Freshwater Microbiology: Recent Updates and Prospects

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Abstract

Along with bacteria, other microorganisms are also key components of microbiota structure of freshwater, and those include archaea, microbial eukaryotes, and viruses that together build the diverse community of microorganisms. All those microorganisms play key ecological functions and can have important impacts on animal, plant, and human health. The traditional culture-based microbiology methods have been mostly replaced, or complemented, by molecular methods, due to their accuracy, fast response, and capability to detect microorganisms that are quite difficult to grow in culture media. This chapter addresses some of the current tendencies on microbial structure and its analysis. Those methods included the "omic-based" DNA studies that allow better and more comprehensive characterization of bacterial communities, leading to metagenomic studies that help to elucidate the population structure of microbiomes in aquatic environments. Strategies for detecting water-borne pathogens are focused on detecting bacteria, protozoa, yeast, and

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virus in different water sources, and these methods can also be complemented by molecular tools such as molecular marker (RAPD, Microsatellites, RFLPs, and AFLPs). Those methods also allow to detect pathogenic bacteria, fungus, and viruses of public health interest, such as the enterovirus and, more recently, the coronavirus. Water bodies can also be important reservoirs of antibiotic-resistant bacteria, and the group of ESKAPE bacteria represents an important study model for antibiotic resistance and its distribution. The acronym "ESKAPE" is associated with this group due to their ability to escape antimicrobial activity and develop high levels of resistance to multiple antibiotics (multidrug resistance). Water bodies play a central role on the selective pressure and is the most important cause of the dissemination and extension of resistance and has contributed to the genetic diversification of resistance genes also affecting ecological microbial structures.

1.1   Introduction

The biological role of freshwater microorganisms in the carbon cycle and its association with the uptake and emission of greenhouse gases is well documented as well as the impact that disturbance of microbial communities as a consequence of anthropogenic activities have on the climate and global change (Regnier et al. 2013; Premke et al. 2022).

Bacteria are prokaryote, unicellular, taxonomically diverse, and ubiquitous microorganisms. They are the most abundant organisms on earth and their habitats include virtually every ecosystem on the planet, even extreme aquatic environments such as hot springs and ice layers in the Arctic and Antarctic regions, under high pressures or extreme acidic environments (Takai et al. 2008; Dalmasso et al. 2016; Merino et al. 2019). The adaptation capabilities of bacteria to adverse environments explain their abundance within the different ecosystems on the earth; it is estimated that the average number of bacterial cells per gram of soil is around 40 million, whereas 1 million diverse bacterial cells can be found in 1 mL of environmental freshwater and it is even higher in estuaries and open ocean waters (Whitman et al. 1998; Anas et al. 2021).

In aquatic environments, the cross-feeding and metabolite interchange represent an important class of interactions, and thus, photosynthetically fixed dissolved carbon could produce chemotactic responses (Seymour et al. 2010). Early experimental evidence suggested the existence of strong positive and negative interactions among the photoautotroph and heterotroph microorganisms (Cole 1982). Besides bacteria, there are various microorganisms in fresh water, such as fungi, algae, and viruses, although they are not microorganisms. Their importance will be addressed

in this chapter. The traditional culture-based microbiology methods have been mostly replaced, or complemented, by molecular methods, due to their accuracy and fast response. Here, some of the current tendencies on microbial structure and its analysis will be addressed.

1.2 Microbiome and DNA-Based Omic Studies

As we move forward to a better understanding at microorganic system level, complexity increases as the number of microorganisms interacting do. For this reason, it is important to develop pipelines to accurately characterize the diversity of bacteria and other microorganisms in specific niches and elucidate their role within the community and the ecosystem. Water is an essential natural resource for all living organisms, and the pollution consequence of human activities has a great impact ranging from environmental effects (sometime negative), animal and human health impact to important economic losses in food production. The causes of contamination of rivers, lakes, and ponds are diverse. Still, contamination with untreated or poorly treated sewage and residual waters containing heavy metals and other xenobiotics from industry, agriculture, and livestock production are among the main drivers that serve as disturbers of native bacteria communities. In the first case, sewage serves as an important source of enteric bacteria and viruses, whereas industrial residual waters modify the physical and chemical properties of the water itself, and these changes impact the bacterial population structure affecting the carbon flux between freshwater bodies and the environment among other important aspects (Schreiber et al. 2015).

Like other ecosystems, in freshwater bodies, bacteria and other microorganisms live in a complex multispecies structure. Begon et al. (1986) defined the microbial communities as the set of microorganisms coexisting in the same space and time. However, the characterization of bacterial communities was limited to microorganisms grown in culture media, and their study was focused mainly on morphological and biochemical profiles. In 1977, Woese and Fox proposed the 16S ribosomal RNA genes as a potential marker to establish phylogenetic relationships between bacteria, and it was only after the development of Sanger sequencing technology that the 16S ribosomal RNA genes analyses was used as the benchmark to fine-tune the classification system of microorganisms.

Later in 1998, Handelsman et al. proposed the term *metagenomics* in their research, which involved the isolation of total DNA from soil bacteria without previous culture on artificial media, and their cloning using BAC vectors in order to transform competent *E. coli* cells and then sequence the genomes and express genes detected, allowing a deep understanding of the bacterial population structures. The arrival of high-throughput next-generation sequencing (NGS) techniques in 2005 with the pyrosequencing method from Roche was a milestone in the microbial communities' characterization because of its relative low cost and reliable methodology to rapid sequencing of short fragments of DNA. However, this platform is discontinued today by more efficient sequencing strategies such as synthesis

sequencing, initially proposed from ion torrent, followed and enhanced then by Illumina and PacBio, all collectively called next-generation sequencing (NGS), that now represents the most accurate and used technique to characterize the metagenome of environmental samples at a different resolution of taxonomic levels; these sequencing technologies allow the rapid detection of perturbations in populations of native bacteria in freshwater deposits under different conditions that can be associated to ecological phenomena (Escobar-Zepeda et al. 2015). In the past few years, through NGS techniques, researchers worldwide have been able to elucidate the population structure of microbiomes in aquatic environments under different and extreme conditions (Table 1.1).

