Water Research 191 (2021) 116767

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Review

Expanding ecological assessment by integrating microorganisms into routine freshwater biomonitoring

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ARTICLE INFO

Article history:
Received 27 August 2020
Revised 14 December 2020
Accepted 19 December 2020
Available online 23 December 2020

ABSTRACT

Bioindication has become an indispensable part of water quality monitoring in most countries of the world, with the presence and abundance of bioindicator taxa, mostly multicellular eukaryotes, used for biotic indices. In contrast, microbes (bacteria, archaea and protists) are seldom used as bioindicators in routine assessments, although they have been recognized for their importance in environmental processes. Recently, the use of molecular methods has revealed unexpected diversity within known functional groups and novel metabolic pathways that are particularly important in energy and nutrient cycling. In various habitats, microbial communities respond to eutrophication, metals, and natural or anthropogenic organic pollutants through changes in diversity and function. In this review, we evaluated the common trends in these changes, documenting that they have value as bioindicators and can be used not only for monitoring but also for improving our understanding of the major processes in lotic and

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https://doi.org/10.1016/j.watres.2020.116767

lentic environments. Current knowledge provides a solid foundation for exploiting microbial taxa, community structures and diversity, as well as functional genes, in novel monitoring programs. These microbial community measures can also be combined into biotic indices, improving the resolution of individual bioindicators. Here, we assess particular molecular approaches complemented by advanced bioinformatic analysis, as these are the most promising with respect to detailed bioindication value. We conclude that microbial community dynamics are a missing link important for our understanding of rapid changes in the structure and function of aquatic ecosystems, and should be addressed in the future environmental monitoring of freshwater ecosystems.

1. Introduction

1.1. Freshwater ecosystems are rapidly altered by anthropogenic pressures

Freshwater environments support high species diversity and are more functionally diverse than other ecosystems. Surface freshwaters—lakes, ponds, streams and rivers—are among the most extensively and rapidly altered ecosystems, encompassing changes in physical structure, chemistry, biotic characteristics and interactions (Carpenter et al., 2011). This alteration increases threats to global biodiversity and thus also the processes modulating nutrient cycling, community stability and food-web structures (Grill et al., 2019; Vörösmarty et al., 2010).

Ecosystem services provided by prokaryotic and eukaryotic microbes have considerable impacts on rates of energy and nutrient flows as well as the intensity of many trophic interactions. Since aquatic microbes also possess high metabolic and growth rates, they are highly responsive to local and global pressures and are directly influenced by changes in physico-chemical characteristics, including inputs of organic and inorganic compounds and pollutants (Caruso et al., 2016; Pernthaler, 2017; Šimek et al., 2014). In addition, rare microbes can act as a “hidden backbone” and provide resistance and resilience against disturbances (Garcia et al., 2018; Jousset et al., 2017). Therefore, measurable responses to environmental stressors caused by human activities can be predicted through the changes in microbial community diversity, dynamics and activity occurring in disturbed ecosystems (Fig. 1A).

Microbe-based bioindicators can be selected from a broad variety of taxonomic groups mediating processes such as oxygenic photosynthesis or the assimilation of carbon dioxide carried out by e.g. cyanobacteria and algae, or complex microbial food webs forming networks that link many heterotrophic microorganisms to the dissolved carbon pool (Azam et al., 1983; Šimek et al., 2019; Šimek et al., 2014; Sommer et al., 2012). However, other ecosystem services that are not associated with particular taxa require the selection of appropriate bioindicators that target specific metabolic pathways. Examples of these pathways include: anoxygenic photosynthesis, provided by only few bacterial phyla; nitrogen fixation, carried out by only specialized prokaryotes; and methanogenesis, delivered by only syntrophic interactions of *Archaea* (Compte-Port et al., 2020; Waidner and Kirchman, 2008; Yao et al., 2018). Additionally, genes coding for metal or antibiotic resistances might be used for bioindication of the respective pollutants (Roosa et al., 2014). Similarly, biotic interactions can be reflected either in general trophic patterns (Bock et al., 2020) or in very specialized host-parasite/endosymbiont relationships such as in *Chlamydiae* and their amoebae hosts (Pizzetti et al., 2012).

Since the beginning of world-wide water biomonitoring (reviewed in Bonada et al., 2006; Santos et al., 2019), environmental microbiology has experienced enormous conceptual and methodological developments with the implementation of cultivation-independent molecular methods. Many tools have been applied, including high-throughput sequencing, PhyloChip, GeoChip, quantitative PCR, metagenomics, metatranscriptomics and metaproteomics (for details see Supplementary files). Molecular approaches have considerably increased knowledge of aquatic microbial community richness, diversity and functional variability (Mansfeldt et al., 2020). However, a systematic compilation of distinct microbial taxa and functional groups associated with water pollution measures is lacking.

Novel strategies for including environmental genomics in water quality monitoring that reflect ecosystem functions, have been previously proposed (Cordier et al., 2020). Regarding novel microbe-based bioindication , the strategies can be modified to provide assessments of both broad-scale processes, indicated by many prokaryotic and eukaryotic taxa, as well as narrow-scale processes indicated by a limited number of specialized prokaryotes (Apothéloz-Perret-Gentil et al., 2017; Falk et al., 2019; Roosa et al., 2014; Tanentzap et al., 2019; Yao et al., 2018). Thus, the strategies for assessments using microbial bioindicators include the following: (A) tracking of specific taxa, their quantification and precise taxonomic detection, used, e.g., in fecal coliform bacteria or water-borne pathogens; (B) novel community-based bioindicators such as diversity indices, employed in determining changes of broad ecosystem services; and (C) functional community/population assessments, focused on specific metabolic pathways or specialized microbial activities. Furthermore, the value of using microbial responses to environmental stressors, compared to bioindication by multicellular eukaryotes or direct chemical measurements, lies in the possibility to identify contamination transfers through aquatic ecosystems. Those are reflected in altered nutrient cycling, metabolic pathways and trophic links, which are then clearly attributable to specific microorganisms. Consequently, this review aims to establish a solid foundation for the widespread adoption of microbes as bioindicators and/or variables in biotic indices in freshwater ecosystems. Additionally, we propose using microorganisms to distinguish the effects of environmental stressors that cannot be determined by other presently used indicators.

2. Current legislation deals only with cultivable pathogenic and toxic microorganisms

Current biomonitoring programs for aquatic ecosystems do not generally include both wide environmental screening and narrow critical pathways that are detectable only in certain prokaryotic and eukaryotic microorganisms. Usually, only selected microorganisms and their products are used, in particular fecal indicator bacteria, water-borne pathogens or the quantification of toxins. In Europe, the Drinking Water Directive (2015/1787, 98/83/EC) (EC, 2015, 1998) and the Bathing Water Directive (2006/7/EC) (EC, 2006), as well as related legislation in the USA (EPA 815-F-08–001, EPA/600/R-12/551, EPA 910-R-15–002, CCL4 2016) (EPA, 2016),

Fig. 1. Aquatic environments of populated areas: A) Inputs of pollutants from human activities and affected ecosystem processes. B) Bioindicators of pollutants include microbial taxa and functional genes of processes involved in their degradation. White arrows indicate proportional increases/decreases. Suggested methods for assessment of pollutants are presented in white bubbles. For description of functional genes see Table 3.

Canada (1970), Australian and New Zealand Environment and Conservation Council (2000), India (2012), South Africa (2015) and State Council of the People's Republic of China (2015) include a limited number of pathogenic or opportunistic pathogenic bacteria. Surveillance of bacteria such as *Escherichia coli* and enterococci, as well as protists such as *Cryptosporidium, Giardia* and *Naegleria fowleri*, is mandatory in many countries in the EU and EEE (World Health Organization guidelines for drinking-water quality, WHO, 2011). Since gastrointestinal illnesses associated with exposure to contaminated waters can vary according to the host organism, information on the origin of contamination is essential for risk mitigation. Therefore, some Directives also require the identifica-

tion of contamination sources in international watercourses (Directive 95/308/EC) (EC, 1995). For these organisms, the directives rely on a cultivation-based approach built on the counting and characterization of viable cells on differential/selective media under defined cultivation conditions.

Cyanobacteria are another microbial group included in legislation because they produce toxins, scums and odors (Ferreira et al., 2011; Kahru and Elmgren, 2014) that negatively influence bathing water quality, with some toxins also having severe effects on drinking water resources (Cheung et al., 2013; Li et al., 2017; Paerl et al., 2011). The importance of certain cyanobacteria has been recognized by the World Health Organization guidelines for drinking-water quality (WHO, 2011), which provides provisional guideline values for Microcystin-LR, the most frequently occurring and toxic of microcystin congeners. While in drinking water individual toxins are monitored, risk assessments and management in recreational waters regulate just the presence of cyanobacteria (quantified as cell numbers or biomass) (Ibelings et al., 2014). Again, the preferred approach suggested by the guidelines is a visual inspection including microscopy for potentially microcystin-containing genera. The Drinking Water Directive of the EU does not address cyanobacterial toxins, and health authorities have turned to the provisional WHO guideline values to assess whether the concentrations are “hazardous” (Li et al., 2017).

