore, the indicator values contribute to the assessment with different weights, the highest weight being assigned to an indicator representing a large SAU with a small number of indicators, and within it a HBT with a small number of indicators. The relative weights of the indicator values were used to identify the indicators that contribute 90% of the weight of the final assessment. In addition, the relative weight of each ecosystem component in each case study assessment was calculated. These summary statistics highlighted differences in aggregating information among case studies.

To test the sensitivity of the case study assessments to the selection and number of indicator values, a sensitivity analysis was performed by running the assessment using randomly selected indicator values. The number of indicator values included into the assessment varied from 1 to the maximum number of indicators in the case study minus one. This process was repeated 100 times for each number of indicator values. For example, take a case study with 120 indicator values. First, one random indicator value is selected and the assessment is done using only that indicator. This procedure is repeated 100 times. Then, two indicator values are picked at random, and the assessment is run using them; this again is repeated 100 times. This procedure is repeated for all numbers of indicator values up to 119. This results in a large number of values whose divergence can be analyzed to see if any patterns can be identified.

Results

Assessment design

The number of SAUs as well as how many hierarchical levels were used in these varied widely between the case studies. The number of SAUs included in the Gulf of Finland and Portugal continental sub-division cases were much higher (>60) than in all other case studies which included, on average, 9 different SAUs. Excluding these two case studies, larger areas were usually assessed using more SAUs. The number of hierarchical SAU levels varied between 1 and 5, but in 7 out of 10 cases, there were 3 or 4 levels (Table 2, Figure 2). The total number of HBTs included in the assessment varied between 3 and 9, and 9 out of 10 case studies had 2 or 3 hierarchical HBT levels (Table 2).

Not all SAUs necessarily included all habitat types, and indicators or data may not exist for all defined HBT types for each SAU. The number of SAU-HBT combinations that were assessed by at least one indicator value, varied between 6 and 132 (Table 2).

The number of ecosystem components included in the analyses varied between 5 and 9, with an average of 7.3 (Table 2). It has to be noted that all ecosystem components identified in this study were not applicable to all areas; an example being reptiles that do not occur in most of the study sites.

The number of unique indicators applied in each case study area varied between 11 and 116 (Table 2, Supplementary Material S11). The number of indicator values varied greatly with 466 values at the higher end in Portugal continental sub-division and between 20 and 200 values in all other case studies (Table 2).

Table 2: Synthesis of the structure used by the different case studies for the nested assessment. SAU: spatial assessment unit. HBT: habitats. The case studies are ordered according to their latitude.

Case study name	Area (km^2)	Number of SAU levels (total number of SAU)	Number of HBT levels (total number of HBT)	Number of SAU*HBT combinations with data	Number of ecosystem components included	Number of unique indicators (see Supplement 11)	Number of indicator values
Norwegian Barents Sea–Lofoten	821 478	4 (13)	3 (9)	21	7	40	74
Gulf of Finland	22 482	5 (60)	2 (3)	103	8	25	147
Lithuanian marine waters	6 426	2 (4)	2 (7)	6	9	27	50
Kattegat	17 440	3 (11)	2 (7)	21	8	31	69
Dutch North Sea	57 000	1 (1)	3 (6)	6	6	15	31
Basque Coast	10 794	3 (8)	3 (6)	22	9	48	109
Portuguese continental subdivision	268 645	4 (61)	4 (7)	132	7	14	466
Black Sea coast (Large Varna Bay)	1 434	3 (7)	2 (4)	15	7	35	112
Saronikos Gulf	2 907	3 (4)	3 (6)	10	7	17	29
Adriatic Sea	138 600	3 (10)	2 (5)	17	5	116	177
Mean	134 721	3.1 (17.9)	2.6 (6.0)	35.3	7.3	39.2	126.4
Stdev	255 807	1.1 (22.7)	0.7 (1.7)	44.3	1.3	30.7	128.8

Biological diversity status

The summary of the test NEAT assessments of the 10 case study areas is presented in Figure 3. The assessment resulted in GES for the Basque EEZ and the Barents Sea-Lofoten, with 100 and 66% confidence, respectively, the remaining eight case studies presented non-GES (i.e., bad, poor, or moderate; Figure 3). Lithuanian coast has the potential for being in GES, but with a low confidence of 20% (Figure 3). For the other case studies, this probability of achieving GES was <1% (Figure 3).

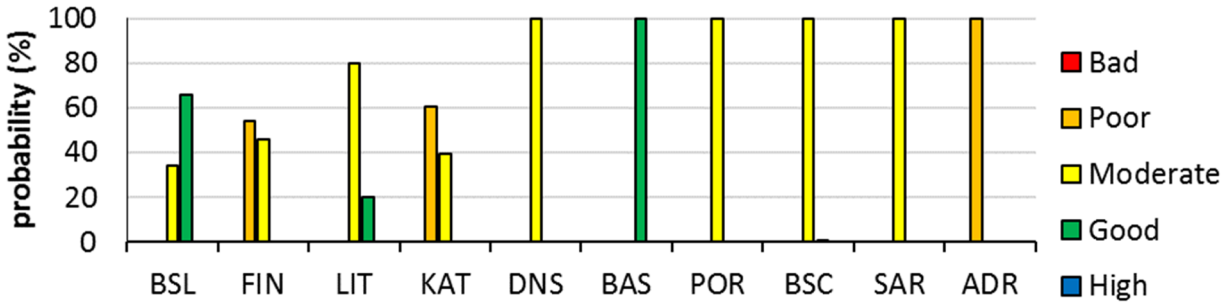

Figure 3. Probabilities for the five environmental status classes for each of the 10 case study assessments. Good environmental status is assumed attained if the cumulative probability of "Good" and "High" is higher than the cumulative probability of "Moderate," "Poor," and "Bad." If opposite, the Good environmental status is not attained. For case study codes see Table 1.

The different ecosystem components showed different status in the case study areas (Figure 4). No strong pattern among the ecosystem components could be detected, but some commonalities were found: Indicators based on marine mammals generally indicated degraded situation in 6 cases out of 7 (Figure 4). When included, birds and benthic vegetation indicators as well as water column indicators of physico-chemical status also indicated degraded situation in 5 cases out of 7. Indicators encompassing several ecosystem components ("AT," on Figure 4) always indicated degraded situations.

On the other hand, indicators of benthic habitats' physico-chemical status and of zooplankton community status indicated GES when they were included in the assessment (Table 1, Figure 4).

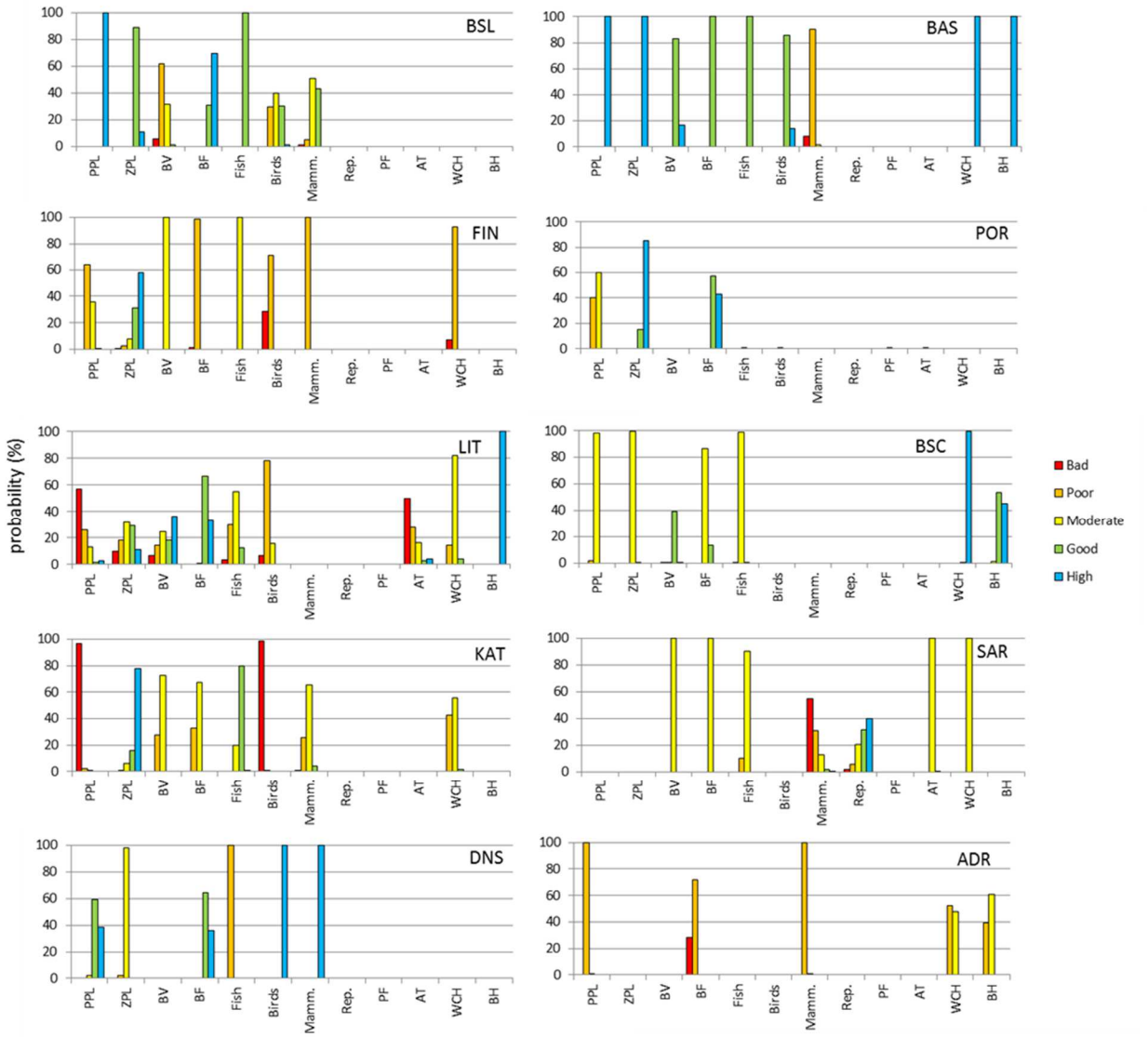

Figure 4. Probabilities for the five environmental status classes separated by ecosystem component for each of the 10 case studies (for codes see Table 1). Ecosystem components considered are: Phytoplankton (PPL), zooplankton (ZPL), benthic vegetation (BV) benthic fauna (BF), fish, birds, mammals (Mamm.), reptiles (Rep.), pelagic fauna (PF), all taxa (AT), water column habitat (WCH), benthic habitat (BH).

Relative contribution of indicator values and biodiversity components

The indicator values contributed differently to the final assessment result (Figure 5); indicator values defined for larger SAUs tend to have more weight, particularly if there are only few indicators defined for these SAUs. In 7 out of the 10 case studies, <10 indicator values already contributed to more than 50% of the final assessment result. For 9 case studies, <50 indicator values contributed to >90% of the final assessment. This 90% of the final assessment was reached with <20 indicator values in five case studies (Figure 5). The five indicator values that made the

highest contribution to the final assessments of each case study are listed in Table 3. These indicator values were dominated by mammal, bird, fish, and benthic fauna indicators.

The 12 different ecosystem components' contribution to the final assessment result did not correspond to the number of indicator values defined for each component (Table 4). For example, most case studies had a large proportion of benthic fauna indicator values (average: 22.4% of indicators values), which ultimately did not reflect proportionally in the final assessment (average contribution: 11.7%). In contrast, the proportion of fish and marine mammals indicator values were lower, but these components contributed to a higher proportion of the final assessment. In five case studies (i.e., Barents-Lofoten, Gulf of Finland, Dutch North Sea, Saronikos Gulf, and Adriatic Sea), "Benthic fauna" was the component with the highest proportion of indicator values (Table 4); the other five case studies each had a different component with the highest number of indicator values. However, in none of the case studies, benthic fauna was the component with highest contribution to the final assessment (Table 4); in five (i.e., Gulf of Finland, Dutch North Sea, Basque coast, Portuguese continental sub-division, and Black Sea coast) and two case studies (i.e., Barents Sea–Lofoten and Adriatic Sea) respectively, fish and mammals were the components carrying the highest weight to the final assessment (Table 4). However, other ecosystem components, that overall did not contribute to many case study assessments, were very relevant for specific case studies (e.g., the composite group "all taxa" in the Saronikos Gulf and benthic habitat in the Lithuanian coast).

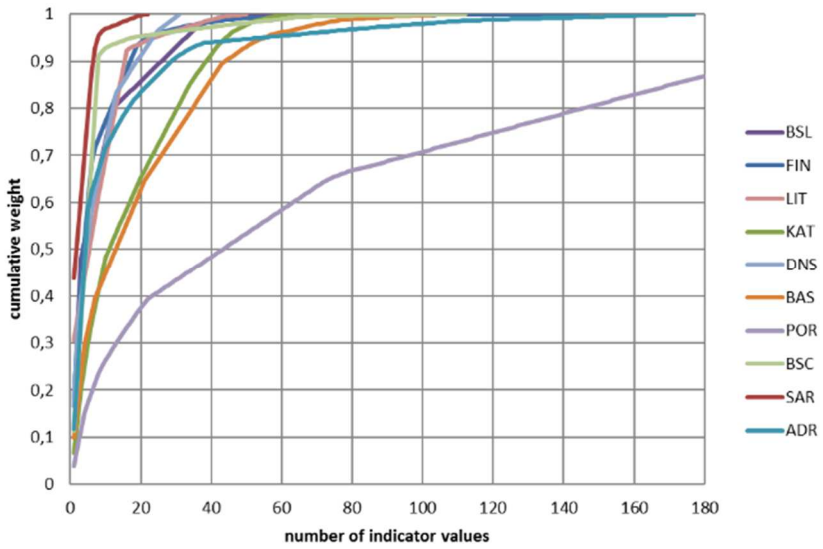

Figure 5. Cumulative contribution to the final assessment (cumulative weight) in relation to the number of indicator values. For each case study, values have been ranked from the most important indicator value (highest weight) to the least important value (lowest weight). The X-axis has been cut; Portuguese case study has a total of 466 indicator values. For case study codes, see Table 1.

Table 3. List of the top-five indicator values contributing the most to the overall assessment for each case study. In case of equal contribution of several indicator values, all the indicator values are given. The contribution to the overall assessment (in %) of each indicator value is given. Numerical values are rounded. ES100: expected number of species in 100 individuals; AMBI: AZTI's Marine Biotic Index; BQI: Benthic Quality Index; DKI: Danish Index; CIMPAL: Cumulative IMPacts of invasive ALien species; M-AMBI: multivariate AMBI; GES: good environmental status.

Spatial Assessment Unit (SAU)	Habitat	Ecosystem component	Indicator name	% contribution to assessment
BARENTS SEA LOFOTEN			***cumulative contrib. of top-five indicators***	***56***
BARENTS SEA - LOFOTEN	sea-ice	Mammals	Harp seal, *Pagophilus groenlandicus* stock size	16.6
Ice-free zone	pelagic	Birds	Kittiwake*, Rissa tridactyla* breeding success over last 5 yr	16.4
Seasonal-ice zone	sea-ice	Mammals	Proportion of non-threatened pagophiletic mammals	15
Offshore (ice-free zone)	shelf (muddy sediments)	Benthic Fauna	4 indicators related to macrobenthic fauna: ES100, abundance level, evenness and AMBI	3.7 (each)
GULF OF FINLAND			***cumulative contrib. of top-five indicators***	**60**
Open sea	Benthic	Benthic Fauna	Average regional diversity	18.4
Gulf of Finland	Pelagic	Fish	Abundance of salmon spawners and smolt	12.5
Gulf of Finland	Pelagic	Fish	Herring, Spawning stock biomass	12.5
Gulf of Finland	Marine (general)	Mammals	3 indicators: Grey seal population growth rate, Grey seal pregnancy rate, Ringed seal population growth rate	8.3 (each)
LITHUANIAN COAST			***cumulative contrib. of top-five indicators***	**47**
Territorial sea	Benthic	Benthic habitat	Extent of the seabed significantly affected by human activities	31
Territorial sea	Marine (general)	Others	Biopollution level index (invasive species)	4
Territorial sea	Marine (general)	Birds	Abundance of wintering populations of seabirds: (1) Red-throated Diver + Black-throated Loon, (2) Great Crested Grebe (Podiceps cristatus), (3) Common Merganser (Megus merganser), (4) Velvet Scotter (Melanitta fusca), (5) Long-tailed Duck (Clangula hyemalis) and (6) Common Goldeneye (Bucephala clangula)	4 (each)

KATTEGAT DK			***cumulative contrib. of top-five indicators***	**30**
KATTEGAT, central parts	Marine (general)	Birds	Fulmar winter abundance (encounter rate)	6.6
KATTEGAT, central parts	Marine (general)	Birds	Kittiwake winter abundance (encounter rate)	6.6
KATTEGAT, central parts	Marine (general)	Birds	Guillemot winter abundance (encounter rate)	6.6
KATTEGAT, central parts	Benthic	Benthic Fauna	BQI	5
KATTEGAT, central parts	Benthic	Benthic Fauna	DKI	5
DUTCH NORTH SEA			***cumulative contrib. of top-five indicators***	**21**
Dutch EEZ	Benthic	Benthic Fauna	Benthic invertebrates total number of species	16.7
Dutch EEZ	Muddy deep bottom	Benthic Fauna	3 indicators related to typological group sensitive to seafloor physical impact, based on (1) density, (2) biomass, (3) number of species	1.1 (each)
Dutch EEZ	Muddy deep bottom	Benthic Fauna	4 indicators related to typological group highly sensitive to seafloor physical impact, based on (1) density, (2) biomass, (3) number of species	1.1 (each)
BASQUE EEZ			***cumulative contrib. of top-five indicators***	**33**
Offshore waters (>200 m depth)	Benthic	Benthic habitat	Seabed affected by human activities	9.9
Offshore waters (>200 m depth)	Pelagic	Birds	Biological value Seabirds	6.6
Offshore waters (>200 m depth)	Pelagic	Mammals	Biological value Mammals	6.6
Offshore waters (>200 m depth)	Pelagic	Phytoplankton	eutrophication indicator: Chlorophyll a, 90th percentile	6.6
Offshore waters (>200 m depth)	Sedimentary	Benthic Fauna	2 indicators: (1) M-AMBI, (2) AMBI	3.3 (each)
Offshore waters (>200 m depth)	Sedimentary	Fish	Biological value Demersal Fish	3.3
PORTUGUESE CONTINENTAL SUBDIVISION			***cumulative contrib. of top-five indicators***	**17**
Continental_A2_600	Marine (general)	Birds	Biological Value Marine Birds	3.9
Continental_A2_600	Pelagic	Zooplankton	Biological Value Zooplankton	3.9
Continental_B4_600	Sedimentary	Benthic Fauna	Biological Value Benthic communities	3.7
Continental_B4_600	Marine (general)	Birds	Biological Value Marine Birds	3.7
Continental_B5_600	Marine (general)	Birds	Biological Value Marine Birds	2.2

COASTAL BLACK SEA			***cumulative contrib. of top-five indicators***	**57**
Black Sea coastal	Pelagic	Fish	2 indicators: Mean length of (1) *Sprattus sprattus*, (2) *Scophtalmus maximus*	11.4 (each)
Black Sea coastal	Pelagic	Fish	2 indicators: Catch/Biomass ratio of (1) *Sprattus sprattus*, (2) *Scophtalmus maximus*	11.4 (each)
Black Sea coastal	Pelagic	Fish	2 indicators: Biomass of (1) *Sprattus sprattus*, (2) *Scophtalmus maximus*	11.4 (each)
Black Sea coastal	Pelagic	Fish	2 indicators: Sexually mature specimen of (1) *Sprattus sprattus*, (2) *Scophtalmus maximus*	11.4 (each)
SARONIKOS GULF			***cumulative contrib. of top-five indicators***	**62**
Saronikos Gulf	Marine (general)	All Taxa	CIMPAL index (alien species)	44
Saronikos Gulf	Pelagic	Reptiles	% loss of spawning areas of sea turtle *Caretta caretta*	4.4
Saronikos Gulf	Pelagic	Fish	4 indicators: Fisging mortality for (1) *Engraulis encrasicolus*, (2) *Sardina pilchardus*, (3) *Merluccius merluccius*, (4) *Mullus barbatus*	4.4 (each)
Saronikos Gulf	Pelagic	Mammals	% Threatened mammals	4.4
Saronikos Gulf	Pelagic	Fish	% Threatened sharks	4.4
Saronikos Gulf	Pelagic	Fish	% of stocks that meet GES based on fishing mortality	4.4
Saronikos Gulf	Pelagic	Fish	% of stocks that meet GES based on reproductive capacity	4.4
Saronikos Gulf	Pelagic	Fish	% of stocks that meet GES based on reproductive capacity and biomass indices	4.4
ADRIATIC SEA			***cumulative contrib. of top-five indicators***	**58**
Adriatic Sea	Pelagic	Mammals	*Tursiops truncatus*, distributional range	11.7
Adriatic Sea	Pelagic	Mammals	*Stenella coeruleoalba*, distributional range	11.7
Adriatic Sea	Pelagic	Mammals	*Grampus griseus*, distributional range	11.7
Adriatic Sea	Pelagic	Mammals	*Ziphius cavirostris*, distributional range	11.7
Adriatic Sea	Pelagic	Mammals	*Tursiops truncatus*, abundance	11.7

Table 4. Number of indicator values per ecosystem component, and the relative weight of ecosystem components in each case study.

	Number of indicator values												% of total weight											
	Phytoplankton	Zooplankton	Benthic Veg	Benthic Fauna	Fish	Birds	Mammals	Reptiles	Pelagic Fauna	All Taxa	Water column habitat	Benthic habitat	Phytoplankton (%)	Zooplankton (%)	Benthic Veg (%)	Benthic Fauna (%)	Fish (%)	Birds (%)	Mammals (%)	Reptiles (%)	Pelagic Fauna (%)	All Taxa (%)	Water column habitat (%)	Benthic habitat (%)
Norwegian Barents Sea–Lofoten	1	4	3	37	9	14	6	0	0	0	0	0	0	3	0	20	14	28	34					
Gulf of Finland	53	2	34	46	4	2	3	0	0	0	3	0	10	4	2	20	30	3	25				6	
Lithuanian marine waters	3	6	1	1	6	19	0	0	0	3	8	3	4	9	0	0	1	29				5	20	32
Kattegat	5	1	25	9	11	3	1	0	0	0	14	0	2	0	26	14	16	20	1				21	
Dutch North Sea	2	2		19	6	1	1	0	0	0	0	0	7	7		33	45	4	4					
Basque Coast	8	5	5	19	42	6	6	0	0	0	12	6	8	1	0	10	54	8	8				1	11
Portuguese continental subdivision	44	42		27	108	44	0	0	177	24	0	0	8	9		8	29	21			25	1		
Black Sea coast (Large Varna Bay)	36	24	4	12	12	0	0	0	0	0	20	4	2	1	2	2	92						1	0
Saronikos Gulf	0	0	2	12	8	0	1	1	0	1	4	0			3	3	26		9	9		44	6	
Adriatic Sea	3	0	0	100	0	0	30	0	0	0	37	7	0			8			89				3	0

Sensitivity analysis

The sensitivity analysis shows that there are major differences in how much the result varies if only a subset of the indicator values is included in the assessment (Figure 6). For example, if only a small number (close to 0) indicators were included, the assessment results in all studies could be anywhere between high and bad status, except in Barents Sea and Portuguese continental subdivision, where they could range from poor to high status. As more indicator values are added, the range of outcomes narrows down. However, how steeply that happens when indicator values are added varies between the case study areas (Figure 6).

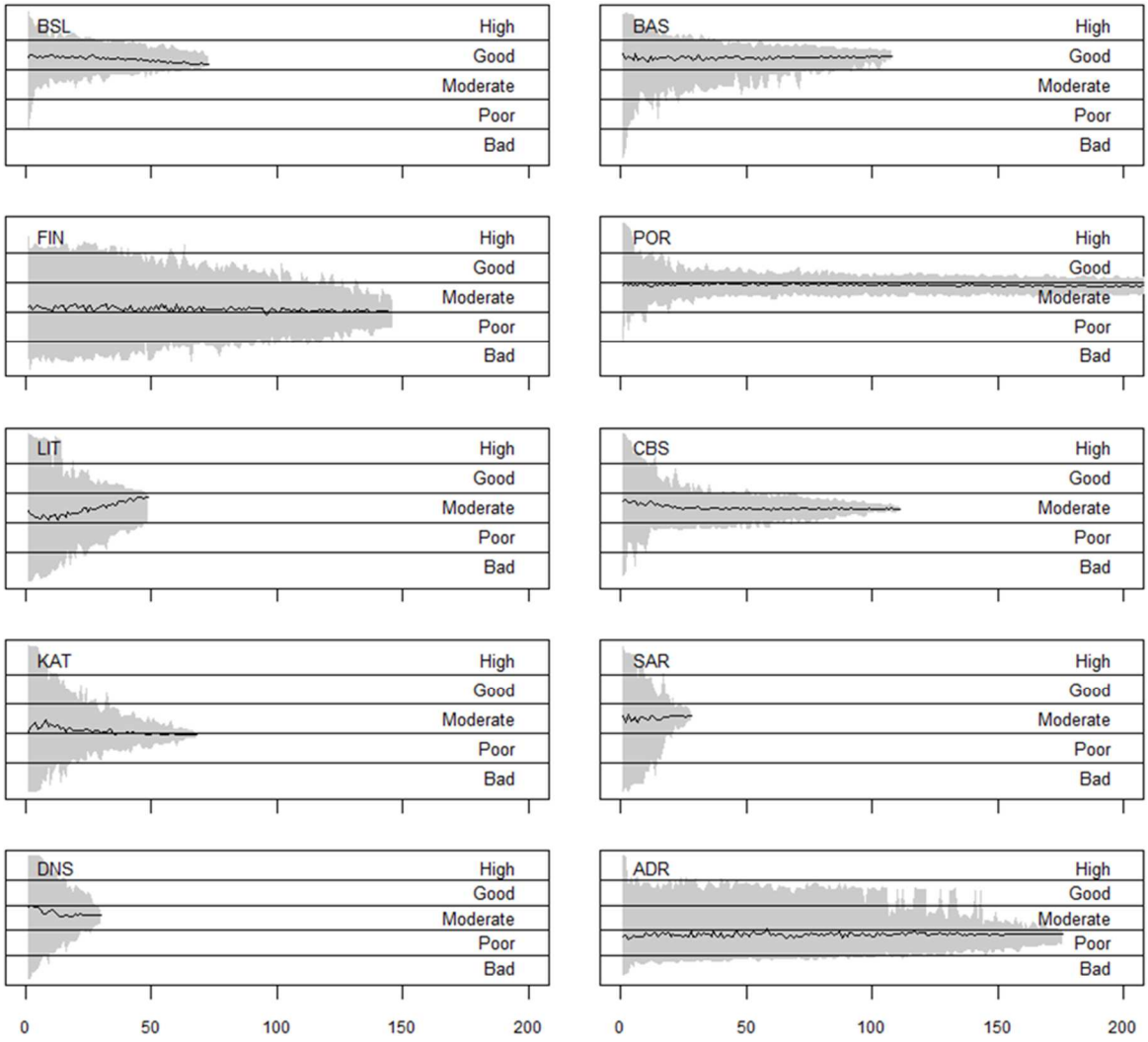

Figure 6. Variation of the overall assessment results in the case studies, if only a subset of the indicator values is taken into account. The x axis indicates how many indicators are included into the assessment, and the gray area spans the assessment result values that emerged. The black line shows the mean assessment result across the 100 runs conducted for each number of indicator values. Note that the x axis goes only up to 200 indicator values; the Portuguese continental subdivision study included 466 indicator values in total.

Discussion

The current NEAT-based assessment demonstrates a large-scale marine biodiversity assessment, providing a feasible solution to the apparent problem pointed out by the European Commission, in their evaluation of the EU member states' reports on the MSFD initial assessments carried out in 2010– 2012 (Palialexis et al., 2014). This problem was the apparent lack of coherence and comparability in indicators used and in the final evaluation of the overall status between the countries and within all regional seas (Palialexis et al., 2014). Despite the available guidance and Commission Decision (European Union, 2010) on GES descriptors, criteria and indicators, the overall picture in assessments was patchy and non-coherent (European Commission, 2014). The use of NEAT, and its validation in different regional seas and case study areas, is a crucial contribution from the DEVOTES project to provide a harmonized approach and methodology for a coherent and comparable environmental status assessment across the European regional seas. It also shows that although the regional seas have different characteristics and human pressures impacting those (Claudet and Fraschetti, 2010; Micheli et al., 2013a; Andersen et al., 2015), a coherent assessment framework can be employed to evaluate differences in the environmental status and the ecological components that are impacted by different pressures.

The study and the comparison of the case studies brought into light several issues that need attention in order to improve the coherent and comparable "biodiversity status" assessments of the European regional seas. These issues are related to the data and indicator availability, how the assessments are structured, how the integrative assessment should be structured, and how this structure should be taken into account when defining the spatial resolution and indicator selection of the assessments. The current study revealed that while these assessments could be carried out, there are two major problems in achieving the objectives of the MSFD assessments: (i) there are still multiple gaps in the availability and coverage of indicators in the various areas, and (ii) comparability of the status assessments across different regions would benefit from a more unified assessment framework, even if indicators suitable for each area remained different. NEAT provides a general framework that could be accompanied with guidelines for the selection of SAUs, HBTs, and indicators.

Each of the case studies was initially designed with the best available selection of spatial units, habitats, and indicators, adhering to the NEAT methodology but without specific guidelines for the indicator selection, target level setting, etc. This situation resembles the situation where the new users would start using NEAT on their area. For the purposes of this study, the assessments were evaluated and harmonized to some degree, e.g., if the same indicator appeared in multiple case studies, it was

ensured that it was associated to the same biodiversity component (e.g., chlorophyll a levels would be assigned to phytoplankton). Despite this harmonizing, there were major differences in how the case studies were constructed in terms of spatial resolution, habitats, and indicator definition. The current assessment is based on best available data and evaluation of the experts participating within this exercise, and the biodiversity status results of this study should be considered as indicative, not definitive.

The indicators selected for the assessments are designed or adapted for each area separately, including the geographical and habitat specification and the target level, i.e., which values are considered good and which less than good in any given area and habitat. This means that the "good" status is scaled according to the area: In areas with a naturally low biodiversity, lower biodiversity is also considered "good" than in areas with naturally high diversity. This makes the assessment relevant for each area, and the result must be interpreted to be in relation to undisturbed condition of that area rather than in absolute terms of diversity.

According to a categorization of rules or methods for combining or aggregating indicators or criteria within a given descriptor (Prins et al., 2013; Borja et al., 2014), NEAT is classified as a high-level integration method which reduces the risks associated to the "one out, all out" principle of the Water Framework Directive approach (Borja and Rodríguez, 2010) while giving an overall and specific (to descriptors and components) assessment.

According to the relevant guidance document for the MSFD (Prins et al., 2013), the spatial scales are not the same for all indicators within the biodiversity descriptor, where depending on the species or habitat a different spatial scale may be used. It is also recommended to address uncertainties and assess confidence of the classification result (as a secondary assessment). In our study, the NEAT software treats equally all assessment elements assigning equal weights, but gives more weight in cases of larger spatial coverage, with higher data representativeness, in that way incorporating the spatial scales issue and the confidence level into the assessment. This could be the reason for which some ecosystem components (e.g., seabirds, mammals, and fishes) have more weight in the final assessment, since they are normally assessed at large scale spatial areas, which have more weight when aggregating (e.g., Saronikos gulf). However, NEAT also includes the possibility to weight indicators differently.

Implications of the assessment design

Most of the case study areas lacked indicators regarding one or several biodiversity components and habitats (Table 1, Figure 4), even those that were deemed important in the area. The lack of indicators

stemmed either from lack of monitoring data regarding the area or biological diversity component (e.g., birds, reptiles, pelagic fauna), or from obstacles in the indicator development, including the lack of expert time to develop indicators, or insufficient knowledge about the target levels due to lack of long-term or reference condition data (Hummel et al., 2015). In some cases, more basic ecological research is needed in order to understand the ecological processes well enough to develop indicators. In fact, most of the assessments undertaken until now by member states is more qualitative than quantitative (Hummel et al., 2015), representing a challenge for the assessment.

The habitats and biodiversity components for which no indicators are available potentially affect the final assessment result. It is entirely possible that adding even one indicator that would represent a poorly-represented, large area or habitat, would change the overall assessment for better or for worse. Therefore, in order to make a reliable assessment of the status of the biological diversity, the critical gaps in each assessment case need to be evaluated for their potential to affect the overall result. If such high-leverage gaps exist, the assessment result must be taken with caution.

Different indicator values and spatial assessment units had varying weights in the final assessment result in all of the cases (Table 3, Figure 2). The differences in the indicator value weights stem from the fact that the default NEAT assessment first assesses the result for each SAU, giving equal weight to each HBT with similar hierarchy, and combines these SAUs hierarchically so that each SAU is given weight according to its area. Therefore, if a SAU has a large surface area and only a small number of indicators per one or several of its habitat types, these indicator values end up contributing strongly to the final assessment.

This emphasizes the importance of the balanced nature of the indicator set, and particularly the reliable assessment of indicators that are used to assess the status of large areas, and particularly their habitats with only few indicators (Feary et al., 2014). Therefore, particular attention should be paid to both the observed value, the boundary values between the classes, and the uncertainty estimation of these most influential indicator values.

The fact that the SAUs are weighted according to their surface area in the default mode of NEAT also emphasizes the need for careful consideration of the definition of the SAUs. Ideally, the SAUs should be defined in the manner that an indicator value defined for a SAU can be expected to reasonably represent all of the SAU. On the other hand, if the assessment area is split into several sub-SAUs and only a fraction of them actually has indicator data, their value will be generalized to represent the whole super-area in the hierarchical assessment anyway.

In NEAT, it is possible to weight the SAUs according to their perceived ecological relevance instead of their surface area; for example, biodiversity hotspots, important reproduction areas, marine

protected areas, etc., could be given a higher weight than their area alone would imply. In this study, this option was not used in any of the case studies.

Uncertainty of the results is assessed based on Monte Carlo simulations, using the observed value as mean and the standard error value as the standard deviations, assuming a Gaussian distribution (Carstensen and Lindegarth, 2016). Based on these simulations, NEAT determines how often the sampled value falls into each of the five classes, and this distribution is reported. Therefore, the standard error values assigned to the indicators play a major role in the uncertainty associated with the final assessment result. This emphasizes the importance of careful evaluation of the standard deviation, particularly with indicators that have a high weight in the assessment.

Evaluation of the assessment results

There are other tools to assess the status of marine systems, e.g., the Ocean Health Index (OHI; Halpern et al., 2012). This index has different concept and a much broader spatial scale, and a comparison between NEAT and OHI results (BD values presented in Table S6 in Selig et al., 2013) shows that the results are quite different (Table 5).

The OHI tends to give a more reduced range of status values (74–97) than those provided by NEAT (0.37–0.69) for these areas. The OHI does not provide a GES/non-GES status, but in general provides higher values than those by NEAT. The OHI study (Selig et al., 2013) has been applied globally, and includes a large variety of worldwide cases with great differences in setting and problems. In that context, e.g., the Mediterranean and the Baltic Sea seem to be in a (seemingly more homogenous) better state than e.g., waters around Africa or Indonesia and Philippines.

An interesting observation is that there is a negative rather than a positive correlation between these results, and those areas ranked low in NEAT (such as the Gulf of Finland and Kattegat) get high scores in OHI, while the best-scoring area in NEAT (Basque EEZ) gets lowest score in OHI (Table 5). This discrepancy is partly due to the fact that the OHI scores are given by country, thus covering larger areas than the case studies assessed here with NEAT. Therefore, the local status of a case study area may be masked by the results from the rest of the country in OHI. The NEAT results are reported here for the entirety of each of the case study areas, but where the case study area includes smaller SAUs, the results can be viewed for each of them separately as well, yielding even a more detailed geographical resolution.

Another factor possibly contributing to this discrepancy is the use of different indicators; the OHI assessment used publically available data with little local/regional detail, which can vary the final assessment when applying to regional scales (Halpern et al., 2014), while the current NEAT

assessment used indicators specifically designed for marine status assessment. The species scores of OHI focused on the extinction risk of marine species (Selig et al., 2013), while the indicators in the NEAT assessments included a wider spectrum of indicators of species status. The OHI habitat scores were based on condition estimates of mangroves, coral reefs, seagrass beds, salt marshes, sea ice, and subtidal soft-bottom (Selig et al., 2013) while the NEAT assessments were tailored for each area.

The NEAT assessment results were in most cases in line with previous regional/local assessments, understanding, or known pressure gradients (Table 1, Figure 4). For example, The Baltic Sea biodiversity has been assessed by HELCOM (2009a, 2010) to be in poor to bad status in all of the three Baltic case study areas included in this analysis (Gulf of Finland, Lithuanian marine waters, Kattegat), being similar to the NEAT results but not to the OHI assessments. The difference between the NEAT and OHI results in these cases is probably largely due to eutrophication, which is documented to be major pressure threatening the ecosystem functioning of the Baltic Sea (HELCOM, 2009b, 2010). While it is reflected in the status of phytoplankton and water column habitats, and also affects the higher trophic levels of the food web (Österblom et al., 2007) and the seafloor (Karlson et al., 2002), it is not likely to be strongly reflected in the extinction threat of marine species (used in OHI), although it does affect the habitat scores, particularly seagrasses (Table S1 in Selig et al., 2013).Another factor affecting the discrepancy in the case of Finland is that the Gulf of Finland area has poorer biodiversity status than the Finnish marine waters on average (HELCOM, 2010).

Table 5. Comparison of the biodiversity assessments obtained using the Ocean Health Index (OHI; data from Selig et al., 2013) and the Nested Environmental status Assessment Tool (NEAT) (this study) in the countries for which NEAT has case studies.

Country (Case study)	Status (NEAT)	Biodiversity score (OHI)
Norway (Barents Sea)	0.646	90
Finland (Finland)	0.401	97
Lithuania (Lithuania)	0.583	89
Denmark (Kattegat)	0.394	93
Netherlands (North Sea)	0.523	85
Spain (Basque Country)	0.689	74
Portugal	0.566	83
Bulgaria (Bulgaria)	0.495	87
Greece (Saronikos Gulf)	0.520	91
Italy/Croatia (Adriatic)	0.370	86/89

In the North Sea, fishing is considered the main pressure, and the results show fish to be the ecosystem component in poorest status; the other assessed ecosystem components (birds, mammals, benthic fauna, and phytoplankton) were assessed to be in GES, with the exception of zooplankton that showed sub-GES (moderate) status (Figure 4). The Black Sea Coast case results obtained in this study also corresponded very well to known pressure gradients, such as nutrient enrichment affecting the status of the plankton community (Figure 4). Phytoplankton and benthic vegetation assessments correspond to category "poor" in the Varna Bay itself (Dencheva and Doncheva, 2014; Moncheva et al., 2015) as the most affected by anthropogenic pressure among the BSC sub-SAUs (Shtereva et al., 2012). The lowest benthic fauna score is also found there, which is fully in compliance with recently published results (National Report on the State and Protection of the Environment in Bulgaria, 2014). Similarly, the Basque area, which was previously assessed as being in good status, using a different methodology (Borja et al., 2011) also results in good status after applying NEAT; only mammals were assessed to be in sub-GES status (Figure 4).

In Saronikos Gulf the assessment results correspond to the ecological status categorization according to the WFD which is poor in the sewage outfall area and moderate in the inner central gulf (Simboura et al., 2014, 2015, 2016). Aliens, fish including threatened sharks, and mammals contributed to the moderate status seen for the outer Saronikos and overall Saronikos. In general, the respective assessment results, although not definitive, are in line with pertinent studies (Frantzis, 2009; Katsanevakis et al., 2013; Papaconstantinou, 2014; Vasilakopoulos et al., 2015; Zeneto6 et al., 2015; Simboura et al., 2016) regarding the Greek marine waters. The Saronikos Gulf result obtained in this analysis was lower than the OHI assessment of the Greek waters, which was to be expected, as the Gulf is intensely exploited.

Results from the Norwegian part of the Barents Sea indicated a general good status, which is in accordance with indicators of fish status on exploited large marine ecosystems (Kleisner et al., 2014; Coll et al., 2015), the report on the Barents Sea management plan (Sunnana et al., 2010) and the work from Certain et al. (2011). Nevertheless, several indicators indicated potentially degraded situations both in the coastal area and in the area of seasonal ice presence: (1) Along northern Norway coast, the current extent of kelp forest, an important component of fjords ecosystem and coastal landscape, cannot be considered as good in northern Norway. Kelp forests along the Norwegian and Russian coast were indeed dramatically grazed during the early 1970s and replaced by barren grounds dominated by sea urchins (Norderhaug and Christie, 2009). Though a progressive northward recovery of kelp forests extent is observed, its recovery status is still partial in northern Norway (Sivertsen, 2006; Rinde et al., 2014). (2) In northernmost part of the Barents Sea, sea-ice extent is undergoing a

particularly dramatic decrease (Parkinson et al., 1999) with a significant decrease rate of −3.5% per decade of winter ice extent (Sorteberg and Kvingedal, 2006) as a response to climate warming (Boitsov et al., 2014). This dramatic loss of habitat has consequences on the associated communities (Kovacs et al., 2011) as well as in the functioning of the Barents Sea ecosystem as a whole (Wassmann et al., 2006). The growing evidence of impacts of climate change on this area rises the issue of exogenic unmanaged pressures on this system and the issue of shifting baselines for the definition of target values. In addition, there are still no indicators of the impact of trawling activities included in this assessment (see however Jørgensen et al., 2016).

For the Portuguese coast, the initial assessment officially provided in the scope of the MSFD (MAMAOT, 2012), presented a general environmental quality status higher than the NEAT results calculated in this study. This may be partly due to the fact that the present assessment did not include some special areas with a higher degree of protection (such as Berlengas' Marine Reserve and Professor Luiz Saldanha's Marine Park or Goringe Seafloor). These areas, which have restricted access by the public, are important for marine high trophic level species (e.g., marine birds, mammals), some of which were not included in the present assessment. Due to inconsistencies in the data (now being improved by projects such as MARPRO— Conservation of Marine Protected Species in mainland Portugal, http://marprolife.org), marine mammals, reptiles and benthic vegetation were not included in the current NEAT assessment, which may also contribute to the lower environmental quality results achieved by NEAT. The higher result reported by the OHI may be related to the methodology used for the scores' calculation, and may reflect more specifically the trend than the present environmental status.

An exception to the good correspondence between the current and previous assessments is the Adriatic Sea, where the assessment provided by NEAT appears too low considering the current trends, also reported in the scientific literature, and available information from expert opinions (Coll et al., 2010; Bastari et al., 2016). Despite the historical impacts on this shallow water basin, the Adriatic Sea is still characterized by a wide diversity of habitats, including rocky and soft bottoms, large estuaries and lagoons, seagrass meadows and in, its southern part, also deep-water environments. The habitat richness is reflected by a high biodiversity (Coll et al., 2012; Micheli et al., 2013b), with approximately 49% of the species described for the Mediterranean Sea (Boudouresque et al., 2009; UNEP, 2015) and a variety of endemic species (e.g., 18% of the endemic fish species of the Mediterranean; UNEP/MAP-RAC/SPA, 2015). Human activities and multiple stressors, and in particular bottom trawling, hydraulic dredging and habitat loss, are certainly still impacting the Adriatic Sea (Micheli et al., 2013a; Pusceddu et al., 2014). However, the overall

environmental condition is not worsening with respect to the past decade. Eutrophication and dystrophic crises, related to the high nutrient discharge from the Po River combined with an alteration in water circulation, have caused hypoxia, anoxia and massive mucilage events, with consequent mortality of the benthic organisms, but the frequency of these events decreased significantly (or even disappeared) in the last decade (Degobbis et al., 2000; Danovaro et al., 2009). Thus, we hypothesize that the assessment of the environmental status obtained by using NEAT can be affected by the number and typology of data included in the specific exercise. An improvement of the number and type of the biological indicators (e.g., species or ecosystem functioning) could be crucial to obtain a more realistic classification of the marine environmental health of the Adriatic Sea.

Birds and mammals were found to be in poor status in many of the case study areas. This reflects the fact that seabirds are indeed considered as more threatened than any other comparable groups of bird species in general and display a faster trend of decline than other bird species during the last decades (Croxall et al., 2012). In addition, using IUCN Red list categories, it has been evidenced that, among seabirds, pelagic species of seabirds are disproportionately more threatened than coastal resident or coastal non-breeding visitor species (Croxall et al., 2012). Pelagic seabirds are particularly sensitive to disturbance as most species lay only a single egg, adults do not reproduce every year and usually reproduce several years after reaching sexual maturity (Furness and Camphuysen, 1997). Most seabird species display very large home range and thus integrate the state of the environment and impacts of pressures over larger scale.

The conservation status of marine mammals is of particular concern with an estimated proportion of threatened species ranging worldwide between 23 and 61% of species (Schipper et al., 2008). The North Atlantic region, which includes several of the cases studied here, is one of the areas where the proportion of threatened marine mammals is the highest, as shown by the low quality values in Barents Sea, Kattegat, and Basque case studies (Figure 4). The main reported threats explaining the bad status of marine mammals are a long history of harvesting, accidental mortalities through bycatch and collisions with vessel as well as a very large panel of pollutions (from sound pollution to contaminants and marine debris) and climate change (Schipper et al., 2008). The sensitivity of these species to changes in their environment might be related to their very slow population dynamics, low densities in correlation with their large body-size (Cardillo et al., 2008). Those life traits are also related with relatively large home range. As a consequence, indicators of marine mammals are usually measured over large scale, and they are difficult to monitor with precision, leading to higher uncertainty on many indicators (Taylor et al., 2007).

In two of the areas (Lithuania and Basque coasts), the indicator contributing the most to the final assessment was "the extent of the seabed significantly affected by human activities," which is a direct indicator of pressure. This is interesting since some authors (e.g., Borja et al., 2013) have supported the use of pressures instead of assessing the environmental status, if there are not enough indicators. This should be done under the premise that if an area has no obvious pressures then any changes in the area must be due to natural changes which are outside the control of management and vice versa.

Sensitivity analysis

The sensitivity analysis results show differences among the case studies in terms of how many indicator values are needed before the assessment results will show approximately the same results regardless of which indicator values are selected into the assessment (Figure 6). This implies that there is no universally sufficient number of indicator values needed to make a reliable assessment, but that the number varies among case studies. No clear patterns could be found among the 10 cases evaluated in this study that would indicate a number of indicator values of biodiversity components that can be considered sufficient regardless of the case study and its structure.

The variation in the assessment result depends on the set of indicator values that is available for the assessment. If the indicator values are close to each other, i.e., all indicating similar status, the variation in the results is naturally smaller. In contrast, if the different indicator values indicate very different status, e.g., some areas or biodiversity components are in good status while others are in bad, this naturally incurs a larger variation when a subset of these variables are selected, as e.g., in the Gulf of Finland.

These observations lead to the conclusions that if there is variation among the status of the geographical or biodiversity components in the study area, all of them should be covered by indicators if possible. Particularly the inclusion of high-leverage indicator values, i.e., those that have high weight and whose value differs from the overall mean, can change the assessment result. Therefore, the careful evaluation of the value and class limits of these indicators should be a priority.

Conclusions

The structured assessment forces us to critically evaluate the available indicator set in terms of ecological and spatial representativeness of each indicator. This framework highlights the gaps in the assessment as well as those parts that are well-represented by current monitoring and available indicators. This, in turn, helps in determining the best way to improve the quality of the assessment: (i) via developing additional indicators to fill in the gaps within the ecosystem approach (i.e., if not all the important trophic levels of key species/ groups are covered in the existing indicator set), (ii) working to determine the optimal SAU for different categories of indicators that are targeted to assess various trophic levels and functions in the food web, as well as the HBT classification for each area, and (iii) working toward improving specificity, robustness, and pressure relevance of the indicators and enabling estimation of their standard errors.

The development of NEAT and this extensive testing with 10 case studies in very different European marine areas offers insight both to the status of the marine waters and to the state-of-the art of the available indicator assemblages as well as the development needs of the marine biological diversity assessment. The application of the tool will make the improvement and harmonization needs of the assessments visible and pave the way toward a harmonized assessment across large geographical scales.

In conclusion, we propose the following recommendations for the best practice in performing the environmental status assessment using NEAT:

- Careful attention needs to be paid particularly to the current status and class boundaries of the indicators that cover large geographical areas (such as mobile birds and mammals), as they tend to carry a lot of weight in the final assessment.
- In order to make the assessment comparable between the different sub-regions and areas in the regional seas and provide a harmonized assessment among the regional seas, the design of the assessment needs to be harmonized. Attention must be paid to the selection of ecosystem components, and definition of size and hierarchy of the spatial assessment units as well as the definition of habitats.
- Consider the possibility of using different weighting for the individual indicator values, if that is ecologically more justified than using the weight based on the spatial area and habitat weighting.
- Contextualize the outputs on the basis of existing data. Different ecosystem components may present quite different data coverage, frequency, and data quality for the evaluation, and that may be reflected in the results. Consider carefully the standard deviation assigned to the indicators, but also consider how well the available indicators represent the ecosystem component and/or area as a whole.

