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Marine protist diversity in European coastal waters and sediments as revealed by high-throughput sequencing

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Running title: Protist diversity in European coastal areas

Summary

Although protists are critical components of marine ecosystems they are still poorly characterized. Here we analyzed the taxonomic diversity of planktonic and benthic protist communities collected in six distant European coastal sites. Environmental DNA and RNA from three size-fractions (pico-, nano-, and micro/meso-plankton) as well as from dissolved DNA and surface sediments were used as templates for tag pyrosequencing of the V4 region of the 18S rDNA. β-diversity analyses split the protist community structure into three main clusters: picoplankton-nanoplankton-dissolved DNA, micro/mesoplankton, and sediments. Within each cluster, protist communities from the same site and time clustered together, while communities from the same site but different seasons were unrelated. Both DNA and RNA-based surveys provided similar relative abundances for most class-level taxonomic groups. Yet, particular groups were overrepresented in one of the two templates, such as MALV-I and MALV-II that were much more abundant in DNA surveys. Overall, the groups displaying the highest relative contribution were Dinophyceae, Diatomea, Ciliophora and Acantharia. Also well represented were Mamiellophyceae, Cryptomonadales, MALV and MAST in the picoplankton, and Monadofilosa and basal Fungi in sediments. Our extensive and systematic sequencing of geographically separated sites provides the most comprehensive molecular description of coastal marine protist diversity to date.

Key words: 18S rDNA / dissolved DNA / environmental DNA and RNA extracts / marine protist diversity / pyrosequencing / taxonomy

Introduction

Protists or unicellular eukaryotes cover a wide spectrum of cell sizes, shapes, and taxonomic affiliations (Schaechter, 2012). They represent the majority of eukaryotic lineages, so studying their diversity is of primary interest for understanding the eukaryotic tree of life (Keeling *et al*., 2005; Burki, 2014). Moreover, protists play a variety of crucial roles in marine ecosystems from primary producers, predators, decomposers to parasites (Sherr *et al*., 2007), leading to much effort in quantifying particular species and inferring their ecological functions. A vast literature exists in which species of dinoflagellates (e.g. Graham *et al*., 2004), diatoms (e.g. Olguín *et al*., 2006) and ciliates (e.g. Dolan *et al*., 2013) have been studied based on morphological features observable in light microscopy (LM), a task that requires considerable expertise and time to key out species accurately. Even for these relatively visible groups, examples are known of morphologically similar individuals belonging to different cryptic species (Amato et *al*., 2007) or morphologically distinct types from the same species (Pizay *et al*., 2009). Accurate identification may thus not always be discerned from cell morphology alone, and this is more critical for protists below 20 µm in size that often lack conspicuous shapes (Massana, 2011). Over the last decades, DNA sequencing of environmental phylogenetic markers has changed our perception of microbial diversity in most ecosystems. These molecular surveys have been instrumental in decoding the large protist diversity and in unveiling new lineages, such as Picozoa (Not *et al*., 2007; Seenivasan *et al*., 2013), MALV (Marine Alveolates) clades (Guillou *et al*., 2008) and MAST (Marine Stramenopiles) clades (Massana *et al*., 2004; 2014).

Earlier molecular surveys were based on clone libraries of near full-length 18S rDNA genes followed by Sanger sequencing of a subset of the clones (Díez *et al*., 2001; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001). The resulting high-quality, often manually checked environmental sequences have been crucial for the phylogenetic placement of novel clades and, together with sequences from monoclonal cultures, are the basis of reference rDNA databases (Guillou *et al*., 2013; Pernice *et al*., 2013). However, traditional clone libraries only capture the most dominant species in the community (Pedrós-Alió, 2006), a limitation bypassed by high-throughput sequencing (HTS) methods. By providing the deep inventories needed both for taxonomic descriptions and sample comparisons, HTS has enabled microbial ecology to advance greatly. HTS has been applied to study protist diversity in a wide variety of systems, including surface and deep marine waters (Amaral-Zettler *et al*., 2009; Cheung *et al*., 2010; Edgcomb *et al*., 2011; de Vargas *et al*., 2015), marine sediments (Bik *et al*., 2012), lakes (Mangot *et al*., 2013), soils (Bates *et al*., 2013), and metazoan hosts (He *et al*., 2014). In the case of marine protists, most studies have targeted a specific size-fraction or a particular location. In addition, these surveys generally used environmental DNA as template for PCR amplification, and it has been shown that using RNA extracts instead can provide a different picture of biodiversity (Stoeck *et al*., 2007; Not *et al*., 2009; Lejzerowicz *et al*., 2013) and useful complementary information (Blazewicz *et al*., 2013).

The present study is an investigation of benthic-pelagic protists in marine habitats along the European coastline, sampled between 2009 and 2010 during the research program *BioMarKs*. The 95 different pyrosequenced samples analyzed herein address total protist diversity from benthic and planktonic (size-fractionated) communities using an eukaryotic "universal" primer set to PCR amplify the V4 rDNA pre-barcode (Pawlowski *et al*., 2012) from both DNA and RNA extracts. Previous studies using this sequencing dataset focused on particular taxonomic groups, such as uncultured MAST (Logares *et al*., 2012), cercozoan amoebae (Berney *et al*., 2013) or diatoms (Nanjappa *et al*., 2014). More recently, we used a subset of the samples (23 planktonic RNA samples) and newly collected HTS reads (Illumina sequencing of the V9 18S rDNA region) to investigate the patterns of a particular community property, the rare biosphere (Logares *et al*., 2014). Here we analyze the complete 454 dataset from a taxonomic community perspective to address the following questions: How different are the protist communities found in the pico-, nano-, micro/mesoplankton and sediments? Does the dissolved DNA fraction originate from particular taxonomic groups and/or organismal size-fractions? Do DNA and RNA surveys provide similar protist diversity profiles? Which taxonomic groups are differentially represented in either survey? Which groups dominate in each plankton organismal size fraction and associated sediments? Overall, our study highlights fundamental questions on the diversity of protists, an important but less known component of marine microbial ecosystems (Caron *et al*., 2009).

Results

As a product of the joint effort within the *BioMarKs* project we sampled six European coastal sites in the water column and sediments (Table 1). The thirteen planktonic communities were size fractionated (pico-, nano-, micro/mesoplankton and dissolved DNA) and, together with 7 benthic communities, used to obtain environmental DNA and RNA for pyrosequencing the V4 region of the 18S rDNA. Most assayed templates were successful, and we obtained 26 pyrosequenced samples for picoplankton, 26 for nanoplankton, 21 for micro/mesoplankton, 8 for dissolved DNA and 14 for sediments (Table 1). The pyrotag number for each 454-sample is detailed in Table S1 (~9000 pyrotags per sample on average). Pyrotags from these 95 samples clustered in 15,295 OTU_{97} (Table S2), which were classified into 99 eukaryotic taxonomic groups (excluding unknown, metazoan and nucleomorphs). The broad coverage of this dataset across ecological and taxonomic scales provided a unique opportunity to study the diversity of both planktonic and benthic marine coastal protists.