3. Detection of pathogenic and toxic microbial contamination by cultivation independent methods

3.1. Pathogen tracking

Important ecosystem services of natural water bodies includes the supply of clean drinking water, often accumulated in dam reservoirs from wide catchment areas, as well as recreational (bathing) waters. The use of these water resources is regularly affected by microbiological contamination, which is caused by potentially harmful microorganisms from intestinal flora. Various sources can contribute to fecal pollution, including wildlife (deer, boar, rodents, birds), farms and domestic animals (poultry, cattle, sheep, cats, dogs), and humans (Devane et al., 2018). However, the currently used methods do not appropriately assess the genetic variability of fecal microbes and resulting changes in their pathogenicity and in the activity of respective functional genes. The most relevant present-day pathogen tracking in freshwater ecosystems utilizes microbial source tracking (MST), a technique that uses quantitative PCR methods to detect and quantify gene markers associated with fecal microorganisms (e.g. Brooks et al., 2020; McKee et al., 2020). Culture independent approaches have been introduced to provide a sensitive and specific detection of fecal contamination indicators such as *E. coli, Salmonella, Shigella* and *Enterococcus* spp., as well as *Campylobacter* spp., *Bacteroides* spp. and viruses or eukaryotes such as *Giardia lamblia* (Brooks et al., 2020; Verweij et al., 2004). Surveyed marker genes traditionally include taxonomic identification using 16S and 23S rRNA genes for prokaryotes and 18S rRNA genes for eukaryotes. They have also been recently expanded to include functional genes that encode the activities traditionally used for identification by selective media such as *lacZ* (β-galactosidase), *lamB* (maltose outer membrane porin) and *uidA* (β-D-glucuronidase, specific for *E. coli*). Furthermore, methods for the elimination of extracellular DNA have been developed (e.g. propidium monoazide) to avoid the false positive detection of dead cells, which is critical for the monitoring of water bacteriological quality (Gensberger et al., 2014; Truchado et al., 2016).

Likewise, due to the importance of water as a medium for disseminating human waterborne diseases, environmental DNA (eDNA) protocols have also been established and successfully applied for the detection of other pathogenic microbes causing severe parasite-induced human diseases such as *Leptospira* (Sato et al., 2019). However, none of these monitoring approaches for fecal contamination has yet provided information about geographic origin and fecal contamination sources, as these indicators are universally and indiscriminately found in warm-blooded animals. Recent research on the co-evolution between hosts and their gut microbiota (i.e. the host-specific microbiome) can provide a solution (Ley et al., 2008). More specifically, for some taxa such as *Clostridiales* and *Bacteroidales*, host-specific bacterial classifiers have been developed and used to differentiate the distributions of microbes among human, cow, deer, pig, cat, and dog sources (Brooks et al., 2020; McKee et al., 2020; Roguet et al., 2018).

3.2. Cyanobacteria toxin tracking

Molecular studies of cyanobacteria have mainly focused on evaluating the spatial and temporal variability of cyanobacterial communities. Similarly to studies of other prokaryotes, amplicon sequencing is used either for the identification of particular taxa or operational taxonomic units (OTUs) representing the sequence variability of marker gene(s) (e.g. Guedes et al., 2014; Jiang et al., 2017). Amplicon sequencing has the potential to monitor toxigenic genotypes of cyanobacteria (Kurmayer et al., 2017; Scherer et al., 2017), although no single approach has been recommended in cyanobacterial bloom monitoring and cyanotoxin detection due to the variability in their resolution and comparability of results (Gaget et al., 2017; Srivastava et al., 2012). In addition, studies have included the detection of potentially toxic cyanobacteria using quantitative PCR-based (qPCR) approaches (e.g. Al-Tebrineh et al., 2010; Chiu et al., 2017). This method can be used for the quantification and distinction of toxin-producing and non-producing types of cyanobacteria, e.g. *Microcystis* (Zuo et al., 2018), or the quantification of toxin biosynthesis genes that can be connected with toxin concentrations for assessing toxicity (Panksep et al., 2020; Scherer et al., 2017). These methods, together with traditional approaches (microscopy, cyanotoxin analysis), have been employed for studying the effects of trophic status based on cyanobacterial community composition (Panksep et al., 2020; Wood et al., 2017) and diversity (Eiler et al., 2013; Lee et al., 2017; Wood et al., 2017; Yoon et al., 2016) (Table 1).

4. Microbial bioindicators of anthropogenic impacts

4.1. Microbial eukaryotes (protists) for biomonitoring

In the European Water Framework Directive (WFD), phytoplankton and phytobenthos communities are acknowledged and integrated as Biological Quality Elements (BQE) for the environmental monitoring of freshwater ecosystems (EC, 2000). Even though many of these bioindicators have been applied in the context of scientific (academic) research, predominantly diatoms (phylum Bacillariophyta) have succeeded in making the transition as BQEs from the academic world into the “real world” of applied freshwater (mostly river) biomonitoring. For these BQEs, standard protocols have been established (European Committee for Standardization, 2016). Similarly to the other already established microbial bioindicators, the challenges of microscopic diatom surveys mostly include long analysis times and high demand on expert knowledge for taxon identification. Inevitably, differences between individual approaches resulted in variation of taxa inventories among laboratories (Kahlert et al., 2012; Kelly et al., 2018). This initiated the development of less expensive, more efficient and robust eDNA metabarcoding protocols for diatoms, especially in lotic ecosystems

Table 1
Studies focusing on changes in cyanobacteria communities.

Water body / habitat	Country / climate zone	Site pollution specificity	Taxa	Parameter / method	Effect	Reference
A. Species diversity and abundance						
rivers / water	Germany / temperate	total P	cyanobacteria	biovolume / microscopy	increase	(Mischke et al., 2011)
lakes / water	Germany / temperate	total N, total P	cyanobacteria	biovolume / microscopy	increase	(Dolman et al., 2012)
		N:P ratio	*Cylindrospermopsis raciborskii, Limnothrix, Planktolyngbya, Pseudanabaena*	biovolume / microscopy	increase	"
		N:P ratio	*Anabaena, Anabaenopsis, Aphanizomenon flosaquae, A. issatschenkoi, Microcystis, Planktothrix agardhii*	biovolume / microscopy	decrease	"
river / biofilm	Iran / dry	nitrates	cyanobacteria	species richness / microscopy	decrease	(Soltani et al., 2012)
		nitrates, sulfates	*Chroococcus minor, Lyngbya kuetzingii, Oscillatoria chlorina, Phormidium tenue*	abundance / microscopy	increase	"
		nitrates, sulfates	*Lyngbya infixa,* L. *mesotrichia, Chroococcus turgidus, Homeothrix juliana*	abundance / microscopy	decrease	"
streams / biofilm	USA, CA / dry, temperate	sulfates, nitrates, total N, ortho-phosphate, total P	heterocystous cyanobacteria	relative biovolume and species number / microscopy	decrease	(Stancheva et al., 2013)
		nitrates, ammonium, N:P ratio	Calothrix, Nostoc, endosymbionts of Epithemia, Rhopalodia	rel. abundance / microscopy, reverse transcriptase-qPCR *nifK*,16S rRNA	decrease	"
rivers / biofilm	Norway / temperate, continental	high total P	*Chamaesiphon incrustans, C. polymorphus*	occurrence / microscopy	present	(Schneider et al., 2013)
		low total P	*Capsosira brebissonii, Clastidium setigerum, Hapalosiphon hibernicus, Scytonematopsis starmachii, Stigonema, Tolypothrix distorta*	occurrence / microscopy	present	"
river / biofilm	Spain / temperate	dissolved inorganic N (DIN), soluble reactive P (SRP)	cyanobacteria	band richness / TGGE, 16S rRNA gene	decrease	(Loza et al., 2013b)
		low DIN, SRP	*Chamaesiphon investiens, Chroococcus minor, Leptolyngbya tenuis, L. nostocorum, Nostoc piscinale, N. punctiforme, Phormidium, Tolypothrix tenuis*	occurrence / microscopy, TGGE, 16S rRNA gene sequencing	present	"
		high DIN, SRP	*Aphanocapsa muscicola, Chamaesiphon, Cyanobium, Leptolyngbya boryana, Phormidium, Pleurocapsa, Pseudanabaena*	occurrence / microscopy, TGGE, 16S rRNA gene sequencing	present	"
rivers / biofilm	Spain / temperate	low N (NO_3^-, NO_2^-, NH_4^+), P (PO_4^{3-})	*Phormidium aerugineocaeruleum, P. corium*	occurrence / microscopy, 16S rRNA gene sequencing	present	(Loza et al., 2013a)
		high N (NO_3^-, NO_2^-, NH_4^+), P (PO_4^{3-})	*Oscillatoria tenuis, Phormidium terebriforme*	occurrence / microscopy, 16S rRNA gene sequencing	present	"
pond / water	India / temperate	P (total P, total particulate P)	potentially microcystin producing *Anabaena, Microcystis, Planktothrix*	*mcyA* copy number / qPCR	increase	(Srivastava et al., 2012)
lake / water	USA, WA / temperate	phosphates, silicates	cyanobacteria, *Microcystis*	16S rRNA, *mcyE* gene copies / qPCR	increase	(Lee et al., 2015)
lake / water	China / temperate	total P	potentially toxic *Microcystis*	*mcyA* gene / qPCR	increase	(Li et al., 2014a)
lake / water	China / temperate	P (total, PO_4^{3-}) N (total, NO_3^-, NH_4^+)	*Microcystis*	16S rRNA, *mcyD* gene copies / qPCR	increase	(Li et al., 2014b)
"		dissolved inorganic C	*Microcystis*	"	decrease	"
lakes / water	Germany / temperate	total P	potentially toxic *Microcystis*	*mcyB* copy number / qPCR	increase	(Scherer et al., 2017)
"	"	inorganic N:P ratio	potentially toxic *Microcystis*	"	decrease	"
"	"	nitrates, total P	*Dolichospermum*	OTU proportion / 16S rRNA gene Illumina sequencing	decrease	"
"	"	nitrates, total P	*Microcystis, Synechococcus, Snowella*	"	increase	"
lake, river est. / water	USA, FL / temperate	total N	potentially toxic *Microcystis*	*mcyE* copy number / qPCR, metagenomics	increase	(Kramer et al., 2018)
B. Morphological and physiological characteristics						
rivers / biofilm	Spain / temperate	P bio-availability	*Chamaesiphon, Nostoc, Phormidium, Tolypothrix*	alkaline phosphatase / enz. Assay	decrease	(Muñoz-Martín et al., 2014)
			Chamaesiphon, Nostoc, Phormidium, Rivularia, Tolypothrix	polyphosphate granules / microscopy	increase	"
			Phormidium, Rivularia	hairs, calyptras / microscopy	decrease	"

