Marine Genomics 24 (2015) 11–20

Contents lists available at ScienceDirect

Marine Genomics

Structure Regulation Evolution

Review

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

Laura Carugati [a], Cinzia Corinaldesi [a], Antonio Dell'Anno [a], Roberto Danovaro [a,b,*]

[a] Department of Life and Environmental Sciences, Polytechnic University of Marche, via Brecce Bianche, 60131 Ancona, Italy
[b] Stazione Zoologica Anton Dohrn, Naples, Italy

ARTICLE INFO

Article history:
Received 9 March 2015
Received in revised form 27 April 2015
Accepted 27 April 2015
Available online 7 May 2015

Keywords:
Meiofauna
Biodiversity
Metagenetic
High-throughput sequencing
18S rRNA gene

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.

Contents

* Corresponding author at: Department of Life and Environmental Sciences, Polytechnic University of Marche, via Brecce Bianche, 60131 Ancona, Italy. Tel.: +39 0712204654; fax: +39 071 2204650.

http://dx.doi.org/10.1016/j.margen.2015.04.010

1. Introduction

1.1. 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 amongst 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 to 10^6 individuals per m^2) and high diversity either at the level of higher taxa or 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).

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	32,000–40,000	85,000–105,000	No data	23–32
Polychaeta	12,632	6320	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	8853	2625–34,000	No data	21–77
Amphipoda	6947	20,000	No data	26
Nematoda (free-living)	6900	50,000	No data	12
Isopoda	6345	63,400–123,400	No data	5–9
Foraminifera	6000	1500	No data	80
Hydrozoa	3426	1550–4100	4976–7526	46–69
Cumacea	1444	6045	No data	19
Nemertea	1285	700–1400	1985–2685	48–65
Acarina	1218	1470–2130	No data	36–45
Tanaidacea	1130	23,500–57,400	No data	2–5
Oligochaeta	910	5900–16,900	No data	5–13
Gastrotricha	434	1810–2810	2244–3244	13–19
Kinorhyncha	228	1250–2350	No data	9–15
Entoprocta	193	1030	1223	16
Tardigrada	183	1120	1303	14
Sipuncula	150	43–230	193–380	39–78
Rotifera	114	320–2520	434–2634	4–26
Loricifera	32	1123	No data	3
Priapulida	19	No data	No data	No data

1.2. Classical taxonomic vs molecular approaches

A major bottleneck in meiofaunal taxonomy is related to the analysis of distinctive morphological characters by using light microscopy. Amongst 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 or 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 characterized (Appeltans et al., 2012; Table 1). 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

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 amongst meiofaunal organisms is still largely unknown, the application of Sanger chain-termination sequencing can make light on biodiversity distributional patterns of marine meiofaunal species.

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 is 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.

2. Methodological steps for assessing meiofaunal biodiversity through molecular-based approaches

2.1. 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).

2.2. 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 g cm^{-3}; Heip et al., 1985). Alternatively, DNA can be extracted from sediment samples without sorting the 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 optimized 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).

