

## Minireview

# High-throughput molecular analyses of microbiomes as a tool to monitor the wellbeing of aquatic environments

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## Summary

**Aquatic environments are the recipients of many sources of environmental stress that trigger both local and global changes. To evaluate the associated risks to organisms and ecosystems more sensitive and accurate strategies are required. The analysis of the microbiome is one of the most promising candidates for environmental diagnosis of aquatic systems. Culture-independent interconnected meta-omic approaches are being increasing used to fill the gaps that classical microbial approaches cannot resolve. Here, we provide a prospective view of the increasing application of these high-throughput molecular technologies to evaluate the structure and functional activity of microbial communities in response to changes and disturbances in the environment, mostly of anthropogenic origin. Some relevant topics are reviewed, such as: (i) the use of microorganisms for water quality assessment, highlighting the incidence of antimicrobial resistance as an increasingly serious threat to global public health; (ii) the crucial role of microorganisms and**

**their complex relationships with the ongoing climate change, and other stress threats; (iii) the responses of the environmental microbiome to extreme pollution conditions, such as acid mine drainage or oil spills. Moreover, protists and viruses, due to their huge impacts on the structure of microbial communities, are emerging candidates for the assessment of aquatic environmental health.**

## Introduction

Freshwater and marine ecosystems are subjected to many drivers of environmental changes. In most cases, they are associated with human activities, which also result in global changes, such as an increase in temperature, acidification, salinity or reduction of the oxygen content. In other cases, the changes at local or regional scales are a consequence of the human population growth and the increasing use of littoral areas. Impacting human activities have risen especially in coastal and shelf areas, which are the most productive areas of the seas (Ramírez *et al.*, 2018). One of the main consequences of the human activities is the release to the ocean and coastal areas of chemical stressors such as persistent organic compounds (including PAHs, PCBs and pesticides), metals and emerging pollutants (e.g. pharmaceuticals -antibiotics, anti-inflammatory, anti-depressives-, personal care products, nanomaterials, micro-/nano-plastics, marine biotoxins; Sauvé and Desrosiers, 2014; Geissen *et al.*, 2015). Due to lack of sanitation or to inefficient treatments, a high proportion of pollutants (e.g. antibiotics) from urban or industrial wastewater treatment plants (WWTP) are directly discharged into aquatic systems (Geissen *et al.*, 2015).

Given the thousands of chemicals released into the aquatic environment, major efforts are necessary to evaluate the associated risks to organisms and ecosystems (Fernández-Cisnal *et al.*, 2017). Additionally, in the natural environment, pollutants are not found individually, and they occur as complex mixtures of hazardous interacting substances. The action of these compounds should thus be assessed considering their cumulative effects and interactions (European Marine Board, 2019).

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Besides chemical stress, multi-stress related to other environmental issues, many of them as result of climate change (e.g. acidification, increase in temperature and UV), can jointly affect the resiliency and health of aquatic ecosystems (Holmstrup *et al.*, 2010; Bejaoui *et al.*, 2020). To assess the impact of pollution and other sources of environmental stress, a relation between the occurrence and the effect should be measured. Aquatic ecotoxicological studies need to find appropriate model organisms (used as biomonitors) that respond sensitively to complex environmental threats (Zhou *et al.*, 2008; Gago-Tinoco *et al.*, 2014; Parmar *et al.*, 2016; Fernández-Cisnal *et al.*, 2017). Invertebrates, fish, mammals and plants have been usually used as bioindicator organisms of pollution (Burger, 2006; Zhou *et al.*, 2008; Li *et al.*, 2019).

The analysis of the microbiome is one of the most promising candidates for environmental diagnosis of aquatic ecosystems due to: (i) the ubiquitous presence of microbial organisms in any environmental compartment, (ii) their direct and immediate contact with the pollutants and other stressors, (iii) their sensitive response to environmental stresses expressed by changes in their structure, diversity and functional activity, which affects matter and flow energy in the ecosystems, and (iv) the ongoing increasing availability of high-throughput techniques to elucidate those changes in both the microbial community structure and its metabolic patterns (Caruso, 2013; Bouchez *et al.*, 2016; Wang *et al.*, 2016).

Use of microbial parameters to monitor environmental pollution started in the 80s, mainly to analyse the quality of drinking water, as most aquatic pollution was produced by unprocessed faecal discharges or uncontrolled effluents from WWTP (Bae and Park, 2014). In fact, the quality of drinking water is routinely monitored by the determination of enterobacteria (Caruso, 2013; Jang *et al.*, 2017; Gorski *et al.*, 2019). Traditionally, bacteria levels in such waters have been quantified by the cultivation of samples under laboratory conditions or even microscopic counting, but these methodologies, although sensitive, are relative slow and do not allow for a quick detection and, thus, an immediate response to remediate the pollution events (Storey *et al.*, 2011; Caruso, 2013; Gorski *et al.*, 2019). Several bioassays to measure pollution have been developed based on the changes in physicochemical parameters triggered by one or multiple toxic chemicals. Common determinations include O<sub>2</sub> consumption/CO<sub>2</sub> production, bioluminescence, activity of microbial enzymes, or the transformation of elements such as carbon, sulphur, or nitrogen (Tothill and Turner, 1996; Bae and Park, 2014; Hassan *et al.*, 2016). Whole-cell biosensors devices have been designed to detect the presence of biological analytes, including pollutants, using living organisms that respond in some measurable

and predictable way. Although these devices are being used widely to determine aquatic pollution, they are not pollutant-specific, and their determinations can be biased by the presence of growth inhibitory chemicals in the samples or by the microbial biosensor profile (OECD, 2010; Hassan *et al.*, 2016; Gorski *et al.*, 2019). Recent methodological innovations to enumerate enteric pathogens in water include sensitive nucleic acid-based methods, being quantitative PCR (qPCR) the most often used. Nevertheless, these methodologies also have their drawbacks, the main one being the detection of false positives due to the persistence of dead cells or naked DNA in the water samples. Finally, metagenomics has significant potential for bacterial quantification, although the application of this technique for water testing is still under development (Gorski *et al.*, 2019).

One of the main issues in ecotoxicology is the development and use of much more sensitive tools for the in-field monitoring of the impact and sublethal effects of pollutants and other sources of environmental stress. Advances in this area are undoubtedly related to the technical progress of specific methodologies. Next, we will review the use of advanced molecular methodologies, particularly those designed to fill the gaps that classical microbial approaches cannot resolve, to assess the main drivers and pressures on aquatic ecosystems.

