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Protist metabarcoding and environmental biomonitoring: Time for change

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Abstract

High-throughput amplicon sequencing of environmental DNA and/or RNA proved to be a powerful tool to describe protist diversity. This new approach called also the metabarcoding has totally transformed our view of protist diversity, revealing a large number of novel lineages and expanding the range of protist phylogenetic diversity at almost every taxonomic level. However, until now the objectives of the vast majority of metabarcoding studies were purely academic. Practical applications of protist metabarcoding are surprisingly scarce, despite the fact that several groups of protists are commonly used as bioindicators of environmental impacts in freshwater or marine ecosystems. Here, we are reviewing studies that examine the ecological applications of metabarcoding for two groups of well-known protist bioindicators: diatoms and foraminifera. The results of these studies show that despite some biological and technical biases, molecular data quite faithfully reflect the morphology-based biotic indices and provide a similar assessment of ecosystem status. In view of these results, protist metabarcoding appears as a rapid and accurate tool for the evaluation of the quality of aquatic ecosystems. Hence, we plead for integration of protist metabarcoding in future biomonitoring projects as a complement of traditional methods and a source of new biosensors for environmental impact assessment.

Keywords: Bioindicators; Diatoms; Environmental DNA; Foraminifera; Metabarcoding; Microbial eukaryotes

Protists as ecological bio-indicators

The composition and structure of protist communities provide powerful tools for measuring the impacts of pollution and other human activities on biological quality of ecosystems. Several biological indices have been established in order to measure species diversity and to relate changes in community composition with environmental disturbance. Most of these indices are based on empirical studies that provide an autoecological value to species or groups of species according to their function or trophic position. Currently, four groups of protists are widely recognized as ecological indicators: diatoms, foraminifera, ciliates and testate amoebae (Table 1; see also Foissner 2016).

Diatoms are routinely used for the assessment of ecological quality in freshwater ecosystems. Together with aquatic invertebrates, they are considered as the best indicators of disturbance related to changes in physical, chemical or biological conditions of watercourses (Stevenson et al. 2010). Numerous studies are reporting using benthic diatoms for biomonitoring of rivers and streams (Almeida et al. 2014; Lavoie et al. 2014; Martin and Reyes Fernandez 2012; Rimet

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Table 1. Major groups of protist bioindicators with selected recent references.

Taxonomic group	Ecosystem	Environmental impact/parameters	References
Diatoms	Rivers, streams	Eutrophication	Lobo et al. 2004; Tan et al. 2015
		Heavy metal contamination	Leguay et al. 2015
	Lakes	Eutrophication	Poulickova et al. 2004
		Heavy metal contamination	Cattaneo et al. 2011; Cantonati et al. 2014
	Marine benthos	Various environmental parameters	Weckström and Juggins 2006; Desrosiers et al. 2013 (review)
	Marine biofilm	Eutrophication	Cibic and Blasutto 2011
Forams	Sea bottom	Off-shore drilling	Mojtahid et al. 2008; Denoyelle et al. 2010
		Organic enrichment associated with aquaculture	Debenay et al. 2009; Vidovic et al. 2009, 2014
		Heavy metal contamination	Le Cadre et al. 2003; Bergin et al. 2006
		Baseline conditions	Alve et al. 2009
Ciliates	Activated sludge	Wastewater treatment	Nicolau et al. 2001; Martín-González et al. 2006
	Marine coastal	Eutrophication, chemical pollution	Xu et al. 2014; Jiang et al. 2013
	Mangrove	Sewage treatment	Chen et al. 2008
	Rivers	Eutrophication	Madoni and Bassanini 1999
		Chemical pollution	Madoni and Braghiroli 2007; Sola et al. 1996
	Soil	Heavy metal contamination	Diaz et al. 2006
Testate amoebae	Peatlands	Restoration	Valentine et al. 2013
		Atmospheric pollution	Nguyen-Viet et al. 2004, 2007
	Moorlands	Burning	Turner and Swindles 2012
	Groundwater, streams	Heavy metal contamination	Yang et al. 2011
	Lakes	Coal mining	Patterson et al. 2013
		Road salt contamination	Roe and Patterson 2014
	Soil	Coal mining	Wanner and Dunger 2001