To this day, metagenomic characterization is mostly descriptive and without a careful approach design it does not provide insights into genes of interest or metabolic integration pathways in the analyzed environments. However, a comprehensive approach integrating every member of the microbial community transcriptome (metatranscriptomics) studies and the total set of proteins (metaproteomics) could improve the insight into the role played by every microbial member at an ecosystem level establishing accurate relationships between taxonomy and functionality profiles within microorganisms.

1.3 Current Strategies for Detection of Water-Borne Pathogens

1.3.1 Incidence of Bacterial Pathogens in Water

Water bodies, such as rivers, lakes, irrigation channels, damps, and hospital and industrial wastewaters, harbor a wide variety of microorganisms. The microorganisms commonly found in wastewater are enteric anaerobic bacteria, some yeasts and fungi, and some protists especially those resistant to harsh environments. Regarding the bacteria present in water bodies, it has been estimated that 90% of the genera and species of bacteria characterized belong to 4 phyla: Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes. Some of the bacteria can be defined as pathogens such as *Escherichia coli* (Rice and Johnson 2000), *Pseudomonas* spp. (Pirnay et al. 2005), and *Stenotrophomonas maltophilia* (Brooke 2012). These bacteria, along with the emergence of antibiotic-resistant bacteria and its wide distribution in freshwater, is a concerning matter that will be addressed further in this chapter with the ESKAPE group.

1.3.2 Incidence of Yeast and Filamentous Fungi Pathogens in Water

The presence of these microorganisms in water has important ecological roles in organic matter transformation, and some have an additional impact, because these microorganisms can be pathogenic or display pathogenic properties and well as

Table 1.1 Metagenomic analyses from selected freshwater vessels under diverse physicochemical conditions

Water body	Location	Water samples	Metagenome composition	Conditions	Sequencing platform
Lake Baikal[a]	Siberia, Russia	2, from 5 and 20 m depth	Verrucomicrobia	High oxygen levels.	Illumina HiSeq
			Actinobacteria		
			Proteobacteria		
			Acidobacteria		
			Bacteroidetes		
			Pseudomonadota		
Fetsui reservoir[b]	Taiwan	Samples taken from 5 m depth	Bacteroidetes	Evidence showed that typhoons disrupt the thermal stratification of the water column and change dissolved oxygen level by delivering oxygen-rich water to the reservoir.	454 Roche
			Actinobacteria		
			Cyanobacteria		
			Flavobacteria		
			Sphingobacteria		
			Alphaproteobacteria		
Lake Tanganyka[c]	DRC, Tanzania, Burundi, Zambia	Tanzanian samples from surface to 1100 m depth	Bacteroidetes	Anoxic water, stratified. Metagenomes were compared from rich oxygen surface to dark, nonoxygen, nutrient-rich depth.	Illumina HiSeq
			Ignavibacteria		
			Betaproteobacteria		
			Gammaproteobacteria		
			Alphaproteobacteria		
			Verrucomicrobia		
			Planctomycetes		
			Chloroflexi		
			Actinobacteria		
			°Tanganyka bacteria		
			Cyanobacteria		
Lake Tai[d]	China	10 cm depth	Proteobacteria	Water body heavily contaminated with population residual water.	Illumina HiSeq
			Firmicutes		
			Actinobacteria		
			Bacteroides		
			Acidobacteria		
El chichon[e]	Mexico	Water column and sediment samples	Found at sediment 50 °C:	Crater lake of an active volcano, formed in 1982. Geothermal activity	Illumina NextSeq
			Actinobacteria		
			Proteobacteria		
			Acidobacteria		
			Found at sediment 92 °C		

Table 1.1 (continued)

Water body	Location	Water samples	Metagenome composition	Conditions	Sequencing platform
			Firmicutes (*Alcylobacillus* and *Sulfobacillus*)	including acidification, presence of sulfur compounds and metals, extreme water temperature.	
			Proteobacteria (*Bradtrhizobium*, *Methylobacterium*, *Sediminibacterium*)		
Lake Untersee[f]	Antarctica	80 m depth	Proteobacteria	High concentration of dissolved oxygen. High pH 9.8–12.1.	Illumina HiSeq
			Bacteroidetes		
Lonar Lake[g]	India	Surface water samples	Proteobacteria	Hyperalkaline and hypersaline basin.	Illumina HiSeq
			Firmicutes		
			Bacteroidetes		
Lake Michigan[h]	U.S.A.	Samples from river, river mouth, near-shore, and offshore, at 45 cm depth	Proteobacteria	Bacterial contamination originated from the Grand Calumet River.	Illumina
			Actinobacteria		
			Bacteroidetes		

[a] Cabello-Yeves et al. (2017)
[b] Tseng et al. (2013)
[c] Tran et al. (2021)
[d] Chen et al. (2019)
[e] Peña-Ocaña et al. (2022)
[f] Koo et al. (2018)
[g] Chakraborty et al. (2020)
[h] Nakatsu et al. (2019)

antifungal resistance (Monapathi et al. 2020). Some species of yeast that have been identified in water bodies include *Candida*, *Clavispora*, *Cyberlindnera*, *Cryptococcus*, *Hanseniaspora,* and *Yarrowia.* Yeast detected in cities water distribution systems and tap water include *Candida*, *Clavispora*, *Cryptococcus*, *Debaromyces*, *Meyerozyma*, and *Pichia*. Yeast isolates identified in sewage treatment plants are *Candida*, *Cryptococcus*, *Debaryomyces*, and *Wickerhamonyces* (Monapathi et al. 2020). Yeasts isolated from different water samples recognized as pathogenic include *Candida* spp. *Cyberlindnera*, and *Cryptococcus*. Fungal infections caused by *Candida albicans* and other *Candida* spp. are important complications in immunosuppressed patients, since frequently, candidiasis in mucosal tissues (oral, gastrointestinal, and vaginal) represent an early sign of immune system malfunction

(Panizo and Reviákina 2001). *Cryptococcus neoformans* is an encapsulated yeast, and the cause of opportunistic infections, such as meningoencephalitis in immuno-compromised patients (Pini et al. 2017).