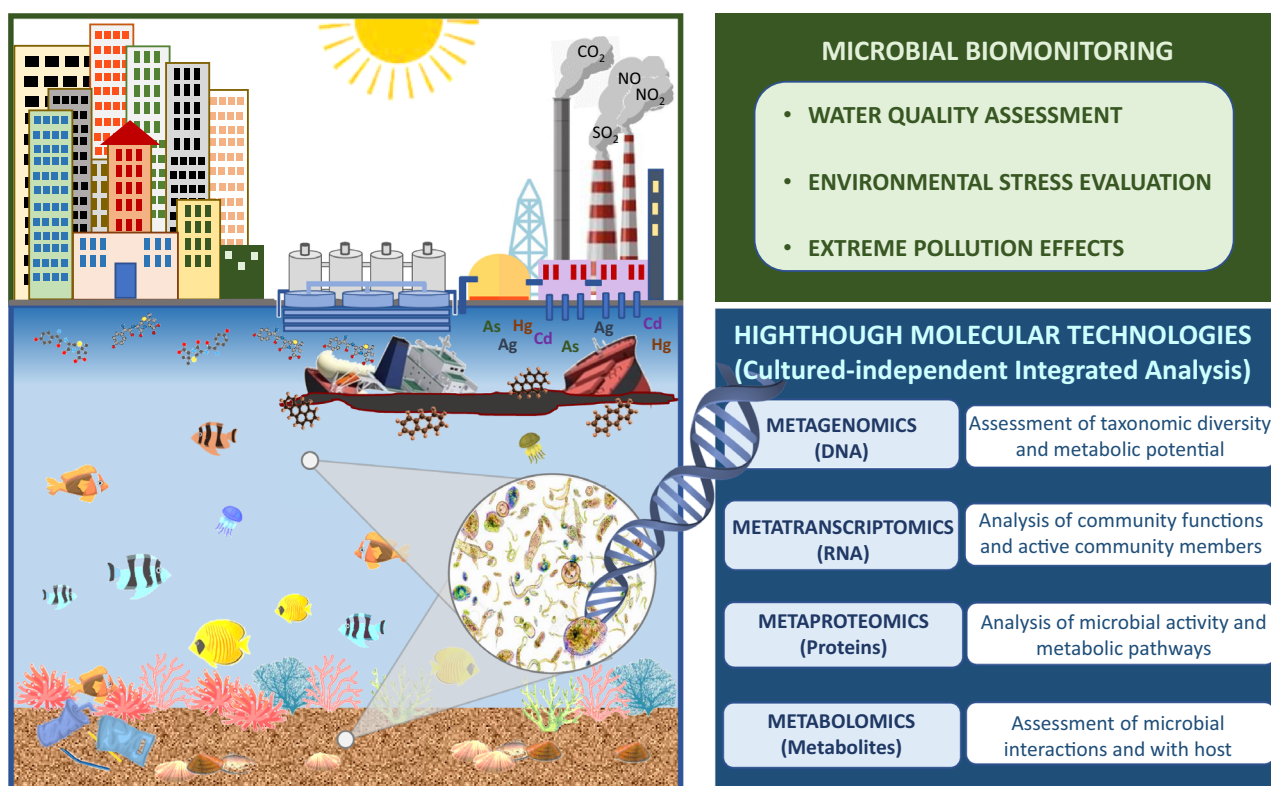
### Application of high-throughput molecular microbiological tools

Considering that uncultured microorganisms represent the great majority of the planet's biodiversity, culture-independent methods are essential to understand the genetic diversity, population structure, metabolic activities, and environmental roles of microbes (Riesenfeld *et al.*, 2004; Lloyd *et al.*, 2018). In this sense, the emergence and increasingly extensive use of new high-throughput sequencing (HTS) technologies, together with the rapidly growing of metagenomic sequence data from different environments, and the application of new user-friendly bioinformatics and statistical tools, are facilitating our understanding of environmental microbial communities (Riesenfeld *et al.*, 2004; Glöckner and Joint, 2010; Heidelberg *et al.*, 2010; Delmont *et al.*, 2011; Barberán *et al.*, 2012; Neelakanta and Sultana, 2013; Coutinho *et al.*, 2015; Zuñiga *et al.*, 2017; Coutinho *et al.*, 2018; Tangherlini *et al.*, 2018). For instance, when comparing the diversity and distribution of 77 metagenomes, the microbial profiles from different ecosystems (e.g. oceans, coral atolls, deep oceans, Antarctic aquatic environments, Arctic snows, soils, hypersaline sediments, sludge, microbial cell biofilms, acid mine biofilms, polluted air and animal microbial populations) were found to be clearly different (Delmont *et al.*, 2011). Thus,

environmental responses can be evaluated by comparing the metagenomes from different conditions, for example their involvement in, and response to, climate change and environmental stress, their response to pollutants and biodegradation capacities, as well as the risks they represent to human health.

The integration of culture-independent, interconnected omic approaches can provide detailed information concerning: how microbial communities assemble and interact, their predominant metabolic activities, their progress over time, and their responses to environmental perturbations (Fig. 1; Ram *et al.*, 2005; Simon and Daniel, 2011; Hettich *et al.*, 2012; Zarraindia *et al.*, 2013; Franzosa *et al.*, 2015; Tan *et al.*, 2015). While metagenomics identifies the potential function of a microbial community in the environment, metatranscriptomics determines which microbes are active and which genes are transcribed, while metaproteomics informs of which proteins are actually expressed; finally, metabolomics may be used to evaluate changes in metabolic fluxes and metabolite levels which are the final result of complex interactions (Wilmes and Bond, 2009; Siggins *et al.*, 2012; Zarraindia *et al.*, 2013; Wang *et al.*, 2014; Aguiar-Pulido *et al.*, 2016; Herbst *et al.*, 2016; Wang *et al.*, 2016; Zuñiga *et al.*, 2017).

Below we provide a prospective view of the increasing application of high-throughput molecular technologies to evaluate the structure and activity of microbial communities in response to changes and disturbances in the environment, mostly of anthropogenic origin (Fig. 1). In relation to this, recently, the Microbiome Stress Project, an open access database of environmental and host-associated 16S rRNA, has collected cross-study analyses of microbial community responses to stressors (Rocca *et al.*, 2019). Here, we address the application of HTS technologies to microbial water quality assessment, highlighting the incidence of antimicrobial resistance as an increasingly serious threat to global public health. Next, we review approaches trying to evaluate the crucial role of microorganisms and their complex relationships with the ongoing climate change, and other environmental stress conditions. Finally, we will assess how the environmental microbiome responds to extreme pollution conditions, such as acid mine drainage or oil spills. The unprecedented advances in these areas represent a valuable source of information that is increasingly being incorporated into the improvement of bioprocesses and environmental engineering applications (e.g. wastewater treatment, bioremediation) to achieve a more sustainable environment (Czaplicki and Gunsch, 2016; Techtmann and Hazen, 2016). Table 1 includes a bibliographic



**Fig. 1.** Application of high-throughput molecular technologies for the microbial biomonitoring of aquatic environments.

compilation of the application of HTS technologies to microbial water quality assessment.

