Title: Bacterial diversity and antibiotic resistance in water habitats: searching the links with the human microbiome

One-sentence summary: In this review antibiotic resistance dissemination is discussed based on bacterial diversity and ecology in water habitats and in the human body.

Ivone Vaz-Moreira[1], Olga C. Nunes[2], Célia M. Manaia[1*]

[1] CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa/Porto, Rua Dr. António Bernardino Almeida, 4200-072 Porto, Portugal

[2]LEPAE – Laboratório de Engenharia de Processos, Ambiente e Energia, Departamento de Engenharia Química, Faculdade de Engenharia, Universidade do Porto, Rua Dr. Roberto Frias, 4200-465 Porto, Portugal

Author for correspondence (*):
C.M. Manaia, PhD
Escola Superior de Biotecnologia
Universidade Católica Portuguesa
Rua Dr. António Bernardino de Almeida
4200-072 Porto, Portugal
Tel: +351 22 5580059
Fax: +351 22 5090351
e-mail: cmanaia@porto.ucp.pt

Running title: Antibiotic resistance in water and human-associated microbiome

Keywords: drinking water, wastewater, urban water cycle, microbiome intersections; natural resistome; contaminant resistome

Abstract

Water is one of the most important bacterial habitats on Earth. As such, water represents also a major way of dissemination of bacteria between different environmental compartments. Human activities led to the creation of the so-called urban water cycle, comprising different sectors (waste-, surface, drinking water), among which bacteria can hypothetically be exchanged. Therefore, bacteria can be mobilized between unclean water habitats (e.g. wastewater) and clean or pristine water environments (e.g. disinfected and spring drinking water) and eventually reach humans. In addition, bacteria can also transfer mobile genetic elements between different water types, other environments (e.g. soil) and humans. These processes may involve antibiotic resistant bacteria and antibiotic resistance genes. In this review, the hypothesis that some bacteria may share different water compartments and be also hosted by humans is discussed based on the comparison of the bacterial diversity in different types of water and with the human-associated microbiome. The role of such bacteria as potential disseminators of antibiotic resistance and the inference that currently only a small fraction of the clinically relevant antibiotic resistome may be known is discussed.

Introduction

The development and spread of antibiotic resistance among bacteria is considered a universal threat to human, animal and environmental health. Numerous studies have demonstrated the importance of the environmental settings (e.g. water or soil) on the cycling of antibiotic resistance in nature, either because antibiotic resistance mechanisms can originate in environmental bacteria or because human and animal commensals and pathogens can contaminate the environment (Riesenfeld *et al.*, 2004; Baquero *et al.*, 2008; Martinez, 2008; Zhang *et al.*, 2009; Allen *et al.*, 2010).

Water is one of the most important bacterial habitats on Earth, is a major way of dissemination of microorganisms in nature and has been recognized as a significant reservoir of antibiotic resistance (Baquero *et al.*, 2008; Zhang *et al.*, 2009; Rizzo *et al.*, 2013). As a microbial habitat, water may represent the origin of resistance genes, be an amplifier and/or reservoir of genes already acquired by human pathogens and released as pollutants in the environment or act as a bioreactor, facilitating the interchange of resistance genes between pathogenic and non-pathogenic bacteria (Poirel *et al.*, 2005; Baquero *et al.*, 2008; Rizzo *et al.*, 2013). However, and in spite of the intense research in this area over the last years, it is not clear under which circumstances water bacteria are important sources of novel mechanisms of antibiotic resistance or when do they act as carriers or helper elements that, somehow, facilitate the spread of antibiotic resistance.

Another question, still unanswered, regards the modes by which antibiotic resistance in water may be relevant for human health. Because antibiotic resistance is harbored and transferred by bacteria, a better understanding of the bacterial diversity and ecology may bring interesting insights into the modes of resistance dissemination from and into humans. This approach is now possible because numerous studies conducted worldwide

have explored the bacterial diversity in water habitats over the last decades. In parallel, the human microbiome project has stimulated the thorough characterization of the diversity of bacteria that permanently or transiently can colonize the human body. The combination of both datasets may bring interesting information for the discussion of antibiotic resistance transmission from water to humans and vice-versa.

This work discusses the hypothesis that bacteria sharing different water compartments and also the human body may represent important pieces in the network of antibiotic resistance dissemination. In addition, the cross-comparison of the bacterial diversity in human and water habitats *versus* the currently identified antibiotic resistance genes is used to sustain the hypothesis that an important fraction of the clinically relevant antibiotic resistome may be yet to be unveiled.

The urban water cycle

Over the centuries, humans settled their lives preferentially in sites around water reservoirs, creating high population densities in these areas and also major sources of pollution. The implementation of sanitation processes capable of removing contaminants (chemical pollutants, organic matter, microorganisms) from wastewater before its discharge into the natural environment became a priority. In the same way, the supplying of clean and safe drinking water, often requiring purification and disinfection, is nowadays regarded as a basic human right, essential for an effective policy for health protection (WHO & UNICEF, 2000). Throughout the years, the scientific knowledge and numerous technologic advances contributed to the continuous improvement of processes for the provision of safe water and appropriate disposal and treatment of wastewater. These two stages constitute the man-made or urban water cycle.

Bacterial diversity in water habitats

Freshwater habitats are amongst the natural habitats that harbour the richest bacterial diversity (Tamames *et al.*, 2010). In a comparative study involving 16S rRNA gene sequences from 3502 sampling experiments of natural and artificial bacterial habitats, Tamames *et al.* (2010) concluded that soil and freshwater, represented by aquifers, groundwater, lakes, rivers, drinking water and wastewater, are the natural habitats that harbour the largest number and most diverse group of bacterial lineages. In the current study the bacterial diversity in different freshwater habitats within the urban water cycle was compared (Fig. 1 and Table S1). This comparison was based on studies published after 1995 in journals indexed to the ISI – Web of Knowledge, in which the major objective was the analysis of the water bacterial diversity, supported by 16S rRNA gene sequence analysis.

At high taxonomic ranks of phylum or class, in general, the most predominant bacteria belong to the phyla *Proteobacteria* (mainly of the classes *Alpha-*, *Beta-* and *Gammaproteobacteria*), *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, irrespective of the type of water – surface (lakes, rivers, wetlands), mineral, drinking and wastewater (Fig. 1, Table S1 and Table S1 references). However, different types of water present distinct patterns of bacterial diversity at lower taxonomic ranks, of genus or species. At least this was the conclusion drawn whenever, according to the publications supporting this comparison, the 16S rRNA gene sequence analysis allowed such a discrimination. An apparent specificity for some types of water was observed. For example, members of the class *Betaproteobacteria* and of the phylum *Bacteroidetes* were frequently detected in surface, mineral and drinking water, but not so often in wastewater. In turn, *Firmicutes* were frequently reported in wastewater. Ubiquitous bacteria are those with low specificity, occurring in different environments, including throughout the urban

water cycle or in the interface air-water-soil (Tamames *et al.*, 2010) (Fig. 1 and Table S1). At the genus rank, examples of the most ubiquitous bacteria in water habitats, *i.e.* those detected in wastewater, surface- and drinking water, are members of the genera *Acidovorax*, *Curvibacter*, *Sphingomonas*, *Aeromonas*, *Acinetobacter*, *Pseudomonas*, *Legionella*, *Rhodococcus*, *Gordonia*, *Mycobacterium*, *Flavobacterium*, *Bacillus* and *Clostridium* (Fig. 1 and Table S1). Bacteria belonging to these groups, and others still unidentified, are probably capable of circulating between different aquatic habitats, spanning the whole urban water cycle.

The use of culture independent approaches, mainly the high throughput sequencing methods, brought a renewed perspective of the bacterial diversity in water habitats, in which less than 0.1% of bacteria can be cultivated (Amann *et al.*, 1995; Simon & Daniel, 2011; Vaz-Moreira *et al.*, 2013). These approaches revealed that bacteria still unidentified below the phylum or class levels are detected in every type of water (Table S1). This is particularly notorious for some bacterial phyla/classes, which despite the apparent poor culturability are common water inhabitants. Good examples of groups almost or exclusively detected by culture-independent methods are members of *Delta-* and *Epsilonproteobacteria*, *Acidobacteria*, *Verrucomicrobia*, *Cyanobacteria*, *Nitrospirae*, *Planctomycetes*, *Chloroflexi*, *Chlorobi*, *Gemmatimonadetes*, *Spirochaetes*, *Chlamydiae*, *Aquificae*, *Thermotogae*, *Fusobacteria*, *Synergistetes* and *Tenericutes*, some of them including bacteria ubiquitous in water habitats (Fig. 1, Table S1). Nevertheless, culture-independent methods, even high throughput sequencing, may fail on the detection of some bacterial groups, in particular the less abundant organisms (Pinto & Raskin, 2012). Different biases (e.g., DNA extraction, PCR or sequence data analysis) may hamper the detection of certain community members. On the other hand, the 16S rRNA gene sequence analysis, particularly of small gene fragments as those

147 generated with high throughput sequencing methods, may not allow a reliable
148 identification of bacteria (e.g. Clarridge, 2004). These arguments may explain why
149 bacteria of the genera *Escherichia* or *Enterococcus*, used as indicators of faecal
150 contamination, and frequently detected in wastewater habitats at counts as high as 10^4-
151 10^6 colony forming units per mL (Garcia-Armisen & Servais, 2004; Ferreira da Silva *et*
152 *al.*, 2007; Levantesi *et al.*, 2010) are not detected in studies surveying the bacterial
153 diversity, as those summarized in Fig. 1. The low abundance of these bacteria in water
154 habitats, even in those with faecal contamination, is also suggested by cultivation
155 procedures. Indeed, the cultivation of *Escherichia* or *Enterococcus* usually requires the
156 use of selective culture media, while on general culture media, such as Plate Count
157 Agar, if isolated, they represent a small fraction of the cultivable populations. Although
158 both approaches are truly complementary to explore the bacterial diversity of an
159 ecosystem, the current state of the art suggests a poor synchronization between culture-
160 independent and culture-dependent methods. This represents a serious limitation in a
161 comprehensive analysis of the bacterial diversity, mainly when the assessment of the
162 features such as metabolism, physiology, genetics, virulence and antibiotic resistance of
163 a specific group is under discussion. Expectably, one of the major outcomes of the
164 implementation of culture-independent methods will be the improvement of cultivation
165 methods and the strengthening of studies based on pure cultures (Anonymous, 2012;
166 Lagier *et al.*, 2012; Prakash *et al.*, 2013). These advances will be indispensable to the
167 thorough assessment of possible intersections between distinct microbiomes, for
168 example, environmental and human.