Table 2
Micro-eukaryotic Indices.

Index	Habitat	Method	Reference
Specific pollution-sensitivity index (SPI)	stream and river epilithon	numbers of diatom species determined by microscopy and clustered into sensitivity classes and indicator value groups	(Coste in Cemagref, 1982)
Diatom biological index (BDI)			(Prygiel and Coste, 1993)
Trophic diatom index (TDI)			(Kelly and Whitton, 1995)
Rott's Trophic and Saprobic Index (Rott TI and SI)			(Rott et al., 2003)
Index of biotic integrity (IBI)			(Wang et al., 2005)
Taxonomy-free molecular diatom index (DI-MOLTAXFREE)		amplicon sequencing of V4 region of the 18S rRNA gene	(Apothéloz-Perret-Gentil et al., 2017)

(Bailet et al., 2020; Kermarrec et al., 2014; Pérez-Burillo et al., 2020; Tapolczai et al., 2019; Vasselon et al., 2019; Visco et al., 2015; Zimmermann et al., 2015).

In contrast to the diatom eDNA metabarcoding protocols for river monitoring, barely any of these protocols exist for other taxonomic groups of microbial eukaryotes (Pawlowski et al., 2018, 2016b, 2016a). This is surprising because numerous studies have demonstrated the sensitivity of various protistan taxonomic groups to environmental stressors in natural as well as artificial freshwater ecosystems (Foissner, 2016; Stoeck et al., 2018b). For example, similar to diatoms, green algae and chrysophytes are also excellent bioindicators of nutrient fortification. This is due to their frequent species-specific nutrient requirements for growth and reproduction (Gökçe, 2016), as well as other environmental parameters such as conductivity (Çelekli and Külköylüoğlu, 2007), oxygen and pH (Çelekli et al., 2014). Similarly, dinoflagellates are also suitable indicators of nutrient conditions in freshwater systems (Moore et al., 2013).

Furthermore, non-photosynthetic protists, which are highly responsive to environmental disturbances, are omitted in the WFD (Keck et al., 2017). Of these, amoebas are highly sensitive to various forms of environmental change including sulfur, nitrogen, carbon dioxide, heavy metals and particulate pollutants (Payne, 2013). Ciliates (phylum Ciliophora) are sensitive to local environmental conditions, including low oxygen concentrations, organic enrichment, phytoplankton composition, acidification, heavy metal pollution and various other chemical stressors (see references in Lynn, 2008). Accordingly, ciliate morphospecies, despite not being acknowledged as a BQE in the EU WFD, are frequently used as suitable biological indicators for the applied environmental compliance monitoring of rivers in Austria (Berger et al., 1997; Berger and Foissner, 2003). They are also used as indicators of activated sludge performance in many European countries (Foissner and Berger, 1996; Madoni, 2011).

Traditional methods for protist detection include staining and microscopy. The diagnosis of protists for the purpose of environmental monitoring using high-throughput sequencing technologies is still a relatively young research field (Pawlowski et al., 2016b). However, the few available studies, which have all focused on entire protistan communities rather than individual taxa have demonstrated the strengths of this approach for modern biomonitoring (Uyaguari-Diaz et al., 2016; Xie et al., 2017).

While for some taxonomic groups, such as diatoms, non-ribosomal genes are preferred as genetic markers (e.g. Vasselon et al., 2019), the taxonomic diversity of the whole community is best captured with ribosomal genes. In the past, the V4 and the V9 hypervariable region of the 18S rRNA gene have proved suitable for the eDNA profiling of protistan communities (Debroas et al., 2017; Pawlowski et al., 2012; Stoeck et al., 2010). A multiple primer approach has been recommended to capture the full protistan diversity (Bradley et al., 2016; Stoeck et al., 2006). However, this approach also increases the costs of monitoring as well as the complexity and time in sample and data analyses. Therefore, the most suitable choice of PCR primers may have to be evaluated for each individual ecosystem (e.g. lake, pond, river, or creek) and the specific monitoring target (e.g. nutrient pollution, organic enrichment, heavy metal pollution).

Two well-known shortcomings in using eDNA metabarcoding of protistan communities for biomonitoring are taxonomic assignments of sequences and inferring relative abundances of individual indicator taxa from the sequence data. Due to incomplete reference databases, large proportions of sequences remain taxonomically unassigned (e.g. up to 70% of sequences (Mortágua et al., 2019)). However, ecological quality inference from the remaining (taxonomically assigned) sequences results in highly similar biological quality status as that obtained from morphospecies data. This has recently been demonstrated in river monitoring studies using diatoms (Mortágua et al., 2019; Rivera et al., 2020). Nevertheless, a taxonomy-free approach that allows descriptions of diatom community structure and diversity without assigning sequences to taxa seems preferable for the evaluation of the environmental (health and quality) status of water bodies (Apothéloz-Perret-Gentil et al., 2017; Cordier et al., 2019; Feio et al., 2020).

Some studies have shown an acceptable agreement between morphospecies abundance or biomass and relative sequence abundance based on sequencing for at least some taxonomic groups (Pitsch et al., 2019; Weber and Pawlowski, 2013). Yet, variations in gene copy numbers and genome size among protists (Zhu et al., 2005) can introduce artifacts that bias relative abundance estimates in protistan communities (Leray and Knowlton, 2016; Piwosz et al., 2020).

A suggested solution to this problem is to focus on individual protistan taxon groups rather than on a whole community so as to reduce the impact of copy number variation (Stoeck et al., 2018a). Furthermore, it is much easier to avoid quantification bias for individual taxonomic groups through the application of a cell biovolume correction factor, as has been successfully shown for diatoms (Vasselon et al., 2018). Accordingly, relative sequence abundances can represent an efficient and reliable measure when inferring the ecological status of ecosystems based on individual protistan taxon groups such as ciliates (Forster et al., 2019; Stoeck et al., 2018a) or foraminiferans (Pawlowski et al., 2016a, 2014). Moreover, recent progress in sequencing allows for the cultivation-independent specific targeting of other planktonic protists by newly designed fluorescence *in situ* hybridization (FISH) probes (e.g. Massana et al., 2009; Šimek et al., 2020). However, while FISH is a promising tool, it is also laborious and thus only suitable for providing specific information. This includes visualization of important but morphologically almost indistinguishable protistan taxa *in situ* as well as the inspection of food vacuoles to determine their feeding modes and major trophic interactions.

In conclusion, a whole protistan community survey can encompass the taxonomic and potentially functional diversity, thus providing a more complete picture of the ecosystem status. A substantial amount of research remains to be done, however, before the broad application of either of these approaches in environmental monitoring can be initiated (Mortágua et al., 2019) (Supplementary Table S2; Table 2).