Reproducibility of PCR and 454 reactions at the OTU level

We explored the reproducibility of sequencing outputs using a subset of nucleic acid extracts (11 DNA and 14 RNA) derived from three cruises (2 from Blanes, 11 from Naples-2009 and 12 from Oslo-2009) and including the four compartments (8 picoplankton, 7 nanoplankton, 5 micro/mesoplankton and 5 sediments). Each duplicated pair (same nucleic acid template and separate PCR and 454 reactions) was selected from the OTU table of 120 samples (Table S2), and linear regressions were performed by plotting OTU abundances in each pair. The pyrotag ratio between duplicates ranged from ~1 to 0.05 and was used to display the statistics of all regressions at once (Fig. 1). The R^2 coefficients (Fig. 1A) were always high (0.89 on average) and independent of pyrotag ratios. This indicated that the same abundant OTUs were found at similar relative abundances in duplicates. The regression slopes were explained by pyrotag ratios (R^2 of 0.92; slope of 0.84; $p<0.001$), indicating that OTU abundances increased proportionally with the number of pyrotags in the sample. Next we explored the pyrotags and OTUs shared between duplicates and calculated the shared percentages of the pair with fewer pyrotags (Fig. 1B). The amount of shared OTUs ranged from 32 to 89% and was higher at low pyrotag ratios, displaying a significant relationship ($R^2=0.52$, $p<0.001$). Indeed, it was easier to find OTUs from the sample with less pyrotags in its duplicate with higher sequencing effort. The percentage of pyrotags included in the set of shared OTUs was always high (average of 91%) and its variation was poorly related to pyrotag ratios ($p=0.048$), indicating that OTUs appearing in only one duplicate contained few pyrotags. Thus, as expected, most differences between duplicates are found among the rare OTUs.

Comparisons between communities

ß-diversity patterns of protist assemblages were explored in a dendrogram with 92 samples. Structuring patterns revealed three main groups of samples: *(i)* picoplankton, nanoplankton and dissolved DNA, *(ii)* micro/mesoplankton, and *(iii)* sediments (Fig. 2). Within the planktonic size fractions, samples from each sampling event grouped together (large grey dots in Fig. 2), regardless of water depth or nucleic acid template (only Naples-2010 did not form a cluster due to the atypical sample "DCM_picoplankton_DNA"). Planktonic samples from the same site collected at different seasons and years did not cluster together, indicating the significant impact of seasonal community changes. By contrast, sediment samples collected in different periods in Naples or Oslo clustered together, suggesting a more stable community in sediments than in plankton.

Protist community structure derived from DNA or RNA-based exhibited only minor differences. In total, 24 of the 41 DNA/RNA pairs appeared closest in the dendrogram (small black dots in Fig. 2). Most of the other pairs were still very close, but their coupling was less tight than that between pico- and nanoplankton from the same site, micro/mesoplankton from surface and DCM, or sediment cores from the same site. Only in two cases (the DCM picoplankton in Naples-2010 and Oslo-2010) the DNA and RNA samples were rather different, although still belonging to the same geographic cluster.

The dendrogram showed a close association of dissolved DNA samples with the pico- and nanoplankton from the same water body (Fig. 2). We then analyzed the overlap of individual OTUs amongst size fractions in the planktonic samples that had a complete sequence report of the four fractions in the DNA survey (Fig. 3). Many of the OTUs from dissolved samples (59% on average) were shared with the pico- and nanoplankton (alone, combined, or together with the micro/mesoplankton). Still, a substantial number of OTUs (35%) remained unique to dissolved samples, whereas only a few (6%) were shared with the micro/mesoplankton. We then computed the number of pyrotags from dissolved samples within each shared subarea (Fig. 3). OTUs shared with the small size fractions explained 89% of pyrotags while unique

OTUs accounted for 8% of pyrotags. Thus, the dissolved DNA composition largely derived from the pico- and nanoplankton fractions.

Taxonomic representations derived from DNA and RNA surveys

Relative abundances of taxonomic groups seen in DNA and RNA-based surveys were analyzed separately for picoplankton (13 DNA/RNA pairs), nanoplankton (13 pairs), micro/mesoplankton (10 pairs), and sediment (7 pairs) samples (Fig. 4). Within the picoplankton we focused in the 26 taxonomic groups more abundant (96.4% pyrotags on average; range of 92.1% to 99.2% in the 26 samples). Most were equally represented in DNA and RNA surveys, as shown by their placement near the 1:1 line (Fig. 4A). Two groups, MALV-I and MALV-II, were notably overrepresented in DNA surveys, averaging 15.4% and 19.7% of DNA pyrotags but only 1.8% and 2.0% of RNA pyrotags. Conversely, seven groups were overrepresented in RNA surveys. These RNA-prevalent groups were Prymnesiophyceae (averaged RNA/DNA pyrotag ratio of 7.4), MOCH-2 (6.0), Pelagophyceae (4.6), Telonema (2.6), Choanomonada (2.1), Ciliophora (2.0) and Chrysophyceae (1.6). In the nanoplankton, we identified 23 taxonomic groups that accounted for 97.1% of pyrotags (between 94.7% and 99.6% amongst samples), and many were equally represented in both surveys (Fig. 4B). As for the picoplankton fraction, MALV-I and -II were prevalent in nanoplankton DNA surveys (9.8% of DNA signal versus 2.3% in RNA), while virtually the same groups were prevalent in RNA surveys: Prymnesiophyceae (RNA/DNA pyrotag ratio of 8.6), MOCH-2 (5.4), Pelagophyceae (5.3), Choanomonada (3.6), Ciliophora (2.9), and Chrysophyceae (3.7). In the micro/mesoplankton, 9 taxonomic groups explained 98.6% of pyrotags (96.6% to 99.9%). Within this compartment MALV-I was overrepresented in DNA surveys and Acantharia showed the opposite trend (Fig. 4C). In sediments, 28 groups accounted for 95.8% of pyrotags (91.4 to 98.4%), and the DNA versus RNA-based taxonomic abundances were more biased than in pelagic samples (Fig. 4D). Seven groups were more abundant in DNA surveys

(Apicomplexa, basal Fungi, Dinophyceae, MALV-I, MALV-II, Peronosporomycetes and RAD-B) while 4 were more abundant in RNA surveys (Bicosoecida, Chrysophyceae, Ciliophora and Tubulinea). In general, taxonomic groups exhibited a consistent RNA/DNA trend across the four major compartments analyzed.

An overview of marine coastal protist diversity based on total RNA extracts

We averaged the contribution of each taxonomic group for all samples within the 9 different combinations of planktonic size fractions, sediments, and nucleic acid templates (Fig. S1). Here we used the RNA survey to provide an overview of protist diversity within the different ecological compartments (Fig. 5), based on the 40 most prevalent taxonomic groups (which account for 97.7% pyrotags in picoplankton, 98.5% in nanoplankton, 99.4% in micro/mesoplankton and 95.2% in sediment samples). Within the picoplankton, Dinophyceae (25.3%), Ciliophora (11.9%) and Mamiellophyceae (9.3%) were the most abundant, followed by 19 additional groups with 1 to 8% relative abundance. The taxonomic composition of the nanoplankton was similar to that of the picoplankton, being dominated by Dinophyceae (39.3%), Diatomea (24.9%), and 13 additional groups at 1-8% abundance. Some contributed similarly in both size fractions, but others were clearly overrepresented in the picoplankton (Mamiellophyceae, Chrysophyceae, Pelagophyceae, Picozoa, Chlorarachniophyta, MAST-7, and MAST-4) or in the nanoplankton (Diatomea, Telonema, Katablepharidae and Trebouxiophyceae). The micro/mesoplankton was dominated by Acantharia (35.1%), Dinophyceae (28.9%) and Diatomea (22.4%), and only Ciliophora and MALV-IV displayed 1-8% abundance. Finally, sediments showed remarkable differences with the plankton. Diatomea (40.9%), Ciliophora (19.4%) and Monadofilosa (11.8%) were dominant in these samples, whereas 8 additional groups appeared at 1-8% abundances, seven of them being very rare in the plankton: basal Fungi, Labyrinthulomycetes, MAST-6, Granofilosea, Apusomonadida, Apicomplexa and Centrohelida.