*Water quality assessment: antimicrobial resistance as a global threat to public health*

The prevalence and dissemination of antimicrobial resistance (AMR) is an increasingly serious threat to global public health (WHO, 2015; Qiao *et al.*, 2018; Thomas *et al.*, 2020), that has turned into an emergency in low-income countries (Bastarud *et al.*, 2020). Consequently, a significant reduction in the effectiveness of antibiotics and other antimicrobial agents (antifungals, antivirals, antimalarials, and anthelmintics) threatens our ability to treat common infectious diseases, resulting in prolonged illness, disability, and death (Wright, 2010; Almakki *et al.*, 2019). Over the past two decades, the misuse and overuse of antimicrobials have accelerated this process (Almakki *et al.*, 2019). Antibiotics are poorly metabolized by humans and animals and are readily excreted, entering the environment through wastewater and manure (Qiao *et al.*, 2018). Several studies have reported on the co-selection of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in contaminated environments (Almakki *et al.*, 2019; Chen *et al.*, 2019; Thomas *et al.*, 2020). Antibiotic production plants, livestock farms and aquaculture, and discharges from wastewater treatment plants (WWTP) contribute to the increase of ARBs and ARGs reported in aquatic environments (Qiao *et al.*, 2018). Thus, ARGs have been detected in natural water bodies and drinking water, soils and even deep ocean sediments (Yang *et al.*, 2014), and their increased prevalence as a result of human activities has led to their characterization as an emerging environmental contaminant of growing concern (Pruden *et al.*, 2006; Yang *et al.*, 2014). Table 2 contains a compilation of the ARGs detected in WWTP, showing that genes encoding resistance to all classes of antibiotics are widespread in influent, effluent and activated sludge of WWTP. A detailed review of available literature on the methods of analysis of the different genes is included in Pazda *et al.*, 2019. The state of the art of the environmental antibiotic resistance in China has also been recently reviewed (Qiao *et al.*, 2018). DNA-based techniques are being increasingly used to detect and quantify resistant genes in environmental samples. Compared to the former, amplification-based approaches (PCR, qPCR), which target a limited number of primers-available ARGs, HTS-based analysis surveys a more complete metagenomic profiling of ARGs in environmental samples (Qiao *et al.*, 2018; Sukhum *et al.*, 2019). The development of annotated and manually curated databases of ARGs (e.g. ARBD, CARD, SNC-ARBD) has allowed for the rapid screening of metagenomic data sets for resistance

(Yang *et al.*, 2014; Li *et al.*, 2015; Sukhum *et al.*, 2019). Thus, comparative metagenomics has shown the spread of ARGs in river environments because of the selective pressure resulting from antibiotic use (Yang *et al.*, 2014; Chen *et al.*, 2019). Anthropogenic activities contributed > 93% for sulphonamide, beta-lactam, and aminoglycoside ARGs (Chen *et al.*, 2019). Shotgun metagenomics also showed a higher abundance of ARGs in the Lake Michigan sediments identical to those present in the effluents of two different WWTP that discharge into the lake; most abundant genes (i.e., *strA*, *dfrE*, *acrB*, *adeJ*, *mexB* and *semeE*) were attributed to organisms belonging to *Helicobacteraceae* (*Helicobacter*), *Legionellaceae* (*Legionella*), *Moraxellaceae* (*Acinetobacter* and *Moraxella*), and *Neisseriaceae* (*Neisseria*) (Chu *et al.*, 2018). An HTS-based metagenomic approach was also used to investigate the wide-spectrum profiles of ARGs in 50 samples from 10 environments. Network analysis showed that the trend of total ARGs abundances matched well with the levels of anthropogenic impacts on the different environments. Based on the co-occurrence pattern revealed by network analysis, *tetM* and aminoglycoside resistance protein were proposed as indicators to evaluate the quantity of other 23 co-occurring ARGs in multiple environmental samples. Five bacterial genera (*Blautia*, *Clostridium*, *Enterococcus*, *Bacteroides* and *Escherichia*) and one archaea (*Methanobrevibacter*) were speculated as the possible ARGs host (Li *et al.*, 2015).

Metagenomic sequencing has shown that mobile genetic elements-associated ARGs dominated the resistome in WWTP (Chu *et al.*, 2018; Che *et al.*, 2019; Ju *et al.*, 2019; Pazda *et al.*, 2019). Thus, by combining Oxford Nanopore and Illumina sequencing it was shown that most of the ARGs detected in WWTP were carried by plasmids, and integrative and conjugative elements (ICEs). Particularly, *tetA* and *sul1* genes carried by plasmids, and *cfxA*, *mefA/E*, *tetQ* and *tetM* genes carried by ICEs, had a persistent prevalence. Remarkably, four potential antimicrobial-resistant pathogens (i.e. *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*), harbouring a high diversity of ARGs, were identified (Che *et al.*, 2019). Conventional biological treatment processes, such as activate sludge and anaerobic digestion, do not only no efficiently reduce ARGs, but these can even proliferate throughout the biological treatment stages and their abundance increase in the effluent (Rizzo *et al.*, 2013; Yang *et al.*, 2014; Mao *et al.*, 2015; Rafrat *et al.*, 2016; Ju *et al.*, 2019; Pazda *et al.*, 2019; Yin *et al.*, 2019). WWTP are thus recognized as hotspots of vertical and horizontal gene transfer of ARGs (Che *et al.*, 2019; Pazda *et al.*, 2019). The amount of ARBs and ARGs discharged by WWTP into the environment

**Table 1.** Most relevant examples of the application of high-throughput molecular technologies to microbial water quality assessment.