169

170 **Evidences of the natural antibiotic resistome**

Over the last 70 years, clinically-relevant antibiotic resistance, *i.e.* in pathogens and opportunistic bacteria, increased to worrisome levels, mainly in areas with strong human intervention (Baquero *et al.*, 2008; Martinez, 2009; Andersson & Hughes, 2011; Cantón & Morosini, 2011). Nevertheless, antibiotic resistance is a natural property of bacteria, occurring in environments with reduced or null anthropogenic impacts, such as wild life or remote Earth zones (Riesenfeld *et al.*, 2004; D'Costa *et al.*, 2006; Dantas *et al.*, 2008; Allen *et al.*, 2010; D'Costa *et al.*, 2011; Segawa *et al.*, 2013). In part this can be due to the fact that antibiotics production is ancient in nature, with more than 10^6-10^9 years (D'Costa *et al.*, 2011). Functions, as diverse as molecular signaling, transcription activation, enhanced gene transfer, stimulation of bacterial adhesion, increased mutation frequency or virulence suppression, have been attributed to antibiotics produced in nature (Davies *et al.*, 2006; Wright, 2007; Dantas *et al.*, 2008; Sengupta *et al.*, 2013). Eventually these functions will vary among the target bacteria and will depend on the genetic and physiological environment of the cell. Accordingly, natural antibiotic resistance mechanisms are those that make these molecules compatible with the normal cell function (Wright, 2007; Sengupta *et al.*, 2013). Natural antibiotic resistance has been studied in depth in soil bacteria of the phyla *Actinobacteria*, *Proteobacteria*, or *Bacteroidetes*, mainly in those yielding antibiotic production or degradation activity (Riesenfeld *et al.*, 2004; D'Costa *et al.*, 2006; Dantas *et al.*, 2008; D'Costa *et al.*, 2011; Forsberg *et al.*, 2012). However, natural antibiotic resistance is not restricted to soil bacteria, being also reported in other environments, including water.

Mineral and spring waters are good examples of natural water habitats, since these aquifers originate in ground water sources and are protected from human intervention (Rosenberg, 2003; European Comission, 2009). Unlike tap water, mineral and spring water cannot be disinfected by any kind of treatment to remove or destroy

196 microorganisms (European Comission, 2009) and, thus, its microbiota mirrors the
197 natural populations of the aquifer. Because this type of water is known to contain a rich
198 microbiota and it is destined to human consumption, several studies have searched the
199 presence of antibiotic resistant bacteria (Rosenberg & Duquino, 1989; Massa *et al.*,
200 1995; Mary *et al.*, 2000; Messi *et al.*, 2005; Zeenat *et al.*, 2009; Falcone-Dias *et al.*,
201 2012). Although in some of these studies the experiments were not designed to survey
202 bacterial diversity and antibiotic resistance, it is possible to infer about the wide
203 diversity of antibiotic resistance patterns and the frequent occurrence of multi-resistance
204 phenotypes. Mineral or spring bottled waters commercialized in Italy, Portugal, France
205 and other world regions contained bacteria resistant to multiple antibiotics, distributed
206 by several genera and species (*Afipia*, *Bosea*, *Brevundimonas*, *Ochrobactrum*,
207 *Curvibacter*, *Ralstonia*, *Variovorax*, *Acinetobacter*, *Klebsiella*, *Moraxella*,
208 *Pseudomonas*, *Flavobacterium*, *Pedobacter*, *Arthrobacter*, *Corynebacterium*,
209 *Microbacterium*, *Micrococcus*, *Bacillus*, *Kurthia*, and *Staphylococcus*) (Massa *et al.*,
210 1995; Mary *et al.*, 2000; Messi *et al.*, 2005; Zeenat *et al.*, 2009; Falcone-Dias *et al.*,
211 2012). Bottled spring water bacteria can reach densities as high as 10^2 colony forming
212 units per mL and display resistance to more than 20 antibiotics belonging to eight
213 different classes, including 3th generation cephalosporins, carbapenems and
214 fluoroquinolones (Falcone-Dias *et al.*, 2012). It is remarkable that, in general, studies
215 conducted in different geographic areas and in different occasions demonstrate that the
216 natural microbiota of mineral and spring waters contains a myriad of antibiotic resistant
217 bacteria, as was observed before for pristine soils or ancient permafrost samples (e.g.
218 D'Costa *et al.*, 2006, 2011; Allen *et al.*, 2009). Many of these (multi-)drug resistance
219 phenotypes are probably intrinsic in these bacteria, and resistance transfer to human-

220 related bacteria can be considered highly unlikely. These considerations require a
221 further discussion about the nature of the environmental antibiotic resistome.

222

223 **Acquired, intrinsic and silent resistance: different assets in the same game**

224 Most of the discussions on antibiotic resistance are centered on acquired resistance,
225 resultant from gene mutation or genetic recombination by horizontal gene transfer
226 (conjugation, transformation or transduction) (Martinez & Baquero, 2000; Livermore,
227 2003; Tenover, 2006; Zhang *et al.*, 2009; Davies & Davies, 2010). Although these can
228 be random processes, in the presence of selective pressures, such as antimicrobial
229 residues, bacterial lineages with acquired antibiotic resistance will have an improved
230 fitness (i.e. a better capacity to survive and reproduce in comparison with bacteria
231 without acquired resistance), becoming more prevalent in the community (Barbosa &
232 Levy, 2000; Martinez 2009; Andersson & Hughes, 2011).
233 In contrast, the intrinsic resistome is described as an ensemble of non-acquired genes
234 with influence on the susceptibility to antibiotics (Fajardo *et al.*, 2008; Baquero *et al.*,
235 2013). This form of resistance comprises diverse mechanisms that can be related with
236 structural, physiological or biochemical properties of bacteria, such as reduced
237 permeability, metabolic functions, efflux systems, among others (Fajardo *et al.*, 2008;
238 Martinez *et al.*, 2008; Wright, 2010; Alvarez-Ortega *et al.*, 2011; Baquero *et al.*, 2013).
239 Intrinsic antibiotic resistance represents a characteristic phenotype of a species or
240 organism, resultant from multiple genes and, hence, is not easily transferable by
241 horizontal gene transfer. In the same way, it is not the direct consequence of adaptation
242 to antibiotics (Alvarez-Ortega *et al.*, 2011).
243 Since about 3% of the genes in a bacterial genome may be related with intrinsic
244 resistance processes (Fajardo *et al.*, 2008), it is expected that this native resistance form

represents an important fraction of the environmental antibiotic resistome. A well characterized intrinsic resistome belongs to the opportunistic pathogen *Pseudomonas aeruginosa*, which displays intrinsic resistance to a wide variety of antibiotics, resultant from a complex network of genes (Fajardo *et al.*, 2008; Alvarez-Ortega *et al.*, 2011; Breidenstein *et al.*, 2011). The low permeability of the external membrane, 12–100 times lower in *P. aeruginosa* than in *E. coli*, and the presence of some proteins involved in the alteration of cell metabolism, leading, for instance, to changes in the cell growth state, are supposed to represent the most important mechanisms of intrinsic resistance in this organism (Hancock, 1998; Alvarez-Ortega *et al.*, 2011; Breidenstein *et al.*, 2011). Although intrinsic resistance may be characteristic of a species, it is not necessarily common to all species members. In *E. coli*, point mutations in different *loci* were observed to promote reduced susceptibility to antibiotics such as ciprofloxacin, rifampin, vancomycin, ampicillin, sulfamethoxazole, gentamicin, or metronidazole (Tamae *et al.*, 2008). The potential of some members of a species to mutate towards significant reduction or increase in antibiotic susceptibility was observed in different species (e.g. *Helicobacter pylori*, *Acinetobacter baylyi*, *P. aeruginosa*), being probably species-specific (Gomez & Neyfakh, 2006; Fajardo *et al.*, 2008; Girgis *et al.*, 2009; Liu *et al.*, 2010). This kind of genome variations in bacterial populations is probably common in nature and may have interesting implications on the ecology of antibiotic resistant bacteria.

The implications of the intrinsic resistome on the evolution of acquired antibiotic resistance are not completely understood. However, the characterization of the intrinsic resistome genes may bring important contributes to predict the stability, emergence and evolution of antibiotic resistance (Martinez *et al.*, 2007; Fajardo *et al.*, 2008). In a community, it is possible that intrinsic resistance will drive bacterial selection, leading

to community rearrangements, mainly when selective pressures, as those imposed by antibiotics, are present (Baquero *et al.*, 2013). Hypothetically, if a bacterial population is intrinsically resistant, it will have higher chances to survive in the presence of antimicrobial residues, and to get in contact with potential resistance donors, proliferating more and faster than non-intrinsically resistant organisms. Thus, it can be hypothesized that intrinsic resistance, at least in some highly ubiquitous bacteria, may represent an advantage for resistance acquisition. A good example of how intrinsic resistance may favor resistance acquisition may be represented by *P. aeruginosa*, one of the opportunistic pathogens with highest potential to acquire antibiotic resistance (Breidenstein *et al.*, 2011).

A major question may be whether genes related with intrinsic resistance phenotypes may be transferred horizontally. Although such an event is not supposed to occur, at least at a high frequency, conceivably, it is not impossible. Other resistance determinants not included in the classical antibiotic-resistance genes, may also occur in nature, and bring interesting insights into the ecology of antibiotic resistance. Silent resistance genes are hidden forms of antibiotic resistance that do not confer resistance to its native host, although are capable of conferring resistance when expressed in other hosts (Dantas & Sommer, 2012).

In summary, the natural antibiotic resistome comprises three categories: i) those designated as acquired resistance genes, which correspond to the classical antibiotic-resistance genes, ii) the genes related with intrinsic resistance and iii) the silent resistance genes. Because some of these genes may respond to unspecific stimuli, and not only to antibiotics, they may contribute to the selection of the antibiotic unsusceptible populations (Dantas & Sommer, 2012; Baquero *et al.*, 2013). These

arguments reinforce the need to study antibiotic resistance in a global perspective either in the context of the cell genome or the whole bacterial community.