4.2. Prokaryote taxa and functional genes for biomonitoring

Prokaryotes respond to environmental conditions by changes in their proportional abundances within the community and diversity, as well as through expressing various functions. These responses are characteristic of individual stressors due to the different physiology of various bacterial and archaeal lineages in various freshwater ecosystems (e.g. Garcia et al., 2018; Jacquiod et al., 2018; Newton et al., 2011).

4.2.1. Nitrogen input

High amounts of nitrogen added to the environment by synthetic and organic fertilizers as well as through the burning of fossil fuels heavily contribute to anthropogenic eutrophication. In many studies, concentrations of various nitrogen forms, ammonium, nitrite and nitrates, and total nitrogen (NH_4^+-N, N_{ox}-N, and TN) accounted for considerable shifts in bacterial community structures in the sediments of both rivers and lakes (Fig. 2, Supplementary Table S3). This indicates that nitrogen is particularly important in shaping the diversity patterns of bacterial communities in aquatic environments (Wan et al., 2017). In the available literature, mostly *Nitrospirae, Betaproteobacteriales, Chloroflexi* and *Sphingobacteriales* proportionally increased with nitrogen concentrations (Fig. 2, Supplementary Table S3A).

Nitrospirae are important nitrite oxidizers in freshwater lake sediments. Consequently, these bacteria flourish in high nitrogen conditions (Wan et al., 2017), and notably a higher concentrations of NH_4^+ promote the growth of *Nitrospirales* (Wan et al., 2017). *Betaproteobacteriales* (former class *Betaprotobacteria*) are functionally diverse, but this order encompasses the majority of ammonia oxidizing bacteria (Yang et al., 2016a). It seems that the chemolithoautotrophic members of this group may particularly benefit from increased concentrations of reduced inorganic nitrogen compounds including ammonium. Furthermore, *Betaproteobacteriales* play a role in nitrogen fixation occurring in sediments (Wu et al., 2009). Chloroflexi are nitrogen fixing bacteria (Dos Santos et al., 2012), and nitrate has been negatively correlated with *Chloroflexi* communities (Zhang et al., 2015). Finally, *Sphingobacteriales*, which also increase with elevated nitrogen, are typically obligatory and facultative aerobic chemoorganotrophs. Their increased occurrence in the community is likely related to the conditions of waste water treatment plant effluents described below (Drury et al., 2013) (Fig. 2, Supplementary Table S3A, B).

A large number of *Archaea* are also active in processing nitrogen in surface waters and sediments (Wang et al., 2018). Interestingly, while sediment nitrate contents can negatively affect bacterial diversity, one study found that archaeal community diversity was not sensitive to any of the environmental factors measured (Zhang et al., 2015).

4.2.2. Anthropogenic organic pollution

The effects of waste water treatment plant (WWTP) effluents on microbial activity and diversity have frequently been studied, especially in lotic environments. All studies have found bacterial communities to be strongly influenced by WWTPs or similar pollution sources (Chonova et al., 2018; Drury et al., 2013; Ibekwe et al., 2016). Commonly, increases in the proportions of *Nitrospirae, Sphingobacteriales* (*Bacteroidetes*) and *Spirochaetes*, and a general decrease of *Actinobacteria* (see below), have been observed in the communities downstream of WWTPs (Fig. 2, Supplementary Table S3B).

Nitrospirae and some *Bacteroidetes* are the dominant taxa of activated sludge (Huang et al., 2017; Wan et al., 2017). Therefore, their increased proportion in microbial communities directly indicates the influence of WWTP effluents. In contrast, *Sphingobacteriales* (*Bacteroidetes*) seem to proliferate after the WWTP inflow, possibly in response to increased resources for the river community, which stimulates the growth of bacteria and higher organisms (Hladilek et al., 2016; Korajkic et al., 2015). *Spirochaetes*, another commonly observed taxon increased in WWTP effluents, are known as abundant endosymbionts, often associated with invertebrate gut contents, where they break down lignocellulose and fix nitrogen (Van De Water et al., 2016). *Actinobacteria* might generally represent a good bioindicator because the proportion of their particular clades in microbial communities across all habitats are highly responsive to changing environmental factors (Meziti et al., 2016; Neuenschwander et al., 2018; Newton et al., 2011; Youssef and Elshahed, 2009). In particular, pelagic actinobacteria seem to be sensitive to eutrophication (e.g. Liu et al., 2019), although they are also capable of degrading complex organic compounds and pollutants in the sediment (Alvarez et al., 2017; Zhang et al., 2019) (Fig. 2, Supplementary Table S3B, C).

4.2.3. Metals, pharmaceuticals and personal care products

River and lake sediments act as a sink for persistent anthropogenic contaminants such as heavy metals, antibiotics, other pharmaceuticals, pesticides, disinfectants and their residues. These compounds are released slowly and pose potential threats to aquatic life and human health for an extended period of time (Kerrigan et al., 2018; Liao et al., 2019).

Metals. The toxicity of some metals is a result of their prooxidative activity (Gauthier et al., 2014; Giner-Lamia et al., 2014), the modification of conformation structures of nucleic acids and proteins, and their interference with oxidative phosphorylation and osmotic balance (Nies, 1999). Although metals clearly interfere with microbial life, no trend has been found for the effects of metal pollution on bacterial communities in the studies reviewed here (Fig. 2, Supplementary Table S3D).

Metal contamination of sediments results in the increased abundance of genes involved in metal homeostasis, including genes for metal import, mobilization, storage and efflux. Efflux pumps, along with other resistance mechanisms, can be quite general, since they transport many harmful substances from the cell (Chandrangsu et al., 2017; Roosa et al., 2014; Yin et al., 2015). Thus, heavy metals can co-select for resistance to antibiotics, as documented in a study which retrospectively researched over a century's worth of anthropogenic pollution (Dickinson et al., 2019). Since metal resistance is often plasmid-bound their presence does not correspond to a specific bacterial taxon due to the inherent ephemeral nature of these genetic elements, and they should be assessed in a taxonomy-independent way. However, this approach may be complicated by the common association of heavy metal and antibiotic resistance genes in the same plasmids (Rahube et al., 2014). Heavy metals also induce horizontal gene transfer and DNA uptake by bacteria, which is evidenced by increased integron activity and diversity (Nemergut et al., 2004) offering another bioindication approach (see below).

Pharmaceuticals and personal care products (PCPs) present particularly in WWTP effluents accumulate in sediments (Barra Caracciolo et al., 2015) resulting in response of local microbial communities (Supplementary Table S3E). The response of taxa seems to be dependent on both the chemical structure of the degraded compound as well as their relationship to oxygen concentration (Wolff et al., 2018). Antibiotic resistance genes (ARGs) are either selected from the community (Aminov and Mackie, 2007) or transferred to the environment with bacteria in waste water (Amos et al., 2014; Rodriguez-Mozaz et al., 2015). For the major classes of antibiotics used in human and veterinary medicine, a variety of resistance determinants exist, which, makes their targeted assessment or quantitation difficult (Aminov et al., 2002)

Gammaproteobacteria and *Cytophaga* (*Bacteroidetes*) increased in several systems in response to the presence of these chem-

Fig. 2. Microbial taxa (including respective phyla) responding to major pollution types in the water column (blue) and sediment (orange). Upward arrows indicate a proportional increase and downward arrows a proportional decrease of the given taxon within the bacterial community. Taxa highlighted in red were identified with the same trend i.e. increase/decrease in multiple studies. The other taxa were found associated with the pollution type in at least one study (see refs. Garrido et al., 2014; Guo et al., 2019; Lu and Lu, 2014; Sharuddin et al., 2017; Sun et al., 2016; Ung et al., 2019). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Specific microbial processes and marker genes.