Among filamentous fungi, some of the complications caused by some virulent species belonging to *Aspergillus, Fusarium,* and *Alternaria* may range from allergic to invasive syndromes. Immunocompetence facilitates clearance by initiating innate and adaptive host responses despite constant spore inhalation (Chotirmall et al. 2014). Regarding *Aspergillus* section Flavi includes 22 species, some of them with potentially pathogenic such as *Aspergillus felis*, *A. fischeri*, *A. fumigatiaffinis*, *A. fumisynnematus*, *A. hiratsukae, A. laciniosus*, *A. lentulus*, *A. novofumigatus*, *A. parafelis*, *A. pseudofelis*, *A. pseudoviridinutans*, *A. spinosus*, *A. thermomutatus*, and *A. udagawae*. The pathogenic species can display a wide range of differences in the frequency of production of toxins or toxic compounds (Tamayo-Ordóñez et al. 2021). Even though fewer pathogenic yeasts and molds have been reported in different water species compared to bacterial genera, their importance stands out, since their contact with humans with a compromised immune system could lead to serious complications and even cause death.

1.3.3   Identifying Virus in Water Samples

In raw wastewater, viruses may be ubiquitous and persistent and even after treatment, some viruses continue being distributed in treated wastewater that later arrive at water bodies. Adenovirus (HAdV), rotavirus (RoV), hepatitis A virus (HAV), and other enteric viruses, such as noroviruses (NoV), coxsackievirus, echovirus, reovirus, and astrovirus, are commonly detected in wastewater as well as some superficial water (rivers and irrigation channels) and are the principal human viral pathogens transmissible via water exposition (Corpuz et al. 2020). More dangerous viruses such as poliovirus can also be present in wastewaters. Enterovirus causing waterborne diseases such as diarrhea in children and adults are often associated with viral disease outbreaks, leading to several clinical manifestations (nausea, vomiting, and fever) and other gastrointestinal diseases, and some enteric viruses have also been related to respiratory, central nervous system (poliovirus), and other diseases (Corpuz et al. 2020).

Recently, a novel Coronavirus disease 2019 (COVID-19), which began in December 2019, that is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Guarner 2020) was reported. Since this virus can invade the gastrointestinal mucosa, it can be released in feces, and the presence of nucleic acid of SARS-CoV-2 has been reported in raw wastewater (Randazzo et al. 2020), sewage samples collected from hospitals (Wang et al. 2020b), and wastewater sample after secondary treatment (Randazzo et al. 2020). However, infection caused by exposition to water is not been documented, and its detection functions as an early warning system to indirectly detect SARS-Cov-2 infections in the population.

1.4 Molecular Markers for Genotyping and Identification of Bacteria in Water Bodies

1.4.1 Intraspecific and Interspecific Variability and Genotyping

Molecular markers are DNA fragments sequences associated with a physiological trait, and are used to identify a particular DNA sequence related to a function or a trait of interest (Langridge and Chalmers 2004). Different types of markers are distinguished by their ability to detect polymorphisms at single or multiple loci, or sequence patterns and can be of dominant or codominant type. Below we describe some of the molecular markers that have been most used to determine intra- and interspecific variability in bacterial genera isolated from different bodies of water.

RAPDs RAPDs (randomly amplified DNA polymorphisms) are markers that randomly amplify segments of DNA in a wide variety of species. RAPDs are based on the statistical probability of complementary sites to the oligonucleotide of ten base pairs (bp) throughout the genome. The polymorphism of the bands between individuals is due to changes in the sequence of nucleotides at oligonucleotide coupling sites and by inserting or deleting fragments at these sites. These markers are dominant; they cannot discern the dominant homozygous or heterozygous for a particular segment, so allelic frequencies must be estimated indirectly, assuming Hardy Weinberg equilibrium. Among some of the applications of these molecular markers in bacteria, it has successfully demonstrated different RAPD profiles in multidrug-resistant coliform bacteria isolated from sewage samples of Ghaziabad city, India (Raj 2012). Since RAPD-derived markers include a genome-wide analysis, they can be a very good source of physiological markers that can be obtained even if little is known about the sequence of the microorganism being studied. Their application could be considered one of the first "genomic-wide" analysis performed before the massive sequencing technology was available; however, several problems with reproducibility limited its applications out of basic research.

Microsatellites Simple repeat sequences (SSRs) or ISBPs (Insertion Site-Based Polymorphisms) are DNA sequences made up of 1–4 base pairs (mononucleotides (TT)n, dinucleotides (AT)n, or tetranucleotides (AAGG)n). These loci are found both in coding and noncoding regions of DNA, formed by breakage events that generate polymorphisms with values greater than 90%. Challagundla et al. (2018) analyzed 598 genome sequences of *Staphylococcus aureus*; this study showed that depending on the geographical area of the isolate, certain mutations in the CC5-MRSA marker are present allowing a deeply analysis of the epidemiology of these strains. A particular important application of simple repeat sequences was on *M. tuberculosis* research, because this allowed the design of a classification system of *M. tuberculosis* strains, based on a VNTR (variable number of tandem repeats) that was so specific to *M. tuberculosis* that later these were called mycobacterial interspersed repetitive units or MIRUs, allowing a great advance on mycobacterial isolates classifications, both from clinical and environmental sources. Dangerous

strains such as the Beijing lineage were spotted with these markers and later with complementary genomic analyses on Beijing strains obtained from different clinical samples, waters, and substrates: this allowed us to know its biogeographic structure and evolutionary history of the Beijing lineage worldwide through the SNPs analysis of 4987 isolates from 99 countries (Merker et al. 2015).

RFLPs This method allows the detection of specific DNA differences recognized by restriction enzymes. Each of the endonucleases (of bacterial origin) recognizes and cuts only a specific sequence in DNA, as long as they are not protected (methylated). Therefore, any DNA that is not methylated can be recognized and cut into fragments of defined length; any mutation within those sites could change the pattern of the fragment and allow an RFLP to be detected when comparing two or more genomes. Originally, RFLP were important tools to detect and related mutations and molecular marker to key physiological functions and to design the initial molecular diagnosis strategies, since sequencing was required only to confirm the design but not to apply the technique and had very good reproducibility results. RFLPs are still currently applied to understand bacterial community dynamics in wastewater treatment systems. Wang et al. (2010) investigated how bacterial communities change in treatment plants over a year using specific PCR followed by terminal restriction fragment length polymorphism (T-RFLP) of the 16S rRNA gene. The T-RFLP results indicated lack of stability in the bacterial community structures in 2 full-scale wastewater treatment systems, with 15 days average change rates observed in the 2 systems. On the other hand, through digestion of the 16S rRNA gene with the endonuclease *Mse* I, it was shown that distinctive patterns for *Acrobacter* species are observed, indicating variation in the population structure of the genera (Figueras et al. 2012).