and Bouchez 2012, see also Table 1). Fewer studies examine the usefulness of diatoms for biomonitoring of lakes (Cattaneo et al. 2011; Larras et al. 2014; Poulickova et al. 2004). Various biotic indices were developed to infer levels of water pollution relying on composition, abundance, ecological preferences and tolerance of benthic diatoms. Following the EU Water Framework Directive (Directive 2000), several European countries introduced biological indices based on diatoms diversity: the Biological Diatom Index in France (Coste et al. 2009; Lenoir and Coste 1996), the Trophic Diatom Index in UK (Kelly et al. 2001), or the Swiss Diatom Index (DI-CH) in Switzerland (Hürlimann and Niederhauser 2007). Although benthic diatoms are abundant and ubiquitous in marine environment, their use as bioindicators is limited and the possibility to establish a marine diatom index for the bioassessment of coastal waters is just being considered (Desrosiers et al. 2013).

Benthic foraminifera are common protist bioindicators in marine environments (Alve 1995; Murray 2006; Nigam et al. 2006; Romano and Bergamin 2009; Yanko et al. 1999). They are highly responsive to environmental perturbations such as organic enrichment, physical disturbances and chemical pollution. Among others, forams have been used to assess the impact of pollution due to oil drilling and spills (e.g. Denoyelle et al. 2010; Jorissen et al. 2009; Schwing et al. 2015), heavy metals (e.g. Bergin et al. 2006; Le Cadre et al. 2003; Frontalini et al. 2009), and industrial aquaculture (Vidovic et al. 2009, 2014). Guidelines to process samples for soft bottom benthic foraminiferal monitoring were recently established by the FOBIMO group (Schönfeld et al. 2012). All these surveys are based on hard-tested foraminifera, which are preserved in the sedimentary record. In many studies, the morphological abnormalities of preserved tests are used as indication of environmental impact (Debenay et al. 2009; Geslin et al. 2002). The fossilized tests can also be used to establish pre-disturbance baseline conditions (Alve et al. 2009; Gooday 2009).

Ciliates are mainly used as bioindicators in wastewater treatment plants, where they are routinely employed for monitoring the activated sludge operation (Nicolau et al. 2001). However, several papers also highlight the importance of ciliates as water quality indicators in rivers and lakes (Berger and Foissner 2003; Foissner and Berger 1996; Sola et al. 1996, see also Table 1). More recently, their role as bioindicators of environmental impact in marine coastal waters has been extensively studied (Jiang et al. 2013; Xu et al. 2014; Zhang et al. 2014). Ciliates have also been proposed as soil bioindicators, using different molecular tools for their identification (Jousset et al. 2010; Lara and Acosta-Mercado 2012).

Testate amoebae are mainly used as proxies for inferring past conditions in paleoecological and paleoclimatic reconstructions of Holocene wetlands (Hendon et al. 2001).

However, there is a growing interest in using testate amoebae also for biomonitoring of modern ecosystem changes (Mitchell et al. 2007). For example, several studies demonstrated their usefulness for peatland management and monitoring its regeneration (Davis and Wilkinson 2004; Valentine et al. 2013). Testate ameobae have also been proposed for the monitoring of moorland ecosystems (Turner and Swindles 2012), as well as lakes contaminated by road salt (Roe and Patterson 2014). Finally, several studies demonstrate their sensitivity to soil, water and atmospheric pollution (Nguyen-Viet et al. 2004, 2007; Wanner and Dunger 2001; Yang et al. 2011).

The advantages of using protists in biomonitoring are numerous. Payne (2013) listed seven key reasons making protists useful as bioindicators: environmental sensitivity, functional importance, widespread distribution, small size and abundance, short response times, ease of analysis and preservation potential. Among these reasons, the high environmental sensitivity and short generation time are the main factors that drive a quick response of protist communities to the environmental disturbances. The fact that protists may form large populations increases the chance for a small sample to yield enough individuals and thus ensures statistical power. Moreover, protists are often characterized by exceptionally high species richness that may reach up to 100,000 species only for diatoms (Mann and Vanormelingen 2013), which additionally increase the chances to select suitable bioindicators.

One of the main limiting factors of using protists as bioindicators is the difficulty in their identification. At present, microscopic observation is the main way to distinguish species and obviously only those protists that are characterized by distinctive morphological features are used as bioindicators. This evidently limits the range of potential indicator taxa. Moreover, the identification of species based on morphological characters is generally time consuming and requires an excellent taxonomic expertise, which is increasingly rare. Given the scarcity of experienced morphotaxonomists and the lack of academic incentives to maintain the training courses in morphological systematics, there is an urgent need to find alternative ways to identify indicator species that could be used for environmental biomonitoring.