Quality assessment tasks	HTS technologies used	Main goals	References
Antimicrobial resistance	Metagenomic (Illumina sequencing) analysis	The trend of the total ARG abundances in different environments matched well with the levels of anthropogenic impacts WWTP effluents substantially influence on the dispersal of microorganisms and ARGs in receiving aquatic sediments In WWTP, removal efficiency of ARGs in anaerobic sludge treatment is much less efficient than the sewage treatment	Li <i>et al.</i> (2015) Chu <i>et al.</i> (2018) Yang <i>et al.</i> (2014)
	16S rRNA, metagenomic & metatranscriptomic (Illumina sequencing) analyses	Conventional treatment process within WWTP strongly influences resistance genes and their transcriptional activities	Ju <i>et al.</i> (2019)
	Metagenomic (Oxford Nanopore & Illumina sequencing) analyses	Most ARGs detected in all compartments of WWTP are carried by plasmids, which have a significant role in facilitating the survival and persistence of multidrug resistant bacteria	Che <i>et al.</i> (2019)
Relationships with climate change and environmental stress	Metagenomic (Sanger shotgun sequencing) and metaproteomic (MS/MS) analyses	Metabolic components (nitrification, anaerobic ammonium oxidation, denitrification, and inorganic C fixation) were differentially expressed in ocean OMZ, and covaried with ubiquitous microbes	Hawley <i>et al.</i> (2014)
	16S rRNA & metagenomic (Illumina sequencing) analyses	Free-living microbial communities in coral reefs have a high potential to infer environmental parameters due to their environmental sensitivity and predictability Microbiome flexibility facilitates rapid responses of corals to environmental changes (e.g. high temperature) and increases stress tolerance	Glasl <i>et al.</i> (2019) Ziegler <i>et al.</i> (2017)
		Corals microbiota plays a critical role in the defence of their host against pathogens	Grottoli <i>et al.</i> (2018)
	Metagenomic (FLX Titanium bar-coded pyrosequencing) analysis	Reduced pH and oil contamination can adversely affect the structure and functioning of sediment benthic communities, including bacteria	Coelho <i>et al.</i> (2015, 2016)
	qPCR, 16S rRNA & metagenomic (Illumina sequencing) analyses	Marine benthic microorganisms are susceptible to changes in ocean carbonate chemistry and seawater temperature	Currie <i>et al.</i> (2017)
	16S rRNA & metagenomic (FLX pyrosequencing) analyses	Exposure to oil-contaminated sediments led to distinct shifts in commensal bacterial population structures in the gill and intestine of the southern flounder fish	Brown-Peterson <i>et al.</i> (2015)
	Metagenomic (Illumina sequencing) analysis	The endogenous xenobiotic metabolism of the Manila clam hepatopancreas is impacted by its microbiome, which is a key component in the host response to chemical stress	Milan <i>et al.</i> (2018)
	16S rRNA & metagenomic (Ion Torrent sequencing) analyses	TiO <sub>2</sub> nanoparticles shift the haemolymph microbiome composition of the bivalve <i>Mytilus galloprovincialis</i> , which may affect its immune system	Auguste <i>et al.</i> (2019)

Table 1. (Continued)

Quality assessment tasks	HTS technologies used	Main goals	References
Responses to extremely polluted environments	Bar-coded 16S rRNA & metagenomic (GS-FLX Titanium pyrosequencing) analyses	Solution pH is the major factor explaining microbial community differences in AMD environments. Microbial communities develop a critical strategy of stress adaptation to highly acidic/metal-rich conditions, by increasing the expression of genes involved in many key functions	Kuang <i>et al.</i> (2013, 2016)
	Metagenomic & metatranscriptomic (sequencing) analyses	Gene transcriptional profiles of microorganisms, as response and adaptation mechanisms, are closely related to the physicochemical characteristics of AMD sites	Chen <i>et al.</i> (2015)
	Bar-coded 16S rRNA, metagenomic & metatranscriptomic (sequencing) analyses	In prokaryotes, multiple strategies for resource acquisition and energy generation, and mechanisms of adaptation and response to AMD environmental stress, are revealed.	Hua <i>et al.</i> (2015)
	16S rRNA, metagenomics & metatranscriptomic (454 GS-FLX Titanium sequencing) analyses	The identity of metabolically active microbes from DWH oil spill and their roles in petroleum consumption are revealed	Rivers <i>et al.</i> (2013)
	16S rRNA & metagenomic (Illumina sequencing) analyses	Surface sediment-associated microbes can account for rapid depletion of aliphatic and simple aromatics of the DWH oil spill, while PAHs are more recalcitrant to degradation After DWH oil spill, early microbial responders which degrade AHs were replaced by populations capable of PAHs decomposition, and at longer times a typical beach community was re-established	Mason <i>et al.</i> (2014) Rodriguez-R <i>et al.</i> (2015)

Used abbreviations: AHs, aliphatic hydrocarbons; AMD, acid mine drainage; ARGs, antibiotic resistance genes; DWH, Deepwater Horizon; HTS; high-throughput sequencing; OMZ, oxygen minimum zones; PAHs, polycyclic aromatic hydrocarbons; WWTP, wastewater treatment plants.

depends on the bacterial biomass remaining in the final effluent (Ju *et al.*, 2019). Thus, urgent measures are necessary to limit the use of antimicrobials in medicine and animal production, and to increase the efficiency of wastewater treatments (Rizzo *et al.*, 2013; Pazda *et al.*, 2019). In that regard, more advanced technologies such as Advanced Oxidation Processes (AOPs) have been developed to effectively degrade antibiotics and improve the removal of ARBs and ARGs in WWTP (Alexander *et al.*, 2016; Pazda *et al.*, 2019; Rodriguez-Chueca *et al.*, 2019; Shen *et al.*, 2019; Wang and Zhuan, 2020).

Several studies have shown that heavy metal contamination can play an important role in the proliferation of antibiotic resistance by inducing ARGs, and metal-resistant genes (MRGs), via co-selection mechanisms (Seiler and Berendonk, 2012; Mao *et al.*, 2015; Li *et al.*, 2017; Ju *et al.*, 2019; Thomas *et al.*, 2020). Thus, metagenomic analysis, using 16S rRNA gene amplicon sequencing, showed significant differences in the relative abundance and diversity of certain ARGs (and MRGs) in metal/radionuclide contaminated soils (Thomas *et al.*,

2020). Recently, flow cytometry and 16S rRNA sequencing have shown that silver nanoparticles (AgNPs) clearly affect the microbial community structure and suggest a potential negative impact on WWTP functions (Guo *et al.*, 2019). Finally, given the increased presence of microplastics as aquatic emerging water pollutants, their role as potential vectors for harmful microbes and propagation of ARBs and ARGs is a matter of growing concern (Bastarud *et al.*, 2020; Song *et al.*, 2020; Zhang *et al.*, 2020).