Dominant OTUs in RNA surveys

All previous analyses were done with OTUs clustered at 97% similarity, which sometimes enclose more than one species or genera. In our dataset, this was obvious within Dinophyceae (the most abundant OTU_{97} retrieved 44 genera above 97% similarity), Diatomea and Cryptomonadales. Consequently, we used OTUs clustered at 99% similarity (a total of 63,365 OTU_{99}) to report the dominant taxa (>0.8% of pyrotags) from the RNA survey (Table 2). Dominant OTU_{99} were often 100% similar to described species. Picoplankton samples were the most distinct, containing small-sized genera like *Micromonas*, *Pelagomonas*, *Florenciella*, and *Minorisa* and several MAST and MALV phytotypes. They also included species within the Acantharia, Ciliophora and Dinophyceae known to belong to the microplankton, so their detection could be due to the presence of small life-cycle stages or to filtration artifacts. Dominant OTU_{99} within the other ecological compartments belonged mostly to Diatomea, Dinophyceae, Ciliophora and Acantharia. Of particular interest were the MALV-IV OTUs in the micro/mesoplankton (MALV-IV are crustaceans' parasites), and OTUs of uncultured basal Fungi, MAST-6 and Monadofilosa in sediments.

Picoplanktonic dominant OTU_{99} tended to exhibit a high frequency across the investigated sites (Table 2). On average they occurred in 74% of the sites, while the occurrence of dominant OTUs in the other compartments was lower, with on average 65% of sites in the nanoplankton, 56% in the micro/mesoplankton and only 55% in sediments. On note, while the dominant OTU_{99} of Ciliophora, Acantharia and Diatomea generally appeared in half of the samples, those of Dinophyceae were generally more widespread (86% of sites).

Discussion

Molecular surveys of marine protist diversity initiated in 2001 (Díez *et al*., 2001; López-García *et al*., 2001; Moon-van der Staay *et al*., 2001), and developed together with the availability of new molecular tools including HTS (Amaral-Zettler *et al*., 2009; Cheung *et al*., 2010; Edgcomb *et al*., 2011; Logares *et al*., 2014). Previous surveys were partial, restricted to a cell-size fraction, a given geographic area, or a single nucleic acid template. We study here planktonic and benthic protist diversity inhabiting distant coastal places, from the smallest picoeukaryotes to larger colonial cells up to 2 mm in size (including also dissolved DNA), and derived from DNA and RNA templates. Given that the sampling plan was designed to maximize the ecological space covered, the emerging patterns are likely generalizable to other similar coastal locations (in terms of latitude and/or bathymetry). Seasonal studies at each site, required for a complete diversity description (Nolte *et al*., 2010), were not addressed in our survey, but time-series HTS datasets are underway in most sites. Moreover, HTS studies using group-specific primers are being conducted to obtain a finer phylogenetic resolution of particular taxonomic groups (Egge *et al.*, 2015). Altogether, our study gives an improved view of marine protists diversity and clearly illustrates the differences between the taxonomic composition derived in DNA and RNA surveys.

Technical considerations

There has been concern about the lack of replication in molecular surveys (Prosser, 2010; Robasky *et al.,* 2014), and our strategy was to test the reproducibility of OTU profiles in a set of 25 samples processed twice for the PCR and pyrosequencing steps. Duplicated pairs were usually very similar (range of R^2 from 0.64 to 1.00) and systematically retrieved the same dominant OTUs at comparable relative abundances. Thus, our molecular surveys were well suited for obtaining robust ß-diversity and taxonomic descriptions. Nevertheless, many low abundant OTUs were found in only one pair, implying that undersampling was affecting the richness observed and/or the finding of different errors in each pair (Decelle *et al.*, 2014).

During the preparation of the OTU table for downstream analysis we removed unknown OTUs that contributed substantially to the OTU number (~13%) but little to the pyrotag number (~1%). This removed sequencing artifacts and chimeras while did not compromise our goal of a comprehensive protist description since they represented few pyrotags. Novel diversity was likely removed, however investigating novel branches on the tree of life was not the aim of this study and deserves a separate analysis. We also removed OTUs affiliating to metazoans, which contributed 1-10% of pyrotags in picoplankton and nanoplankton and about 50% in micro/mesoplankton and sediment samples. The presence of metazoans in smaller fractions could be due to minute life-cycle stages but also to the breakdown of animals during filtration. The later certainly occurs in some cases, such as picoplankton OTUs affiliating to copepods (these have internal fecundation and never release gametes). So, although metazoans are worthy targets for molecular studies (Fonseca *et al.*, 2010), we preferred to exclude them from our analysis. Finally, our survey aimed to cover all eukaryotic lineages but it is important to keep in mind that universal primers may miss some relevant taxonomic groups. In our case, this negative selection was certain for Foraminifera, Prymnesiophyceae, and several excavate lineages.

DNA versus RNA-based surveys of total marine protist diversity

Comparing diversity surveys using environmental DNA or RNA is a common practice in microbial ecology (Weinbauer *et al.*, 2002). Initially, the rDNA/rRNA ratios measured from a given microbial population were proposed as proxies of *in situ* growth rates (Poulsen *et al.*, 1993), but soon this moved to a more qualitative scenario where OTUs found in DNA surveys indicated species present, while OTUs found in RNA represented active species (Stoeck *et al.*, 2007; Jones and Lennon, 2010). Even this simplified view might not be universal, and differences in life histories, life strategies, and non-growth activities among species might confound this interpretation (Blazewicz *et al.*, 2013). Nevertheless, protistologists have generally accepted this scenario, and RNA surveys were recently used to identify the active protist community (Stoeck *et al.*, 2007; Not *et al.*, 2009; Lejzerowicz *et al.*, 2013). However, there is an additional factor that may severely affect rDNA/rRNA comparisons in eukaryotes, which is the large variation (up to orders of magnitude) in the rDNA copy number among species (Zhu *et al.*, 2005; Weber and Pawlowski, 2013).

In our dataset, community structure derived from DNA or RNA templates was similar, as paired samples clustered very close in the dendrogram and many taxonomic groups exhibited similar relative abundances. Among the groups with differential abundance, the most extreme were MALV-I and MALV-II that dominated picoplankton DNA surveys as in other studies (Massana, 2011) and contributed little to RNA surveys. One explanation for this discrepancy is that the MALV I-II parasites are abundant in the picoplankton as dispersal, relatively inactive stages with few ribosomes. However, FISH (Fluorescence In situ Hybridization) direct cell counts showed relative abundances much lower than in DNA surveys (Siano *et al.*, 2010), so a most plausible explanation is that MALV I-II cells have a higher genomic rDNA copy number than other picoeukaryotes. Similarly, groups more abundant in RNA surveys may have lower rDNA copy numbers. Given the consistent trends found across size-fractions, our DNA/RNA ratios could reflect different genomic architectures among taxonomic groups, and not necessarily different relative activities. At any rate, RNA surveys should provide a better representation of *in situ* protist biomass and diversity, since labile RNA likely derives from living cells, in contrast with DNA that can be preserved in dead cells or in the dissolved extracellular pool (Karl and Bailiff, 2012). Our analysis of the eukaryotic diversity derived from dissolved DNA samples showed that, in contrast to what was found in anoxic deep-sea sediments (Corinaldesi *et al*., 2011), these samples did not contain a particular phylogenetic signature. Dissolved DNA samples from these coastal sites resembled the pico- and nanoplankton from the same seawater, suggesting cell breakage during filtration.

Marine protist diversity across organismal size-fractions and habitats

Our analysis confirms a well-known observation that planktonic and benthic protists are different (Lee and Patterson, 1998; Bik *et al*., 2012). This was evident in the ß-diversity analysis as well as in the taxonomic list, where 13 out of 40 groups were much more abundant in sediments than in the water column. Sediments have also been considered seed banks of planktonic biodiversity, out of which taxa can re-emerge into the plankton at appropriate seasons (Satta *et al*., 2010). Our data is consistent with this view, since dominant OTUs in the plankton generally were also found, at lower abundances, in sediments.