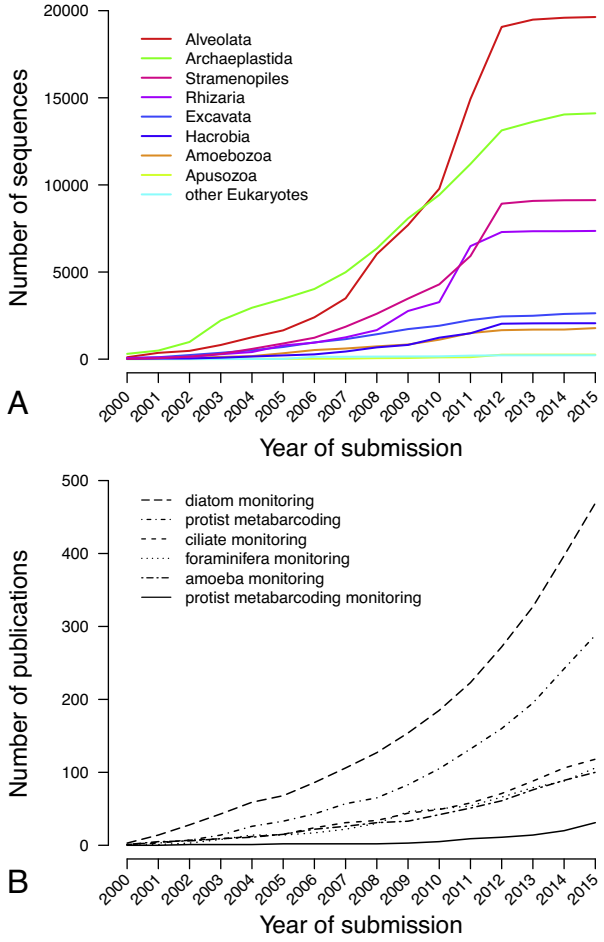

Fig. 1. Genetic database of protists diversity and their use for biomonitoring. (A) Cumulative number of sequences deposited in GenBank and validated in the PR2 database for each of the major protistan supergroup. (B) Cumulative number of publications targeting the four major groups of protist bioindicators and number of papers reporting protist metabarcoding applied or not to biomonitoring. The references were retrieved automatically by querying the Medline Database (Entrez PubMed) with combinations of keywords and corrected manually.

Molecular inventory of protist environmental diversity

The last decade has seen a dramatic increase of the number of DNA barcodes for protist diversity. The reference database experienced a major burst as illustrated by the number of protist rDNA sequences deposited in the GenBank and validated in the PR2 database (Guillou et al. 2013) (Fig. 1A). The number of sequences increased up to tenfold for some eukaryotic supergroups (Alveolata, Stramenopiles, Rhizaria). Yet, with the advent of high-throughput sequencing (HTS) technologies, a continuous flow of new unidentified eukaryotic sequences makes it clear that the protist barcoding reference database remains extremely limited, lagging far behind that devoted to animal, plant or fungal species (del Campo et al. 2014, 2016; Pawlowski et al. 2012).

The HTS-based metabarcoding surveys have profoundly changed our view on protist diversity, not only by unraveling the immense gaps in our taxonomic and phylogenetic knowledge but also by expanding the information about protist biogeography and ecology. The main objective of the early metabarcoding studies was to explore the unknown diversity of protists. Some studies attempted to estimate global protist diversity for planktonic and benthic biomes (de Vargas et al. 2015; Logares et al. 2014; Massana et al. 2015). The others focused on the diversity of particular taxonomic

Table 2. Diatoms DNA barcodes and their use in metabarcoding.

Gene	Number of barcode sequences	Reference DNA barcode	Reference metabarcoding
rbcL	523	Hamsher et al. 2011; MacGillivary and Kaczmarska 2011; Rimet et al. 2014; Stepanek and Kociolek 2014; Guo et al. 2015; Hamilton et al. 2015	Kermarrec et al. 2013; Stoof-Leichsenring et al. 2012; Villanueva et al. 2014; Zimmermann et al. 2014, 2015
V4 18S	455	Zimmermann et al. 2011; Luddington et al. 2012; Rimet et al. 2014; Stepanek and Kociolek 2014; Guo et al. 2015; Hamilton et al. 2015	Kermarrec et al. 2013; Zimmermann et al. 2014, 2015; Visco et al. 2015
ITS	260	Evans et al. 2009; Moniz and Kaczmarska 2009, 2010; Guo et al. 2015	
cox1	195	Evans et al. 2007, 2009; Moniz and Kaczmarska 2009; Hamsher et al. 2011; Rimet et al. 2014; Guo et al. 2015	Kermarrec et al. 2013
D2/D3 28S	123	Hamsher et al. 2011; Moniz and Kaczmarska 2009, 2010; Rimet et al. 2014	