- Consider not only the overall assessment, but the partial assessments (e.g., biological components or MSFD descriptors), as partial assessments can contribute to increased understanding of results and defining management measures for specific issues or areas.

Authors and contributors

LU, HB, and AB conceived the paper. The following partners provided the case studies: HB, SC (Barents Sea – Lofoten), LU, VK, HN (Gulf on Finland), GS (Lithuanian marine waters), CM, JA, JC (Kattegat), OB (Dutch North Sea), AB, MU (Basque Coast), JN (Portuguese continental subdivision), SM (Black Sea Coast), NP, MP, NS (Saronikos Gulf), RD, LC, AC, SB (Adriatic Sea). LU wrote the first draft. TB and CM contributed to the calculations and sensitivity analyses. LU, HB, AB, SC, AH, and MU contributed largely to the introduction and discussion. All co-authors contributed to the last draft and to the discussions.

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Supplementary Material

Here we provide the "Case study area description: Adriatic Sea" (Supplement 1) and the "List of unique indicators used in each case study" (Supplement 11). The Supplementary Material S2-S10 can be found online at: http://journal.frontiersin.org/article/10.3389/fmars.2016.00159

Supplement 1

Case study area description: Adriatic Sea

Map

Biogeographical characteristics

The Adriatic Sea is an 800 km long and 200 km wide basin, located in the North-Central Mediterranean Sea and connected to the Eastern Mediterranean Sea. It is a semi-enclosed basin representing about 5% of the entire Mediterranean Sea and ranging from 35 m (Northern sector) to 1200 m (Southern sector) water depth (Artegiani et al 1997a). The Adriatic Sea can be divided into three sub-basins: the Northern, the Central and the Southern sectors, with increasing water depth from North to South, different biogeographical characteristics and topographic gradients (Bonaldo et al. 2016). The mean temperature of the Adriatic Sea ranges from 18 to 25 °C. Salinity typically ranges from 37 to 38.9 and it is strongly influenced by the Po river and other rivers outflow, in particular in the surface of the North-western sector. Overall, the Adriatic Sea is characterized by a cyclonic superficial circulation, however seasonal fluctuations of oceanographic and biological conditions can be observed, mainly due to atmospheric forcing, freshwater discharges, variable intrusion of high salinity waters and a variable and complex circulation (Artegiani et al 1997a). The Adriatic Sea is one of the most productive basins of the entire Mediterranean Sea: the high nutrient load along the western coast causes high primary production (100-350 gC m^{-2} y^{-1}; Vilibic et al 2009).

Biodiversity

49 % of the known life forms in the Mediterranean are present in the Adriatic Sea (Boudouresque et al 2009; UNEP, 2015). Regarding the biodiversity of macroalgae, 358 species belonging to Rhodophyta or Phaeophyta have been observed (Coll et al 2010). As far as the benthos is concerned, a total of 270 foraminiferal species were documented in the basin. Nematodes are generally the most abundant taxon of metazoan meiofauna and the most diverse, with 263 species, followed by Copepoda Harpacticoida, with 118 species (Balsamo et al 2010). Regarding the most important macrofaunal taxa, the Adriatic Sea hosts 462 species of Gasteropoda, 293 of Decapoda, 242 of Amphipoda, 230 of Porifera, 101 of Echinodermata, 100 of Anthozoa, 47 of Isopoda, 45 of Cephalopoda, 36 of Sipuncula, 34 of Mysidiacea, 17 of Cirripeda, 13 of Cumacea, 12 of Euphasiacea (Coll et al 2010). The Adriatic Sea hosts 452 fish species (53 of whom are Elasmobranchii, Dulčić & Lipej 2015) and it is one of the largest areas of occurrence of demersal and small pelagic fishes. In addition, 3 species of sea turtles and 8 species of cetaceans are present and 2 species of cetaceans are considered occasional (UNEP, 2015). Because of the shallow depth of the most of the Adriatic, seabirds are scarce and represented by 7 species (UNEP, 2015).

Human activities, pressures and their effects

The Adriatic Sea has been recently reported as medium-highly or very highly impacted area (Micheli et al 2013). This basin has been impacted by anthropogenic-driven pressures over the last 50 years (Russo et al, 2002 and 2005). Due to inputs from the Po river, the sediments of the Adriatic Sea are characterized by the accumulation of organic loads and locally experienced hypoxic crises, increased frequency of red tides, intensification of mucilage formation, possibly enhancing the spread of pathogens (Danovaro et al 2009). Nevertheless, during last few years a partial regression of the eutrophication has been observed, related to a progressive decrease of the primary production observed since the last decade or more and possibly associated to the decreasing nutrients input (Ribera D'Alcalà et al 2004; Cozzi and Giani 2011).

The North-central Adriatic Sea constitutes the widest continental shelf in the Mediterranean Sea and it is of a great value for fishing within the Italian and European coasts. Catches of small pelagic fishes strongly increased from the mid-1970s to the mid-1980s and, as a consequence, a strong decrease of their stocks has been reported (in particular *Engraulis encrasicolus* and *Sardina pilchardus*; Coll et al 2007). The eastern Adriatic coast has experienced introduction of alien and thermophilic species (due to aquaculture activities and shipping) from other Mediterranean subregions (Pecarevic et al 2013). Moreover, in the last 30 years, the Adriatic Sea has experienced changes in the trophic regime, structure and organization of pelagic and benthic communities, also in response to current climate shifts (Kamburska & Fonda Umani 2006; Danovaro et al 2009; Conversi et al 2010; Mozetič et al 2012; Giani et al 2012; Di Camillo & Cerrano 2015).

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Supplement 11

List of unique indicators used in each case study

Adriatic Sea (ADR)

- Abra alba
- Abra nitida
- Acanthochitona fascicularis
- Acarina
- Amphipholis squamata
- Amphiura chiajei
- Amphiura filiformis
- Anfipoda
- Anomia ephippium
- Antalis inaequicostata
- Antalis inaequicostata
- Aporrhais pespelecani
- Bela brachystoma
- Bivalvia
- BPC
- Calyptraea chinensis
- CHL (500 m from the coastline)
- Chla
- CHO
- Copepoda
- Corbula gibba
- CPE
- Cumacea
- Cylichna cylindracea
- Diplodonta rotundata
- Dosinia lupinus
- Eulima glabra
- Euspira nitida
- Feo
- Funiculina quadrangularis
- Gari fervensis
- Gastrochaena dubia
- Gnathostomulida
- Grampus griseus abundance
- Grampus griseus distributional range
- Hiatella arctica
- Holothuroidea
- Hyala vitrea
- Hydroid juveniles
- Isopoda
- Kellia suborbicularis
- Kinorhyncha
- Kurtiella bidentata

- Laevicardium crassum
- LIP
- Loripes lucinalis
- Lucinella divaricata
- Mangelia attenuata
- Mangelia coarctata
- Megastomia conoidea
- Monia patelliformis
- Myrtea spinifera
- N inorg (500 m from the coastline)
- N TOT (3000 m from the coastline)
- Nassarius mutabilis
- Nassarius pygmaeus
- Nauplii
- Nematoda
- Neolepton obliquatum
- NH3 (500 m from the coastline)
- NITRITES (3000 m from the coastline)
- Nucula nitidosa
- Nuculana pella
- Oligochaeta
- Ophiura albida
- Ophiura carnea
- Ophiura grubei
- Ophiuroidea juveniles
- Ostracoda
- Ova canaliferus
- OX (3000 m from the coastline)
- Papillicardium papillosum
- Parvicardium exiguum
- Parvicardium minimum
- Pennatula rubra
- pH (3000 m from the coastline)
- Pharus legumen
- Phaxas adriaticus
- Phaxasa adriaticus
- PHOSPHATES (3000 m from the coastline)
- Pitar rudis
- Polychaeta
- Priapulida larvae
- PRT
- Ptereoides spinosum
- P-TOT (3000 m from the coastline)
- Retusa umbilicata
- Saccella commutata
- Saccella illirica
- SALT (500 m from the coastline)
- Secchi depth (3000 m from the coastline)
- SiO2 (500 m from the coastline)

- SiO4 (3000 m from the coastline)
- Spisula subtruncata
- Stenella coeruleoalba abundance
- Stenella coeruleoalba abundance
- Stenella coeruleoalba distributional range
- Tellina albicans
- Tellina distorta
- Tellina donacina
- Tellina pulchella
- Tellina serrata
- Thracia phaseolina
- Thyasira flexuosa
- Timoclea ovata
- Trachythyone elongata
- Trophonopsis muricata
- Turbonilla rufa
- Turritella communis
- Tursiops truncatus abundance
- Tursiops truncatus by catch
- Tursiops truncatus distributional range
- Venerupis corrugata
- Virgularia mirabilis
- Ziphius cavirostris distributional range
- Ziphius cavirostris risk of exposure

Basque EEZ (BAS)

- AMBI
- Ammonia
- Biological value Demersal Fish
- Biological value Macroalgae
- Biological value Macroinvertebrates
- Biological value Mammals
- Biological value Seabirds
- Biological value Zooplankton
- Blooms of any phytoplankton taxa
- Catch-biomass ratio Engraulis
- CFR
- Chlorophyll a 90th percentile
- Fish larger than mean size 1st sexual maturation Trachurus
- Fishing mortality L. boscii (8c9a)
- Fishing mortality L. budegassa (VIIIC & Ixa)
- Fishing mortality L. piscatorius
- Fishing mortality L. whiffiagonis (8c9a)
- Fishing Mortality Merluccius Iberian Waters
- Fishing mortality Merluccius BoB+North
- Fishing mortality Micromesistius (I-IX)
- Fishing mortality Sardine VIIIc+Ixa

- Fishing mortality Scomber (NEAM)
- Fishing mortality T. alalunga
- Fishing mortality T. thynnus
- Fishing mortality Trachurus
- M-AMBI
- Nitrate
- Oxygen saturation
- Phosphate
- Proportion of fish larger than 1000 mm
- Ratio NIS/native species macroalgae
- Ratio NIS/native species macroinvertebrates
- Seabed affected by human activities
- Spawning-stock biomass Engraulis
- Spawning-stock biomass L. boscii (8c9a)
- Spawning-stock biomass L. budegassa BoBiscay
- Spawning-stock biomass L. piscatorius BoBiscay
- Spawning-stock biomass L. whiffiagonis (8c9a)
- Spawning-stock biomass Merluccius BoBiscay+North
- Spawning-stock biomass Merluccius Iberian Waters
- Spawning-stock biomass Micromesistius
- Spawning-stock biomass Sardine VIIIc-Ixa
- Spawning-stock biomass Scomber (NEAM)
- Spawning-stock biomass T. alalunga (Natlantic stock)
- Spawning-stock biomass T. thynnus East Atlantic + Med)
- Spawning-stock biomass Trachurus
- Suspended solids
- Turbidity

Black Sea Coast (BSC)

- AMBI
- B mesozooplankton, spring
- B mesozooplankton, summer
- BAC:DIN biomass, spring
- chl.a, spring
- chl.a, summer
- DE% spring
- DE% summer
- DIN-spring
- DIN-summer
- EEIc
- H index mesozoo spring
- H index mesozoo summer
- H index fish
- H index, zoobenthos
- IBI, spring
- IBI, summer
- index taxonomic distance Δ*

- M.leyidii summer
- M-AMBI
- Menhinick index, spring
- Menhinick index, summer
- N. scintilans, spring
- O2 saturation bottom, summer
- O2 saturation surface, spring
- P-PO4 spring
- P-PO4 summer
- Scophtalmus maximus biomass
- Scophtalmus maximus mean length
- Scophtalmus maximus ratio catch/biomass
- Scophtalmus maximus sexually mature specimen
- Sprattus spratu sexually mature specimen
- Sprattus spratus ratio catch/biomass
- Sprattus spratus biomass
- Sprattus spratus mean length

Barents – Lofoten (BSL)

- abundance level [macrofauna]
- Abundance level of Calanus finmarchicus
- Abundance of Alle alle at sea (Little auk, sighting data)
- Abundance of Fratercula arctica at sea (atlantic puffin, sighting data)
- Abundance of Fulmarus glacialis at sea (Northern fulmar, sighting data)
- Abundance of Rissa tridactyla at sea (black-legged kittiwake, sighting data)
- Abundance of Uria aalge at sea (common guillemot, sighting data)
- Abundance of Uria lomvia at sea (polar guillemot, sighting data)
- AMBI
- Balaenoptera acutorostrata Abundance level in the Barents Sea compared to 1989-1995 period (MINKE WHALE 2002-2007 abundanceof "Eastern" (E) stock (according to Bothun et al. (2008) and values reported by the IWC)
- Biomass of zooplankton [>2 mm]
- Biomass of zooplankton [0.18 mm-1 mm]
- Biomass of zooplankton [1 mm-2 mm]
- Boreogadus saida total stock Biomass (ARCTIC COD after data in Hop & Gjaeseter, 2013)
- Clupea harengus SSB (HERRING in Subareas I, II, and V, and in Divisions IVa and XIVa)
- coastal Gadus morhua SSB (NORWEGAIN COASTAL WATER COD in Subareas I and II)
- ES100
- eutrophication Chl-a value
- evenness [macrofauna]
- Fratercula arctica Population trend over last 5 yr
- Gadus morhua SSB (COD in Subareas I and II)
- Hippoglosoides platessoides 0-group abundance (Long rough dab - 0-group - Abundance indices)
- Kelp forest extent
- Mallottus villosus SSB (CAPELIN in Subareas I and II, excluding Division IIa west of 5°W)

- Melanogrammus aeglefinus SSB (HADDOCK in Subareas I and II)
- Micromesistius poutassou SSB (BLUE WHITING in Subareas I–IX, XII, and XIV)
- Pagophilus groenlandicus stock size (HARP SEAL from Greenland area + White Sea/Barents Sea area; ICES data)
- Proportion of non-threatened Benthos-feeding seabird species
- Proportion of non-threatened Cetacean species (from Norwegian Red list)
- Proportion of non-threatened coastal mammal species (3 species)
- Proportion of non-threatened coastal mammal species (3 species)
- Proportion of non-threatened Fish species (according to Norwegian red list)
- Proportion of non-threatened pagophiletic Mammals
- Proportion of non-threatened Pelagos-feeding seabird species
- Rissa tridactyla Breeding success over last 5 yr
- Rissa tridactyla Population trend over last 5 yr
- Stock of Pandalus borealis (NORTHERN SHRIMP IN THE BARENTS SEA (ICES SUB-AREAS I AND II))
- Uria aalge Population trend over last 5 yr
- Uria lomvia Breeding success over last 3 yr
- Uria lomvia Population trend over last 5 yr

Dutch North Sea (DNS)

- Benthic invertebrates species richness
- Benthic species richness
- Benthopelagic species richness
- Bird species richness
- Chlorophyll a concentration
- Copepod concentration
- LFI
- Mammal species richness
- MML
- MTL
- Pelagic species richness
- Phytoplankton species richness
- Typological group highly sensitive to seafloor physical impact
- Typological group sensitive to seafloor physical impact
- Zooplankton species richness

Gulf of Finland (FIN)

- Copepod biomass
- Average regional diversity
- BBI-index
- Chlorophyll a concentration
- Cladophora glomerata growth rate
- Cyanobacterial surface accumulations - the CSA-index
- Depth distribution of selected perennial macroalgae
- Fucus growth depth, open
- Fucus growth depth, sheltered

- HELCOM Core: Abundance of key fish species
- HELCOM Core: Abundance of salmon spawners and smolt
- HELCOM core: Abundance of waterbirds in the breeding season
- HELCOM core: Abundance of waterbirds in the wintering season
- HELCOM Core: Population growth rate, abundance and distribution of marine mammals: grey seal population growth rate
- HELCOM Core: Population growth rate, abundance and distribution of marine mammals: ringed seal population growth rate
- HELCOM Core: Pregnancy rates of the marine mammals: grey seal
- HELCOM Eutrophication indicator: Chlorophyll-a
- HELCOM Eutrophication indicator: Nutrients: Nitrogen
- HELCOM Eutrophication indicator: Nutrients: Phosphorus
- HELCOM Eutrophication indicator: Oxygen debt
- HELCOM Eutrophication indicator: Water clarity
- Microphagous mesozooplankton biomass
- Phytoplankton taxonomic diversity (Shannon95)
- Spawning stock biomass of herring
- Spawning stock biomass of sprat

Kattegat (KAT)

- Fulmar winter abundance (encounter rate)
- BQI
- Chl-a, inner parts
- Chla-a, summer mean
- DKI index
- Eelgrass depth limit
- F of Herring in IIIa and sd 22-24
- F of Sole in IIIa and the Belts
- Guillemot winter abundance (encounter rate)
- Harbour porpoise summer abundance (encounter rate
- Invertebrate biomass
- Kittiwake winter abundance (encounter rate)
- Large Fish Indicator (LFI)
- Log(Number of long lived fish)
- Macroalgae species richness, outer parts
- Phytoplankton biomass
- SAV, total cover, Venø Bay
- Secchi depth
- Size spectra height
- Size spectra slope
- Species eveness
- Species richness
- SSB of Cod in Kattegat
- SSB of Herring in IIIa and sd 22-24
- SSB of Sole in IIIa and the Belts
- TN, annual mean
- TN, summer mean, outer parts

- TP, anual mean
- TP, summer mean, inner parts
- Zoobenthos species richness, outer parts
- Zooplankton biomass

Lithuanian coast (LIT)

- Abundance of wintering Great Crested Grebe (Podiceps cristatus)
- Abundance of wintering populations of seabirds Common Goldeneye (Bucephala clangula)
- Abundance of wintering populations of seabirds Common Merganser (Megus merganser)
- Abundance of wintering populations of seabirds Long-tailed Duck (Clangula hyemalis)
- Abundance of wintering populations of seabirds Red-throated Diver and Black-throated Loon
- Abundance of wintering populations of seabirds Velvet Scotter (Melanitta fusca)
- Annual average total nitrogen concentration
- Annual average total phosphorus concentration
- Bentic quality index (BQI)
- Biomass of copepods
- Biomass of microphagous zooplankton
- Biopollution level index
- Extent of the seabed significantly affected by human activities
- Fish community abundance index
- Fish community size index
- Fish community trophic index
- Fishing mortality Fmsy of Baltic cod
- Fishing mortality Fmsy of Baltic herring
- Fishing mortality Fmsy of Baltic sprat
- Maximum macrophyte distribution depth
- Proportion of oiled seabirds
- Secchi depth
- Summertime average chlorophyll a concentration
- Summertime average total nitrogen concentration
- Summertime average total phosphorus concentration
- Wintertime dissolved inorganic nitrogen concentration
- Wintertime dissolved inorganic phosphorus compounds concentration

Portuguese EEZ (POR)

- Benthic Communities Marine Biological Value
- Bottom Fish
- Coastal Fish Marine Biological Value
- Deep Coastal Fish Crustacea Cephalopods Marine Biological Value
- Deep Pelagic Cephalopods
- Deepsea Selaceos
- Demersal Coastal Fish Crustacea Cephalopods Marine Biological Value
- Demersal Fish Marine Biological Value
- Elasmobran Fish
- Marine Birds Marine Biological Value

- Pelagic Cephalopods
- Pelagic Coastal Fish Crustacea Cephalopods Marine Biological Value
- Phytoplankton Marine Biological Value
- Zooplankton Marine Biological Value

Saronikos gulf (SAR)

- CIMPAL index (alien species)
- % Threatened mammals
- % Threatened sharks
- % of stocks that meet GES based on reproductive capacity
- % of stocks that meet GES based on reproductive capacity and biomass indices
- % loss of spawning areas of sea turtle Caretta caretta
- Eutrophication Index (EI)
- Benthic Vegetation (EEI EQR)
- Benthic Fauna (Multimetric BENTIX)
- Benthic Fauna (BENTIX)
- S index
- % of stocks that meet GES based on fishing mortality
- (F-FMSY)/FMSY of Engraulis encrasicolus
- (F-FMSY)/FMSY of Sardina pilchardus
- (F-FMSY)/FMSY of Merluccius merluccius
- (F-FMSY)/FMSY of Mullus barbatus
- Physicochemical Status (FA)

CHAPTER 2. METHODOLOGICAL IMPLEMENTATION

2.1 Implementing and innovating marine monitoring approaches for the assessment of the environmental status

Ref: Danovaro R. *, **Carugati L.** *, Berzano M., Cahill A.E., Carvalho S., Chenuil A., Corinaldesi C., Cristina S., David R., Dell'Anno A., Dzhembekova N., Garcés E., Gasol J.M., Goela P., Féral J.-P., Ferrera I., Forster R.M., Kurekin A.A., Rastelli E., Marinova V., Miller P.I., Moncheva S., Newton A., Pearman J.K., Pitois S.G., Reñé A., Rodríguez-Ezpeleta N., Saggiomo V., Simis S.G.H., Stefanova K., Wilson C., Lo Martire M., Greco S., Cochrane S.K.J., Mangoni O. and Borja A. (2016). Implementing and Innovating Marine Monitoring Approaches for Assessing Marine Environmental Status. Front. Mar.Sci.3:213. doi: 10.3389/fmars.2016.00213

* These authors have contributed equally to this work

Abstract

Marine environmental monitoring has tended to focus on site-specific methods of investigation. These traditional methods have low spatial and temporal resolution and are relatively labor intensive per unit area/time that they cover. To implement the Marine Strategy Framework Directive (MSFD), European Member States are required to improve marine monitoring and design monitoring networks. This can be achieved by developing and testing innovative and cost-effective monitoring systems, as well as indicators of environmental status. Here, we present several recently developed methodologies and technologies to improve marine biodiversity indicators and monitoring methods. The innovative tools are discussed concerning the technologies presently utilized as well as the advantages and disadvantages of their use in routine monitoring. In particular, the present analysis focuses on: (i) molecular approaches, including microarray, Real Time quantitative PCR (qPCR), and metagenetic (metabarcoding) tools; (ii) optical (remote) sensing and acoustic methods; and (iii) in situ monitoring instruments. We also discuss their applications in marine monitoring within the MSFD through the analysis of case studies in order to evaluate their potential utilization in future routine marine monitoring. We show that these recently-developed technologies can present clear advantages in accuracy, efficiency and cost.

Keywords: Marine Monitoring – Marine Strategy Framework Directive – Marine biodiversity – Molecular approaches – *In situ* monitoring

Introduction

Marine ecosystems are subject to a multitude of direct human pressures, such as overexploitation, eutrophication, pollution and species introductions (Halpern et al., 2008; Hoegh-Guldberg and Bruno, 2010; Burrows et al., 2011), including the effects of global impacts, namely ocean acidification and climate change (Doney et al., 2012). These stressors can have synergistic effects on marine ecosystems (Mora et al., 2013; Griffen et al., 2016), altering their functioning and ability to provide goods and services (Worm et al., 2006; Crain et al., 2008). Their impact is expected to be even stronger in enclosed and semi-enclosed basins with high population density, tourism flow and maritime activities (Danovaro, 2003). Improved knowledge on the consequences of the effects of multiple stressors on marine biodiversity and ecosystem functioning is urgently required (Danovaro and Pusceddu, 2007; Zeidberg and Robison, 2007; Danovaro et al., 2008; Nõges et al., 2016; Zeppilli et al., 2016). In 2008, the European Commission enacted the Marine Strategy Framework Directive (MSFD; 2008/56/EC), which aims to manage the European seas by using an ecosystem-based approach in order to gain a healthy and productive state (so called good environmental status; GES; see Box 1 for the list of acronyms) (Borja et al., 2013).

The MSFD particularly aims at investigating the functioning of ecosystems (Cardoso et al., 2010; Borja et al., 2011), making a shift from structural, site-specific approaches to a functional, whole-sea system of monitoring (Borja and Elliott, 2013). An overarching aim is to promote regional harmonization of monitoring methods, used to assess marine environmental health and to obtain complete and long-term datasets from multiple ecosystem components, ranging from microbes to large marine mammals (Caruso et al., 2015).

Traditional methods applied to analyse marine biodiversity (e.g., morphological species identification, laboratory culture, toxicological analyses) are based on morphological identification and observational surveys, which are costly, time consuming and characterized by low upscaling potential to resolve change. One of the most evident limitations of traditional approaches is the identification and quantification of rare species and the ability to distinguish morphologically close or identical species (i.e., cryptic species), or poorly characterized juvenile stages of known species.

Box 1. List of the acronyms used.

Acronym	Definition
ACI	Acoustic Complexity Index
AMBI	AZTI Marine Biotic Index
ARMS	Autonomous Reef Monitoring Structure
ASU	Artificial Substrate Unit
AUV	Autonomous Underwater Vehicle
BLAST	Basic Local Alignment Search Tool
CALPS	Continuous Automated Litter and Plankton Sampler
CLEAN SEA	Continuous Long-term Environmental and Asset iNtegrity monitoring at SEA
COI	Cytochrome Oxidase c Subunit 1
CTD	Conductivity, Temperature, Depth
mtDNA	Mitochondrial Deoxyribonucleic Acid
rDNA	Ribosomal Deoxyribonucleic Acid
FCM	Flow Cytometry
GES	Good Environmental Status
HAB	Harmful Algal Bloom
HFNI	High Frequency Non-Invasive
HPLC	High-Performance Liquid Chromatography
HTS	High-Throughput Sequencing
MCZ	Marine Conservation Zone
MSFD	Marine Strategy Framework Directive
OHI	Ocean Health Index
OSD	Ocean Sampling Day
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
qPCR	Real Time Quantitative PCR
rRNA	Ribosomal Ribonucleic acid
ROV	Remotely Operated Vehicle
SST	Sea Surface Temperature

Recently developed technologies present a wide variety of advantages including a higher taxonomic resolution and the capability to rapidly provide, often in near real time, information regarding wide geographic areas (remote sensing) or large temporal scales (e.g., autonomous observation platforms—buoys, moorings, ships-of-opportunity). As a result, the technological advancement is evolving in two main directions: (i) innovative molecular approaches for rapid biodiversity assessment (Bourlat et al., 2013); and (ii) autonomous and sensitive (optical) sensor systems, which allow us to operate and collect data in situ over wide spatial and temporal scales (She et al., 2016). Methods able to combine both requirements are thus highly desirable.

Innovative molecular technologies have fundamentally changed our understanding of biodiversity, particularly for microbes, rare species, “soft-species” or extremely small specimens, which are difficult to identify and cryptic species (to be studied combining molecular and

morphological information; e.g., Derycke et al., 2005; Sogin et al., 2006) and new sensors and in situ technologies have already been applied to identify new forms of life in remote deep-sea habitats (Danovaro et al., 2014). However, most of the approaches/tools still need to be tested prior to their application in routine marine monitoring (e.g., EU project DEVOTES DEVelopment Of innovative Tools for understanding marine biodiversity and assessing good Environmental Status). In this overview, we investigate the potential applications of various innovative tools and approaches in order to evaluate their applicability to routine marine monitoring, with a special focus on three main categories, which seem to be the most promising: (i) molecular approaches; (ii) innovative systems for in situ analysis; and (iii) remote sensing.

Molecular approaches to assess marine biodiversity: from microbes to macrofauna

Morphological identification of species is heavily dependent on taxonomic experts, who are generally specialized on some specific groups of organisms (McManus and Katz, 2009; Bacher, 2012), and in some cases, the identification is impossible (e.g., cryptic and microbial species). Moreover, traditional taxonomy is generally time-consuming (Bourlat et al., 2013; Carugati et al., 2015), making large-scale and intense monitoring programs difficult to be undertaken. Molecular techniques are more universal (e.g., can target a broader range of taxa in a single analysis) and are less influenced by taxonomic expertise. Hence, molecular approaches have the potential to contribute to a large number of MSFD Descriptors (Table 1) and are promising tools to analyse the biodiversity of different biotic components (e.g., from prokaryotes, micro-eukaryotes to metazoans; Table 2), to identify species with different phenotypes or through the different stages of the life cycles (still unknown for the majority of marine species).

Use of metabarcoding to study marine biodiversity

The term "metabarcoding" refers to large-scale analyses of biodiversity through the amplification and sequencing of marker genes (e.g., 18S and 16S rDNA, Creer et al., 2010) and may also apply to capture-enrichment approach (Taberlet et al., 2012). Originally, most of the studies based on metabarcoding focused on prokaryotes (e.g., Sogin et al., 2006; Gilbert et al., 2009; Brazelton et al., 2010; Salazar et al., 2016), but, more recently, eukaryotes have also been investigated, including marine protists (e.g., Amaral-Zettler et al., 2009; Stoeck et al., 2010; Logares et al., 2014a; de Vargas et al., 2015; Massana et al., 2015) and metazoans (Thomsen et al., 2012; Lindeque et al., 2013; Hirai et al., 2015; Pearman and Irigoien, 2015). The development of high-throughput sequencing (HTS)

technologies and of standardized procedures could allow metabarcoding analyses to be included in routine monitoring programmes (Visco et al., 2015; Zaiko et al., 2015a, b).

Morphology-based studies target a limited range of taxa (e.g., meiofauna or macrofauna). These biotic components host a potentially large number of cryptic and rare species (Ainsworth et al., 2010), which could be contextually detected using universal primers, targeting a broad range of taxa at the same time. This could lead to the incorporation of novel candidates for indicator species. For example, Chariton et al. (2010) suggested that phyla such as Kinorhyncha could be sensitive to contamination and used as an indicator. Metabarcoding could also be applied to assess changes in community structure along a disturbance gradient (Hewitt et al., 2005), or to detect non-native transient species (Jerde et al., 2011; Dejean et al., 2012; Cowart et al., 2015; Viard et al., 2016), allowing for better planning and implementation of conservation approaches. An interesting potential development of molecular techniques is the detection of sequences of eukaryotes from ancient DNA, or from the extracellular DNA pools, which enable the comparison between living species and species that were present in the same area in the (even recent) past (Corinaldesi et al., 2008, 2011, 2014; Pearman et al., 2016b). In addition, the progressive reduction of the costs of sequencing over time makes large-scale metabarcoding more feasible (e.g., de Vargas et al., 2015; Salazar et al., 2016).

Although metabarcoding can represent a useful tool for the census of marine biodiversity, there are still different shortcomings and pitfalls that prevent its extensive use in marine monitoring programmes. Metabarcoding can indeed provide an inaccurate or wrong estimation (under/over estimation) of the actual biodiversity of the sample due to variability in primers, PCR conditions, sequencing technology and bioinformatics pathways used.

The use of different marker genes could give different results in terms of taxonomic composition. Different gene regions vary in both taxonomic coverage and species-resolving power, leading to the introduction of errors in the identification and estimates of taxon relative abundance (Bik et al., 2013). The mitochondrial gene encoding for the cytochrome oxidase c subunit 1 (COI), is one of the preferred candidate loci for standard DNA barcoding projects (e.g., the International Barcode of Life, http://ibol.org). However, alternative genomic regions (e.g., nuclear 16S/18S rRNA genes, 12S mtDNA) characterized by more conserved priming sites have been identified as more appropriate for "metabarcoding" studies allowing to broader scale amplification of biodiversity across the eukaryotic taxa (Deagle et al., 2014). Nevertheless, for some taxa, these markers provide little resolving power at the species level. A possible alternative is represented by D2–D3 "diversity loop" region of 28S rRNA. A possible way forward to address this issue is represented by the multi-

barcode approach (i.e., using a cocktail of gene markers for the same sample), which could help to improve taxonomic coverage and resolution.

Table 1. List of monitoring tools and MSFD Descriptors covered in this review (D1: Descriptor 1. Biodiversity is maintained; D2: Descriptor 2. Non-indigenous species do not adversely alter the ecosystem; D3: Descriptor 3. The population of commercial fish species is healthy; D4: Descriptor 4. Elements of food webs ensure long-term abundance and reproduction; D5: Descriptor 5. Eutrophication is minimised; D6: Descriptor 6. The sea floor integrity ensures functioning of the ecosystem; D7: Descriptor 7. Permanent alteration of hydrographical conditions does not adversely affect the ecosystem; D8: Descriptor 8. Concentrations of contaminants give no effects; D9: Descriptor 9. Contaminants in seafood are below safe levels; D10: Descriptor 10. Marine litter does not cause harm; D11: Descriptor 11. Introduction of energy (including underwater noise) does not adversely affect the ecosystem.

Monitoring approaches	**Descriptors**
Metabarcoding	D1, D2, D3, D4, D5
Microarrays	D1, D2, D3, D4, D5
qPCR	D1, D2, D4, D5
Chemical sensors	D8, D10
ROVs and AUVs (e.g., Clean Sea System)	D1, D2, D3, D4, D6, D10
Acoustic monitoring	D1, D3, D6, D7, D11
Flow cytometry, HPLC, Chemtax	D1, D5
Remote sensing of ocean colour (i.e., satellite data)	D1, D5
Multibeam survey	D1, D6, D7
ARMS and ASUs	D1, D2, D3, D4
High resolution sampling instruments (e.g., CALPS)	D1, D2

Setting the best PCR conditions to recover the organisms present in an environmental sample is crucial for a successful application of metabarcoding to routine marine monitoring. A recent study demonstrated that different PCR conditions could affect the final taxonomic assignment in metabarcoding studies. A constant low annealing temperature (46 or 50◦C) provides more accurate taxonomic inferences compared to the touch down profile (Aylagas et al., 2016). Conversely, increasing the number of PCR cycles leads to the increase in the number of spurious sequences and chimeras formed (Haas et al., 2011). Chimeras can inflate the overall biodiversity estimates and be eliminated by comparing the length of matched bases from the top hit in a MEGABLAST search to the length of the query sequence. As long as the database sequence is longer than the query sequence and a portion of the 3′ end does not match, it is likely that the query is a recombinant. Chimeras can be removed also by using other algorithms, including Perseus (Quince et al., 2011), UCHIME (Edgar et al., 2011) and USEARCH (Edgar, 2010).

The choice of the sequencing platform is strictly linked to the aim of the research (Carugati et al., 2015). Recently Illumina platforms have become more appealing than the Roche 454 to assess metazoan biodiversity, because of their increasing read lengths, lower per base cost, production of tens to thousands times more sequences, and lower error rates (0.1% vs. 1%, Glenn, 2011).

Metabarcoding is not exempt from errors: i) during the processing of the samples (e.g., DNA amplification steps producing "chimeras," see above; Cline et al., 1996; Smyth et al., 2010), (ii) during sequencing (Glenn, 2011), and/or (iii) presence of multi-copy genes within a single species (e.g., Telford and Holland, 1997; Alverson and Kolnick, 2005; Bik et al., 2012). Metabarcoding based on PCR cannot yet provide reliable biodiversity indices since, especially for eukaryotes, it does not supply information on the abundance of every single species detected (Lindeque et al., 2013; Hirai et al., 2015). Most of the studies aimed at evaluating the relationships between species abundance and metabarcoding data obtained looser associations (Carew et al., 2013; Zhou et al., 2013; Hirai et al., 2015). Conversely, stronger relationships have been reported between biomass and read proportions (Elbrecht and Leese, 2015). Measure of relative abundance within metabarcoding samples need to be carefully considered. Nevertheless, in the absence of primer bias, a species characterized by larger biomass should be reflected by a greater proportion of sequence reads. Conversely, if the species is smaller or rarer, then fewer reads are likely to be obtained (Creer et al., 2016).

We are at the very beginning of applying this approach to analyse marine eukaryotic biodiversity. Further studies associated with the recent progress made in DNA sequencing technologies will allow elimination of DNA amplification steps and could open new perspectives to use metabarcoding in marine monitoring programmes. A recently developed approach, which could avoid PCR biases is based on the Illumina-sequencing of environmental metagenomes (mitags) (Logares et al., 2014b). We suggest that this method could represent, in the future, a powerful alternative to 18S rDNA amplicon sequencing and a useful tool to obtain simultaneously information on taxonomic and functional diversity.

An additional limitation of metabarcoding is that it does not differentiate between life stages, and thus juvenile stages and adults are pooled together. Further, species lists produced through metabarcoding currently are presence-absence based, and lack relative abundance data. Thus, traditional community analyses used for impact detection cannot be applied in the traditional manner, and instead the focus will be on overall species richness and presence of indicator species.

Another issue is represented by the still limited availability of sequences in public databases (Carugati et al., 2015). In some cases, operational taxonomic units (OTUs) can not be taxonomically assigned to a species, or even to a genus, due to the paucity of data in reference databases and the

lack of taxonomic resolution at the species level of the marker gene (Dell'Anno et al., 2015; Leray and Knowlton, 2016). Thus, exploiting the data will require the continued refinement of database resources and bioinformatic pipelines (Minster and Connolly, 2006; Hajibabaei et al., 2011; Bik et al., 2012; Radom et al., 2012).

Table 2. List of monitoring tools and ecosystem (abiotic and biotic) components to be used for.

Monitoring approaches	**Main target components**
Metabarcoding	Benthic assemblages (Micro-, meio-, macrofauna); Plankton assemblages (prokaryotic pico-plankton, eukaryotic pico-, nano-, micro-, meso- macro-, megaplankton)
Microarrays	Phytoplankton (i.e., harmful algal blooms)
qPCR	Water and sediment pathogens (e.g., *Escherichia coli*, fecal Enterococci, *Salmonella*)
Chemical sensors	Heavy metals, organic pollutants, algal toxins
ROVs and AUVs (e.g., Clean Sea System)	Physical and chemical parameters, trace pollutants. Benthic assemblages (macrofauna and megafauna), ichthyofauna.
Acoustic monitoring	Zooplankton and fish standing stocks
Flow cytometry, HPLC, Chemtax	Phytoplankton, picoplankton, virioplankton
Satellite data	Phytoplankton assemblage structure and biomass (Chl a), Suspended Particulate Matter (SPM), Dissolved Organic Matter (CDOM).
Multibeam survey	Seafloor, Hydrographical Conditions
ARMS and ASUs	Meio-, macro-, megafauna, microalgae, macrophytes
High resolution sampling instruments (e.g., CALPS)	Environmental parameters, zooplankton

Consequently, the collaboration between molecular ecologists and taxonomists is required for the accurate characterization of species and for the deposition of quality assured barcode sequences in public databases (Jenner, 2004). The improvement of reference databases and thus the ability to assign OTUs to known species will enable metabarcoding techniques to be more reliably used in monitoring surveys, with high potential for the detection of non-indigenous species. It is also important to underline that relating sequences to taxonomically described species is not a necessity for many applications since in monitoring the focus is in pattern changes, not on taxonomic composition per se. We suggest that, in order to apply metabarcoding for the purposes of the MSFD (e.g., Descriptor 1), an attempt could be made using the overall species richness. For instance, significant changes in the species richness of the community can be a useful warning indicator and assessing such changes does not require that each molecular OTU is assigned to a precise taxon. The

Biodiversity Descriptor of the MSFD does not explicitly require that species are all taxonomically identified. Furthermore, molecular barcodes of a species, even when the species is not in the reference database, generally allow identification at the genus or family level if other species of the same genus or family are present in the reference database.

Case study 1. Microbes. HTS approaches have been recently applied to study the biodiversity of marine viruses (Tangherlini et al., 2012), bacterioplankton (Bacteria and Archaea) (e.g., Sogin et al., 2006; Gilbert et al., 2009; Brazelton et al., 2010), eukaryotic pico- (0.2–3 µm) (e.g., Shi et al., 2009; Massana et al., 2015), nano- (3–20 µm) (e.g., de Vargas et al., 2015; Massana et al., 2015), and microplankton (20–200 µm) (e.g., de Vargas et al., 2015). Data on their abundance and diversity may provide useful information on the impact of human pressures. Protists have been recurrently proposed as bioindicators (Payne, 2013). Nevertheless, the bacterioplankton component is still neglected by the MSFD (Caruso et al., 2015). The use of HTS allows the analysis of microbial biodiversity at an unprecedented scale, greatly expanding our knowledge on the microbiomes of marine ecosystems (Caporaso et al., 2011). These approaches provide relatively fast and cost efficient observations of the microbial component, and thus, may be suitable tools in biodiversity monitoring programs (Bourlat et al., 2013). Application of recently developed sequencing methodologies (e.g., Illumina technologies) to the analysis of the 16S rRNA gene for bacteria and of the 18S rRNA gene for eukaryotes in samples taken along the Barcelona coast (NW Mediterranean Sea) suggests that certain taxa (i.e., members of the Gammaproteobacteria) as well as the ratio between some phylogenetic groups may be good indicators of ecosystem health status. However, the robustness of these indicators needs to be explored by gathering data on plankton diversity in coastal areas subjected to different degrees of anthropogenic pressure over various temporal and spatial scales. Seasonality seems to play a major role in shaping bacterioplankton biodiversity and community structure (Gilbert et al., 2012; Cram et al., 2015) which could overwhelm the effects of human-induced pressures. Thus, despite being extremely promising, the suitability of incorporating prokaryotic/eukaryotic biodiversity into MSFD descriptors needs to be further explored in order to discriminate between changes resulting from human activities and the natural variability of the marine environment (Ferrera et al., 2016).

Case study 2. Meiofauna. Small metazoans belonging to the meiofauna are sensitive to environmental changes and are increasingly used in monitoring studies for the assessment of environmental quality (Moreno et al., 2011; Pusceddu et al., 2011). However, meiofaunal diversity is so large that the analysis of a single phylum, such as Nematoda, requires huge investments of time from highly specialized taxonomists. Metabarcoding could facilitate the census of biodiversity,

especially for meiofauna, for which morphological identification is difficult. The typical metabarcoding workflow used to study meiofaunal biodiversity in marine benthic ecosystems is reported in Figure 1. Recent investigations of shallow and deep-sea nematodes based on 454 sequencing and classical morphological identification revealed that, at the order-family level, metabarcoding assignments Although metabarcoding is a useful tool to explore the diversity of marine meiofaunal organisms, it still presents some gaps. Indeed, not all species in a sample are detected and a certain percentage remains unidentified due to the limited coverage of public sequence repositories for meiofaunal taxa (Carugati et al., 2015). This applies particularly to the deep sea, where most of the taxa are still unknown (Appeltans et al., 2012). Thus, we suggest to continue combining morphological identification performed though light microscopy with molecular analyses, in order to feed or even create local database, at least for marine protected area or high priority areas. To more accurately delineate species in metabarcoding datasets major efforts should be devoted to understanding the actual variability of the 18S rRNA gene amongst individuals of the same species and amongst different species taking into account the contribution of potential biases due to PCR and sequencing steps in such variability. There is also the urgent need to identify alternative single copy markers, nuclear or mitochondrial, less subjected to such intra-specific variability. Finally, alternative solutions can be the use of non-PCR-based metabarcoding approaches, using capture probes, which are much less sensitive to mismatches between probe/primer and target and may replace PCR-metabarcoding. Future investigations are needed to address these issues in order to facilitate the inclusion of meiofaunal diversity in marine monitoring programs.

Case study 3. Macrofauna. Marine benthic macroinvertebrates are commonly used as indicators of ecosystem health; yet, calculation of biotic indices based on macro-invertebrate taxonomic composition (e.g., AMBI) requires each sample to be sorted and each specimen to be taxonomically identified by an expert taxonomist. This is a tedious, expensive and time-consuming process, which has limitations, particularly when cryptic species, damaged specimens or immature life stages are present (Ranasinghe et al., 2012). Metabarcoding is a promising alternative to overcome the limitations of traditional taxonomy and can help in ensuring the accomplishment of temporarily and spatially comprehensive monitoring. However, before routine implementation of this approach, the development of standardized practices at each step of the procedure (Aylagas and Rodríguez-Ezpeleta, 2016) and the increase of the reference libraries for taxonomic assignment are required (Aylagas et al., 2014). Additionally, in order to ensure accurate biotic indices derived from metabarcoding, the ability to detect the majority of organisms representing the full gradient of tolerance to pollution is necessary. With the aim of benchmarking metabarcoding against traditional

taxonomy in the context of biotic index calculation, Aylagas et al. (2016) performed a thorough experiment comparing taxonomic inferences and biotic indices derived from samples of known species composition analyzed using alternative metabarcoding protocols. The work resulted in a series of guidelines for the application of metabarcoding for macroinvertebrate monitoring.

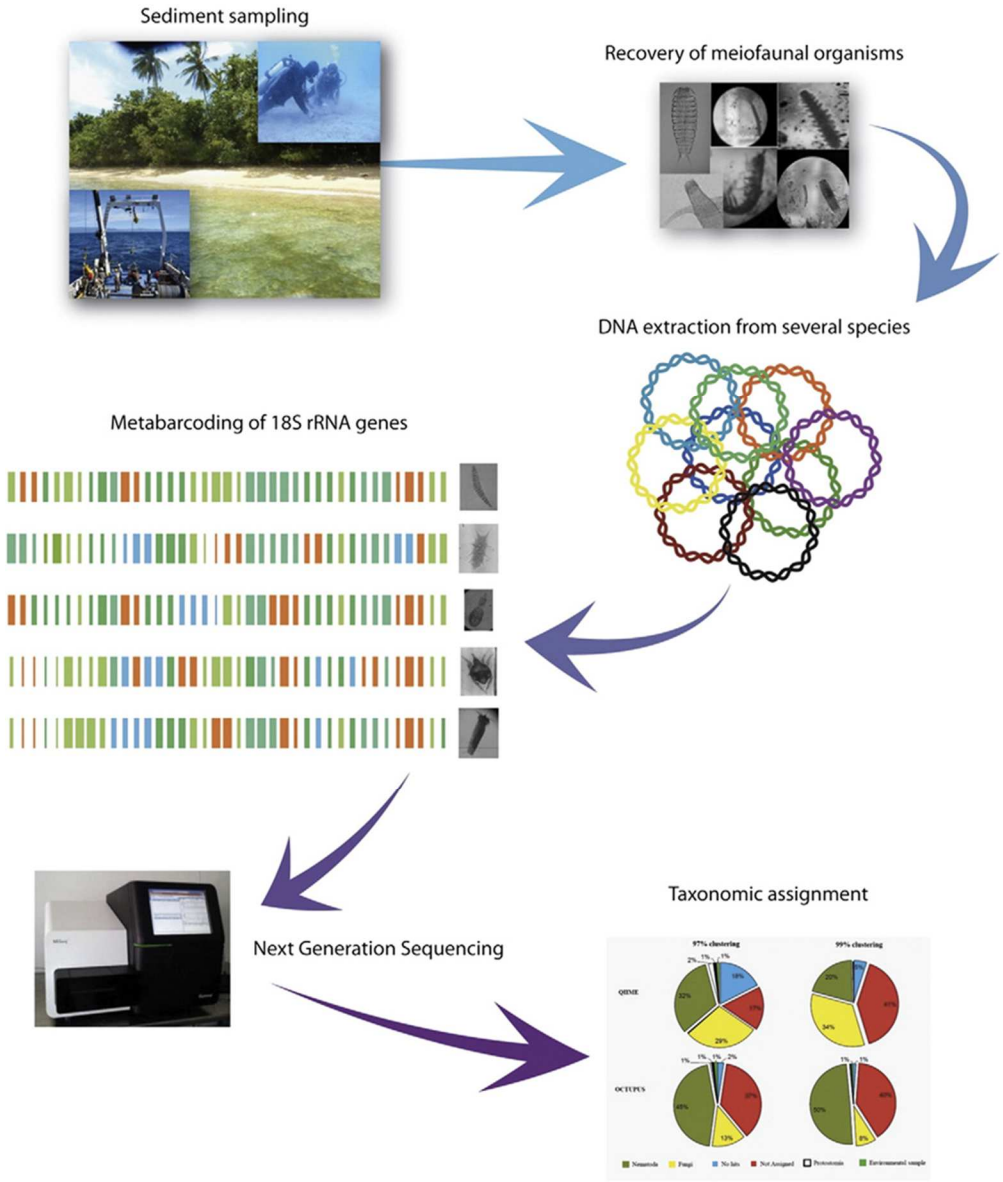

Figure 1. Standardized workflow to study meiofaunal biodiversity in marine benthic ecosystems using high-throughput sequencing. Sediment samples (from shallow to deep-sea environments) are collected and subsequently frozen (−20 °C or −80 °C). In the laboratory, meiofaunal organisms are recovered from the sediments and their DNA extracted and purified. Following the PCR amplification of marker genes (e.g. 18S rRNA), high-throughput sequencing can be conducted on Roche 454 or Illumina platforms. Raw reads are processed and then clustered into operational taxonomic units (OTUs) under a range of pairwise identity cutoffs. After the BLAST-match of the obtained OTUs against public nucleotide databases, analysis of α- and β-diversity and phylogeography are performed. Image of Illumina MiSeq platform: Source: Wikipedia, Author: K. Förstner (Carugati et al., 2015).