#### *Microbiome relationships with global climate change and environmental stress*

Global climate change due to human-induced stressors (e.g. increased sea surface temperature, ocean acidification and reduced oxygen content, increased ultraviolet radiation, reduced salinity) has the potential to affect marine ecosystems adversely (Coelho *et al.*, 2013; FAQ, 2017; Cavicchioli *et al.*, 2019). Different microbial components respond with different sensitivity to changes in

**Table 2.** Antibiotic resistance genes detected in wastewater treatment plants (Compiled from Rizzo *et al.*, 2013; Pazda *et al.*, 2019).

Antibiotic class	Mechanism of action	Mechanism of bacterial resistance	Gene name <sup>a</sup>
Aminoglycosides	Inhibiting protein synthesis	Drug modification by adenylation Drug modification by phosphorylation	<i>aac</i> [A1, A2, A13, B] <i>aph</i> [A, A-3, A-6, 2]; <i>str</i> [A, B]; <i>strB</i>
Beta-lactams	Interfering with the bacterial cell wall biosynthesis by inactivation of penicillin-binding proteins	Penicillin-binding protein Inactivation by beta-lactamases (Drug degradation)	<i>mecA</i> <i>amp</i> [C, F]; <i>bla</i> [CIT, CMY, CTX, FOX, GES, IMP, NPS, OXA, PER, PSE, SHV, TEM, TLA, VEB, VIM]; <i>cfx</i> [A, A3]
Glycopeptides	Interfering with cell wall biosynthesis	Produces a modified peptidoglycan pentapeptide	<i>vanA</i>
Macrolides	Inhibiting early stages of protein synthesis (binding to the 50S bacterial ribosome subunit)	Methylation of 23S rRNA target site Cleavage of the drug lactone ring Inactivation of macrolide phosphotransferases	<i>erm</i> [A, B, C, F, O] <i>ere</i> [A, A2, B] <i>mph</i> [A, B], <i>BM</i> <i>mef</i> [A, E]; <i>mel</i> ; <i>msrA</i>
Quinolones	Inhibiting bacterial DNA replication enzymes (DNA gyrase & topoisomerase IV)	Active pumping of the drug Modification of target enzymes DNA gyrase and topoisomerase IV protection	<i>gyr</i> [A, B]; <i>parC</i> <i>qnr</i> [A, A3, B, B1, B2, B4, B5, D, S, S1, S2, VC] <i>aacA6-ib-cr</i>
Sulphonamides	Inhibiting the dihydropteroate synthase, enzyme involved in the folic acid synthesis	Modification by acetylation Competitive inhibitors of dihydropteroate synthase, enzyme involved in folic acid synthesis	<i>su</i> [1, 2, 3, A]
Tetracyclines	Inhibiting bacterial protein synthesis (binding to the 30S ribosomal subunit and preventing the aminoacyl tRNA association)	Ribosome protection Drug export by membrane associated proteins	<i>ortA</i> , <i>tet</i> [B(P), M, O, Q, S, T, W, X] <i>tet</i> [A, A(C), A(P), B, C, D, E, G, H, J, K, L, V, Y, Z, 31, 35, 36, 39]
Trimethoprim	Inhibiting Dihydrofolate reductase (DHFR)	Enzymatic inactivation of the drug Increased synthesis of DHFR	<i>tetX</i> <i>dhfr</i> [II, V, XIII, 13, 16, 17, A19, B2, A20, A3, A12, A13, A21, A22, A33, B1, B5, B6, B8, D, E]; <i>dhfr</i> [V, VII, VIII, IX, XII, XV, A1, A14]
Multidrug	Multidrug resistance	Accumulation of many genes encoding resistance to a single drug and multidrug efflux pumps operation	<i>acr</i> [B, D]; <i>ade</i> [A, J]; <i>amrB</i> ; <i>mdt</i> [F, G, H, N, O]; <i>mex</i> [B, D, F, I, W, Y]; <i>norM</i> ; <i>orf11</i> ; <i>qac</i> [B, EA1, EA1-01, F, G2, H]; <i>sedY</i> ; <i>sme</i> [B, E]

a. For simplicity, gene variations are presented within square brackets.

ocean temperature, oxygen concentration, pH and food supply, providing evidence that global change will affect different groups of microorganisms differentially (Danovaro *et al.*, 2016; Currie *et al.*, 2017; Danovaro *et al.*, 2017). Comparative analysis of bacteria and archaea based on qPCR allowed to conclude that climate change will primarily affect deep-sea benthic archaea, with important consequences concerning global biogeochemical cycles (Danovaro *et al.*, 2016).

Climate-induced warming of the upper ocean is contributing to the reduction of the oxygen content of the global ocean and to the expansion of oxygen minimum zones (OMZ) (Ulloa *et al.*, 2012; Hawley *et al.*, 2014; Khan *et al.*, 2018). Multi-omic molecular approaches have shown that diverse microbial communities are contributing there to major losses of fixed nitrogen and the production of climate active greenhouse gases (Ulloa *et al.*, 2012; Hawley *et al.*, 2014; Hawley *et al.*, 2017). The combined application of metagenomics and metaproteomics showed that the expression of metabolic pathway components for nitrification, anaerobic ammonium oxidation (anammox), denitrification, and inorganic carbon fixation was differentially expressed in these

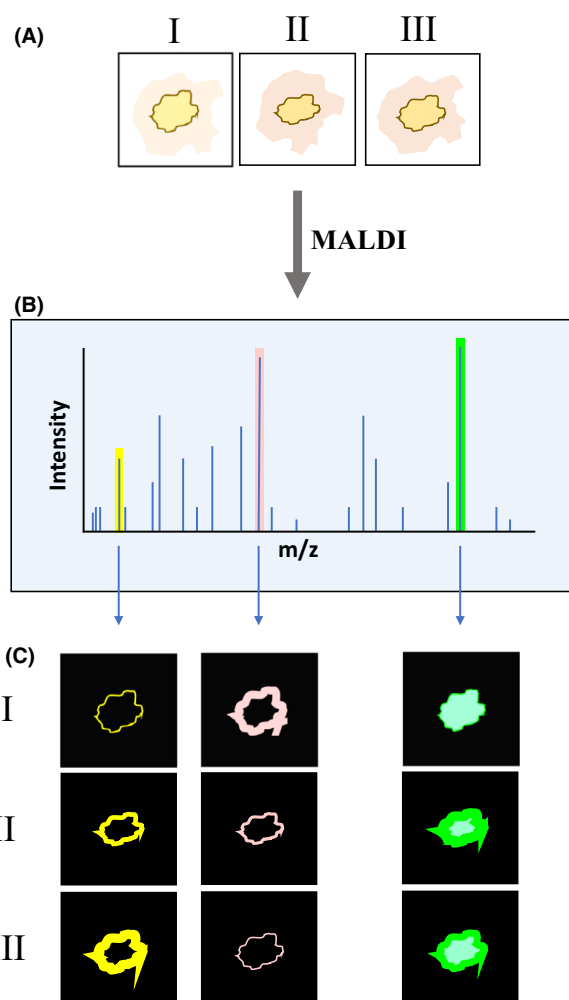
areas, and covaried with ubiquitous OMZ microbes (e.g. *Thaumarchaeota*, *Nitrospina*, *Nitrospira*, *Planctomycetes*; Hawley *et al.*, 2014).