Antibiotic resistance in wastewater

Among the man-made environments, wastewater treatment plants (WWTP) are the most important receptors and suppliers of human derived antibiotic resistance (Manaia *et al.*, 2012; Rizzo *et al.*, 2013). The indicators of faecal contamination, *E. coli* and *Enterococcus* spp., are often used to monitor antibiotic resistance prevalence in urban wastewaters (Ur-WW). In these groups, high resistance prevalence values have been observed for antibiotics with a long history of use, such as aminopenicillins, sulfonamides and tetracyclines for *E. coli* or tetracycline and erythromycin for enterococci (Manaia *et al.*, 2012). Moreover, it is shown that conventional wastewater treatment does not contribute to reduce the fraction of antibiotic resistant bacteria, leading, sometimes, to its increase in the final effluent (Ferreira da Silva *et al.*, 2006, 2007; Łuczkiewicz *et al.*, 2010; Novo *et al.*, 2013). It is impressive that in different world regions and using distinct types of wastewater treatment, WWTP are responsible for the discharge of about one billion of culturable antibiotic resistant coliforms per minute to the environment (exemplified for ciprofloxacin resistance in Fig. 2). Despite the relevance of *E. coli* and *Enterococcus* as indicators of human faecal contamination, apparently these bacteria are not the most prevalent bacterial groups in sewage sludge or in wastewater (Sanapareddy *et al.*, 2009; McLellan *et al.*, 2010; Xia *et al.*, 2010b; Yang *et al.*, 2011; Wang *et al.*, 2012; Ye & Zhang, 2012; Zhang *et al.*, 2012) (Fig. 1). Indeed, *E. coli* and enterococci are probably minor representatives of the water bacterial communities. This conclusion leads us to a new dilemma. If most of the well-known bacteria in terms of antibiotic resistance are minor representatives of wastewater

communities, it is reasonable to argue that other community members, mainly the most abundant, may play also important roles as donors, receptors or simply mediators of antibiotic resistance dissemination.

Antibiotic resistance in aquaculture environments

In aquaculture, antimicrobials are routinely used through the direct addition into the water body. However, the negative impacts of this procedure have been demonstrated and include the persistence of antimicrobial residues in water and fish and the selection and spread of resistance genes, with the consequent contamination of the environment and the human food-chain (Sørum, 1998; Cabello *et al.*, 2006; Taylor *et al.*, 2011; Tamminen *et al.*, 2011). The spread of antibiotic resistance among fish pathogens has economic impacts on aquaculture productivity and increases the possibilities of the dissemination of resistance determinants to other bacteria, including human pathogens (Rhodes *et al.*, 2000; Cabello *et al.*, 2006). The long term effects are demonstrated by the fact that, even in the absence of selective pressures, when the antibiotic used was banned from an aquaculture system, genes conferring low susceptibility to that antibiotic will persist (Tamminen *et al.*, 2011). Bacterial diversity studies in aquaculture water bodies are scant, but the presence of some genera, such as *Yersinia*, *Vibrio*, *Photobacterium*, *Pseudomonas* and *Aeromonas*, is consistently reported (Sørum, 1998; Schulze *et al.*, 2006; Ozaktas *et al.*, 2012; Rodríguez-Blanco *et al.*, 2012). These genera comprise also some bacteria with important roles on antibiotic resistance spread, for example *qnr*A, encoding a DNA topoisomerase protector and the extended-spectrum beta-lactamase PER-6 (Poirel *et al.*, 2005; Girlich *et al.*, 2010a). Moreover, the dissemination of antimicrobial resistance in aquaculture environments may be

343 associated with other resistance determinants such as heavy metals or biocides
344 (Akinbowale *et al.*, 2007; Rodríguez-Blanco *et al.*, 2012, Seiler & Berendonk, 2012;
345 Cabello *et al.*, 2013).
346

347 **Antibiotic resistance in disinfected drinking water**

348 Despite the scarce information regarding antibiotic resistance in disinfected drinking
349 water, it was already demonstrated that it may contain bacteria, such as those of the
350 genera *Sphingobium*, *Sphingomonas*, *Pseudomonas* and *Acinetobacter* or non-faecal
351 *Enterobacteriaceae* capable of resisting different antibiotics (Faria *et al.*, 2009; Xi *et*
352 *al.*, 2009; Vaz-Moreira *et al.*, 2011b, 2012; Figueira *et al.*, 2012; Narciso-da-Rocha *et*
353 *al.*, 2013) (Table S2). For instance, *Sphingomonadaceae*, a bacterial group recognizedly
354 ubiquitous, rich in mobile genetic elements, and comprising common inhabitants of
355 environments contaminated with xenobiotcs, can be highly prevalent in disinfected
356 drinking water (Koskinen *et al.*, 2000; Furuhata *et al.*, 2007; Stolz *et al.*, 2009; Aylward
357 *et al.*, 2013). Tap water *Sphingomonadaceae* yield a rich and diversified resistance
358 pattern to penicillins, cephalosporins, carbapenems and aminoglycosides (Vaz-Moreira
359 *et al.*, 2011b), but their relevance on the spread of antibiotic resistance is unknown.

360 Independent studies have demonstrated that antibiotic resistant bacteria, at least for
361 some classes of antibiotics, may be more prevalent in tap than in the water source (Xi *et*
362 *al.*, 2009; Gomez-Alvarez *et al.*, 2012; Vaz-Moreira *et al.*, 2012; Narciso-da-Rocha *et*
363 *al.*, 2013). Such an effect may be due either to the selective effect of the disinfection
364 processes or to the income of antibiotic resistant bacteria downstream the disinfection
365 point (Gomez-Alvarez *et al.*, 2012; Vaz-Moreira *et al.*, 2013). This is a fundamental and
366 difficult to answer question, given the complex rearrangements in the bacterial

communities that result from the disinfection processes (Hoefel *et al.*, 2005; Eichler *et al.*, 2006; Kormas *et al.*, 2010; Figueira *et al.*, 2011; Vaz-Moreira *et al.*, 2013). However, strain tracking approaches do not support the conclusion that the water source is the most probable origin of the antibiotic resistance detected in tap water (Vaz-Moreira *et al.*, 2011b, 2012; Narciso-da-Rocha *et al.*, 2013). Regarding the origin of the antibiotic resistance found in drinking water, it has been observed that the majority of the resistance phenotypes in bacteria of groups such as *Sphingomonadaceae*, *Pseudomonas* or *Acinetobacter* is species dependent. This observation suggests a pattern of vertical inheritance of resistance and, thus, it can be hypothesized that antibiotic resistance in these organisms is probably intrinsic (Shehabi *et al.*, 2005; Vaz-Moreira *et al.*, 2011b, 2012; Narciso-da-Rocha *et al.*, 2013). Either being acquired or intrinsic, the impacts that antibiotic resistant bacteria present in drinking water may have on human-health are still unknown.

Antibiotic resistance genes throughout the urban water cycle

The tracking of antibiotic resistance genes in different environmental compartments is an important tool to assess the ecology and epidemiology of antibiotic resistance. Antibiotic resistance genes, encoding every known type of mechanism (target protection, target modification, drug modification, reduced permeability or efflux), are found throughout the urban water cycle (Table S2). These genes have been detected either in bacterial isolates or in total genomic DNA samples, using, most of the times, primers or probes targeting antibiotic resistance genes that are already known. Most of such primers and probes were designed based on genome sequences of bacterial isolates yielding a given resistance phenotype. Therefore, the vast majority of surveys of

391 antibiotic resistance genes rely, directly or indirectly, on cultivable bacteria recognized
392 as opportunists or pathogens. Examples of the most common hosts of the well-known
393 antibiotic resistance genes are members of the family *Enterobacteriaceae* (e.g. genera
394 *Klebsiella, Citrobacter, Enterobacter, Raoultella*) or the genera *Acinetobacter,*
395 *Aeromonas, Burkholderia*, *Pseudomonas, Enterococcus, Staphylococcus* and some
396 other that in total represent a humble fraction of the bacterial groups thriving in water
397 habitats.

398 Wastewater, in particular raw, is the richest water habitat in known antibiotic resistance
399 genes. There, can be found a typical signature of genes encoding resistance to "old"
400 antibiotics such as tetracyclines, sulfonamides, aminoglycosides and beta-lactams (e.g.
401 *tet*, *aac*, *dfr*, *sul*, class A beta-lactamases) (Table S2). Most of these genes are located in
402 plasmids and some are part of the variable gene cassettes of integrons and, probably,
403 can easily be mobilized amongst bacteria (Garcillán-Barcia *et al.*, 2011; Partridge,
404 2011). Recently, Zhang *et al.* (2011) demonstrated that plasmids, mainly harbored by
405 *Proteobacteria* of the classes *Alpha-*, *Beta-* and *Gamma-* and members of the genera
406 *Bacillus, Mycobacterium* and *Nocardiopsis*, some of which are abundant in wastewater
407 habitats, are relevant vectors of tetracycline, macrolide and multidrug resistance genes
408 in these environmental niches.

409 Studies reporting the diversity and abundance of antibiotic resistance genes in drinking
410 water are scarce. However, the occurrence of genes also detected in clinical isolates,
411 encoding resistance to beta-lactams, aminoglycosides, macrolides or sulfonamides is
412 described even in disinfected water (Table S2) (Faria *et al.*, 2009; Xi *et al.*, 2009;
413 Figueira *et al.*, 2012). The origin of these resistance genes in drinking water is still
414 unknown, being unclear in which cases it results from environmental contamination. A

major limitation to answer this question is related with the fact that most of the drinking water bacteria are of environmental origin and poorly or not at all characterized in terms of antibiotic resistance genes (Fig. 1, Table S1).