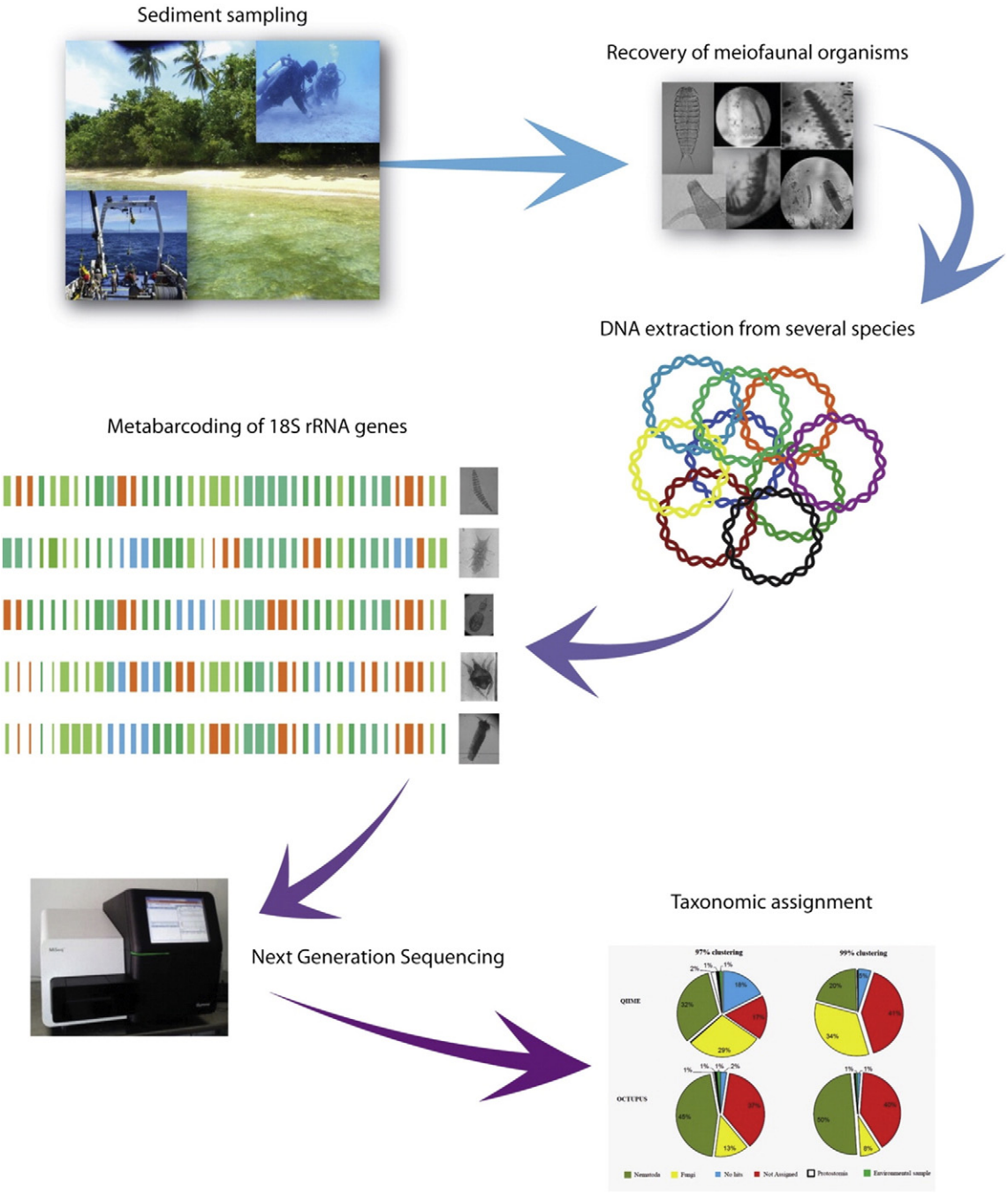

Fig. 1. Standardized workflow to study meiofaunal biodiversity in marine benthic ecosystems using high-throughput sequencing. Sediment samples (from shallow to deep-sea environments) are collected and subsequently frozen (−20 °C or −80 °C). In the laboratory, meiofaunal organisms are recovered from the 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.

2.3. 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 metabarcording, 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.

2.4. 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, 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 700 Mb, within 24 h. 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, Ilumina 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 2 × 150 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 2 × 300 bp amplicon, generating an output up to 15 Gb in 55 h. 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). Till 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 h) 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 terms 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, 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, 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.

3. 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.

3.1. 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, till 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 or 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.

3.2. 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, 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).

4. Shortcomings and pitfalls

4.1. Amplification and sequencing errors

Despite the high potential in species identification, metagenetic approach presents different shortcomings. Amongst 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 "chimaeras" 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, chimaeras tend to appear in richer and more genetically diverse samples, where molecules from two different origins artificially combine together (Meyerhans et al., 1990). Since chimaeras 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 chimaera 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 chimaeras, include Perseus (Quince et al., 2011), UCHIME (Edgar et al., 2011) and USEARCH (Edgar, 2010). Both Perseus and UCHIME assume that chimaeras 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 chimaera 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 amongst different sequencing platforms used (Glenn, 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 generates tens (MiSeq) to thousands (HiSeq 4000) of times more data per run, needs other approaches, such as quality-filtering strategies (Bokulich et al., 2013).

4.2. 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).

4.3. 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 amongst 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.

5. 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

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

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 amongst individuals of the same species and amongst 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 amongst 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 datasets 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.

Acknowledgements

This paper was produced thanks to the financial support of DEVOTES (*DEVelopment Of innovative Tools for understanding marine biodiversity and assessing good Environmental Status*) project, funded by the European Union under the 7th Framework Programme, 'The Ocean of Tomorrow' Theme (grant agreement no. 308392, www.devotes-project.eu).