Process	Gene	Activity	Method	Main hosts	Study	Site	Reference
CO_2 fixation							
CBB-cycle	*cbbL, cbbM*	RubisCO	PCR	ubiquitous	water column, ratio of oxic and anoxic zones within a water body	European lakes	(Alfreider et al., 2017)
4-hydroxy-butyrate cycles (HP/HB and DC/HB)	*hcd*	4-hydroxy-butyryl-CoA dehydratase	qPCR	*Archaea*			
rTCA pathway	*aclA*	ATP citrate lyase	PCR	*Bacteria*			
anoxygenic photosynthesis	*pufM*	M subunit of photosynthetic reaction center	qPCR	*γ-Proteobacteria*	sediments, salinity, nutrients, oxygen variation	Delaware estuary, USA	(Waidner and Kirchman, 2008)
methanogeny/ methanotrophy							
CH_4 production	*mcrA*	methyl coenzyme Q reductase	qPCR	*Euryarchaeota*	sediments, positively correlated with methane yield; methanogens diversity; eutrophication, sediment O_2, organic matter	Dianchi and Erhai Lakes, Yunnan Plateau, China; Lake Bourget, France	(Billard et al., 2015; Wilkins et al., 2015; Yang et al., 2020)
CH_4 oxidation	*pmoA*	methane monooxygenase	qPCR	*γ-Proteobacteria*	negative correlation with ammonia nitrogen, increase with eutrophication	Dianchi and Erhai Lakes, China	(Knief, 2015; Yang et al., 2019b; Yang et al., 2016b)
N- cycling							
denitrification	*narG*	nitrate reductase	qPCR	*Proteobacteria, Firmicutes, Actinobacteria*	higher diversity and relative abundance of nitrate reducers in macrophyte rhizospheres compared to unvegetated sediment	Lake Hampen, Denmark	(Chon et al., 2011; Kofoed et al., 2012; Liu et al., 2018; Smith et al., 2007)
	nirK, nirS	nitrite reductase	qPCR	*Proteobacteria, Firmicutes, Bacteroidetes*	sediment, negative correlation with oxygen concentration, positive to NO_3 concentration	Yangtze Lakes	
nitrite reduction to ammonia	*nrfA*	nitrite reductase	qPCR	*Bacteria*	sediment, positive correlation with TOC, sulfide, cyanobacteria bloom	Chinese lakes	(Li et al., 2020; Welsh et al., 2014)
N fixation	*nifH*	nitrogenase	qPCR	*Cyanobacteria, Proteobacteria, Actinobacteria*	N fixation decrease with DIN increase (USA rivers), sediment N fixation low	Lake Taihu, China	(Gaby et al., 2018)
nitrification	*amoA*	ammonia monooxygenase	qPCR	*Proteobacteria, Thaumarchaeota* (*Nitrososphaeria*)	lake sediment AOA and AOB communities were regulated by trophic state, main contribution of total N and P	Dianchi and Erhai Lakes, China	(Alves et al., 2018; Rotthauwe et al., 1997; Yang et al., 2016a)
sulfate reduction	*apsA*	adenosine phosphosulfate reductase	PCR	*δ-Proteobacteria, Firmicutes, Nitrospirae, Euryarchaeota*	abundance of sulfate-reducing bacteria in industrial waste water; presence of diverse and active SRB communities in extreme ecosystems of hypersaline soda lakes	soda lakes, Kulunda Steppe, Siberia, Russia	(Ben-Dov et al., 2007; Foti et al., 2007; Watanabe et al., 2016)
	dsrAB	sulfite reductase	PCR				
horizontal gene transfer	*intI1*	class 1 integron integrase	qPCR	*Bacteria* (widespread)	relationship between anthropogenic impacts and the abundance of intI1; assessment of the effect of anthropogenic activities on watersheds; strong association between intI1 and ARGs	watersheds with different land use, British Columbia, Canada	(Gillings et al., 2015; Uyaguari-Díaz et al., 2018)

icals, while *Betaproteobacteriales* decreased (Grenni et al., 2014; Kim et al., 2017). The degradation of pharmaceuticals also usually increases nitrification, which likely reflects the utilization of nitrogen from these compounds by various taxa (Men et al., 2017; Nsenga Kumwimba and Meng, 2019; Xu et al., 2016). The most common group of enzymes degrading pharmaceuticals and PCPs are monooxygenases. These enzymes are relatively rare and not widespread among different bacterial species, which makes them useful bioindicators (Achermann et al., 2020).

4.2.4. Functional groups and marker genes

Monitoring the dynamics of functional groups is a preferred approach for study aquatic ecosystems because their assessment targets precisely the provided ecosystem services. This approach is not generally used for eukaryotic microbes because they are not as metabolically diversified and also often difficult to cultivate, so the metabolism of many taxa has not been examined. Likewise, archaea are often only studied in specific situations, so their use in functional diversity assessments needs to be researched further (Compte-Port et al., 2020; Zou et al., 2019).

Traditionally, bacteria are assigned to physiological groups according to substrate utilization, products of metabolism, growth condition preferences and biotic interactions. However, functions are unevenly distributed within prokaryotic taxa and thus need to be assessed by tools focusing directly on metabolic pathways (e.g. Falk et al., 2019; Yin et al., 2015). Moreover, most taxonomic groups are not cultivable, so molecular methods are the only cultivation-independent option for their accurate quantification. A variety of functional genes have already been extensively studied, their activities are well characterized and primers for their direct quantification by qPCR have been validated by studies in different habitats (Table 3). In the future, the selection of marker genes can be based on shotgun metagenomic analyses of contrasting sites to complement the knowledge on metabolic pathways assessed from

isolated microbes, their physiological studies and whole genome sequences.

4.2.5. Carbon cycling

Carbon dioxide and methane are currently responsible for approximately 80% of the radiative forcing from long-lived greenhouse gasses. These two gasses represent key metabolic pathways in carbon recycling because they represent a transfer link between inorganic and organic carbon forms (Levine et al., 2011). In freshwater ecosystems, heterotrophic bacteria, despite their tiny size, regulate the freshwater carbon cycle by processing globally-significant amounts of carbon. Therefore, it is essential to understand the factors that control microbial activity – i.e. the underlying context for why freshwater ecosystems serve as hot spots for carbon cycling (Schmidt et al., 2020).

Carbon sequestration. The most important global carbon sink is photosynthetic carbon dioxide fixation (CO_2). However, the chemolithotrophic fixation of CO_2 is also important and distributed through different phyla of bacteria and archaea (Berg, 2011). Since the known pathways of CO_2 fixation are respective to either phototrophic microorganisms or chemolithotrophic bacteria and archaea, monitoring of genes specific to these pathways may reveal the different proportions of assimilated CO_2 between oxygenated and oxygen-deficient environments (Alfreider et al., 2017) (Table 3).

Methanogeny and methanotrophy. Methanogenesis contributes to the removal of organic matters and is the central process in the carbon cycle within anoxic layers of water and sediments (Lüders, 2001; Staley et al., 2014; Yang et al., 2020). The methyl coenzyme-M reductase α-subunit encoding gene (*mcrA*) has been used as a marker to assess the abundance and diversity of methanogen populations, and the gene encoding monooxygenase subunit A (*pmoA*) as a marker of methanotrophic communities (Yang et al., 2019b, 2020). Changes in the abundance of methanogens may indicate a limited oxygen availability in the sediments (Horton et al., 2019; Wang et al., 2018) (Table 3). Targeting methano- and methylotrophy is essential for determining the potential of methane release (Knief, 2015).

4.2.6. Nitrogen cycling pathways

The biogeochemical cycling of nitrogen and carbon appear to be most heavily impacted by human activities (Gruber and Galloway, 2008). The high availability of various nitrogen forms in both sediment and water bodies is often reflected by an increased proportion and expression of genes encoding specific processes involved in nitrogen cycling. For example, nitrification, anammox, denitrification, or dissimilatory nitrate reduction to ammonium have been detected by metagenomic/metatranscriptomic and targeted quantitative studies (Fan et al., 2019; Pang et al., 2019; Yang et al., 2016a; Yao et al., 2018; Yin et al., 2019) (Table 3). The balance between N sinks (e.g. denitrification, riverine outputs and sediment burial) and sources (e.g., N_2 fixation, sediment release, riverine inputs and atmospheric deposition) determines the N budget in an ecosystem (Yao et al., 2018).

Nitrogen fixation. Cyanobacteria include many N_2-fixing taxa but heterotrophic, chemolithotrophic and chemoorganotrophic bacteria and archaea can also carry out N_2 fixation. Thus, the potential for sediment N_2 fixation is widespread (Severin et al., 2012; Yao et al., 2018). Nitrogen fixation activities have been correlated with sediment water content, density, TN content and TP content, as well as with the quantity and quality of organic matter in sediments (Newell et al., 2016). In one study, nitrogen fixation was significantly negatively correlated with nitrate levels, dissolved inorganic N levels and denitrification rates (Caton et al., 2018), while another study correlated N fixing *nifH* genes with NH_4^+ (Fan et al., 2019). Interestingly, it seems that decreased nitrogen fixation in sediments can predict cyanobacterial blooms (Yao et al., 2018).

Nitrification and denitrification. Long-term high N loading affects the central processes of the N cycle by increasing rates of nitrification and denitrification and decreasing the efficiency of N use by community members. The relative abundances of denitrification genes, nitrification genes, and all P related genes have showed a significantly positive correlation with the levels of nutrients, such as TN, TP, TOC and NH_4^+ (Fan et al., 2019). The ammonia oxidizing genes of *Archaea* and *Bacteria* are involved in the nitrification process, and bacterial ammonia oxidizers seem to be less sensitive to eutrophication than archaeal ones (Yang et al., 2016a). Furthermore, nitrate reduction and methanogenesis appear to be primary processes in the contaminant transformations of persistent organic pollutants (Falk et al., 2019).