AFLPs AFLPs (amplified fragment length polymorphisms) is a technique that combines the digestion of two restriction enzymes, generally MseI and EcoRI, within a sequence, involving a selective amplification of restriction fragment lengths from a genome using PCR, and followed by gel electrophoresis to separate the amplified fragments and obtain a banding pattern, which can be used to identify genetic differences between individuals or populations, and can also be used for genetic mapping and phylogenetic analysis (Tamayo-Ordoñez et al. 2012).

This technique has been used to determine the biodiversity of the bacterium *Pseudomonas aeruginosa* in an aquatic environment in a study including 100 isolates of *P. aeruginosa*, where the *oprL* gene was analyzed, and a DNA-based fingerprint (AFLP) indicated a positive relationship between pollution and prevalence of *P. aeruginosa*. In the Woluwe River, *P. aeruginosa* community was almost as diverse as the global *P. aeruginosa* population. These findings illustrate the significance of river water as a stable reservoir and source for the distribution of potentially pathogenic *P. aeruginosa* strains (Pirnay et al. 2005).

In another recent study, antimicrobial tolerance to oxytetracycline and taxonomic diversity relationship in the culturable oxytetracycline-resistant (Otr) isolates of

heterotrophic bacteria in two Belgian aquatic sites receiving wastewater was investigated. Profiles for ampicillin and kanamicin tolerance were detected from the taxonomic differences in the Otr bacteria detected at genera and subgenera level. In addition, *Enterobacter* sp., *Stenotrophomonas maltophilia*, and *A. veronii* resulted as potential indicator organisms to help to assess microbial tolerance in various compartments of the aquatic environment (Huys et al. 2001).

1.4.2 Identification of Species Through Sequencing of Molecular Markers

To date, depending on the purpose of each investigation, different genetic regions can be used to identify species from various samples. Among the most explored genes used for species identification are the 16S rRNA, 23S rRNA, *rpo*B, *gyr*B, *dna*K, *dsr*AB, *amo*A, *amo*B, *mip*, *hor*A, *hit*A, *rec*A, *ica*, *frc*, *oxc*, 16S–23S rDNA ISR, and IS256.

Amplification and sequencing of 16S (V3–V4 of 16S rRNA) is commonly applied to study microbial communities with third-generation sequencers (MinION from Oxford Nanopore Technologies) and this made it possible to analyze the full length of the 16S rRNA gene, and this allows a deeper identification even to species level identification and at relative low cost. The analysis of nine indigenous bacteria that can be related to food poisoning and act as opportunistic infections was carried out with due diligence. *Enterococcus faecalis* and *Enterococcus hirae* were identified at the species level with an accuracy of 96.4–97.5%. Also, using these technologies, it is possible to evaluate the antibiotic sensitivities of multiple bacteria simultaneously. Kawai et al. (2022), using these technologies, allowed rapid evaluation of antibiotic activity spectrum at the species level containing a wide variety of bacteria, such as biofilm bacteria and gut microbiota. Even though the sequencing of these genes is a powerful tool to identify bacteria, it has some limitations when it comes to related bacterial complexes that can be difficult to distinguish.

Since the sequencing and analysis of 16S rDNA ribosomal regions is the most used molecular marker, we decided to find out the abundance of bacteria identified in bodies of water and of these, which accessions have been reported in the Gen Bank from NCBI. It was possible to identify 103 sequences of 16S rDNA regions of bacteria present in different bodies of water (Fig. 1.1). After analysis, a total of 58 bacterial genera were identified.

Conclusively, we want to emphasize that the use of molecular markers allows to identify and genotype a wide variety of microorganisms from different water samples, highlighting the importance of molecular markers in the clinical and environmental areas.
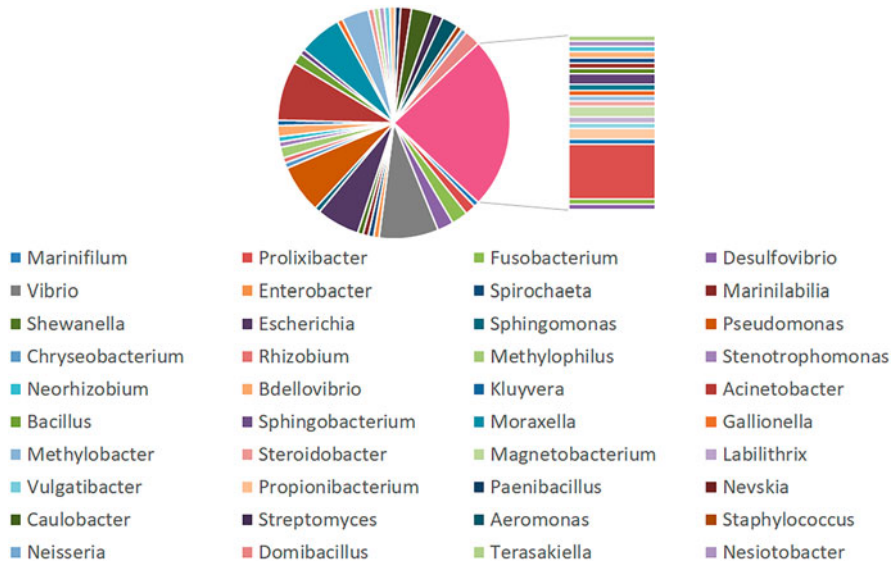
Fig. 1.1 Representation of the bacterial species identified in water bodies. The bacterial species were identified by searching in the sequences reported in the NCBI

1.4.3   Methods of Detection of Viruses in Waters

Viruses are important components of aquatic systems, since they are heavy regulators of bacteria populations, and some of them can be related to plants, animal, and human diseases, so their detection is crucial as indicated in numerous studies, including different water matrices such as surface water, treated wastewater, and irrigation water (Wang et al. 2020a; Ji et al. 2020; Rusiñol et al. 2020). Several methods to directly and indirectly detect and quantify viruses found in wastewater include epifluorescence microscopy, transmission electronic microscopy, pulsed-field gel electrophoresis, immunofluorescence assay, flow cytometry, traditional cell-culture, and molecular detection (Corpuz et al. 2020). Among the molecular techniques, PCR and especially real-time PCR are currently used.