References

Angly, F.E., Felts, B., Breitbart, M., et al., 2006. The marine viromes of four oceanic regions. PLoS Biol. 4, e368.
Appeltans, W., Ahyong, S.T., Anderson, G., et al., 2012. The magnitude of global marine species diversity. Curr. Biol. 22 (23), 2189–2202.
Armstrong, M.R., Blok, V.C., Phillips, M.S., 2000. A multipartite mitochondrial genome in the potato cyst nematode *Globodera pallida*. Genetics 154, 181–192.
Avise, J.C., 1994. Molecular Markers, Natural History and Evolution. Chapman and Hall, New York.
Avise, J.C., 2000. Phylogeography: The History and Formation of Species. Harvard University Press, Cambridge, MA, USA (447 pp.).
Baas Becking, L.G.M., 1934. Geobiologie of inleiding tot de milieukunde. VP Van Stockkum and Zoon. The Hague, The Netherlands.
Bartram, A.K., Lynch, M.D.J., Stearns, J.C., Moreno-Hagelsieb, G., Neufeld, J.D., 2011. Generation of multimillion-sequence 16S rRNA gene libraries from complex microbial communities by assembling paired-end Illumina reads. Appl. Environ. Microbiol. 77, 3846–3852.
Bhadury, P., Austen, M.C., Bilton, D.T., Lambshead, P.J.D., Rogers, A.D., Smerdon, G.R., 2006. Development and evaluation of a DNA-barcoding approach for the rapid identification of nematodes. Mar. Ecol. Prog. Ser. 320, 1–9.
Bhadury, P., Austen, M.C., Bilton, D.T., Lambshead, P.J.D., Rogers, A.D., Smerdon, G.R., 2007. Exploitation of archived marine nematodes—a hot lysis DNA extraction protocol for molecular studies. Zool. Scr. 36, 93–98.
Bhadury, P., Austen, M.C., Bilton, D.T., Lambshead, P., Rogers, A., Smerdon, G., 2008. Evaluation of combined morphological and molecular techniques for marine nematode (*Terschellingia* spp.) identification. Mar. Biol. 154, 509–518.
Bhadury, P., Bik, H., Lambshead, J.D., Austen, M.C., Smerdon, G.R., Rogers, A.D., 2011. Molecular diversity of fungal phylotypes co-amplified alongside nematodes from coastal and deep-sea marine environments. PLoS One 6, e26445.
Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K.L., Meier, R., Winker, K., Ingram, K.K., Das, I., 2007. Cryptic species as a window on diversity and conservation. Trends Ecol. Evol. 22, 148–155.
Bik, H.M., Thomas, W.K., Lunt, D.H., Lambshead, P.J.D., 2010. Low endemism, continued deep-shallow interchanges, and evidence for cosmopolitan distributions in free-living marine nematodes (order Enoplida). BMC Evol. Biol. 10, 389.
Bik, H.M., Sung, W., De Ley, P., et al., 2011. Metagenetic community analysis of microbial eukaryotes illuminates biogeographic patterns in deep-sea and shallow water sediments. Mol. Ecol. 21, 1048–1059.
Bik, H.M., Sung, W., de Ley, P., Baldwin, J.G., Sharma, J., Rocha-Olivares, A., Thomas, W.K., 2012. Metagenetic community analysis of microbial eukaryotes illuminates biogeographic patterns in deep-sea and shallow water sediments. Mol. Ecol. 21, 1048–1059.
Bik, H.M., Fournier, D., Sung, W., Bergeron, R.D., Thomas, W.K., 2013. Intra-genomic variation in the ribosomal repeats of nematodes. PLoS One 8 (10), e78230. http://dx.doi.org/10.1371/journal.pone.0078230.
Blaxter, M., 2003. Counting angels with DNA. Nature 421, 122–124.
Blaxter, M.L., De Ley, P., Garey, J.R., et al., 1998. A molecular evolutionary framework for the phylum Nematoda. Nature 392, 71–75.
Blaxter, M., Floyd, R., Abebe, E., 2003. Molecular barcoding for nematode identification and diversity studies. J. Nematol. 35, 326.
Blaxter, M., Mann, J., Chapman, T., et al., 2005. Defining operational taxonomic units using DNA barcode data. Philos. Trans. R. Soc. B 360, 1935–1943.
Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10 (1), 57–59.

Bunge, J., Fitzpatrick, M., 1993. Estimating the number of species: a review. J. Am. Stat. Assoc. 88, 364–373.

Caporaso, J.G., Lauber, C.L., Walters, W.A., et al., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 6, 1621–1624.

Carvalho, G., Creer, S., Allen, M., et al., 2010. Genomics in the discovery and monitoring of marine biodiversity. In: Boyen, C., Cock, M. (Eds.), An Introduction to Marine Genomics. Springer-Verlag, pp. 1–32.

Castro, C.E., Thomason, I.J., 1973. Permeation dynamics and osmoregulation in *Aphelenchus avenae*. Nematologica 19, 100–108.

Cline, J., Braman, J.C., Hogrefe, H.H., 1996. PCR fidelity of Pfu DNA polymerase and other thermostable DNA polymerases. Nucleic Acids Res. 24, 3546–3551.

Corinaldesi, C., Beolchini, F., Dell'anno, A., 2008. Damage and degradation rates of extracellular DNA in marine sediments: implications for the preservation of gene sequences. Mol. Ecol. 17, 3939–3951. http://dx.doi.org/10.1111/j.1365-294X.2008.03880.x.