4.2.7. Phosphorus and sulfur cycling pathways

Phosphorus is a limiting factor for organisms in terrestrial, oceanic and freshwater environments, particularly during the growing season because biologically available inorganic phosphate tends to get depleted (Janssen et al., 2019). Under such circumstances, microorganisms utilizing P-containing organic compounds have an advantage. The major enzymes required for this activity are alkaline phosphatases (APases) of PhoA, PhoX and PhoD families. These differentiate the functional groups into phototrophic *Cyanobacteria* and heterotrophic *Proteobacteria* and *Firmicutes*, the other phyla possessing these enzyme activities (Kageyama et al., 2011) (Table 3). Different abundance distribution patterns of various phosphatases have been observed in summer and winter, while phosphorus solubilization was shown to be one of the most important processes in summer (Fan et al., 2019).

Sulfate reducers are key organisms in the decomposition of organic matter in anoxic environments because they have the highest affinity for acetate, the final product of fermentation, and recycle it to CO_2, while also releasing hydrogen sulfide (Lovley et al., 1982). Genes from sulfate reduction pathways, in particular the *apsA* (*aprA*) gene coding for adenosine phosphosulfate reductase and *dsrAB* sulfite reductase, have been proposed as bioindicators (Watanabe et al., 2016). The increased occurrence of genes from sulfate-reducing pathways is typically connected to metal pollution (Yin et al., 2015); however, the genes are also indicative of other pollutants such as microplastics (Pinnell and Turner, 2019) (Table 3).

4.2.8. Mobile genetic elements

Of the mobile elements participating in horizontal gene transfer, integrons have received attention for reacting to different types of anthropogenic pollution (Gillings et al., 2015). They were first characterized as elements disseminating antibiotic resistance; however, molecular studies have revealed that integrons occur in all environments, are able to move between species and lineages, and appear to play a general role in bacterial adaptation and genome evolution (Gillings, 2014). Furthermore, these genes reside in diverse bacterial species, often on mobile genetic elements, and are often linked to antibiotic resistance, disinfectants and heavy metals. Quantification of a relatively conserved variant of class 1 integron-integrase gene *intI1* spreading in clinical environments was successfully used in the monitoring of anthropogenic pollution. Also gene abundance can rapidly change in response to a broad range of environmental pressures, including polycyclic aromatic hydrocarbons (Wang et al., 2017) and pesticides (Dealtry et al., 2014). Both integrases *intl1* and *intl2* also increased in biofilms colonizing microplastic particles (Wang et al., 2020).

5. Microbial indices

Microbial indices based on eukaryotes are mainly focused on diatoms in streams and rivers (Rimet, 2012; Stevenson et al., 2010).

Table 4
Prokaryotic indices.

Index	Habitat	Definition	Reference
bacterial eutrophic index (BEI)	freshwater, water column	ratio of abundance of *Cyanobacteria* and *Actinobacteria*	(Ji et al., 2019)
AOA/AOB proportion	freshwater, lake surface sediments	proportion of ammonia-oxidizing archaea (AOA) and bacteria (AOB)	(Yang et al., 2016a)
bacterial community index (BCI)	freshwater, stream biofilms	based on clustering the bacterial community profiles generated by Automated Ribosomal Intergenic Spacer Analysis (ARISA)	(Lau et al., 2015)
microbial community-based index of biotic integrity (MC-IBI)	freshwater, river sediments	% FCA (percentage of *Firmicutes, Chloroflexi* and *Acidobacteria*); log 16S rRNA/ARG (log the ratio of 16S rRNA gene to antibiotic resistance gene); log AOB/AOA (log the ratio of ammonium oxidizing bacteria to ammonium oxidizing archaea); *nirK*/*nirS* (ratio of *nirK* gene to *nirS* gene); BFG/A (ratio of *Bacteroidetes, Firmicutes* and *Gammaproteobacteria* to *Alphaproteobacteria*)	(Niu et al., 2018)
microbial community-based index of biotic integrity (Ba-IBI)	freshwater, river sediments	*Bacilli, Bacteroidetes*, and *Clostridia* to *Alphaproteobacteria* (BBC/A) *Oxalobacteraceae*, methanotrophs, thermophiles	(Yang et al., 2019a)

The standardized indices are based on traditional morphological identifications (Kelly et al., 2009). Recently, eDNA-based approaches have been developed, a curated reference database has been established (e.g. Rimet et al., 2019), and up-scaling for monitoring networks has been processed (e.g. Vasselon et al., 2019). Standardization is now in progress at the European Committee for Standardization (CEN, 2018a, 2018b). However, these approaches are mostly based on existing morpho-taxonomic indices, changing only the methodology used to obtain species lists. DI-MOLTAXFREE (Apothéloz-Perret-Gentil et al., 2017), based on amplicon sequencing of 18S rRNA, is the only currently proposed taxonomy-free diatom index (Table 2).

Several types of prokaryotic indices have been proposed to date, based on abundances or proportions of selected taxa responsive to certain environmental conditions, or on taxa providing a specific function in the water column or sediment (Table 4). The indices have already been used for the identification of characteristics such as nutrient levels, urbanization intensity, land use, etc., and have successfully been correlated with those based on eukaryotic taxonomic groups such as macrophytes, macroinvertebrates or fishes. However, as different taxonomic indicators may highlight distinct stressors on aquatic systems, indicators based on different biological groups can conflict in their assessment of ecosystem health status (Horton et al., 2019).

In freshwater environments, Yang et al. (2016a) observed a difference in the proportion of ammonia-oxidizing archaea (AOA) and bacteria (AOB) in lakes differing in their trophic status, with AOB being more abundant in a hypertrophic lake. Niu et al. (2018) proposed a complex microbial community-based index of biotic integrity (MCIBI) for assessment of the ecological status of rivers based on prokaryotic communities in the sediment. The index includes five core metrics (Table 4). Among the included metrics, the highest discriminative power is provided by log AOB/AOA (log of the ratio of ammonium oxidizing bacteria to ammonium oxidizing archaea) and BFG/A (the ratio of *Bacteroidetes, Firmicutes* and *Gammaproteobacteria* to *Alphaproteobacteria*), followed by % FCA (percentage of *Firmicutes, Chloroflexi* and *Acidobacteria*). Ji et al. (2019) proposed a Bacterial Eutrophic Index (BEI) to quantitatively describe water quality, which was defined as the ratio of the relative abundance of *Cyanobacteria* and *Actinobacteria* OTUs in the water column. At the studied sites, the BEI index ranged between 0.55 and 2.65 for lakes of the lowest and the highest trophic levels, respectively. Novel indices can be proposed using a combination of the above-mentioned taxa with high bioindication values and functional genes. In such indices, general variables such as microbial diversity or the relative proportion of primary producers or their quantity (defined by chlorophyll a concentration) might be combined with more specific indicators reflecting individual functional groups. Together these may then more precisely assess the quality of an environment (Niu et al., 2018; Yang et al., 2019a).

6. Microbial communities

6.1. Responses to multiple stressors

Environmental stressors and disturbances include impacts from diffuse sources. For example, the runoff of inorganic nutrients from agriculture and other land use, natural organic compounds arising from animal husbandry or urbanized areas, or more specific compounds coming from storm-water runoff, WWTP effluents, ground water, or even underground constructions such as pipes or trenches (Hatt et al., 2004; Ibekwe et al., 2016; Lemaire et al., 2020; Wiest et al., 2018).

These stressors co-occur in ecosystems and often interact to produce effects that can be less than the sum of their individual effects (antagonistic – overall decreased stress) or greater than the sum of their individual effects (synergistic – overall increased stress) (Birk et al., 2020; Folt et al., 1999; Piggott et al., 2015b). Non-additive effects are frequent, often being the rule rather than the exception in aquatic systems (Orr et al., 2020). However, current legislation on biomonitoring and the management of aquatic ecosystems is often based on the assumption that the effects of multiple stressors are additive (Villar-Argaiz et al., 2018; Jackson et al., 2016; Crain et al., 2008), which may result in an underestimation of impacts.

Multiple stressors may depend on an organism's mode of nutrition, i.e., autotrophs may tend to respond antagonistically, while heterotrophs tend to respond more synergistically (Nuy et al., 2018). Antagonistic effects of stressors seem more common in primary producers and freshwaters, while a synergy of stressors is more common in marine environments. Synergistic responses still occur in freshwater systems, but need further investigations to ensure sufficient management (Crain, et al., 2008; Jackson et al., 2016). Antagonisms seem to be the more common stressor-induced interaction at the community level, likely due to the co-tolerance of taxa (Nuy et al., 2018; Salis et al., 2017) as a tolerance to one stressor can improve the tolerance to a second stressor that acts through similar mechanisms (Vinebrooke et al., 2004).