PCR is an in vitro enzymatic reaction that amplifies or generates millions of copies of a specific DNA sequence during several repeated cycles in which the target sequence is faithfully copied. This technique uses thermostable DNA polymerase to replicate DNA strands, for which alternate high and low temperature cycles are used to separate the newly formed DNA strands after each replication phase. Among the components used in this in vitro reaction are amplification template (DNA or complementary DNA, or RNA in the case of viruses), oligonucleotides, DNA polymerase (Taq polymerase), polymerase cofactor ($MgCl_2$), PCR buffer,

deoxyribonucleotide triphosphates, water, and equipment (thermocycler). Since viruses have DNA or RNA genomes, PCR is an important tool for detecting them in water samples. For the detection of RNA genome viruses, it is necessary to carry out an RT-PCR (Reverse Transcription-Polymerase Chain Reaction) that is a derived technique with a step where a RNA complementary DNA is generated that can be used directly in the PCR (Kadri 2020). An important derivative technique for virus study is the Real-time PCR or quantitative PCR that is a method that allows us to monitor the evolution (simultaneous amplification and quantification) of the PCR while it is being carried out. Cycle-by-cycle analysis of the changes in the accumulation of the PCR product, detected by a change in the fluorescent signal generated in the three steps of the PCR, is done with this technique and allows two types of determinations: the quantification absolute and quantitative comparison between samples. The quantification absolute determines the exact number of DNA or RNA molecules present in each sample. Applying this quantification method requires generating a standard curve and using the comparative Ct method. The other type of determination is the quantitative comparison of target nucleic acid, expressed in orders of magnitude concerning a calibrator.

Variants have been derived from both techniques, such as Multiplex PCR and ICC-PCR (integrated-cell culture PCR) (Corpuz et al. 2020). The application of multiplex PCR allows the simultaneous detection of multiple viruses present in a single sample using more than one set of primers in one reaction. Multiplex qPCR also enables the detection of different specific viruses using more than one fluorescent reporter. In the ICC-PCR method, the samples in which the viruses may be present are used to infect cell culture media and incubated for several days. Then the samples of cell culture are subjected to freezing to cause lysis of the cells. If viable viruses were present in the original sample and invaded the culture cells, those are from their host cells when lysis occurs. After this, PCR-based amplification is applied to the lysate (Corpuz et al. 2020), so the ICC-PCR is an effective enrichment method for virus detection.

1.4.3.1 Detection of Coronaviruses in Samples of Waters by Molecular Methods

There are extensive reviews in the literature describing the persistence of coronavirus in different types of wastewaters. The integrity of the coronavirus particle in water depends upon the wastewater characteristics, presence of suspended solids, organic matter, and temperature (Mandal et al. 2020). The methods of early detection of the virus on waters help to detect future immediate outbreaks, allow to implement measures to minimize the infections and deaths caused by COVID-19, and new molecular techniques are currently being developed that allow the effective detection of other viruses relevant for public health such as adenovirus and poliovirus,

1.5   ESKAPE Bacteria as a Model of Antibiotic-Resistant Bacteria Distribution in Water

1.5.1   Antibiotics and Drug Resistance

The use of antibiotic-producing microbes to prevent disease goes to back more than 2000 years ago from the use of moldy bread to treat open wounds (Hutchings et al. 2019). Still, it was not until the twentieth century that the microbiologist Alexander Fleming discovered the origin of Penicillin in 1928, a substance produced by mold (*Penicillum notatum*), that was lethal to several bacteria giving rise to the birth of the antibiotic era (Patel 2016), but also to the next milestone in history "antibiotic resistance" statement made by Alexander Fleming during his Nobel Prize acceptance speech (1945) (Podolsky 2018) predicting the extent and severity of antibiotic resistance over the next 10 years, statement that then became a reality, with hundreds of cases of resistance recorded throughout the world (Rosales Magallanes 2018).

Currently, antimicrobial resistance is one of the greatest threats to global health (Maillard et al. 2020). The widespread and inappropriate use of antibiotics has led to an increased prevalence of multidrug-resistant (MDR) bacteria in the environment. MDR infections led to 700,000 deaths globally in 2016 and are expected to rise to10 million by 2050 (Brooks et al. 2018). The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeroginosa,* and *Enterobacter* species) (Tigabu and Getaneh 2021) are a group of common opportunistic pathogens associated mainly with nosocomial infections (Santaniello et al. 2020), the acronym "ESKAPE" is associated to this group due to their ability to escape the antimicrobial activity and development of resistance to multiple antibiotics (multidrug-resistance) (Founou et al. 2018). The World Health Organization (WHO) listed in the critical priority list carbapenem-resistant *A. baumannii, P. aeruginosa, K. pneumoniae*, and *Enterobacter* spp., whereas vancomycin-resistant *E. faecium* (VRE) and methicillin/vancomycin-resistant *S. aureus* (MRSA or VRSA) in the list of high priority group (Mulani et al. 2019). The presence of the ESKAPE pathogens in the environment is likely due to contamination via sewage spills, hospital waste that has been discarded incorrectly; consequently, several antibiotic-resistant microorganisms and antibiotic-resistance genes (ARGs) have been detected in water bodies (Denissen et al. 2022). The water sources represent a reservoir of various chemical and microbiological pollutants such as chemical, heavy metal, organic pollutants, residues of pharmaceuticals (Voigt et al. 2020), including horizontal gene transfer of resistance genes, the misuse or overuse of antibiotics, and the contamination through livestock slurry and plant wastewater (Daniel et al. 2017) are factors that contribute to genetic selection pressure for the development of MDR bacterial infections in the community (Wang et al. 2018).