groups, such as foraminifera (Lecroq et al. 2011), haptophytes (Bittner et al. 2013; Egge et al. 2013), ciliates (Filker et al. 2015; Stoeck et al. 2014), stramenopiles (Massana et al. 2014), or unicellular opisthokonts (del Campo et al. 2015). A special attention was given to address technical issues aiming at the optimization of protist metabarcoding, such as the choice of barcoding region (Drummond et al. 2015; Dunthorn et al. 2012; Tanabe et al. 2015) and the selection of PCR primers (Hadziavdic et al. 2014; Hugerth et al. 2014; Stoeck et al. 2010). Significant efforts were also made to improve the accuracy of HTS analysis through refined bioinformatics treatments, and notably for the robust multiplexing of hundreds of environmental samples (Esling et al. 2015; Herbold et al. 2015; Kozich et al. 2013; Schirmer et al. 2015).

Until now, practical applications of protist metabarcoding are very limited. Indeed, the number of applied metabarcoding studies is still very low compared to that dealing with protists as bioindicators of environmental impacts (Fig. 1B). The usefulness of eDNA metabarcoding for environmental biomonitoring is well recognized (Baird and Hajibabaei 2012; Bohmann et al. 2014; Valentini et al. 2015). Yet, its application is generally restricted to multicellular organisms. The majority of biomonitoring – oriented metabarcoding studies focus on animal bioindicators, such as aquatic insects (Hajibabaei et al. 2012) or marine macro-invertebrates (Cowart et al. 2015). Some studies aim at testing the congruence between species inventories inferred from HTS and morphological studies of fixed bulk samples (Carew et al. 2013; Stein et al. 2013; Yu et al. 2012; Zhou et al. 2013). Others analyze the preservation of invertebrate DNA in environmental samples (Deiner and Altermatt 2014; Mächler et al. 2014). Several studies propose using global eukaryotes diversity inferred from HTS eDNA data for the assessment of environmental impact (Bik et al. 2012) or for the monitoring of various environments, such as estuaries (Chariton et al. 2010, 2015), freshwater lakes (Eiler et al. 2013), or deep-sea canyons (Guardiola et al. 2015). However, only few groups of protists have been the subjects of more extensive studies regarding the practical applications of metabarcoding approach. We will focus here on diatoms and forams, but it worth also to mention the metabarcoding studies conducted on other protist bioindicators, such as soil euglyphids (Seppey et al. 2015; Lara et al. 2015).

Diatoms metabarcoding

Molecular identification of diatoms has been subject of several studies searching for suitable DNA barcodes. Five molecular markers (cox1, rbcL, 18S, ITS, and 28S) were examined as potential barcodes that could offer a satisfying species taxonomic resolution and be retrieved with high amplification success (Evans et al. 2007; Guo et al. 2015; Hamsher et al. 2011; Zimmermann et al. 2011). Because no ideal diatom DNA barcode was found, it has been proposed that different markers are used for different purposes. Indeed, the highly variable cox1, ITS and 28S genes were considered more suitable for taxonomic studies, while more conserved 18S and *rbcL* genes seem more appropriate for biomonitoring (Mann et al. 2010) (Table 2).

In early diatom metabarcoding studies the accuracy of different barcodes (18S, cox1, rbcL) applied to mock communities or environmental samples was tested using pyrosequencing technology (Kermarrec et al. 2013, 2014). Another study compared the identification of diatoms from environmental samples through traditional microscopic approach and metabarcoding analysis of 18S V4 region (Zimmermann et al. 2015). All these studies recognize the potential of diatom metabarcoding as a tool for water quality surveys, but raised several issues concerning the discrepancies between the abundance of HTS reads and abundance of specimens. Moreover, difficulties were encountered for