Commonly used arguments to explain the evolutionary success of acquired antibiotic resistance

Acquired antibiotic resistance is an emblematic example of biological evolution, driven by two major mechanisms – genetic variability (mutation and recombination) and selection (Thomas & Nielsen, 2005; Andersson & Hughes, 2010; Wiedenbeck & Cohan, 2011). Genetic variability results from gene mutation and horizontal gene transfer, in which the latter has more dramatic implications on the physiology and ecology of bacteria (Hausner & Wuertz, 1999; Arber, 2000; Miyahara *et al.*, 2011). On the other hand, antibiotics, even at sub-inhibitory concentrations, or other micro-pollutants such as heavy metals, contribute for the selection of resistant bacteria (Alonso *et al.*, 2001; Davies *et al.*, 2006; Tello *et al.*, 2012). However, the selection of antibiotic resistant bacteria may not represent the only consequence of the environmental contamination with antibiotics. Actually, the residues of antibiotics at environmental concentrations (often sub-inhibitory) are also correlated with disturbances on the structure and composition of bacterial communities in water habitats (Huerta *et al.*, 2013; Novo *et al.*, 2013). Moreover, in the environment, pollutants occur in complex mixtures, which make it difficult to predict their effects on the microbial communities. Processes of co- or cross-resistance, for instance, due to genetic linkage or to broad enzyme specificity, may lead to the selection of resistance genes in the absence of a selective pressure by antibiotics (Baker-Austin *et al.*, 2006; Harada & Asai, 2010). If

the above mentioned arguments could explain antibiotic resistance proliferation, acquired antibiotic resistance would be detected only in habitats such as wastewater or in the animal or human body, mainly in the gut, during antibiotherapy periods. However, this is not the case and antibiotic resistance determinants are found in environments where none of the above mentioned pressures are present (Harada & Asai, 2010). The strongest argument to explain the occurrence of recognized clinically relevant resistance genes in environments with no apparent selective pressure refers to the low fitness costs of antibiotic resistance genes (*i.e.* when antibiotic resistance acquisition do not reduce the survival and proliferation of a bacterium, even in the absence of selective pressures) (Andersson & Hughes, 2010; Gullberg *et al.*, 2011). The influence of compensatory mutations on the reduction of fitness costs imposed by acquired antibiotic resistance has been demonstrated (Björkman et al., 2000; Maisnier-Patin & Andersson, 2004; Handel *et al.*, 2006; Andersson & Hughes, 2010; Schulz zur Wiesch *et al.*, 2010; Tanaka & Valckenborgh, 2011). Since compensatory mutations may alleviate the fitness costs associated with a given acquired resistance, resistant and susceptible bacteria will display a comparable fitness in the environment, although with different levels of tolerance to antibiotics. As a consequence, strains harboring resistance and compensatory mutations may have a selective advantage in the environment, mainly in the presence of antimicrobial residues (Björkman et al., 2000; Handel *et al.*, 2006; Andersson & Hughes, 2010; Schulz zur Wiesch *et al.*, 2010). The importance of the environmental conditions on the selection of resistance and compensatory mutations is suggested by the fact that different fitness-compensating mutations are observed in bacteria thriving in mice or in a laboratory medium (Björkman et al., 2000). These evidences emphasize the complexity of the ecology of antibiotic resistance, although it seems reasonable to assume that as long as bacteria

464 and/or genetic elements are able to move across different water habitats, cross-
465 resistance and low fitness costs may explain why acquired antibiotic resistance can
466 reach habitats such as drinking water.

467 **Intersections between the water and the human-associated microbiome**

468 Increasing evidences on the diversity, metabolic and functional capabilities of the
469 microbiota associated with the human body show that microbial consortia play
470 important roles in disease and health conditions, although their roles are not yet
471 completely understood (Turnbaugh et al., 2007; Eloe-Fadrosh & Rasko, 2013).
472 Microorganisms colonizing or infecting humans may derive from different primary
473 habitats, and not only the human body, and play distinct roles in health or disease status.
474 The expression "human-associated microbiome" is herein used to refer to all
475 microorganisms capable of colonizing or infecting a human host independently of
476 which is their primary habitat.

477 Two types of intersection between the human-associated microbiome and water habitats
478 are expected. One refers to the release of bacteria from humans to wastewater. The other
479 comprises bacteria that being present in drinking water are also reported in the human-
480 associated microbiome. The first type of intersection was comprehensively analysed by
481 McLellan *et al*. (2010) who concluded that, as expected, only a small fraction of
482 bacteria excreted by humans were represented in sewage and even less were found in
483 surface water. Among the bacterial lineages found throughout these compartments, the
484 predominant were *Lachnospiraceae*, *Bacteroidaceae* and *Ruminococcaceae* (McLellan
485 *et al.*, 2010), groups poorly characterized in terms of antibiotic resistance. Other
486 intersections are widely known as those of the indicators *E. coli* and enterococci, which
487 representativeness in water and human-associated microbiomes is not so evident as

could be expected (Table S1) (Qin *et al.*, 2010; Arumugam *et al.*, 2011; The Human Microbiome Project Consortium, 2012).

The assessment of the second type of intersection is even more difficult. The occurrence of antibiotic resistant bacteria in drinking water may be important because of the harmful effects that this could have in the human health. In such case, transmission could be directly of water bacteria to humans or, indirectly, via transmission of resistance genes from water bacteria to human related bacteria (Fig. 3). Lee *et al.* (2010) used germ-free mice to demonstrate a correlation between the microbiota of drinking water and its presence in the gastrointestinal tract. However, this approach hardly can be used to infer about the fate of antibiotic resistant bacteria in the human gastrointestinal tract, given the richness and diversity of such habitat. Considering the value of taxonomy and phylogeny in the prediction of the ecology and physiology of bacteria, the currently available information about human and environmental microbiomes may allow interesting inferences. Using this rationale, the occurrence of the same bacterial lineages in drinking water and in the human-associated microbiome may be an indication of the fitness of those bacteria to the human body. In addition, it may suggest its potential to, under favorable conditions, *e.g.* antibiotherapy, suffer positive selection or promote horizontal gene transfer. The search of bacterial groups found in water habitats (Table S1) in the NIH Human Microbiome Project catalog (http://www.hmpdacc-resources.org/hmp_catalog) revealed that 35 groups, distributed by five phyla (*Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Cyanobacteria*), found in treated drinking water can also be detected in the human-associated microbiome (e.g. in the gastrointestinal tract, oral cavity or skin, including lesions). Identically, 19 lineages distributed by three phyla (*Proteobacteria*, *Actinobacteria* and *Firmicutes*), found in mineral water can also be found in the human-

associated microbiome (Table S1; Fig. 1). Probably, in the future, when more data are made available, more bacterial groups will be observed to be common to water environments and the human body. Nevertheless, it is already worthy of note that bacteria of the genera *Burkholderia*, *Acinetobacter*, *Aeromonas*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas* or *Clostridium* (Table S1), all of them with high potential to acquire antibiotic resistance genes (Zhang *et al.*, 2009), can be found in drinking water and in the human-associated microbiome. Others such as members of the genera *Sphingomonas* or *Methylobacterium* which exhibit resistance to several antibiotics, but about which almost nothing is known about antibiotic resistance genetics (Furuhata *et al.*, 2006, 2007; Vaz-Moreira *et al.*, 2011b), can also be found in water habitats and in the human-associated microbiome. The meaning of these evidences is still unclear but it may hint a link between water habitats and the human body, giving support to the hypothesis that water habitats may, directly or indirectly, supply antibiotic resistant bacteria for the human-associated microbiome (Fig. 3).

Missing links between natural and contaminant antibiotic resistance

Water and soil are regarded as important potential antibiotic resistance reservoirs, either natural or due to animal (and manure used as fertilizer) and human derived environmental contamination (Bush *et al.*, 2011; Forsberg *et al.*, 2012). However, except in a few well documented cases (e.g. *qnr* and $bla_{\text{CTX-M}}$) (Poirel *et al.*, 2002, 2005), it is difficult to demonstrate the passage of resistance genes from the environment to clinically-relevant bacteria or to clarify the mechanisms that made such a gene transfer possible. Previous studies have demonstrated that the human gut antibiotic resistome comprises an impressive myriad of antibiotic resistance genes not

identified before and evolutionarily distant from the currently known resistance genes (Sommer *et al.*, 2009). The increasing number of complete bacterial genome sequences, support this observation (http://www.ncbi.nlm.nih.gov/genome). Putative annotation data, available in public databases, suggests that multidrug resistance as well as other specific resistance mechanisms are widespread in *Bacteria*. However, the annotated function encoded by these genome sequences is not reliable to infer with accuracy the expected phenotypes, mainly because the phenotype encoded by a gene may depend on the genetic and physiological environment (e.g. silent resistance genes, Dantas & Sommer, 2012). Probably, most of the still unknown resistome is composed by resistance genes not yet validly annotated and others which expression is host-dependent. However, the clinical relevance of these genetic determinants as well as their influence on antibiotic resistance emergence is not clear yet. Although it can be hypothesised that the "unkown" human resistant microbiome may represent the missing link between the environment and the human pathogens, evidences that ingested products (food and water) can be the major sources of antibiotic resistance genes are still missing.

Antibiotic therapy imposes profound and long lasting rearrangements in the human-associated microbiome, characterized by the increase of *Proteobacteria* and the simultaneous reduction of other groups such as *Bacteroidetes* or *Firmicutes* (Young & Schmidt, 2004; Antonopoulos *et al.*, 2009; Jakobsson *et al.*, 2010; Jernberg *et al.*, 2010). Eventually, it can be argued that, under specific conditions (e.g. antibiotherapy), minor or silenced parts of the human antibiotic resistome may lead important microbial and genomic rearrangements responsible for resistance development. Apparently, the environmental and pathogenic resistomes are not distinct, with the same genes being detected in both, although with higher prevalence in the pathogenic resistome (D'Costa

562 *et al.*, 2006; Allen *et al.*, 2010; Forsberg *et al.*, 2012) (Fig. 3). Indeed, antibiotic
563 resistance genes and gene mobilization cassettes, many of which without recognized
564 clinical relevance, are widespread in nature, spanning numerous lineages of the bacterial
565 world (Cantón, 2009; Allen *et al.*, 2010). However, apparently only a small fraction of
566 these genetic elements was successfully spread through animals, humans and the
567 environment, representing a public health threat. Which are the genetic characteristics
568 or the external conditions that support the evolutionary success of an antibiotic
569 resistance gene is still a major question.
570

571 **Concluding remarks**

572 In summary, the previous discussion on the diversity and ecology of water bacteria and
573 antibiotic resistance led to a few conclusions and raised some new hypothesis:

574 1. Water habitats host an impressive bacterial diversity. However, only a few lineages
575 are known to harbor antibiotic resistance genes of already recognized clinical relevance.
576 The hypothesis that many bacterial lineages, some of them still unculturable, inhabiting
577 water may represent a reservoir of new or emerging antibiotic resistance determinants
578 cannot be discarded;

579 2. Bacteria belonging to the same bacterial lineages inhabit different types of water,
580 including pristine water, disinfected water and raw wastewater. The hypothesis that
581 these lineages can transfer relevant properties, mainly those that can be acquired by
582 horizontal gene transfer, from unclean water habitats to clean environments, cannot be
583 discarded;

584 3. Only a few groups of bacteria found in waters were, so far, identified in the human-
585 associated microbiome. Although it is still uncertain in which cases the same species

and strain can live in water and colonize humans, it is arguable that at least some of those lineages can represent a link between the water habitats and humans. In such case, those bacteria may be involved in the direct or indirect transfer of properties, including antibiotic resistance;

4. Well known human commensal (as coliforms or enterococci) and pathogenic bacteria are minor and often undetected representatives of the water microbial communities assessed based on metagenomic analysis. Therefore, metagenomic approaches may be of limited value to detect antibiotic resistance determinants already described in these organisms, unless enrichment or targeted methods are used.