In a climate change scenario, increased sea surface temperatures and ocean acidification also contribute significantly to the ongoing decline of coral reef ecosystems. Increasingly serious consequences are predicted, given that coral reefs are among the most biologically diverse and economically important ecosystems (Hoegh-Guldberg *et al.*, 2007; Sharp and Ritchie, 2012). Within corals, microbes play a critical role, ultimately governing the overall health and resilience of these reef systems and influencing their response to environmental changes (Sharp and Ritchie, 2012; Chiarello *et al.*, 2020). HTS methods have allowed the study of the composition of coral microbiomes, its spatial-temporal variability, plus its role in corals' health and in response to environmental changes, in a broad range of coral specimens (Sharp and Ritchie, 2012; Hernandez-Agreda *et al.*, 2017). Thus, recently, a comprehensive microbial database of multiple coral reef microbiomes was generated by 16S rRNA gene sequencing. When this database was coupled to environmental parameters, seawater



microbiomes were shown to be good indicators since they respond in a very sensitive and predictable way to the environmental perturbations (Glasl *et al.*, 2019). Network analysis of metagenomic data has shown that microbial species commonly found in corals play potential roles in host nutrient metabolism, carbon, nitrogen and sulphur cycles, and host detoxification (Wegley *et al.*, 2007; Sharp and Ritchie, 2012; Cai *et al.*, 2018). The response to environmental changes differs according to the composition of the microbial community and suggests a key role of the microbiome in the acclimatization/adaptive responses of the coral host to the stress conditions (Ziegler *et al.*, 2017; Voolstra and Ziegler, 2020). It is no wonder that stress-tolerant corals have a more stable and diverse microbiome and are also more physiologically resilient to ocean warming and acidification (Grottoli *et al.*, 2018). Microbiota that inhabits corals also play a critical role in the defence of their host against pathogens. Thus, the presence of a high number of genes involved in antibacterial compound biosynthesis has been detected in the metagenomes from corals (Thurber *et al.*, 2009). Additionally, high resolution mass spectrometry imaging (MALDI-MSI) has allowed the detection of the metabolites responsible for the antifungal properties of coral microbiota (Fig. 2; Moree *et al.*, 2013; Moree *et al.*, 2014).

Mass spectrometry imaging (MSI) has been proposed as a general tool to study the metabolic exchange patterns of microorganisms, which may have important implications based on the hypothesis that microbes could have an impact on their environmental niches or receive the impact of environmental changes (Gonzalez *et al.*, 2012; Maloof *et al.*, 2020). In the same way, HTS technologies have been used to study the impacts of different stresses on different tissues' microbial communities and its associated consequences on the host's metabolism and health, for example the effects of oil-contaminated sediments in the gill and intestine of the juvenile southern flounder (Brown-Peterson *et al.*, 2015), hypoxia in the digestive gland of the eastern oyster (Khan *et al.*, 2018), chemical contamination and seasonal fluctuations in the hepatopancreas of the Manila clam (Milan *et al.*, 2018), and nTiO<sub>2</sub> in the haemolymph of a marine bivalve (Auguste *et al.*, 2019). In this sense, it has been shown that host's microbial abundance/diversity and/or its metabolic activity, are significantly affected by stressful conditions and may contribute to their toxicity (Brown-Peterson *et al.*, 2015; Khan *et al.*, 2018; Milan *et al.*, 2018), thus compromising host's health status and susceptibility to diseases (Auguste *et al.*, 2019). Then, to understand how animals respond to chemical stress a key component of such response, the microbiota, must be taken into account (Milan *et al.*, 2018).



**Fig. 2.** Overview of MALDI-MSI applied to the study of metabolic exchange interactions of microorganisms. A. Schematic representation of microorganisms (e.g. bacterial, and fungal species isolated from a coral specimen) grown under different conditions (I–III). B. The average signal from the  $m/z$  of all the spectra obtained in the imaging runs. C. Separate signals of three specific  $m/z$  ions obtained in each of the studied environmental conditions. The signals correspond to the different metabolites produced by the bacterial and fungal species at the site of interaction. Metabolites with antifungal properties, antibiotic compounds, and signalling molecules can be identified.

Climate change conditions (ocean warming and acidification, increased UV radiation) can alter the speciation, solubility and bioavailability of various pollutants, and, when coexist, they exert combined effects on the functions and services of marine ecosystems (Coelho *et al.*, 2013; Louvado *et al.*, 2015; Zeng *et al.*, 2015; Coelho *et al.*, 2016). For instance, on the one hand acidification increases the biotoxicity of heavy metals and reduces the degradation of organic pollutants, on the other hand heavy metals and oils could decrease the photosynthesis rate and increase the respiration of marine organisms, both leading to ocean acidification (Zeng *et al.*,



2015). Metagenomic analysis showed that the interaction between reduced seawater pH and oil hydrocarbon contamination significantly altered the microbial estuarine community, greatly reducing the relative abundance of oil-degrading sulphate-reducing bacteria (order *Desulfobacterales*, and *Desulfosarcina/Desulfococcus* clade) (Coelho *et al.*, 2015; Coelho *et al.*, 2016). A reduction in specific archaeal groups and an increase of several hydrocarbonoclastic fungi was also found. All together this points to an impairment of the ability of the ecosystems to recover from acute oil contamination events in future acidified marine environments (Coelho *et al.*, 2016). Significantly, despite its potentially harmful effects, UV-B radiation appears to minimize the synergistic effects of seawater acidification and oil pollution (Coelho *et al.*, 2015).

#### *Microbial responses to extremely polluted environments*

Microorganisms living under extreme conditions have attracted considerable attention because of their peculiar physiology and ecology. In response to extreme pollution conditions, microbes can undergo significant structural and metabolic adaptations to survive (Desai *et al.*, 2010; Bouhaja *et al.*, 2016; Malla *et al.*, 2018).