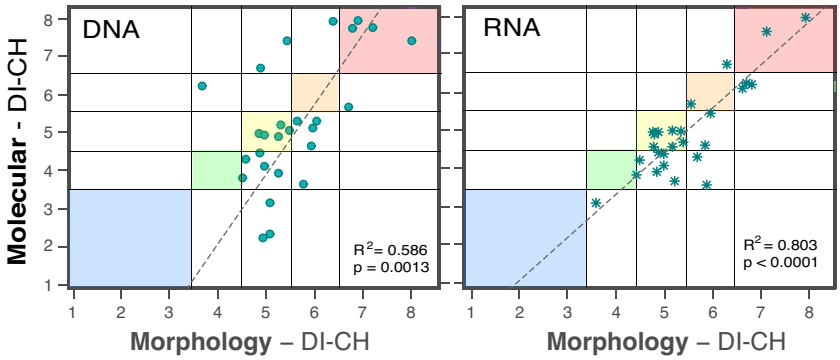

Fig. 2. Relationships between the DI-CH inferred from morphological and DNA or RNA data. Each point corresponds to the value of the DI-CH found in one site for morphological (*x*-axis) or molecular (*y*-axis) analysis. Colored boxes represent the ecological status given by the DI-CH (blue: very good, green: good, yellow: average, orange: poor, red: bad). The regression line for all samples is represented by dashed line and the R^2 and *p*-value are indicated for each graph.

the taxonomic assignment of eDNA sequences because the variability of diatom DNA barcodes does not always match that observed morphologically, a confusing situation which is aggravated by the gaps in reference database. Completing the taxonomic reference database was considered as the most important step toward applying diatoms in metabarcoding (Zimmermann et al. 2014).

The incompleteness of diatom barcoding database was also evidenced by another recent study, which compared the values of Swiss Diatom Index (DI-CH) obtained for 27 river sites based on sequencing of the 18S V4 region and microscopic quantification of diatom morphospecies (Visco et al. 2015). The authors of this study analyzed separately the eDNA and eRNA data in order to evaluate the bias that could be due to the presence of extracellular DNA in the biofilm samples. Because the calculation of the diatom index requires autoecological values and weighting factors associated with morphospecies, only a small fraction (30%) of the sequences that could be assigned to morphospecies were included in the analyses. Interestingly, despite the gaps in reference database and the variations in relative abundance of analyzed species, the diatom index shows a significant correlation between morphological and molecular data indicating similar biological quality status for the majority of sites (Fig. 2).

Forams metabarcoding

Compared to diatoms, the molecular identification of foraminifera is based on a single DNA barcode corresponding to the 3′ fragment of the 18S rDNA (Pawlowski 2000; Pawlowski and Holzmann 2014). This fragment varies in length between 800 and 1000 nucleotides and is composed of six hypervariable regions corresponding to stem-loop structure of the rRNA. These regions allow the distinction of practically all species that have been sequenced up to now (Pawlowski and Lecroq 2010). A database of foraminiferal 18S barcodes, which includes photographs of processed specimens, taxonomic references, and DNA barcode sequences, is publicly available at http://forambarcoding.unige.ch/. Partial sequences of other rRNA genes were occasionally used to examine the intraspecific or intrageneric variability in foraminifera (Hayward et al. 2004; Pawlowski et al. 2007; Tsuchiya et al. 2008). However, the lack of foraminiferal mitochondrial genes sequences prevents us from testing other potentially highly variable barcodes that could provide better markers of ecological diversity than nuclear ribosomal genes.

All metabarcoding studies of foraminifera are based on the hypervariable region corresponding to the helix 37F situated at the 5′ end of the 18S rDNA barcoding fragment (Lecroq et al. 2011; Lejzerowicz et al. 2014; Pawlowski et al. 2014a; Pochon et al. 2015). This short region was initially selected because it complied with size limitations of early version of Illumina HTS technology (Lecroq et al. 2011). Although the overall genetic divergence information contained in other variable regions is equally high, detailed analyses of their resolutions across foraminiferal lineages showed that the 37F region is more relevant for species-level identification than the others (Pawlowski and Lecroq 2010). Therefore, this region remained a standard of foraminiferal metabarcoding, despite the significant increase of the length of sequences generated by the latest Illumina platforms. The fact that the same region is used in all metabarcoding studies has several advantages, among others allows building up a large dataset of potentially high informative value for large-scale comparative studies of different ecosystems and geographic areas.