Many microbes are substrate-flexible and metabolize both inorganic and organic compounds of anthropogenic origin (e.g. pollutants), mitigating some of the anthropogenic impact (Kiersztyn et al., 2019). However, the degradation byproducts of pollutants can sometimes be more toxic than the original compound (Varjani et al., 2017), so specialized microbes with appropriate metabolic capabilities may be needed for their further biodegradation (Chakraborty and Das, 2016). The synergy or an-

tagonism of multiple stressors must be considered when searching for bioindicators because some stressors may produce common effects. The response of organisms to multiple stressors seems to be dependent on the environmental conditions of aquatic systems (Richardson et al., 2018). In line with this, effects of stressors on biofilm formation were found to depend on sediment and nutrient conditions (Piggott et al., 2015a). Similarly, ecosystem processes were observed to be more vulnerable to stress in naturally harsh environments usually inhabited by specialized microbes (Tolkkinen et al., 2015). Since microbes are abundant and functionally diversified, even a small change in the composition of primary producers leads to significant changes in the quality of their metabolites and exudates, and thus may cause large changes in specialized microbes. In such situations, specialized groups may be used for disentangling the combined effects of various stressors (e.g. Horňák et al., 2017).

6.2. Biotic interactions

Microbial community patterns are strongly influenced by complex interactions between biotic and abiotic factors, as well as typical doubling times of microbes of hours to days (Pernthaler, 2017; Šimek et al., 2014). Environmental and geographic factors explain only a part of the biological variation (Bock et al., 2020). For example, interactions include the bacterial incorporation of organic carbon exudated by phytoplankton (Horňák et al., 2017; Sarmento et al., 2016; Šimek et al., 2011; Woodhouse et al., 2018), or grazing by heterotrophic protists (Grossart et al., 2008; Grujčić et al., 2018; Pernthaler, 2005; Salcher et al., 2016; Šimek et al., 2013). Notably, even small changes in characteristics of the bacterial prey community have been found to rapidly cascade up to the food chain via a considerable alteration in the trophic level of protistan predator communities (Grujčić et al., 2018; Šimek et al., 2020, 2013). Moreover, heterotrophic bacteria can produce growth-promoting compounds such as vitamins and can improve nutrient supplies for phytoplankton (Cole, 1982; Croft et al., 2005). The presence and abundance of distinct species thus affects the occurrence of other species due to biotic interactions including trophic relationships, symbiosis, parasitism, allelopathy or chemical communication. Some species with similar preferences may co-occur in the same habitats or exclude each other by competition in microniches, and some replace each other by chance (Grossmann et al., 2016; Louca et al., 2018), which complicates the interpretation of co-occurrence patterns.

Due to the significance of random processes in community assemblies, multi-species and community approaches are likely to be more reproducible and more reliable for biomonitoring than single-species approaches. Assigning OTUs to trophic guilds or functional traits is a straightforward basis for tracking down groups of OTUs that have a similar bioindication value. However, such assignments are currently limited to very general traits (Ramond et al., 2019), while only a very minor fraction of amplicon diversity can be linked to more specific traits given the vast gaps in linking microbial sequences to species and species to ecology. Co-occurrence networks and related tools are indicative of interactions without an *a priori* knowledge about the species identity and ecology behind distinct OTUs (Bock et al., 2020; Steele et al., 2011). Furthermore, parameters elucidated by network analyses, such as the connectedness and the presence or absence of certain interactive links, might be strong indicators for habitat characteristics (Schloter et al., 2018).

7. Recommendations for biomonitoring

Molecular methods based on the extraction of nucleic acids or proteins from environmental samples enable a complex view of microbial communities. As a result, their composition, structure and diversity have been connected to environmental traits and stressors (Newton and McLellan, 2015; Tanentzap et al., 2019), with these measures able to be used as broad scale bioindicators.

However, even in this broad scheme, sediment or water column habitats should be sampled separately because their microbial communities differ substantially (Ibekwe et al., 2016; Zeglin, 2015). In addition, the responses to stressors differ according to long-term and short-term events. Long-term continuous impacts typically lead to a changes in community compositions (e.g. Gillan et al., 2015). The effects of combined stressors on microbial community are also clearly influenced by functional redundancy and various resistance mechanisms, which differ considerably between taxonomic and functional groups (Delgado-Baquerizo et al., 2017; Deng et al., 2020; Jacquiod et al., 2018). Thus, functions commonly provided by many taxa are less affected by the community composition. In comparison, shared resistance within related taxonomic groups may ensure group survival, which can result in differences in the resistance and resilience of various taxa, also leading to community alteration (Cabrerizo et al., 2019). Finally, different growth rates reflecting optimal conditions for non-affected taxa may further modify the resulting community composition (Pernthaler, 2017).

For more precise bioindication, the methodological approach should be selected depending on the goal, i.e. the type of assessed pollution (Achermann et al., 2020). Therefore, the expected driver(s) of environmental changes, such as water level fluctuations, eutrophication from agriculture, WWTP effluents, diffuse pollution, metals, etc. should be identified *a priori* for adequate water quality evaluation. Apart from the already-mentioned monitoring strategy, focusing on measures of community composition, structure and diversity (see introduction; strategy B), the tracking and quantification of individual microbial taxa (strategy A), or the targeting of specific functional groups or genes (strategy C), may also be used depending on the particular situation. Strategy A possibly represents the easiest modification to current routine protocols because monitoring of those organisms is already part of legislation. The other strategies can be applied to various sources of pollution bioindication as follows:

Eutrophication and diffuse pollution seem to be most appropriately monitored using the bacterial community structure in the water column. In both lakes and rivers, eutrophication leads to an increase in bacteria OTU richness (Henson et al., 2018; Ibekwe et al., 2016; Kiersztyn et al., 2019), which implies that community richness is a general bioindicator of eutrophication. The taxonomic composition of bacteria is strongly influenced by the presence of planktonic cyanobacteria, which proportionally increase with eutrophication (for European lakes, see Kiersztyn et al., 2019), while pelagic actinobacteria prefer oligotrophic habitats and decrease with eutrophication (Zeglin, 2015). This suggests the ratio between cyanobacteria and actinobacteria OTUs in open water is another community bioindicator, which has been already tested in the monitoring of some Chinese lakes (Ji et al., 2019).

Yet, only the short-term impacts of eutrophication can be appropriately determined using bacterial communities. That is due to the fact that longer exposure to eutrophication leads to the adaption of bacteria to increased nutrient levels, which stabilizes the bacterial community and the influence of eutrophication is diminished (Jacquiod et al., 2018; Kiersztyn et al., 2019). Despite this, the long-term effect of continuous eutrophication can result in loss of beta diversity (Monchamp et al. 2018).

In addition to eutrophication, augmented levels of nitrogen can be indicated using the bacterial community structure in both lakes and rivers. In particular, *Betaproteobacteriales* increase proportionally with N concentrations (Horton et al., 2019; Wan et al., 2017).

In both rivers and lakes, *Nitrospirae* seem to systematically positively correlate to increased nitrogen, not only with respect to dispersed pollution but also in response to WWTP or point source effluents, which makes them a good indicator of hidden inputs of waste water (e.g. Chu et al., 2018; Drury et al., 2013; Huang et al., 2019).

Organic pollution and compounds associated with organic matter such as pharmaceuticals and cosmetics seem to be best monitored in sediments where both natural organic compounds and anthropogenic organic pollutants accumulate, making sediments an accumulation hotspot (Jacquiod et al., 2018). In sediment prokaryotic communities, the proportion of *Actinobacteria* may be the simplest indicator of organic pollution since *Actinobacteria* are adapted to high concentrations of organic matter (Zeglin, 2015) and consistently increase with organic contamination deposited in sediments (Beale et al., 2017; Ibekwe et al., 2016). Furthermore, a decrease in richness and other diversity measures can be a measure of organic pollution, because the reduced taxonomic and potentially also functional diversity is a result of biotic homogenization arising from major pollutant discharges or through the introduction of non-indigenous bacteria (Drury et al., 2013). A more specific approach in the determination of organic pollution could use the class 1 integron integrase *intI1* gene as a marker of horizontal gene transfer, the frequency of which increases in organically polluted waters (Gillings et al., 2015).

Microbial functional groups offer an elegant *de novo* approach to biomonitoring through the quantification of individual nutrient cycling pathways. The most important are the carbon and nitrogen flows and sinks. Methane production is a key pathway in the carbon cycle and methane metabolism is correlated to organic pollution through oxygen availability. Quantification of *mcrA* and *pmoA* genes, which respectively mark methanogeny and methanotrophy, have already been applied in the Ba-IBI index (Yang et al., 2019a). Our suggestion is to add measures/proxies of primary production either by the respective genes or simply by quantifying chlorophyll a to assess the proportion of CO_2 fixation to the methane cycle as a measure of carbon flow. Since *Euryarchaeota* are the only methanogens, their proportion in the prokaryotic community may be used instead of gene quantification for the community structure-based approach. For nitrogen, the key pathways include N_2 fixation and denitrification, and thus the proportional quantity of the respective genes may represent a novel bioindicator of nitrogen flow.