Regarding planktonic assemblages, our data show a broad differentiation among larger (micro/mesoplankton) and smaller (pico- and nanoplankton) protists. This could be due partly to the different collection protocols (net tows and pressure filtration, respectively), but also from the known different cell size of given species. Within the micro/mesoplankton, only four taxonomic groups dominated, Acantharia, Dinophyceae, Diatomea and Ciliophora, and we did not find other relevant groups, consistent with classical inverted microscopy inspections of the plankton. At the other end of the size spectrum, many taxonomic groups were identified, highlighting the high-rank diversity of small marine protists (Massana, 2011). Picoplankton and nanoplankton assemblages from the same seawater were always related, but a closer inspection revealed marked differences in the relative abundances of key groups. The diversity of both large and small protists was very different in each coastal site, so a standard protist community could not be delineated. Samples from the same site but different seasons were also unrelated, indicating the impact of seasonal successions and highlighting that one or few samples cannot represent the complete diversity at one site.

Molecular surveys of protist diversity have often used size-fractionated samples to discern among different size classes. This step may introduce artifacts, derived from cell breakage and retention of smaller fragments in the smaller size fraction, or from the retention of smaller cells in filters whose larger pores have been obstructed after processing large sample volumes. Our study has ample evidences of the first case, since about 40% of picoplankton pyrotags comes from the four dominating micro/mesoplankton groups. Although part of this signal might derive from gametes or spores (Amato *et al*., 2007; Kimoto *et al*., 2011), the most plausible explanation is that larger cells from these groups (typically >10 µm) are broken during the filtration (Sørensen *et al*., 2013). This bias could have been even more dramatic had we used a smaller pore-size filter (such as 0.2 µm) for picoplankton collection (Sørensen *et al*., 2013). Picoeukaryote diversity excluding this microplanktonic signal would be composed by a set of photosynthetic groups (58% of pyrotags), heterotrophic groups (16%), MALV clades (15%) and MAST clades (11%). Following this reasoning, it is expected that nanoplanktonic cells are also represented in the picoplankton (such as Cryptomonadales). The second case of filtration artifact, smaller cells retained in larger size fractions, is not obvious from our data, and groups known to be picoeukaryotes (like Mamiellophyceae, MAST-4 or MAST-7) are only minoritary in the nanoplankton. So, our data show clear evidence of larger cells collected in smaller size fractions but little support of the other way around. Filtration artifacts need to be considered when interpreting molecular surveys, and cell sizes should be established by direct cell observations by FISH (Siano *et al*., 2010, Massana, 2011).

Concluding remarks

This study presents a description of marine coastal protist diversity obtained from 95 independent HTS samples. Our data show that larger protists affiliate to a few taxonomic groups well studied by microscopy for decades, whereas smaller protists, known as "small flagellates", include a wide variety of less-known taxonomic groups. Our results provide answers to the initial questions, with a special emphasis on *(i)* the general similarity between DNA and RNA surveys despite clear biases in few groups, *(ii)* the taxonomic discontinuity between micro/mesoplankton and the two smaller size fractions, *(iii)* the phylogenetic signature of dissolved DNA deriving from the smallest cells in the sample, and *(iv)* the distinct composition of planktonic and benthic communities. Our data also suggests a higher seasonal variability in the plankton compared to the benthos. These patterns emerging from the large spatial sampling strategy adopted here would likely apply to other coastal sites, while open ocean assemblages require similar approaches (de Vargas *et al*., 2015). In the future, diversity studies based on sequencing surveys, complementary FISH counts and SAGs (Single Amplified Genomes) analyses will contribute to infer the genetic potential and ecological roles of the key protist players.

Experimental Procedures

Sampling

Marine samples were collected through the *BioMarKs* project (http://biomarks.eu/) in coastal sites near Blanes (Balearic Sea, Spain), Gijon (Gulf of Biscay, Spain), Naples (Tyrrhenian Sea, Italy), Oslo (Skagerrak, Oslofjorden, Norway), Roscoff (Western English Channel, France) and Varna (Black Sea, Bulgaria) (Table 1). Some sites are long-term observatories, the Blanes Bay Microbial Observatory (BBMO), the Long Term Ecological Research station MareChiara (Naples), and the SOMLIT-Astan site (Roscoff). Seawater temperature, salinity and chlorophyll *a* concentration were recorded with CTD (conductivity-temperature-depth) and fluorometer sensors. Water samples were collected with Niskin bottles at the sea surface in all sites (and at the DCM [deep chlorophyll maximum] in a few sites), and passed through a 20 µm metallic sieve. The nanoplankton (3-20 µm) and picoplankton (0.8-3 µm) fractions were sampled from ~20 liters of seawater filtered sequentially with a peristaltic pump through 3 µm and 0.8 µm polycarbonate (PC) membranes (142 mm diameter) for less than 40 min to minimize RNA degradation. For dissolved DNA, 20 liters of 0.2 µm-filtered seawater were mixed with 400 mL of 0.5% CTAB (pH=8) for 5 h and filtered onto 0.2 µm PC membranes (142 mm). A plankton net of 20-µm mesh-size was towed for 5-15 min and the large protists collected were rinsed with 0.2 µm filtered seawater, passed through a 2000 µm metallic sieve and filtered on 12 µm PC membranes (47 mm), to collect the micro- (20-200 µm) and meso- (200-2000 µm) planktonic fractions (micro/mesoplankton). Filters were flash-frozen and stored at -80° C until processed. Finally, sediment cores were taken with a Multicorer sampler or by Scuba divers and kept at -80°C until processed.

Nucleic acid extraction and pyrosequencing

Total DNA and RNA were extracted simultaneously from a complete filter using the NucleoSpin® RNA kit (Macherey-Nagel) or from 2.5 g of surface sediment (~1 cm upper layer) using the Power Soil RNA kit (MoBio). Extracts were quantified using a Nanodrop ND-1000 Spectrophotometer and checked on a 1.5% agarose gel. Contaminating DNA was removed from RNA extracts using the Turbo DNA-free kit (Ambion). Complete DNA removal was verified by PCR using eukaryotic primers, and in the few cases of positive PCR we did a second DNase treatment. One hundred ng of extracted RNA were immediately reverse transcribed to cDNA using the RT Superscript III random primers kit (Invitrogen). Both DNA extracts and cDNA products were kept at -80°C until processed.

Environmental DNA or cDNA were used as templates for PCR amplification of the V4 region of the 18S rDNA (~380 bp) using primers TAReuk454FWD1 and TAReukREV3 (Stoeck *et al*., 2010) that amplify most eukaryotic groups. The forward primer had a barcode and both primers were adapted for 454 sequencing. PCR reactions (25 µl) contained 1x Master Mix Phusion® High-Fidelity DNA Polymerase (Finnzymes), 0.35 µM of each primer, 3% DMSO, and 5 ng of DNA or cDNA. The PCR program had an initial denaturation step at 98°C during 30 sec, 10 cycles of 10 sec at 98°C, 30 sec at 53°C and 30 sec at 72°C, then 15 similar cycles but with 48°C annealing temperature, and a final step at 72°C for 10 min. PCR triplicates were purified and eluted (30 µl) with NucleoSpin® Gel and PCR Clean-Up kit (Macherey-Nagel), and quantified with the Quant-It™ PicoGreen® dsDNA Assay kit (Invitrogen). About 1 µg of pooled amplicons were sent to Genoscope (http://www.genoscope.cns.fr, France) for pyrosequencing on a 454 GS FLX Titanium system (454 Life Sciences, USA). The complete sequencing dataset is available at the European Nucleotide Archive (ENA) under the study accession number PRJEB9133 (http://www.ebi.ac.uk/ena/data/view/PRJEB9133).