Like for many other protists, the main objective of early foraminiferal metabarcoding studies was to explore the exceptionally high diversity of environmental lineages. Efforts focused on the poorly known single-chambered monothalamids, which huge phylogenetic diversity was revealed based on cloning and Sanger sequencing approach (Habura et al. 2004; Pawlowski et al. 2011). The first Illumina HTS study confirmed that the vast majority of foraminiferal eDNA sequences belong to the monothalamous phylotypes (Lecroq et al. 2011). Further metabarcoding studies examined the distribution of sequences at various spatial scales in order

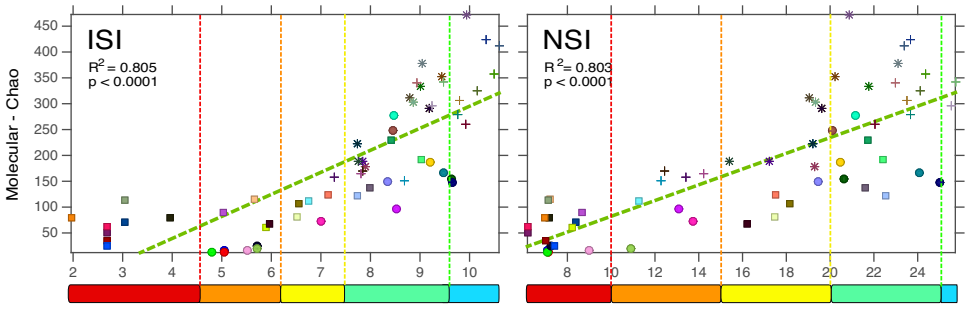

Fig. 3. Correlation between ecological quality statuses inferred from macrofauna-based ISI (Indicator Species Index) and NSI (Norwegian Sensitivity Index) biotic indices and eDNA forams diversity estimated using Chao index. Each point corresponds to the value of the Chao index based on molecular analysis (*y*-axis) and ISI or NSI index based on morphological counts (*x*-axis). The shape of the points indicates the different sampling sites. Colored boxes below the graphs represent the ecological status given by the biotic indices (blue: very good, green: good, yellow: average, orange: poor, red: bad). A dashed line represents the regression line for all samples and the R^2 and *p*-value are indicated for each graph. The figure is based on analysis of four salmon farm sites in Norway (Pawlowski et al. submitted for publication).

to document the micro-distribution of foraminifera in patchy deep-sea sediments (Lejzerowicz et al. 2014) as well as to test the preservation of ancient foraminiferal DNA in downcore sediments (Lejzerowicz et al. 2013; Pawlowska et al. 2014, 2015).

Practical application of forams metabarcoding was tested in the case of benthic monitoring of the impact of organic enrichment associated with salmon farming. The studies conducted in Scotland (Pawlowski et al. 2014a), New Zealand (Pochon et al. 2015) and Norway (Pawlowski et al. submitted for publication) showed a correspondence of microscopic and molecular analyses in terms of taxonomic composition and species abundance. Some foraminiferal species (e.g. *Leptohyalis scotti*) were found in higher abundance near the fish cages, in agreement with previous morphology-based studies (Scott et al. 1995). These species appear as potential candidates for indicators of organic enrichment, but this still needs to be confirmed by comparison of their distribution in distant geographic areas.

In view of metabarcoding studies, the foraminiferal diversity seems to respond strongly to fish farms impact. The analyses of beta diversity show that foraminiferal communities in stations highly impacted by organic discharge were more similar to each other than to those in reference stations (Pawlowski et al. 2014a). In many sites, the foraminiferal OTUs richness decreased with increasing enrichment (Pawlowski et al. submitted for publication), but the correlation between the richness and enrichment stage was not always strong (Pochon et al. 2015). Comparative analysis of foraminiferal eDNA and eRNA showed that environmental variables such as redox, total organic matter and sulphides were generally better correlated based on eRNA rather than on eDNA material. Strong correlation was also observed between foraminiferal eRNA data and macrofauna-based biotic indices (AMBI, BQI, Shanon-Wiener). The assessment of ecological quality based on macrofaunal indices used for the bioassessment in Norway was also well correlated with foraminiferal diversity inferred from eDNA data (Fig. 3).

Challenges and perspectives

Diatoms and forams provide an excellent material for testing the application of HTS technologies to protist biomonitoring. As shown above, the pilot studies on both groups give very promising results. Yet, there are still some technical issues that need to be resolved in order to increase the accuracy of protist metabarcoding applied to biomonitoring. The major technical challenges include: (i) the distinction between active and inactive cells, (ii) the definition of a molecular unit that would be the most relevant for ecological analyses, and (iii) the interpretation of HTS quantitative data.