The application of microarrays for the detection of harmful algal blooms

Microarrays have been applied for *in situ* detection of harmful algal bloom (HAB) species (Descriptors D1, D2, D5 in the MSFD; see Tab. 1 for more details). This method is especially useful for the rapid identification of toxic algae (Tab. 2) that can have serious consequences on human health (Bricker et al., 2007). The European project MIDTAL (Microarrays for the detection of toxic algae) has developed a microarray to target major HAB species including toxic dinoflagellates, raphidophytes, prymnesiophytes, Dichtyocophyceae and the diatom Pseudonitzschia (Lewis et al., 2012). Microarrays are made of coated solid surfaces onto which a large number of selected DNA probes (specific for a taxon) can be spotted. Each probe is fluorescently labeled and when the probe hybridizes with a sample, the sample/probe complex fluoresces in UV light. An advantage of this approach is that no PCR step is required when total RNA is selected and this reduces the bias of any unknown inhibitors in the sample. Because microarrays rely on DNA probes for detection of HAB species, the potential for new indicators could be nearly unlimited. This chip has been tested on selected seawater samples previously morphologically identified. Microarrays have shown high sensitivity and several species not identified under light microscope have been recognized by the probes on board the microarray. Thus, microarray could be a potentially useful tool to provide quick evaluation on the presence of toxic algae. However, the use of microarray presents a series of limits. Some of the algal species morphologically identified in a sample could not be detected by the molecular probes. Moreover, the sensitivity of selected probes was confirmed at genus level, but at species level the results were less satisfactory. The costs of the MIDTAL microarray chip plus reagents and consumables is still high. Thus, further attempts are needed to make convenient and accurate the results provided by the use of the microarray approach and we recommend the use of the microarray in monitoring programs only if combined with microscopy analyses. The combined approach between current monitoring practices and microarrays could be applied in the MSFD (e.g., Descriptor 5) in order to provide quick and reliable information on the presence of algae potentially toxic for human health.

Quantification of pathogens by means of Real Time quantitative PCR (qPCR)

Real-time polymerase chain reaction (qPCR) consists of the amplification and quantification of a gene sequence specific to the organism(s) of interest. The correlation of the amount of DNA obtained with the number of individuals allows the quantification of the investigated organisms in a given sample. This procedure could be applied only to unicellular organisms that contain a known number of copies of the gene under study. Exponential amplification of the target sequence is followed in

real-time by means of a fluorescent dye or a fluorescently labeled DNA probe. Quantification is performed by comparison to a standard curve, which is run concurrently with samples using reference material consisting of pre-enumerated cells or DNA. qPCR has been recently tested to evaluate the quality of the freshwater and marine environment (Descriptors D1, D2, D5 in the MSFD, Table 1; Newton et al., 2011; Harwood et al., 2014; Lu et al., 2015). Traditionally, the classical microbiological analyses include the investigation, by using cultivation techniques, of the abundance of fecal indicator bacteria such as Escherichia coli and Enterococci in water samples, and E. coli, Enterococci and Salmonella in sediment samples (Table 2). The determination of total prokaryotic abundances could be also performed through epifluorescence microscopy. Such a technique allows the determination of the whole quantitative relevance of marine microbes contrary to the cultural techniques, which can only detect less than 1% of the actual abundance of prokaryotes (Staley and Konopka, 1985). Epifluorescence microscopy could be utilized in combination with qPCR of the prokaryotic 16S rRNA genes. The combined use of qPCR and metabarcoding could open new perspectives to investigate the biodiversity of the microbial community in seawater and sediment samples and in particular the relevance of human pathogens, going beyond the limits of the traditional approaches.

In situ instruments to monitor marine abiotic and biotic variables

Some of the best approaches to meet current demands in marine monitoring are represented by novel in situ technologies, which provide high-frequency (continuous or semi-continuous) observations. So far, most of in situ instruments have been developed to monitor marine hydrological and physico-chemical variables, whereas the monitoring of the biotic variables is still mostly dependent on non-remote or automatic devices. An example is the system of SmartBuoys, which house a range of instruments for measuring salinity, temperature, turbidity, chlorophyll fluorescence, oxygen saturation and nitrate concentration. Such instruments enable the creation of wide-scale international networks of environmental data acquisition and sharing, as implemented in the framework of the ongoing S&T Med European project (http://stmedproject. eu/). Nonetheless, technological limitations are at the base of the presently scarce modeling capacity regarding population/stock and biodiversity assessments as well as ecosystem functioning.

Chemical sensors

There are few sensors currently in use for monitoring concentrations of heavy metals, organic pollutants and algal toxins. An in situ analyzer has been developed to measure nitrate plus nitrite and

total sulfide in deep-sea areas close to hydrothermal vents (Le Bris et al., 2000). More recently, Vuillemin et al. (2009) developed an in situ analyzer (the CHEMINI system) which measures analytes at even greater depths. However, as for any instrument deployed at sea, especially in nutrient rich environments, it is subjected by a rapid biological colonization (biofouling), which can limit overall deployment times (Mills and Fones, 2012).

Seabed observatories

Marine observatories allow the collection of long-term time series of environmental parameters, but have yet not been commonly used. It is widely recognized that underwater technology could open new and interesting opportunities to ensure continuous, long-term, execution of monitoring. In particular, during the last decades, underwater video technologies have gained considerable importance in all fields of marine science. They represent a powerful, non-destructive and useful tool to study the dynamics and the interactions between benthic organisms, especially on hard-bottom sediments where traditional grab methods are ineffective. The use of underwater visual surveillance is becoming increasingly accessible for monitoring activities since it is versatile, serving as an "underwater eye" for researchers. Video cameras can be mounted on various vehicles ranging from simple towed platforms, Remotely Operated Towed Vehicles (ROTVs), to more advanced systems such as Remotely Operated Vehicles (ROVs) or Autonomous Underwater Vehicles (AUVs). Stills photos can be acquired using drop cameras, mounted on ROVs or by diver at shallow depths, and long-term data series can be used to study the links between biodiversity and climatic variations, for example correlating changes in biodiversity related to the North Atlantic Oscillation (NAO) index (Beuchel et al., 2006). In coastal benthic and pelagic systems at shallow depth, SmartBuoys equipped with underwater cameras can enable such time-series studies, contextually monitoring multiple environmental parameters to complement visual information. In general, video surveys produce indicators of overall sediment conditions and frequency of occurrence of the most visible taxa. Indicators from stills images focus on small-scale observations and automated image recognition techniques can be employed to quantify both presence and abundance of organisms but also extent of coverage or various proxies for biomass (Beuchel et al., 2006).

The increasing use of ROVs, AUVs and non-permanent camera stations have provided new insights on the biodiversity and ecosystem functioning of continental margin and deep-sea ecosystems (Solan et al., 2003; Stoner et al., 2008). However, challenges emerge in that inherently qualitative information needs to be converted into quantitative data from which indicators can be developed. ROV technology is available at all offshore petroleum installations, and biological visual

seabed surveys frequently are carried out in potentially sensitive habitats both before and after the drilling event. Using a set of customized visual indicators, the extent of seabed smothering can be quantified and appropriate mitigation measurements planned based on the information collected during these surveys. Autonomous and cabled observatories are receiving increasing attention in marine science and have been demonstrated as capable platforms for collecting data remotely, and increasing insight into the functioning of remote marine ecosystems (Taylor, 2009; Best et al., 2013). Such cabled systems are expected to become an important tool in marine monitoring and management (Aguzzi et al., 2012a).

A possible limit of the use of video-imaging systems is that the lights necessary to acquire the images may influence the behavior of the organisms being observed. Operational lifetimes of remotely deployed instruments are often limited by the available power supplies. Cabled observatories can provide the power to operate for long-term periods. However, the establishment of the infrastructure is still expensive and therefore limited in scope. Many in situ instruments still rely on commercially available batteries, which could limit they autonomy. Small wireless autonomous devices, such as remote marine sensors can be less energy consuming thus allowing longer deployments (Mills and Fones, 2012). Another challenge is represented by the large amount of data generated, which need to be stored and processed. Cabled multiparametric seafloor observatories are usually connected to the shore to transmit data in real-time. Data could be delivered via cable, automatically streamed to an internet socket, uploaded onto the website and automatically processed (Aguzzi et al., 2012b).

Underwater autonomous and integrated monitoring

An interesting, recently developed technology is the CLEAN SEA (Continuous Long-term Environmental and Asset iNtegrity monitoring at SEA; Figure 2), which uses a commercially available AUV, upgraded with technologies enabling off shore monitoring of seafloor integrity and pollution (Table 1). This vehicle is characterized by a set of sensors able to measure both physical and chemical parameters and carry out in situ analysis of trace pollutants (Table 2). The CLEAN SEA system can also collect discrete water samples in situ. It is developed to perform acoustic surveys of the seabed and pipelines/flowlines as well as to detect hydrocarbon leakage. The CLEAN SEA system can also perform benthic community survey with detailed photographic/video coverage of the investigated area in order to determine the abundance and biodiversity of benthic assemblages and their temporal variations (Table 2). CLEAN SEA is characterized by wireless underwater communication for mission data downloading and wireless power recharge for increased autonomy.

This may enable a “permanent” operation subsea independently of support from surface. CLEAN SEA seems to be a powerful technology for future environmental monitoring around oil and gas infrastructures and to gain long-term data on abiotic and biotic variables.

Figure 2. The CLEAN SEA (Continuous Long-term Environmental and Asset iNtegrity monitoring at SEA). The Clean Sea system, launched by Eni E&P and its subsidiary Eni Norge, in cooperation with Tecnomare, is a commercially available AUV, properly upgraded with key enabling technologies, for the execution of environmental monitoring and asset integrity in offshore fields.

Biosensors

High frequency non-invasive (HFNI) valvometers have been utilized as a potential tool for long-term marine monitoring and assessments (Andrade et al., 2016). The principle of the method is based on the regular gaping behavior (closing and opening of the valves) of bivalve molluscs and the fact that physical or chemical stressors disrupt that gaping reference pattern. Bivalve gaping behavior is monitored in the natural environment, remotely, continuously over a long-time period (e.g., years), requirements that must be fulfilled if bivalve behavior is to be a useful biomonitoring tool. We here suggest the potential application of the HFNI valvometry as a biosensor to monitor and provide early-warning alerts of changes in water quality, such as temperature increase, releases of contaminants and toxic algal blooms. Finally, HFNI valvometry could be used in the MSFD for routine monitoring of areas impacted by anthropogenic activities such as bathing beaches and harbors, oil platforms and aquaculture installations.

Acoustic monitoring

An alternative method for studying marine organisms is a non-invasive acoustic approach. Active and passive hydroacoustics have explored a wide range of ecological subjects, such as pelagic communities, behavior, predator–prey interactions, and fish standing stock. The use of passive acoustic technologies (e.g., hydrophones) may solve problems of photic disturbance or limitation and provide useful results for the Descriptor 11 of the MSFD (Table 1). Most marine organisms produce sounds (marine mammals, fishes, invertebrates) to accomplish important ecological processes (e.g., communication, reproduction, foraging, predation, detection of predators and habitat selection; Van Opzeeland and Slabbekoorn, 2012). Understanding normal levels of variations in acoustic complexity is crucial for conservation efforts, enabling managers to decide whether changes in acoustic dynamics need further investigation. However, quantifying and characterizing the acoustic production of animals in marine soundscapes can sometimes be a challenging task to address. Active acoustic scattering techniques have potential to study the zooplankton and fish distributions, as they provide remote and non-intrusive samples at high resolution over large ranges (Figure 3), which is difficult to achieve using traditional net or other underwater systems alone. Multiple frequency scientific echosounders with split-beams and resulting echo-trace analysis (using frequency responses) can provide information on the sizes of animals, thus allowing some distinctions to be made. Despite the fact that the underwater acoustic instruments do not allow species classification (Knudsen and Larsson, 2009), they could be useful to gain information on pelagic and semi-demersal species as well as on zooplankton assemblages (Trenkel et al., 2011; Table 2). The Acoustic Complexity Index (ACI) (Pieretti et al., 2011) coupled with a software dedicated to soundscape analysis (Farina et al., 2011) can be used to elaborate collected acoustic files, in order to track the various biological signals, their daily and nightly dynamics and distinguish them from noise pollution. Anthropogenic noise usually has specific frequency ranges (typically <1 kHz) which overlaps with the frequencies used by fishes for communication and other processes. We suggest that the ACI seems to be a promising tool to analyse marine soundscape filtering out noises and biological sounds.

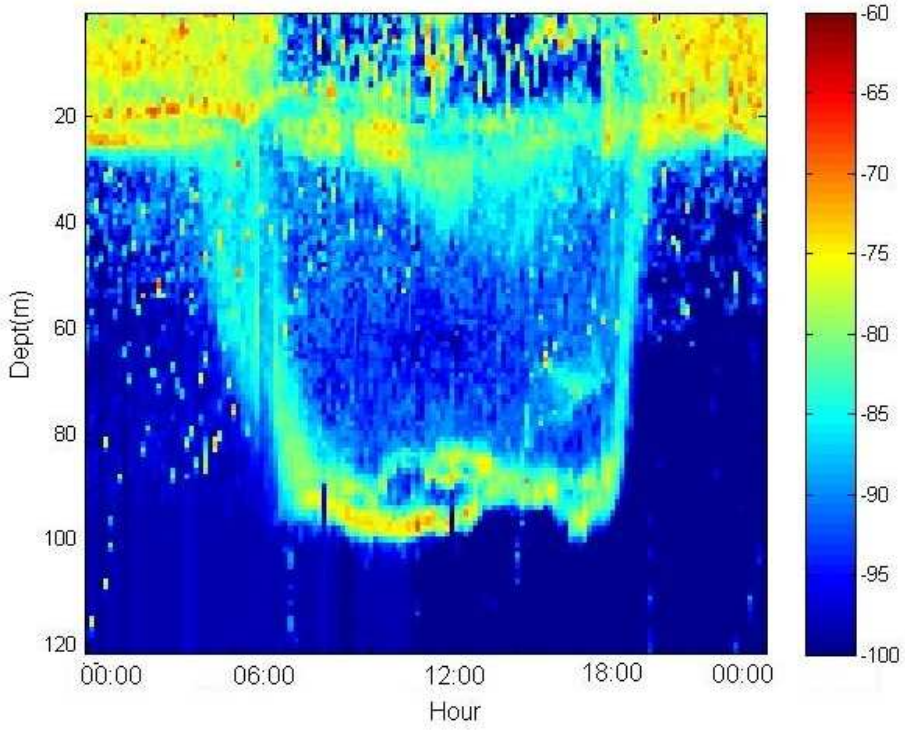

Figure 3. Echogram of diel vertical migration of a deep sound scattering layer impacted by small pelagic fish (*Sprattus sprattus*) and zooplankton (*Calanus euxinus*), Western Black Sea (Source: Institute of Oceanology, IO-BAS).

New methodologies for marine monitoring

Comparison of methods for identifying phytoplankton diversity

Considering the objectives of the MSFD, it becomes important to evaluate emerging methods to enhance the efficacy and cost-efficiency of monitoring approaches, in particular non-intrusive, relatively low-cost methods based on optics. The optical metrics of phytoplankton include the size, shape, dimensions and complexity of the phytoplankton cell, as well as its light absorption, scattering and fluorescence characteristics, which are influenced by cell size, material and pigmentation. Each optical method shows some degree of selectivity or bias, either for a cell size range, pigment concentration range, or the ability to discern individual cell characteristics vs. a population of cells in a volume as a whole. Furthermore, it is recognized that the optical attributes of phytoplankton taxa are subject to natural variability regarding pigmentation, cell size, and colony formation within species.

Light microscopy is precise with regard to taxonomic determination, but less sensitive to rare species and practically limited to cells larger than 1–2 µm. Both fresh and stored samples can be analyzed, even if for some protists, fixatives deform the cells, making difficult their identification. The main limitation of this method is the time spent by an expert analysing a single sample, which is

in the order of 1/day.limitation of this method is the time spent by an expert analysing a single sample, which is in the order of 1/day.

Flow cytometry analysis can be considered a combination of particle based and pigment analysis methods. The taxonomic distinction of each investigated particle is dependent on the number of lasers (usually 1 or 2 in benchtop instruments), detectors (4–8 in modern configurations) and is limited to those pigments that exhibit autofluorescence (chlorophylls and phycobilipigments). Besides fluorescence, flow-cytometers record forward- and side-scattering parameters, allowing basic size and shape characterization. Direct comparison of phytoplankton biodiversity obtained by using light microscopy, HPLC pigment and flow cytometry resulting from a multi-year sampling campaign in the productive season in the Baltic Sea revealed no meaningful correlation between the three methods (Figure 4). In this case, the lack of correspondence between the three methods can be explained by different lag times in the response of pigmentation, particle size distribution, or species composition to environmental changes. In other two studies a relatively good correspondence has been observed between the various methods (Casamayor et al., 2007; Christaki et al., 2011).

Pigment high-performance liquid chromatography (HPLC), has been for a long time a useful tool for obtaining information on taxonomic composition of phytoplankton, based on presence/absence of diagnostic pigments (Smith et al., 2010; Roy et al., 2011). Computational approaches, such as the statistical fitting tool CHEMTAX, have been used to determine phytoplankton biodiversity by estimating the relative contribution of different taxa to the total chlorophyll a (TChla) concentration in a sample (Mackey et al., 1996; Gibb et al., 2001; Goela et al., 2015). Although the software is fully developed, an a priori knowledge of the classes existent in the samples is required, as well as an appropriate choice of the ratios of pigment:Chla, considering the characteristics of the investigated geographical region (i.e., light availability; Higgins et al., 2011). As the inferences of this technique are based on the chemical composition of a sample and not on the direct observation of the phytoplankton cells, it has an improved capability to differentiate among organisms in smaller size classes, which in traditional methods such as microscopy fall into the category of unidentified flagellates (Goela et al., 2014). A recent application of this approach to oceanic regions, where populations of small organisms can be dominant, has proven to be particularly useful to distinguish the contribution of cryptophytes, prymnesiophytes, and prasinophytes to TChla concentration (Goela et al., 2014). Thus, the use of chemotaxonomic methods in combination with the classical methods (e.g., microscope enumeration, phytoplankton size-structure) would be useful to evaluate and characterize Descriptor 5 of the MSFD (Mangoni et al., 2013; Cristina et al., 2015; Goela et al., 2015; Table 1). Once the HPLC methodology is implemented and running, CHEMTAX

offers a rapid and cost-effective way to assess the taxonomic composition of a sample, used as a first assessment of the phytoplankton assemblage. It might provide valuable insights on the potential presence of specific groups (e.g., harmful species), especially when there is previous knowledge of the classes that are likely to contain HAB species (Mangoni et al., 2011; Liu et al., 2014).

The major caveats applied to the use of the method are often observed in phytoplankton classes which contains no diagnostic pigments or in which the diagnostic pigment is not present in all the species of the class. That is the case, for example, of dinoflagellates. Often, the marker pigment used in CHEMTAX for dinoflagellates class is peridinin, which is only present in some of the auto- or mixotrophic species of dinoflagellates (Throndsen, 1997). This might lead to the underestimation in areas where most of the dinoflagellates are heterotrophic (e.g., Goela et al., 2014). In those cases, a more reliable CHEMTAX analysis would involve a careful examination of the typical pigment profiling of the local dinoflagellates community, namely the combinations between different diagnostic pigments, or the search for species specific diagnostic pigments (e.g., Örnólfsdóttir et al., 2003; Smith et al., 2010; Roy et al., 2011). The versatility of the method, that is, the possibility to run the software with different combinations and values of pigment:Chla ratios is, in fact, one of the major advantages of the method, allowing easily to locally adapted pigment profile schemes. Recently, several studies have focused on the effective and successful use of CHEMTAX to detect HABs (e.g., Örnólfsdóttir et al., 2003), although pigment profiling studies, such as Liu et al. (2014), in other areas of the globe would be beneficial to the fulfilment of this objective.

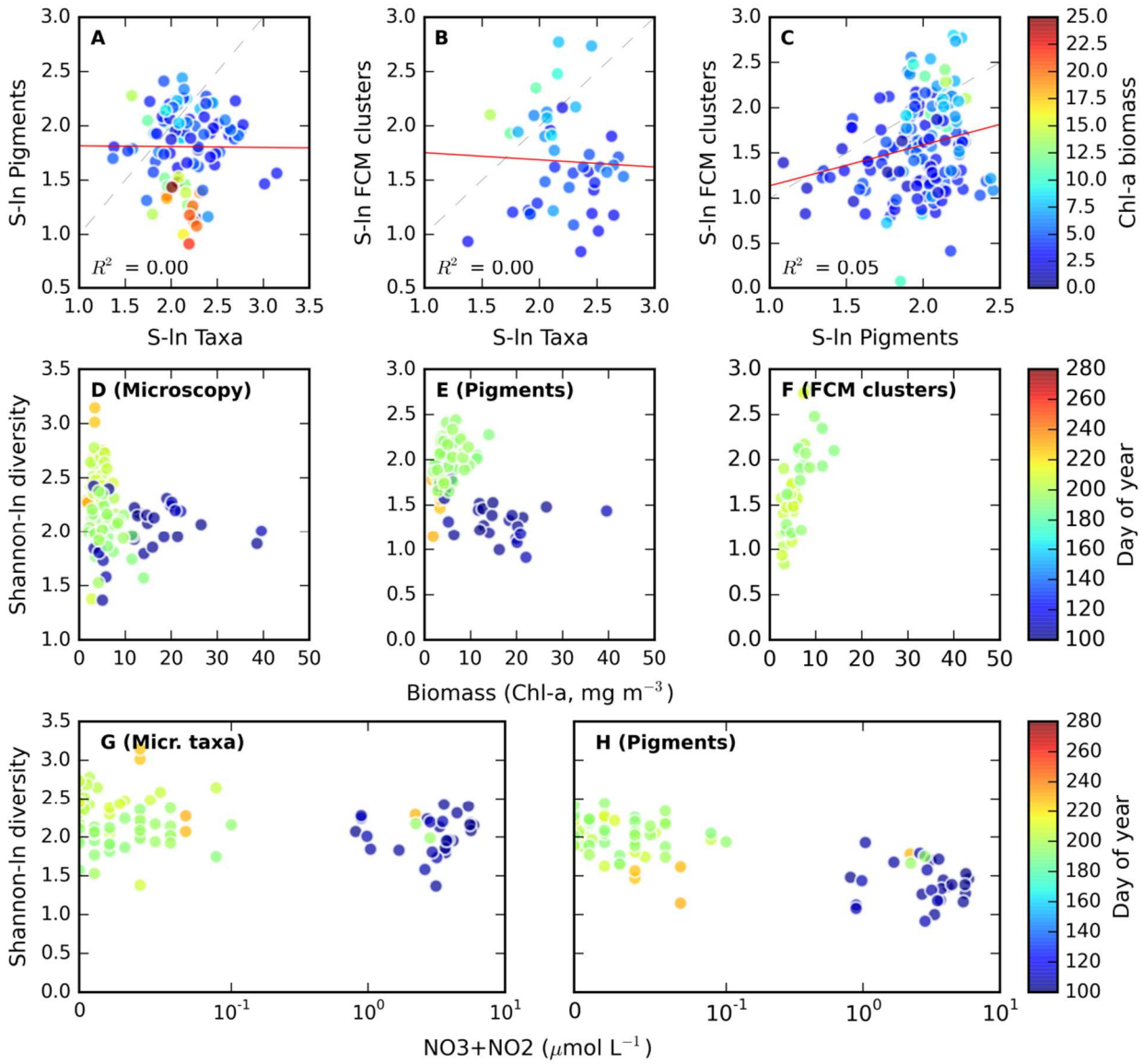

Figure 4. (A-C) Scatter plots comparing the Shannon diversity metrics obtained with HPLC Pigments (Pigments), Flow Cytometry cluster analysis (FCM clusters), and light microscopy determined to the most detailed taxonomic level (Taxa) from samples collected in the productive season in the Baltic Sea. Linear least-squares regression lines are drawn in red, dashed lines indicate unity. The colour scale applied to each data point indicates the chlorophyll-a biomass of the sample (units mg m^{-3}). (D-F) Shannon diversity derived from Microscopy, Pigments, or Flow cytometry cluster analysis, as a function of biomass and sampling time (colour scale). (G-H) Shannon diversity derived from Microscopy and Pigments as a function of inorganic nitrogen concentration and sampling time (colour scale) (Source: Plymouth Marine Laboratory, Finnish Environment Institute).

Analysis of planktonic microbial diversity by flow cytometry

In plankton microbial flow cytometry, small sample volumes are circulated in front of a laser with a fluidics system that forces each cell to pass in front of the laser, which is typically blue, red or UV. The instruments can observe thousands of cells per second, so a few minutes of operation enables inspection of several hundred thousand cells. Both the cells and the abiotic particles disperse the laser light and generate fluorescence after the excitation. Since scattered light is proportional to cell size (and cell internal rugosity) and fluorescence is proportional to pigment content, it is possible to differentiate various groups of phototrophic oxic (Marie et al., 2005) and anoxic (Casamayor et al., 2007) microorganisms according to their average cell size, types of pigments and pigment ratios. In addition, it is possible to stain the nucleic acids of heterotrophic prokaryotes (Gasol and del Giorgio, 2000), heterotrophic eukaryotes (Christaki et al., 2011) and viruses (Brussaard et al., 2000) and simple activity probes can be used to obtain indication of the relative physiological state of prokaryotes and phytoplankton (del Giorgio and Gasol, 2008). This method allows easy fingerprinting of the microbial assemblages and a fast indication of how they respond to disturbances.

Besides the cost of instrumentation, which is progressively decreasing in recent years, the total cost is on the order of a few euros per analysis and can be done and processed in less than an hour. Moreover, sample collecting, processing, flow cytometry and data analysis can be automated (Besmer et al., 2014) and even commercial (Dubelaar et al., 1999) and non-commercial (Olson and Sosik, 2007; Swalwell et al., 2011) instruments can be submerged and send the data via cabling or radio. This allows their inclusion in environmental monitoring systems such as SmartBuoys, whose multiple sensors provide complementary information of the environmental settings in which cytometry data are acquired.

There are at least four different ways in which flow cytometric data can be used to infer ecosystem properties or environmental status (Gasol and Morán, 2015): (i) Presence/absence of specific microbial assemblages (e.g., presence of red-fluorescing cyanobacteria is generally associated with turbid low-light environments, whereas high abundances of Prochlorococcus or dominance of pico-eukaryotes with nutrient-rich environments; Stomp et al., 2007); (ii) Estimates of cytometric diversity (Li, 1997) of either pico-phytoplankton and heterotrophic prokaryotes; (iii) Population size and pigment content (e.g., temperatures lead to total phytoplankton and bacterioplankton decreases in cell size; Morán et al., 2010, 2015); and (iv) Ratios between populations abundance (e.g., the ratio between picocyanobacteria and eukaryotic picophytoplankters has been used to indicate nutrient levels as cyanobacteria are more likely to be abundant in low

nutrient oligotrophic environments while eukaryotes tend to dominate in high nutrient conditions; Calvo-Díaz et al., 2008).

While the potential for these methods to work exists and a cost-savings potential is clearly demonstrated, additional testing is needed to determine how robust the methods are to detect physiological changes, such as those caused by nutrient and light availability. Sensitivity of these methods to cell physiological constrains may for example introduce undesirable seasonal or geographical bias which traditional (e.g., microscopy) methods would not show. Further studies are therefore needed to derive robust indicators of environmental status, preferably based on a multitude of complementary methods. Gathering data over various temporal and spatial scales in order to distinguish natural variability from that resulting from anthropogenic pressures will help validate these indicators, in order to subsequently develop highly automated tools for rapid assessment of marine environmental status.

Remote sensing

Remote sensing of optical, thermal and radar images from airborne and satellite sensors offers many new opportunities for the direct monitoring of biodiversity, for observing patterns in the land and sea which relate directly to biodiversity, or for the provision of environmental data layers which are needed in order to build predictive models of species and habitat distributions (Turner et al., 2003; Pettorelli et al., 2014). A new impetus has been given to the field of satellite remote sensing by the European Union's Copernicus programme in which the first of a series of Earth-observing sensors on the Sentinel satellites have been successfully launched. Sentinel 1 is a radar satellite with cloud-penetrating ability, in orbit since April 2014, and now delivering images that relate to marine and maritime needs, such as sea-ice extent, oil-spill monitoring and ship detection for maritime security. Radar images are very useful for determining the extent and composition of intertidal and salt-marsh habitats (Van Der Wal and Herman, 2007). Sentinel-2 for high resolution optical images of the coastal zone, as with Sentinel-1, will greatly enhance our ability to detect changes in intertidal and shallow subtidal habitats (Van der Wal et al., 2008). The final recent launch was that of Sentinel-3 for wide-field ocean color viewing, altimetry and sea surface temperature on 16th February 2016. Sentinel-3 will continue the progress made by other ocean-viewing satellites such as SEAWIFS, MERIS and MODIS and ensure continuity of ocean color measurements (Le Traon et al., 2015). The use of remote sensing represents a cost-effective tool supplementing conventional in situ sampling. The in situ measurements are typically based on oceanographic cruises that provide discrete data sets with often spatial and temporal coverage, which could limit the analysis of the dynamics of the phytoplankton

in relation to human activities (Rivas et al., 2006). Remote sensing can provide highly valuable data bridging the spatial and temporal gaps in observations complementing the in situ measurements. These are the major advantages of remote sensing as compared to in situ observation systems (Blondeau-Patissier et al., 2004). However, ocean color remote sensing also present some limitations as: (i) satellite-derived Chla concentrations estimates of phytoplankton biomass content are based on conversion factors (Rivas et al., 2006); (ii) information about the surface parameters can be obtained only during cloud free conditions, limiting spatial and temporal coverage, especially in high latitudes and the tropics (Blondeau-Patissier et al., 2004; Peters et al., 2005); (iii) the confidence of the estimated values based on global algorithms has to be validated with in situ observations, which are essential to ensure the optimal quality of the data retrieved by satellite remote sensing, in particular in coastal and estuarine systems due to the optical complexity of such waters (Aurin and Dierssen, 2012).

Selected uses of satellite Earth observation in the field of marine biodiversity are presented in the sections below.

Satellite data for the implementation of MSFD with respect to Eutrophication (D5)

The use of remote sensing allows a cost-effective and synoptic monitoring of extensive oceanic and coastal areas (IOCCG, 2009). The products acquired by ocean color remote sensing can be quantified by bio-optical algorithms that retrieve the concentration of Chlorophyll a (Chla), Suspended Particulate Matter (SPM) and the absorption of the Colored Dissolved Organic Matter (CDOM). These indicators of the status of the marine ecosystems give information about the phytoplankton biomass (Chla), the water transparency or turbidity (SPM) and about the terrestrial inputs of freshwater (CDOM) (Vantrepotte and Mélin, 2010; Table 2).

Several studies have been carried out in European waters for the validation of remote sensing satellite products in a wide range of geographical areas (Sørensen et al., 2007; Antoine et al., 2008; Kratzer et al., 2008; Petersen et al., 2008; Cristina et al., 2009, 2014; Zibordi et al., 2013). These studies demonstrate the accuracy and the precision of the technique to provide good quality data and to identify what are the main sources that influence the complexity of these waters.

The advantages of this tool are evident for countries that have limited resources to monitor one of the largest marine zones of regional seas (Cristina et al., 2015). An ocean color remote sensing product (Chla) can be used to detect and track the development of algal blooms in coastal and marine waters. Thus, this tool can support the implementation of the MSFD with respect to Descriptor 5: eutrophication, as demonstrated in Sagres, southwest Iberia (Cristina et al., 2015, Table 1).

Furthermore, it allows distinguishing whether the eutrophication is natural, driven by upwelling, or due to land-based inputs. The implementation of a regional algorithm increases the accuracy of the remote sensing data produced to retrieve the Chla, particularly during upwelling events when the highest concentrations of Chla occur (Cristina et al., 2016). This is supported by studies in the Baltic Sea (Harvey et al., 2015), also showing the advantages of using satellite remote sensing for monitoring and eutrophication assessment and for the status classifications of water basins. These studies show that this tool can be applied for both national, European and Regional Seas monitoring plans as well as the implementation of the MSFD and the Water Framework Directive (Gohin et al., 2008; Novoa et al., 2012). In summary, the use of remote sensing can be an efficient tool providing a synoptic view of the products (e.g., phytoplankton biomass), showing their distribution over an extended period, identifying seasonal patterns and showing the effect of changes in marine ecosystems promoted by human pressures and by environmental changes.

However, the eutrophication of the benthic compartment and its effects on the biota, which have been investigated repeatedly in the last decade (Danovaro et al., 2000, 2004; Danovaro and Gambi, 2002; Dell'Anno et al., 2002; Pusceddu et al., 2007, 2009) cannot be assessed through remote sensing.

Satellite imaging of harmful algal blooms

Harmful algal blooms (HABs) adversely affect the marine environments by releasing toxins, decreasing food availability for higher trophic levels, and reducing oxygen levels in water, potentially causing mass mortality of marine organisms (Silke et al., 2005). HAB species may dominate the phytoplankton community, with very high chlorophyll concentration that can be detected from satellite sensors (Miller et al., 2006). Hence satellite monitoring of HABs is a novel method to detect undesirable (reduced biodiversity) water quality events, which may sometimes be related to eutrophication as described above. The remote sensing of chlorophyll concentration product has been successfully used to identify algal bloom events in the marine and coastal waters (Babin et al., 2008). However, the algal bloom of potentially harmful species could not be identified from analysis of chlorophyll concentration (Babin et al., 2008).

The method developed at Plymouth Marine Laboratory (PML), UK, uses measurements of water reflectance and inherent properties (IOPS) for automatic detection of HABs in satellite optical images (Kurekin et al., 2014). It is based on the relationships between water absorption properties and algal pigment composition, and between water backscatter and phytoplankton cell size, as features for HAB discrimination. The features were classified by Linear Discriminant Analysis (LDA) technique to produce HAB risk maps, as shown in Figure 5.

The method has been trained to discriminate *Karenia mikimotoi* and *Pseudo-nitzschia sp.* in the UK coastal waters, as well as *Phaeocistis globosa* algal blooms in the Southern North Sea. Measurements on board the RV Cefas Endeavor, provided by CEFAS, were integrated in the assessment of HAB risk. Joint analysis of satellite ocean color and Ferrybox data has been successfully applied for detection of a Karenia mikimotoi bloom off the North East of Scotland in August-September in 2013 and in 2014. The experiment has confirmed a strong correlation between satellite observations of HAB risk (Kurekin et al., 2014) with measurements of CTD profiles (including fluorescence and oxygen profiles) and in-situ samples (algal pigments, chlorophyll-a, cell count by microscopy and flow cytometry).

This method allows daily estimation of certain HABs over a wide area, depending on cloud cover. However, it is limited to phytoplankton species that produce high biomass blooms with a characteristic surface water coloring, whereas many toxin-producing algae are harmful in low concentrations. HAB risk maps are already operational for early warning of blooms affecting Scottish salmon farms, so it would be practical to extend the method toward further monitoring programs. The method is dependent upon the quality training data available for each HAB type, and so this aspect requires ongoing development.

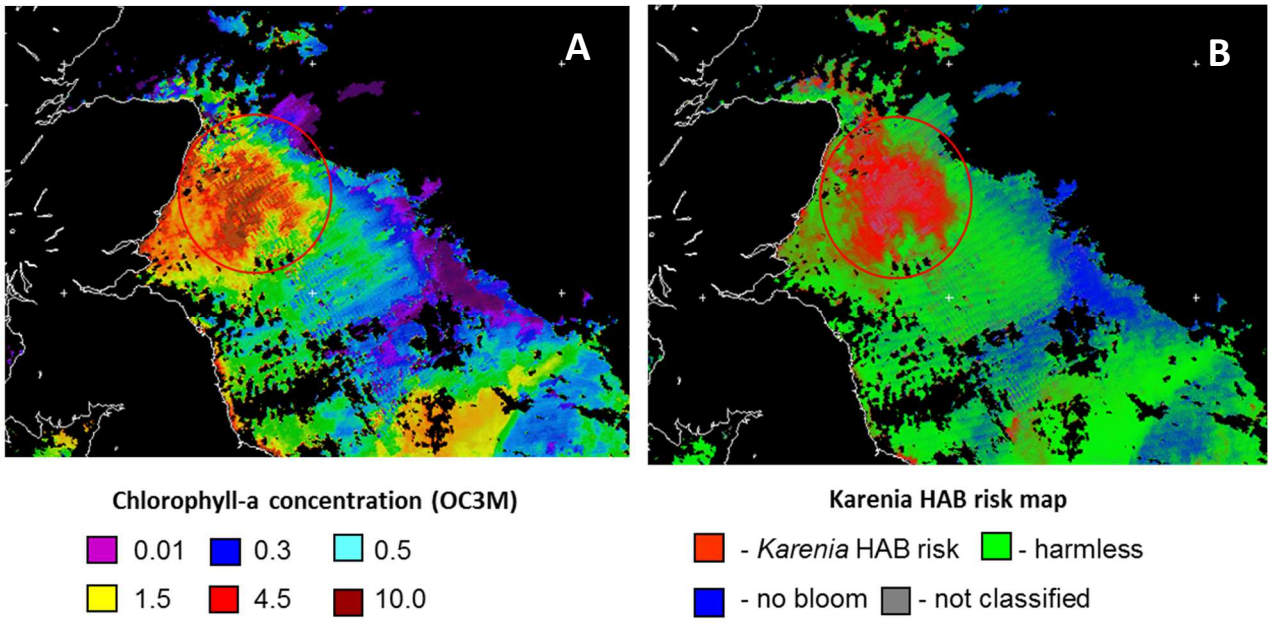

Figure 5. *Karenia mikimotoi* harmful algal bloom off the North East of Scotland in Sept. 2013 detected by MODIS AQUA sensor: (A) Chlorophyll-*a* concentration map, OC3M algorithm; (B) *Karenia* HAB risk map. High-risk areas are given in red, harmless areas - in green and no bloom areas – in blue (Source: Plymouth Marine Laboratory).

Remote sensing of shelf-sea fronts for estimating pelagic biodiversity

A novel approach to the mapping of pelagic diversity has been implemented for the UK continental shelf, using a long time-series of remotely-sensed SST data to automatically detect thermal ocean fronts and then aggregating observations into climatological seasonal metrics (Miller and Christodoulou, 2014). These metrics have characterized the spatial, seasonal and interannual variability of fronts observed in 30,000 satellite passes over a 10-year period. Many researchers have determined that fronts are related to the abundance and diversity of pelagic vertebrates such as seabirds and cetaceans (reviewed by Scales et al., 2014). The resulting front maps were successfully applied as a proxy of pelagic diversity to the UK Marine Conservation Zone (MCZ) project—a key element of efforts to improve environmental status of European seas, and this influenced the designation of 11 of the recommended MCZs (Miller and Christodoulou, 2014) (Figure 6).

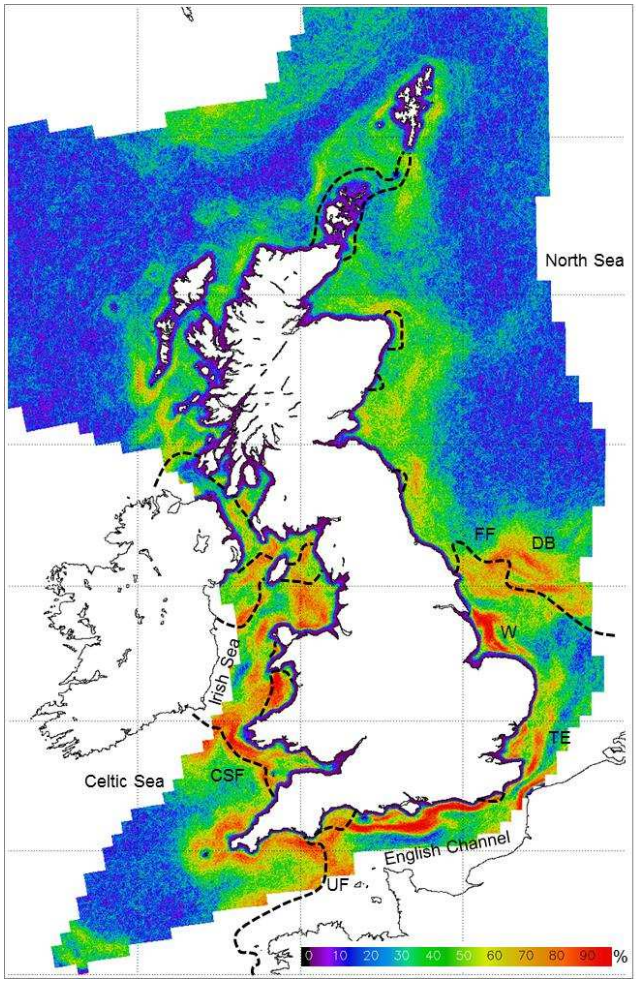

Figure 6. Summer frequent front map based on a 10-year time-series of satellite SST data, compared with fronts predicted by a numerical model based on tidal currents and bathymetry (dashed lines where Simpson-Hunter stratification parameter S=1.5). FF=Flamborough front, UF=Ushant front, CF=Celtic Sea front, DB=Dogger Bank, W=Wash, TE=Thames Estuary. (From Miller and Christodoulou, 2014, UKCS region, 1.2 km resolution, 1999-2008 data).

Although seasonal locations of frequent fronts were found to be fairly consistent, there are considerable interannual and week-to-week variations in the location and frequency of fronts, with consequential changes in the water column likely to affect species distributions. Hence satellite monitoring of shelf-sea fronts can serve as a proxy for certain mobile pelagic animals and as a physical boundary that structures other components such as zooplankton. Real-time front maps can be compared and integrated with other tools such as Ferrybox to assess aspects of the ecosystem and its biodiversity. Real-time satellite front maps have been applied to a UK project to optimize the MCZ/MSFD monitoring strategy using sea gliders and autonomous underwater vehicles across frontal biodiversity gradients (Suberg et al., 2014).

Hence the key benefits of this technique for marine monitoring are to assist the optimization of sampling strategies and to inform predictions of the abundance of fish and other pelagic animals that are difficult to measure directly.

Broadscale seabed mapping using opportunistic, high-resolution seafloor acoustic data

One of the core requirements of the MSFD is the use of habitat maps at the regional or sub-regional scale (Annex III, Table 1). In addition, there is an expectation that the assessment takes account of environmental conditions when deciding assessment boundaries [Article 3(2)] and this involves an understanding of predominant habitat types, including the structure and substrata composition of the seabed. The importance of knowing the changes in seabed conditions in detail are particularly relevant for the directives Habitats (D1), Seabed Integrity (D6), and changes to Hydrographical Conditions (D7) (Tables 1, 2). So whilst assessments must be reported on at the regional level the actual scale of assessment is on subdivisions of the subregions (European Commission, 2014). Determining the relevant scale for assessment is especially important when we consider that these must be aggregated and reported at a higher level, so that errors and uncertainties will propagate up from the minimum assessment areas (Dong et al., 2015). So whilst identifying the most appropriate assessment method for indicators is a challenge in itself (Berg et al., 2015), the spatial component fundamentally affects our ability to accurately assess ecosystem components.

For the benthic environment we are severely restricted as to the amount of existing data we have to define ecologically relevant areas. The failure of market-value to adequately represent the societal importance of the marine environment has been widely recognized (Brouwer et al., 2016) and the practical reality is that there is less short-term economic incentive to collect seabed information (compared to terrestrial remote sensing), as a result little of the European seabed has been mapped using modern methods. A direct consequence of such data deficiency is that 76% of seabed

habitats are in unknown status (EEA, 2015) and there are no systematic habitat mapping programmes in place at national or Pan-European scales.

In the absence of adequate seabed data, the urgent need to define seabed habitats for management has resulted in the construction of modelled seabed data such as UKSeaMap (Connor et al., 2006). These existing broadscale maps will inevitably contain errors due to data deficiencies and generalizations. However, the alternative of using the scattering of existing high-resolution maps, does not address our needs to define biogeographical limits of species or overall habitat distribution at a regional scale. To overcome this difficulty (of high resolution data only existing as a localized patchwork) and make best use of existing resources, the novel strategy of continuously logging high-resolution multibeam data during existing monitoring cruises has been adopted on the RV Cefas Endeavor using the Olex software programme. This allows non-hydrographers to automatically mosaic and navigate around the seafloor data in real time through a simple graphical interface. It is then possible to use the data operationally rather than waiting for it to be processed and made available in an accessible format. As there are no dedicated personnel required and the system has no adverse effect on existing operations, large amounts of high-resolution data are collected with negligible additional cost (continuous operation is not expected to reduce its serviceable life expectancy of sonar systems).

Integrating the high resolution bathymetry and backscatter data with existing broadscale environmental data (such as modelled currents and seabed morphology) using random-forest models (e.g., Hengl et al., 2015), it is then possible to create a complete coverage map of the seabed conditions (Figure 7). By using only acoustic data in our study the modelled variables produced (whilst not ground-truthed) are repeatable, provide outputs at a uniform resolution, and allow a consistent assessment of uncertainty to be made across the area (Mascaro et al., 2014). These properties are valuable when addressing questions of map interpretation (Steiniger and Weibel, 2005) and ecosystem status at regional scale (Walz and Syrbe, 2013; Galparsoro et al., 2015a). It is possible to use these data to produce categorical maps. However, there are concerns as to the validity of categorizing continuous environmental variables for habitat delimitation (Wilson et al., 1999; Orpin and Kostylev, 2006; Galparsoro et al., 2015b). Defining a fixed set of conditions which delimit the extent of a single species is conceptually problematic (Randin et al., 2006; Heads, 2015), and, as habitats are taxon and scale-specific (Mairota et al., 2015; Mathewson and Morrison, 2015), the use of existing, readily available, categorical GIS habitat maps for biotope assessments should not be considered as scientifically defensible.

Using the method outlined above to collect large quantities of high-resolution data over a broad extent, we can also directly map highly localized features and impacts, such as the direct mapping of species distribution and condition of biogenic reefs. In this way we have a direct relationship between sonar image and species distribution without the need to go through the process of inferring their distribution from correlations. Models can be used to identify areas where the feature is likely to be present and additional monitoring effort can be deployed as necessary, both to monitor condition, as well as to better define their extent (as required by the relevant indicators).

There is no practical hindrance to the collection of spatially-extensive, high-resolution data from a wide range of platforms already conducting regular monitoring activities. The challenge is in recognizing the benefits of such data in supporting the spatial assessment of multiple indicators, implementing the necessary routines and then incorporating the outputs into monitoring, assessment, and management strategies.

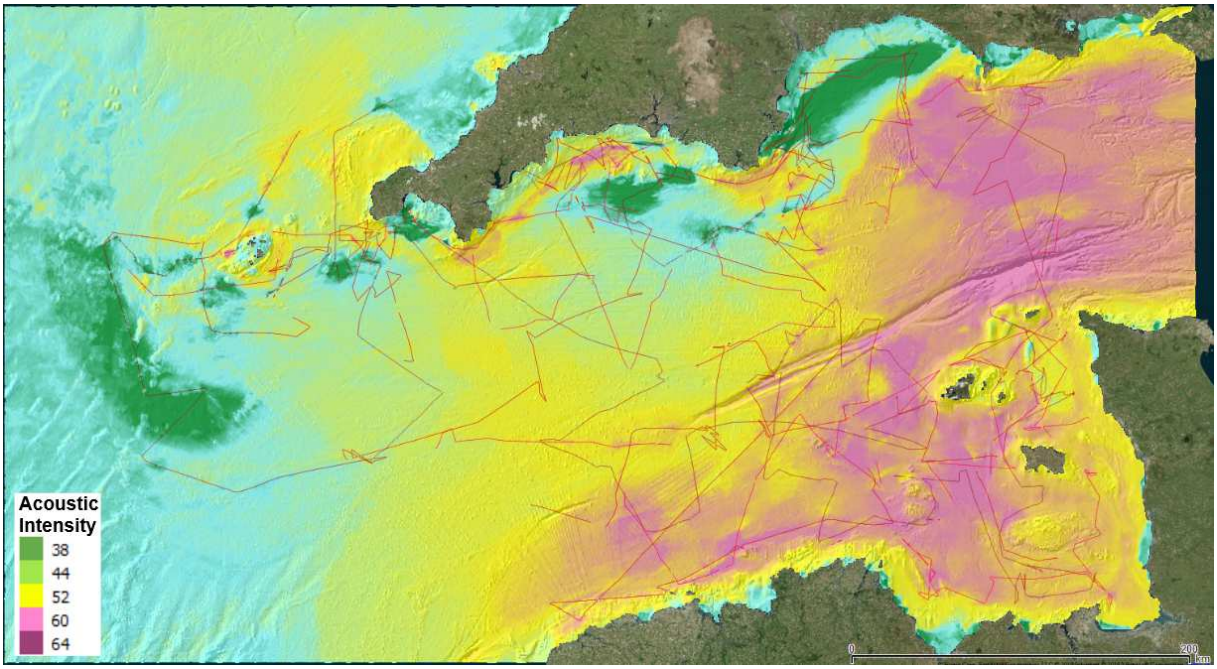

Figure 7. Random forest model of seabed acoustic intensity, extrapolated from high-resolution multibeam data collected opportunistically during fisheries research cruises (ships tracks as red lines) (Source: OceanDTM).

Innovative sampling methods

Here we summarized the experience made on innovative sampling methods, some of which have been applied for the first time in European seas. These include methods to monitor hard-bottom substrata, but also the use of citizen science to obtain massive information.