Corinaldesi, C., Barucca, M., Luna, G.M., Dell'anno, A., 2011. Preservation, origin and genetic imprint of extracellular DNA in permanently anoxic deep-sea sediments. Mol. Ecol. 20, 642–654. http://dx.doi.org/10.1111/j.1365-294X.2010.04958.x.

Coull, B.C., Chandler, G.T., 1992. Pollution and meiofauna: field, laboratory and mesocosm studies. Oceanogr. Mar. Biol. Annu. Rev. 30, 191–271.

Creer, S., Fonseca, V.G., Porazinska, D.L., et al., 2010. Ultrasequencing of the meiofaunal biosphere: practice, pitfalls, and promises. Mol. Ecol. 19, 4–20.

Curini-Galletti, M., Artois, T., Delogu, V., et al., 2012. Patterns of diversity in soft-bodied meiofauna: dispersal ability and body size matter. PLoS One 7, e33801.

Danovaro, R., Fraschetti, S., 2002. Meiofaunal vertical zonation on hard bottoms: comparison with soft-bottom meiofauna. Mar. Ecol. Progr. Ser. 230, 159–169.

Danovaro, R., Dell'Anno, A., Fabiano, M., Pusceddu, A., Tselepides, A., 2001. Deep-sea ecosystem response to climate changes: the eastern Mediterranean case study. Trends Ecol. Evol. 16, 505–510.

Danovaro, R., Gambi, C., Dell'Anno, A., et al., 2008. Exponential decline of deep-sea ecosystem functioning linked to benthic biodiversity loss. Curr. Biol. 18, 1–8.

Danovaro, R., Company, J.B., Corinaldesi, C., et al., 2010. Deep-sea biodiversity in the Mediterranean Sea: the known, the unknown, and the unknowable. PLoS One 5 (8), e11832. http://dx.doi.org/10.1371/journal.pone.0011832.

De Ley, P., Félix, M.A., Frisse, L.M., Nadler, S.A., Sternber, P.W., Thomas, W.K., 1999. Molecular and morphological characterization of two reproductively isolated species with mirror-image anatomy (Nematoda: Cephalobidae). Nematology 1, 591–612.

De Ley, P., De Ley, I.T., Morris, K., et al., 2005. An integrated approach to fast and informative morphological vouchering of nematodes for applications in molecular barcoding. Philos. Trans. R. Soc. Lond. B 360, 1945–1958.

De Wit, R., Bouvier, T., 2006. "Everything is everywhere, but, the environment selects"; what did Baas-Becking and Beijerinck really say? Environ. Microbiol. 8, 755-748.

Decraemer, W., Gourbault, N., Helleouet, M.N., 2001. Cosmopolitanism among nematodes: examples from Epsilonematidae. Vie Milieu 51, 11–19.

Degnan, P.H., Ochman, H., 2011. Illumina-based analysis of microbial community diversity. ISME J. 6, 183–194.

Deiner, K., Walser, J.C., Mächler, E., Altermatt, F., 2015. Choice of capture and extraction methods affect detection of freshwater biodiversity from environmental DNA. Biol. Conserv. 183, 53–63.

Derycke, S., Remerie, T., Vierstraete, Backeljau, A.T., Vanfleteren, J., Vincx, M., Moens, T., 2005. Mitochondrial DNA variation and cryptic speciation within the free-living marine nematode *Pellioditis marina*. Mar. Ecol. Progr. Ser. 300, 91–103.

Derycke, S., Backeljau, T., Vlaeminck, C., et al., 2007. Spatiotemporal analysis of population genetic structure in *Geomonhystera disjuncta* (Nematoda, Monhysteridae) reveals high levels of molecular diversity. Mar. Biol. 151, 1799–1812.

Derycke, S., Fonseca, G., Vierstraete, A., Vanfleteren, J., Vincx, M., Moens, T., 2008a. Disentangling taxonomy within the Rhabditis (*Pellioditis*) *marina* (Nematoda, Rhabditidae) species complex using molecular and morphological tools. Zool. J. Linn. Soc. Lond. 152, 1–15.

Derycke, S., Remerie, T., Backeljau, T., et al., 2008b. Phylogeography of the Rhabditis (*Pellioditis*) *marina* species complex: evidence for long-distance dispersal, and for range expansions and restricted gene flow in the northeast Atlantic. Mol. Ecol. 17, 3306–3322.

Derycke, S., De Ley, P., De Ley, I.T., Holovachov, O., Rigaux, A., Moens, T., 2010a. Linking DNA sequences to morphology: cryptic diversity and population genetic structure in the marine nematode *Thoracostoma trachygaster* (Nematoda, Leptosomatidae). Zool. Scr. 39, 276–289.