The fact that environmental DNA comprises both intra- and extra-cellular DNA is a major issue. Presence of extra-cellular DNA can be very useful for biomonitoring and is widely used for the detection of traces of invasive and endangered species of aquatic vertebrates (Bohmann et al. 2014). However, in the case of bioindication the distinction between living and dead or inactive cells is necessary to analyze the impact of environmental changes on community composition and structure. In this case, the presence of extra-cellular DNA preserved in sediment for long periods of time, for example thousands of years in the case of deep-sea bottom (Lejzerowicz et al. 2013), may create a noise that will obscure the ecological signal. The transportation of extra-cellular DNA in flowing water can also be a source of errors in interpretation of eDNA data for biomonitoring (Deiner and Altermatt 2014). To overcome these biases, the cited above studies of diatoms and forams proposed to analyze eRNA as better proxy for active cells. Indeed, the correlation between eRNA datasets and environmental variables was stronger compared to eDNA datasets (Pochon et al. 2015; Visco et al. 2015). Yet, there is some evidence that the RNA may not be as fragile as commonly

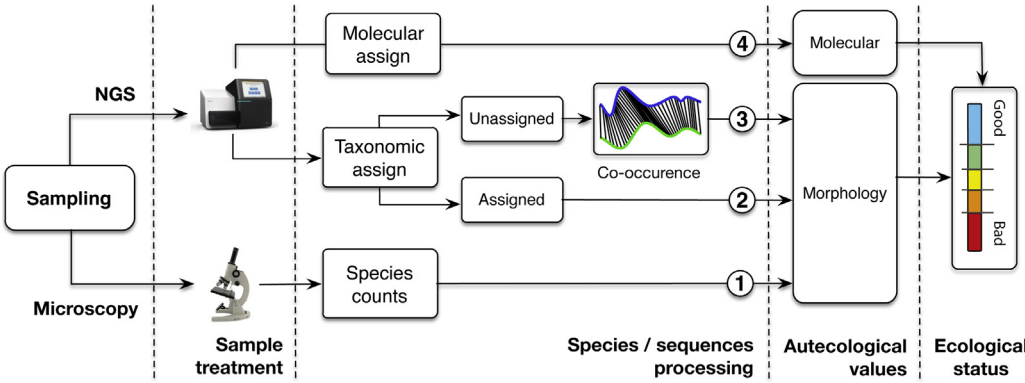

Fig. 4. Workflow illustrating the microscopy-based (1) and HTS-based (2–4) pathways of protist biomonitoring. The HTS pathways include the analysis performed exclusively on taxonomically assigned sequences (2), the analysis including unassigned sequences that have been given autoecological values based on co-occurrence patterns (3), and the analysis of sequences assigned to molecular index values (4).

accepted and its short fragments may be preserved in sediments for a certain time (Orsi et al. 2013). An alternative solution would be to avoid the presence of extra-cellular DNA by increasing the size of amplified eDNA fragments, but then the size limitations of HTS platforms might be an issue.

The important problem raised by previous studies is how to define species in metabarcoding data. Currently, the molecular equivalent of species is the OTU (Operational Taxonomic Unit), sometimes called MOTU (Molecular Operational Taxonomic Unit, Blaxter 2004), which corresponds to a group of sequences defined usually by a fixed genetic divergence threshold. However, the conception of OTU may not provide satisfying results for protists (Grattepanche et al. 2014). Indeed, there exists no universal threshold that would account for all protists taxa because they evolve at very different rates (Pernice et al. 2013). A solution would be to specify different thresholds to group the environmental sequences of each taxon independently, as recently recommended for zooplankton communities (Brown et al. 2015) and already proposed for the clustering of foraminiferal environmental sequences (e.g. Lejzerowicz et al. 2014; Pawlowski et al. 2014b). However, the presence of artifactual sequences creates numerous rare OTUs that are difficult to diagnose and discard, especially for protists given the potentially high diversity of its mostly undescribed "rare biosphere" (Bachy et al. 2013). To overcome these problems, different OTU clustering procedures were recently developed that rely on the sequence nucleotide composition and alignment (Mahé et al. 2014) or are alignment-free (La Rosa et al. 2013); or are based on the distribution and counts of sequence units in the samples (e.g. Preheim et al. 2013).