Artificial Structures to monitor hard-bottom benthic biodiversity

ARMS. Small invertebrates, including sessile and encrusting organisms as well as mobile specimens inhabiting ecological niches in hidden spaces, represent most of the benthic biodiversity in rocky areas. Despite its importance for ecosystem functioning, a considerable percentage of benthic biodiversity is untargeted during traditional surveys and thus likely to be unreported (Pearman et al., 2016a). In the current scenario of global change, caused by natural and anthropogenic pressures, species may be pushed to extinction even before their identities and roles in ecosystem functioning can be understood (Costello and Wilson, 2011).

To overcome the difficulty in obtaining standardized and comparable information on benthic biodiversity from different habitats and regions, the Coral Reef Ecosystem Division (CRED) of the United States National Oceanic and Atmospheric Administration (NOAA) developed a standardized biodiversity assessment tool called an "Autonomous Reef Monitoring Structure" (ARMS; Figure 8A). This device consists of nine 23 × 23 cm gray, Type I PVC plates stacked in an alternating series of layers that are either open to the current or obstructed, which are intended to mimic the three-dimensional structure of the reef environment. They should be deployed for 1–3 years and colonized by bacteria, algae and sessile and mobile fauna, including cryptic species, of different size ranges (meiofauna, 20–500 µm; macrofauna, >500 µm; large macrofauna, >2000 µm). After recovery, both sides of each plate are photographed, and then surfaces are scraped, homogenized and analyzed using barcoding and metabarcoding techniques. The ARMS processing protocol applies a combination of morphology (for organisms >2000 µm) and molecular-based (all components) identification approaches to assess species richness (Leray and Knowlton, 2015).

The use of a standard sampling unit and the application of homogeneous protocols for morphological and molecular identifications can produce comparable datasets over different geographical areas. Despite some limitations of the metabarcoding technique (Carugati et al., 2015; see metabarcoding section), such as the incompleteness of reference databases, the sequence inventory obtained is already valuable for biodiversity assessment that be further improved in the future without additional laboratory work by rerunning the bioinformatics analyses on updated reference databases. Over a deployment of 1–3 years, colonization and succession patterns could be

affected by changes in environmental conditions, making ARMS proper tools for marine monitoring of coastal areas. ARMS can be also re-deployed in the same locations and used to assess biodiversity changes over time. The characterization of the surrounding environment where ARMS units are deployed should be carried out for a comparison with natural assemblages. Temporal variability in key environmental variables, such as temperature, nutrients and chlorophyll a, should be investigated during the deployment period. Combining the use of ARMS with standard surveys, generally targeting fish and conspicuous invertebrates (Table 2), it is possible to obtain a comprehensive picture of the biodiversity and more accurate information on the health status of the system.

The use of ARMS for routine marine monitoring presents some problems that need to be addressed. Although the costs of sequencing are dropping, and even if the ARMS-based approach is more cost effective than morphological-based one (Hayes et al., 2005), overall costs may still be high. Moreover, protocols for the assessment of biodiversity associated to ARMS rely upon the use of molecular approaches and thus the use of such devices present the same problems described above for metabarcoding. The ARMS protocol of Leray and Knowlton (2015) proposed the use of the mt COI gene. However, the database for this gene is highly biased toward metazoans and may thus be limited in the detection of other groups (such as algae and unicellular eukaryotes). Other genes have been targeted for ecological studies (e.g., 18S rDNA, Logares et al., 2014a, 28S rDNA, Hirai et al., 2015, and the ITS region Tonge et al., 2014) and a combination of these genes and COI may give a more comprehensive assessment of diversity. In the future, molecular studies using ARMS may also investigate the functional ability of the assemblage using shotgun metagenomic techniques.

ASUs. Another example of standardized sampling devices for marine biodiversity assessment is represented by Artificial Substrate Units (ASUs; Figure 8B). ASUs are nylon pot scrubbers, which have been used to study recruitment and taxonomic composition for over 20 years (Menge et al., 1994, 2002, 2009; Gobin and Warwick, 2006; Underwood and Chapman, 2006; Hale et al., 2011). They are particularly used to mimic filamentous algae or kelp holdfasts (Menge et al., 1994), a preferred habitat for recruits of many species (e.g., mussels, Paine, 1974).

After their recovery, ASUs are traditionally processed to identify species by using their morphological characters (Menge et al., 2002; Underwood and Chapman, 2006; Hale et al., 2011). With the advent of metabarcoding, the diversity associated with ASUs has been assessed by combining morphological and molecular methods.

The advantages and disadvantages of ASUs are similar to those of the ARMS, which are detailed above. Comparing the two structures, ASUs are easier to deploy than ARMS and the materials needed to construct an ASU are less expensive than those used to build ARMS. Moreover,

the processing of an ASU takes fewer person-hours per unit (18 person-hours per ARMS vs. 6 per ASU). This makes ASUs more amenable to fine-scale sampling, for instance to measure temporal changes in biodiversity. They would be a valuable contribution to current monitoring programs, which require intensive samplings. The use of ASUs in monitoring programs can be relatively simple (e.g., Hale et al., 2011). Another consequence of simpler processing is that there are fewer risks of deviation from standardized procedures for ASUs than for ARMS during the processing of samples. However, they do not sample the same ecosystem component as the ARMS, since the two devices mimic different habitats. The small size of the ASUs relative to the ARMS imposes a selection for smaller organisms and species, such that large-bodied organisms cannot be collected by using the ASUs.

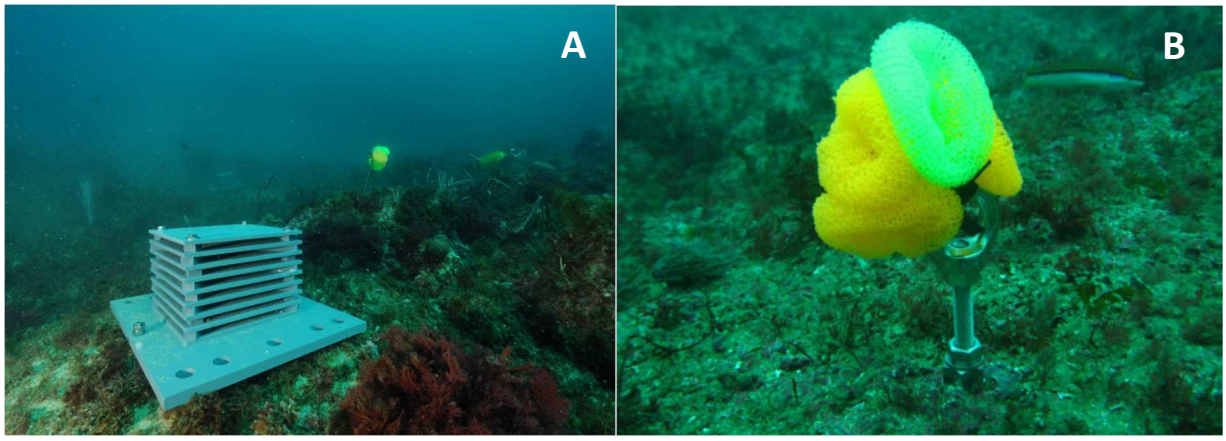

Figure 8. Standardized sampling devices to monitor hard-bottom benthic biodiversity. (A) Autonomous Reef Monitoring Structure (ARMS), which recreate the 3D structure of a natural reef environment. (B) Artificial Substrate Unit (ASU) developed to mimic the filamentous algae or kelp holdfasts.

High Resolution Sampling

Recent advances in robotic technologies provide new opportunities to conduct high-resolution sampling of patchily distributed organisms (such as zooplankton), by using AUV, carrying bottles for collecting discrete seawater samples and a sensor for gathering contextual environmental data. Environmental Sample Processors have been developed as stationary (moored) devices able to conduct in situ molecular assays (sandwich hybridization assay) by using 18S ribosomal RNA oligonucleotide probes, in order to detect actual plankton diversity (from calanoid and podoplean copepods, to larvae of barnacles, mussels, polychaete worms, brachyuran crabs, and invasive green crabs; Carcinus maenas; Harvey et al., 2012).

The Continuous Automated Litter and Plankton Sampler (CALPS) is a custom-made semiautomatic sampler which collects water using a pump system at a single depth along a

predetermined transect as the ship sails. The system consists of a pump system and additional elements fitted onto the research vessel. The additional elements include a water inlet of 20 cm diameter, a flowmeter, 6 cylinder traps and associated valves and level detectors to prevent overflowing and the system is controlled by computer (Figure 9). When activated, the system pumps sea water from a depth of 4 m at rates of between 35 and 45 L per minute, and distributes the water into one or more of the 6 possible traps. Each trap consists of a PVC cylinder (height: 73.3 cm, diameter: 28.0 cm) containing a plankton net (length 66.0 cm and diameter 26.5 cm) of chosen mesh-size. The volume of water filtered is measured with an electronic flowmeter. The performance of the CALPS against traditional vertical net sampling was evaluated in a study by Pitois et al. (2016). The authors concluded that the CALPS is suited to describe broad geographic patterns in zooplankton biodiversity and taxonomic composition; its particular advantage over more traditional vertical sampling methods is that it can be integrated within existing multidisciplinary surveys at little extra cost and without requiring additional survey time. These features make the CALPS a particularly useful tool as part of integrated monitoring of environmental status to underpin policy areas such as the MSFD.

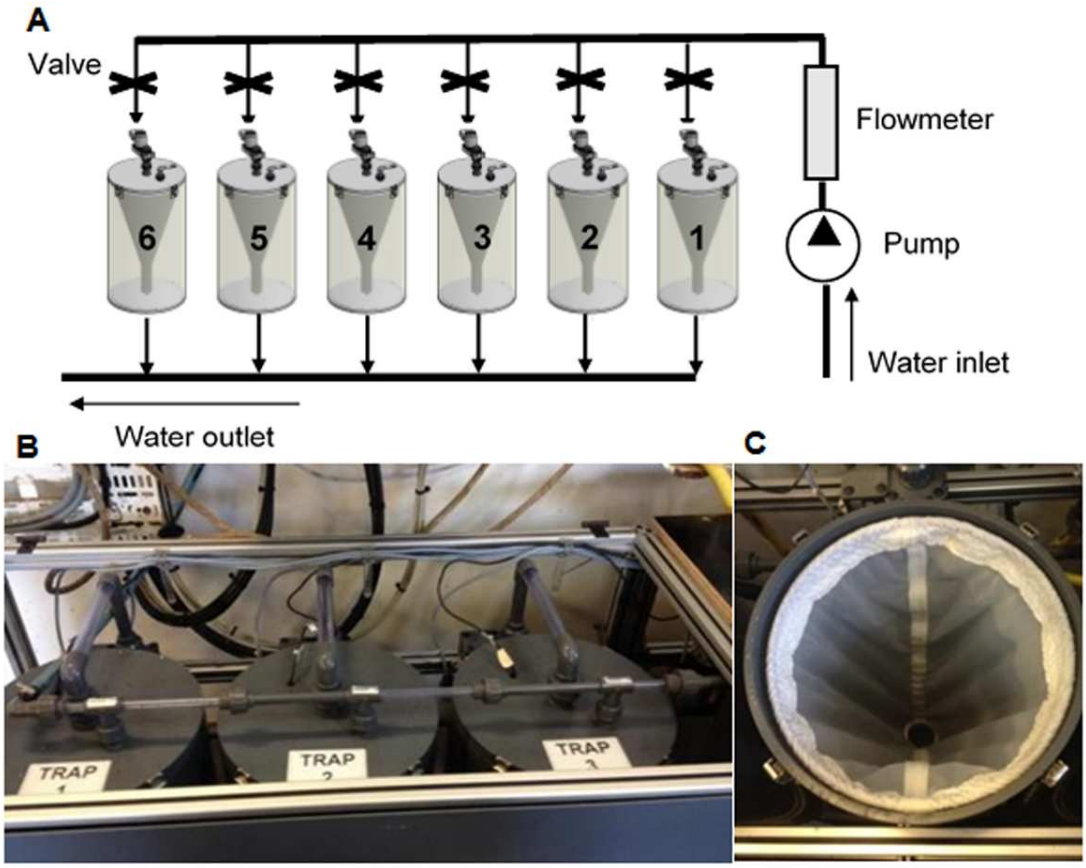

Figure 9. CALPS system. (A) Schematic illustration of the CALPS system. (B,C) are photographs of the Trap system and inside plankton net (Pitois et al., 2016).

Ocean Sampling Day

The Ocean Sampling Day (OSD) is a simultaneous sampling campaign of the world's coastal oceans which took place for the first time on the summer solstice (June 21st) in the year 2014 and was repeated in 2015 and 2016 (Kopf et al., 2015). In this way, the collected samples related in time, space and environmental parameters, will provide new insights regarding microbial diversity and function and contribute to the blue economy through the identification of novel, ocean-derived biotechnologies. Micro B3's OSD project aims to generate, in a single day and in a cost-effective way, the largest standardized marine microbial data set, complementary to what obtained by other large-scale sequencing projects. The standardized procedure including a centralized hub for laboratory work and data processing via the Micro B3 Information System, ensures the collection and the processing of sea water samples with a high level of interoperability and consistency between data points worldwide. All OSD data (i.e., sequences and contextual data) are archived and immediately made openly accessible without an embargo period (Ten Hoopen and Cochrane, 2014). OSD sampling sites are typically located in coastal regions within exclusive economic zones (EEZ) and thus the OSD data set provides a unique opportunity to test anthropogenic influences on microbial assemblages. The final aim is to create an OSD time-series indicators to assess environmental vulnerability and resilience of ecosystems and climatic impacts. In the long term such indicators may be incorporated into the Ocean Health Index (OHI) (Halpern et al., 2012), which currently does not include microorganisms due to the lack of reliable data. OSD has the potential to close that gap expanding oceanic monitoring toward microbes. This could lead to a global system of harmonized observations to inform scientists and policy-makers, but also to raise public awareness for the major, unseen component of world's oceans.

Conclusions

There is an urgent need to improve our knowledge of the spatio-temporal variations of marine biodiversity and of the consequences of global changes on marine ecosystems. This should be done quickly, in real time, using harmonized, standardized and low-cost tools (Borja and Elliott, 2013), and extending our ability to monitor the deep-sea ecosystems (Danovaro et al., 2014; Corinaldesi, 2015). Recently developed technologies and instruments should help to determine not only the biodiversity but also the functioning of ecosystems, feeding the needs of the recently enacted Marine Strategy Framework Directive (Cardoso et al., 2010).

Some of the innovative methodologies and technologies described here (e.g., AUVs, high-resolution sampling instruments) are tested and validated in different geographical areas and they can help to

achieve in real time information on different ecosystem components (from microbes to megafauna), rapidly and in a rigorous way, at a lower cost than traditional ones. Other tools, especially molecular ones, e.g., metabarcoding, need further evaluation (Bourlat et al., 2013).

In this context, such innovative approaches for marine monitoring need to be further implemented through: (i) defining standardized manuals and protocols for sampling and sample processing; (ii) developing new indicator metrics and indices fitting the new approaches and also useful for policy and decision-making; (iii) integrating, in monitoring surveys, information on biodiversity with other data sources (CTD, remote sensing, multibeam, taxonomy databases) for an holistic marine ecosystem assessment.

Authors contributions

RD and LC conceived the paper. All authors have contributed equally to the Introduction. RDan, LC, MB, SCar, AC, CC, AD, EG, JG, JF, IF, JP, AR, NR, and AB contributed to the section of molecular approaches. RDan, LC, ND, VM, SM, KS, ER, SCon, SG, SS contributed to the section of in situ instruments. RDan, LC, SCri, RDav, PG, RF, AK, PM, AN, EG, JG, IF, AR, CW, VS, OM contributed to the section of remote sensing. RDan, LC, SCar, JP, ML, AEC, SP, SG, SC, and AB contributed to the section of innovative sampling methods. All authors have contributed equally to the discussion and conclusions. All authors reviewed the manuscript.

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2.2 Metagenetic tools for the census of marine meiofaunal biodiversity: An overview

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Abstract

Marine organisms belonging to meiofauna (size range: 20-500 μm) are amongst the most abundant and highly diversified metazoans on Earth including 22 over 35 known animal Phyla and accounting for more than 2/3 of the abundance of metazoan organisms. In any marine system, meiofauna play a key role in the functioning of the food webs and sustain important ecological processes. Estimates of meiofaunal biodiversity have been so far almost exclusively based on morphological analyses, but the very small size of these organisms and, in some cases, the insufficient morphological distinctive features limit considerably the census of the biodiversity of this component. Molecular approaches recently applied also to small invertebrates (including meiofauna) can offer a new momentum for the census of meiofaunal biodiversity. Here, we provide an overview on the application of metagenetic approaches based on the use of next generation sequencing platforms to study meiofaunal biodiversity, with a special focus on marine nematodes. Our overview shows that, although such approaches can represent a useful tool for the census of meiofaunal biodiversity, there are still different shortcomings and pitfalls that prevent their extensive use without the support of the classical taxonomic identification. Future investigations are needed to address these problems and to provide a good match between the contrasting findings emerging from classical taxonomic and molecular/bioinformatic tools.

Keywords: meiofauna, biodiversity, metagenetic, high-throughput sequencing, 18S rRNA gene

Introduction

Meiofauna in marine ecosystems

The importance of estimating species richness in both terrestrial and marine environments is still a priority in several research fields, from ecology to evolutionary and conservation biology (Gaston, 2009). Spatial patterns of biodiversity have been primarily focused on large (macroscopic) organisms (Gaston, 2000; Tittensor et al., 2010) rather than on small-sized organisms, which include the majority of animal phyla represented in the poly-phyletic group of meiofauna (Lambshead and Boucher, 2003; Giere, 2009). Meiofauna are defined on the basis of body size as organisms passing through 500 μm mesh net and retained by a 20 μm mesh net (Giere, 2009). Metazoan meiofauna are widely distributed in all benthic habitats/ecosystems of the world oceans, and include also a variety of parasitic forms. Meiofaunal organisms represent the numerically dominant component among benthic metazoans in all marine systems, from intertidal beaches down to the ocean floor, and colonize all substrates from muds to the coarsest shell gravels and rocks (Danovaro and Fraschetti, 2002; Giere, 2009). Meiofaunal assemblages are dominated by nematodes, which in benthic deep-sea ecosystems (the largest biome of the biosphere) represent more than 90% of the total meiofaunal abundance (Lambshead and Boucher, 2003; Lambshead, 2004). Meiofauna are characterized by high abundances (up 10^6 individuals per m^2) and high diversity either at the level of higher taxa and at the genus/species level (Giere, 2009; Curini-Galletti et al., 2012). Meiofaunal organisms show a high sensitivity to environmental changes and are increasingly used also in monitoring studies for the assessment of environmental quality (Coull and Chandler, 1992; Moreno et al., 2011; Pusceddu et al., 2011). They are also important as a functional link between macrofauna and microbial assemblages and are thus considered a suitable model for the study of species distribution and biodiversity patterns in marine environments (Snelgrove, 1999; Danovaro et al., 2001, 2008).

Classical taxonomic vs molecular approaches

A major bottleneck in meiofaunal taxonomy is related to the analysis of distinctive morphological characters by using light microscopy. Among meiofaunal organisms, marine free-living nematodes are expected to have a high species richness, whose estimate ranges from ca. 61,000 species to >100,000 species (based on expert evaluation), but so far only 11,400 species (including either parasitic and free-living forms) have been fully described and formally taxonomically accepted (Appeltans et al., 2012). Possibly more than 80% of marine free-living nematode species remain to be discovered and characterised (Appeltans et al., 2012; Table 1).

Table 1. Estimates of known and unknown marine meiofaunal species. The data reported are: number of currently described and taxonomically accepted species, total species unknown (undescribed + undiscovered based on expert opinions), total estimated number of species based on expert-opinion, estimated percent of all existing species that are currently described (% known). Data from Appeltans et al., 2012.

	Described (Accepted)	**Total Unknown (Experts)**	**Total Estimated**	**% Known**
Gastropoda	32,000-40000	85,000-105,000	no data	23-32
Polychaeta	12,632	6,320	no data	67
Platyhelminthes	11,690	23,606- 61,751	35,296-73,441	16-33
Copepoda	10,000	30,125-50,125	no data	17-25
Ostracoda	8,853	2,625-34,000	no data	21-77
Amphipoda	6,947	20,000	no data	26
Nematoda (free-living)	6,900	50,000	no data	12
Isopoda	6,345	63,400-123,400	no data	5-9
Foraminifera	6,000	1,500	no data	80
Hydrozoa	3,426	1,550-4,100	4,976-7,526	46-69
Cumacea	1,444	6,045	no data	19
Nemertea	1,285	700-1,400	1,985-2,685	48-65
Acarina	1,218	1,470-2,130	no data	36-45
Tanaidacea	1,130	23,500-57,400	no data	2-5
Oligochaeta	910	5,900-16,900	no data	5-13
Gastrotricha	434	1,810-2,810	2,244-3,244	13-19
Kinorhyncha	228	1,250-2,350	no data	9-15
Entoprocta	193	1,030	1,223	16
Tardigrada	183	1,120	1,303	14
Sipuncula	150	43-230	193-380	39-78
Rotifera	114	320-2,520	434-2,634	4-26
Loricifera	32	1123	no data	3
Priapulida	19	no data	no data	no data

Meiofaunal diversity is so large that the analysis of a single Phylum, such as Nematoda, requires huge investments of time of highly specialized personnel. As an example, the morphological identification of 10% of nematodes encountered in a sample requires an effort 120 times higher than that requested to successfully identify all vertebrate morphospecies in tropical forests (Lawton et al., 1998). However, the analysis of meiofaunal diversity is not just time-consuming and laborious, but most importantly does not allow the identification of closely related (similar) species (**Box 1;** Derycke et al., 2005, 2008a; Bhadury et al., 2008; Fontaneto et al., 2009; Creer et al., 2010). During the last years, DNA-based approaches for species identification based on distances (e.g., DNA barcoding and Automatic barcode gap discovery), branching rates (e.g., K/θ, the generalized mixed Yule–coalescent model and Poisson tree process model) and on heterozygosis (haplowebs) have been widely applied to several meiofaunal groups (including rotifers, copepods, gastrotrichs, ostracods, molluscs, nemerteans; Fontaneto et al., 2015). However, the use of these approaches to delineate species boundaries has never been exhaustively performed on nematodes.

In the last decade it has been proposed to use Sanger chain-termination sequencing to identify marine nematodes (Rogers and Lambshead, 2004; De Ley et al., 2005; Bhadury et al., 2006). This because Sanger sequencing of nuclear and mitochondrial genes can allow us also to study the cryptic diversity within marine nematode morphospecies (Derycke et al., 2007; Meldal et al., 2007; Bhadury et al., 2008). Recent genetic surveys (Derycke et al., 2005, 2008a,b; Fonseca et al., 2008) revealed a significant population genetic structure of species, which so far were considered single morphospecies (e.g., the Rhabditis, *Pellioditis marina* and the *Halomonhystera disjuncta* species complex; Derycke et al., 2005, 2007, 2008b). At the same time, the morphological identification remains a crucial step prior to molecular analysis, in particular when new barcodes have to be produced (Derycke et al., 2010a).

Given the high abundance and diversity of meiofauna, standard barcoding (based on Sanger sequencing) is not an ideal tool for investigating meiofaunal biodiversity at large spatial scales. At the beginning of 2000s, the advent of high-throughput sequencing platforms, capable of producing hundreds of thousands or even millions of sequences per run, led to a revolution in the field of ecology. Indeed, the advent of *en mass* molecular identification trough next generation sequencing (NGS) platforms may significantly enhance our ability to assess meiofaunal biodiversity (Creer et al., 2010; Fonseca et al., 2010; Pawlowski et al., 2011; Porazinska et al. 2010, 2012; Fonseca et al., 2014). "Metagenetics" or "Metabarcoding" refer to large-scale analyses of biodiversity through the amplification and sequencing of homologous genes (Creer et al., 2010).

Here we provide an overview of metagenetic workflow used to study marine meiofaunal biodiversity (Fig. 1), describing the different methodological steps required, the outputs provided by high-throughput sequencing analyses and highlighting pitfalls associated with these molecular approaches. In particular, a special focus is addressed to the application of metabarcoding to marine nematodes to assess their biodiversity from shallow-water to deep-sea ecosystems.

Box 1. The problem of cryptic species

Although marine nematodes have low dispersal abilities, studies performed so far demonstrated that most of the species are cosmopolitan, being characterized by widespread distribution (Decraemer et al., 2001; Lambshead and Boucher, 2003). This concept is known as "meiofauna-paradox", but relies exclusively on a classically oriented concept of species coming from morphological identification, which unlikely allows us to identify genetically closely related species (Ristau et al., 2013). Therefore, it has been supposed that "meiofauna-paradox" could be explained by the presence of cryptic species, organisms that are morphologically similar, but belong to genetically distinct species. Such cryptic diversity occurs in a variety of metazoan taxa and biogeographical regions (Pfenninger and Schwenk, 2007) and seems to be particularly relevant in the marine environment (Knowlton, 2000). Morphological similarity can be the result of strong divergent selection on non-visual mating signals (Bickford et al., 2007) or, alternatively, of ecological constraints, where adaptive evolution favours similar phenotypes (Wellborn and Broughton, 2008). Whatever the speciation process, the knowledge of cryptic diversity is crucial to better understand biogeographical and ecological patterns of marine organisms (Bickford et al., 2007). Indeed, the occurrence of cryptic species can transform what was thought to be generalist species into several specialist species with more restricted distributions (Giere, 1993; Vanelslander et al., 2009). The presence of cryptic diversity has been reported in nematodes belonging to different orders from freshwater habitats (Ristau et al., 2013) to marine ecosystems (Derycke et al., 2005, 2007, 2008a, 2010a). In the latter, cryptic diversity appears in individuals with different life histories and from different areas, suggesting that it could be a common phenomenon, not correlated with life history traits. Since the number of cryptic species among meiofaunal organisms is still largely unknown, the application of Sanger chain-termination sequencing can make light on biodiversity distributional patterns of marine meiofaunal species.

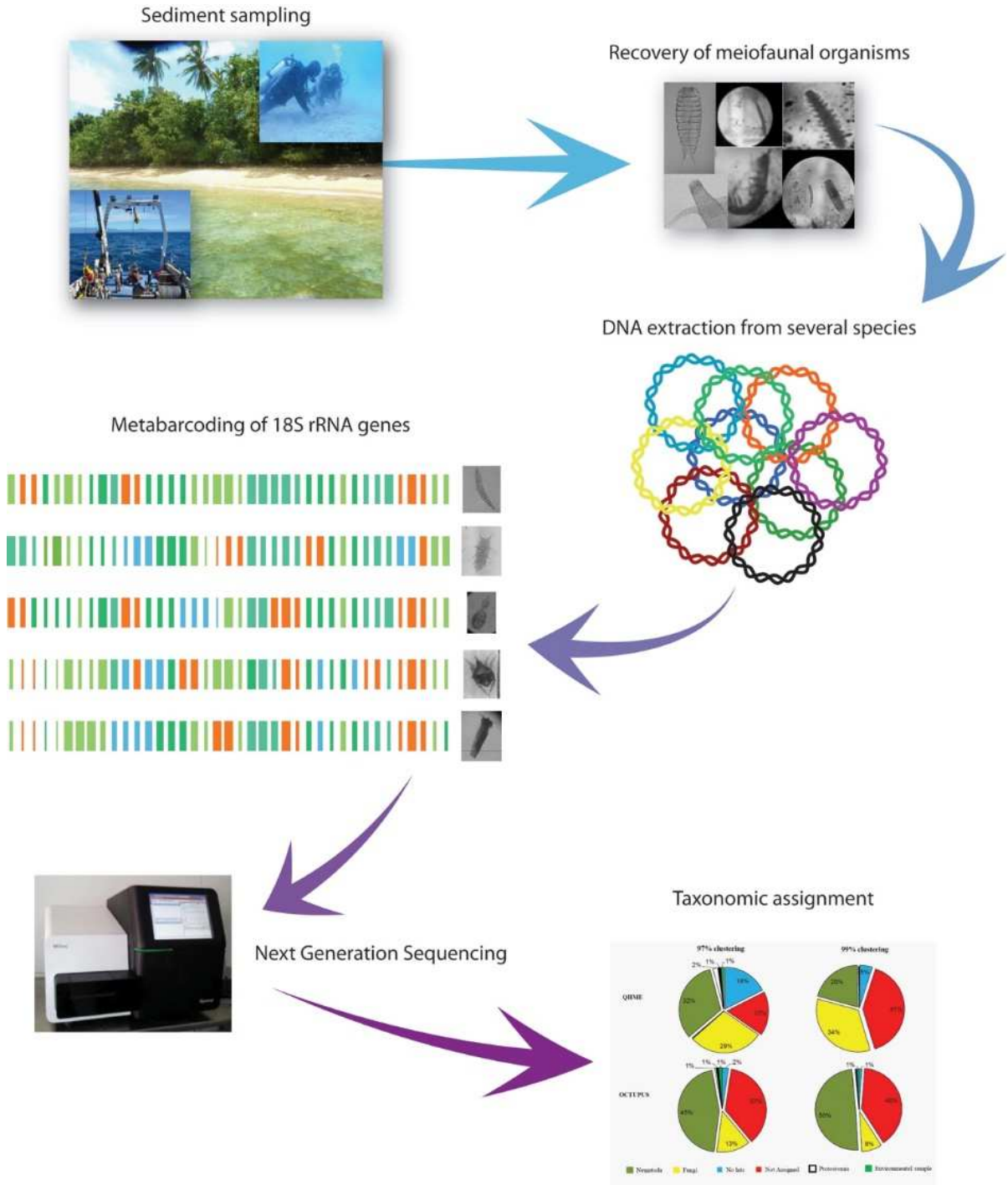

Figure 1. Standardized workflow to study meiofaunal biodiversity in marine benthic ecosystems using high-throughput sequencing. Sediment samples (from shallow to deep-sea environments) are collected and subsequently frozen (–20°C or –80 °C). In the laboratory, meiofaunal organisms are recovered from the sediments and their DNA extracted and purified. Following the PCR amplification of marker genes (e.g. 18S rRNA), high-throughput sequencing can be conducted on Roche 454 or Illumina platforms. Raw reads are processed and then clustered into operational taxonomic units (OTUs) under a range of pairwise identity cutoffs. After the BLAST-match of the obtained OTUs against public nucleotide databases, analysis of α- and β- diversity and phylogeography are performed. Image of Illumina MiSeq platform: Source: Wikipedia, Author: Konrad Förstner.

Methodological steps for assessing meiofaunal biodiversity through molecular-based approaches

Sample preservation

After collection, sediment samples should be adequately preserved to allow a proper morphological identification of the meiofaunal organisms (i.e. by preserving the morphological characteristics) and, at the same time, the recovery of DNA suitable for molecular analysis.

Sediment samples used for meiofaunal analysis by classical taxonomic approach are commonly preserved using 4% buffered formalin solution, which keeps the morphological structures of animals intact (Heip et al., 1985). However, formalin (i.e., formaldehyde) promotes the formation of DNA-formaldehyde complexes, cross-links between DNA and proteins and DNA fragmentation (Gagna et al., 1997; Serth et al., 2000), thus limiting the application of molecular analyses. Conversely, cryo- and ethanol preservation have proven to be more appropriate procedures for molecular studies (e.g., Seutin et al., 1991; Reiss et al., 1995; Liu et al., 2001). However, cryopreservation is not easy to manage when transportation is needed, whereas ethanol may dehydrate tissues and cause significant damage of anatomical features of animals, hampering proper morphological identification (Castro and Thomason, 1973). Recently, Fonseca and Fehlauer-Ale (2012) demonstrated that a solution of dimethyl sulfoxide (DMSO), EDTA, and NaCl salts, known as DESS (Yoder et al., 2006), can be more effective for preserving both morphological characteristics and DNA integrity in small invertebrates, including meiofaunal specimens. DESS, indeed, inactivates nucleases by a combination of osmotic shocks, followed by rapid transportation of disodium EDTA and NaCl into tissues facilitated by DMSO (Creer et al., 2010; Fonseca et al., 2010; Fonseca et al., 2014).

Besides formalin, Rose Bengal (a protein stain) is also used to facilitate the sorting of animals from the sediment grains (Heip et al., 1985). However, in case of molecular analyses, Rose Bengal should be avoided as it inhibits the polymerization of DNA molecules during polymerase chain reaction (PCR) analyses, thus hampering DNA amplification and sequencing (Srivastava and Modak, 1983). Even though a plethora of stains could be used, present knowledge on the impact of these stains on PCR is still limited and from a conservative point of view it would be recommended to avoid any use of stains (Fonseca and Fehlauer-Ale, 2012).

DNA extraction

The recovery of DNA from meiofaunal organisms suitable for PCR amplification represents the crucial step for the assessment of their biodiversity based on molecular approaches.

Two different approaches can be used to recover DNA from meiofauna (Fontaneto et al., 2015). One approach is based on DNA extraction from meiofaunal organisms, previously separated from sediment through a 20-30 μm mesh net. The filtration step is usually followed by gradient centrifugation using Ludox (arranged to a final density of ca 1.18 cm^{-3}; Heip et al., 1985). Alternatively, DNA can be extracted from sediment samples without sorting animals (Creer et al., 2010; Fonseca et al., 2010, 2014). The first approach allows us to link morphological information to each individual in a sample. However, it can be time consuming when meiofaunal abundance is very high and it could determine an under-representation of soft-bodied organisms, which can be damaged during meiofaunal extraction. The second approach is more powerful to efficiently detect rare species and to study their distribution (Zhan and MacIsaac, 2015). However, when meiofaunal abundance is low, a high amount of sediment has to be processed, thus increasing the costs. An additional problem is related to the presence of extracellular DNA in the sediment that can lead to an overestimation of the actual meiofaunal biodiversity (Corinaldesi et al., 2008, 2011). Since different DNA extraction approaches can result in different estimates of biodiversity, this issue should be taken into account when results obtained from different DNA extraction procedures are compared (Deiner et al., 2015).

Previous molecular investigations carried out on marine nematodes have used different DNA extraction procedures (Floyd et al., 2002; De Ley et al., 2005; Derycke et al., 2005; Bhadury et al., 2006, 2007, 2008, 2011; Bik et al., 2010; Creer et al., 2010; Fonseca et al., 2008, 2010, 2014). Some approaches were based on the use of an alkaline solution (NaOH) and freezing-thawing steps (Floyd et al., 2002) and modifications (Bhadury et al., 2006, 2011); others were based on the use of lysis buffer containing proteinase K followed (Bhadury et al., 2007, 2008) or not (De Ley et al., 2005; Derycke et al., 2005; Fonseca et al., 2008) by DNA purification with commercial kits. So far, the performance of these extraction procedures has never been compared, and this hampers the identification of the most suitable protocol for DNA extraction to be used for the analysis of meiofaunal biodiversity using molecular-based approaches. Indeed, the PCR amplification efficiency relies from one side on the amount of DNA template and from the other from its purity (i.e. lack of inhibitors for polymerase reactions). Thus, DNA extraction procedures have to be optimised in order to obtain a sufficient amount of DNA and to remove compounds (e.g., humic substances and/or metabolites) potentially inhibiting PCR and sequencing reactions (Creer et al., 2010 and references cited therein).

Choice of genomic loci

The choice of genomic loci is closely dependent on the objective of the study. For the study of biodiversity at community level through metabarcoding, genetic markers should have at least two important characteristics. First, they should mutate at just the right rate so that closely related species sequences differ for a few characters (typically ≥ 2%), but sequences from members of the same species should present differences <2%. Secondly, in order to successfully amplify the targeted locus, the flanking regions of barcode sequence should display a limited variation, so that it is easier to design a universal primer set. The mitochondrial gene encoding for the cytochrome oxidase c subunit 1 (COI), having a haploid mode of inheritance, is one of the preferred candidate locus for "universal" barcoding (Lorenz et al., 2005). Moreover, it shows high rates of molecular evolution, no introns and limited recombination rate (Wilson et al., 1985; Avise, 1994; Piganeau et al., 2004). The molecular evolution of COI usually facilitates the species discrimination and in the meantime allows us to reconstruct phylogenetic relationships, gene-flow patterns and to recognize the presence of cryptic diversity (Hebert et al., 2003). Unfortunately, nematode mitochondrial genomes are characterized by high levels of recombination (Lunt and Hyman, 1997), editing by insertion (Vanfleteren and Vierstraete, 1999) and multi-partitioning (Armstrong et al., 2000). For all these reasons, the amplification of the Folmer region (M1-M6 partition of the COI gene; Folmer et al., 1994), the most-used for animal barcoding studies, is difficult and unreliable in marine nematodes (Bhadury et al., 2006). A recent study developed a modified primer set (JB3-JB5), which amplifies satisfactorily another COI partition, (e.g., I3-M11 partition) in species belonging to the family *Rhabditidae* and *Leptosomatidae* (Derycke et al., 2010b). However, amplification is still difficult for species belonging to other nematode families (e.g., *Monhysteridae*) and hence, it remains unclear to what extent this region of COI can be used as a more general DNA barcoding fragment for marine nematodes (Derycke et al., 2010b). On the other hand, nuclear genes present lower mutation rate and a four-fold larger effective size than mitochondrial ones, and consequently evolve more slowly (Avise, 2000). Considering the lack of universal COI priming sites for marine nematodes and the dominance of this taxon within meiofaunal communities, it has been suggested that nuclear genes, both 18S small subunit (nSSU) and 28S large subunit (nLSU) rRNA genes could be more efficient markers to study meiofaunal biodiversity in metagenetic surveys (Blaxter, 2003; Blaxter et al., 2003). 18S and 28S rRNA genes are strongly conserved due to their role in the assemblage of proteins in the ribosome, facilitating the design of conserved primers (Floyd et al., 2005; Markmann and Tautz 2005; Carvalho et al., 2010). In particular, 18S rRNA gene seems to be more appropriate to study meiofaunal diversity than 28S rRNA gene due to its greater abundance in the genome and larger size. The PCR

amplification of 18S rRNA gene with universal primer set is more consistent, and public databases notably contain more 18S than 28S sequences (e.g., GenBank database contains 904,189 18S entries vs. 568,268 28S entries; 17,189 18S entries vs. 11,963 28S entries for nematodes). The SSU rRNA gene is variable enough to permit the differentiation between families or genera and in just a few cases, between species (Holterman et al., 2006; Fontanilla and Wade, 2008). Considering that some meiofaunal species have identical 18S sequences, this genetic marker remains of limited utility to detect patterns at the species level, to distinguish between closely related species or to discriminate cryptic species (Tang et al., 2012; Morgan et al., 2014). Tang et al. (2012) reported that the estimated number of taxa using 18S rDNA is lower than the number obtained by using COI and morphological identification. The authors concluded that the use of 18S rDNA is not a reliable marker for the assessment of meiofaunal diversity at the species level and it can underestimate the actual species richness (Tang et al., 2012).

A more variable portion is represented by D2-D3 "diversity loop" region of 28S rRNA. Due to conserved regions alternating with D2 and D3, primer set has a high success rate when applied to the entire phylum of nematodes (De Ley et al., 2005; Ye et al., 2007; Subbotin et al., 2008; Derycke et al., 2008a, 2010a). Moreover, since D2-D3 loop is not known to be subjected to intraspecific polymorphisms, it also allows us to identify cryptic species in some groups (De Ley et al., 1999). These results suggest that there is a need of identifying genes (or a combination of genetic markers) suitable for metagenetic analyses of meiofaunal biodiversity, that are also able to uncover the possible cryptic diversity.

Amplification and sequencing

At present, the NGS platform most frequently utilised for metagenetic studies on meiofauna is the Roche 454 (**Box 2**). The portion of 18S rDNA to be sequenced is amplified using the primer set SSU_F04 and SSU_R22 towards the 5' end (Blaxter et al., 1998; Creer et al., 2010; Fonseca et al., 2010, 2014; Bik et al., 2011). The selection of such set of primers is based on a combination of factors, namely: i) maximum length of amplicon recommended for Roche 454 (ca. 600 bp), ii) the best resolving power of the target regions and iii) the level of primer sequence conservation across meiofaunal organisms (Porazinska et al., 2009a; Creer et al., 2010). At the same time, Roche 454 is still expensive due to the high cost of reagents and has an error rate of ca. 1% per base within each single read (Glenn et al., 2011). Moreover, the error rate is more frequent in stretches of identical nucleotides (homopolymers; Huse et al., 2007). Specific and time-consuming software packages are

needed to remove these systematic errors prior to further analysis (Huse et al., 2010; Reeder and Knight, 2010; Quince et al., 2011).

Box 2. NGS platforms

Roche 454 was the first commercial NGS platform successfully used in metagenetic surveys of metazoan biodiversity (Fonseca et al., 2010; Creer et al., 2010). Roche 454 uses beads that start with a single template molecule, which is amplified via emulsion PCR (emPCR). In the early 2000s, when Roche 454 has been launched, the sequencer was able to produce 20,0000 reads with a maximum read length of 100–150 bp, and could output 20 Mb per run. An important development was made with the launching of 454 GS FLX Titanium systems, such as the new GS FLX Titanium XL+, which currently could generate 1 million sequences/run with read length of 700 bp, an output of 700Mb, within 24 hours. Compared with other NGS platforms, Roche 454 presents many advantages such as the speed and the higher read length, but the cost of reagents is still a challenge. However, the library construction can be automated, and the emPCR can be semiautomated which could reduce the manpower in a great extent.

The 2nd commercial NGS platform was developed by Solexa, subsequently acquired by Illumina, which uses a solid glass surface (similar to a microscope slide) to capture individual molecules and bridge PCR to amplify DNA into small clusters of identical molecules. In early 2010, Illumina launched Illumina HiSeq 2000. Basically, compared with Roche 454, llumina HiSeq 2000 has the advantages of biggest output at lower cost. The new Illumina HiSeq 4000 recently launched, could generate 2.5 billion reads/Flow Cell, with a maximum read length of 2x150 bp and an output of 1,5 T/run in less than 5 days. In 2011, Illumina developed Illumina MiSeq, which shared most technologies with HiSeq and it is especially convenient for amplicon and bacterial sample sequencing. At the time of writing, Illumina MiSeq system can sequence 2x300 bp amplicon, generating an output up to 15 Gb in 55 hours. Library preparation and their concentration measurement can both be automated.

SOLiD was the 3rd commercial NGS platform, using emPCR to amplify templates (as Roche 454). Until the most recent release of Illumina's software and reagents, SOLiD produces more reads, at lower cost than Illumina. Another two NGS platforms recently launched are i) Ion Torrent which uses a sequencing strategy similar to Roche 454, and ii) PacBio which is an instrument able to sequence individual DNA molecules in real time,

using individual DNA polymerases which are attached to the surface of microscope slides. Ion Torrent is suitable for microbial sequencing and targeted sequencing, but present higher cost per Mb, and longer sample preparation time compared to other NextGen platforms. PacBio produces a lower number of reads/run but with longer read length (till 1100 bp), at lower cost per sample and faster run times (less than 2 hours) than other NGS systems.

All NGS systems developed so far have traditionally split their focus into long reads (e.g., 454) vs. short reads (e.g., Illumina and SOLiD). However, considering the recent developments, the choice of the system that should be used for biodiversity surveys is strictly linked to the aim of the research, knowledge of the systems and to the researcher's ability to adapt techniques to obtain data efficiently.

Another NGS sequencing platform similar to Roche 454 in term of sequencing strategy is Ion Torrent, which has been recently used to study the diversity of marine eukaryotic organisms (Leray and Knowlton, 2015). However, it has a high cost per megabase and displays an error rate similar to Roche 454 (1%, Glenn et al., 2011).

For all of these reasons, Illumina sequencing platforms (**Box 2**) have been increasingly used due to the lower costs and lower per-base error rate (ca. 0.1% per base within single reads, Glenn et al., 2011). Moreover, Illumina is not so susceptible to indel errors in homopolymer stretches (Loman et al., 2012). Illumina sequencing platforms also improve sequencing protocols and library, increasing significantly the quality of the results (Caporaso et al., 2012). For instance, the length and quality of Illumina sequenced amplicons can be further improved by a process known as "read merging", aligning and combining each set of paired end reads into a single contig. In the past, the main constraint of Illumina was represented by the limited length of sequences generated (30–100 bp), but at present this problem has been overcame by the Illumina MiSeq platform, which generates reads with a length up to ca. 600 bp. These implementations allow the sequencing of amplicons of length similar to those produced by Roche 454; this in combination with the lower cost per sequence has oriented several scientists to prefer Illumina for sequencing 16S rRNA gene amplicons (Gloor et al., 2010; Bartram et al., 2011; Degnan and Ochman, 2011; Ram et al., 2011; Caporaso et al., 2012; Eren et al., 2013; Nelson et al., 2014). This leads us to hypothesise that soon Illumina MiSeq will replace Roche 454 also for 18S rRNA gene sequencing.

Emerging insights into meiofaunal biodiversity from metagenetic analysis

High-throughput sequencing technologies, producing large amounts of sequence data in a very short time, provide an unique opportunity to identify different species from complex communities at lower costs than both Sanger sequencing analyses (e.g. Leininger et al., 2006; Poinar et al., 2006; Sogin et al., 2006) and morphological identification procedures. High-throughput sequencing approach has been traditionally applied to studies dealing with microbial diversity (Angly et al., 2006; Sogin et al., 2006; Huber et al., 2007; Desnues et al., 2008; Zinger et al., 2011). However, an increasing number of NGS studies (based on the 18S rRNA gene) investigated the diversity of protists and small metazoans (Porazinska et al., 2009a, 2010, 2012; Stoeck et al., 2009, 2010; Creer et al., 2010; Fonseca et al., 2010, 2014; Medinger et al., 2010; Bik et al., 2011; Edgcomb et al., 2011; Lindeque et al., 2013; Pearman et al., 2014; Hirai et al., 2015). Although metabarcoding is an emerging field, recent metagenetic surveys of marine metazoan biodiversity have already provided new insights into taxonomic composition and spatial diversity patterns of eukaryotic communities from different marine environments.

Taxonomic composition and species richness of meiofaunal assemblages

Recent investigations of shallow and deep-sea meiofauna based on the use of 454 sequencing and classical morphological identification provided different results (Fonseca et al., 2010; Bik et al., 2011). On the basis of morphological analyses, nematodes, for instance, have been repeatedly reported as the dominant taxon of meiofaunal communities, both in terms of abundance and species richness (Lambshead, 2004; Schmidt-Rhaesa, 2014). However, the results obtained from 454 sequencing showed an equally dominant role of Platyhelminthes, which are traditionally considered a rare taxon and in most cases absent in benthic deep-sea ecosystems (Fonseca et al., 2010; Bik et al., 2011). This discrepancy can be explained by the fact that "soft body" organisms, such as Platyhelminthes, are not well preserved or are lost during meiofaunal sorting (Fonseca et al., 2010). Thus, sorting animals prior to DNA extraction can lead to an underestimation of the diversity of those organisms with more delicate body structures (e.g., Platyhelminthes). Since most of the Platyhelminthes living in marine benthic ecosystems are predators (Reise, 1988), these findings, if confirmed, could change our view of the functioning of trophic food webs in shallow and deep-sea benthic ecosystems (Giere, 2009). However, these considerations leave open the debate on the interpretation of the results obtained through metabarcoding, which could be affected by potential amplification and sequencing errors, therefore further studies are needed to better evaluate and clarify these issues.

In all ecological studies the number of species increases with increasing number of sampled individuals, until saturation occurs (Bunge and Fitzpatrick, 1993). However, recent metagenetic investigations of meiofaunal communities based on 18S rDNA revealed that, despite the massive sequencing effort, rarefaction curves of the OTUs do not reach the saturation, leading to hypothesise that the biodiversity is far higher than expected (Fonseca et al., 2010, 2014; Bik et al., 2011). The lack of saturation of the rarefaction curves could be also caused by the low resolution of 18S rDNA in discriminating meiofauna at the species level (Tang et al., 2012).

Despite the nSSU is the genomic region most frequently used for meiofaunal barcoding (Floyd et al., 2002; Blaxter et al., 2005), a large fraction of the OTUs obtained in metagenetic studies still shows limited sequence identity (<95%) with sequences deposited in the public databases. This is due to the limited number of deposited sequences belonging to described species. This applies particularly to the deep sea, where most of the taxa, from either ecological models, empirical data and expert evaluations are still unknown (Danovaro et al., 2010; Mora et al., 2011; Appeltans et al., 2012).

The increase of public database coverage combined with deeper sampling effort could in the future improve the accuracy of marine biodiversity estimates based on metagenetic surveys.

Biogeographic patterns

The knowledge of biogeographic patterns of these microscopic metazoan taxa is fundamental to better understand the global marine diversity and the spatial distribution of marine organisms. High-throughput sequencing is an ideal tool to conduct biogeographic studies, as it can facilitate the identification of the mechanisms driving species' distribution, their geographic dispersion to an accuracy that is unfeasible at present using classical morphological identification alone. An example is provided by studies on the microbial component. Bacteria, for a long time, have been hypothesised to exhibit cosmopolitan distributions and the lack of spatial patterns (Baas Becking 1934), but the results of biogeographic patterns based on 16S rDNA sequencing have shown that this is not true (De Wit and Bouvier, 2006; Hughes Martiny et al., 2006).