Derycke, S., Vanaverbeke, J., Rigaux, A., Backeljau, T., Moens, T., 2010b. Exploring the use of cytochrome oxidase c subunit 1 (COI) for DNA barcoding of free-living marine nematodes. PLoS One 5, e13716.

Desnues, C., Rodriguez-Brito, B., Rayhawk, S., et al., 2008. Biodiversity and biogeography of phages in modern stromatolites and thrombolites. Nature 452, 340–345.

Dover, G., 1982. Molecular drive: a cohesive mode of species evolution. Nature 299, 111–117.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26 (19), 2460–2461.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.

Edgcomb, V., Orsi, W., Bunge, J., et al., 2011. Protistan microbial observatory in the Cariaco Basin, Caribbean. I. Pyrosequencing vs Sanger insights into species richness. ISME J. 5, 1344–1356.

Eren, A.M., Vineis, J.H., Morrison, H.G., Sogin, M.L., 2013. A filtering method to generate high quality short reads using Illumina paired-end technology. PLoS One 8, e66643.

Floyd, R., Abebe, E., Papert, A., Blaxter, M., 2002. Molecular barcodes for soil nematode identification. Mol. Ecol. 11, 839–850.

Floyd, R.M., Rogers, A.D., Lambshead, P.J.D., Smith, C.R., 2005. Nematode-specific PCR primers for the 18S small subunit rRNA gene. Mol. Ecol. Notes 5, 611–612.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoeck, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Mol. Mar. Biol. Biotech. 3, 294–299.

Fonseca, G., Fehlauer-Ale, K.H., 2012. Three in one: fixing marine nematodes for ecological, molecular, and morphological studies. Limnol. Oceanogr. Methods 10, 516–523.

Fonseca, G., Derycke, S., Moens, T., 2008. Integrative taxonomy in two free-living nematode species complexes. Biol. J. Linn. Soc. 94, 737–753.

Fonseca, V.G., Carvalho, G.R., Sung, W., et al., 2010. Second-generation environmental sequencing unmasks marine metazoan biodiversity. Nat. Commun. 1, 98.

Fonseca, V.G., Carvalho, G.R., Nichols, B., et al., 2014. Metagenetic analysis of patterns of distribution and diversity of marine meiobenthic eukaryotes. Global Ecol. Biogeogr. http://dx.doi.org/10.1111/geb.12223.

Fontaneto, D., Kaya, M., Herniou, E.A., Barraclough, T.G., 2009. Extreme levels of hidden diversity in microscopic animals (Rotifera) revealed by DNA taxonomy. Mol. Phylogenet. Evol. 53, 182–189.

Fontaneto, D., Flot, J.F., Tang, C.Q., 2015. Guidelines for DNA taxonomy, with a focus on the meiofauna. Mar. Biodivers. http://dx.doi.org/10.1007/s12526-015-0319-7.

Fontanilla, I.K.C., Wade, C.M., 2008. The small subunit (SSU) ribosomal (r) RNA gene as a genetic marker for identifying infective 3rd juvenile stage *Angiostrongylus cantonensis*. Acta Trop. 105, 181–186.

Gagna, C.E., Lambert, W.C., Kuo, H.R., Farnsworth, P.N., 1997. Localization of B-DNA and Z-DNA in terminally differentiating fiber cells in the adult lens. J. Histochem. Cytochem. 45, 1511–1521.

Gaston, K.J., 2000. Global patterns in biodiversity. Nature 405, 220–227.

Gaston, K.J., 2009. Geographic range limits of species. Proc. R. Soc. Lond. B Biol. 276, 1391–1393.

Giere, O., 1993. Meiobenthology, The Microscopic Fauna in Aquatic Sediments. Springer-Verlag, Berlin.

Giere, O., 2009. Meiobenthology: The Microscopic Motile Fauna of Aquatic Sediments. Springer-Verlag, Heidelberg.

Glenn, T.C., 2011. Field guide to next-generation DNA sequencers. Mol. Ecol. Resour. 11, 759–769.

Gloor, G.B., Hummelen, R., Macklaim, J.M., et al., 2010. Microbiome profiling by Illumina sequencing of combinatorial sequence-tagged PCR products. PLoS One 5, e15406.

Hebert, P.D.N., Ratnasingham, S., DeWaard, J.R., 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B Biol. 270, S96–S99.

Heip, C.H.R., Vincx, M., Vranken, G., 1985. The ecology of marine nematodes. Oceanogr. Mar. Biol. Annu. Rev. 23, 399–489.

Hirai, J., Kuriyama, M., Ichikawa, T., Hidaka, K., Tsuda, A., 2015. A metagenetic approach for revealing community structure of marine planktonic copepods. Mol. Ecol. Resour. 15, 68–80.