From biomonitoring perspective, the main challenge is to maintain the ecological relevance of OTUs without over-interpreting genetic polymorphism. Indeed, several recent studies have drawn attention to the high intra-genomic divergence of ribosomal copies present in some ciliates and foraminifera (Gong et al. 2013; Weber and Pawlowski 2014). Although accounting for intra-genomic polymorphisms may strengthen a species ecological signal, it is also important to not miss the ecologically distinctive genotypes by overestimating the level of polymorphism. Indeed, the sequences of such genotypes may diverge by only one sequence position change (Eren et al. 2013), which is not necessarily entailed in very short barcodes as was recently pinpointed based on robust time-series data (Tikhonov et al. 2015).

Another controversial aspect of metabarcoding is the interpretation of HTS quantitative data. Many ecological studies show the discrepancies between the abundance of specimens in microscopic studies and the abundance of reads in eDNA datasets (Medinger et al. 2010; Stoeck et al. 2014). Despite the problems related to the accuracy of taxonomic assignment of sequences (see below), these discrepancies may be a result of biological variations such as different number of nuclei or gene copies, different genome size or biovolume variation; or technical artifacts related to the PCR or sequencing conditions (Weber and Pawlowski 2013). Given the panoply of biasing factors, one cannot expect an ideal match between the number of reads and specimens in terms of absolute abundance. Some authors prefer to ignore sequence abundance information and perform all their analyses based only on presence-absence data (Chariton et al. 2010, 2014, 2015; Zaiko et al. 2015). However, most of biotic indices formulas require relative abundance values. Although the relative values may also be biased by various factors in microscopic analyses (Wall et al. 2010), it can be assumed that both molecular and morphological biases will be constant across samples and shall not interfere with the comparison between different ecological conditions.

The last issue that merits to be discussed here is the importance of reference database and taxonomic assignment of HTS data. Until now, all applied metabarcoding studies used the morphological taxonomy as a baseline to ascribe ecological values to the OTUs generated by HTS data. As mentioned above this taxonomic assignment is one of the main controversial points concerning the interpretation of HTS data. Genetic variations are usually higher than morphological and the correspondence between morphotypes and phylotypes is not always straightforward. Moreover, for many groups

of protists the morphology-based taxonomic description is restricted to very few species. As shown by all environmental diversity surveys, the vast majority of protists remained undescribed. Isolation and taxonomic description of all these species, would take hundreds of years. This time span would be reduced if only potential bioindicators species are morphologically described, but even this task sounds gigantic.

Here, we argue that taxonomic identification might not be absolutely necessary for using protists as bioindicators. In fact, from the perspective of biomonitoring, the morphological species identification is used only for assigning the correct ecological value, because the ecological observations are exclusively based on the morphospecies concept. However, this could change if ecological values could be inferred from HTS data or connected directly to OTUs as was proposed for planktonic microbial communities (Steele et al. 2011). For example, one way to do it would be to calculate a new molecular index, in which the morphospecies will be replaced by OTUs, and which will be calibrated with the index values of a morphology-based study conducted in parallel. The main advantage of such approach (illustrated as path 4 in Fig. 4) is the fact that the number of potential bioindicators will explode, including hundreds of unknown protist taxa that might have an important ecological role, but are currently ignored because they cannot be morphologically identified. The same approach is currently being applied in studies of human microbiome that associate bacteria to human health diagnosis without worrying too much about taxonomic identity of bacterial sequences. There is no reason why the same cannot be done for assessing ecosystem health using protists identified only through their sequence data.

Concluding remarks

To conclude, we think that it is time to change the way protists are used in environmental biomonitoring and to integrate protist DNA barcoding and metabarcoding to the routine ecological diagnosis. This plea is addressed to the ecologists and environmental managers who are studying sensitivity and response of protist communities to environmental changes and who are using them as bioindicators. In their work, they often face various taxonomic problems related to morphological identification that could be easily overcome by using DNA barcoding approach. This paper is also addressed to molecular taxonomists and geneticists that are making formidable efforts to explore the environmental diversity of protists using HTS metabarcoding. Over the last decade their work totally changed our view on protist biodiversity and biogeography, contributing the discovery of seemingly endless number of new lineages and phylotypes. Some of these protists could probably become precious bioindicators. However, significant efforts have to be made to establish more active collaboration between the researchers involved in protist biomonitoring and metabarcoding.

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