The hypothesis of cosmopolitism for meiofaunal assemblages has been tested through recent metagenetic studies in different habitat types, from rainforest to deep-sea environments (Porazinska et al., 2010, 2012, Bik et al., 2011; Fonseca et al., 2014). These studies showed that nematode assemblages are characterized by the presence of endemic species in all investigated ecosystems, with higher species richness at tropical than at temperate latitudes (Porazinska et al., 2009a and 2010). In marine systems, it has been reported that there is a clear distinction between shallow-water and deep-

sea nematode communities. A recent study carried out along bathymetric gradients, from shallow down to abyssal depths in the Pacific and Atlantic Oceans, reported that eukaryotic organisms display a higher genetic divergence along depth gradients of the same oceanic region than between the two regions at similar bathyal/abyssal depths (Bik et al., 2011). This could be explained by a relatively recent geographic isolation between deep-sea eukaryotic taxa inhabiting the two oceanic regions, or by a slower rate of evolution of rRNA genes in the deep-sea meiofauna (Bik et al., 2011).

A highly debated and still open issue in ecology is whether the patterns of distribution of biodiversity are caused by spatially limited dispersal or by niche-related factors (Fonseca et al., 2014). A high-throughput sequencing analysis of 66 marine sediment samples collected at 23 sites (from UK, France, Spain, Portugal and Gambia) showed that meiofaunal assemblage structure is mainly shaped by dispersal limitation and habitat features. The presence of unique OTUs with narrow range sizes independent from sample size, suggests that, in addition to abiotic factors, biotic interactions and local adaptation could influence local patterns of diversity and community composition of the meiobenthos (Fonseca et al., 2014).

Shortcomings and pitfalls

Amplification and sequencing errors

Despite the high potential in species identification, metagenetic approach presents different shortcomings. Among these, biases in the output sequences can be generated during DNA amplification and sequencing steps. PCR amplification can introduce substitutions, insertions or deletions (Cline et al., 1996). The formation of PCR artefacts, known as "chimeras" is an additional problem. These in vitro recombinant DNA molecules are usually generated during the first PCR step by the amplification of homologous regions from a large number of potentially highly related organisms (von Wintzingerode et al., 1997; Qiu et al., 2001). In metagenetic analyses of eukaryotes, chimeras tend to appear in richer and more genetically diverse samples, where molecules from two different origins artificially combine together (Meyerhans et al., 1990). Since chimeras can inflate the overall biodiversity estimates, it is crucial to identify and remove these PCR artefacts, or, even better, to avoid their creation. A quick and efficient method used to flag a putative chimera is to compare the length of matched bases from the top hit in a MEGABLAST search to the length of the query sequence (Creer et al., 2010 and references cited therein). If the database sequence is longer than the query sequence, and the portion of the 3' end does not match, it is likely that the query is a recombinant. Other algorithms, currently used for the identification and removal of chimeras, include Perseus (Quince et al., 2011), UCHIME (Edgar et al., 2011) and USEARCH (Edgar, 2010). Both

Perseus and UCHIME assume that chimeras should be less frequent than parental sequences. USEARCH can identify chimeric sequences against a user provided reference database (such as ChimeraSlayer algorithm), but also can perform *de novo* chimera detection based upon abundances of input sequences.

However, it is also important to reduce the level of DNA recombination during PCR amplification performing a 'gentle' DNA extraction (enzymatic digestion and using spinning wheels; Huber et al., 2002), increasing polymerase extension times and reducing the number of PCR cycles to the minimum (e.g. 20; Meyerhans et al., 1990; von Wintzingerode et al., 1997; Qiu et al., 2001).

Sequencing errors appear to be less abundant than those produced during amplification step (Taberlet et al., 2012). Sequencing error type and rate vary among different sequencing platforms used (Glenn et al., 2011). To correct sequencing errors of Roche 454, it is usually suggested, as a pre-processing step, to denoise the raw reads using tools such as AmpliconNoise (Quince et al., 2011) or Denoiser (Reeder and Knight, 2010). "Denoising" is specific to 454 platform's error profile and could be useful to avoid concerns regarding specific biological questions, such as species counts. Illumina technology, which generate tens (MiSeq) to thousands (HiSeq4000) of times more data per run, needs other approaches, such as quality-filtering strategies (Bokulich et al., 2013).

The need for optimized cocktail of primer sets

The amplification and sequencing of a single diagnostic locus (e.g., 18S rRNA) unlikely cover all of the biodiversity present in a sample (Creer et al., 2010; Bik et al., 2011). Therefore, in order to identify most of the taxa, it is suggested to use a cocktail of primer sets optimized to sequence alternate loci (e.g. nLSU and COI; Bhadury et al., 2006; Porazinska et al., 2009a; Fontaneto et al., 2015). The comparison of metagenetic results obtained from different genes could help to choose the best combination of genetic markers for eukaryotic metagenetic surveys. This issue is even more important if we consider a recent study showing that the use of 18S rRNA for meiofaunal surveys could largely underestimate the actual species richness. This could be due to the fact that 18S rDNA is highly conserved between meiofaunal species belonging to the same genus (Tang et al., 2012; Morgan et al., 2014). The use of 18S rRNA gene could be useful for comparing the levels of relative diversity at higher taxonomic levels, but at the species level, COI appears to be more robust and able to efficiently identify different morphospecies, and eventually cryptic species (Derycke et al., 2005; Fonseca et al., 2008; Tang et al., 2012).

Arbitrary OTUs and unreliable inferences on species richness

Due to the huge amount of raw sequences coming out from high-throughput sequencing, filtering and clustering steps are needed to decrease the downstream computational requests. In this sense, clustering raw reads in Operational Taxonomic Units (OTUs) can be viewed simply as an important processing step, but OTU generation based on different cut-offs results in very different estimates of sample richness (Creer et al., 2010; Fontaneto et al., 2015). OTU cut-off level that broadly correlates with species, typically occurs between 95% and 99% of the 18S sequence similarity, but there will be exceptions according to species in environmental samples. Lower cut-offs are known to lump taxonomic genera or even orders together, while the most stringent cut-offs (e.g. 99%) can substantially over split different individuals from a single species (Porazinska et al., 2010). Further studies are therefore needed to better understand the effect of OTU clustering on biodiversity surveys of meiofauna.

Metagenetic analyses on prokaryotes are based on the assumption that the numbers of OTUs reflect the species richness (Kemp and Aller, 2004). The same assumption, however, cannot be applied to multicellular organisms. In particular, despite concerted evolution predicts lower levels of divergence between intraspecific copies, and higher levels of divergence among interspecific gene copies (Dover, 1982), it has been demonstrated that ribosomal RNA gene copies in nucleus and the number of nuclei vary dramatically between individuals belonging to the same species (Bik et al., 2012). Although new mutations should be rapidly propagated across the rRNA gene copies within a species, ribosomal variation within the same species is extensive in some cases (James et al., 2009; Bik et al., 2013). Clustering analysis incorporates this variation in OTUs picking, hampering the accurate quantification of the abundance of individuals and species richness (Porazinska et al., 2009a,b; Porazinska et al., 2010).

Moreover, some nematode species differ significantly at the level of the single nucleotide in the 18S rRNA gene (Porazinska et al., 2010). This does not allow distinguishing the sequencing errors from the presence of ‘rare species” in environmental samples (Sogin et al., 2006). As a consequence, all data derived from metagenetic analyses on meiofauna surveys should be critically evaluated. In consideration of the present degree of uncertainty some authors suggested that the OTU analysis could be useful to compare the relative diversity between different samples even without explicitly referring to species (Creer et al., 2010).

Recent metagenetic studies carried on zooplankton assemblages revealed that the number of sequences is better related to biomass than to individual abundance (Lindeque et al., 2013; Hirai et al., 2015), thus providing new insights for the assessment of the relative contribution of each taxon

from metabarcoding analysis. However, this relationship needs to be tested for meiofaunal assemblages.

Conclusions and future outlooks

Molecular approaches will allow us soon to process a larger number of samples at low cost, and produce a breadth of data that are unimaginable by using the classical morphological identification. The study of marine meiofaunal biodiversity using high-throughput approach presents several advantages and disadvantages compared to morphological identification (Table 2). Not all species in a sample are detected, and a certain percentage remains under-estimated due to two major issues: i) the limited coverage of public sequence repositories, and ii) the impossibility to link the OTU number with the abundance of individuals belonging to each species as observed on the basis of 18S rRNA gene pyrosequencing (Porazinska et al., 2010).

Databases for small-size eukaryotes (18S rRNA genes) are far from been exhaustive and this problem is amplified for other loci (e.g., 28S rRNA) for which databases are still more limited (Pruesse, et al., 2007). Meiofaunal taxa, indeed, have been historically under-represented in public databases: for example, for some meiofaunal taxa, few 18S rRNA sequences have been published (e.g. two 18S rRNA sequences for the phylum Loricifera). This issue has a variety of consequences on meiofaunal biodiversity surveys since the accuracy of taxonomy assignment derived from BLAST match depends on the database coverage. For the few well-sampled groups (e.g. Arthropoda or Annelida) it is possible to obtain genus-level accuracy, whereas for less-studied phyla (e.g. Loricifera, Gnathostomulida), only phylum-level accuracy might be possible.

Considering all these evidences, there is an urgent need to extensively collect full-length eukaryotic sequences or whole genomes in order to "feed" the current databases and provide a stronger link between sequence data and morphology.

To overcome the obstacle of linking meiofaunal OTU numbers with the abundance of individuals per species, major effort should be devoted to understand the actual variability of the 18S rRNA gene among individuals of the same species and among different species taking into account the contribution of potential biases due to PCR and sequencing in such a variability. We need to set up PCR methods that reduce amplification biases, to improve the performance of sequencing platforms in order to obtain more accurate and longer sequences, and to elaborate more reliable bioinformatic pipelines, which can take advantage of recent advances in sequencing technology. If these gaps will be filled in the near future, the use of metabarcoding will increase enormously our ability to provide more accurate estimates of biodiversity.

For all these reasons, there is the urgency to implement the collaboration among traditional taxonomists, molecular and computational biologists.

Nowadays a key challenge for high-throughput studies is to move beyond the pure ecological descriptions of biodiversity patterns and move towards understanding the whole ecosystem functions, linking metabarcoding data sets with the knowledge of species function (e.g., meta-transcriptomics). An alternative approach to avoid PCR biases and obtain simultaneously information on taxonomic and functional diversity is based on the use of Illumina-sequenced environmental metagenomes ($_{mi}$tags) (Logares et al., 2014). This approach could represent, in the future, a powerful tool to investigate the biodiversity of meiofaunal assemblages.

The power offered by high-throughput surveys to monitor biodiversity rapidly may open new perspectives to investigate the micro-eukaryotic biosphere and also increase the effectiveness of institutions responsible for monitoring, protecting and conserving biodiversity worldwide.

Table 2. Primary advantages and disadvantages of morphological identification and high-throughput sequencing approaches to study meiofaunal biodiversity.

Morphological identification	High-throughput sequencing
Providing species abundance information in a sample	Not providing a reliable estimate of species abundances
Not allowing the study of cryptic diversity	Identifying morphologically cryptic species
Providing biodiversity estimates at greater taxonomic resolution: most of the taxa are identified at genus or even at species level	Most of the OTUs could be assigned to the order level at best and very few to family, genus, and species level. Not all species are detected
Need of taxonomist experts and morphological identification could be subjective	No need of taxonomist experts
Time-consuming method	Allowing large-scale biodiversity surveys in a relatively short time scale
Traditionally used to determine biodiversity indexes for basic and applied ecological research	Not allowing the determination of reliable biodiversity indexes

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CHAPTER 3. IMPLEMENTATION OF METAGENETIC ANALYSIS OF MEIOFAUNAL BIODIVERSITY

3.1 Sensitivity of metagenetic analysis of meiofaunal biodiversity

Abstract

In the context of the sixth wave of extinction and global changes, reliable surveys of marine biodiversity are urgently needed to infer the consequences of species loss on ecosystem functioning. Meiofaunal organisms are increasingly used as indicators of the status of marine ecosystems because of their high sensitivity to environmental changes and anthropogenic impacts. Traditional benthic monitoring, relying on morphological identification of species, is time-consuming and expertise-demanding. High-throughput sequencing of rDNA barcodes (namely metabarcoding) has been recently proposed to speed up the assessment of benthic biodiversity. Here we tested the reliability of metabarcoding by comparing traditional approach and 18S rDNA sequencing of marine meiofaunal organisms, previously morphologically identified at higher taxa level. Our results indicate that in order to assess meiofaunal biodiversity at higher taxa level, the use of a more relaxed clustering as a conservative threshold value (97%) is recommended, rather than risking an inflation of Operational Taxonomic Units (OTUs) by using more stringent cut-offs. Despite this, we show that independently from the cut-off criteria used, the number of OTUs largely exceeds the number of individuals, hampering the use of metabarcoding to quantitative ecological studies. Metabarcoding proves powerful by revealing a remarkable level of diversity that vastly exceeded the morphological survey. The retrieval of 50% of taxa morphologically identified, suggest that the remaining were not detected by the molecular screening due to primer biases and the incompleteness of public database. Our findings highlight the need of comprehensive and well-curated taxonomic reference libraries and multi-gene barcoding. Morphological and molecular-based surveys provide different information; thus they should be used in synergy to obtain better evaluations of the actual marine benthic biodiversity.

Keywords: marine ecosystems, meiofauna, metabarcoding, high-throughput sequencing, 18S rDNA

Introduction

Meiofauna (size range between 20 and 500 µm) are amongst the most abundant (up 10^6 individuals per m^2) and diversified group in the marine realm, including 24 of the 35 animal phyla (Giere, 2009). Due to their widespread distribution and the important role played in the functioning of the food webs, meiofaunal organisms are considered suitable model to study marine biodiversity patterns (Snelgrove, 1999; Danovaro et al. 2008). Meiofauna are also increasingly used as indicators of the status of marine ecosystems because of their high sensitivity to environmental changes and anthropogenic impacts (Balsamo et al. 2012; Schönfeld et al. 2012; Moens et al. 2014, Zeppilli et al., 2015).

Estimates of meiofaunal biodiversity are traditionally based on morphological identification, which is time-consuming, requires taxonomic experts and does not allow to identify cryptic or closely related species (Fontaneto et al., 2009; Creer et al., 2010). The advent of DNA-based approaches (e.g., Sanger sequencing) has brought significant help to reinforce taxonomic identification and to distinguish morphologically similar species (Derycke et al., 2010). During the last decade, researchers have applied high-throughput sequencing (HTS) analysis targeting specific gene regions (i.e. 18S rDNA) to study meiofaunal taxonomic composition (Chariton et al., 2010; Creer et al., 2010; Fonseca et al., 2010, 2014; Creer and Sinniger, 2012; Bik et al., 2012a; Brannock et al., 2014; Lallias et al., 2015; Lejzerowicz et al., 2015; Sinniger et al., 2016). Such approach, namely "metagenetic" or "metabarcoding" (Taberlet et al., 2012), allows for a more comprehensive study of meiofaunal assemblage as a whole that is not constrained by taxonomic expertise and handling time. The ability to assess species richness in a rapid and cost-effective way, over large spatial and temporal scales, is particularly important in the deep sea, the biggest ecosystem on earth. It has been recently demonstrated that metabarcoding could increase our ability to investigate deep-sea biodiversity and thus improve our knowledge about the potential consequences of species loss on ecosystem functioning (Danovaro et al., 2008; Dell'Anno et al., 2015; Guardiola et al., 2015).

So far, metabarcoding analyses carried out to assess marine metazoan biodiversity have been primarily based on protocols and methods implemented from prokaryotic studies. However, how such approaches could be transferred over to the metazoan assemblages has not been extensively explored. Morphological and molecular approaches have long been considered complementary, but doubts still exist regarding the accuracy and reliability of metabarcoding. Some attempts to compare morphological and metabarcoding-based taxonomic identification have been recently performed on macrofauna. Results showed that molecular barcodes provided a level of diversity that vastly exceeded the assessment based on morphological identification, and a large proportion of species (up to 60%) identified by using microscopy were not detected by the molecular screening (Cowart et al., 2015; Aylagas et al., 2016). A rigorous comparison of the efficiency of molecular approach versus

morphological identification is still lacking for meiofaunal taxa. Most of previous studies carried out to assess meiofaunal biodiversity by using metabarcoding have recovered DNA from sediment (Lejzerowicz et al., 2015; Sinniger et al., 2016), or from a sieved fraction of the sediment (Creer et al., 2010; Fonseca et al., 2010, 2014; Brannock et al., 2014), without sorting and identified animals prior to molecular analysis. Only few studies, focusing on deep-sea (Dell'Anno et al., 2015) and terrestrial (Porazinska et al., 2009, 2010, 2012) nematodes, recovered DNA from organisms previously morphologically identified. We still lack a pilot study, aiming at evaluating the ability of metabarcoding to identify the different meiofaunal taxa, by carrying out morphological and molecular identification on the same individuals.

In the present work we tested the sensibility of Illumina MiSeq sequencing analyses to identify meiofaunal organisms at higher taxa level (collected along two deep-sea canyons and the adjacent western open slope, in the Ligurian Sea, Western Mediterranean Sea). We here provided one of the first rigorous comparison of morphological identification versus Illumina MiSeq sequencing of 18S rDNA for assessing the biodiversity of deep-sea meiofaunal organisms previously morphologically identified at higher taxa level. We also analysed pitfalls related to molecular approach and provided suggestions for its implementation, including the need for more complete reference databases to unravel the broadest possible taxonomic diversity.

Material and methods

Study area and sample collection

In the framework of project RITMARE, sediment samples have been collected during the BioLig cruise (7^{th} - 20^{th} May 2013) on board *R/V* Minerva, by means of a NIOZ-type box-corer, along Polcevera and Bisagno Canyons and the adjacent western open slope, in the Ligurian Sea (North Western Mediterranean Sea). Overall, eleven stations have been selected from 200 to 2000 m water depth: three stations have been sampled along each of the two canyons and five stations along the open slope. Sediment samples were collected by using sterile Plexiglas cores (inner diameter 3.6 cm) and then were immediately stored at -20°C, without the use of any preservative, until further processing.

Recovery of meiofaunal organisms from sediment

Each sample was treated with ultrasound (for 1 min 3 times, with 30 s intervals) to detach organisms from the grain particle surface and, then, sieved through a 1000-µm and a 20-µm mesh net to retain the smallest organisms. The fraction remaining on the latter sieve was re-suspended and centrifuged three times with Ludox HS40 diluted with water to a final density of 1.18 gcm^{-3} (Heip et al., 1985).

All meiobenthic animals were sorted under a stereomicroscope and identified at higher taxa level. After identification, a single individual of each taxon was carefully picked and placed into a 2 ml sterile tube, until further molecular analysis. Microscopic observations revealed that the storage at -20°C does not alter the morphological features used for taxonomic identification.

DNA extraction and sequencing

DNA was extracted using a commercial kit for tissue DNA extraction (QIAGEN DNeasy Blood and Tissue Kit), following the manufacturer's protocol. Once extracted and purified, DNA was amplified using the primer pairs SSUF04 (5'-GCTTGTCTCAAAGATTAAGCC-3') and SSUR22 (5' – CCTGCTGCCTTCCTTGGA-3') (Blaxter et al., 1998) targeting the 18S rRNA gene and suitable, in term of amplicon length (450 bp), for metabarcoding analyses, according to the needs of the sequencing platform utilised in the present study. Sequencing analysis was carried out on Illumina MiSeq platform by POLO GGB, by using Nextera XT DNA Sample Preparation Guide (Polo d'Innovazione di Genomica, Genetica e Biologia Società Consortile R.L., Perugia, Italy).

Bioinformatic analyses

Automatic demultiplexing of samples occurred on the MiSeq sequencing machine and forward and reverse reads were joined using the FLASH software (Magoc and Salzberg, 2011). All overlapped sequences were quality-filtered using USEARCH (version 8.0) algorithm (Edgar, 2010) with the maximum expected error (-fastq_maxee) set to 1 (Brannock and Halanych, 2015) and trimmed to a length of 350 bp. Operational Taxonomic Units (OTUs) were generated by using a clustering threshold ranging from 1 to 3%, through USEARCH -cluster_otus workflow within the UPARSE (Edgar, 2010) clustering method. Chimeras and sequences appear < 10 times in the dataset were automatically identified and removed. Sequences were then mapped against the OTUs to generate an OTU table with the USEARCH –usearch_global function, with a minimum identity percentage of 97%. After generation of the OTU table, representative OTU sequences were then taxonomically assigned using MegaBLAST (sequence identity ≥ 90%, e-value 1.0^{-5}) against the nt sequence database (latest version as of July, 2016).

Data analysis

Once the taxa list was obtained, further processes were carried out to refine the dataset. A table was constructed for each sequenced sample, with the list of the OTUs and their relative BLAST match name and the number of reads mapping against each OTU. The dataset was used both qualitatively and quantitatively. We grouped OTUs obtained at 97, 98 and 99% cut offs following the major Super-

Groups of eukaryotes suggested by Guillou et al. (2013), with the only exception of Opisthokonta which were splitted into Metazoa, Fungi and other Opisthokonta. As we were particularly interested in the Meiofauna, we splitted Metazoa into the main meiofaunal taxa. We grouped reads mapping against each OTU, following the same criteria.

First, we calculated the percentage contribution to the whole molecular dataset (by using the number of OTUs and the number of mapping reads) of meiofaunal taxa, non-metazoan meiofaunal-eukaryotic taxa and Fungi. Then, we manually eliminated sequences matching to Fungi, to clearly non-marine organisms (these could be contaminations or DNA of continental origin present in the sediment) and to non-metazoan meiofaunal eukaryotes and thus we analysed meiofaunal taxonomic composition on the final retained sequences.

Results

Morphological identification

Morphological identification of sediment samples allowed us to collect 55 individuals belonging to 13 meiofaunal taxa: Acarina, Amphipoda, Copepoda, Cumacea, Cladocera, Gastrotricha, Isopoda, Kinorhyncha, Nematoda, Nemertina, Oligochaeta, Ostracoda, Polychaeta (Fig. 1).

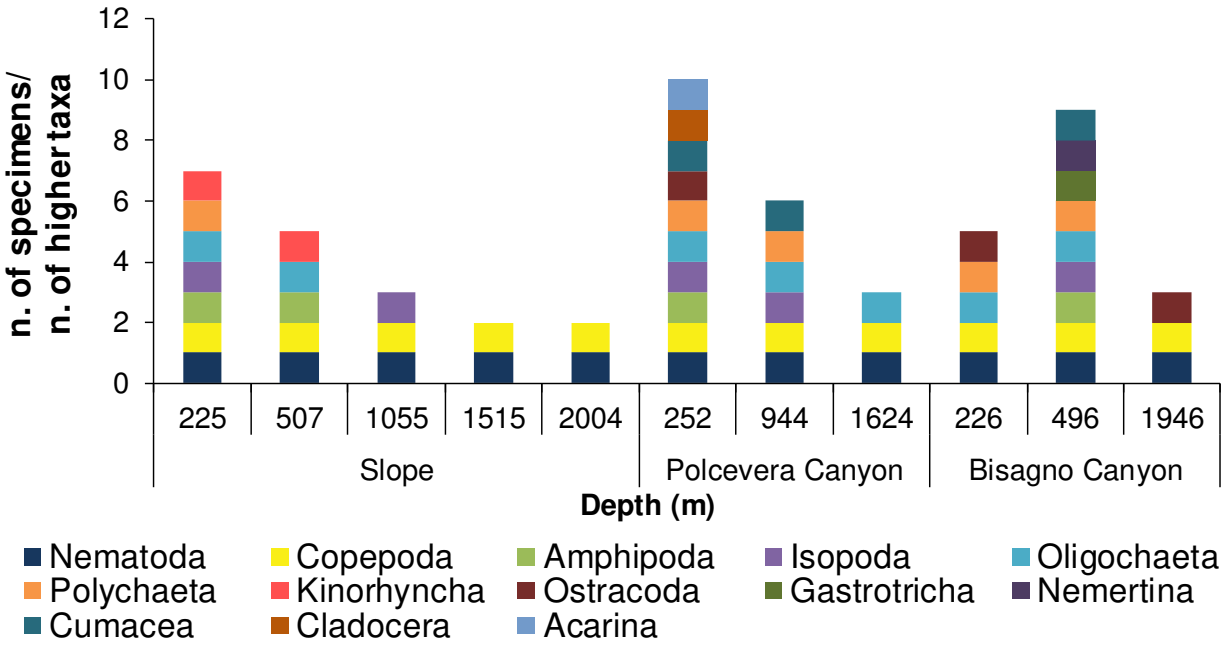

Figure 1. Meiofaunal higher taxa identified by morphological analysis for each sampling site.

Overall, the total number of taxa pooled together for each sample ranged from 2 to 10, for the two deepest stations along the slope (1515 m and 2004 m depth) and for the shallowest station (252 m depth) along the Polcevera Canyon, respectively. Nematodes and copepods occurred in all pools. Polychaetes were present in five out of eleven samples, at the shallowest stations along the open slope

and two canyons. Oligochaetes occurred at all sampling sites along the Polcevera Canyon, and at the two shallowest stations along the open slope and the Bisagno Canyon. Cladocera and Acarina were found only at 252 m depth along the Polcevera Canyon, whereas Gastrotricha and Nemertina were found only at 496 m depth along the Bisagno Canyon. Kinorhyncha were exclusively identified along the open slope, at the two shallowest stations, whereas Ostracoda were found only along the two canyons.

High-throughput sequencing data

Illumina MiSeq sequencing of 18S gene produced a total of 3.253.599 paired raw reads which were quality filtered to 1.657.588 reads. Clustering of reads resulted in a number of OTUs ranging from 12 to 82 at 97% cut off, from 13 to 95 at 98% and from 13 to 145 at 99% clustering thresholds (Fig. 2).

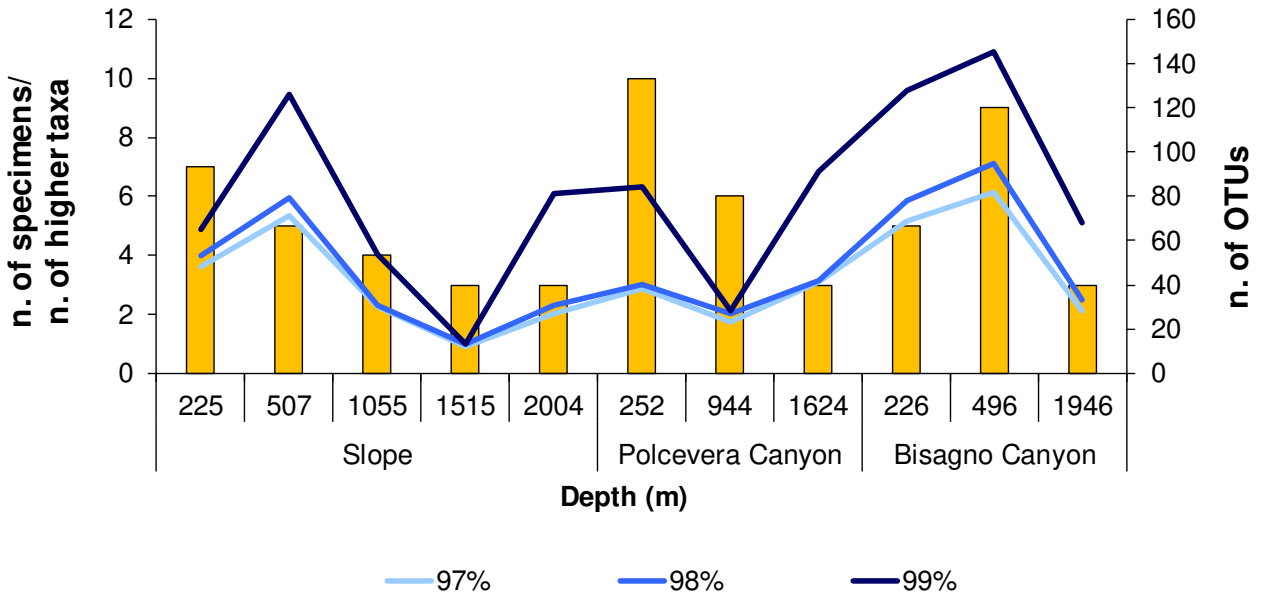

Figure 2. Number of specimens and higher taxa morphologically identified and pooled together for each sampling site, plotted against the number of OTUs obtained by 18S rDNA metabarcoding analysis. OTUs are created at 97%, 98% and 99% clustering thresholds.

Overall, the number of OTUs increased with increasing the percentage of similarity used for clustering. The lowest number of OTUs at the three different cut-offs were recorded for meiofaunal pool belonging to sediments collected at 1515 m depth along the slope, whereas the highest number of OTUs was found for the sample collected at 496 m depth along the Bisagno Canyon (Fig. 2).

The number reads mapping against OTUs created at 97, 98, 99% cut offs, ranged from 23305 to 252212, for meiofaunal pools belonging to sediment samples collected at 1515-m and 2004-m depth along the slope, respectively.

Throughout the whole dataset, only two OTUs (BLAST matching to *Bipalponephtys cornuta*, Polychaeta and to *Momordica charantia*, Spermatophyta) were shared between the eleven samples at both 97% and 98% clustering thresholds. Just one OTU (BLAST matching with *Momordica charantia*, Spermatophyta) was shared between all meiofaunal pools at 99% cut off. A substantial proportion of the recovered OTUs (55, 66, 135 OTUs at 97, 98, 99% clustering thresholds, respectively) were unique and most of them BLAST matched to "uncultured eukaryotes". Moreover, at all clustering thresholds, 5 phyla were represented by only 1 OTU (Acarina, Choanoflagellida, Cryptophyta, Rhizaria, Loricifera), constituted by a relative low number of reads (ranging from 41 for Cryptophyta to 121 for Rhizaria).

Molecular taxonomic assessment

We focused on the results of taxonomic composition obtained at both relaxed (97%) and more stringent (99%) clustering thresholds.

97% clustering threshold. At 97% similarity cut-off, we obtained 43 OTUs as average between all eleven samples. OTUs were assigned to 25 different taxa, including 12 belonging to metazoan meiofauna (Acarina, Amphipoda, Bivalvia, Brachiopoda, Branchiopoda, Cnidaria, Copepoda, Isopoda, Loricifera, Nematoda, Ostracoda, Polychaeta). Most of the retrieved OTUs matched to metazoan meiofaunal organisms, whose contribution ranged between 37% and 70%, at 253 and 944 m depth along the Polcevera Canyon, respectively. OTUs matching to eukaryotes different than metazoan meiofaunal taxa contributed from 17% to 52%, at 2004 m and 507 m depth along the open slope, respectively. OTUs matching to Fungi represented, as average, 17% (Fig. 3A). More in detail, the molecular assemblages were dominated, by "uncultured eukaryotes" (22%), followed by nematodes (17%), fungi and polychaetes (16%). Considering all of the OTUs retrieved, other eukaryotic phyla such as Isopoda, Spermatophyta and Cnidaria contributed with lower proportion (6%, 4% and 4%, respectively).

Looking at reads mapping against OTUs, our results showed that molecular assemblage was dominated by metazoans meiofaunal taxa (92%), followed by other eukaryotes (7%) and Fungi (2%) (Fig. 4). Sequences matched to polychaetes contributed for 68% to the total number of reads, followed by those assigned to nematodes (19%) and to "uncultured eukaryotes" (3%). Taxonomic composition based on reads mapping to OTUs at more stringent cut offs was very similar and thus not reported.

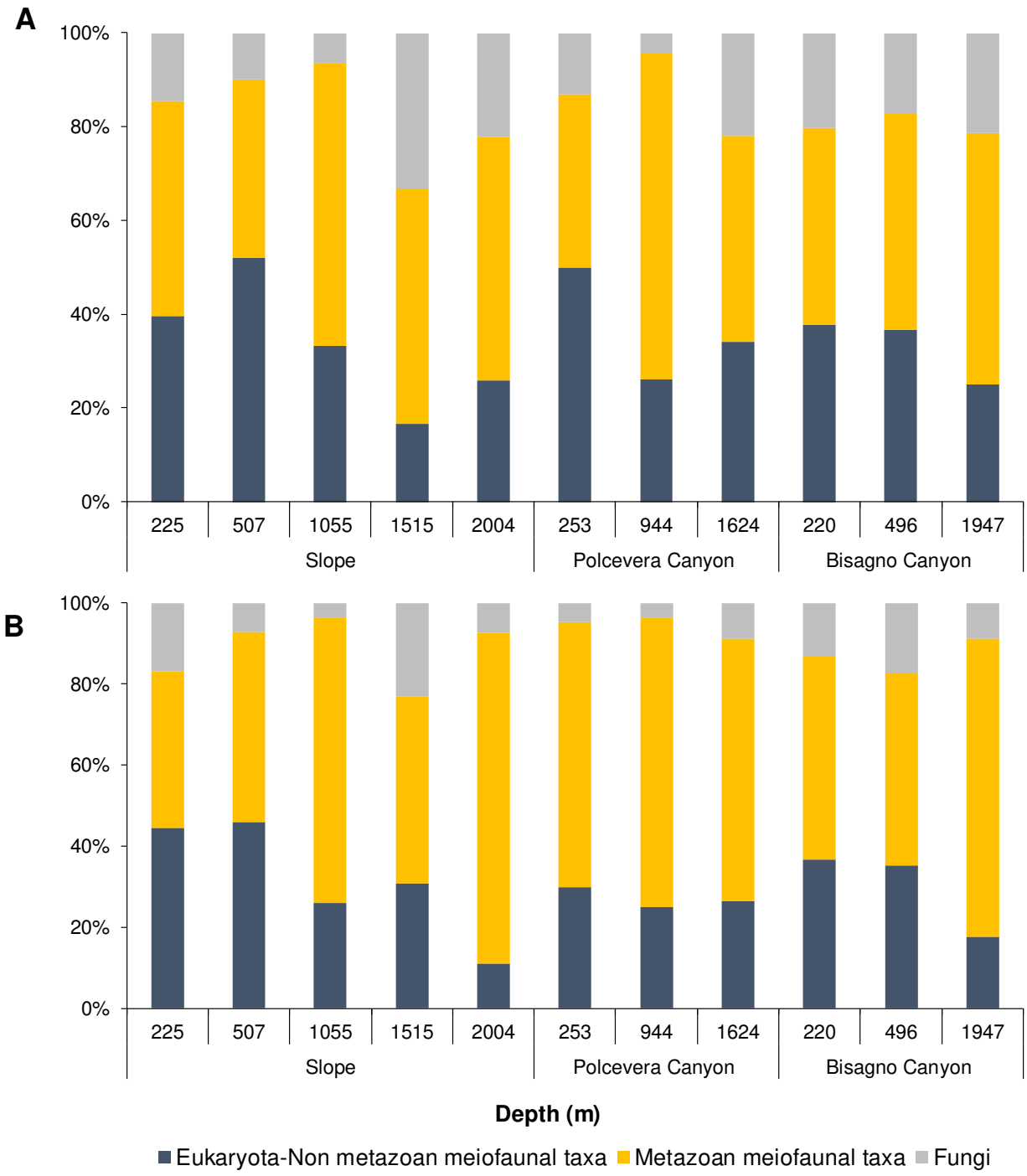

Figure 3. Contribution of Non-Metazoan meiofaunal Eukaryotes (blue), Metazoan meiofaunal taxa (yellow), and Fungi (grey) to the total number of OTUs. Graphs are based on the proportion of OTUs (expressed as percentage) obtained by 18S rDNA metabarcoding analysis. The contributions are calculated from filtered, non-chimeric OTUs created at 97% (A) and 99% (B) cut offs and classified by means of a MegaBLAST match against NCBI public nucleotide (nt) database.

99% clustering threshold. At 99% similarity cut-off, we obtained, as average, 80 OTUs which matched to 26 different taxa. All taxa recovered by the most stringent clustering threshold were the same previously found at 97% cut off, plus the taxon of Gastropoda. OTUs matching to metazoan meiofaunal taxa represented 59% as average, of the total number of OTUs, ranging from 39% to 82%, at 225 m and 2004 m water depth along the slop, respectively. Eukaryotes different than metazoan meiofauna represented a lower percentage (30%), ranging from 11% to 46% in the samples collected at 2004 m and 507 m water depth along the open slope. Fungi represented, as average, a lower percentage compared to what found at 97% cut off (10% vs 17%) (Fig. 3B). Unlikely to what previously reported for more relaxed clustering threshold, the molecular assemblage of OTUs obtained at 99% cut off is dominated by polychaetes (31%), followed by "uncultured eukaryote" (18%), nematodes (16%) and fungi (10%). A lower contribution to OTUs richness was represented by Streptophyta (6%), Isopoda (3%) and Brachiopoda (3%).

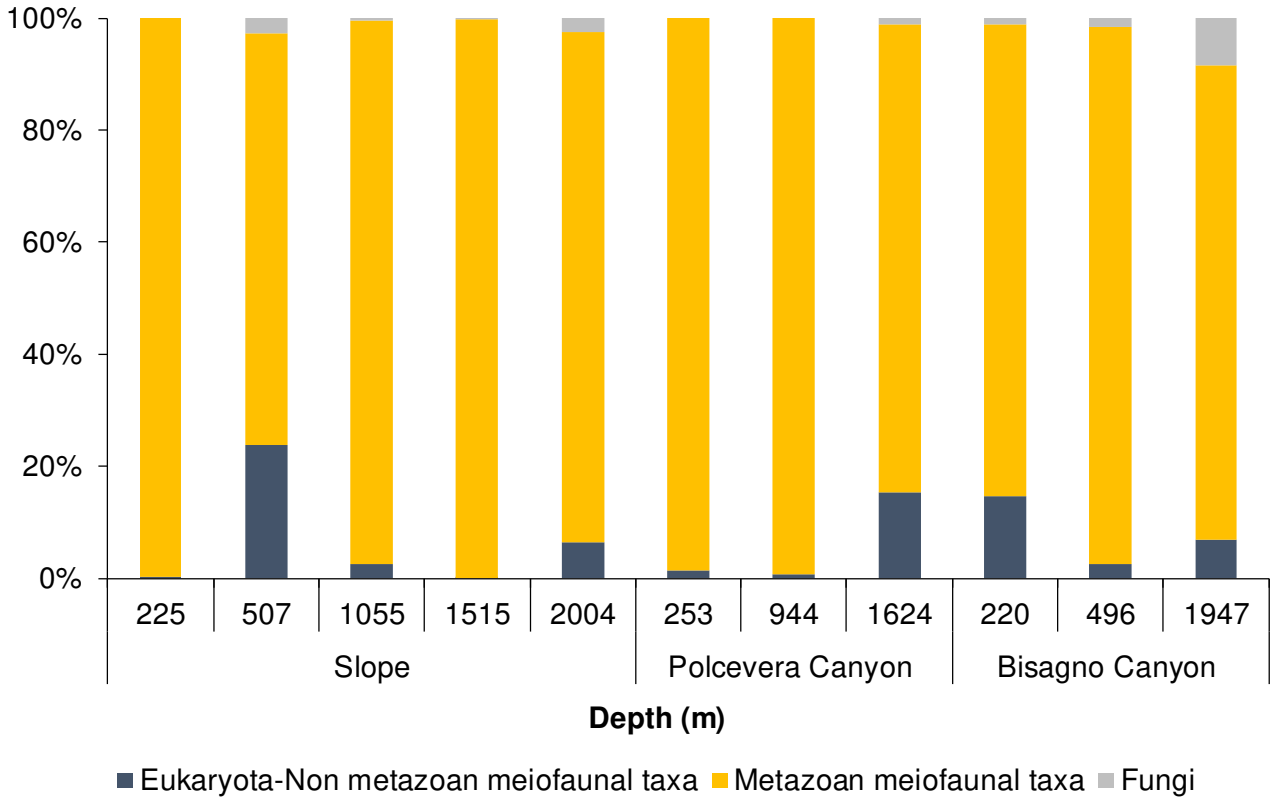

Figure 4. Contributions of Non-Metazoan meiofaunal Eukaryotes (blue), Metazoan meiofaunal taxa (yellow), and Fungi (grey) to the total number of mapping reads. Graph is based on the proportion of reads (expressed as percentage) obtained by 18S rDNA metabarcoding analysis. The contributions are calculated from reads mapping against OTUs created at 97% cut off; OTUs are classified by means of a MegaBLAST match against NCBI public nucleotide (nt) database. Results of the analyses carried out by mapping sequences against OTUs created at more stringent cut offs are very similar to these and thus are not shown.

Meiofaunal taxonomic composition based on HTS data

As the morphological analysis focused on meiofaunal organisms, in order to accurately compare the results obtained from morphological and molecular approaches, we manually eliminated sequences matching to fungi, to clearly non-marine organisms (these could be contaminations or DNA of continental origin present in the sediment) and to non-metazoan meiofaunal eukaryotes. Thus we analysed the taxonomic composition on the final retained sequences matching to metazoan meiofaunal taxa.

Table 1 reported the list of taxa morphologically identified, those not recovered by molecular approach and those detected only by high-throughput sequencing analysis but not identified by using microscope. Five phyla (Cladocera, Cumacea, Gastrotricha, Oligochaeta, Nemertina) occurring in the morpho-taxonomic analysis were not detected by high-throughput sequencing analysis, neither at 97% nor at 98% or 99% clustering thresholds. Conversely, several phyla (e.g., Bivalvia, Brachiopoda, Branchiopoda, Cnidaria, Gastropoda, Loricifera) that are not identified by using microscopy were detected through metabarcoding. The taxon of Nematoda were correctly recovered in all samples, by molecular approach. Copepods and ostracods were detected by molecular approach, even if not in all samples where they were morphologically identified. The taxon of Copepoda was represented by only 1 OTU, BLAST matched to *Itunella muelleri*. The most striking discrepancy between morphological and molecular data is the high number of OTUs and reads assigned to Polychaeta even when this taxon was not morphologically identified.

97% clustering threshold. Figure 5A reported the number of OTUs assigned to each specific taxon, after MegaBLAST match against NCBI public database. Metazoan meiofaunal diversity was dominated, in terms of number of OTUs, by nematodes (39% as average), which represented from 7% to 71%, in the pools belonging to sediment samples collected at 226 m depth along the Bisagno Canyon and at 2004 m depth along the open slope, respectively. The second most OUT-rich taxon was Polychaetes (33% as average), contributing from 17% to 69% in the pools belonging to sediment samples collected at 1515 m depth along the open slope and at 226 m depth along the Bisagno Canyon, respectively. Isopods were the third most abundant taxon in terms of number OTUs, representing 12% as average between all samples, followed by Cnidaria (6%), Amphipoda (3%) and Branchiopoda (2%). Copepoda were not recovered in all samples and their contribution was up to 7% in two of the three pools where they were retrieved by molecular approach. We found the same pattern for Ostracoda, which contribution was up to 7% in the sample belonging to sediments collected at 226 m depth along the Bisagno Canyon. Acarina and Loricifera represented less than 0,5% as average between all the investigated meiofaunal pools.

Table 1. Number of meiofaunal higher taxa morphologically identified. Identification mismatch: list of taxa identified only through microscopy and not by metabarcoding and viceversa.

	Depth (m)	Meiofaunal higher taxa morphologically identified	Identification mismatch	
			Only by Microscopy	**Only by Metabarcoding**
Slope	225	7	Amphipoda, Copepoda, Kinorhyncha, Oligochaeta	Bivalvia, Brachiopoda, Branchiopoda, Cnidaria
	507	5	Copepoda, Kinorhyncha, Oligochaeta	Bivalvia, Brachiopoda, Cnidaria, Isopoda, Polychaeta
	1055	3	Copepoda	Polychaeta
	1515	2	Copepoda	Isopoda, Polychaeta
	2004	2	Copepoda	Brachiopoda, Polychaeta
Polcevera Canyon	252	10	Acarina, Cladocera, Cumacea, Oligochaeta	Bivalvia, Brachiopoda
	944	6	Copepoda, Cumacea, Oligochaeta	na
	1624	3	Copepoda, Oligochaeta	Brachiopoda, Cnidaria, Isopoda, Polychaeta
Bisagno Canyon	226	5	Oligochaeta	Amphipoda, Brachiopoda, Branchiopoda, Cnidaria, Isopoda
	496	9	Copepoda, Cumacea, Gastrotricha, Nemertina, Oligochaeta	Acarina, Brachiopoda, Branchiopoda, Cnidaria, Loricifera, Ostracoda
	1946	3	Ostracoda	Brachiopoda, Isopoda, Polychaeta

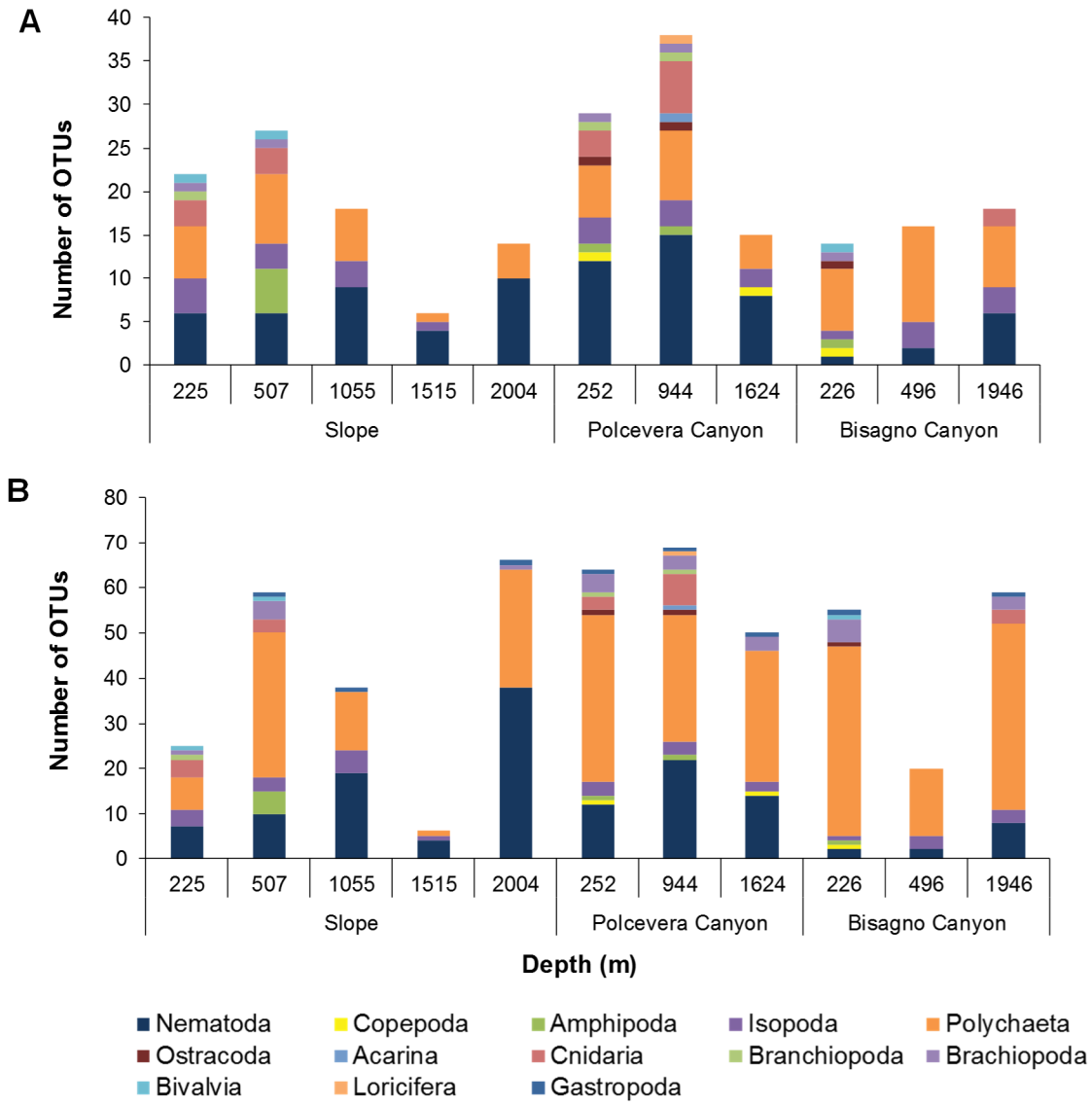

Figure 5. Meiofaunal taxonomic composition obtained by using molecular approach. Graphs are based on the number of OTUs assigned to each meiofaunal taxon and obtained by 18S rDNA metabarcoding analysis. OTUs are created at 97% (A) and 99% clustering thresholds (B), and classified by means of a MegaBLAST match against NCBI public nucleotide (nt) database.