Pyrotag processing and OTU tables

Sequences obtained through 454 sequencing (pyrotags) were demultiplexed using the barcode identifier in the forward primer. Pyrotags 150-600 bp long, with exact primer sequences, and with homopolymers no longer than 8 bases, were retained. Identical pyrotags within each sample were dereplicated to keep a single representative sequence. For quality check, errors were computed in sliding windows of 50 bp and pyrotags containing a window with an error >1% and appearing only once in the dataset were removed. Chimera check was run with UCHIME (Edgar *et al*., 2011), using *de novo* and reference-based chimera searches against the SILVA108 release (Quast *et al.*, 2013). Additional chimera searches were done using ChimeraSlayer (Haas *et al.*, 2011) and SILVA108. A local BLAST search (Altschul *et al.*, 1990) against SILVA108 was used to exclude 16S rDNA prokaryotic or plastidial sequences.

This initial procedure yielded ~1.3 million curated pyrotags derived from 120 samples (95 distinct samples, 25 of them in duplicate [same nucleic acid extract but separate PCR and sequencing reactions]). These were clustered into OTUs (Operational Taxonomic Units) at 97% similarity (OTU_{97}) with USEARCH (Edgar, 2010). A local BLAST was done to compare the representative sequence of each OTU (the most abundant one) against the GenBank Release 183.0. Distant OTUs having an e-value $>10^{-100}$ (below ~85% similarity) were considered as "unknown" and removed. Taxonomic OTU assignation was performed by best hit BLAST against two reference databases, PR^2 (Guillou *et al.*, 2013) and a smaller and phylogenetically fully-validated database (Pernice *et al.*, 2013). Metazoans and nucleomorphs were removed, leaving OTUs classified into 99 eukaryotic groups, including 64 described groups generally at class level (Adl *et al.*, 2012), 27 environmental ribogroups (Guillou *et al.*, 2008; Massana *et al.*, 2014), and 8 unidentified categories within each supergroup. The initial OTU table (120 samples) was used to extract duplicated samples. A second table with the distinct 95 samples was prepared for the general diversity analyses. A third table (only the 44 RNA samples) was used for the taxonomic description (see Table S2 for the number of OTUs and pyrotags within these tables).

For beta diversity analyses, three samples with few 300-500 pyrotags (Table S1) were removed from the OTU table and the remaining 92 samples were subsampled to 1402 pyrotags using the tool "rrarefy" of the Vegan R package (Oksanen *et al.,* 2008). Pyrotag counts were log transformed to diminish the effect of the most abundant OTUs, and the table subsampled again with rrarefy (after multiplying the log-transformed values times 1000) to obtain the same signal per sample. A distance matrix was computed with the Bray-Curtis index and a dendrogram was constructed using the UPGMA function in Vegan. Venn diagrams were generated with the R package VennDiagram.

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Conflict of Interest

The authors declare no conflict of interest.

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Table legends

Table 1. Coastal marine sites visited and water column (surface and DCM) and sediment samples taken for investigating global protist diversity by pyrosequencing. Each planktonic community is analyzed in up to seven 454-samples (pico, nano and micro/mesoplankton by RNA/DNA plus dissolved DNA) and each sediment sample in two (by RNA/DNA). Cases with no sequencing results are noted. See Table S1 for details on the number of pyrotags for each 454-sample.

Table 2. Averaged relative abundance (% tags), occurrence (number of times detected), and taxonomic affiliation of the most abundant OTU_{99} derived from the RNA survey in the four ecological compartments. Relative abundances in the other compartments (Pic= Picoplankton; Nan= Nanoplankton: Mic= Micro/mesoplankton; Sed= Sediments) are also shown (in grey when >0.8%).

Figure legends

Fig. 1. Summary of the comparison of duplicated 454-samples (n=25) shown in relation to the pyrotag ratio between duplicates. A. Statistics of the linear regressions between the OTU abundance in each duplicate: R^2 coefficients (white dots) and slope values (black dots; regression line shown). B. Percentage of OTUs shared in duplicate pairs (black dots; regression line shown) and percentage of pyrotags within these shared OTUs (white dots). These later percentages apply to the pair with less pyrotags.

Fig. 2. UPGMA dendrogram based on the Bray-Curtis dissimilarity matrix from an OTU table with 92 samples (subsampled to 1402 pyrotags and log-transformed). Sample names have four components: sampling cruise, depth, size fraction, and nucleic acid template. The three main clusters are highlighted. Large grey dots indicate grouping of samples from the same sampling event, while small black dots indicate a closest relation of the same assemblage analyzed by DNA and RNA.

Fig. 3. Number of shared OTUs among plankton size-fractions derived from the DNA survey in five cases. Areas covering the dissolved samples are shaded. Below the number of shared OTUs, the percentages of pyrotags they represent in dissolved samples are shown.

Fig. 4. Relative pyrotag abundance in RNA and DNA surveys of taxonomic groups in picoplankton (A), nanoplankton (B), micro/mesoplankton (C) and sediment (D) samples. Each point represents paired percentages (in logarithmic scales) of a given taxonomic group in a given sample. Groups selected are those with highest pyrotag abundance (together account for 96-98% of pyrotags). Groups overrepresented in DNA surveys appear above the 1:1 line as colored triangles, those overrepresented in RNA surveys appear below the line as colored circles, and those equally represented appear as small black circles.

Fig. 5. Percentages of pyrotags (average and standard error) of taxonomic groups in RNA surveys in the picoplankton (13 samples), nanoplankton (13 samples), micro/mesoplankton (11 samples) and sediments (7 samples). Note the different vertical scale in the four plots. Groups are ordered by their overall pyrotag abundance in the whole dataset.

Supporting Information

Table S1. Number of pyrotags per 454-sample in the global survey of protist diversity in the plankton (several organismal size-fractions) and sediments analyzed in DNA and RNA surveys (total of 95 samples). See Table 1 for additional information on sampling cruises.

Table S2. Number of OTUs and pyrotags included in OTU97 tables, showing the initial numbers and the final numbers after excluding unknown, metazoan, and nucleomorph OTUs. Three OTU tables are used in this study: 120 samples (all datasets including duplicates), 95 samples (duplicates removed), and 44 samples (only the RNA survey).

Fig. S1. Percentages of pyrotags (average and standard error) of taxonomic groups in RNA and DNA surveys in the picoplankton, nanoplankton, micro/mesoplankton, sediments and the dissolved fraction (the latter only DNA survey). Note the different vertical scale in the four plots. Groups are ordered by their overall pyrotag abundance in the whole dataset.