5. Studies designed to survey the phylogeny of the antibiotic resistance genes and tracking the same gene types over different environmental compartments may contribute to shade some light on the relevance of environmental bacteria on the spread and transfer to humans of antibiotic resistance.

Acknowledgements:

Authors gratefully acknowledge the National Funds from FCT – Fundação para a Ciência e a Tecnologia, Portugal, through projects PEst-OE/EQB/LA0016/2011, PTDC/AAC-AMB/113840/2009, and PTDC/AAC-AMB/113091/2009, and the IVM grant SFRH/BPD/87360/2012.

Conflict of interest:

None to declare

610 **References:**

611 Aizenberg-Gershtein Y, Vaizel-Ohayon D & Halpern M (2012) Structure of bacterial
612 communities in diverse freshwater habitats. *Can J Microbiol* **58**: 326-335.
613 Akinbowale OL, Peng H, Grant P & Barton MD (2007) Antibiotic and heavy metal
614 resistance in motile aeromonads and pseudomonads from rainbow trout
615 (*Oncorhynchus mykiss*) farms in Australia. *Int J Antimicrob Agents* **30**: 177–182.
616 Allen HK, Moe LA, Rodbumrer J, Gaarder A & Handelsman J (2009) Functional
617 metagenomics reveals diverse beta-lactamases in a remote Alaskan soil. *ISME J*
618 **3(2)**:243-251.
619 Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J & Handelsman J (2010)
620 Call of the wild: antibiotic resistance genes in natural environments. *Nat Rev*
621 *Microbiol* **8**: 251-259.
622 Alonso A, Sanchez P & Martinez JL (2001) Environmental selection of antibiotic
623 resistance genes. *Environ Microbiol* **3**: 1-9.
624 Alvarez-Ortega C, Wiegand I, Olivares J, Hancock REW &Martínez JL (2011) The
625 intrinsic resistome of *Pseudomonas aeruginosa* to β-lactams. *Virulence* **2(2)**:144-146.
626 Amann RI, Ludwig W & Schleifer KH (1995) Phylogenetic identification and *in situ*
627 detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169.
628 Andersson DI & Hughes D (2010) Antibiotic resistance and its cost: is it possible to
629 reverse resistance? *Nat Rev Microbiol* **8**: 260-271.
630 Andersson DI & Hughes D (2011) Persistence of antibiotic resistance in bacterial
631 populations. *FEMS Microbiol Rev* **35**: 901-911.
632 Anonymous (2012) The cultural revolution. *Nature Rev Microbiol* **11**: 1-1.
633 doi:10.1038/nrmicro2948.

634 Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML & Young VB
635 (2009) Reproducible community dynamics of the gastrointestinal microbiota following
636 antibiotic perturbation. *Infect Immun* **77**: 2367-2375.
637 Araújo C, Torres C, Silva N, *et al.* (2010) Vancomycin-resistant enterococci from
638 Portuguese wastewater treatment plants. *J Basic Microbiol* **50**: 605-609.
639 Arber W (2000) Genetic variation: molecular mechanisms and impact on microbial
640 evolution. *FEMS Microbiol Rev* **24**: 1-7.
641 Arumugam M, Raes J, Pelletier E, *et al.* (2011) Enterotypes of the human gut
642 microbiome. *Nature* **473**: 174-180.
643 Auerbach EA, Seyfried EE & McMahon KD (2007) Tetracycline resistance genes in
644 activated sludge wastewater treatment plants. *Water Res* **41**: 1143-1151.
645 Aylward FO, McDonald BR, Adams SM, Valenzuela A, Schmidt RA, Goodwin LA,
646 Woyke T, Currie CR, Suen G & Poulsen M (2013) Comparison of 26 Sphingomonad
647 genomes reveals diverse environmental adaptations and biodegradative capabilities.
648 *Appl Environ Microbiol* **79(12)**: 3724-2733.
649 Baik KS, Park SC, Kim EM, Bae KS, Ahn JH, Ka JO, Chun J & Seong CN (2008)
650 Diversity of bacterial community in freshwater of Woopo wetland. *J Microbiol* **46**:
651 647-655.
652 Baker-Austin C, Wright MS, Stepanauskas R & McArthur JV (2006) Co-selection of
653 antibiotic and metal resistance. *Trends Microbiol* **14**: 176-182.
654 Baquero F, Martínez JL & Cantón R (2008) Antibiotics and antibiotic resistance in
655 water environments. *Curr Opin Biotechnol* **19**: 260-265.
656 Baquero F, Tedim AP & Coque TM (2013) Antibiotic resistance shaping multi-level
657 population biology of bacteria. *Front Microbiol* **4(15)**, doi:10.3389/fmicb.2013.00015.

Barbosa TM & Levy SB (2000) The impact of antibiotic use on resistance development and persistence. *Drug Resist Updat* **3**: 303–311.

Barker-Reid F, Fox EM & Faggian R (2010) Occurrence of antibiotic resistance genes in reclaimed water and river water in the Werribee Basin, Australia. *J Water Health* **8**: 521-531.

Björkman J, Nagaev I, Berg OG, Hughes D &AnderssonDI (2000) Effects of environment on compensatory mutations to ameliorate costs of antibiotic resistance. *Science* **287**: 1479-1482.

Böckelmann U, Dörries HH, Ayuso-Gabella MN, *et al*. (2009) Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. *Appl Environ Microbiol* **75**: 154-163.

Borjesson S, Mattsson A & Lindgren PE (2010) Genes encoding tetracycline resistance in a full-scale municipal wastewater treatment plant investigated during one year. *J Water Health* **8**: 247-256.

Borjesson S, Melin S, Matussek A & Lindgren PE (2009) A seasonal study of the *mec*A gene and *Staphylococcus aureus* including methicillin-resistant *S. aureus* in a municipal wastewater treatment plant. *Water Res* **43**: 925-932.

Breidenstein EBM, de la Fuente-Núñez C & Hancock REW (2011) *Pseudomonas aeruginosa*: all roads lead to resistance. *Trends Microbiol* **19(8)**: 419-426.

Bush K, Courvalin P, Dantas G, *et al.* (2011) Tackling antibiotic resistance. *Nat Rev Microbiol* **9**: 894-896.

Cabello FC (2006) Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ Microbiol* **8(7)**: 1137-1144.

682 Cabello FC, Godfrey HP, Tomova A, Ivanova L, Dölz H, Millanao A & Buschmann
683 AH (2013) Antimicrobial use in aquaculture re-examined: its relevance to
684 antimicrobial resistance and to animal and human health. *Environ Microbiol* **15(7)**:
685 1917–1942.
686 Cantón R (2009) Antibiotic resistance genes from the environment: a perspective
687 through newly identified antibiotic resistance mechanisms in the clinical setting. *Clin*
688 *Microbiol Infect* **15 Suppl 1**: 20-25.
689 Cantón R & Morosini MI (2011) Emergence and spread of antibiotic resistance
690 following exposure to antibiotics. *FEMS Microbiol Rev* **35**: 977-991.
691 Casanovas-Massana A & Blanch AR (2012) Diversity of the heterotrophic microbial
692 populations for distinguishing natural mineral waters. *Int J Food Microbiol* **153**: 38-
693 44.
694 Cattoir V, Poirel L, Aubert C, Soussy CJ & Nordmann P (2008) Unexpected occurrence
695 of plasmid-mediated quinolone resistance determinants in environmental *Aeromonas*
696 spp. *Emerg Infect Dis* **14**: 231-237.
697 Chen H, Shu W, Chang X, Chen JA, Guo Y & Tan Y (2010) The profile of antibiotics
698 resistance and integrons of extended-spectrum beta-lactamase producing
699 thermotolerant coliforms isolated from the Yangtze River basin in Chongqing.
700 *Environ Pollut* **158**: 2459-2464.
701 Chen Z, Zhou Z, Peng X, Xiang H, Xiang S & Jiang Z (2013) Effects of wet and dry
702 seasons on the aquatic bacterial community structure of the Three Gorges Reservoir.
703 *World J Microbiol Biotechnol* **29**: 841-853.
704 Chouchani C, Marrakchi R, Henriques I & Correia A (2013) Occurrence of IMP-8,
705 IMP-10, and IMP-13 metallo-beta-lactamases located on class 1 integrons and other

706 extended-spectrum beta-lactamases in bacterial isolates from Tunisian rivers. *Scand J*
707 *Infect Dis* **45**: 95-103.
708 Clarridge JE, 3rd (2004) Impact of 16S rRNA gene sequence analysis for identification
709 of bacteria on clinical microbiology and infectious diseases. *Clin Microbiol Rev* **17**:
710 840-862, table of contents.
711 Colomer-Lluch M, Jofre J & Muniesa M (2011) Antibiotic resistance genes in the
712 bacteriophage DNA fraction of environmental samples. *PLoS One* **6**: e17549.
713 Cottrell MT, Waidner LA, Yu L & Kirchman DL (2005) Bacterial diversity of
714 metagenomic and PCR libraries from the Delaware River. *Environ Microbiol* **7**: 1883-
715 1895.
716 Czekalski N, Berthold T, Caucci S, Egli A & Burgmann H (2012) Increased levels of
717 multiresistant bacteria and resistance genes after wastewater treatment and their
718 dissemination into lake geneva, Switzerland. *Front Microbiol* **3**: 106, doi:
719 10.3389/fmicb.2012.00106
720 D'Costa VM, McGrann KM, Hughes DW & Wright GD (2006) Sampling the Antibiotic
721 Resistome. *Science* **311**: 374-377.
722 D'Costa VM, King CE, Kalan L, *et al.* (2011) Antibiotic resistance is ancient. *Nature*
723 **477**: 457-461.
724 Dantas G & Sommer MO (2012) Context matters - the complex interplay between
725 resistome genotypes and resistance phenotypes. *Curr Opin Microbiol* **15**: 577-582.
726 Dantas G, Sommer MO, Oluwasegun RD & Church GM (2008) Bacteria subsisting on
727 antibiotics. *Science* **320**: 100-103.
728 Davies J & Davies D (2010) Origins and evolution of antibiotic resistance. *Microbiol*
729 *Mol Biol Rev* **74(3)**: 417–433.