The meiofaunal taxonomic composition based on number of reads mapped against OTUs clustered at 97% similarity threshold was reported in Figure 6. The distribution of reads within the different meiofaunal taxa was highly variable between eleven samples with a large dominance of polychaetes (68%), followed by nematodes (26%) and isopods (3%). Polychaetes represented from 0.08% to 99.5% in the pool belonging to sediments collected at 1515 m depth along the slope and at 496 m depth along the Bisagno Canyon, respectively. The number of reads matching to nematodes peaked (99.9%) in the pool belonging to sediment sample collected at 1515 m depth along the slope, whereas the lowest value (0.01%) was found at 226 m depth along the Bisagno Canyon. The third most represented taxon in terms of number of reads was Isopoda, whose contribution ranged from 0% to 23% at 2004 and 1055 m depth along the slope,

respectively. All the other taxa contributed less than 1% as average to the total number of reads. Bivalvia and Amphipoda peaked (12% and 5%, respectively) in the pool belonging to samples collected at 507 m depth along the slope. Cnidaria represented 5% at 944 m depth along the Polcevera Canyon.
Results of meiofaunal taxonomic composition based on reads mapping against OTUs obtained at more stringent cut offs (98 and 99%) were very similar to these and thus not reported.

99% clustering threshold. Our results showed that half of the number of metazoan meiofaunal OTUs matched to polychaetes, which ranged from 17% to 76% in the pools belonging to sediment samples collected at 1515 m depth along the slope and at 226 m depth along the Bisagno Canyon, respectively. Nematoda represented, as average, 30% followed by Isopoda (8%), Branchiopoda and Cnidaria (4%). Gastropods, that were not identified by morphological approach, were detected in 8 samples out of eleven. Overall gastropods represented a low percentage as average between eleven samples (1%), but their contribution was up to 3% in the pool belonging to sediments collected at 1055 m depth along the slope. Amphipods represented more than 8% at 507 m depth along the slope. All the other taxa, contributed less than 1% to the total number of OTUs. Copepods represented as average 0,4% between all eleven samples, and their contribution was up to 2% in two of the three pools where they were retrieved. The same pattern was found for Ostracoda which contributed with a low percentage to the overall molecular dataset (0,4% as average between all samples), but were up to 2% in the sample belonging to sediments collected at 226 m depth along the Bisagno Canyon (Fig. 5B).

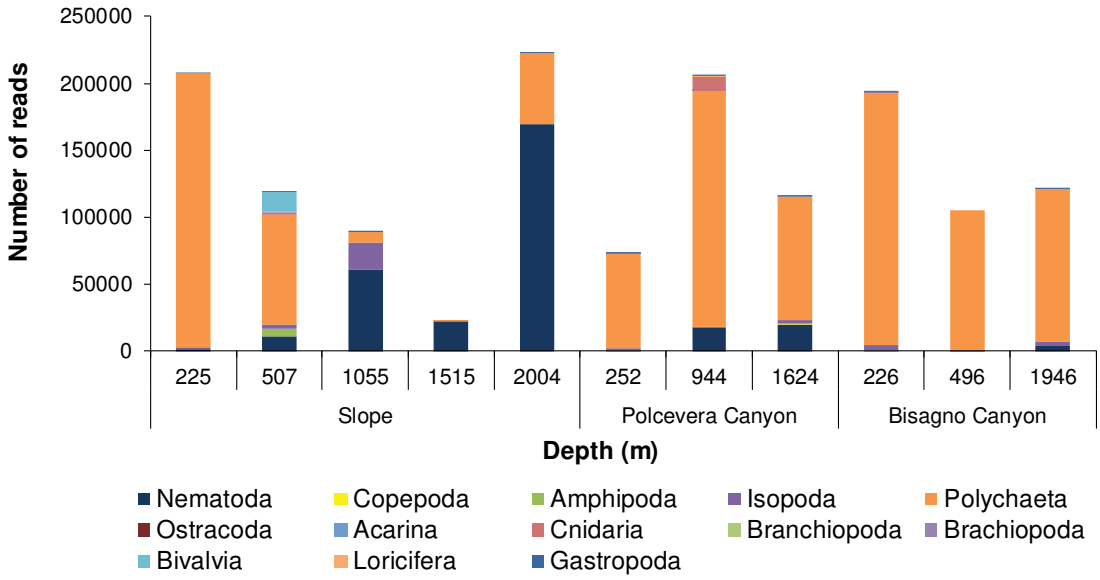

Figure 6. Meiofaunal taxonomic composition obtained by using molecular approach. Graph is based on the number of reads mapping against OTUs. OTUs are created at 97% cut off and classified by means of a MegaBLAST match against NCBI public nucleotide (nt) database. Results of the analyses carried out by mapping sequences against OTUs created at more stringent cut offs are very similar to these and are not shown.

Discussion

The optimal similarity threshold for meiofaunal higher taxa identification

The use of DNA-based barcodes as a tool for species identification was proposed almost a decade ago (Floyd et al. 2002; Hebert et al. 2003) and it has been recently applied in ecological studies aiming to investigate the biodiversity of microscopic hyperabundant and hyper-diverse groups, where taxonomic expertise is scarce (Fonseca et al., 2010).

The DNA-based identification approach uses molecular Operational Taxonomic Units (OTUs) and employs similarity cut-offs to distinguish intraspecific from interspecific variation. It is important to cluster taxonomic units in a biologically meaningful manner, as even slight differences in similarity cut-offs can result in significantly different estimates of richness (Fonseca et al., 2010). Previous studies on both terrestrial and marine nematodes demonstrated that the intra-specific variation can be detected using more stringent thresholds (99%), whereas genera and order can be grouped using more conservative cut-off (97%) (Porazinska et al., 2010; Dell'Anno et al., 2015). In an attempt to test the relationship between groupings at different similarity levels and meiofaunal biodiversity (in terms of higher taxa richness), we clustered Illumina MiSeq sequencing reads at similarity levels (97-99% clustering threshold) typically used in other metabarcoding studies (e.g. Creer et al., 2010; Fonseca et al., 2010, 2014). We here found that different percentage identity cut-offs for OTUs generation (i.e. a different level of similarity for OTU clustering) provided different number of OTUs and estimates of taxon richness. The number of generated OTUs increased with the increasing level of similarity percentage. Although clusters (OTUs) created at 99% cut-off would be the best for achieving species-equivalent level resolution, OTU data overestimated the number of taxa/specimens in the samples (e.g., 145 OTUs vs 9 taxa/specimens). Our results showed that the use of 97% similarity threshold provided a comparable resolution in assigning sequences to the higher taxa level compared to what found at 99% cut-off, but avoided the risk of inflated diversity estimates. Thus, we here suggest that the threshold of 3% is recommended to study meiofaunal biodiversity at higher taxa level.

Linking OTUs to the abundance of meiofaunal higher taxa

High-throughput sequencing abundance data depend on many technical and biological biases and their exploitation for quantitative analyses of biodiversity in ecological studies remains a major issue.

In the present study the total number of OTUs was considerably higher than the actual number of individuals. We found a variable number of OTUs among the eleven samples analyzed, up to 82 using 97% cut-off in the sample constituted by 9 individuals. This discrepancy could be due to i) methodological concerns (i.e., amplification biases of primers) and ii) the presence of pseudogenes and intra-individual polymorphisms in 18S rDNA (Decelle et al., 2014; Dell'Anno et al., 2015), which can lead to inflated

diversity metrics by increasing the number of predicted OTUs (Leray and Knowlton, 2016). It has been demonstrated that marine nematodes are characterized by the presence of polymorphisms among rRNA gene copies. Previous studies reported that the number of OTUs matching nematode species collected from the deep sea varied from 1 to 5 (using 97% cut-off; Dell'Anno et al., 2015). Also for soil nematodes, the number of OTUs for each species ranged from 2 to 95 (using 99% cut-off; Porazinska et al., 2010). In our study, each nematode was represented by a number of OTUs ranging from 1 to 15, at the most conservative clustering threshold used (97%). Not only nematodes, but also Polychaeta were represented by a high number of OTUs ranging from 1 to 11 at 97% clustering threshold. This issue hampers the possibility to use metabarcoding to infer quantitative estimates of biodiversity (Cowart et al., 2015; Dell'Anno et al., 2015; Guardiola et al., 2015; Aylagas et al., 2016). Further studies are needed to elucidate the extent of the polymorphism of 18S rRNA gene in different meiofaunal taxa and among different individuals of the same species/taxon. Moreover, the advancement of HTS platforms along with the possibility to avoid the amplification step will allow us to reduce the errors and thus to provide more confident estimates of the actual OTU number for each species/taxon.

Meiofaunal biodiversity: comparison between morphological versus HTS analyses

Our results showed that metazoa was the most abundant group in terms of number of OTUs and number of reads mapping against OTUs. In particular, meiofaunal taxa had a disproportionally higher abundance when considering relative read numbers, likely due to their bigger sizes, which translate into higher ribosomal DNA presence (Cowart et al., 2015). Non-meiofaunal taxa represented a small portion considering proportion of reads, but they contributed to a higher extend in terms of OTU number.

Metabarcoding analysis uncovered half of the taxa morphologically identified in each sample, letting us to hypothesise that the other 50% i) were present, but the tissue contained degraded DNA; ii) were not amplified with the primers employed, or iii) were not recognized through the taxonomic assignment, as not adequately homologous to taxa present in the reference database. It has been previously demonstrated that the success of PCR amplification and further sequencing steps relies on one side on the amount of DNA and on the other from its purity. Thus, it is crucial to start the metabarcoding workflow from non-degraded DNA, free of potential inhibitors (Dell'Anno et al., 2015). Secondly, at present, most of the metabarcoding studies aiming at analysing meiofaunal biodiversity, are based on amplification and sequencing of 18S rRNA gene. Although this marker present different advantages, suffers limitations related to database incompleteness and primer specificities. Thus, we here suggest that the combined use of 18S rDNA along with other loci, such as COI, ITS or D2/D3 region of 28S rDNA, can provide better estimates of benthic biodiversity (Cowart et al., 2015; Sinniger et al., 2016). Finally, deriving accurate taxonomy using current repositories is difficult due to the paucity and low reliability of public available

taxonomic databases, especially for organisms of small class sizes (Creer et al., 2010; Fonseca et al., 2010; Bik et al., 2012b; Lindeque et al., 2013; Carugati et al., 2015). The compromise between reliability and efficiency of sequence repositories is not easy to reach (Becheler et al., 2014), but the need for comprehensive reference databases should be stressed. This issue is confirmed by the high percentage of "not assigned" OTUs (i.e., uncultured eukaryotes; sequences with hits in the public database, but without an assigned taxonomy) here recovered. All these findings provide evidence of the paucity of public repositories and highlight the need to improve the information contained in databases, linking DNA sequences to formally described species.

Molecular method did not recover all the taxa morphologically identified, but, overall, unmasked a higher amount of diversity, providing a broader view of the taxa present in the community. Comparisons between molecular and morphological surveys showed that a number of taxa, such as Bivalvia, Brachiopoda, Branchiopoda, Cnidaria, Loricifera were recovered only by high-throughput sequencing analysis of 18S rDNA and not by morphological classification. This could be explained considering that molecular surveys are particularly adept at recovering animals common in smaller class sizes, e.g. Loricifera, in addition to identifying eggs or juveniles of larger animals, e.g. Bivalvia, (the so called "temporary meiofauna" typically found in the mesh size <1mm; McIntyre AD, 1969). Metabarcoding surveys may also include the transitory presence of individuals having left biological footprints through partial remains such as feces, scales, mucus, spines, as in the case of Cnidaria (Cowart et al., 2015; Mohrbeck et al., 2015). The presence of small parasites living on the cuticle of meiofaunal taxa, not detectable by using morphological approach, but amplifiable along with the host, can also explain the higher diversity found with metabarcoding analysis (Bhadury et al., 2011; Leray and Knowlton, 2015).

Meiofaunal taxonomic composition

Our results showed that the different taxonomic groups contributed to a different extend to the whole dataset, in terms of both number of OTUs and reads mapping against OTUs. This is not expected since there was one individual per taxon in each sample. Different reasons can be invoked to explain such differences, including i) different successful rate of DNA extraction and amplification of different taxa; ii) primer specificity (leading to unequal amplification success in different taxa) and iii) the presence of polymorphisms among 18S rRNA gene copies.

Within metazoans, the most OTU-rich phylum was Nematoda, followed by Polychaeta. While annelids ranked second in number of OTUs, they dominated when considering proportion of reads. The number of reads depend on differences in rDNA copy number among groups (Medinger et al., 2010; Bik et al., 2012b) and on the potential primer bias (Lejzerowicz et al., 2015). In addition, such differences could be also linked to the size of the organisms, letting us to hypothesise that the relative number of reads

may capture relative biomass of the groups (Guardiola et al., 2015). Further studies are needed to elucidate the relationships between meiofaunal biomass and number of reads, as already carried out for other marine eukaryotes (i.e., zooplankton, Lindeque et al., 2013; Hirai et al., 2015).

Conclusion

The synergies afforded by the advent of HTS platforms (Glenn, 2011) and reference libraries (Pruesse et al., 2007) present an unprecedented opportunity to assess the biodiversity of meiofaunal assemblages. Our findings suggested that a comparison between "double inventories" (morphological and molecular) using standardized protocols is important in order to check the accuracy of molecular protocols for the targeted meiofaunal assemblages. Results presented here support metabarcoding as a reliable approach for expeditiously uncovering meiofaunal biodiversity at higher taxa level.

Beyond its many advantages, the routine application of metabarcoding for benthic monitoring requires overcoming some limitations. The main shortcomings involve the incompleteness of reference sequence databases. Despite considerable barcoding efforts, reference sequences are still very rare for benthic meiofaunal species. In order to overcome limitations related to the paucity of database and primer specificities, it is also important that molecular workflow includes more than one marker gene. It is necessary to implement our knowledge on gene copy numbers and polymorphisms among individuals belonging to the same taxon and to explore the relationship between read numbers and biomass. With this new information, it will be possible to get quantitative ecological inferences of biodiversity by using HTS technology.

Finally, given the cosmopolitanism and high abundance of meiofauna in marine habitats, it is crucial to further describe potential meiofaunal bioindicator taxa not only through their genetic identification but also to specify their ecological values and functions.

If these gaps will be filled in the near future, metabarcoding could be routinely applied in monitoring programs. Until then, the synergistic relationship between molecular and morphological identifications could provide better evaluations of the actual marine biodiversity.

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3.2 Unveiling the Biodiversity of Deep-Sea Nematodes through Metabarcoding: Are We Ready to Bypass the Classical Taxonomy?

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* These authors have contributed equally to this work

Abstract

Nematodes inhabiting benthic deep-sea ecosystems account for >90% of the total metazoan abundances and they have been hypothesised to be hyper-diverse, but their biodiversity is still largely unknown. Metabarcoding could facilitate the census of biodiversity, especially for those tiny metazoans for which morphological identification is difficult. We compared, for the first time, different DNA extraction procedures based on the use of two commercial kits and a previously published laboratory protocol and tested their suitability for sequencing analyses of 18S rDNA of marine nematodes. We also investigated the reliability of Roche 454 sequencing analyses for assessing the biodiversity of deep-sea nematode assemblages previously morphologically identified. Finally, intra-genomic variation in 18S rRNA gene repeats was investigated by Illumina MiSeq in different deep-sea nematode morphospecies to assess the influence of polymorphisms on nematode biodiversity estimates. Our results indicate that the two commercial kits should be preferred for the molecular analysis of biodiversity of deep-sea nematodes since they consistently provide amplifiable DNA suitable for sequencing. We report that the morphological identification of deep-sea nematodes matches the results obtained by metabarcoding analysis only at the order-family level and that a large portion of Operational Clustered Taxonomic Units (OCTUs) was not assigned. We also show that independently from the cut-off criteria and bioinformatic pipelines used, the number of OCTUs largely exceeds the number of individuals and that 18S rRNA gene of different morpho-species of nematodes displayed intra-genomic polymorphisms. Our results indicate that metabarcoding is an important tool to explore the diversity of deep-sea nematodes, but still fails in identifying most of the species due to limited number of sequences deposited in the public databases, and in providing quantitative data on the species encountered. These aspects should be carefully taken into account before using metabarcoding in quantitative ecological research and monitoring programmes of marine biodiversity.

Keywords: deep-sea ecosystems, nematode biodiversity, DNA extraction, high-throughput sequencing

Introduction

Field and theoretical studies increasingly argue that biodiversity influences ecosystem functions that are responsible for the production of natural goods and services for the entire biosphere (e.g., biomass production and nutrient regeneration; Cardinale et al., 2012). This appears to be particularly evident in the deep sea, where positive interactions (e.g., facilitation) apparently dominate among interspecific ecological interactions (Danovaro et al., 2008). Thus, the ability to assess biodiversity over large spatial and temporal scales is crucial for a better understanding of the potential consequences of species loss on ecosystem functioning.

Deep-sea ecosystems cover ca. 65% of the surface of the globe and host a large fraction of the global biodiversity (Appeltans et al., 2012; Danovaro et al., 2014). In benthic deep-sea ecosystems, nematodes, which account for >90% of the total metazoan abundances (Vincx et al., 1994; Danovaro et al., 2014) are probably the best model organisms for exploring the relationship between biodiversity and ecosystem functioning (Danovaro et al., 2008; Snelgrove et al., 2014). Nematodes have been hypothesised to be hyper-diverse, and potentially representing one of the most diverse animal phyla (Lambshead, 2004), but at present, their biodiversity is still largely unknown. Despite many nematode taxa are morphologically highly diverse (De ley, 2006), the diversity at genus and species level often remains under-appreciated being time consuming and requiring highly specialised taxonomic expertise (Platt and Warwick, 1988; Deprez, 2005). In addition, the use of morphological criteria can prevent the correct identification of nematode species since some of them show phenotypic plasticity and single morpho-species can hide cryptic diversity (De Ley et al., 1999; Derycke et al., 2005, 2007, 2010). Molecular methods can solve these uncertainties and accelerate the difficult morphological identification of marine nematode species, thus allowing us to address key questions on cosmopolitanism, cryptic biodiversity and phylogeny (Meldal et al., 2007; Bhadury et al., 2008). DNA barcoding, based on the analysis of nuclear and mitochondrial genes, has been proposed since a decade for the assessment of the diversity of marine nematodes (DeLey et al., 2005; Bhadury et al., 2006). Molecular markers such as the nuclear small subunit and large subunit ribosomal RNA genes (i.e. 18S rRNA, 28S rRNA) and the mitochondrial cytochrome oxidase subunit 1 gene (COI) have been selected for nematode barcoding studies (Deprez, 2005; Bhadury et al., 2006; Derycke et al., 2010). Linking molecular and morphological identification of nematode species can overcome taxonomic uncertainties and create a link between ecological/morphological investigations and community-based DNA analyses (Floyd et al., 2002, 2005; De Ley et al., 2005; Bhadury et al., 2006; Bik et al., 2010; Derycke et al., 2010). However, standard barcoding approaches based on Sanger sequencing are not appropriate for large-scale ecological investigations. In the last years, we are witnessing the rise in the use of High-Throughput Sequencing (HTS) platforms for the study of biodiversity of tiny metazoans, including nematodes, using homologous gene regions (Creer et al., 2010;

Fonseca et al., 2010; 2014; Porazinska et al., 2010a, 2012). Nevertheless, the application of molecular tools to study benthic deep-sea nematodes is still in its infancy (Bik et al., 2010; 2012), partly due to the difficulty in collecting deep-sea nematodes and amplifying their DNA (Lecroq et al., 2011). Thus there is an urgent need to identify reliable procedures for the recovery of DNA from marine nematodes suitable for sequencing analyses. Another critical issue is to estimate the number of nematode species and their relative proportion in a sample from the number of Operational Clustered Taxonomic Units (OCTUs) obtained from HTS (Porazinska et al., 2009, 2010a, b). Terrestrial nematodes have a number of rRNA gene copies which can vary dramatically between species (Bik et al., 2013), hampering the possibility to correlate the number of OCTUs with the number of individuals. This issue has never been investigated in marine nematode assemblages.

In this study we compared, for the first time, different procedures for the recovery of DNA from marine nematode cultures and free-living nematodes collected from both coastal and deep-sea sediments suitable for Sanger and HTS analyses. Once extraction and amplification conditions of DNA were optimised, we investigated the reliability of Roche 454 sequencing analyses for assessing the biodiversity of deep-sea nematode assemblages (collected at two different benthic deep-sea sites) previously morphologically identified. Finally, intra-genomic variation in 18S rRNA gene repeats was investigated for different deep-sea nematode morphospecies in order to assess the influence of polymorphisms on nematode biodiversity estimates.

Materials and methods

Ethics statement

All of the field activities were approved by the local national authorities (Spanish and Italian Ministry). The sampling areas were not privately owned or protected in any way, and no endangered or protected species were involved in this study.

Sample collection and processing

Three cultured species of marine nematodes and free-living nematodes collected from coastal and deep-sea sediments were used to identify the most efficient procedure for the recovery of DNA suitable for Sanger and HTS analyses. Nematodes belonging to the species *Diplolaimelloides oschei*, *Pellioditis marina* and *Plectus sp.* were grown in Petri dishes containing 0.75% bacto-agar medium and maintained under controlled conditions at 20°C in the dark (Moens and Vincx, 1998). Benthic shallow-water nematodes were extracted from sediment samples collected using manual corers at ca. 2 m depth in the central Adriatic Sea (Mediterranean Sea). Deep-sea nematodes were recovered from sediment samples collected using a NIOZ-type box-corer in the NW Mediterranean Sea (Gulf of Lions) and in the Central

Mediterranean Sea (Sicily Channel) both at ca. 500 m depth. After collection, sediment samples were stored without the use of any preservative at −20°C until further processing. Nematodes were recovered from the sediments using a 20 μm mesh net and then the fraction retained on the sieve was resuspended and centrifuged 3 times using Ludox HS40 (density 1.31 g cm^{-3}; Heip et al., 1985). After centrifugation, all meiobenthic animals were sorted under a stereomicroscope. Then, each nematode was picked by using a sterile needle, and temporarily mounted on a slide using a drop of autoclaved MilliQ water and covered with a cover slip. Each nematode has been identified under light microscope to the genus or species-level when possible (indicated as sp1, sp2, sp3, etc., due to the presence of several unknown deep-sea species) according to previous studies (Platt and Warwick, 1983, 1988) and the recent literature dealing with new nematode genera and species (i.e. NeMys database; Deprez, 2005). After identification, each specimen was carefully picked from the slide and placed into a 2 ml sterile tube until further molecular analysis. Microscopic observations revealed that the storage at -20°C does not alter the morphological features used for taxonomic classification.

Morphological and molecular analyses on single nematodes

To test for DNA extraction efficiency, different protocols were applied on single nematodes belonging to the same species and with a similar size extracted from the deep-sea and coastal sediments, and from cultures. Among free-living nematodes, individuals of *Sphaerolaimus uncinatus* were selected because this is one of the few formally described deep-sea nematode species (Vincx et al., 1994) and it is distributed across different benthic habitats and ecosystems (Deprez, 2005). A total of 60 individuals, including: i) 10 organisms belonging to each of the three different species of cultured nematodes, ii) 15 individuals from benthic shallow systems and iii) 15 from deep-sea sediments, were processed and used for assessing DNA extraction efficiency. DNA from a single nematode was extracted using three different methods: i) a physical-chemical procedure (hereafter defined as "NaOH procedure"), ii) a commercial kit for tissue DNA extraction (QIAGEN DNeasy Blood & Tissue Kit), and iii) a commercial kit for DNA extraction from soils (PowerSoil DNA Isolation Kit, MoBio). The physical-chemical procedure was based on a method previously utilised for DNA extraction from nematodes (Floyd et al., 2002) with slight modifications. Briefly, nematodes were added to 20 μL of 0.25 M NaOH and incubated overnight at -20°C. After incubation samples were heated at 60 °C for 3 h followed by an incubation at 99 °C for 3 min and then 4 μL HCl (1M), 10 μL Tris-HCl (0.5M, pH 8.0) and 5 μL Triton X-100 (2%) were added. Samples were again incubated at 99 °C for 3 min and then stored at –20 °C until further analysis (i.e. PCR amplification). DNA extraction from individual nematodes using the commercial kits (hereafter defined as QIAGEN kit and MoBio kit) was carried out following the instructions provided by the manufactures. After extraction, DNA was analysed fluorometrically using a NanoDrop ND-3300 Fluorospectrometer

and SYBR Gold (Invitrogen) as fluorochrome. The fluorescence of DNA in the sample was converted into concentrations using calibration curves obtained from standard solutions of calf thymus DNA (from 2 to 50 pg μL^{-1}). Differences in DNA concentrations obtained by using the different extraction procedures were checked by using the Mann–Whitney U test. To assess whether the extracted DNA was amplifiable, PCR analyses of DNA from single nematodes were carried out using the primer pairs Nem_18S_F (5'-CGCGAATRGCTCATTACAACAGC-3') and Nem_18S_R (5'-GGGCGGTATCTGATCGCC-3') (Floyd et al., 2005) suitable for Sanger sequencing (amplicon length 900 bp) which is commonly used for phylogeographic and cryptic diversity analyses of nematodes. Details on PCR analyses are reported in the Supporting Information, Appendix S1. Sanger sequencing analyses were carried out by MACROGEN sequencing service (Macrogen Inc., Europe). Since the procedure based on the QIAGEN kit applied on deep-sea nematodes investigated in the present study provided DNA which was always successfully amplified when compared to the NaOH procedure (see the result section), all subsequent molecular analyses carried out on deep-sea nematode assemblages were based on the use of the QIAGEN kit.

Morphological and molecular analyses on deep-sea nematode assemblages

To test the consistency and reliability of metabarcoding, nematodes extracted from deep-sea sediments were randomly picked, identified to species level (Supporting Information, Table S1 and S2) and then pooled before DNA extraction. DNA extraction was carried out on two assemblages of 10 and 100 individuals recovered from sediment samples collected in the NW Mediterranean and Central Mediterranean, respectively. Once extracted and purified, DNA was amplified using the primer pairs SSUF04 (5'-GCTTGTCTCAAAGATTAAGCC-3') and SSUR22 (5' –CCTGCTGCCTTCCTTGGA-3') (Blaxter et al., 1998) targeting the 18S rRNA gene and suitable, in term of amplicon length (450 bp), for metabarcoding analyses, according to the needs of the sequencing platforms utilised in the present study (see below). PCR reactions were carried out using the conditions and thermal protocol described in the Supporting Information, Appendix S1, but using ca. 1 ng DNA as template. Sequencing analysis was performed on a Roche 454 GS FLX Titanium platform by using Lib-L kit, by MACROGEN sequencing service (Macrogen Inc., Korea).

Intra-genomic variability of 18S rRNA gene repeats within deep-sea nematodes

To investigate the intra-genomic variability of 18S rRNA gene repeats in marine nematodes, 5 different morphospecies have been collected from deep-sea sediments of the Central Mediterranean Sea, and DNA was extracted from each specimen using the QIAGEN kit. The 18S rRNA gene was amplified using the primer set SSUF04 (5'-GCTTGTCTCAAAGATTAAGCC-3') and SSUR22 (5' – CCTGCTGCCTTCCTTGGA-3') (Blaxter et al., 1998) and sequenced on the Illumina MiSeq platform (2 × 300 bp reads). The Illumina MiSeq platform has been selected because it has a sequencing error rate lower than Roche 454 (Glenn, 2011), thus providing a more robust view of the actual variability of 18S rRNA gene repeats. Illumina MiSeq sequencing analysis was carried out by LGC Genomics (Germany).

Bioinformatic analyses

Sanger sequences. Sequences obtained (both forward and reverse) by the Sanger procedure were manually checked using BioEdit v.7.2.0. Similarity search analysis of these sequences were performed against the GenBank database using MEGABLAST with default parameters.

Roche 454 data. Reads obtained by Roche 454 sequencing were processed using three different bioinformatic pipelines: i) OCTUPUS (Fonseca et al., 2010), ii) AmpliconNoise (Quince et al., 2011), as implemented in Mothur (Schloss et al., 2009) and iii) QIIME (Caporaso et al., 2010). Using OCTUPUS, after a quality check of sequences using Lucy (Chou and Holmes, 2001) with default parameters and screening for a minimum length of 200 bp, sequences were clustered in OCTUs (Operational Clustered Taxonomic Units) both at 97% and 99% similarity to generate a list of consensus OCTUs. The obtained OCTUs were then BLAST-matched against the nucleotide GenBank and SILVA NR 119 databases. Taxonomic assignments for each OCTU were derived from the top-scoring BLAST match (exhibiting >90% pairwise identity). All OCTUs were analysed for the presence of putative chimeras using a frequency and length dependent algorithm incorporated into the OCTUPUS pipeline. All OCTUs identified as chimeras were removed from the analysis.

The same reads obtained from Roche 454 sequencing were also analysed using the AmpliconNoise (Quince et al., 2011), as implemented in Mothur (Schloss et al., 2009), to remove 454 sequencing errors and PCR single base errors using default settings. Cleaned reads were analysed with Mothur using Perseus (Quince et al., 2011) to detect chimeras and the furthest neighbour method for clustering. OCTUs obtained at 97% and 99% clustering thresholds were BLAST-matched against the nucleotide GenBank and SILVA NR 119 databases and the top-scoring BLAST hit (exhibiting >90% pairwise identity) was used for taxonomic assignment.

Finally, the raw reads obtained from Roche 454 sequencing were analysed using QIIME pipeline (Caporaso et al., 2010). Screening for a minimum length of 200 bp was performed by using split.libraries.

Sorting, chimera detection and OCTUs picking were performed by using the USEARCH tool (Edgar, 2010) implemented within the QIIME pipeline (Caporaso et al., 2010). OCTUs obtained at 97% and 99% clustering thresholds were BLAST-matched against the Genbank and SILVA NR 119 databases and the top-scoring BLAST hit (exhibiting >90% pair-wise identity) was used for the taxonomic assignment.

A χ2-test with Yates continuity correction was used to check for the presence of statistical differences between the relative contributions of OCTUs assigned to nematode genera by metabarcoding and the relative contribution of individuals of the same genera identified by morphological analysis.

Illumina MiSeq data. QIIME pipeline was used to analyse the Illumina MiSeq sequences obtained from the five nematode morpho-species. Screening for a minimum length of 200 bp was performed by using the PRINSEQ tool (Schmieder and Edwards, 2011). Sorting, chimera detection and OCTUs picking were performed by using the USEARCH tool (Edgar, 2010) implemented within the QIIME pipeline (Caporaso et al., 2010). OCTUs obtained at 97% and 99% clustering thresholds were BLAST-matched against the GenBank database and the top-scoring BLAST hit (exhibiting >90% pairwise identity) was used for the taxonomic assignment.

OCTUs distribution among different taxonomic groups identified by using both Roche 454 and Illumina MiSeq sequencing were analysed by using MEGAN software (Huson et al., 2011), with default parameters.

Results

Recovery of DNA from single nematodes

DNA concentrations of cultured nematodes extracted using the QIAGEN kit were significantly higher than those obtained using the NaOH procedure ($p<0.01$; Fig. 1A). The QIAGEN kit and the NaOH procedure provided DNA concentrations from coastal nematodes 3-4 fold higher than using the MoBio kit (Fig. 1B). DNA concentrations of nematodes collected from deep-sea sediments were significantly higher using the QIAGEN kit than using the two other DNA extraction procedures (Fig. 1B).

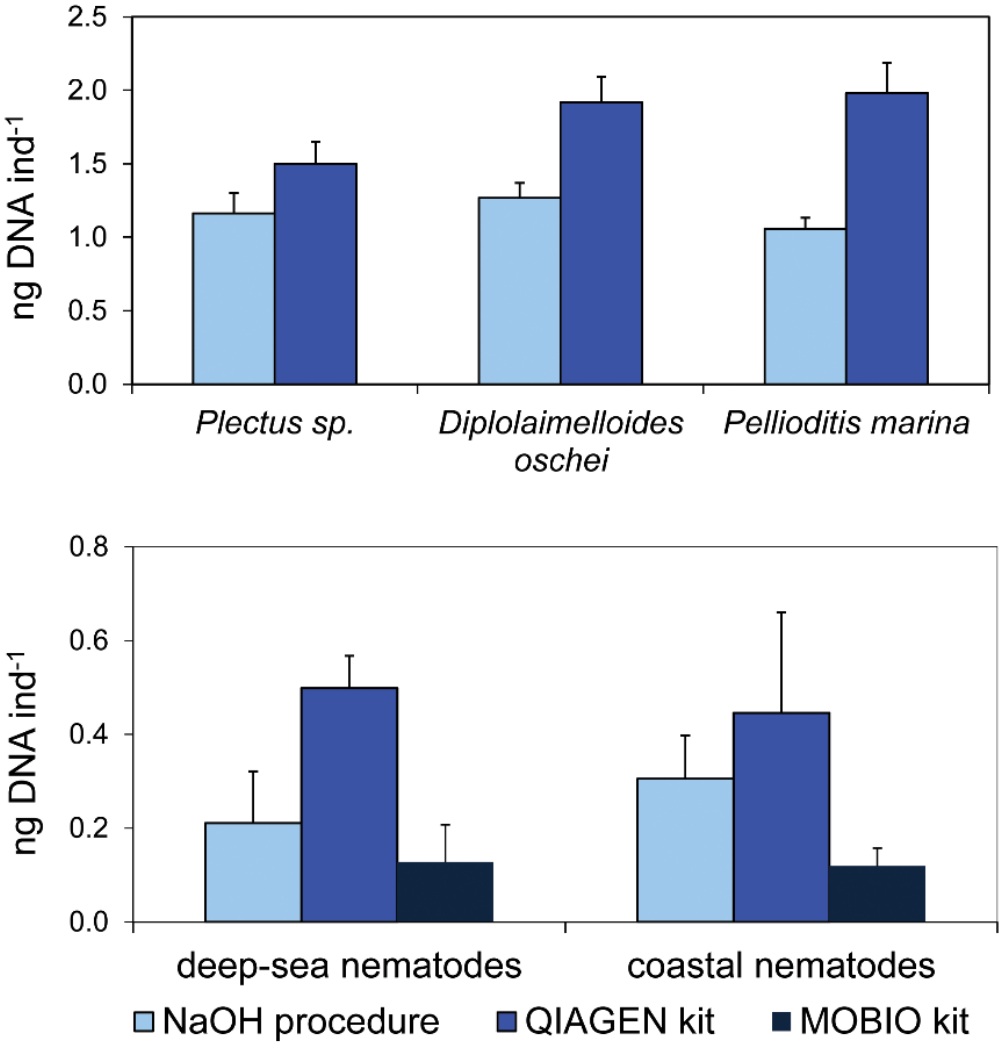

Figure 1. Comparison between DNA concentrations obtained by different DNA extraction procedures from wild and cultured nematodes. (a) Comparison of DNA concentrations obtained using NaOH and QIAGEN kit extraction procedures from cultured nematodes (i.e. *Plectus* sp., *Diplolaimelloides oschei* and *Pellioditis marina*). (b) Comparison of DNA concentrations obtained using NaOH procedure, QIAGEN and MoBio kits from coastal and deep-sea nematodes. Mean (n=5) and standard deviations are reported.

Although DNA extracted from coastal nematodes with both the commercial kits and the NaOH procedure resulted in a successful amplification of 18S rRNA gene (Supporting Information, Fig. S1), the success of PCR amplification of DNA obtained from deep-sea nematodes by using the NaOH procedure was highly stochastic (even after several modifications including the addition of bovin serum albumin (BSA) to the reaction mix and tuning the PCR thermal protocol; Supporting Information, Fig. S2a). This was not the case for the QIAGEN and MoBio kits, which always provided consistent and highly reproducible results in terms of 18S rRNA amplicons suitable for Sanger and HTS analyses (Supporting Information, Fig. S2b and S3).

Biodiversity assessment: comparison between morphological vs. molecular approaches

The list of samples analysed by using Sanger, Roche 454 and Illumina MiSeq sequencing is reported in Table 1.

Table 1. List of samples analysed by using Sanger, Roche 454 and Illumina MiSeq sequencing, and the database against which the BLAST search has been performed. The number of raw reads obtained, the number of reads obtained after trimming (sorting and chimera removal) and the bioinformatic pipelines utilised for data analysis are also reported. na=not applicable.

	Samples	Number of reads	Number of reads after trimming	Pipeline	Database
Sanger	15 deep-sea nematodes	na	na	na	GenBank
	15 shallow-water nematodes	na	na	na	GenBank
	30 cultured nematodes	na	na	na	GenBank
Roche 454	10 deep-sea nematodes from NW Mediterranean	56851	54949	OCTUPUS	GenBank and Silva
			1484	Mothur	GenBank and Silva
			23390	QIIME	GenBank and Silva
Roche 454	100 deep-sea nematodes from Central Mediterranean Sea	61647	58186	OCTUPUS	GenBank and Silva
			3518	Mothur	GenBank and Silva
			24747	QIIME	GenBank and Silva
Illumina MiSeq	*Halichoanolaimus sp1*	237034	2526	QIIME	GenBank
	Sabatieria sp1	157511	2282	QIIME	GenBank
	Metachromadora sp1	232452	3900	QIIME	GenBank
	Viscosia sp1	67677	2200	QIIME	GenBank
	Thoracostoma sp.	195381	2190	QIIME	GenBank

Analyses on single nematodes. The analysis of Sanger sequences obtained from each individual of cultured nematodes, based on a similarity search on GenBank, confirmed the morphological identification. Similarity search of Sanger sequences obtained from each individual of deep-sea nematodes morphologically classified as *Sphaerolaimus uncinatus* indicated 93% similarity with both *Sphaerolaimus hirsutus* and *Terschellingia longicaudata*. The similarity found with *T. longicaudata* could be explained considering that this species has a sister relationship with *S. hirsutus* (e.g., 18S rDNA sequences of *T. longicaudata* from Ras al Barr- Bahrain- are 99% identical with 18S rDNA sequences of S. hirsutus) (Bhadury et al., 2008).

Analyses on deep-sea nematode assemblages from the NW Mediterranean Sea. Roche 454 sequencing generated a total of 56,851 reads (average length 355 bp) from the assemblage of 10 nematodes recovered from the NW Mediterranean Sea (Table 1). The number of OCTUs obtained after chimera removal and the number of chimeras are reported in Table 2.

Table 2. Summary of clustering analysis carried out with OCTUPUS and Mothur pipelines at 97% and 99% thresholds for nematode assemblages recovered from deep-sea sediments of the NW and Central Mediterranean Sea. In parenthesis the percentage of chimeras on the total OCTUs detected.

	% of clustering	OCTUs without chimeras	Chimeras
OCTUPUS			
10 nematodes (NW Mediterranean)	97%	359	325 (48%)
10 nematodes (NW Mediterranean)	99%	5356	1186 (18%)
100 nematodes (Central Mediterranean)	97%	1359	1151 (46%)
100 nematodes (Central Mediterranean)	99%	7128	2806 (28%)
MOTHUR			
10 nematodes (NW Mediterranean)	97%	666	345 (34%)
10 nematodes (NW Mediterranean)	99%	1346	345 (20%)
100 nematodes (Central Mediterranean)	97%	2096	1531 (42%)
100 nematodes (Central Mediterranean)	99%	3177	1553 (33%)

BLAST results against the Genbank database indicated the presence of OCTUs not only with hits for Nematoda, but also for Fungi, Plantae (i.e., Viridiplantae) and Protostomia (Fig. 2). Especially with a clustering threshold of 97%, 115 OCTUs out of 359 (~ 32%) were assigned to the phylum Nematoda. The number of OCTUs assigned to nematodes decreased considerably when using the 99% cut-off (~ 20%) and the number of "not assigned" OCTUs (i.e., OCTUs with hits in the public database but without an assigned taxonomy; e.g., uncultured eukaryotes) increased (Fig. 2; Supporting Information, Table S3). Indeed, the contribution of "not assigned" OCTUs to the total OCTUs was 17% using 97% clustering threshold and 41% using 99%. A fraction of OCTUs (18% and ~5% for 97% and 99% clustering threshold, respectively) displayed no significant match ("no hit", sequence identity <90%) to known ribosomal sequences (Fig. 2; Supporting Information, Table S3).

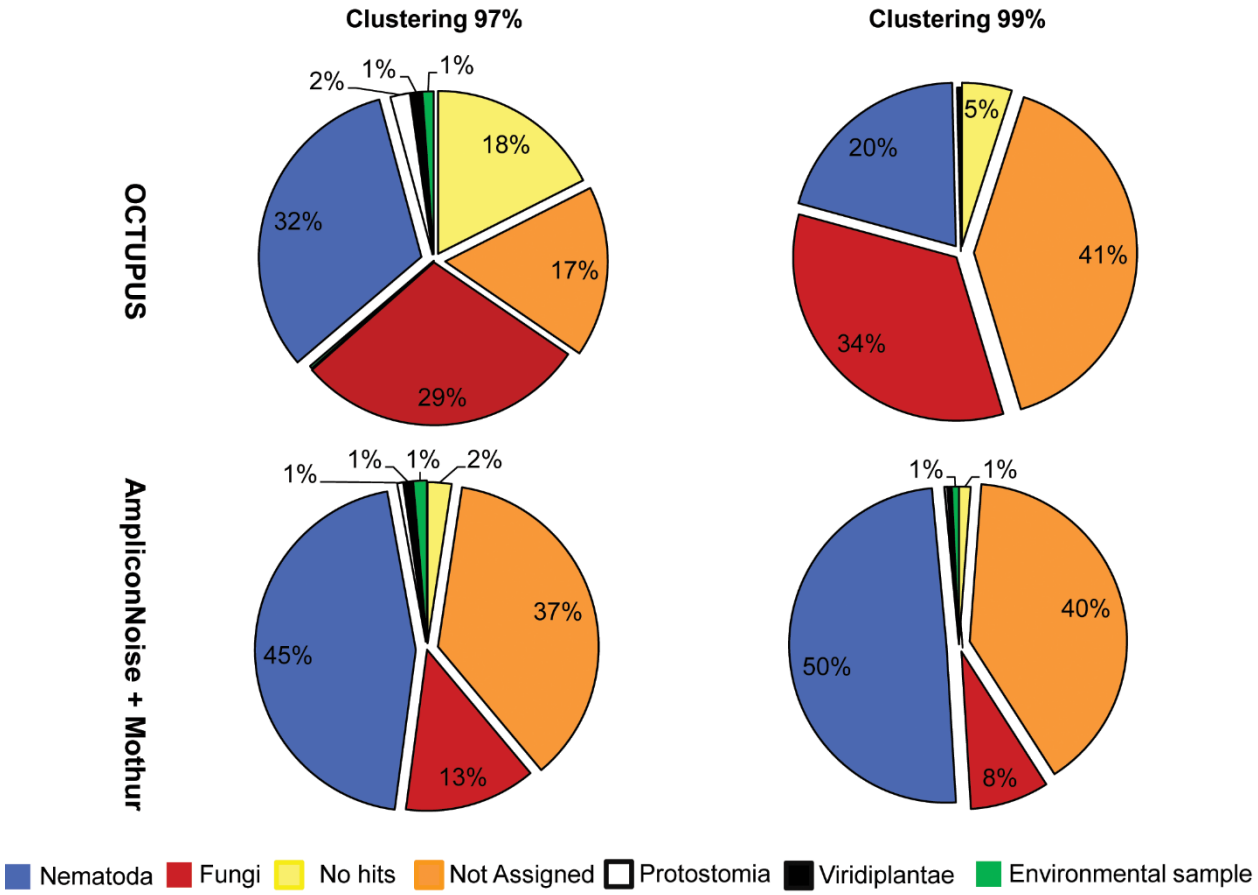

Figure 2. Eukaryotic taxa identified by metagenetic analysis of the nematode assemblage (10 individuals) collected in the NW Mediterranean Sea. Reported are the relative contribution of eukaryotic taxa obtained at 97% and 99% clustering thresholds using the OCTUPUS pipeline and AmpliconNoise plus Mothur programs. The contributions are calculated from non-chimeric Operational Clustered Taxonomic Units (OCTUs) representing forward sequencing reads from 18S rRNA gene. The contributions of the most important components (expressed as percentage) are reported.

The analysis of OCTUs at the genus level (Fig. 3) highlighted the presence of 5 out of 6 genera morphologically identified (the 18S rRNA of *Hopperia sp.* was lacking in the GenBank database) with a different contribution of OCTUs assigned to each genus depending on the clustering threshold used (Supporting Information, Table S4). The relative contribution of OCTUs assigned to *Sabatieria* and *Sphaerolaimus* to the total OCTUs deviated significantly from the contribution of the individuals belonging to these two genera morphologically identified (Table 3).

Table 3. Comparison between the relative contributions of OCTUs at 99% clustering threshold assigned to nematode genera by metabarcoding (using the OCTUPUS pipeline) and the relative contribution of individuals of the same genera identified by morphological analysis. The χ^2-test has been performed with Yates continuity correction to evaluate the significance of the deviation between the two values. *Hopperia* sp. was not identified by sequencing. na= not applicable.

Genus	Molecular identification	Morphological identification	χ^2-test 95% *P*-value
Sabatieria	10%	40%	**0.01**
Setosabatieira	15%	10%	0.994
Sphaerolaimus	48%	10%	**0.03**
Syringolaimus	11%	20%	0.682
Halalaimus	16%	10%	0.956
Hopperia	0%	10%	na

As far as the comparison at the species level is concerned (Fig. 3), the molecular analysis allowed to identify two or three different species (for 97 and 99% clustering threshold, respectively) of *Halalaimus* (although not formally described), although only one morpho-species of *Halalaimus* was included in the nematode assemblage. On the contrary, by using the molecular approach only one species of *Sabatieria* out of four was identified. BLAST search analysis against the Genbank database of reads obtained using the Mothur pipeline indicated the presence of Fungi besides the Nematoda phylum, but the number of OCTUs assigned to this group was much lower (13 and 8% at 97% and 99% clustering threshold, respectively) with respect to the OCTUPUS results. Moreover, the taxonomic differences detected by OCTUPUS when using different clustering thresholds, were not so evident for reads analysed with Mothur (Fig. 2). Considering a clustering threshold of 97%, 300 OCTUs (out of 666, 45%) were assigned to Nematoda, while at a 99% threshold 666 OCTUs (out of 1346, 50%) were identified as Nematoda. OCTUs labelled as "not assigned" represented also in this case a high proportion (37% and 40% at 97% and 99% clustering threshold), while "no hit" OCTUs decreased drastically (1-2%; Fig. 2; Supporting Information, Table S3). At the genus level OCTUs cleaned by using AmpliconNoise yielded a more faithful representation of the genera proportion derived from morphological identification. *Sabatieria* genus represented indeed 14% of the total nematode abundance, *Setosabatieria* sp. the 31%, *Sphaerolaimus* sp. the 29%, *Syringolaimus* sp. 15% and *Halalaimus* sp. 11%. These proportions were not significantly different from those obtained from morphological classification (χ^2-test with Yates continuity correction, ns). However, the use of AmpliconNoise did not improve the identification at the species level, since only one species of *Sabatieria* out of the four morphologically identified were detected. Bioinformatic analyses performed using QIIME resulted in a lower number of genera (4 out of 6 genera morphologically identified) compared to that found using OCTUPUS and Mothur, at both 97% and 99% clustering thresholds.

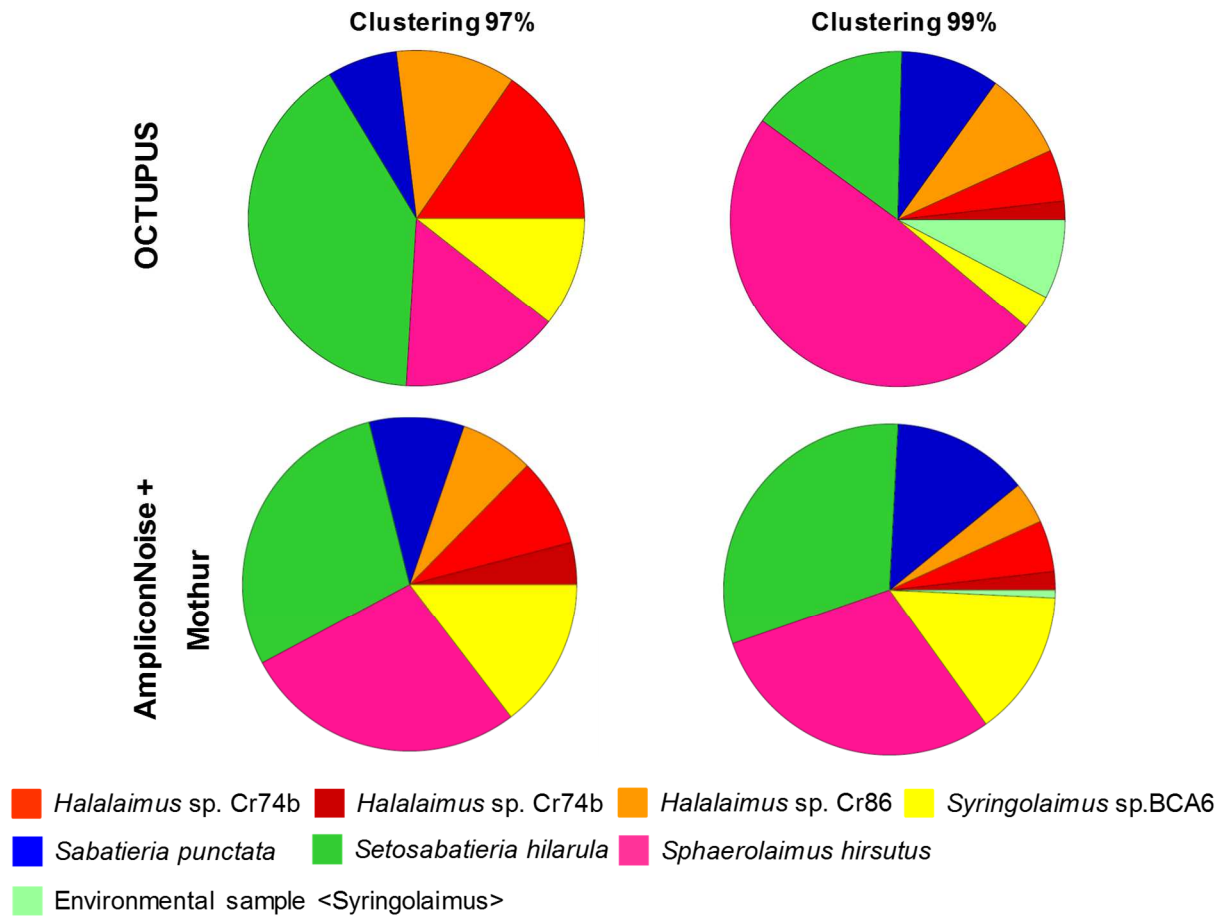

Figure 3. Relative proportion of OCTUs belonging to different nematode species within the nematode assemblage (10 individuals) collected in the NW Mediterranean Sea. Reported are the relative contribution of the different nematode genera obtained at 97% and 99% clustering thresholds using the OCTUPUS pipeline and AmpliconNoise plus Mothur programs. The contributions are calculated from non-chimeric OCTUs representing forward sequencing reads from 18S rRNA gene.