1.5.2 *Enterococcus*

Enterococci is a Gram-positive bacteria that commonly resides in the intestinal tracts of humans and animals but is also found in water, soil, and plants due to their high tolerance to different conditions (Cho et al. 2020), they can survive in a variety of environments, such as soil, water, food, plants, and animals (Castillo-Rojas et al. 2013), and they can survive in a wide range of temperatures (10–45 °C), pH (4.4–9.6), and in a hypersalty media with 6.5% NaCl (Ben Braïek and Smaoui 2019). Nowadays, *Enterococcus* is the leading cause of nosocomial infections, responsible for causing endocarditis, urinary tract infections, and bloodstream infections (Raza et al. 2018). There are more than 50 species of enterococci where *E. faecium* and *E. faecalis* are the most ecology and epidemiological relevant (Ramos et al. 2020). Furthermore, enterococci have intrinsic antimicrobial resistance to aminoglycosides, and cephalosporins antibiotic agents and can adapt to acquire resistance to antimicrobials such as β-lactams and vancomycin from the environment (Lee et al. 2019), by mutation and/or acquisition of genes through the plasmids and transposons (Grassotti et al. 2018). Vancomycin is an antibiotic of last resort used to treat MRSA. The vancomycin-resistance among clinical isolates is a growing health problem that involves the exchange of *van* genes between strains; where *vanA* and *vanB* are the most prevalent genotypes in *E. faecium* in clinical isolates (Melese et al. 2020), where the *vanA* gene encodes an enzyme that modifies the vancomycin binding site through the substitution of D-ala-D-ala by D-ala-D-lactate (Liu et al. 2021). Water ecosystems, in particular wastewater treatment plants, are considered one of the main hotspots of the evolution and spread of antibiotic-resistant bacteria (ARB) and ARGs into the natural environment that leads easily reach communities via aquatic environments (Ekwanzala et al. 2020). Enterococci and antimicrobials are commonly excreted in urine and feces, and most of this waste is treated in wastewater treatment plants where *E. faecalis* and *E. faecium* are dominant species identified before being discharged into surface water mainly during the dry season (Sanderson et al. 2020). *E. faecalis* and *E. faecium* are also used as an indicator of water quality criteria by fecal contamination in freshwater in the United States of America at geometric means of 33 and 35 CFU/100 mL (Wen et al. 2020) due to they are more resistant to stress than *E. coli* and other fecal coliform bacteria (Chidamba and Korsten 2018). Nevertheless, the distribution of resistant bacteria from the environment to humans may occur through contaminated food, manure, and contaminated surface water used for irrigation (Taučer-Kapteijn et al. 2016).

1.5.3 *Staphylococcus*

Staphylococcus aureus is a commensal opportunistic Gram-positive bacterium in humans (Pasachova et al. 2019) and are able to survive at a wide range of temperatures, dryness, dehydration, and low water activity (Silva et al. 2020). The genus *Staphylococcus* comprises more than 50 species, including coagulase-positive (*S. aureus*) and coagulase-negative staphylococci (*E. epidermidis*) (Kosecka-Strojek

et al. 2019). *S. aureus* is one of the main pathogens in hospital and community infections associated with skin infections, pneumonia, and nosocomial bacteremia and represents public problem health (Cheung et al. 2021). The staphylococci became antibiotic resistant through genetic mutations, reducing outer membrane proteins, and acquiring resistance genes through HGT (Guo et al. 2020). The methicillin resistance in *S. aureus* (MRSA) is a significant global health concern determined by the expression of *mecA* gene, which encodes a penicillin-binding protein (PBP2a) with low affinity for β-lactam agents (Watkins et al. 2019). MRSA strains show a multidrug-resistant pattern with resistance to fluoroquinolones, macrolides, aminoglycosides, clindamycin, penicillins, cephalosporins, and carbapenem, making β-lactams ineffective (Gajdács 2019). MRSA is known as the major cause of hospital-/community-acquired infections, and morbidity infections are elevated worldwide. On the other hand, vancomycin has been one of the first-line drugs to treat MRSA infections; nowadays the intermediate and complete vancomycin-resistant *S. aureus* (VRSA) is a severe public health concern (Cong et al. 2020).

Environmental factors such as pH, temperature, nutrient content, salinity, and dissolved oxygen play important roles in biofilm development, influencing their persistence in water, especially in piping due to inefficient water treatment (Silva et al. 2022). Despite this, wastewater treatment reduces the prevalence of *S. aureus*, thus reducing its presence in surface water, a fact attributable to the fact that the effluents are diluted in the river water (Zieliński et al. 2020). Although Staphylococci are less frequent in surface water than *E. coli, Enterococcus,* and other ESKAPE pathogens, they are indicators of antimicrobial resistance in the environment mainly by the presence of *mecC* gen associated with *S. aureus* (Silva et al. 2021).

1.5.4 *Klebsiella pneumoniae*

Klebsiella spp. are Gram-negative and nonmotile bacteria resident in the environment (surface water, sewage, soil, and plants), and on mucosal surfaces such as humans and animals (Bengoechea and Sa Pessoa 2019). In humans, *K. pneumoniae* is a commensal and opportunistic pathogen found in gastrointestinal and respiratory tracts and skin of healthy people, and is responsible for causing community-acquired and nosocomial infections (urinary, respiratory tract infections, and infections of wounds and soft tissue) (Herridge et al. 2020). In contrast to other nosocomial pathogens, *K. pneumoniae* clones are divided into two phenotypic groups characterized by multidrug resistance and hypervirulence (Gonzalez-Ferrer et al. 2021). *K. pneumoniae* shows high resistance to a broad spectrum of antibiotics such as ESBL, carbapenem, quinolones, polymyxin, tigecycline, fluoroquinolones, and aminoglycosides. Recently, the WHO recognized extended-spectrum β-lactam (ESBL) producing and carbapenem-resistant *K. pneumoniae* as a critical public health threat (Wyres et al. 2020).

β-lactam antibiotics are the first-line option for the treatment of infections, but ESBL enzymes hydrolyze β-lactams (penicillins, monobactams, carbapenems, and

cephalosporins) (Pillai 2022); on the other hand, the carbapenems are β-lactam antibiotics of last resort with the broadest spectrum of activity, that, in less than a decade after their use, become a global public health crisis (Cherak et al. 2021), mainly because infections caused by the ESBL/carbapenemase-producing *Enterobacteriaceae* are associated with increased mortality, length stay, and health-care costs (De angelis et al. 2020).

The environment is an important reservoir for disseminating ARB, antibiotic-resistance genes, and mobile genetic elements that interact and spread to other parts or to human and animal hosts (Samreen et al. 2021). Carbapenemase and ESBL-producing *K. pneumoniae* have been reported in aquatic environments, such as rivers, hospital effluents, and posthospital wastewater (Furlan et al. 2020). Some human health concerns associated with antibiotic resistance in the environment are the alteration of the human microbiome toward the emergence of antibiotic resistance and the potential hazard of selective pressure to create an antibiotic resistance environment (Ben et al. 2019). Nevertheless, measurements of the amounts and types of hazards (ARBs and ARGs) associated with environmental sources (water, air, soil, and food) that are related to the acquisition of resistant infections in humans are essential to establish suitable mitigation strategies (Vikesland et al. 2017).