Acid mine drainage (AMD), the wastewater released by the mining industry, represents an extremely harsh environment and a major environmental challenge worldwide. It is characterized by a low pH and high concentrations of metals and sulphates (Méndez-García *et al.*, 2015; Chen *et al.*, 2016). In the last decade, HTS technologies have extensively increased our knowledge of microbial diversity in AMD ecosystems (Kuang *et al.*, 2013; Chen *et al.*, 2016). Phylogenetic differentiation among microbial communities from diverse AMD sites across Southeast China has been widely studied by metagenomics and metatranscriptomics (Kuang *et al.*, 2013; Chen *et al.*, 2015; Kuang *et al.*, 2016). Unexpectedly, a high microbial diversity was found in these extremely acidophilic conditions, highlighting the high transcriptional activities exhibited by the abundant taxa of *Acidithiobacillus*, *Leptospirillum* and *Acidiphilium* (Chen *et al.*, 2015). Studies identified pH as a strong predictor of relative lineage abundance. Thus, *Betaproteobacteria* (dominated by the genus *Ferroplasma*) were predominant under moderate pH conditions, whereas *Alphaproteobacteria*, *Euryarchaeota*, *Gammaproteobacteria* and *Nitrospira* were better adapted to acidic environments (Kuang *et al.*, 2013). Metatranscriptomic analyses have shown that natural acidophilic microbial communities develop elaborate adaptation mechanisms to extreme conditions by regulating the expression of genes involved in many essential key functions. That includes multiple strategies for resource acquisition and

energy generation (carbon, nitrogen and phosphate utilization, iron and sulphur oxidation for energy conservation), and responses to environmental stresses (low pH adaptation, resistance to heavy metals and to oxidative stress) (Chen *et al.*, 2015; Hua *et al.*, 2015; Méndez-García *et al.*, 2015; Kuang *et al.*, 2016). Combining genomic analysis with mass spectrometry-based proteomic methods, essential activities and metabolic functions in a natural AMD microbial biofilm community were studied. A total of 2,033 proteins were identified, 48% of which are from the dominant biofilm organism, *Leptospirillum* group II. Significantly, key proteins for the survival in these extreme environments were highly expressed; *for example*, proteins involved in protein refolding, as chaperones, and in the response to oxidative stress, such as thioredoxins and peroxiredoxins (Ram *et al.*, 2005). Metagenome sequencing and functional gene annotations revealed that heavy metal polluted sediments from the Yellow River (Gansu Province, China) contain a larger number of genes related to DNA recombination and repair, and heavy metal resistance. Additionally, metal polluted sediments had a higher viral abundance, suggesting virus-mediated heavy metal resistance gene transfer as an adaptation mechanism (Chen *et al.*, 2018). Other studies highlight the increased proportion of metal-resistant genes and the presence of mobile genetic elements at the metal polluted sites (Gillan *et al.*, 2015; Jacquiod *et al.*, 2018). The remarkable insights into the composition of microorganisms, and their metabolic adaptations, in AMD extreme ecosystems provide significant clues for biotechnological applications (Chen *et al.*, 2016; Villegas-Plazas *et al.*, 2019), with iron and sulphur-oxidizers being used in biomining, and the activities of sulphate reducers which are essential in the AMD bioremediation systems (Chen *et al.*, 2016).

Increasing global petroleum demand has brought a dramatic increase in oil spills and leakage accidents all over the world, with devastating consequences for the local marine environment, given the high toxicity of petroleum compounds and its derivatives (Xue *et al.*, 2015). This has promoted intensive research to understand how microbial communities respond to hydrocarbon pollution, with the final goal of providing new biotechnological strategies for the recovery of the affected areas (Atlas and Bragg, 2009; Acosta-González *et al.*, 2015; Acosta-González and Marqués, 2016; Bouhaja *et al.*, 2016). The magnitude of accidental spills, such as the *Deepwater Horizon* oil spill (April 20, 2010; northern Gulf of Mexico), encouraged studies with a high level of resolution using meta-omic based molecular analyses, as previously reviewed (King *et al.*, 2015; Bouhaja *et al.*, 2016). For instance, after the spill, the responses in the sediments were evaluated, using a combination of 16S rRNA sequencing and screening of shotgun metagenomic

data, to characterize the microbial community and to explore the nitrogen and hydrocarbon metabolic potentials, respectively. Uncultured *Gammaproteobacterium* and *Colwellia* species were shown to be dominant in the most heavily oil-impacted seawater and sediments, where pathways of denitrification and degradation of aliphatic and simple aromatic compounds were also abundant (Rivers *et al.*, 2013; Mason *et al.*, 2014). Hydrocarbon degraders were dominated by *Gammaproteobacteria*, *Oceanospirillales* in the deep-sea plume and the *Alcanivorax* genus in coastal systems and in sea surface oil, while fungi increased in oil salt marshes and beach sands (King *et al.*, 2015). Complex successional taxonomic and functional patterns in coastal sands persisted when evolution was followed for over one year after the spill. Thus, the microbial community first shifted towards generalist populations of oil-degrading bacteria and then, a year after the disturbance, to a typical beach community, although significantly different from the original one before the spill, with little or no oil hydrocarbon degradation ability and enriched by xenobiotic-sensitive archaeal taxa (Rodriguez-R *et al.*, 2015).

#### **New prospects for development: microbes are more than bacteria**

Although all microorganisms play key roles in aquatic ecosystems, meta-omic studies focused on non-bacterial microbes are scarce due to taxonomic limitations (Pawłowski *et al.*, 2016). Protists can have huge impacts on the structure of microbial communities as they are the most common predators in aquatic environments (Sherr and Sherr, 2002). Furthermore, they are components of the phytoplankton communities that accounts for over half of the global primary production, and, thus, play a vital role in aquatic food webs (Chavez *et al.*, 2011). Additionally, they are generally broadly distributed even within extreme habitats, have short life spans, and respond sensitively to environmental variables (Sherr and Sherr, 2002; Heidelberg *et al.*, 2010). Several non-HTS-based studies have proposed protists for pollution biomonitoring. Thus, significant disturbances were successfully linked to salt, trace metals or organic pollution. Significantly, the presence of pollutants produces a decrease in protists global richness (Shannon Index) and abundance (Roe *et al.*, 2010; Desrosiers *et al.*, 2013; Roe and Patterson, 2014). However, the methodology used in these studies was usually time consuming as the different species were manually identified by microscopy (Roe *et al.*, 2010; Desrosiers *et al.*, 2013; Roe and Patterson, 2014). Some specific taxons have been proposed as suitable bioindicators of certain stresses, for example *Diffugia oblonga* of eutrophication (Roe *et al.*, 2010). Of particular interest is the fact that protists

have proved to be excellent bioindicators to monitor the quality of wastewater effluents (Foissner, 2016). Recently, a combination of HTS and conventional methods (e.g. accessory pigment analysis) has been used to characterize the major phytoplankton groups in an estuarine area (North Caroline, USA). 18S rRNA sequencing showed that nutrients' concentration and salinity influenced the phytoplankton taxonomic composition, being *Trebouxiophyceae* the most dominant family (Gong *et al.*, 2020).