Holterman, M., van der Wurff, A., van den Elsen, S., et al., 2006. Phylum-wide analysis of SSU rDNA reveals deep phylogenetic relationships among nematodes and accelerated evolution towards crown clades. Mol. Biol. Evol. 23, 1792–1800.

Huber, J.A., Butterfield, D.A., Baross, J.A., 2002. Temporal changes in archeal diversity and chemistry in a mid-ocean ridge subseafloor habitat. Appl. Environ. Microbiol. 68, 1585–1594.

Huber, J.A., Mark Welch, D.B., Morrison, H.G., et al., 2007. Microbial population structure in the deep marine biosphere. Science 318, 97–100.

Hughes Martiny, J.B., Bohannan, B.J.M., Brown, J.H., et al., 2006. Microbial biogeography: putting microorganisms on the map. Nat. Rev. Microbiol. 4, 102–112.

Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L., Welch, D.M., 2007. Accuracy and quality of massively parallel DNA pyrosequencing. Genome Biol. 8, R143.

Huse, S.M., Welch, D.M., Morrison, H.G., Sogin, M.L., 2010. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ. Microbiol. 12, 1889–1898.

James, S.A., O'Kelly, M.J., Carter, D.M., Davey, R.P., van Oudenaarden, A., Roberts, I.N., 2009. Repetitive sequence variation and dynamics in the ribosomal DNA array of *Saccharomyces cerevisiae* as revealed by whole-genome resequencing. Genome Res. 19, 626–635.

Kemp, P.F., Aller, J.Y., 2004. Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. FEMS Microbiol. Ecol. 47, 161–177.

Knowlton, N., 2000. Molecular genetic analyses of species boundaries in the sea. Hydrobiologia 420, 73–90.

Lambshead, P.J.D., 2004. Marine nematode biodiversity. In: Chen, Z.X., Chen, S.Y., Dickson, D.W. (Eds.), Nematology: Advances and Perspectives. Nematode Morphology, Physiology and Ecology vol. 1. CABI Publishing, Wallingford, U.K, pp. 436–467.

Lambshead, P.J.D., Boucher, G., 2003. Marine nematode deep-sea biodiversity—hyperdiverse or hype? J. Biogeogr. 30, 475–485.

Lawton, J.H., Bignell, D.E., Bolton, B., et al., 1998. Biodiversity inventories, indicator taxa and effects of habitat modification in tropical forest. Nature 391, 72–76.

Leininger, S., Urich, T., Schloter, M., et al., 2006. Archaea predominate among ammonia-oxidizing prokaryotes in soils. Nature 442, 806–809.

Leray, M., Knowlton, N., 2015. DNA barcoding and metabarcoding of standardized samples reveal patterns of marine benthic diversity. PNAS 112 (7), 2076–2081.

Lindeque, P.K., Parry, H.E., Harmer, R.A., Somerfield, P.J., Atkinson, A., 2013. Next generation sequencing reveals the hidden diversity of zooplankton assemblages. PLoS One 8, e81327.

Liu, B.Z., Song, L.S., Xiang, J.H., 2001. DNA isolation and RAPD analysis of the *Argopecten irradians* tissue preserved with different methods. Mar. Sci. 25, 51–53.

Logares, R., Sunagawa, S., Salazar, G., et al., 2014. Metagenomic 16S rDNA Illumina tags are a powerful alternative to amplicon sequencing to explore diversity and structure of microbial communities. Environ. Microbiol. 16 (9), 2659–2671.

Loman, N.J., Misra, R.V., Dallman, T.J., et al., 2012. Performance comparison of benchtop high-throughput sequencing platforms. Nat. Biotechnol. 30, 434–439.