730 Davies J, Spiegelman GB & Yim G (2006) The world of subinhibitory antibiotic
731 concentrations. *Curr Opin Microbiol* **9**: 445-453.
732 De Boeck H, Miwanda B, Lunguya-Metila O, Muyembe-Tamfum JJ, Stobberingh E,
733 Glupczynski Y & Jacobs J (2012) ESBL-positive Enterobacteria isolates in drinking
734 water. *Emerg Infect Dis* **18**: 1019-1020.
735 de Figueiredo DR, Pereira MJ, Moura A, Silva L, Bárrios S, Fonseca F, Henriques I &
736 Correia A (2007) Bacterial community composition over a dry winter in meso- and
737 eutrophic Portuguese water bodies. *FEMS Microbiol Ecol* **59**: 638-650.
738 Dong X & Reddy GB (2010) Nutrient removal and bacterial communities in swine
739 wastewater lagoon and constructed wetlands. *J Environ Sci Health A Tox Hazard*
740 *Subst Environ Eng* **45**: 1526-1535.
741 Drudge CN, Elliott AV, Plach JM, Ejim LJ, Wright GD, Droppo IG & Warren LA
742 (2012) Diversity of integron- and culture-associated antibiotic resistance genes in
743 freshwater floc. *Appl Environ Microbiol* **78**: 4367-4372.
744 Eichler S, Christen R, Höltje C, Westphal P, Bötel J, Brettar I, Mehling A & Höfle MG
745 (2006) Composition and dynamics of bacterial communities of a drinking water
746 supply system as assessed by RNA- and DNA-based 16S rRNA gene fingerprinting.
747 *Appl Environ Microbiol* **72**: 1858-1872.
748 Eloe-Fadrosh EA & Rasko DA (2013) The Human Microbiome: from symbiosis to
749 pathogenesis. *Annu Rev Med* **64**:145-163.
750 European Comission (2009) DIRECTIVE 2009/54/EC - on the exploitation and
751 marketing of natural mineral waters.
752 Fajardo A, Martínez-Martín N, Mercadillo M, *et al.* (2008). The neglected intrinsic
753 resistome of bacterial pathogens. *PLoS One* **3(2)**:e1619. doi:
754 10.1371/journal.pone.0001619

755 Falcone-Dias M, Vaz-Moreira I & Manaia CM (2012) Bottled mineral water as a
756 potential source of antibiotic resistant bacteria. *Water Res* **46**: 3612-3622.
757 Faria C, Vaz-Moreira I, Serapicos E, Nunes OC & Manaia CM (2009) Antibiotic
758 resistance in coagulase negative staphylococci isolated from wastewater and drinking
759 water. *Sci Total Environ* **407**: 3876-3882.
760 Ferreira da Silva M, Vaz-Moreira I, Gonzalez-Pajuelo M, Nunes OC & Manaia CM
761 (2007) Antimicrobial resistance patterns in *Enterobacteriaceae* isolated from an urban
762 wastewater treatment plant. *FEMS Microbiol Ecol* **60**: 166-176.
763 Ferreira da Silva M, Tiago I, Verissimo A, Boaventura RA, Nunes OC & Manaia CM
764 (2006) Antibiotic resistance of enterococci and related bacteria in an urban wastewater
765 treatment plant. *FEMS Microbiol Ecol* **55**: 322-329.
766 Figueira V, Vaz-Moreira I, Silva M & Manaia CM (2011) Diversity and antibiotic
767 resistance of *Aeromonas* spp. in drinking and waste water treatment plants. *Water Res*
768 **45**: 5599-5611.
769 Figueira V, Serra EA, Vaz-Moreira I, Brandão TRS & Manaia CM (2012) Comparison
770 of ubiquitous antibiotic-resistant *Enterobacteriaceae* populations isolated from
771 wastewaters, surface waters and drinking waters. *J Water Health* **10.1**.
772 Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO & Dantas G (2012) The
773 shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337**: 1107-
774 1111.
775 Furuhata K, Kato Y, Goto K, Hara M, Yoshida S & Fukuyama M (2006) Isolation and
776 identification of *Methylobacterium* species from the tap water in hospitals in Japan and
777 their antibiotic susceptibility. *Microbiol Immunol* **50**: 11-17.

778 Furuhata K, Kato Y, Goto K, Saitou K, Sugiyama J, Hara M & Fukuyama M (2007)
779 Identification of yellow-pigmented bacteria isolated from hospital tap water in Japan
780 and their chlorine resistance. *Biocontrol Sci* **12**: 39-46.
781 Gajan EB, Abashov R, Aghazadeh M, Eslami H, Oskouei SG & Mohammadnejad D
782 (2008) Vancomycin-resistant *Enterococcus faecalis* from a wastewater treatment plant
783 in Tabriz, Iran. *Pak J Biol Sci* **11**: 2443-2446.
784 Galvin S, Boyle F, Hickey P, Vellinga A, Morris D & Cormican M (2010) Enumeration
785 and characterization of antimicrobial-resistant *Escherichia coli* bacteria in effluent
786 from municipal, hospital, and secondary treatment facility sources. *Appl Environ*
787 *Microbiol* **76(14)**: 4772–4779.
788 Garcia-Armisen T & Servais P (2004) Enumeration of viable *E. coli* in rivers and
789 wastewaters by fluorescent in situ hybridization. *J Microbiol Methods* **58**: 269-279.
790 Garcillán-Barcia MP, Alvarado A & de la Cruz F (2011) Identification of bacterial
791 plasmids based on mobility and plasmid population biology. *FEMS Microbiol Rev* **35**:
792 936-956.
793 Gich F, Schubert K, Bruns A, Hoffelner H & Overmann J (2005) Specific detection,
794 isolation, and characterization of selected, previously uncultured members of the
795 freshwater bacterioplankton community. *Appl Environ Microbiol* **71**: 5908-5919.
796 Girgis HS, Hottes AK & Tavazoie S (2009) Genetic architecture of intrinsic antibiotic
797 susceptibility. *PLoS One* **4(5)**: e5629, doi: 10.1371/journal.pone.0005629.
798 Girlich D, Poirel L & Nordmann P (2010a) PER-6, an extended-spectrum beta-
799 lactamase from *Aeromonas allosaccharophila*. *Antimicrob Agents Chemother* **54**:
800 1619-1622.

801 Girlich D, Poirel L & Nordmann P (2010b) Novel ambler class A carbapenem-
802 hydrolyzing beta-lactamase from a *Pseudomonas fluorescens* isolate from the Seine
803 River, Paris, France. *Antimicrob Agents Chemother* **54**: 328-332.
804 Girlich D, Poirel L & Nordmann P (2010c) First isolation of the *bla*OXA-23
805 carbapenemase gene from an environmental *Acinetobacter baumannii* isolate.
806 *Antimicrob Agents Chemother* **54**: 578-579.
807 Girlich D, Poirel L & Nordmann P (2011) Diversity of clavulanic acid-inhibited
808 extended-spectrum beta-lactamases in *Aeromonas* spp. from the Seine River, Paris,
809 France. *Antimicrob Agents Chemother* **55**: 1256-1261.
810 Graham DW, Olivares-Rieumont S, Knapp CW, Lima L, Werner D & Bowen E (2011)
811 Antibiotic resistance gene abundances associated with waste discharges to the
812 Almendares River near Havana, Cuba. *Environ Sci Technol* **45**: 418-424.
813 Gomez-Alvarez V, Revetta RP & Santo Domingo JW (2012) Metagenomic analyses of
814 drinking water receiving different disinfection treatments. *Appl Environ Microbiol* **78**:
815 6095-6102.
816 Gomez MJ & Neyfakh AA (2006) Genes involved in intrinsic antibiotic resistance of
817 *Acinetobacter baylyi*. *Antimicrob Agents Chemother* **50:** 3562–3567.
818 Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, Hughes D & Andersson DI (2011)
819 Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog* **7**:
820 e1002158.
821 Hamelin K, Bruant G, El-Shaarawi A, Hill S, Edge TA, Fairbrother J, Harel J, Maynard
822 C, Masson L & Brousseau R (2007) Occurrence of virulence and antimicrobial
823 resistance genes in *Escherichia coli* isolates from different aquatic ecosystems within
824 the St. Clair River and Detroit River areas. *Appl Environ Microbiol* **73**: 477-484.