1.5.5 *Acinetobacter baumannii*

The genus *Acinetobacter* comprises a group of Gram-negative, nonfermenting, strictly aerobic, catalase-positive and oxidase-negative coccobacillus (Torres et al. 2010). *Acinetobacter* species are widely distributed in the environment, making it possible to recover *Acinetobacter* isolates in almost 100% of the soil and water samples, as these are considered their main ecological niches (Salazar de Vegasa and Nieves 2005). Among the 32 varieties of species of the genus *Acinetobacter*, the most representative species is *Acinetobacter baumanii*, whose, while being distributed in nature, prevalence is strongly associated with the clinical-hospital environment, where some strains can survive in wet or dry inanimate objects in the hospital environment for weeks or months; in addition, it is a microorganism that is part of the normal flora of human skin and is distinguished as the microorganism that is most frequently carried persistently on the skin by hospital personnel (Phillips 2014).

Thus, all these factors increase the chances that patients will be colonized, in addition to promoting contamination of medical equipment, thus playing a determining role in the spread of infection and prolonged hospital outbreaks. The risk factors for *Acinetobacter baumannii* infection will depend on the type of infection (intrahospital, extrahospital) and its drug resistance profile, the latter being the most important. Community-acquired *Acinetobacter baumannii* infections, unlike those acquired in the hospital environment, are reported less frequently, because other risk factors are involved, such as residence in a tropical developing country, alcoholism, smoking, or diseases such as diabetes mellitus and chronic lung disease, conditions that easily allow infection to result in bacteremia or pneumonia.

It is worth mentioning that despite being rarely detected, this type of infection is of greater concern once it is acquired in the community, since it is a more severe infection than nosocomial infection and, in most cases, it is fulminant, dying in 60% of patients (Antunes et al. 2014). The *A. baumannii* genome contains several genes grouped into resistance islands, whose structure, in addition to providing high intrinsic resistance to antimicrobials, facilitates the acquisition of resistance mechanisms from other species of bacteria present in its environment, such as *Pseudomonas* spp. through mobile genetic elements (plasmids, transposons and integrons) (Agodi et al. 2013; Fournier et al. 2006), thus giving rise to an *Acinetobacter* isolate with a multidrug-resistant (MDR) phenotype with the capacity to produce extended-spectrum beta-lactamases, which confers resistance to most of the known antimicrobials, including carbapenems and colistin (Towner 2009). Therapeutic difficulties, added to the great capacity that it must survive for a long time in the hospital environment, together with the potential to develop in an acidic pH and at low temperatures, which allows it to increase its ability to invade devitalized tissues, together with the development of a biofilm ("biofilm") on human surfaces and cells, constitute this microorganism as an emerging public health problem (Gaddy and Actis 2009).

1.5.6 *Pseudomonas aeruginosa*

Bacteria of the genus *Pseudomonas* are Gram-negative microorganisms that can be classified as facultative anaerobes and are characterized as being ubiquitous and preferring humid environments (Luján Roca 2014). Specifically, *Pseudomonas aeruginosa* is a bacterium that successfully colonizes an enormous diversity of niches, as a consequence of its great nutritional versatility, it can survive in extreme environmental conditions; however, naturally, we can find it widely distributed in nature, its reservoir is the moist soil, water, wastewater, vegetation, humans and animals, humans and animals being the main hosts (CDC 2016). *P. aeruginosa* is an opportunistic pathogen responsible for a wide range of infections, mainly nosocomial, which is why it is considered highly prevalent in infections associated with health-care infections (HAIs), since it has a high adaptation to repeated changes in the microenvironment, nutrient availability, resistance mechanisms, and capacities to form biofilms (Villanueva-Ramos et al. 2019).

In humans, the species most at risk from infection is *Pseudomonas aeruginosa*, but infections by other species such as *P. paucimobilis*, *P. putida*, *P. fluorescens*, or *P. acidovorans* may also occur (Dumaru et al. 2019). Invasive infections by *P. aeruginosa* occur mainly in patients with underlying diseases that cause immunosuppression and mostly occur in the hospital setting. Community-acquired infection may occasionally occur in previously healthy patients. These infections are usually localized at the skin level (folliculitis, cellulitis, or ecthyma gangrenosum) without associated bacteremia, or present as severe clinical forms (Brady 2004; De almeida et al. 2002;). In the case of community-acquired infections, such as sepsis due to *Pseudomonas aeruginosa* in previously healthy patients, it is an infrequent

clinical entity that must be considered when ecthyma gangrenosum, neutropenia, and gastrointestinal manifestations are present.

However, there is a possibility that *P. aeruginosa* can colonize parts of the human body; however, the prevalence of this colonization in healthy people is low (Rossolini and Mantengoli 2005). In case of acquiring an infection caused by *P. aeruginosa*, the vast majority of these will be related to the hospital environment, constituting a severe clinical problem, since, in most cases, there is a compromise of the host's defenses (Lyczak et al. 2000). This is because once the infection is established, *P. aeruginosa* produces a series of toxic compounds that cause not only extensive tissue damage but also interfere with the functioning of the immune system. Among the proteins involved in *P. aeruginosa* infection, we find toxins, as well as hydrolytic enzymes that degrade the membranes and connective tissue of various organs. This situation is aggravated by the difficulty in treating *P. aeruginosa* infections, since this bacterium is intrinsically resistant to multiple classes of antibiotics that are not structurally related to each other (Strateva and Yordanov 2009) due to decreased permeability of its outer membrane, the constitutive expression of several efflux pumps, and the production of enzymes that inactivate antibiotics. In addition, it can acquire new resistance mechanisms via mutations (Mesaros et al. 2007). Furthermore, in aqueous environments, this bacterium adheres to surfaces, producing a kind of aggregate called a biofilm. The formation of these accumulations of bacteria and extracellular material represents a health problem, since it contaminates devices implanted inside the body, such as intrauterine devices, catheters, or heart valves. Biofilms also represent a problem in the production process of various industries as they cause clogging and corrosion of connections and filters (Villanueva-Ramos et al. 2019). It is estimated that 65% of bacterial infections are due to the formation of biofilm and its mechanisms of tolerance to antibiotics, which represents a problem in the health field; the appearance of biofilms is strongly associated with recurrent chronic and delayed wound healing (Romeo 2020). When growing in a biofilm state, *Pseudomonas aeruginosa* increases tolerance to antibiotics up to a thousand times more than when it grows in its free form (Torres et al. 2010) and is mainly associated with the following mechanisms: quorum sensing (QS) signal, porins, efflux pumps, gene expression, membrane vesicles, extracellular DNA, and enzymes (Bolívar-Vargas et al. 2021). *P. aeruginosa* appears as an exceptional bacterium; the wide variety of virulence factors, the breadth of infections it causes, and its multiple mechanisms of resistance to antibiotics make it stand out among the pathogenic microorganisms for humans.