Lorenz, J.G., Jackson, W.E., Beck, J.C., Hanner, R., 2005. The problems and promise of DNA barcodes for species diagnosis of primate biomaterials. Philos. Trans. R. Soc. Lond. B 360, 1869–1877.
Lunt, D.H., Hyman, B.C., 1997. Animal mitochondrial DNA recombination. Nature 387, 247.
Markmann, M., Tautz, D., 2005. Reverse taxonomy: an approach towards determining the diversity of meiobenthic organisms based on ribosomal RNA signature sequences. Philos. Trans. R. Soc. Lond. B 360, 1917–1924.
Medinger, R., Nolte, V., Pandey, R.V., et al., 2010. Diversity in a hidden world: potential and limitation of next-generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. Mol. Ecol. 19, 32–40.
Meldal, B.H.M., Debenham, N.J., De Ley, P., et al., 2007. An improved molecular phylogeny of the Nematoda with special emphasis on marine taxa. Mol. Phylogenet. Evol. 42, 622–636.
Meyerhans, A., Vartanian, J.P., Wain-Hobson, S., 1990. DNA recombination during PCR. Nucleic Acids Res. 18, 1687–1691.
Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B., Worm, B., 2011. How many species are there on Earth and in the ocean? PLoS Biol. 9, e1001127.
Moreno, M., Semprucci, F., Vezzulli, L., Balsamo, M., Fabiano, M., 2011. The use of nematodes in assessing ecological quality status in the Mediterranean coastal ecosystems. Ecol. Indic. 11, 328–336.
Morgan, M.J., Bass, D., Bik, H.M., et al., 2014. A critique of Rossberg et al.: noise obscures the genetic signal of meiobiotal ecospecies in ecogenomic datasets. Proc. R. Soc. Lond. B 281 (20133076).
Nelson, M.C., Morrison, H.G., Benjamino, J., Grim, S.L., Graf, J., 2014. Analysis, optimization and verification of Illumina-generated 16S rRNA gene amplicon surveys. PLoS One 9 (4), e94249. http://dx.doi.org/10.1371/journal.pone.0094249.
Pawlowski, J., Christen, R., Lecroq, B., et al., 2011. Eukaryotic richness in the abyss: insights from pyrotag sequencing. PLoS One 6, e18169.
Pearman, J.K., El-Sherbiny, M.M., Lanzén, A., Al-Aidaroos, A.M., Irigoien, X., 2014. Zooplankton diversity across three Red Sea reefs using pyrosequencing. Front. Mater. Sci. 1, 27. http://dx.doi.org/10.3389/fmars.2014.00027.
Pfenninger, M., Schwenk, K., 2007. Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. BMC Evol. Biol. 7, 121.
Piganeau, G., Gardner, M., Eyre-Walker, A., 2004. A broad survey of recombination in animal mitochondria. Mol. Biol. Evol. 21, 2319–2325.
Poinar, H.N., Schwarz, C., Qi, J., et al., 2006. Metagenomics to paleo-genomics: large-scale sequencing of mammoth DNA. Science 311, 392–394.
Porazinska, D., Giblin-Davis, R.M., Faller, L., et al., 2009a. Evaluating high-throughput sequencing as a method for metagenomic analysis of nematode diversity. Mol. Ecol. Resour. 9, 1439–1450.
Porazinska, D.L., Sung, W., Giblin-Davis, R.M., Thomas, W.K., 2009b. Reproducibility of read numbers in high-throughput sequencing analysis of nematode community composition and structure. Mol. Ecol. Resour. 10, 666–676.
Porazinska, D.L., Giblin-Davis, R.M., Sung, W., Thomas, W.K., 2010. Linking operational clustered taxonomic units (OCTUs) from parallel ultrasequencing (PUS) to nematode species. Zootaxa 2427, 55–63.
Porazinska, D.L., Giblin-Davis, R.M., Powers, T.O., Thomas, W.K., 2012. Nematode spatial and ecological patterns from tropical and temperate rainforests. PLoS One 7, e44641.
Pruesse, E., Quast, C., Knitte, K., Fuchs, B.M., Ludwig, W., Peplies, J., Glöckner, F.O., 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res. 35, 7188–7196.
Pusceddu, A., Bianchelli, S., Gambi, C., Danovaro, R., 2011. Assessment of benthic trophic status of marine coastal ecosystems: significance of meiofaunal rare taxa. Estuar. Coast. Shelf Sci. 93, 420–430.
Qiu, X., Wu, L., Huang, H., et al., 2001. Evaluation of PCR generated chimeras, mutations, and heteroduplexes with 16S rRNA gene-based cloning. Appl. Environ. Microbiol. 67, 880–887.
Quince, C., Lanzen, A., Davenport, R.J., Turnbaugh, P.J., 2011. Removing noise from pyrosequenced amplicons. BMC Bioinf. 12, 38.
Ram, J.L., Karim, A.S., Sendler, E.D., Kato, I., 2011. Strategy for microbiome analysis using 16S rRNA gene sequence analysis on the Illumina sequencing platform. Syst. Biol. Reprod. Med. 57, 162–170.
Reeder, J., Knight, R., 2010. Rapidly denoising pyrosequencing amplicon reads by exploiting rank-abundance distributions. Nat. Methods 7, 668–669.
Reise, K., 1988. Plathelminth diversity in littoral sediments around the island of Sylt in the North Sea. Prog. Zool. 36, 469–480.