825 Hancock REW (1998) Resistance mechanisms in *Pseudomonas aeruginosa* and other
826 nonfermentative Gram-negative bacteria. *Clin Infect Dis* **27**(Suppl. 1): S93–S99.
827 Hancock REW & Brinkman FSL (2002) Function of *Pseudomonas* porins in uptake and
828 efflux. *Ann Rev Microbiol* **56**: 17-38.
829 Handel A, Regoes RR & Antia R (2006) The role of compensatory mutations in the
830 emergence of drug resistance. *PLoS Comput Biol* **2(10)**: e137.
831 Harada K & Asai T (2010) Role of antimicrobial selective pressure and secondary
832 factors on antimicrobial resistance prevalence in *Escherichia coli* from food-producing
833 animals in Japan. *J Biomed Biotechnol* **2010**: 180682.
834 Hausner M & Wuertz S (1999) High rates of conjugation in bacterial biofilms as
835 determined by quantitative in situ analysis. *Appl Environ Microbiol* **65**: 3710-3713.
836 Henriques I, Moura A, Alves A, Saavedra MJ & Correia A (2006a) Analysing diversity
837 among beta-lactamase encoding genes in aquatic environments. *FEMS Microbiol Ecol*
838 **56**: 418-429.
839 Henriques IS, Fonseca F, Alves A, Saavedra MJ & Correia A (2006b) Occurrence and
840 diversity of integrons and beta-lactamase genes among ampicillin-resistant isolates
841 from estuarine waters. *Res Microbiol* **157**: 938-947.
842 Henriques IS, Fonseca F, Alves A, Saavedra MJ & Correia A (2008) Tetracycline-
843 resistance genes in gram-negative isolates from estuarine waters. *Lett Appl Microbiol*
844 **47**: 526-533.
845 Henriques IS, Araújo S, Azevedo JSN, Alves MS, Chouchani C, Pereira A & Correia A
846 (2012) Prevalence and diversity of carbapenem-resistant bacteria in untreated drinking
847 water in Portugal. *Microb Drug Resist* **18(5)**:531-537.

Hiorns WD, Methe BA, Nierzwicki-Bauer SA & Zehr JP (1997) Bacterial diversity in Adirondack mountain lakes as revealed by 16S rRNA gene sequences. *Appl Environ Microbiol* **63**: 2957-2960.

Hoefel D, Monis PT, Grooby WL, Andrews S & Saint CP (2005) Profiling bacterial survival through a water treatment process and subsequent distribution system. *J Appl Microbiol* **99**: 175-186.

Huerta B, Marti E, Gros M, López P. Pompêo M, Armengol J, Barceló D, Balcázar JL, Rodríguez-Mozaz S & Marcé R (2013) Exploring the links between antibiotic occurrence, antibiotic resistance, and bacterial communities in water supply reservoirs. *Sci Total Environ* **456-457**: 161-170.

Jakobsson HE, Jernberg C, Andersson AF, Sjolund-Karlsson M, Jansson JK & Engstrand L (2010) Short-term antibiotic treatment has differing long-term impacts on the human throat and gut microbiome. *PLoS One* **5**: e9836.

Jernberg C, Lofmark S, Edlund C & Jansson JK (2010) Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiol* **156**: 3216-3223.

Kassem, II, Esseili MA & Sigler V (2008) Occurrence of *mec*A in nonstaphylococcal pathogens in surface waters. *J Clin Microbiol* **46**: 3868-3869.

Kim J, Kang HY & Lee Y (2008) The identification of CTX-M-14, TEM-52, and CMY-1 enzymes in *Escherichia coli* isolated from the Han River in Korea. *J Microbiol* **46**: 478-481.

Kormas KA, Neofitou C, Pachiadaki M & Koufostathi E (2010) Changes of the bacterial assemblages throughout an urban drinking water distribution system. *Environ Monit Assess* **165**: 27-38.

871 Koskinen R, Ali-Vehmas T, Kämpfer P, Laurikkala M, Tsitko I, Kostyal E, Atroshi F &
872 Salkinoja-Salonen M (2000) Characterization of *Sphingomonas* isolates from Finnish
873 and Swedish drinking water distribution systems. *J Appl Microbiol* **89**: 687-696.
874 Lachmayr KL, Kerkhof LJ, Dirienzo AG, Cavanaugh CM & Ford TE (2009)
875 Quantifying nonspecific TEM beta-lactamase (*bla*TEM) genes in a wastewater stream.
876 *Appl Environ Microbiol* **75**: 203-211.
877 Lagier JC, Armougom F, Million M, *et al.* (2012) Microbial culturomics: paradigm shift
878 in the human gut microbiome study. *Clin Microbiol Infect* **18**: 1185-1193.
879 LaPara TM, Burch TR, McNamara PJ, Tan DT, Yan M & Eichmiller JJ (2011)
880 Tertiary-treated municipal wastewater is a significant point source of antibiotic
881 resistance genes into Duluth-Superior Harbor. *Environ Sci Technol* **45**: 9543-9549.
882 Lee J, Lee CS, Hugunin KM, Maute CJ & Dysko RC (2010) Bacteria from drinking
883 water supply and their fate in gastrointestinal tracts of germ-free mice: a phylogenetic
884 comparison study. *Water Res* **44**: 5050-5058.
885 Letunic I & Bork P (2007) Interactive Tree Of Life (iTOL): an online tool for
886 phylogenetic tree display and annotation. *Bioinformatics* **23(1)**:127-128.
887 Letunic I & Bork P (2011) Interactive Tree Of Life v2: online annotation and display of
888 phylogenetic trees made easy. *Nucleic Acids Res* **39(2)**: W475-W478.
889 Levantesi C, La Mantia R, Masciopinto C, Böckelmann U, Ayuso-Gabella MN, Salgot
890 M, Tandoi V, Van Houtte E, Wintgens T & Grohmann E (2010) Quantification of
891 pathogenic microorganisms and microbial indicators in three wastewater reclamation
892 and managed aquifer recharge facilities in Europe. *Sci Total Environ* **408**: 4923-4930.
893 Liu A, Tran L, Becket E, Lee K, Chinn L, Park E, Tran K & Miller JH (2010)
894 Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout

collection: generating an antibiotic bar code. *Antimicrob Agents Chemother* **54(4)**: 1393–1403**.**

Liu B & Pop M (2009) ARDB-Antibiotic Resistance Genes Database. *Nucleic Acids Res* **37**: D443-447.

Livermore DM (2003) Bacterial resistance: origins, epidemiology, and impact. *Clin Infect Dis* **36**(Suppl 1): S11-S23.

Loy A, Beisker W & Meier H (2005) Diversity of bacteria growing in natural mineral water after bottling. *Appl Environ Microbiol* **71**: 3624-3632.

Lu P, Chen C, Wang Q, Wang Z, Zhang X & Xie S (2013) Phylogenetic diversity of microbial communities in real drinking water distribution systems. *Biotechnol Bioproc E* **18**: 119-124.

Łuczkiewicz A, Jankowska K, Fudala-Ksiazek S & Olanczuk-Neyman K (2010) Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Res* **44**: 5089-5097.

Luo Y, Mao D, Rysz M, Zhou Q, Zhang H, Xu L & P JJA (2010) Trends in antibiotic resistance genes occurrence in the Haihe River, China. *Environ Sci Technol* **44**: 7220-7225.

Lymperopoulou DS, Kormas KA & Karagouni AD (2012) Variability of Prokaryotic Community Structure in a Drinking Water Reservoir (Marathonas, Greece). *Microbes Environ* **27**: 1-8.

Macedo AS, Freitas AR, Abreu C, Machado E, Peixe L, Sousa JC & Novais C (2011) Characterization of antibiotic resistant enterococci isolated from untreated waters for human consumption in Portugal. *Int J Food Microbiol* **145**: 315-319.

Maisnier-Patin S & Andersson DI (2004) Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. *Res Microbiol* **155**: 360–369.

Manaia CM, Novo A, Coelho B & Nunes OC (2010) Ciprofloxacin resistance in domestic wastewater treatment plants. *Water Air Soil Pollut* **208**: 335–343.

Manaia CM, Vaz-Moreira I & Nunes OC (2012) Antibiotic resistance in waste water and surface water and human health implications. Chapter 6 In *The Handbook of Environmental Chemistry*,(Barceló D, ed.), vol 20: 173-212. Springer-Verlag, Berlin Heidelberg. DOI: 10.1007/698_2011_118.

Martinez JL (2008) Antibiotics and antibiotic resistance genes in natural environments. *Science* **321**: 365-367.

Martinez JL (2009) Environmental pollution by antibiotics and by antibiotic resistance determinants. *Environ Pollut* **157**: 2893-2902.

Martinez JL & Baquero F (2000) Mutation frequencies and antibiotic resitance. *Antimicrob Agents Chemother* **44(7)**: 1771-1777.

Martinez JL, Baquero F & Andersson DI (2007) Predicting antibiotic resistance. *Nat Rev Microbiol* **5**: 958–965.

Mary P, Defives C & Hornez JP (2000) Occurrence and multiple antibiotic resistance profiles of non-fermentative Gram-negative microflora in five brands of non-carbonated french bottled spring water. *Microb Ecol* **39**: 322-329.

Massa S, Petruccioli M, Fanelli M & Gori L (1995) Drug resistant bacteria in non carbonated mineral waters. *Microbiol Res* **150**: 403-408.

McGarvey JA, Miller WG, Sanchez S & Stanker L (2004) Identification of bacterial populations in dairy wastewaters by use of 16S rRNA gene sequences and other genetic markers. *Appl Environ Microbiol* **70**: 4267-4275.

943 McGarvey JA, Miller WG, Sanchez S, Silva CJ & Whitehand LC (2005) Comparison of
944 bacterial populations and chemical composition of dairy wastewater held in circulated
945 and stagnant lagoons. *J Appl Microbiol* **99**: 867-877.
946 McLellan SL, Huse SM, Mueller-Spitz SR, Andreishcheva EN & Sogin ML (2010)
947 Diversity and population structure of sewage-derived microorganisms in wastewater
948 treatment plant influent. *Environ Microbiol* **12**: 378-392.
949 Messi P, Guerrieri E & Bondi M (2005) Antibiotic resistance and antibacterial activity
950 in heterotrophic bacteria of mineral water origin. *Sci Total Environ* **346**: 213-219.
951 Miyahara E, Nishie M, Takumi S, *et al.* (2011) Environmental mutagens may be
952 implicated in the emergence of drug-resistant microorganisms. *FEMS Microbiol Lett*
953 **317**: 109-116.
954 Moura A, Jove T, Ploy MC, Henriques I & Correia A (2012) Diversity of gene cassette
955 promoters in class 1 integrons from wastewater environments. *Appl Environ Microbiol*
956 **78**: 5413-5416.
957 Moura A, Tacão M, Henriques I, Dias J, Ferreira P & Correia A (2009) Characterization
958 of bacterial diversity in two aerated lagoons of a wastewater treatment plant using
959 PCR-DGGE analysis. *Microbiol Res* **164**: 560-569.
960 Narciso-da-Rocha C, Vaz-Moreira I, Svensson-Stadler L, Moore ERB & Manaia CM
961 (2013) Diversity and antibiotic resistance of *Acinetobacter* spp. in water from the
962 source to the tap. *Appl Microbiol Biotechnol* **97**: 329-340.
963 Navarro-Noya YE, Suarez-Arriaga MC, Rojas-Valdes A, Montoya-Ciriaco NM,
964 Gomez-Acata S, Fernandez-Luqueno F & Dendooven L (2013) Pyrosequencing
965 analysis of the bacterial community in drinking water wells. *Microb Ecol*. DOI
966 10.1007/s00248-013-0222-3.