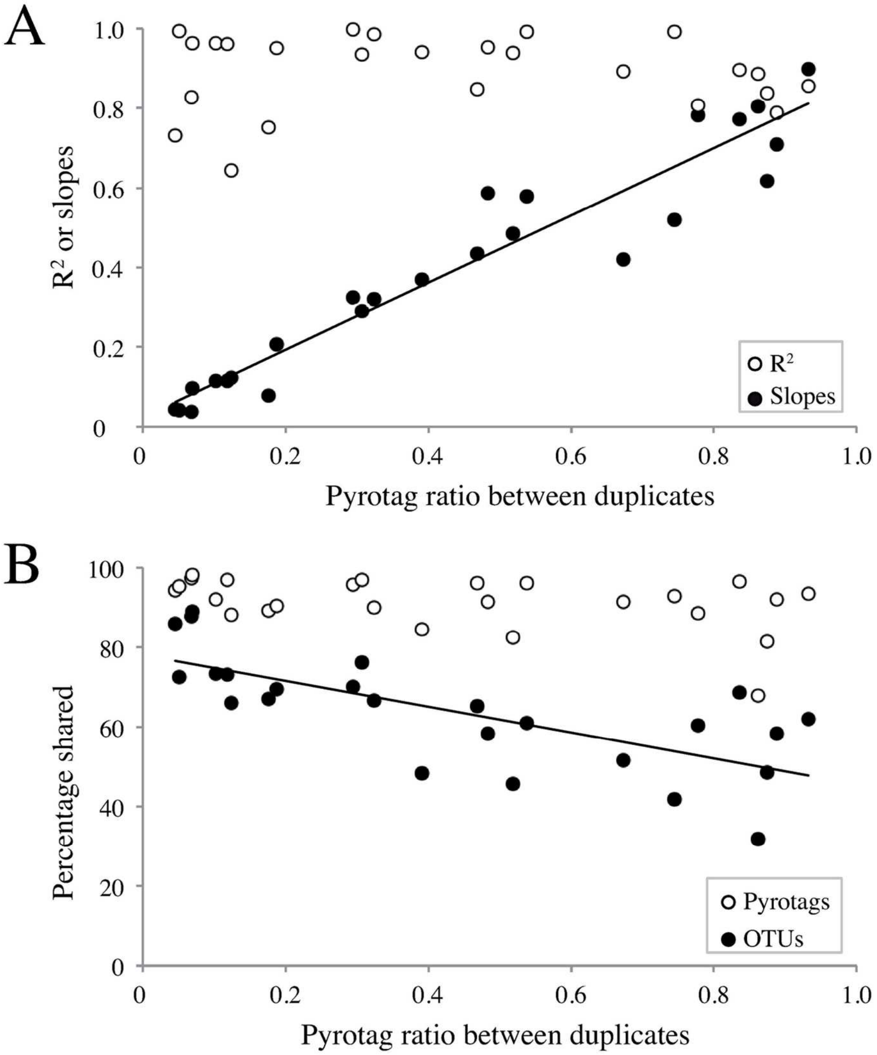

Fig. 1. Summary of the comparison of duplicated 454-samples (n=25) shown in relation to the pyrotag ratio between duplicates. A. Statistics of the linear regressions between the OTU abundance in each duplicate: R2 coefficients (white dots) and slope values (black dots; regression line shown). B. Percentage of OTUs shared in duplicate pairs (black dots; regression line shown) and percentage of pyrotags within these shared OTUs (white dots). These later percentages apply to the pair with less pyrotags.
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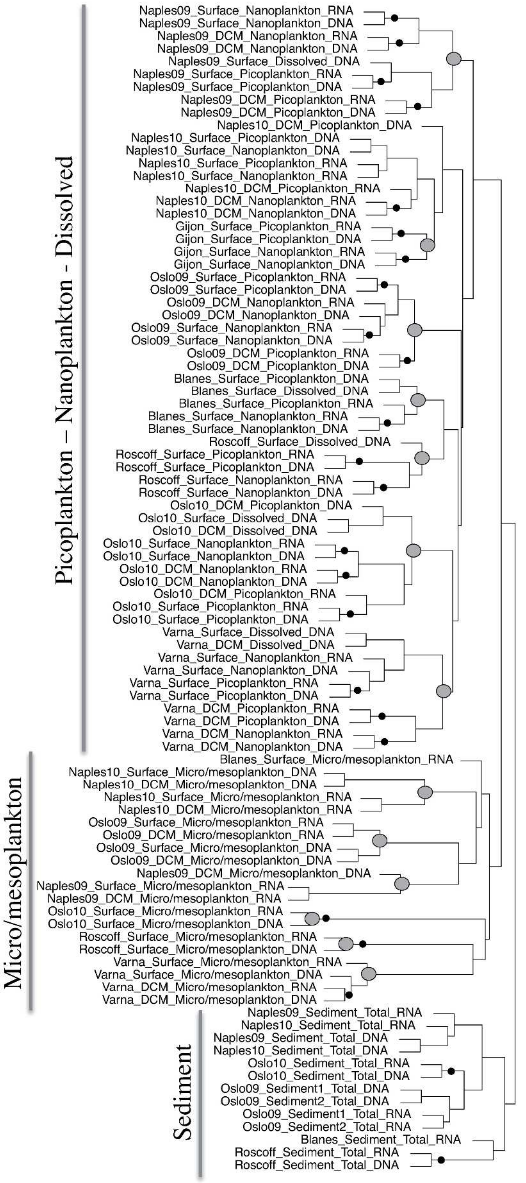

Fig. 2. UPGMA dendrogram based on the Bray-Curtis dissimilarity matrix from an OTU table with 92 samples (subsampled to 1402 pyrotags and log-transformed). Sample names have four components: sampling cruise, depth, size fraction, and nucleic acid template. The three main clusters are highlighted. Large grey dots indicate grouping of samples from the same sampling event, while small black dots indicate a closest relation of the same assemblage analyzed by DNA and RNA.
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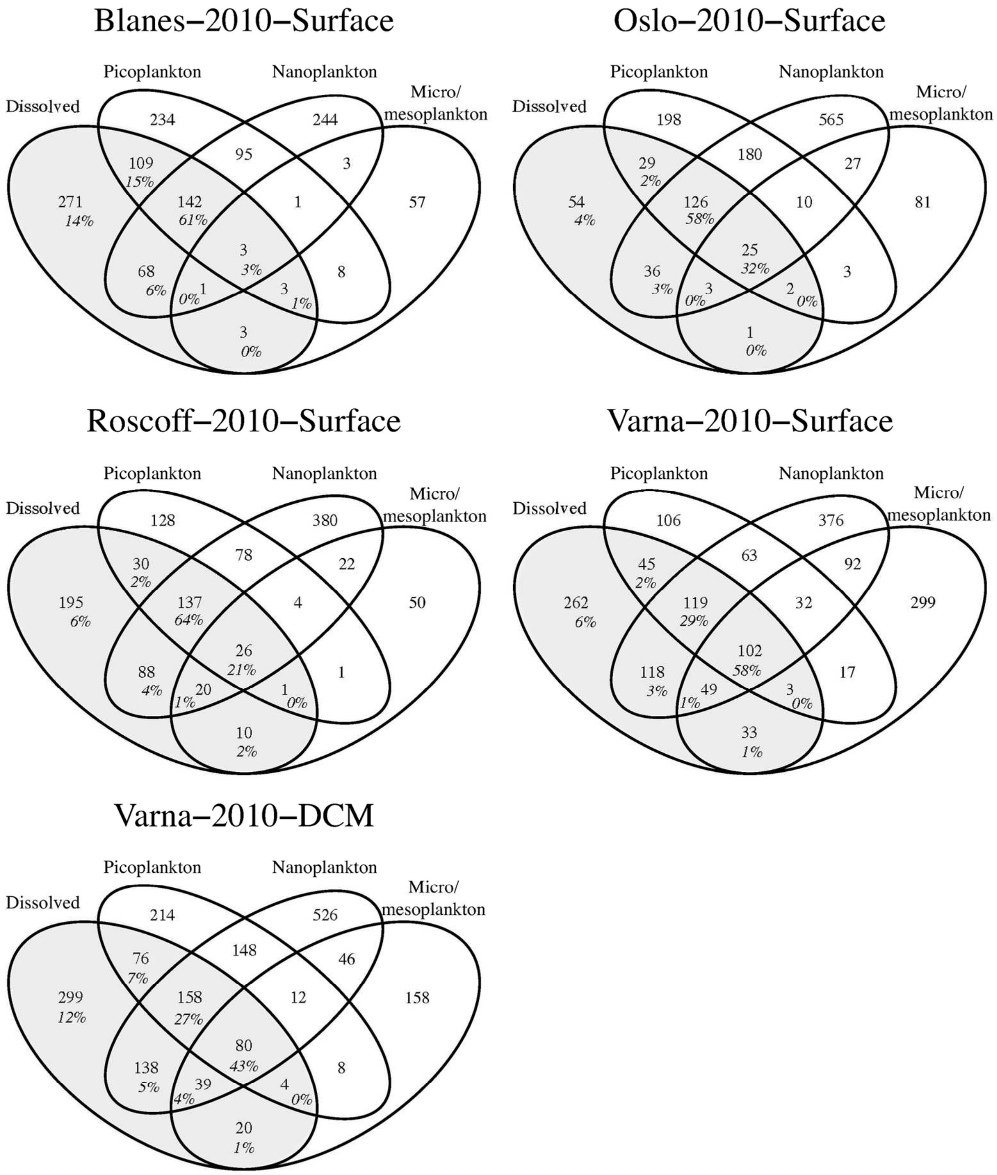