Reiss, R.A., Schwert, P.D., Ashworth, A.C., 1995. Field preservation of Coleoptera for molecular genetic analyses. Environ. Entomol. 24, 716–719.
Ristau, K., Steinfartz, S., Traunspurger, W., 2013. First evidence of cryptic species diversity and significant population structure in a widespread freshwater nematode morphospecies (*Tobrilus gracilis*). Mol. Ecol. 22, 4562–4575.
Rogers, A.D., Lambshead, P.J.D., 2004. Molecular studies of nematode diversity; past, present and future. In: Cook, R., Hunt, D.J. (Eds.), Proc Fourth Int Congr Nematol, 8–13 June 2002. Nematology Monographs and Perspectives vol. 2. Brill Academic Publishing, Hendon, VA.
Schmidt-Rhaesa, A., 2014. Handbook of zoology: Gastrotricha, Cycloneuralia and Gnathifera. Nematoda vol. 2. De Gruyter, Berlin, Germany.
Serth, J., Kuczyk, M.A., Paeslack, U., Lichtinghagen, R., Jonas, U., 2000. Quantitation of DNA extracted after micro-preparation of cells from frozen and formalin-fixed tissue sections. Am. J. Pathol. 156, 1189–1196.
Seutin, G., White, B.N., Boag, P.T., 1991. Preservation of avian blood and tissue samples for DNA analysis. Can. J. Zool. 69, 82–90.
Snelgrove, P.V.R., 1999. Getting to the bottom of marine biodiversity: sedimentary habitats. Bioscience 49, 129–138.
Sogin, M.L., Morrison, H.G., Huber, J.A., et al., 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc. Natl. Acad. Sci. U. S. A. 103, 12115–12120.
Srivastava, S.K., Modak, M.J., 1983. Rose Bengal mediated inhibition of DNA polymerases: mechanism of inhibition of avian myeloblastosis virus reverse transcriptase under photooxidative conditions. Biochemistry 22, 2283–2288.
Stoeck, T., Behnke, A., Christen, R., et al., 2009. Massively parallel tag sequencing reveals the complexity of anaerobic marine protistan communities. BMC Biol. 7, 72.
Stoeck, T., Bass, D., Nebel, M., Christen, R., Jones, M.D., Breiner, H.W., Richards, T.A., 2010. Multiple marker parallel tag enviornmental DNA sequencing reveals a highly complex eukaryotic community in marine anoxic water. Mol. Ecol. 19, 21–31.
Subbotin, S.A., Ragsdale, E.J., Mullens, T., Roberts, P.A., Mundo-Ocampo, M., Baldwin, J.G., 2008. A phylogenetic framework for root lesion nematodes of the genus *Pratylenchus* (Nematoda): evidence from 18S and D2–D3 expansion segments of 28S ribosomal RNA genes and morphological characters. Mol. Phylogenet. Evol. 48, 491–505.
Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E., 2012. Towards next-generation biodiversity assessment using DNA metabarcoding. Mol. Ecol. 21, 2045–2050.
Tang, C.Q., Leasia, F., Oberteggerc, U., Kieneked, A., Barracloughа, T.G., Fontaneto, D., 2012. The widely used small subunit 18S rDNA molecule greatly underestimates true diversity in biodiversity surveys of the meiofauna. Proc. Natl. Acad. Sci. U. S. A. 109 (40), 16208–16212.
Tittensor, D.P., Mora, C., Jetz, W., et al., 2010. Global patterns and predictors of marine biodiversity across taxa. Nature 466, 1098-1001.
Vanelslander, B., Creach, V., Vanormelingen, P., et al., 2009. Ecological differentiation between sympatric pseudocryptic species in the estuarine benthic diatom *Navicula phyllepta* (Bacillariophyceae). J. Phycol. 45, 1278–1289.
Vanfleteren, J.R., Vierstraete, A.R., 1999. Insertional RNA editing in metazoan mitochondria: the cytochrome b gene in the nematode *Teratocephalus lirellus*. RNA 5, 622–624.
von Wintzingerode, F., Gobel, U.B., Stackebrandt, E., 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213–229.
Wellborn, G.A., Broughton, R.E., 2008. Diversification on an ecologically constrained adaptive landscape. Mol. Ecol. 17, 2927–2936.
Wilson, A.C., Cann, R.L., Carr, S.M., et al., 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. Biol. J. Linn. Soc. 26, 375–400.
Ye, W., Giblin-Davis, R., Braasch, H., Morris, K., Thomas, W., 2007. Phylogenetic relationships among *Bursaphelenchus* species (Nematoda: Parasitaphelenchidae) inferred from nuclear ribosomal and mitochondrial DNA sequence data. Mol. Phylogenet. Evol. 43, 1185–1197.
Yoder, M., Tandigan De Ley, I., King, I.W., 2006. DESS: a versatile solution for preserving morphology and extractable DNA of nematodes. Nematology 8, 367–376.
Zhan, A., MacIsaac, H.J., 2015. Rare biosphere exploration using high-throughput sequencing: research progress and perspectives. Conserv. Genet. http://dx.doi.org/10.1007/s10592-014-0678-9.
Zinger, L., Amaral-Zettler, L.A., Fuhrman, J.A., et al., 2011. Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. PLoS One 6, e24570.