Metal pollution can be determined using either community structure, particularly by the increased proportion of *Betaproteobacteriales* together with an altered proportion of other taxa (see Fig. 2), or bacteria typical for sulfur metabolism from the class *Deltaproteobacteria* (*Desulfomicrobiaceae, Desulfobacteraceae*) (Hatam et al., 2019; Liao et al., 2019). Metal pollution can also be detected by genes coding for metal homeostasis, the gene *apsA* coding for adenylyl-sulfate reductase, or other genes involved in the dissimilatory sulfate reduction pathway (Li et al., 2018) (see Fig. 2).

Pharmaceuticals, cosmetics and other anthropogenic organic molecules appear to be most challenging for bioindication, because their individual characteristics cause specific changes in microbial communities, which may be difficult to observe in complex samples. Ecological studies have revealed chronic long-term negative impacts on aquatic habitats rather than immediate toxicity (Barra Caracciolo et al., 2015). Interactions among enzymes and such pollutants have identified several groups of potentially indicative taxa and genes; however, our current knowledge remains limited (Achermann et al., 2020) for routine applications. Therefore, specific bioindication may only be possible through functional community metrics of metagenomics/metatranscriptomics (Fig. 1B).

8. Future possibilities for biomonitoring studies

Metagenomics/metatranscriptomics can predict community functions, even if the function is rare (Achermann et al., 2020). These approaches are thus promising, but relatively few reference datasets are currently available and they often lack a sufficient number of replicates (Achermann et al., 2020; Cordier et al., 2020). Consequently, we propose that the construction of large-scale spatial and temporal metagenomic/metatranscriptomic reference data sets covering habitats relevant to water quality bioindication should proceed, including references to methodologies and data storage. Using this approach, co-variations of functional genes, species and environmental variables can be identified, thus facilitating the selection of appropriate bioindicators. The focus might be placed on 1) genes indicating trophic status that participate in major nutrient pathways, particularly those that enable transitions between inorganic and organic forms (e.g. methanogens and methanotrophs, or nitrogen fixators and denitrifies); or 2) genes indicating toxic pollution, i.e., those participating in horizontal gene transfer, particularly of various resistances. Furthermore, shallow metagenomic techniques are becoming financially more efficient than multiple quantitative PCR, though the results are more complex. However, manipulative laboratory studies targeting the effects of individual and multiple stressors on various parameters of aquatic environments should also be conducted to gain additional insights into the functioning of aquatic microbial communities using metagenomics/metatranscriptomics. Finally, the identification of keystone taxa and rare community members should be included in environmental evaluations to assess aquatic ecosystem stability, long-term adaptation and the resolution of functional redundancy (Herren and McMahon, 2018; Pernthaler, 2017; Poursat et al., 2019).

A further recommended approach to improve the application of environmental genomics-based bioindication by microorganisms is to introduce novel bioinformatic tools. Firstly, network analyses can identify taxa with similar habitat requirements and/or interacting taxa (Banerjee et al., 2016; Bock et al., 2020), and also establish sets of sequence variants indicative of certain habitat characteristics, similar to the current use of indicator morphospecies in, e.g., macrozoobenthos or diatoms. Similarly, supervised machine learning is currently the best solution to classification problems involving multidimensional and noisy datasets (Libbrecht and Noble, 2015), and its use could significantly improve the reliability of microbial community patterns analyses (Cordier et al., 2020, 2019). The application of such techniques requires training datasets, for which model metagenomic/metatranscriptomic studies are most useful because of the unbiased quantification of both community structure and the presence/activity of bioindicating metabolic pathways.

9. Outlook: practical applications

The still-incomplete list of case studies presented in this review highlight the immense added value of microbial indicators for environmental monitoring and process understanding in freshwater ecosystems. While for many important stressors the pressure-response functions are well-known, a profound understanding of the indication value of microbial taxa in the context of novel and multiple stressors is still in need of further validation prior to inclusion in formal regulatory biomonitoring programs. Such method plausibility checks and data validation are needed in the context of larger transdisciplinary networks. Based on the derived results, formal implementation can be initiated. Here, case studies should target long-term ecological monitoring sites (e.g. Haase et al., 2018; Mirtl et al., 2018), routine monitoring sites of regulatory monitoring programs, as well as operational monitoring sites from e.g.

sewage plants or aquacultures (e.g. Pawlowski et al., 2016a, 2014). For such sites, a wealth of information on other environmental parameters, in particular stressors, typically exist. This creates a unique set of metadata to fuel synergies when it comes to developing novel diagnostic tools as part of so-called weight-of-evidence approaches (e.g. Höss et al., 2011; Wolfram et al., 2012).

Besides such coordinated approaches to derive indicators, formal standardization is of critical importance. Scientific standards of microbial community analysis using high-throughput amplicon sequencing have previously been proposed e.g. for soil samples within the Earth Microbiome project (https://earthmicrobiome.org) including DNA isolation (Marotz et al., 2017; Minich et al., 2018), amplification and sequencing (Caporaso et al., 2012, 2011), and for marine water column samples (Jeunen et al., 2019). However, when considering the application of novel indicators into future regulatory monitoring, formal standardization e.g. via the International Organization for Standardization (ISO) or (in parallel) the European Committee for Standardization (CEN CENELEC) is a pivotal step. This starts with decisions on where and how to sample, because great variability exists due to seasons and various unexpected point sources of contaminated water (Lemaire et al., 2020; Meziti et al., 2019; Wan et al., 2017; Yao et al., 2018). Here, however, traditional standards for sampling and propositions for novel eDNA based methods are in the process of development (CEN/TC 230/WG 28).

With respect to laboratory analysis, it is known that the reproducibility of molecular techniques largely depends on the quality and representativeness of environmental DNA including cell lysis and purification steps. Furthermore, the quantification of marker genes by real-time qPCR are especially sensitive to template eDNA quality including fragmentation and the presence of inhibitors, which may be site and sample-specific (Hargreaves et al., 2013). Many formal standards for traditional biomontioring programs exist and are intercalibrated. The same holds true for medical, forensic or classical ecotoxicological analyses. For example, ISO 11063 describes standards for DNA extraction for assessing microbial abundance and community structure in soils, and ISO 22119 on procedures for qPCR validation of food-borne pathogens.

In addition to field and lab standards, the standardization of bioinformatic pipelines will be also needed to achieve interlaboratory comparability of results, as existing pipelines have been shown to provide different outputs (Bailet et al., 2020). By co-extracting variable types and amounts of inhibitory substances, limitations may be diminished by using digital-droplet PCR (ddPCR) (Dingle et al., 2013; for details see Methodological annex). Again, a comparability of results among laboratories requires rigorous standards, which may be inspired by the proposed minimum information for the publication of quantitative PCR experiments for real-time PCR (Bustin et al., 2011) and ddPCR (Huggett et al., 2013).

10. Conclusions

In conclusion, our review demonstrates that the inclusion of microbial bioindicators can provide improved assessments of water quality, including both general and specific stressor diagnostics. Although bioindicators of processes such as eutrophication have already been established by other approaches, the use of microbes, and prokaryotes in particular, represents not only a monitoring tool but also a path toward the deeper understanding of processes underlying water quality parameters. Using microbes for the regular observation of major nutrient pathways and recycling allows the quantification of flows and sinks in the biosphere over long time periods. Finally, studies on microbes have proposed various bioindicators that can be used to create biotic indices, specifically targeting the most important stressors in various regions and habitats. The indices developed so far have shown that a combination of taxon-dependent markers with functional markers is possible, which makes them more powerful than currently-used traditional indices based only on the identification of taxa. Considering all currently available results, we advise that decisions be made towards including microbes, with their high growth and community dynamics, in biomonitoring programs of freshwater environments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The work is the result of DNAqua-Net workshop supported by European Cooperation in Science and Technology (COST) grant CA15219 (DNAqua-Net). MSM and AS were supported by Ministry of Education, Youth and Sports of the Czech Republic, European Regional Development Fund-Project CZ.02.1.01/0.0/0.0/16_019/0000845 and LTC20063. AB and TC were funded by OFB (Office Français de la Biodiversité). JK was supported by Ministry of Agriculture of the Czech Republic, grant RO0418. PN and KP were supported by Estonian Research Council grants PUT PRG709, PUT PRG705 and base financed project P190250PKKH of Estonian University of Life Sciences. SO was partially supported by the project STIM – REI, Contract Number: KK.01.1.1.01.0003, a project funded by the European Union through the European Regional Development Fund – the Operational Programme Competitiveness and Cohesion 2014–2020 (KK.01.1.1.01).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116767.

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