Fig. 3. Number of shared OTUs among plankton size-fractions derived from the DNA survey in five cases. Areas covering the dissolved samples are shaded. Below the number of shared OTUs, the percentages of pyrotags they represent in dissolved samples are shown.
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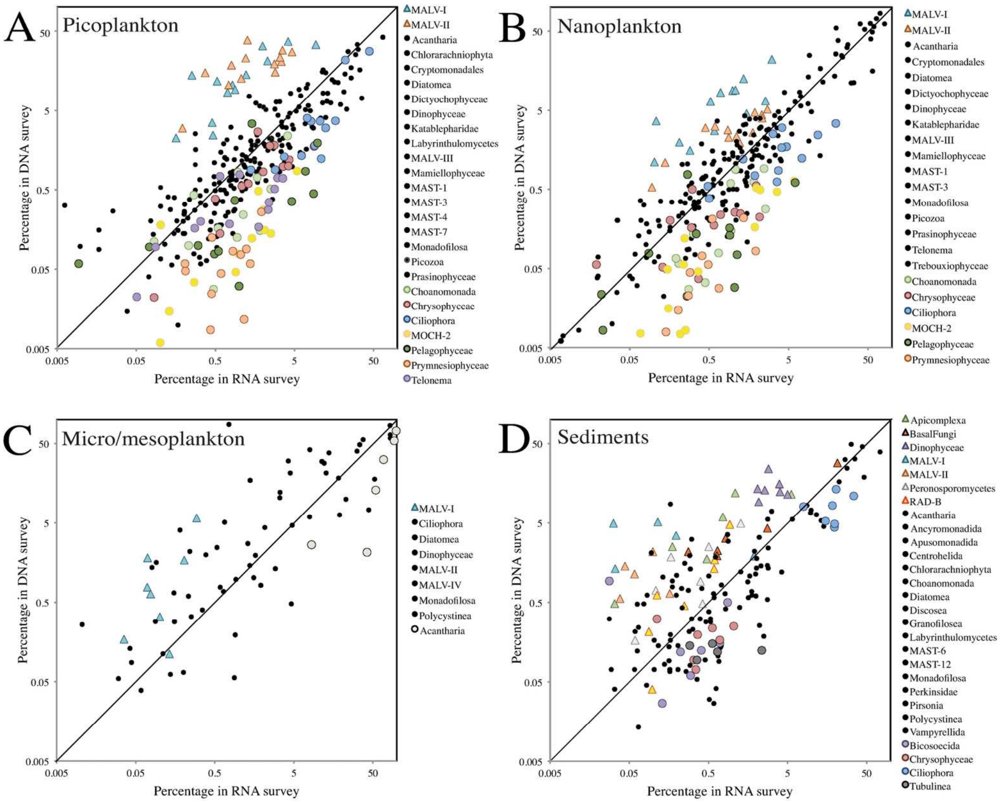

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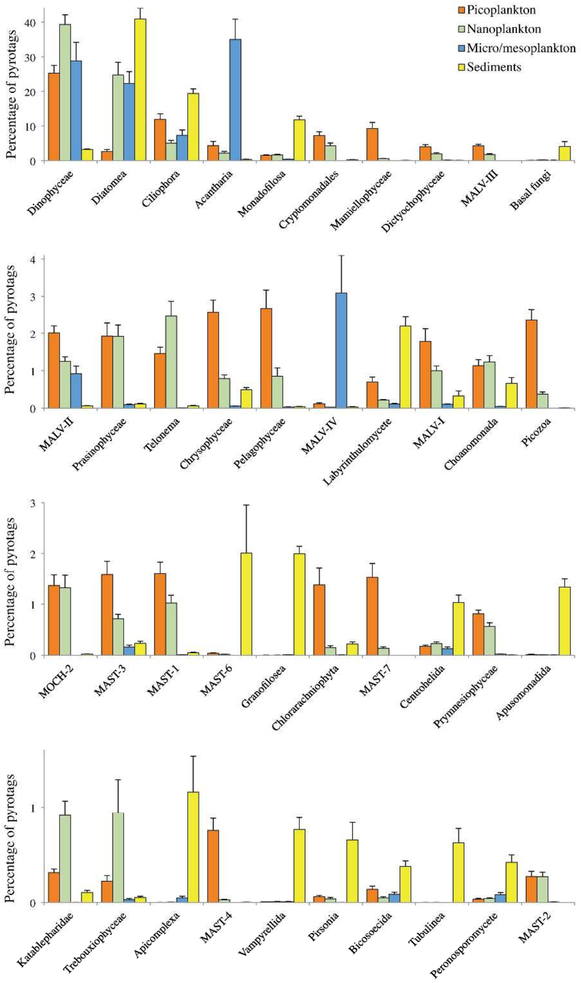

302x425mm (300 x 300 DPI)

Table 1. Coastal marine sites visited and water column (surface and DCM) and sediment samples taken for investigating global protist diversity by pyrosequencing. Each planktonic community is analyzed in up to seven 454-samples (pico, nano and micro/mesoplankton by RNA/DNA plus dissolved DNA) and each sediment sample in two (by RNA/DNA). Cases with no sequencing results are noted. See Table S1 for details on the number of pyrotags for each 454-sample.

Site	Coordinates	Date	Depth (m)	Temperature (°C)	Salinity	Chl a (µg L^{-1})	454-samples	Pyrotags
Blanes	41°40'N, 2°48'E	9/02/2010	Surface (1)	12.5	37.6	0.7	7	28569
			Sediment (20)	12.6	37.8	-	2	3899
Gijon	43°40'N; 5°35'W	14/09/2010	Surface (1)	20.2	35.7	0.6	4 [a,b,c]	49747
Naples	40°48'N, 14°15'E	13/10/2009	Surface (1)	22.8	37.7	1.7	6 [b]	100567
			DCM (26)	19.2	37.9	1.5	7	82327
			Sediment (78)	14.6	37.9	-	2	20545
		14/05/2010	Surface (1)	19.2	37.2	1.1	6 [c]	31185
			DCM (34)	15.5	37.7	1.0	6 [c]	49402
			Sediment (78)	14.0	37.9	-	2	9680
Oslo	59°16'N, 10°43'E	22/09/2009	Surface (1)	15.5	25.2	2.5	6 [c]	61963
			DCM (20)	16.1	29.2	1.1	6 [c]	70006
			Sediment-1 (103)	8.2	35.0	-	2	10974
			Sediment-2 (103)	8.2	35.0	-	2	12661
		22/06/2010	Surface (1)	15.0	21.5	1.1	7	56190
			DCM (10)	11.9	29.5	1.9	5 [a,b]	61067
			Sediment (103)	6.0	35.0	-	2	10480
Roscoff	48°46'N, 3°57'W	20/04/2010	Surface (1)	9.9	34.9	0.2	7	33142
			Sediment (60)	9.9	34.9	-	2	3122
Varna	43°10'N, 28°50'E	27/05/2010	Surface (3)	18.0	16.5	5.2	7	60352
			DCM (40)	8.7	17.9	6.1	7	84620

[a] No results for Micro/mesoplankton-RNA; [b] No results for Micro/mesoplankton-DNA; [c] No results for Dissolved-DNA

Table 2. Averaged relative abundance (% tags), occurrence (number of times detected), and taxonomic affiliation of the most abundant OTU_{99} derived from the RNA survey in the four ecological compartments. Relative abundances in the other compartments (Pic= Picoplankton; Nan= Nanoplankton: Mic= Micro/mesoplankton; Sed= Sediments) are also shown (in grey when >0.8%).