1.5.7 *Enterobacter* spp.

The *Enterobacter* genus is a member of the ESKAPE group, which contains the main resistant bacterial pathogens (Rice 2010). It consists of fermentative, facultative anaerobic, Gram-negative bacterial species belonging to the Enterobacteriaceae family. This genus is associated with a variety of environmental habitats, their presence in the intestinal tract as natural commensals of animal and human intestinal

microbiota resulting in their wide distribution in soil, plants, water, and wastewater (Singh et al. 2018). Among this genus of bacteria, only certain subspecies/species have been associated with hospital-acquired infections and outbreaks. In humans, multiple *Enterobacter* species are known to act as opportunistic pathogens (disease-causing organisms): species, such as *E. cloacae*, *E. aerogenes*, *E. gergoviae*, and *E. agglomerans*, are associated with hospital-acquired infections and outbreaks (Akbari et al. 2016). These bacteria cause a variety of conditions, including eye and skin infections, meningitis, bacteremia (bacterial infection blood), pneumonia, and urinary tract infections.

The *Enterobacter aerogenes*, *E. cloacae*, and *E. hormaechei* species represent the most frequently described isolated species in clinical infections, especially in immunocompromised patients hospitalized in an intensive care unit (ICU), due to the adaptability of these species to antimicrobial agents and their behavior as opportunistic pathogens. These pathogens are frequently associated with a multidrug resistance (MDR) phenotype, mainly due to their adaptation to the hospital setting and their ability to readily acquire numerous mobile genetic elements containing resistance and virulence genes. These species have intrinsic resistance to ampicillin, amoxicillin, first-generation cephalosporins, and cefoxitin due to the expression of a constitutive AmpC β-lactamase. In addition, the production of extended-spectrum β-lactamases has been reported in these bacteria, making their treatment difficult (Davin-Regli et al. 2016). Antibiotic resistance, the regulation of resistance genes, and the expression of extended-spectrum beta-lactamases and carbapenemases significantly reduce therapeutic options, creating a particularly worrisome scenario with this microorganism as the protagonist of a global crisis of multiresistance to antibiotics.

Recent studies show that carbapenem-resistant enterobacteriaceae (CRE) can contaminate aquatic environments such as marine surface waters, rivers, estuaries, and contaminated drinking water (Mahon et al. 2019). CRE can reach aquatic bodies as a consequence of organic contamination from multiple sources (Mathys et al. 2019), including hospital effluents, wastewater treatment plants (PTARs), discharges from livestock and agricultural farms, water seepage, and others. Once mixed with the aquatic body, these effluents can introduce not only resistant microorganisms but also high doses of antibiotics, likely triggering the spread of resistance (Aydin et al. 2019). However, evaluating the link between the clinical and aquatic epidemiology of CRE is often challenging. While some studies have shown that clinical strains can be found in aquatic bodies, others have yet to prove such a link (Piedra-Carrasco et al. 2017).

1.5.8 Current Situation of Resistance

During the last 20 years, an increase in resistance has been observed in the 6 bacteria of the ESKAPE group; this represents a new crisis and a global public health problem (WHO 2015). Specifically, in Latin America, more than 50% of infections acquired in intensive care units (ICU) are caused by ESKAPE bacteria, with a

growing tendency toward extreme drug resistance (resistance to all families of antimicrobials except 2 or 1 of them) and pan drug resistance (resistance to all families of antimicrobials) (WHO 2015).

Selective pressure is the most important cause of the dissemination and extension of resistance; in the last 70 years, the indiscriminate use of antibiotics had contributed to the genetic diversification of resistance genes, as can be seen in the current number of TEM beta-lactamases, where to date, there are at least 187 described, when in 1982 before third-generation cephalosporins were introduced into the clinic, only TEM-1 and TEM-2 were known (Corvec et al. 2013).

Antimicrobial resistance is favored by the inappropriate use of antimicrobials in human medicine, veterinary medicine, agriculture, and aquaculture. Insufficient prevention and control measures for infections associated with hospital care, incomplete treatment by patients, and lack of hygiene and sanitation, are the other factors that complicate global efforts to contain it (Bolívar-Vargas et al. 2021). The prevalence of resistance has not only affected the efforts made to hold it but has also complicated the interpretation of the phenotypic profile and addressing the appropriate treatment, since the association of different resistance mechanisms for the same is increasingly frequent.

Furthermore, the prevalence of resistance has not only increased in infection-causing bacteria. Intestinal colonization of healthy people by ESBL-producing Enterobacteriaceae has reached pandemic levels worldwide in just a few years, and it is estimated that there are 1753 million colonized people worldwide (Phillips 2014; Woerther et al. 2013). Other factors that contribute to the extension and dissemination of resistance are a consequence of the current situation in the world, where the hyperconnection among borders, for example, the food and animal trade, tourism, health and business trips, emigration, and refugees, among other events, allows resistant strains to reach anywhere (Rogers et al. 2011). In addition, wild animals can also act as a reservoir and a potential source of dissemination. An example of this is the presence of bacteria resistant to antibiotics in migratory birds, a situation that could undoubtedly favor the spread of resistance over long distances (Simoes et al. 2010). In all of the above examples, water bodies do play an important role as reservoirs and distribution nodes of antibiotic-resistant bacteria, especially those of the ESKAPE group, which, besides commonly being multidrug resistant, have an enhanced ability to transfer antibiotic-resistance genes to related bacteria present in the water bodies.

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