Analyses on deep-sea nematode assemblages from the Central Mediterranean Sea. Roche 454 sequencing analysis generated a total of 61,647 reads (average length 353bp) from the assemblage of 100 nematodes collected from the Central Mediterranean (Table 1). The number of OCTUs obtained by using OCTUPUS and Mothur pipelines after removing chimeras and the number of chimeras are reported in Table 2. By using OCTUPUS at 99% clustering threshold, 61% of OCTUs was assigned to Nematoda, whereas the number of OCTUs identified as both "not assigned" and "no hit" represented a relatively high percentage of the assemblage (24% and 13%, respectively; Fig. 4; Supporting Information, Table S3). At a 97% clustering threshold a higher percentage of "no hit" OCTUs was detected (26%; Fig. 4), but the number of "not assigned" OCTUs was lower (18%; Fig. 4; Supporting Information, Table S3). Bioinformatic analysis of reads performed with the Mothur pipeline indicated that the percentage of organisms different from Nematoda (i.e., Fungi) was very low (ca. 1% at 97% and 99% clustering thresholds) whereas the percentages of "not assigned" and "no hit" OCTUs were 23-25% and 14-15%,

respectively (Figure 4; Supporting Information, Table S3). In general, the two different methods provided similar results in terms of assignment at higher taxonomy level (i.e. order).

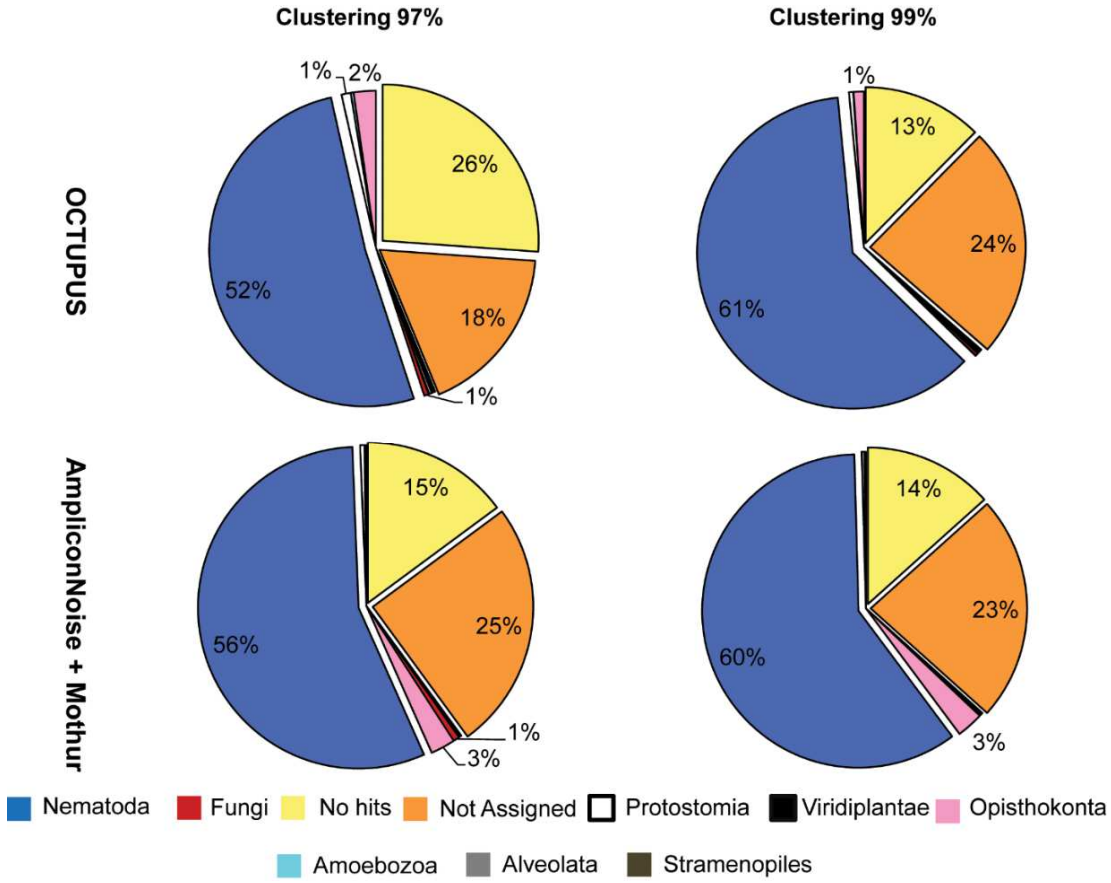

Figure 4. Eukaryotic taxa identified by metagenetic analysis of the nematode assemblage (100 individuals) collected in the Central Mediterranean Sea. Reported are the relative contribution of eukaryotic taxa obtained at 97% and 99% clustering thresholds using the OCTUPUS pipeline and AmpliconNoise plus Mothur programs. The contributions are calculated from non-chimeric Operational Clustered Taxonomic Units (OCTUs) representing forward sequencing reads from 18S rRNA gene. The contributions of the most important components (expressed as percentage) are reported.

Morphological analysis of nematodes collected in the Central Mediterranean allowed to identify 35 morpho-species (Supporting Information, Table S2), whereas taxonomic identification of OCTUs performed with the MEGAN software allowed to retrieve 20 and 33 species using 97% and 99% clustering threshold, respectively by using OCTUPUS and 26 and 30 species using Mothur (Fig. 5; Supporting Information, Table S4). OCTUs obtained with OCTUPUS at 99% clustering threshold correctly detected 6 out of 6 nematode orders (with two further orders lacking using the morphological classification). OCTUs were classified in 19 families (Supporting Information, Table S4) and 1 super-families, and 13 out of 17 families identified through the morphological analysis were properly assigned. Similarly the analysis at the 97% threshold allowed identifying the same orders detected with 99% threshold, but only 11 out of 17 families were assigned (Supporting Information, Table S4). At 99% clustering threshold, 17

genera out of 27 were properly identified. The results obtained with Mothur provided comparable results: both at 97% and 99% clustering, 6 out 6 nematode orders were correctly identified (with three further orders lacking from morphological classification). OCTUs were classified in 12 and 14 families at 97% and 99% clustering threshold respectively (Supporting Information, Table S4), and included 9 out of 17 families detected with the morphological analysis at both cut-offs. Using 99% clustering threshold, 14 genera out of 27 were correctly identified.

The bioinformatic analysis of reads performed using the QIIME pipeline produced a lower number of OCTUs (162 and 191 OCTUs at 97% and 99% cut-off, respectively) compared to that obtained using OCTUPUS and Mothur pipelines. Moreover, analyses using QIIME at 99% clustering threshold provided a lower number of genera (i.e., 16 genera) than using Mothur and OCTUPUS (i.e., 20 and 22 genera, respectively) by using the same taxonomic assignment method (e.g. GenBank BLAST match identity analysis and MEGAN; Carporaso et al., 2010).

BLAST match analyses against the SILVA NR 119 database of OCTUs derived from the OCTUPUS pipeline provided a lower number of genera (i.e., 16 genera at 99% cut-off) compared to that found using the GenBank database (i.e., 22 genera at 99% cut-off; Supporting Information, Table S4). BLAST match analyses against the SILVA NR 119 database of the OCTUs obtained through the Mothur pipeline detected the same number of genera as the GenBank database (i.e., 19 genera at 99% cut-off).

Intra-genomic variability of 18S rRNA gene repeats of deep-sea nematodes

The total number of reads (average length 362 bp) obtained from Illumina MiSeq sequencing and the number of reads remained after trimming for each sequenced nematode morphospecies are reported in Table 1. The 97% and 99% clustering similarity thresholds allowed obtaining 15-102 and 18-108 OCTUs, respectively. The number of OCTUs assigned to the different morphospecies of deep-sea nematodes ranged from 1 to 5 at 97% and from 1 to 18 at 99% cut-off (Table 4).

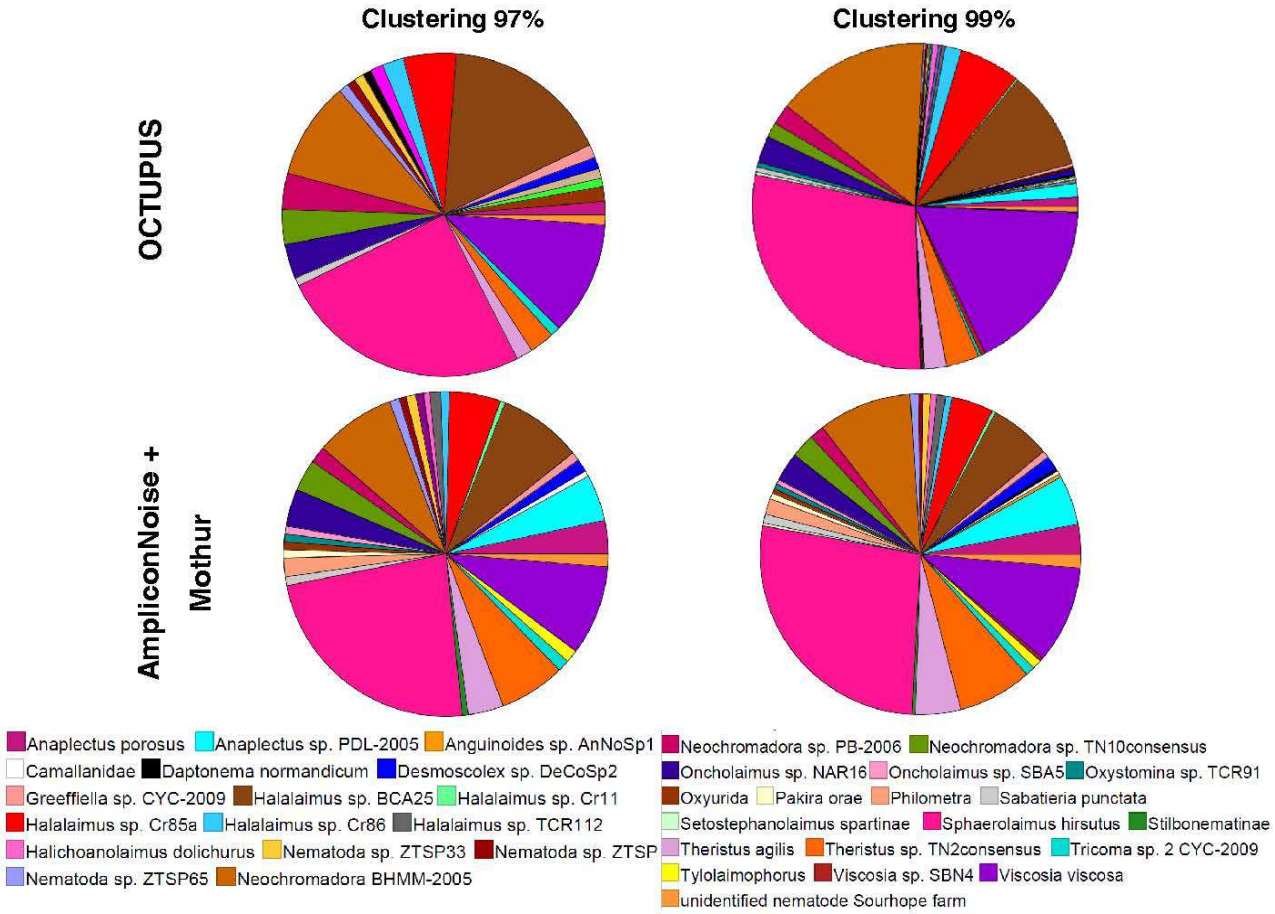

Figure 5. Relative proportion of OCTUs belonging to different nematode species within the nematode assemblage (100 individuals) collected in the Central Mediterranean Sea. Reported are the relative contribution of the different nematode species obtained at 97% and 99% clustering thresholds using the OCTUPUS pipeline and AmpliconNoise plus Mothur programs. The contributions are calculated from non-chimeric OCTUs representing forward sequencing reads from 18S rRNA gene.

Table 4. Number of OCTUs at 97% and 99% clustering thresholds and number of OCTUs correctly assigned to each nematode morphospecies obtained using Illumina MiSeq.

Morphospecies	OCTUs 97% clustering	OCTUs correctly assigned	OCTUs 99% clustering	OCTUs correctly assigned
Halichoanolaimus sp1	21	3	28	9
Sabatieria sp1	28	5	45	18
Metachromadora sp1	18	1	38	6
Viscosia sp1	15	1	18	1
Thoracostoma sp.	102	3	108	5

Discussion

Methods for DNA extraction from marine nematodes suitable for molecular analysis

The recovery of DNA from meiofaunal organisms suitable for PCR amplification represents a crucial step for the assessment of their biodiversity based on molecular approaches. Previous molecular investigations carried out on marine nematodes, have used different DNA extraction procedures (De Ley et al., 2005; Bhadury et al., 2006; Bik et al., 2010; Creer et al., 2010; Derycke et al., 2010; Fonseca et al., 2010; Thomas, 2011). Some approaches are based on the use of an alkaline solution (NaOH) and freezing-thawing steps (Floyd et al., 2002) and modifications (Bhadury et al., 2006); others on the use of a lysis buffer containing proteinase K followed or not by DNA purification with commercial kits (De Ley et al., 2005; Bhadury et al., 2007; Fonseca et al., 2008; Bik et al., 2010). Further studies conducted on terrestrial nematodes have used commercial kits for the extraction and purification of DNA (Porazinska et al., 2009, 2010a, 2012). So far, the performance of these extraction procedures has never been compared, and this hampers the identification of the most suitable DNA extraction protocol for biodiversity analysis through molecular approaches. In this study different procedures were compared for their efficiency of DNA extraction from cultured and free-living nematodes collected from both coastal and deep-sea sediments. Our results indicate that the QIAGEN kit represents an efficient tool, among those tested, for DNA extraction from deep-sea nematodes. Moreover, 18S rDNA sequences, independently from the DNA extraction procedures used, were consistently obtained both in cultured and coastal nematodes. This was not the case for deep-sea nematodes, where only the QIAGEN and MoBio kits provided always 18S rDNA amplicons which can be sequenced by using both Sanger and HTS. This could be explained considering that PCR amplification efficiency relies from one side on the amount of DNA template and from the other from its purity (i.e. lack of inhibitors for polymerase reactions). Therefore, these results indicate that the use of these commercial kits allow us to recover an amount of DNA from marine nematodes free of potential inhibitors, suitable for amplification and as such they should be preferred for the molecular analysis of biodiversity of deep-sea nematodes.

Identification of deep-sea nematodes based on metagenetics

In the present study we found that different cut-offs for OCTUs generation (i.e. a different level of similarity for the identification of OCTUs) using the OCTUPUS pipeline provided different estimates of taxon richness. This is not surprising since the intra-specific variation can be detected using more stringent thresholds (99%), whereas genera and order can be grouped using more conservative thresholds (97%) (Shaw et al., 2008). Such differences in taxon richness between the two clustering thresholds were not so evident using Mothur program. These findings suggest that the use of 99% cut-off provides a better resolution in assigning sequences to the species level (Porazinska et al., 2010a) for deep-sea nematodes.

Our results pointed out also that the number of OCTUs for each genus did not reflect the number of individuals belonging to the same genus morphologically identified in the sample. In particular, for the assemblage of 10 nematodes, by using the OCTUPUS pipeline, two out of five genera (*Sabatieria* and *Sphaerolaimus*) showed a significant deviation from the expected quantitative representation. Different reasons related to PCR amplification can be invoked to explain such differences, including: i) differences in the amplification efficiency of the different genera, which can lead to a different proportion of OCTUs with respect to the number of individuals per genus; ii) occasional failure of nematode templates due to the use of primers that are highly conserved in eukaryotes and iii) loss of some amplicons during the emulsion PCR step (Creer et al., 2010). At the same time, it should be taken into account that the four specimens belonging to the *Sabatieria* genus and identified as morphologically different could be only phenotypically diverse, but genetically identical. Despite this, by using AmpliconNoise and Mothur none of these proportions significantly deviated from the expected values suggesting that the cleaning of sequences before the analysis can reduce the gap between proportions obtained from morphological and molecular analysis. Moreover, we found that using 99% clustering threshold a high number of OCTUs were attributed to *Sphaerolaimus* and *Setosabatieria* despite they were represented by only one individual in the nematode assemblage. These findings suggest either a potential incorrect assignment of sequences belonging to other genera (i.e. *Hopperia sp.*), the presence of intra-individual variation among rRNA gene copies, or errors occurring during amplification and/or sequencing steps.

The taxonomic identification of the OCTUs proceeds through a similarity analysis against public databases, so that the output depends on nematode sequences deposited therein. Indeed, our results show that the number of genera obtained from Roche 454 sequencing analysis of DNA recovered from the nematode assemblage of the NW Mediterranean Sea was lower than that morphologically identified (as 18S rDNA sequences of the genus *Hopperia* are lacking in the public databases). Moreover, a high proportion of OCTUs (41% and 40% at 99% clustering threshold for OCTUPUS and Mothur analysis, respectively) displayed a significant match with unclassified ribosomal sequences ("Not assigned"). Roche 454 sequencing analysis carried out on the nematode assemblage of 100 individuals, provides further evidence of the limits of similarity search tools against databases, which, at present, contain a very low number of marine nematode sequences classified with no ambiguities (i.e., assigned to a genus or species instead of to a general "unknown nematode"). At the same time, the lack of taxonomic resolution at the species level of most marine nematodes (i.e. morphologically classified at the genus level followed by generic labels for the discrimination at species level, i.e. sp.1, sp.2 …) represents an additional problem for the correct assignment of nematode sequences. There is thus a strong need to improve the information contained in public databases linking DNA sequences to formally described species.

Influence of polymorphisms on nematode biodiversity estimates

In the present study the total number of OCTUs assigned to nematodes was considerably higher than the actual number of individuals. This discrepancy could be due to the variability of 18S rRNA gene within nematode species and/or amplification and sequencing errors. Indeed, we found a variable number of OCTUs for the five morphospecies of deep-sea nematodes analysed (up to 5 and 18 using 97 and 99% cut-offs, respectively). Similarly, previous studies reported that the number of OCTUs matching each nematode species extracted from soils varies from 2 to 95 (using 99% cut-off) (Porazinska et al., 2010b). Our results suggest that nematodes inhabiting benthic deep-sea ecosystems can be characterised by the presence of polymorphisms among rRNA gene copies within their genomes, as also reported from whole genome shotgun library analyses carried out on different terrestrial nematode species (Bik et al., 2013). Further studies are needed to elucidate the extent of the polymorphism of 18S rRNA gene in different marine nematode species and among different individuals of the same species. However, polymorphisms do not allow us to completely explain the OCTUs number we found using Roche 454 sequencing analysis, which could be further enhanced by PCR and sequencing errors. Indeed, it is known that Roche 454 is more prone to errors than Illumina MiSeq (1% *vs*. 0.1% per base within single reads; Glenn, 2011). The advancement of HTS platforms along with the possibility to avoid the amplification step will allow us to reduce the errors and thus to provide more confident estimates of the actual OCTU number for each species.

Conclusions

There is increasing evidence that deep-sea ecosystems are highly vulnerable to biodiversity losses, which can have major adverse consequences for key ecological processes (i.e., nutrient regeneration, biomass production; Danovaro et al., 2008, 2014). Since deep-sea ecosystems are already threatened by human impact through trawling, dumping, oil, gas and mineral extraction, and other pollution sources (Ramirez-Llodra et al., 2011), there is an urgent need to develop suitable tools to be used for the quantification of the actual biodiversity in the deep sea. Our results indicate that the use of metabarcoding allows the analysis of the diversity of complex communities in a rapid and cost-effective way, thus increasing enormously our ability to investigate deep-sea nematode biodiversity. However, the identification of nematodes at genus and species level is still problematic, due to the limited number of sequences deposited in public databases. Thus we still need to couple morphological identification and the sequencing of the 18s rRNA gene (typically using sanger sequencing) to implement the available databases. Additional efforts are also needed to understand the actual variability of the 18S rRNA gene repeats among marine nematodes and to identify alternative single copy markers (nuclear or mitochondrial) able to provide quantitative estimates of the relative contribution of each species to the whole assemblage. These aspects

should be carefully taken into account for using metabarcoding in quantitative ecological research and monitoring programmes of marine biodiversity.

Acknowledgments

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Authors contribution

Conceived and designed the experiments: AD CC RD. Performed the experiments: LC GR.

Analyzed the data: AD LC CC GR RD. Wrote the paper: AD LC CC GR RD.

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Data Accessibility

DNA sequences obtained from single individual of deep-sea nematodes is deposited in the GenBank under the accession number KJ755995. DNA sequences obtained from collected individuals of deep-sea nematodes are deposited in the GenBank under the accession numbers SRA: SRX554532, SRX540994. Additional data are available in the Supporting Information.

Supporting material

Appendix S1

PCR reactions using the primer pairs Nem_18S_F and Nem_18S_R were carried out in a total volume of 50 µL containing 50-100 pg DNA as template, 0.4 µM of each primer, 0.05 Units μL^{-1} MyTAQ™ HS DNA Polymerase (Bioline), 1× MyTAQ™ HS reaction buffer (including 3 mM $MgCl_2$), 1 mM dNTPs and 0.5mg ml^{-1} BSA (Applied Biosystems). The adopted thermal cycling conditions were: an initial denaturation at 95°C for 2 min, 35 cycles of amplification (30 s at 94°C, 1,30 min at 54°C, 3 min at 72°C) followed by a final extension at 72°C for 10 min. Negative controls were included for each amplification reaction. All PCR reactions were done in a Veriti thermal cycler (Applied Biosystems) and PCR products of the expected length size (i.e. 900 bp) were checked using 1% agarose gel electrophoresis after staining with GelRed (BIOTIUM). PCR products once purified using ExoSAP-IT (Affimetrix) were also sequenced by Sanger chain-termination sequencing. PCR reactions using the primer pairs SSUF04 and SSUR22 were carried out in a total volume of 50 µL containing 50-100 pg DNA as template, 0.4 µM of each primer, 0.04 Units μL^{-1} PrimeSTAR GXL DNA polymerase (Takara), 1× PrimeSTAR reaction buffer (including 3 mM $MgCl_2$) and 0.8 mM dNTPs. The adopted thermal cycling conditions were similar to those previously published (Fonseca et al., 2010), with slight modifications. After an initial denaturation at 94°C for 10 min, 35 cycles of amplification (1 min. at 94°C, 1,30 min at 57°C, 40 sec at 72°C) followed by a final extension at 72°C for 10 min have been used. Negative controls were included for each amplification reaction. PCR products were checked using 1% agarose gel electrophoresis after staining with GelRed (BIOTIUM).

Table S1. Number of OCTUs classified as "Not Assigned" and "No hit" after BLAST Search using OCTUPUS and Mothur pipelines at 97 and 99% clustering thresholds. In parenthesis the percentage on the total number of OCTUs.

	"Not Assigned" OCTUs	**"No hit" OCTUs**
OCTUPUS		
10 nematodes - 97% clustering	61 (17%)	63 (18%)
10 nematodes - 99% clustering	2164 (41%)	265 (5%)
100 nematodes - 97% clustering	240 (18%)	355 (26%)
100 nematodes - 99% clustering	1709 (24%)	885 (13%)
MOTHUR		
10 nematodes - 97% clustering	243 (37%)	16 (2%)
10 nematodes - 99% clustering	535 (40%)	16 (1%)
100 nematodes - 97% clustering	525 (25%)	312 (15%)
100 nematodes - 99% clustering	739 (23%)	425 (14%)

Table S2. List of morpho-species belonging to different genera of nematodes (10 individuals) collected in deep-sea sediments of the NW Mediterranean Sea utilised for metagenetic analysis. ID= identification number.

ID	Morpho-species	ID	Morpho-species
1	*Sabatieria sp. 1*	**6**	*Syringolaimus sp. 2*
2	*Sabatieria sp. 2*	**7**	*Halalaimus sp. 1*
3	*Sabatieria sp. 3*	**8**	*Sphaerolaimus sp. 1*
4	*Sabatieria sp. 4*	**9**	*Setosabatieria sp. 1*
5	*Syringolaimus sp. 1*	**10**	*Hopperia sp. 1*

Table S3. List of morpho-species belonging to different genera of nematodes (100 individuals) collected in deep-sea sediments of the Central Mediterranean Sea utilised for metagenetic analysis. ID= identification number.

ID	Morpho-species	ID	Morpho-species	ID	Morpho-species	ID	Morpho-species
1	*Halalaimus sp. 1*	**26**	*Desmodora sp. 5*	**51**	*Desmoscolex sp. 4*	**76**	*Sphaerolaimus sp. 1*
2	*Desmoscolex sp. 1*	**27**	*Richtersia sp. 5*	**52**	*Desmoscolex sp. 2*	**77**	*Desmoscolex sp. 4*
3	*Halalaimus sp. 9*	**28**	*Pselionema sp. 1*	**53**	*Sabatieria sp. 1*	**78**	*Halichoanolaimus sp. 4*
4	*Halalaimus sp. 9*	**29**	*Viscosia sp. 1*	**54**	*Sphaerolaimus sp. 1*	**79**	*Parasphaerolaimus sp. 1*
5	*Halalaimus sp. 9*	**30**	*Viscosia sp. 1*	**55**	*Leptolaimus sp. 5*	**80**	*Desmodora sp. 5*
6	*Sphaerolaimus sp. 1*	**31**	*Sphaerolaimus sp. 1*	**56**	*Monhystrella*	**81**	*Halalaimus sp. 9*
7	*Halichoanolaimus sp. 1*	**32**	*Cyatholaimus sp. 1*	**57**	*Theristus sp. 1*	**82**	*Tricoma sp. 1*
8	*Tricoma sp. 1*	**33**	*Tricoma sp. 5*	**58**	*Desmoscolex sp. 4*	**83**	*Halalaimus sp. 1*
9	*Parasphaerolaimus sp. 1*	**34**	*Tricoma sp. 1*	**59**	*Syringolaimus filicaudatus*	**84**	*Halalaimus sp. 9*
10	*Sabatieria sp. 1*	**35**	*Desmodora sp. 1*	**60**	*Neochromadora sp. 1*	**85**	*Richtersia sp. 2*
11	*Procamacolaimus sp. 1*	**36**	*Anaplectus*	**61**	*Halalaimus sp. 1*	**86**	*Sphaerolaimus sp. 1*
12	*Procamacolaimus sp. 1*	**37**	*Bathyeurystomina sp. 1*	**62**	*Daptonema sp. 1*	**87**	*Tricoma sp. 1*
13	*Molgolaimus sp. 1*	**38**	*Sphaerolaimus sp. 1*	**63**	*Tricoma sp. 1*	**88**	*Daptonema sp. 1*
14	*Molgolaimus sp. 1*	**39**	*Oncholaimus*	**64**	*Pselionema sp. 3*	**89**	*Desmoscolex sp. 4*
15	*Procamacolaimus sp. 1*	**40**	*Pselionema sp. 3*	**65**	*Tricoma sp. 1*	**90**	*Richtersia sp. 2*
16	*Sabatieria sp. 1*	**41**	*Desmoscolex sp. 1*	**66**	*Tricoma sp. 1*	**91**	*Daptonema sp. 1*
17	*Desmodora sp. 1*	**42**	*Greeffiella*	**67**	*Spilophorella sp. 1*	**92**	*Desmoscolex sp. 4*
18	*Desmoscolex sp. 2*	**43**	*Theristus sp. 1*	**68**	*Desmoscolex sp. 4*	**93**	*Richtersia sp. 2*
19	*Sphaerolaimus sp. 1*	**44**	*Sphaerolaimus sp. 1*	**69**	*Parasphaerolaimus sp. 1*	**94**	*Spilophorella sp. 1*
20	*Tricoma sp. 1*	**45**	*Tricoma sp. 1*	**70**	*Desmoscolex sp. 4*	**95**	*Spilophorella sp. 1*
21	*Parasphaerolaimus sp. 1*	**46**	*Spilophorella sp. 1*	**71**	*Tricoma sp. 1*	**96**	*Richtersia sp. 2*
22	*Sphaerolaimus sp. 1*	**47**	*Oxystomina sp. 1*	**72**	*Sabatieria sp. 1*	**97**	*Richtersia sp. 2*
23	*Microlaimus sp. 3*	**48**	*Richtersia sp. 5*	**73**	*Spilophorella sp. 1*	**98**	*Tricoma sp. 1*
24	*Richtersia sp. 5*	**49**	*Desmodora sp. 1*	**74**	*Halichoanolaimus sp. 4*	**99**	*Desmoscolex sp. 4*
25	*Microlaimus sp. 3*	**50**	*Halalaimus sp. 9*	**75**	*Spilophorella sp. 1*	**100**	*Richtersia sp. 2*

Figure S1. Gel electrophoresis of PCR products using the primer pairs Nem_18S_F and Nem_18S_R from DNA of nematodes collected from benthic shallow-water systems. Lane M: molecular marker (BIOLINE, 2.5 Kbp); lanes 1-2: 18S rRNA amplicons obtained from DNA of nematodes extracted with the QIAGEN kit; lanes 3-4: 18S rRNA amplicons obtained from DNA of nematodes extracted with NaOH procedure; lane 5: negative control.

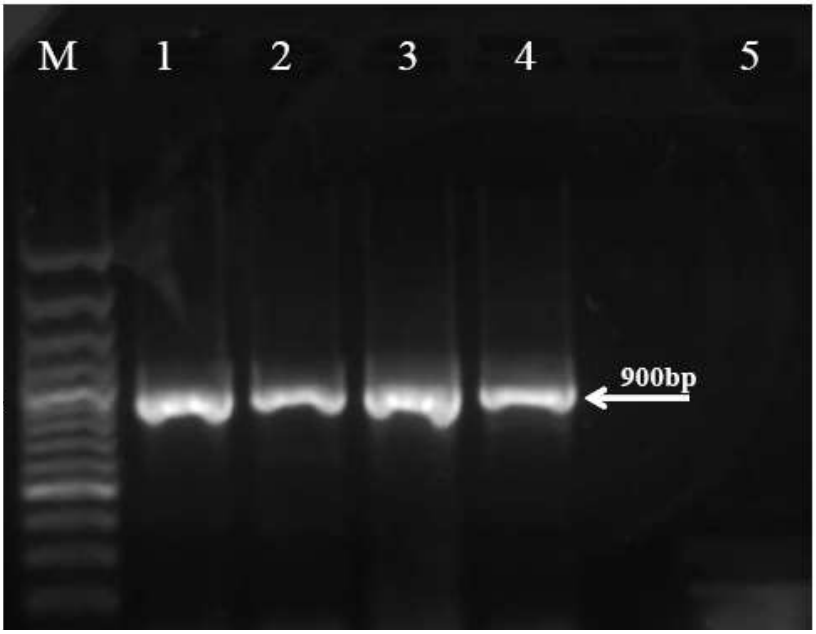


Figure S2. Gel electrophoresis of PCR products using the primer pairs Nem_18S_F and Nem_18S_R from DNA of deep-sea nematodes (*Sphaerolaimus uncinatus*) extracted with NaOH procedure (a) and QIAGEN kit (b). (a) Lane M: molecular marker (BIOLINE, 2.5 Kbp); lanes 1-10: 18S rRNA amplicons of *Sphaerolaimus uncinatus*, lane 11: positive control (obtained from PCR analysis of DNA from shallow water nematodes), lane 12: negative control. (b) Lane M: molecular marker (BIOLINE, 2.5 Kbp), lanes 1-7: 18S rRNA amplicons of *Sphaerolaimus uncinatus*, lane 8: positive control (obtained from PCR analysis of DNA from shallow water nematodes), Lane 9: negative control.

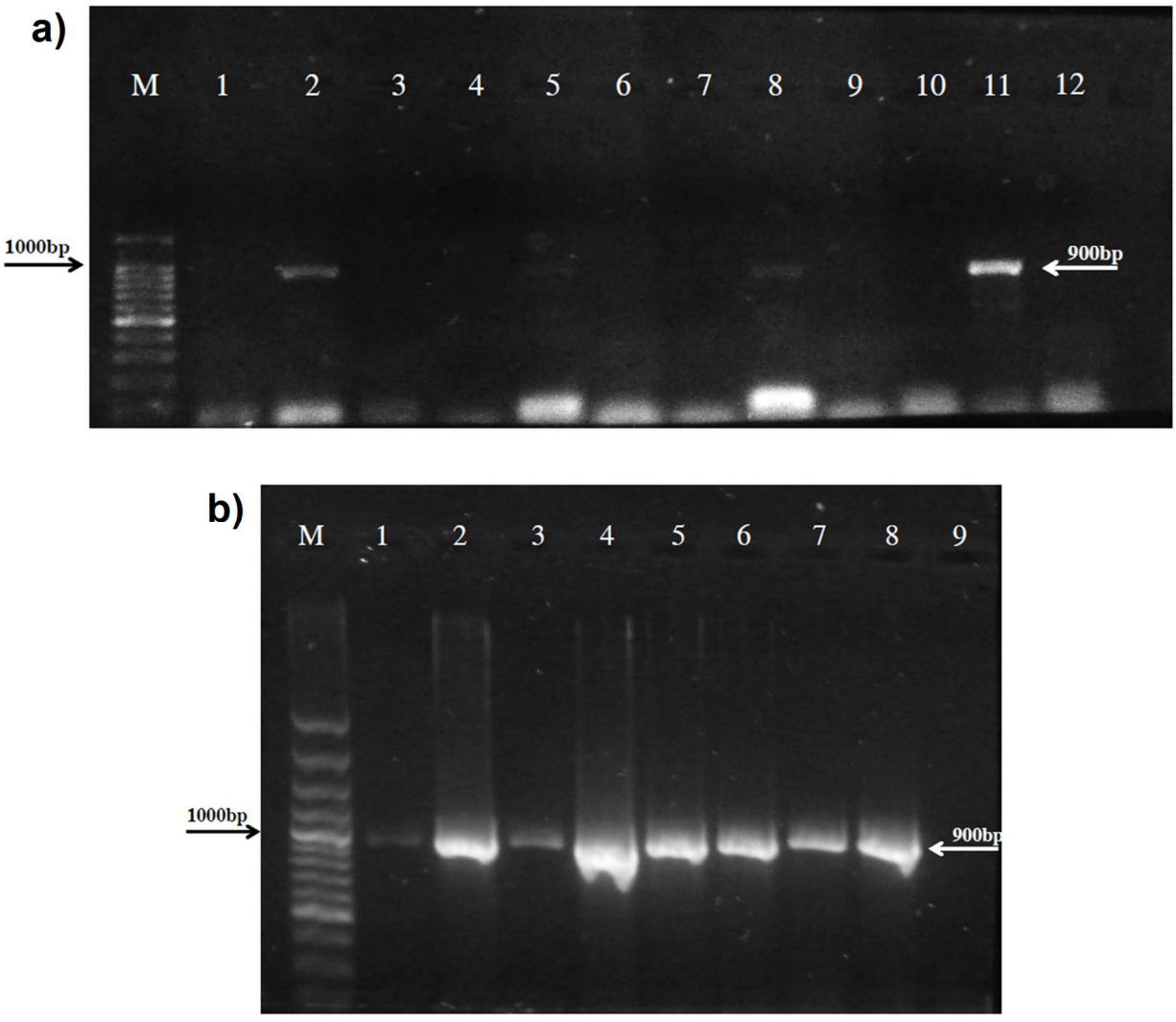
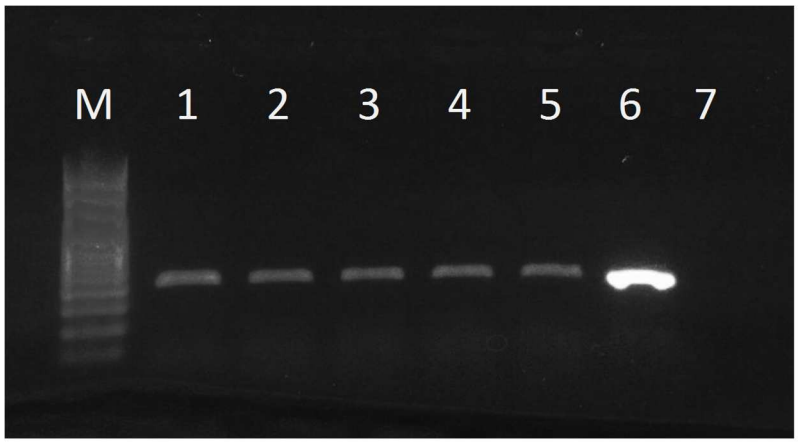


Figure S3. Gel electrophoresis of PCR products using the primer pairs SSUF04 and SSUR22 from DNA of nematodes collected from deep-sea sediments. Lane M: molecular marker (BIOLINE, 2.5 Kbp); lanes 1-2: 18S rRNA amplicons obtained from DNA of nematodes extracted with the QIAGEN kit; lanes 3-5: 18S rRNA amplicons obtained from DNA of nematodes extracted with MoBio kit; lane 6: positive control (obtained from PCR analysis of DNA from shallow water nematodes); lane 7: negative control.



CHAPTER 4. CASE STUDY AND APPLICATION TO MEIOBENTHIC BIODIVERSITY

4.1 Meiofaunal biodiversity in the Ross Sea continental shelf, Antarctica

Abstract

Recent ecological models predicted that most marine ecosystems will be impacted by the ongoing climate changes. Antarctica, in particular, will be affected by water warming causing water stratification and hypoxic zones. Meiofaunal organisms are highly sensitive to temperature shifts and represent one of the most vulnerable component of Antarctic biota to the effects of climate change. The study of Antarctic benthos is hampered by the logistical constraints of remote field work and the traditional method used for the identification of benthic species, which is time-consuming and expertise demanding. Reliable molecular tools are needed for an enhanced support to morphological-based surveys in order to implement our knowledge about Antarctic biodiversity. Here, we compared, for the first time, traditional, morphological identification and metabarcoding to assess the biodiversity of meiofauna collected in the Ross Sea. Our results showed that the molecular data faithfully reflect the microscopy-based analysis, uncovering all the taxa morphologically identified. Metabarcoding proved powerful by revealing a remarkable level of benthic diversity that exceeded the morphological survey. Both approaches identified nematodes as the dominant meiofaunal taxon. The retrieval of ca. 20% of unknown sequences suggested the need of more comprehensive and well-curated taxonomic reference libraries and the importance of multi-gene surveys. We here also provided an efficient protocol for the recovery of amplifiable DNA from meiofaunal organisms fixed in formalin. DNA retrieved has been sequenced by using Illumina MiSeq technology and 40% of the recovered Operational Taxonomic Units were successfully mapped. Despite some hurdles remain, this study represents a step forward for the application of molecular approach to formalin-fixed samples. Overall, our study supports for metabarcoding as a powerful and repeatable method for characterizing Antarctic meiofaunal communities, while also presenting suggestions for its improvement.

Keywords: Antarctica, meiofauna, metabarcoding, high-throughput sequencing, 18S rDNA

Introduction

The Southern Ocean (SO) represents 30% of the global ocean surface (about 34.8 million km^2), contains roughly 11% of the world's continental-shelf area (Zwally et al., 2002) and hosts a significant portion of the planet's marine diversity (ca. 5 % based on Register of Antarctic Marine Species and World Register of Marine Species) (Clarke and Johnston, 2003; Barnes and Peck, 2008; Barnes et al., 2009). Such ecosystems are very susceptible to the growing impact of global climate change. Recent studies predicted that water warming will cause water stratification and hypoxic zones especially in benthic habitats (Matear et al., 2000). In particular, benthic fauna is highly sensitive to environmental shifts, pH (acidification; Orr et al. 2005; Barnes and Peck 2008) and temperature variations (Danovaro et al., 2001; Yasuhara and Danovaro, 2016), thus representing the most vulnerable component of Antarctic biota (Clarke et al., 2007; Ingels et al., 2012).

The knowledge of biodiversity patterns in remote benthic Antarctic habitats is a key issue to understand the biological effects of current global climate change, as well as for conservation efforts. Despite this, a high number of Antarctic benthic species still remain undiscovered due to i) the chronic undersampling caused by logistic difficulties exacerbated by the harsh environmental conditions and ii) the traditional method used for the identification of benthic species, based on morphological characters.

Within benthic assemblages, metazoan meiofauna are excellent candidates to test general ecological hypotheses and theories (Snelgrove, 1999; Danovaro et al. 2008), due to the high abundance and diversity, widespread distribution, rapid generation time and important role played into ecosystem functioning (Jessup et al. 2004; Nascimento et al. 2011, 2012; Bonaglia et al. 2014). Meiofauna can be also valuable to predict global climate changes and anthropogenic impacts, because of their high sensitivity to environmental variations (Zeppilli et al., 2015). Despite this, manual sorting and morphological identification of organisms are time consuming and require taxonomic expertise, especially for the presence of cryptic species (Clarke and Johnston, 2003; Held, 2003; Held and Wagele, 2005; Raupach and Wagele, 2006; Linse et al., 2007; Havermans et al., 2011).

The development of molecular approaches is bringing significant help to reinforce taxonomic identification and to distinguish morphologically similar species and cryptic species. However, standard barcoding analysis based on Sanger sequencing cannot be applied for large-spatial scale ecological investigations. The recent developed high-throughput sequencing (HTS) approach can open new opportunities to collect biodiversity data, in a cost-efficient way and at larger spatial scale (Chown et al., 2015). Such HTS-based studies, namely metabarcoding (sensu Taberlet et al., 2012) have been widely applied in temperate regions to assess marine benthic biodiversity and provided

valuable snapshots of biodiversity for future conservation efforts (Bohmann et al., 2014; Lejzerowicz et al., 2015; Creer et al., 2016). Conversely, in Antarctica, high-throughput sequencing analysis has been mostly applied in terrestrial or lacustrine habitats to study viruses (Lopez-Bueno et al., 2009), bacteria in hypolithic communities (Makhalanyane et al., 2013), soil (Teixeira et al., 2010), air (Bottos et al., 2014), as well as fungi and other eukaryotes (Pointing et al., 2009; Rao et al., 2012; Dreesens et al., 2014; Niederberger et al., 2015; Czechowski et al, 2016). We still lack a pilot study aiming at evaluating the potential use of metabarcoding to study Antarctic marine metazoan biodiversity.

Formalin is the preferred fixative for morphological identification of meiofaunal organisms (Giere, 2009). However, formalin-fixation of specimens damages DNA though i) fragmentation, ii) base modification, and iii) cross-linkage within the DNA itself or between DNA and proteins (Wong et al., 2014; Campos and Gilbert, 2012; Do and Dobrovic, 2012). These effects make DNA molecules unavailable for molecular analysis (Serth et al., 2000). Despite this, a recent study successfully recovered and sequenced DNA from formalin-fixed nematodes (Bhadury et al., 2005). We still need to evaluate the potential use of metabarcoding on meiofaunal organisms preserved in formalin.

In the present study, we evaluated the reliability of Illumina MiSeq sequencing analyses for assessing the biodiversity of Antarctic meiofaunal assemblages, collected at two sampling sites along the continental shelf, in the Ross Sea. Amplicons of 18S rDNA were obtained from meiofaunal organisms, previously morphologically identified at higher taxa level and then sequenced by using Illumina MiSeq platform. Secondly, in order to test the potential application of metabarcoding to formalin-fixed samples, we carried out the same analysis on meiofaunal organisms which have been preserved in 4% formalin for long-term period (i.e., ca. 6 months).

Material and methods

Study area and sample collection

The sediment samples were collected in the Ross Sea (Antarctica) during the XXX Italian Antarctic Expedition carried out on board of Malippo between the 21st to the 30th of January 2015. Samples were taken, by means of a Van Ven grab, from two different sites, at ca 70 m water depth: site 4 (ST4; -74,43.078, 164,07.757) and site 8 (ST8; -74,46.390, 163,57.977). Site 4 is located in Road Bay (close to the Italian Base of Terra Nova Bay), whereas site 8 in the Adelie Cove (Fig. 1).

Sediment samples were collected by using sterile Plexiglass cores (inner diameter 4.6 cm) and then were immediately stored at -20°C, without the use of any preservative, until further processing.

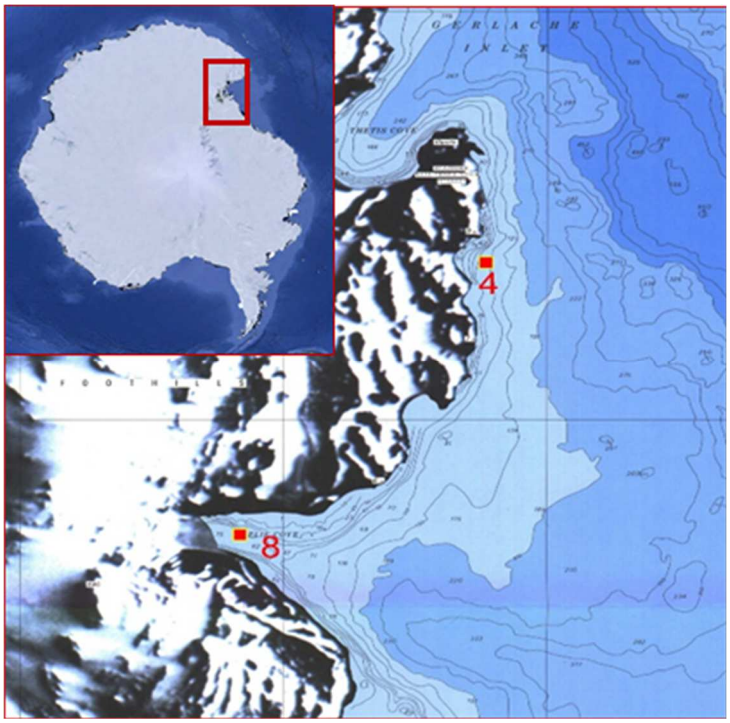


Figure 1. Sampling area and the two investigated sites located in Road Bay (site 4) and Adelie Cove (site 8), in the Ross Sea.

Recovery of meiofaunal organisms from sediment

For each site, we analysed two cores. Meiofaunal organisms were extracted from the superficial layer (0-1 cm) of each core. Each sample was treated with ultrasound (for 1 min 3 times, with 30 s intervals) to detach organisms from the grain particle surface and, then, sieved through a 1000-µm and a 20-µm mesh net to retain the smallest organisms. The fraction remaining on the latter sieve was re-suspended and centrifuged three times with Ludox HS40 diluted with water to a final density of 1.18 gcm-3 (Heip et al., 1985). Meiofaunal organisms extracted from the first core were fixed in 4% formalin, stained with Rose Bengal (0.5 g l^{-1}) and stored for ca. 6 months at room temperature. Hereafter, formalin-fixed samples belonging to site 4 and 8 are called ST4F and ST8F. The same meiofaunal extraction procedure was used for the second core of each station. In the latter case, after Ludox centrifugation, meiofaunal organisms were preserved in milliQ at 4°C and subsequent analyses were performed within two days. Hereafter, those samples are called ST4 and ST8, respectively.

All meiobenthic animals were counted under a stereomicroscope and classified at higher taxa level. After identification, all individuals extracted from the same core were picked and pooled together in a 2ml tube. Microscopic observations of both formalin-fixed and frozen samples revealed that the morphological features used for taxonomic identification were not altered.

DNA extraction and sequencing

DNA was extracted by using a commercial kit for tissue DNA extraction (QIAGEN DNeasy Blood & Tissue Kit), following the manufacturer's protocol. Formalin-fixed samples have been previously washed three times in a solution of PBS 10X to remove the fixative, as suggested by the manufacturer's protocol and scientific literature (e.g., Rubtsova et al., 2005).