A. Picoplankton samples (n=13)

OTU#	% tags	Occurrence	Closest match to described species	%	Taxonomic group	Nan	Mic	Sed
13	4.5	8	*Micromonas pusilla* CCMP1195	100	Mamiellophyceae	0.2	0.0	0.0
1	3.2	7	*Acanthostaurus purpurascens*	100	Acantharia	1.6	25.3	0.0
10	2.2	11	*Teleaulax amphioxeia*	100	Cryptomonadales	1.8	0.0	0.0
36	1.6	6	*Pelagomonas calceolata*	100	Pelagophyceae	0.3	0.0	-
51	1.6	10	*Micromonas pusilla* RCC829	99.5	Mamiellophyceae	0.1	-	0.0
5	1.5	4	*Lynnella semiglobulosa*	100	Ciliophora	0.1	0.0	0.0
14	1.4	11	*Gyrodinium fusiforme*	100	Dinophyceae	1.3	0.9	0.0
28	1.2	4	*Polykrikos kofoidii*	100	Dinophyceae	0.2	0.2	0.2
46	1.2	10	*Pseudotontonia simplicidens*	99.7	Ciliophora	0.4	0.0	-
22	1.2	13	*Katodinium rotundatum*	100	Dinophyceae	1.5	0.0	0.0
44	1.1	12	*Brachidinium capitatum*	93.2	MALV-III	0.7	0.0	-
26	1.1	13	*Azadinium concinnum*	99.7	Dinophyceae	3.2	0.9	0.0
73	1.1	12	*Florenciella parvula*	100	Dictyochophyceae	0.4	0.0	-
9	1.0	10	*Lepidodinium viride*	100	Dinophyceae	2.4	0.2	-
50	0.9	12	*Teleaulax gracilis*	100	Cryptomonadales	0.6	-	-
107	0.9	11	*Pirsonia verrucosa*	86.3	MAST-7B	0.1	-	-
32	0.9	10	*Strombidium* sp. SNB99-2	97.3	Ciliophora	1.2	0.0	0.0
87	0.9	13	*Prorocentrum triestinum*	91.6	MALV-III	0.3	-	-
145	0.8	7	*Minorisa minuta*	100	Chlorarachniophyta	0.1	0.0	-

B. Nanoplankton samples (n=13)

OTU#	% tags	Occurrence	Closest match to described species	%	Taxonomic group	Pic	Mic	Sed
0	4.8	6	*Leptocylindrus aporus*	100	Diatomea	0.4	0.4	0.1
26	3.2	13	*Azadinium concinnum*	99.7	Dinophyceae	1.1	0.9	0.0
9	2.4	11	*Lepidodinium viride*	100	Dinophyceae	1.0	0.2	-
3	2.4	3	*Bacterosira bathyomphala*	100	Diatomea	0.0	0.5	0.9
10	1.8	10	*Teleaulax amphioxeia*	100	Cryptomonadales	2.2	0.0	0.0
1	1.6	8	*Acanthostaurus purpurascens*	100	Acantharia	3.2	25.3	0.0
22	1.5	13	*Katodinium rotundatum*	100	Dinophyceae	1.2	0.0	0.0
14	1.3	12	*Gyrodinium fusiforme*	100	Dinophyceae	1.4	0.9	0.0
32	1.2	9	*Strombidium* sp. SNB99-2	97.3	Ciliophora	0.9	0.0	0.0
12	1.2	6	*Skeletonema marinoi*	100	Diatomea	0.0	0.1	3.6
174	1.1	3	*Thalassiosira profunda*	99.7	Diatomea	0.0	0.1	1.1
15	1.0	8	*Chaetoceros setoense*	100	Diatomea	0.2	0.4	1.2

C. Micro/mesoplankton samples (n=11)

OTU#	% tags	Occurrence	Closest match to described species	%	Taxonomic group	Pic	Nan	Sed
1	25.3	7	*Acanthostaurus purpurascens*	100	Acantharia	3.2	1.6	0.0
17	6.0	5	*Noctiluca scintillans*	100	Dinophyceae	0.0	0.0	-
53	3.2	9	*Neoceratium fusus*	100	Dinophyceae	0.0	0.0	-
24	3.1	5	*Skeletonema pseudocostatum*	100	Diatomea	0.1	0.4	0.9
63	2.2	10	*Neoceratium azoricum*	99.5	Dinophyceae	0.0	0.0	-
18	2.0	2	*Favella markusovszkyi*	100	Ciliophora	-	-	-

77	1.7	4	*Hexaconus serratus*	98.9	Acantharia	0.1	0.0	-
23	1.5	6	*Biddulphia alternans*	86.1	Diatomea	0.1	0.0	0.8
271	1.5	4	*Hematodinium* sp. ex Nephrops	95.3	MALV-IV	0.1	0.0	0.0
52	1.4	11	*Neoceratium furca*	100	Dinophyceae	0.0	0.0	-
54	1.4	5	*Xiphacantha alata*	100	Acantharia	0.0	0.1	0.0
191	1.2	2	*Stenosemella pacifica*	100	Ciliophora	0.0	-	-
284	1.1	1	*Amphorides quadrilineata*	98.9	Ciliophora	0.0	-	-
283	1.1	6	*Thalassiosira rotula*	100	Diatomea	0.0	0.1	0.2
173	1.0	11	*Dinophysis acuminata*	100	Dinophyceae	0.0	0.0	-
26	0.9	11	*Azadinium concinnum*	99.7	Dinophyceae	1.1	3.2	0.0
14	0.9	9	*Gyrodinium fusiforme*	100	Dinophyceae	1.4	1.3	0.0
385	0.8	2	*Thalassiosira anguste-lineata*	99.7	Diatomea	-	0.0	0.1
161	0.8	8	*Syndinium* sp. ex Corycaeus	100	MALV-IV	0.1	0.0	-

D. Sediment samples (n=7)

OTU#	% tags	Occurrence	Closest match to described species	%	Taxonomic group	Pic	Nan	Mic
12	3.6	3	*Skeletonema marinoi*	100	Diatomea	0.0	1.2	0.1
30	2.4	3	*Chaetoceros cf. neogracile*	100	Diatomea	0.0	0.0	0.0
57	2.2	3	*Powellomycetaceae sp.*	85.7	Basal fungi	0.0	0.1	0.1
275	1.3	1	*Pirsonia formosa*	89.4	MAST-6	-	-	-
33	1.3	3	*Monodinium sp.*	98.7	Ciliophora	0.4	0.0	0.0
249	1.3	3	*Protaspis obliqua*	93.6	Monadofilosa	-	-	-
15	1.2	5	*Chaetoceros setoense*	100	Diatomea	0.2	1.0	0.4
623	1.1	6	*Psammodictyon sp.*	99.7	Diatomea	-	0.0	0.0
174	1.1	6	*Thalassiosira profunda*	99.7	Diatomea	0.0	1.1	0.1
24	0.9	4	*Skeletonema pseudocostatum*	100	Diatomea	0.1	0.4	3.1
3	0.9	5	*Bacterosira bathyomphala*	100	Diatomea	0.0	2.4	0.5