As already mentioned, faecal bacteria have been traditionally used to monitor wastewater pollution in aquatic environments, where they are also determined for health check as infectious agents (Jang *et al.*, 2017). Some viruses (e.g. waterborne enteric or human polyomaviruses) are highly resilient to wastewater treatments what makes them excellent biomonitors of faecal pollution (Rachmadi *et al.*, 2016; Farkas *et al.*, 2020). Actually, enteric- and polyoma viruses have proven to be more persistent in the environment than initially thought, even resisting UV, chlorination, pH changes or salty environments (Fong and Lipp, 2005; Rachmadi *et al.*, 2016; Farkas *et al.*, 2020). In contrast, enveloped corona-, Ebola- or influenza-viruses are more susceptible due to their labile outer lipid layer (Polo *et al.*, 2020). In the case of molecular viral determinations, PCR-based methods are being widely used as they are not only quite sensitive but also highly specific. However, nucleic acids detection do not provide information about viral infectivity, as the loss of the cover in enveloped viruses leads to a reduced infection capacity in aqueous systems (Fong and Lipp, 2005; Polo *et al.*, 2020). Thus, polyomavirus can be detected in almost all types of aquatic environments from coastal to tap waters, but there is no proof so far that these can be a source of infection (Rachmadi *et al.*, 2016). Viruses are also major players in the ocean ecosystems where they are estimated to be at least ten times more abundant than the microbiome, and they kill approximately 20% of their microbial biomass daily, which the subsequent impact on nutrient and energy cycles (Suttle, 2007; Dávila-Ramos *et al.*, 2019). There are several methodological bottlenecks linked to the widespread use of viruses as biomonitors, such as the need for: (i) an initial concentration phase, since their concentration is usually low in abiotic matrices because they cannot regrow outside their hosts; (ii) complex methods to extract nucleic acids from matrices that can contain inhibitors of the enzymes used for molecular biology analysis; (iii) good viral databases for analysing massive sequencing results. Nevertheless, several recent studies are focusing on the study of the ocean virome (Hayes *et al.*, 2017; Thurber *et al.*, 2017; Dávila-Ramos *et al.*, 2019), that, although not directly related to environmental stress responses, are

providing new sequences from an enormous diversity of unknown viruses that are greatly improving available databases, for example the Global Ocean Virome dataset. Although viral communities vary according to the depth, season, and distance from the littoral, top levels of richness functional capacity were found in the coastal areas (Hayes *et al.*, 2017), otherwise the most exposed to pollution. Nevertheless, to-date we are not aware of any studies relating virome alterations to pollution. Finally, in the current Sars-CoV2 pandemic situation, the importance of viruses as infective agents and the search for potential viral biomarkers to monitor the quality of waters has become even more urgent. Thus, levels of Sars-CoV2 in wastewaters have proven to be an excellent bioindicator for the COVID infection detection of the global population and to model and monitor the pandemic (Kumar *et al.*, 2020; Peccia *et al.*, 2020; Polo *et al.*, 2020).

### Final remarks

Microorganisms are the most diverse forms of life on Earth, where they play essential roles (e.g. biogeochemical cycles, marine food web, global change) and inhabit all possible environments, including the most extreme and hostile ones. Microbial structure and activity are highly sensitive to natural and anthropogenic stressors, with consequences for global environmental health, and, thus, perfect potential sensors of environmental disturbances. However, most microbial lineages numerically dominant in all major environments on Earth, known as the 'microbial dark matter', have never been characterized in laboratory cultures, and represent a bulk of undiscovered physiologies that may be crucial to the ecosystem (Riesenfeld *et al.*, 2004; Lloyd *et al.*, 2018). Thus, HTS techniques are emerging as a valuable source of information to assess the worrying effects of anthropogenic activity on the environmental microbiota. To achieve this, further improvements in the extraction of biomolecules from complex matrices (e.g. wastewaters, biofilms, sediments, soils), together with greater developments in high-throughput molecular technologies, more consistent annotated databases, and more powerful bioinformatics software for big data analysis, are required. Altogether, these developments will allow us to evaluate the structure and functions of microbial communities better, and their consequences, in response to changes and aggressions on the environment, especially in an evolving climate change scenario.

In addition to the natural environmental studies described in this review, microcosm/mesocosm experiments have been proposed to study the interactive effects of global change on organisms from different trophic levels, including microorganisms. This approach

makes it possible to simulate the exposure of estuarine benthic communities under controlled laboratory conditions to directly associate changes with the source of environmental disturbance or stress (Coelho *et al.*, 2015; Coelho *et al.*, 2016; Louvado *et al.*, 2019). Also, an emergent field of study is the evaluation of how bacterial interactions and their symbiotic relationships with higher-level organisms are affected in response to environmental perturbations, mostly considering the vital importance of microbial communities in the hosts' development and health. Thus, further development and more extensive application of MALDI-MSI to image host-microbiome symbioses and their metabolic interactions look quite promising. In this sense, a spatial metabolomics pipeline (metaFISH) has been recently presented that combines fluorescence in situ hybridization (FISH) microscopy and atmospheric pressure MALDI-MSI to image host-microbiome symbioses and their metabolic interactions (Geier *et al.*, 2020). Another area of future development and application, that is not without important technological problems to solve, is the mass spectral identification of microbes in complex environmental matrices and the evaluation of changes in response to stressful conditions, to be used as fingerprints of the environmental aggression (Welker and Moore, 2011; Maloof *et al.*, 2020).

Nowadays, in a global change scenario with the increase of anthropogenic activities, to get a sustainable development is an essential objective for our societies. The improvement in the management of natural resources and the reduction of the number and quantity of substances released to the environment (e.g. from wastewater, industrial activities, agriculture, etc.) are priority actions. To support these objectives, the ongoing application of molecular technologies can shed light on the factors governing the growth, dynamics, and metabolism of indigenous microbial communities. This knowledge will provide useful tools for a better risk assessment, management, and design of more efficient bio-engineering applications in fields as wastewater treatment or bioremediation.

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### Conflict of interest

The authors have no conflict of interest to declare.

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