Once extracted and purified, DNA was amplified using the primer pairs SSUF04 (5'-GCTTGTCTCAAAGATTAAGCC-3') and SSUR22 (5'– CCTGCTGCCTTCCTTGGA-3') (Blaxter et al., 1998) targeting the 18S rRNA gene and suitable, in term of amplicon length (450 bp), for High-Throughput Sequencing analyses, according to the needs of the sequencing platform chosen in the present study. Sequencing analyses were carried out on Illumina MiSeq platform (2 × 300 bp reads). by LGC Genomics (Germany).

Bioinformatic analyses

Automatic demultiplexing of samples occurred on the MiSeq sequencing machine and forward and reverse reads were joined using the FLASH software (Magoč and Salzberg, 2011). All overlapped sequences were quality-filtered using USEARCH (version 8.0) algorithm (Edgar, 2010) with the maximum expected error (-fastq_maxee) set to 1 (Brannock and Halanych, 2015) and trimmed to a length of 350 bp. Operational Taxonomic Units (OTUs) were generated by using a clustering threshold ranging from 1 to 3%, through USEARCH -cluster_otus workflow within the UPARSE (Edgar, 2010) clustering method. Chimeras and sequences appear < 10 times in the dataset were automatically identified and removed. Sequences were then mapped against the OTUs to generate an OTU table with the USEARCH –usearch_global function, with a minimum identity percentage of 97%. After generation of the OTU table, representative OTU sequences were then taxonomically assigned using MegaBLAST (sequence identity ≥ 90%, e-value 1.0^{-5}) against the nt sequence database (latest version as of July, 2016).

Data analysis

Once the taxa list was obtained, further processes were carried out to refine the dataset. A table was constructed for each sequenced sample, with the list of the OTUs and their relative BLAST match name and the number of reads mapping against each OTU. The dataset was used both qualitatively and quantitatively. We grouped OTUs obtained at 97 and 99% cut offs following the major Super-Groups of eukaryotes suggested by Guillou et al. (2013), with the only exception of Opisthokonta which were splitted into Metazoa, Fungi and other Opisthokonta. As we were particularly interested

in the Meiofauna, we splitted Metazoa into the main meiofaunal taxa. We grouped reads mapping against each OTU, following the same criteria.

First, we calculated the percentage contribution to the whole molecular dataset (by using the number of OTUs and the number of mapping reads) of each taxon. Then, we manually eliminated sequences matching to Fungi, to clearly non-marine organisms (these could be contaminations or DNA of continental origin present in the sediment) and to non-metazoan meiofaunal eukaryotes and thus we analysed meiofaunal taxonomic composition on the final retained sequences.

Results

Morphological identification

We collected 1061 and 714 meiobenthic animals from frozen samples at site 4 and 8, respectively. Whereas, 1010 and 1036 meiofaunal organisms were pooled together for ST4F and ST8F, respectively. The total number of collected taxa was three for both meiofaunal pools belonging to site 8 and for formalin-fixed sample belonging to site 4. Whereas, eight meiofaunal taxa were collected and pooled together for sample ST4 (Fig. 2).

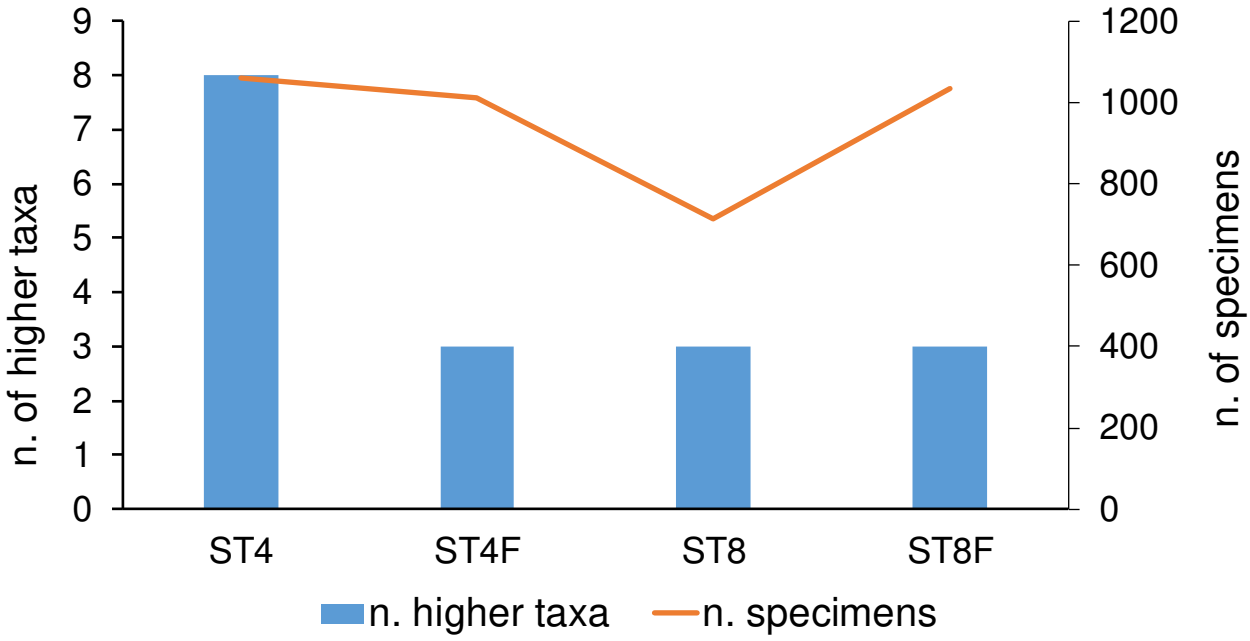


Figure 2. Number of meiofaunal higher taxa and specimens collected for each sample. Frozen samples: ST4 and ST8. Formalin-fixed samples: ST4F and ST8F.

Overall, meiofaunal biodiversity was higher at site 4, where we found eight different taxa: Acarina, Amphipoda, Cladocera, Copepoda, Isopoda, Nematoda, Oligochaeta and Polychaeta. At site 8, considering the diversity found in both frozen and formalin-fixed samples, we found 4 taxa: Acarina, Copepoda, Isopoda and Nematoda. Nematodes and copepods occurred in all samples. Nematodes ranged from 94 to 99% at ST4 and ST4F, respectively. Copepods were the second most abundant taxon, ranging from 1 to 5% at ST4F and ST4, respectively. Amphipoda, Oligochaeta, Polychaeta were found only in the frozen sample collected at site 4, where Amphipoda and Oligochaeta accounted for 0.2%, whereas Polychaeta accounted for 0.1%. Ostracoda were exclusive of site 4 and represented 0.4% and 0.1% in frozen and formalin-fixed sample, respectively. Isopoda were found in frozen samples collected at both sites, and they represented ca. 0.1% of the whole meiofaunal assemblage (Fig. 3).

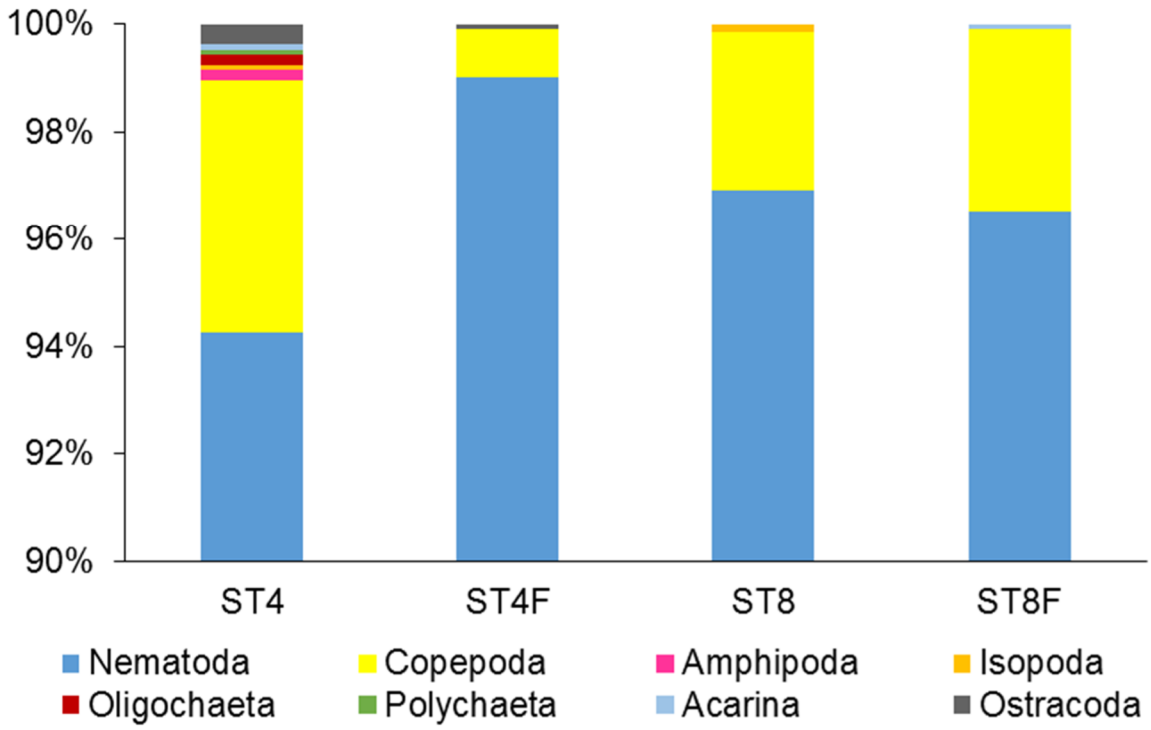


Figure 3. Taxonomic composition of meiofaunal assemblages collected at the two investigated sites, identified by using morphological approach. Frozen samples: ST4 and ST8. Formalin-fixed samples: ST4F and ST8F.

High-throughput sequencing data

The Illumina MiSeq run produced a total of 4.541.140 and 780.374 raw reads from frozen and formalin-fixed samples, respectively. After read overlapping, quality and sequence-length filtering and elimination of chimaeras, we obtained a total of 1.907.857 and 218.702 reads which went into OTU clustering. The number of OTUs obtained for each sample at 97, 98, 99% cut-offs is reported in Figure 4.

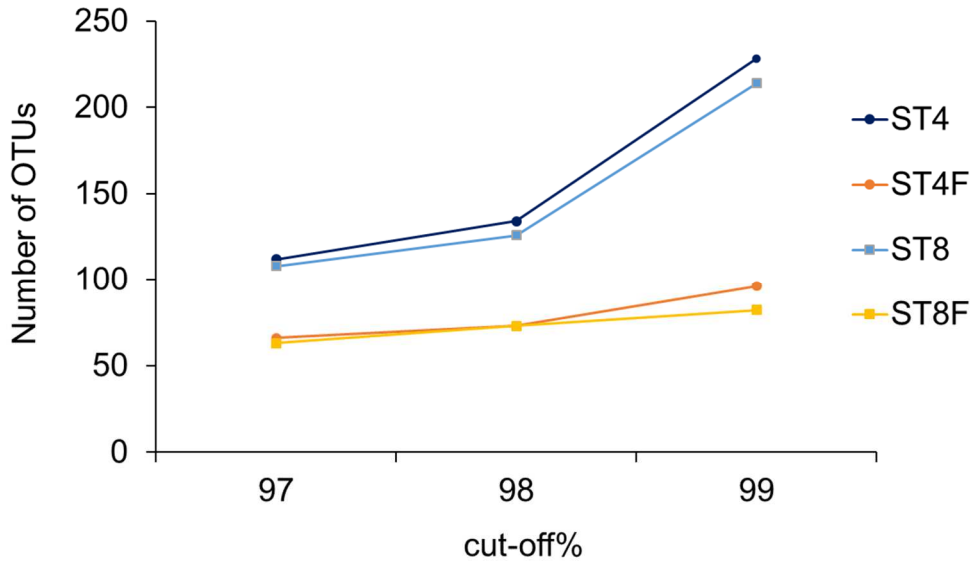


Figure 4. Number of OTUs plotted against percentage identity cut-off (97–99% similarity) generated using non-chimeric sequences (> 350 bases in length) from the two sampling sites (Road Bay and Adelie Cove).

Overall, the number of OTUs increased with increasing the clustering thresholds used. We obtained a lower number of OTUs from formalin-fixed samples compared to those generated from frozen samples. The mean number of OTUs obtained from frozen samples at the three different clustering thresholds were double (154±53) than in formalin-fixed samples (76±12). At 97% clustering threshold, the number of OTUs ranged from 63 to 112 at ST8F and ST4, respectively. By using 98% cut-off, the number of OTUs ranged from 73, for both formalin-fixed samples to 134 for frozen sample belonging to site 4. Finally, at more stringent clustering threshold (99%), OTUs' number ranged from 82 to 228 for ST8F and ST4, respectively. Similarly, we obtained a higher number of reads mapping against OTUs from frozen samples than from formalin-fixed ones. The mean number of mapping reads obtained from frozen samples (941351±87387) were 8 times higher than that obtained from formalin-fixed ones (108818±43341) (Fig. 5).

Throughout the whole dataset, 27 OTUs (most of them BLAST matching to *Nematoda*) were shared between samples. Instead, a substantial proportion of the recovered OTUs (77) were unique.

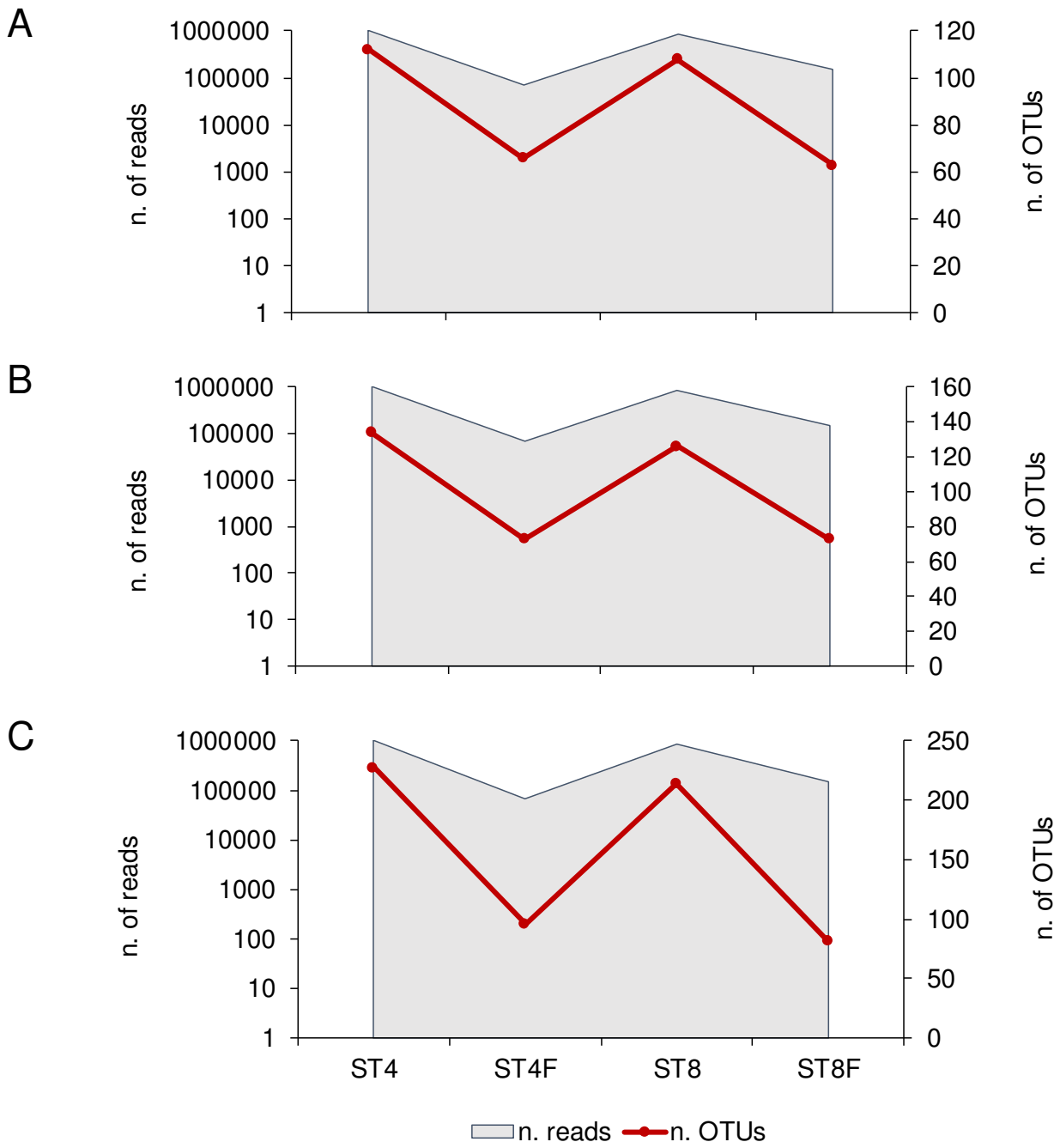


Figure 5. Number of reads mapping against OTUs and number of OTUs recovered under (A) 97% (B) 98% and (C) 99% percentage identity cut-offs. Values reported are calculated from non-chimeric Operational Taxonomic Units (OTUs) representing Illumina MiSeq sequencing reads from 18S rRNA gene.

Analyses of main groups

In order to study meiofaunal biodiversity at higher taxa levels, previous work demonstrated that it is recommended to use a more relaxed clustering as a conservative threshold value (97%), rather than risking an inflation of OTUs, by using more stringent ones (such as 98 or 99%). Thus, we report here the results obtained by using 97% cut-off.

BLAST results against the GenBank database indicated the presence of OTUs not only with hits for meiofauna, but also for Streptophyta and Fungi (Fig. 6). 55 and 37 OTUs out of 112 and 108 (~ 42% as average) were assigned to the phylum Nematoda in the frozen samples collected at ST4 and ST8, respectively. The number of OTUs assigned to nematodes decreased considerably in the formalin-fixed samples (~ 33%) and the number of fungi increased (Fig. 6). Fungi represented 4% and 6% in frozen samples and 9% and 11% in formalin-fixed ones. Not assigned OTUs (i.e., OTUs with hits in the public database but without an assigned taxonomy; e.g., uncultured eukaryotes) represented a high percentage in all samples, being 16% and 18% at ST4 and ST4F, respectively and 25% and 14% at ST8 and ST8F, respectively. Only 2 OTUs in the whole dataset displayed no significant match ("no BLAST hit", sequence identity <90%) to known ribosomal sequences and they were found in frozen sample collected at ST8. At all samples we found a proportion of OTUs matching to Homo sapiens. This proportion is particularly high (35%) in the formalin-fixed sample belonging to site 8, probably due to contamination in formalin (Fig. 6).

The ranking of the different Super-Groups, as per total number of OTUs and relative number of reads, is plotted in Figure 7. Nematoda was the most abundant group for both variables, in all samples, except for ST8F where Streptophyta dominated the dataset in terms of number of reads. In frozen samples, Copepoda, Fungi and Streptophyta were the next most abundant groups in terms of total number of OTUs. Looking at the number of reads, Nematoda were followed by Annelida in ST4 and Isopoda in ST8. The other Super-Groups had a marginal representation in our frozen samples for both variables. In formalin-fixed samples, the most OTU-rich groups after nematodes were Fungi and Streptophyta. The latter dominated the assemblage in terms of number of reads at ST8F. Also in formalin-fixed samples, other groups contributed to a lower extend to the total dataset, for both variables (Fig. 7).

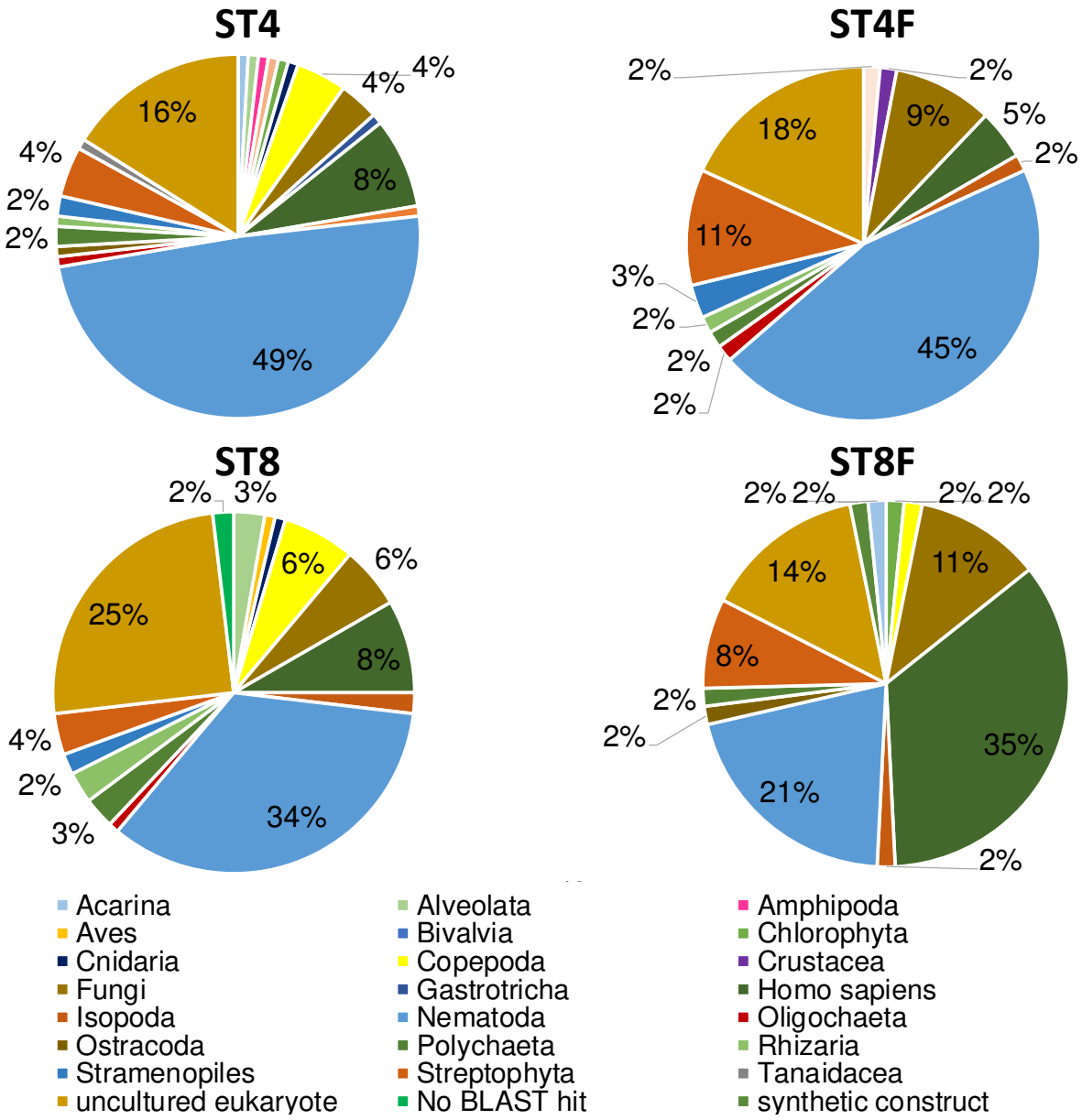


Figure 6. Eukaryotic taxa identified by metabarcoding of the meiofaunal assemblages collected at the two investigated sites (site 4 and 8) in the Ross Sea. Reported are the relative contribution of groups obtained at 97% clustering thresholds using the QIIME pipeline. The contributions are calculated from non-chimeric Operational Taxonomic Units (OTUs) representing Illumina MiSeq sequencing reads from 18S rRNA gene. The contributions of the most important components (expressed as percentage) are reported.

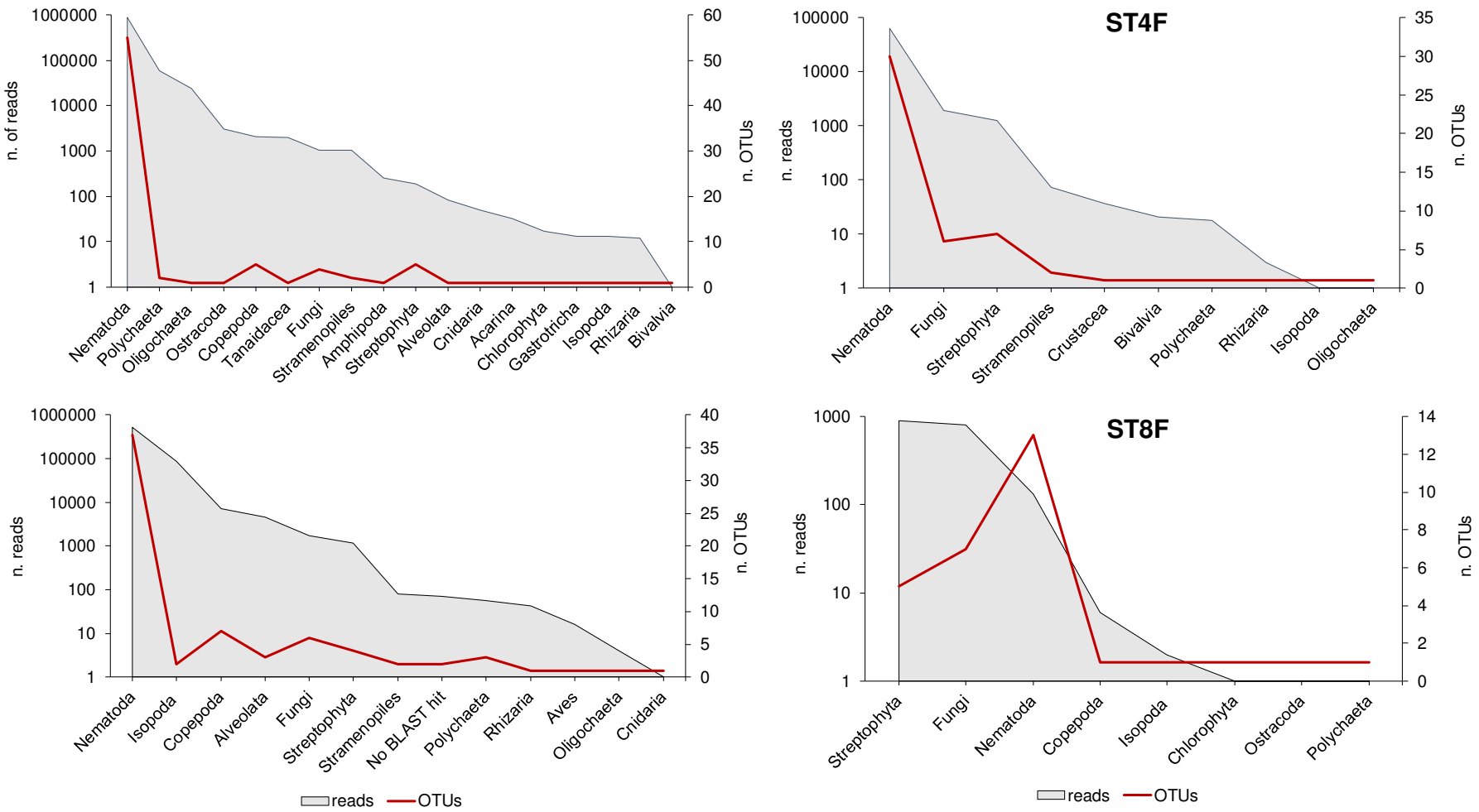


Figure 7. Rank order of the main groups according to total number of reads in the samples. The red line indicates the proportion of OTUs of the taxa in the samples.

Analysis of meiofaunal biodiversity

The contribution of OTUs matching to meiofaunal organisms was higher for frozen samples than for formalin-fixed ones. Indeed, the percentage of OTUs matching to meiofauna was 63% and 47% for ST4 and ST8, respectively and 53% and 27% for ST4F and ST8F, respectively.

In frozen samples, the analysis of OTUs at the higher taxa level (Table 1) highlighted the presence of all the taxa morphologically identified. Conversely, for formalin-fixed samples, molecular analysis did not retrieve all the taxa morphologically classified (Table 1). Nematoda and Amphipoda were correctly recovered by HTS analyses in all samples where they were morphologically identified. Copepods were correctly detected by metabarcoding in all samples, with the only exception of ST4F. The other taxa morphologically identified (as Annelida) were detected by molecular approach also in samples where they were not initially included by morphology-based analysis. The most striking discrepancy between morphological and molecular data is the high number of OTUs and reads assigned to Polychaeta even when this taxon was not morphologically identified. Overall, the relative contribution of OTUs assigned to each taxon to the total OTUs deviated from the contribution of the individuals belonging to this taxon morphologically identified.

Several phyla (e.g., Bivalvia, Cnidaria, Crustacea, Gastrotricha, Tanaidacea), not identified by using microscopy, were detected through metabarcoding.

Table 1. Number of taxa morphologically classified, correctly identified by high-throughput sequencing and list of taxa non recovered by using molecular approach.

Sample	n. of higher taxa morphologically identified	n. of higher taxa correctly identified with molecular approach	Higher taxa not recovered by molecular approach
ST4	8	8	na
ST4F	3	1	Copepoda, Ostracoda
ST8	3	3	na
ST8F	3	2	Acarina

Meiofaunal taxonomic composition

Nematodes dominated meiofaunal assemblages when considering both the number of OTUs and number of reads mapping against OTUs. In terms of number of OTUs, the contribution of nematodes to the total assemblages ranged from 70% to 88% at ST8 and ST4F, respectively. Nematodes were followed by copepods, ranging from 0 to 13% at ST4F and ST8. Isopoda were the third most OTU-rich taxon, and their contribution ranged between 1% to 6% at ST4 and ST8F, respectively (Fig. 8).

The differences between richness (number of OTUs) and relative abundances (number of reads) of the phyla were marked. Indeed, in terms of number of reads mapping against OTUs, nematodes were the most abundant taxon in all samples, and their abundances ranged between 85% to 99% at ST8 and at ST4F, respectively. Polychaeta (6%) and Isopoda (14%) were the second most abundant taxa at ST4 and at ST8, respectively. In formalin-fixed sample belonging to site 4, meiofaunal assemblage were dominated by nematodes (99%). Other taxa, such as Isopoda, Oligochaeta, Polychaeta, represented a minor fraction (<0.1%). In ST8F, nematodes were followed by copepods (4%) and isopods (1%). Polychaeta and Ostracoda represented a small portion (ca. 1%) (Fig. 8).

Discussion

Morphological- versus metabarcoding-based assessment of meiofaunal biodiversity

This study presented one of the first comparative analysis of Antarctic meiofaunal biodiversity, estimated by using morphological identification and metabarcoding approach. Our results showed that DNA metabarcoding of meiobenthic organisms collected in the Ross Sea uncovered higher diversity than morphological analysis. It should be noted that this is mainly due to the inclusion of a variety of taxa, such as Bivalvia, Crustacea, Cnidaria and Gastrotricha. They can leave in the sediments biological footprints, such as faeces, scales, mucus, spines that can be amplified and sequenced by HTS analysis (Cowart et al., 2015; Mohrbeck et al., 2015). Molecular survey is particularly efficient at uncovering animals common in smaller class sizes, such as Gastrotricha, and to identify “temporary meiofauna”, eggs or juvenile of larger animals (e.g., Bivalvia), commonly found in the mesh size <1mm (McIntyre AD, 1969). The higher diversity uncovered by HTS technology could be also due to the presence of small parasites living on the cuticle of meiofaunal taxa (Bhadury et al., 2011; Leray and Knowlton et al., 2015). Overall, DNA-based analysis, performed on frozen samples, was able to retrieve all the taxa identified by the morphological classification, indicating that these taxa were actually collected, amplified, sequenced and present in public database.

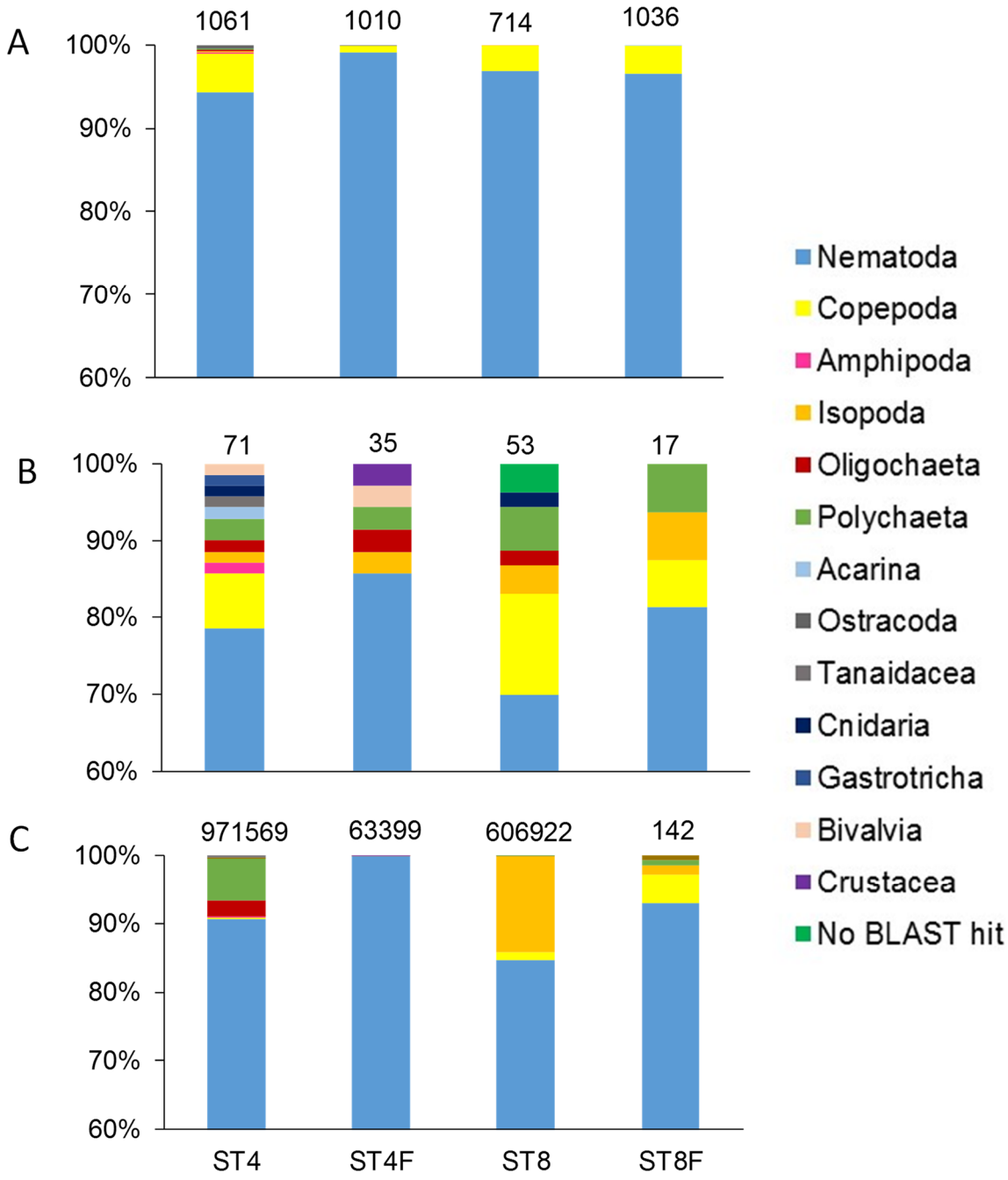


Figure 8. Taxonomic composition of meiofaunal assemblages. The relative contribution of each taxon are calculated using (A) morphology-based dataset, (B) number of OTUs and (C) number of reads mapping against OTUs, obtained by using metabarcoding. In the latter two cases, the contributions are calculated from non-chimeric Operational Taxonomic Units (OTUs) representing Illumina MiSeq sequencing reads from 18S rRNA gene.

The methods and results reported here provide another step towards the development of a reliable, repeatable and cost-effective metabarcoding protocol for baseline studies, while still pointing towards some required improvements. Indeed, the high amount of "not assigned" OTUs (i.e., OTUs with hits in the public database but without an assigned taxonomy) makes metabarcoding a still partly blind method. Our difficulties to uncover ca. 20% of the diversity hidden by "uncultured eukaryote" OTUs underlined the need of i) multi-gene surveys and ii) the synergistic efforts between molecular and morphological expertise to feed and curate reference libraries. First of all, different DNA loci present different inherent characteristics (evolutionary rate, neutrality, and presence of introns, for example). Thus, the use of a multilocus barcoding approach including mitochondrial and nuclear genes, can minimize gene/primer specific biases and enhance resolution at different taxonomic levels (Frézal and Leblois, 2008; Hajibabaei et al., 2011). Secondly, it is crucial to implement the collaboration among traditional taxonomists, molecular and computational biologists in order to refine the reference dataset to target specific groups. This would be useful in ecological research and monitoring programmes where the goals are to provide diversity estimates and taxonomic composition based on reliable taxonomic inferences. Awaiting the results of this improvement, we here suggest a synergistic use of morphological and molecular inventories, at least during this first phase, before safely engaging in longer-term 'blind' metabarcoding surveys. We therefore stress the need of further studies including parallel morphological and molecular analyses, that should be performed to i) test the efficiency of metabarcoding using a cocktail of genetic markers; ii) provide a baseline for a long time-scale investigation of marine biodiversity (which could be studied by using "blind" metabarcode method once taxonomic coverage and assignment are enhanced), and iii) feed public databases against which future metabarcode datasets could be compared. This would allow us to obtain a preliminary set of parameters and information which is needed prior to perform fully "blind" metabarcode studies.

Meiofaunal taxonomic composition

Our results pointed out that meiofaunal taxonomic composition obtained by using molecular approach (both in terms of OTUs or number of reads) showed both overlaps and discrepancies compared to that obtained by using morphological identification (based on number of organisms).

Nematodes dominated meiofaunal assemblages in both morphological and molecular-based surveys. This is not unsuspected since Nematoda are known to be the most abundant taxon in marine sediments (Lambshead, 2004; Bhadury et al., 2006) and dominated meiofaunal assemblages in previous metabarcoding surveys of shallow-water ecosystems (Creer et al., 2010; Fonseca et al., 2010, 2014; Bik et al., 2012; Lallias et al., 2015; Cowart et al., 2015). Microscopy-based identification

reported that, in terms of abundance, nematodes were followed by copepods, at both sampling sites. Metabarcoding revealed that the most OTU-rich phylum was Nematoda, but they were followed by Polychaeta. These findings showed that the number of OTUs for each taxon did not reflect the number of individuals belonging to the same taxon morphologically identified in the sample. In particular, Annelida (i.e., Oligochaeta and Polychaeta) showed a significant deviation from the expected quantitative representation.

Assessing the percentage contribution of each taxon using read number is also risky and needs previous calibration, as the number of reads depends on i) differences in rDNA copy number among groups, ii) different amplification efficiency for the different taxa and on iii) the potential primer bias (leading to unequal amplification success in different taxa) (Creer et al., 2010; Dell'Anno et al., 2015; Guardiola et al., 2015). Our rankings considering number of OTUs and number of reads mapping against OTUs, showed discrepancies that could be related to the size of the organisms (i.e. biomass). The good correlation between read number and biomass has been already demonstrated for other marine eukaryotes (i.e., zooplankton, Lindeque et al., 2013; Hirai et al., 2015). Further studies are thus needed to elucidate this relationship for meiofaunal communities.

The monitoring of marine benthic ecosystems is currently based on abundance metrics which cannot be generated by using metabarcoding approach and protocols currently available. Thus, we suggest that for comparative studies and monitoring programmes, molecular survey and genetic data could be used for presence-absence assessments. These findings confirmed what has been previously reported in literature (Ranasinghe et al., 2012; Aylagas et al., 2014).

The potential use of metabarcoding on meiofaunal formalin-fixed samples

This study highlighted both the opportunities and the challenges of obtaining metabarcoding data from meiofaunal assemblages fixed in formalin. We here developed and tested an efficient and reliable molecular protocol, which allowed us, for the first time, to recover from formalin-fixed meiofaunal organisms, amplifiable DNA, which has been successfully sequenced by Illumina MiSeq platform.

We were able to map 40% of the OTUs obtained from the amplification and sequencing of formalin-fixed samples and reconstructed meiofaunal taxonomic composition. As reported above for frozen samples, metabarcoding uncovered a higher diversity than microscopy-based analysis. However, in this case, it was not able to recover all the taxa morphologically identified. This could be due to degraded or damaged DNA, due to the presence of formalin.

The results of this pilot study highlighted the presence of some pitfalls that should be addressed prior applying "blind" metabarcoding to formalin-fixed samples. We obtained a lower number of raw

reads and OTUs compared to what would be expected with a fresh/frozen sample, containing the same amount of organisms. In addition, we recovered a high proportion of OTUs matching to Fungi (ST4F) and to human (ST8 F), probably linked to the presence of contamination in the formalin. While this method will make many formalin-fixed samples available for metabarcoding studies, we here emphasize the need of further research to refine the protocol.

Despite the work that still needs to be done to fill in these gaps and streamline sequencing of formalin-fixed specimens, the progress made here constitutes a step forward for the application of molecular analysis to samples fixed in formalin. Metabarcoding has the potential to unlock a treasure trove of genetic and genomic information from formalin-fixed specimens and to bring a large fraction of many museum collections into the age of genomics (Hykin et al., 2015).

Conclusions

Most marine ecosystems will be impacted by the ongoing global change and Antarctica will be affected by water warming causing water stratification and hypoxic zones (Matear et al., 2000). Since meiofaunal organisms are highly sensitive to temperature shifts (Danovaro et al., 2001; Yasuhara and Danovaro, 2016), they represent one of the most vulnerable component of Antarctic biota to the effects of global climate change (Clarke et al., 2007). The impact of ongoing global change on the Antarctic benthos is still not extensively explored and is a key priority for future studies in this region. Recent research works highlighted the need of reliable molecular tools for an enhanced support to morphological-based surveys in order to implement our knowledge about Antarctic biodiversity its functional aspects (Ingels et al., 2012). The potential to screen many samples, rapidly and at low cost, using high-throughput sequencing technology will facilitate large-scale biodiversity surveys in Antarctica.

Our results indicated that metabarcoding is a powerful and reliable approach for the study of Antarctic meiofaunal biodiversity, representing an opportunity to overcome the difficulties associated with morphological approaches. This study raised substantial warnings as to the need for more comprehensive and rigorously curated reference libraries in order to shed light on the taxonomic identity of “uncultured eukaryote” OTUs. Given that technical factors likely influence the number of times a sequence/OTU is observed more than the abundance of the corresponding taxon, metabarcoding datasets are still limited to presence/absence data. In addition, since morphological- and DNA-based analyses provide different information about meiofaunal assemblages, they should be used in parallel to obtain more accurate assessment of marine biodiversity.

This work provided also evidence that metabarcoding can be a promising approach to study the biodiversity of meiofaunal samples fixed in formalin. Some gaps still remain, but if they will be filled

in, the value of museum and university collections will increase in the future, since many formalin-fixed specimens will be available for genetic and genomic analysis.

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CHAPTER 5. CONCLUSIONS

5.1 Perspectives in the use of molecular tools for the analysis of marine benthic biodiversity

Global changes and human activities are having an increasing impact on marine biodiversity. Nevertheless, impact assessments and monitoring programmes are based almost exclusively on large and conspicuous species that represent a minor fraction of ocean diversity. Small and cryptic organisms, playing important ecological roles, remain overlooked in biodiversity surveys due to the difficulties associated with the traditional method used for their identification. This highlights a major limitation in our ability to study marine biota: it is difficult to establish biological baselines, quantify changes in biodiversity over time and understand the consequences of biodiversity loss on ecosystem functioning, if most species are unknown to science or cannot be easily surveyed (Leray and Knowlton, 2016).

In order to answer to the needs of the recently enacted MSFD, promoting functional, whole-sea system of monitoring and regional harmonization of protocols, an environmental assessment tool (i.e., NEAT) and a variety of innovative sampling methodologies (e.g., molecular approaches, optical - remote- sensing and acoustic methods, *in situ* monitoring instruments) have been tested in different marine ecosystems. They represent powerful solutions to assess marine environmental status in a holistic way and to obtain long-term datasets from microbes to large marine mammals (Danovaro et al., 2016). All these methodologies present proper advantages and biases and this needs to be recognised in data analysis rather than assume a single conventional method as representative of all biodiversity. Combining the use of different methodologies may be the best way to gain insights into different aspects of biodiversity and obtain a more comprehensive understanding of how biodiversity is changing in the ocean (Costello et al., 2017).

Among recently developed approaches, metabarcoding has a great potential in innovating the analysis of marine biodiversity, especially for tiny organisms, such as meiofauna. Compared to the morphological identification, HTS technology represents a useful tool for the census of meiofaunal biodiversity, allowing us to process a larger number of samples, more rapidly, at lower cost and to produce a breadth of data that are unimaginable by using the classical morphological identification (Carugati et al., 2015). Molecular surveys can uncover a higher taxa richness, potentially extending the scope of the analysis to a much broader benthic diversity. Metabarcoding is also a quick and cost-effective method to investigate the biodiversity of deep-sea nematodes, often neglected due to the presence of cryptic species and the need of highly specialized taxonomic expertise (Dell'Anno et al., 2015).

Beyond its many advantages and ease of use, the routine application of metabarcoding for benthic monitoring requires overcoming some limitations. The main shortcomings involve the incompleteness of reference databases. Despite considerable barcoding efforts, reference sequences are still very poor for benthic meiofaunal species, especially for those living in the deep sea. For this reason, we stress the need for the continuous collaboration between taxonomists, molecular biologists and computer scientists in order to feed open-access repositories. Encouraging the ongoing dialog between these sectors will be crucial for enabling comparative metadata analyses and untangling ecological patterns.

It is also important that molecular surveys include more than one genetic marker and the use of a cocktail of primer sets optimized to sequence different genetic loci. So far, most of metabarcoding studies relied on the amplification and sequencing of 18S rDNA, which exhibits conservation in priming sites resulting in the broad scale amplification of biodiversity across the eukaryotic tree of life. However, for some taxa, this marker provides little resolving power at the species level and is affected by the lack of taxonomic reference material. Therefore, the application of multi-barcode approach (i.e. using different suites of gene markers for the same community) may improve taxonomic coverage and resolution.

We here demonstrated that, in marine nematodes, rRNA gene copies present intraspecific variation which hampered the use of HTS data for quantitative ecological research. Further studies are needed to understand the actual variability of the 18S rRNA gene repeats among marine nematodes and to identify alternative single copy markers (nuclear or mitochondrial) able to provide quantitative estimates of the relative contribution of each species to the whole assemblage.

Another important challenge is to develop biotic indices specifically for HTS data. In the formulas of the currently applied ones (such as Infaunal Trophic Diversity and AZTI Marine Biotic Index), the ecological weight of each taxon morphologically identified is used as a factor of its abundance in the sample. However, metabarcoding data, in terms of number of reads or number of OTUs, depend on many technical and biological biases and their exploitation for quantitative assessments remains a major issue. A particular need in metabarcoding studies is to move from identification and detection of taxa to their quantification in terms of abundance and/or biomass. This will require concerted effort to address biases associated with gene copy number variation and polymorphisms. Moreover, new pilot studies are also needed to elucidate the relationships between meiofaunal biomass and number of reads, as already carried out for other marine eukaryotes (i.e., zooplankton).

Considering the abundances and cosmopolitanisms of meiofaunal taxa, it is crucial to further describe potential meiofaunal bioindicator taxa not only through their genetic identification but also

to specify their ecological values and functions. An alternative approach to avoid technical (i.e. PCR) biases and obtain simultaneously information on taxonomic and functional diversity is based on the use of Illumina-sequenced environmental metagenomes ($_{mi}$tags) (Logares et al., 2014).

Despite the remarkable promise of metabarcoding in yielding fast and informative assessment of meiofaunal diversity, from shallow-water to deep-sea ecosystems, it is critically important to maintain expertise and capacity in morphological taxonomic identification to ensure that metabarcoding approaches can be further validated. Such integrative morphological and molecular taxonomic approach will provide the necessary baseline to apply "blind" metabarcoding in routine monitoring programme in the future.

A holistic understanding of marine biodiversity will only become possible with coordinated efforts, further methodological developments, strict methodological standards and consistency of experimental designs (Duffy et al., 2013). The need for biodiversity baselines cannot be postponed in this rapidly changing world and metabarcoding represents a powerful method. Since morphological- and molecular-based approaches provide different information, they should be used in synergy to obtain better evaluations of the actual marine biodiversity.

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Annex 1- Publications on ISI journal

CHAPTER 1

Hummel H., Frost M., Juanes J.A., Kochmann J., Castellanos Perez Bolde C.F., Aneiros F., Vandenbosch F., Franco J.N., Echavarri B., Guinda X., Puente A., Fernandez C., Galvan C., Merino M., Ramos E., Ernandez P., Pitacco V., Alberte M., Wojcik D., Grabowska M., Jahnke M., Crocetta F., **Carugati L.**, et al. 2015. A comparison of the degree of implementation of marine biodiversity indicators by European countries in relation to the Marine Strategy Framework Directive (MSFD). Journal of the Marine Biological Association of the United Kingdom, doi:10.1017/S0025315415000235

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CHAPTER 2

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