Minireview

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

Carmen Michán,1 Julián Blasco2 and José Alhama3
1Departamento de Bioquímica y Biología Molecular, Campus de Excelencia Internacional Agroalimentario CeIA3, Universidad de Córdoba, Campus de Rabanales, Edificio Severo Ochoa, Córdoba, E-14071, Spain.
2Department of Ecology and Coastal Management, IMAN-CSIC, Campus Río San Pedro, Puerto Real (Cádiz), E-11510, Spain.

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 increasingly 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).
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 O₂ consumption/CO₂ 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 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; Zuniga 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; Zarraonaindia 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; Zarraonaindia et al., 2013; Wang et al., 2014; Aguiar-Pulido et al., 2016; Herbst et al., 2016; Wang et al., 2016; Zuniga 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
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 (Bastaraud 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 (Methanobreivibacter) 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 not 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; Rafraf 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 | Li et al. (2015) |
|                          |                        | WWTP effluents substantially influence on the dispersal of microorganisms and ARGs in receiving aquatic sediments | Chu et al. (2018) |
|                          |                        | In WWTP, removal efficiency of ARGs in anaerobic sludge treatment is much less efficient than the sewage treatment | Yang et al. (2014) |
| 16S rRNA, metagenomic & metatranscriptomic (Illumina sequencing) analyses |                        | Conventional treatment process within WWTP strongly influences resistance genes and their transcriptional activities | Ju et al. (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 et al. (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 et al. (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 | Glasl et al. (2019) |
|                          |                        | Microbiome flexibility facilitates rapid responses of corals to environmental changes (e.g. high temperature) and increases stress tolerance | Ziegler et al. (2017) |
|                          |                        | Corals microbiota plays a critical role in the defence of their host against pathogens | Grottoli et al. (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 et al. (2015, 2016) |
|                          | qPCR, 16S rRNA & metagenomic (Illumina sequencing) analyses | Marine benthic microorganisms are susceptible to changes in ocean carbonate chemistry and sweater temperature | Currie et al. (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 et al. (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 et al. (2018) |
|                          | 16S rRNA & metagenomic (Ion Torrent sequencing) analyses | TiO₂ nanoparticles shift the haemolymph microbiome composition of the bivalve Mytilus galloprovincialis, which may affect its immune system | Auguste et al. (2019) |
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 (Bastaraud 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 | aadA, A1, A2, A13, B |
|                  |                     | Drug modification by phosphorylation | aprA, A, A3, A-6, 6, 2; strA, B; strB |
|                  | Interfering with the bacterial cell wall biosynthesis by inactivation of penicillin-binding proteins | Inactivation by beta-lactamases (Drug degradation) | mecA, ampicillin-binding protein |
| Glycopeptides    | Interfering with cell wall biosynthesis | Produces a modified peptidoglycan pentapeptide | emrA, B, C, F, O |
| Macrolides       | Inhibiting early stages of protein synthesis (binding to the 50S bacterial ribosome subunit) | Methylation of 23S rRNA target side | eryA, A2, B |
|                  |                     | Cleavage of the drug lactone ring | mptA(A), (B), BM |
| Quinolones       | Inhibiting bacterial DNA replication enzymes (DNA gyrase & topoisomerase IV) | Inactivation of target enzymes | mfsA, E; mtt;-msrA |
| Sulphonamides    | Inhibiting the dihydropteroate synthase, enzyme involved in the folic acid synthesis | Modification by acetylation | gyrA, B; parC |
|                  |                     | Competitive inhibitors of dihydropteroate synthase, enzyme involved in folic acid synthesis | qnrA, A3, B, B1, B2, B4, B5, D, S, S1, S2, VC |
| Tetracyclines    | Inhibiting bacterial protein synthesis (binding to the 30S ribosomal subunit and preventing the aminoacyl tRNA association) | Ribosome protection | ortA, teta[P(M), M, O, O, S, T, W, X] |
|                  |                     | Drug export by membrane associated proteins | teta[A(C), A(P), B, C, D, E, G, H, J, K, L, V, Y, Z, 31, 36, 39] |
| Trimethoprim     | Inhibiting Dihydrofolate reductase (DHFR) | Enzymatic inactivation of the drug | tetA, tetB, L, X, XIII, 16, 17, A19, B2, A20, A3, A12, A13, A21, A22, A33, B1, B5, B6, B8, D, E |
|                  |                     | Increased synthesis of DHFR | dfrA(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 | aceB[D], adeA[A, B]; amrB; mdt(F, G, H, N, O); mexB, D, F, I, W, Y; norM; orf11; qadB, E; 1, 01, F, G2, H; sedY; smaB[E, E] |

*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 metatranscriptomics 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 co-varied with ubiquitous OMZ microbes (e.g. *Thaumarchaeota, Nitrospira, 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 nTiO2 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).

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., 2016).

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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.
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 Desulfobacteriales, 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; Bouhajja 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 sulfates (Méndez-Garcia 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 *Ferrovum*) 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-Garcia et al., 2015; Méndez-Garcia 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; Bouhajja 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; Bouhajja 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, metagenomic studies focused on non-bacterial microbes are scarce due to taxonomic limitations (Pawlowski 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 taxa have been proposed as suitable bioindicators of certain stresses, for example Diffugia oblonga of eutrophization (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 biomonitor 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 coronavirus-, 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 biomonitor, 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
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). 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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