967 Novo A, Andre S, Viana P, Nunes OC & Manaia CM (2013) Antibiotic resistance,
968 antimicrobial residues and bacterial community composition in urban wastewater.
969 *Water Res* **47**: 1875-1887.
970 Novo A & Manaia CM (2010) Factors influencing antibiotic resistance burden in
971 municipal wastewater treatment plants. *Appl Microbiol Biotechnol* **87**: 1157–1166.
972 Okoh AI & Igbinosa EO (2010) Antibiotic susceptibility profiles of some *Vibrio* strains
973 isolated from wastewater final effluents in a rural community of the Eastern Cape
974 Province of South Africa. *BMC Microbiol* **10**: 143.
975 Otterholt E & Charnock C (2011) Microbial quality and nutritional aspects of
976 Norwegian brand waters. *Int J Food Microbiol* **144**: 455-463.
977 Ozaktas T, Taskin B & Gozen AG (2012) High level multiple antibiotic resistance
978 among fish surface associated bacterial populations in non-aquaculture freshwater
979 environment. *Water Res* **46**: 6382-6390.
980 Ozgumus OB, Sandalli C, Sevim A, Celik-Sevim E & Sivri N (2009) Class 1 and class
981 2 integrons and plasmid-mediated antibiotic resistance in coliforms isolated from ten
982 rivers in northern Turkey. *J Microbiol* **47**: 19-27.
983 Partridge SR (2011) Analysis of antibiotic resistance regions in Gram-negative bacteria.
984 *FEMS Microbiol Rev* **35**: 820-855.
985 Picão RC, Poirel L, Demarta A, Silva CS, Corvaglia AR, Petrini O & Nordmann P
986 (2008) Plasmid-mediated quinolone resistance in *Aeromonas allosaccharophila*
987 recovered from a Swiss lake. *J Antimicrob Chemother* **62**: 948-950.
988 Pinto AJ & Raskin L (2012) PCR biases distort bacterial and archaeal community
989 structure in pyrosequencing datasets. *PLoS One* **7**: e43093.
990 Pinto AJ, Xi C & Raskin L (2012) Bacterial community structure in the drinking water
991 microbiome is governed by filtration processes. *Environ Sci Technol* **46**: 8851-8859.

992 Poirel L, Kampfer P & Nordmann P (2002) Chromosome-encoded Ambler class A beta-
993 lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M
994 extended-spectrum beta-lactamases. *Antimicrob Agents Chemother* **46**: 4038-4040.
995 Poirel L, Rodriguez-Martinez JM, Mammeri H, Liard A & Nordmann P (2005) Origin
996 of plasmid-mediated quinolone resistance determinant QnrA. *Antimicrob Agents*
997 *Chemother* **49**: 3523-3525.
998 Poitelon JB, Joyeux M, Welte B, Duguet JP, Prestel E, Lespinet O & DuBow MS
999 (2009) Assessment of phylogenetic diversity of bacterial microflora in drinking water
1000 using serial analysis of ribosomal sequence tags. *Water Res* **43**: 4197-4206.
1001 Prakash O, Shouche Y, Jangid K & Kostka JE (2013) Microbial cultivation and the role
1002 of microbial resource centers in the omics era. *Appl Microbiol Biotechnol* **97**:51-62.
1003 Qin J, Li R, Raes J *et al.* (2010) A human gut microbial gene catalogue established by
1004 metagenomic sequencing. *Nature* **464**: 59-67.
1005 Revetta RP, Pemberton A, Lamendella R, Iker B & Santo Domingo JW (2010)
1006 Identification of bacterial populations in drinking water using 16S rRNA-based
1007 sequence analyses. *Water Res* **44**: 1353-1360.
1008 Rhodes G., Huys G, Swings J, McGann P, Hiney M, Smith P & Pickup RW (2000)
1009 Distribution of oxytetracycline resistance plasmids between aeromonads in hospital
1010 and aquaculture environments: implication of Tn*1721* in dissemination of the
1011 tetracycline resistance determinant Tet A. *Appl. Environ Microbiol* **66**: 3883-3890.
1012 Riesenfeld CS, Goodman RM & Handelsman J (2004) Uncultured soil bacteria are a
1013 reservoir of new antibiotic resistance genes. *Environ Microbiol* **6**: 981-989.
1014 Rizzo L, Manaia C, Merlin C, Schwartz T, Dagot C, Ploy MC, Michael I & Fatta-
1015 Kassinos D (2013) Urban wastewater treatment plants as hotspots for antibiotic

1016 resistant bacteria and genes spread into the environment: a review. *Sci Total Environ*
1017 **447**: 345-360.
1018 Rodríguez-Blanco A, Lemos ML & Osorio CR (2012) Integrating conjugative elements
1019 as vectors of antibiotic, mercury, and quaternary ammonium compound resistance in
1020 marine aquaculture environments. *Antimicrob. Agents Chemother* **56(5)**: 2619-2626.
1021 Rosenberg FA (2003) The microbiology of bottled water. *Clin Microbiol Newslett* **26**:
1022 41-44.
1023 Rosenberg FA & Duquino HH (1989) Antibiotic-resistance of *Pseudomonas* from
1024 german mineral waters. *Toxic Assess* **4**: 281-294.
1025 Rudi K, Tannaes T & Vatn M (2009) Temporal and spatial diversity of the tap water
1026 microbiota in a Norwegian hospital. *Appl Environ Microbiol* **75**: 7855-7857.
1027 Samra ZQ, Naseem M, Khan SJ, Dar N & Athar MA (2009) PCR targeting of antibiotic
1028 resistant bacteria in public drinking water of Lahore metropolitan, Pakistan. *Biomed*
1029 *Environ Sci* **22**: 458-463.
1030 Sanapareddy N, Hamp TJ, Gonzalez LC, Hilger HA, Fodor AA & Clinton SM (2009)
1031 Molecular diversity of a North Carolina wastewater treatment plant as revealed by
1032 pyrosequencing. *Appl Environ Microbiol* **75**: 1688-1696.
1033 Schulze AD, Alabi AO, Tattersall-Sheldrake AR & Miller KM (2006) Bacterial
1034 diversity in a marine hatchery: Balance between pathogenic and potentially probiotic
1035 bacterial strains. *Aquaculture* **256**: 50–73.
1036 Schulz zur Wiesch P, Engelstädter J & Bonhoeffer S (2010) Compensation of fitness
1037 costs and reversibility of antibiotic resistance mutations. A*ntimicrob Agents*
1038 *Chemother* **54(5)**: 2085–2095.

1039 Segawa T, Takeuchi N, Rivera A, Yamada A, Yoshimura Y, Barcaza G, Shinbori K,
1040 Motoyama H, Kohshima S & Ushida K (2013) Distribution of antibiotic resistance
1041 genes in glacier environments. *Env Microbiol Rep* **5**: 127-134.
1042 Seiler C & Berendonk TU (2012) Heavy metal driven co-selection of antibiotic
1043 resistance in soil and water bodies impacted by agriculture and aquaculture. *Front*
1044 *Microbiol* **3**, 399. doi: 10.3389/fmicb.2012.00399
1045 Sengupta S, Chattopadhyay MK & Grossart HP (2013) The multifaceted roles of
1046 antibiotics and antibiotic resistance in nature. *Front Microbiol* **4**: 47.
1047 Shehabi AA, Masoud H & Maslamani FA (2005) Common antimicrobial resistance
1048 patterns, biotypes and serotypes found among *Pseudomonas aeruginosa* isolates from
1049 patient's stools and drinking water sources in Jordan. *J Chemother* **17**: 179-183.
1050 Shi P, Jia S, Zhang XX, Zhang T, Cheng S & Li A (2013) Metagenomic insights into
1051 chlorination effects on microbial antibiotic resistance in drinking water. *Water Res* **47**:
1052 111-120.
1053 Simon C & Daniel R (2011) Metagenomic analyses: past and future trends. *Appl*
1054 *Environ Microbiol* **77(4)**: 1153-1161.
1055 Smith P (2008) Antimicrobial resistance in aquaculture. *Rev Sci Tech Off int Epiz* **27(1)**:
1056 243-264.
1057 Sommer MO, Dantas G & Church GM (2009) Functional characterization of the
1058 antibiotic resistance reservoir in the human microflora. *Science* **325**: 1128-1131.
1059 Sørum H (1998) Mobile drug resistance genes among fish bacteria. *APMIS* **84(106)**: 74-
1060 76.
1061 Stoll C, Sidhu JP, Tiehm A & Toze S (2012) Prevalence of clinically relevant antibiotic
1062 resistance genes in surface water samples collected from Germany and Australia.
1063 *Environ Sci Technol* **46**: 9716-9726.

1064 Stolz A (2009) Molecular characteristics of xenobiotic-degrading sphingomonads. *Appl*
1065 *Microbiol Biotechnol* **81**: 793-811.
1066 Storteboom H, Arabi M, Davis JG, Crimi B & Pruden A (2010) Identification of
1067 antibiotic-resistance-gene molecular signatures suitable as tracers of pristine river,
1068 urban, and agricultural sources. *Environ Sci Technol* **44**: 1947-1953.
1069 Su HC, Ying GG, Tao R, Zhang RQ, Zhao JL & Liu YS (2012) Class 1 and 2 integrons,
1070 sul resistance genes and antibiotic resistance in *Escherichia coli* isolated from
1071 Dongjiang River, South China. *Environ Pollut* **169**: 42-49.
1072 Szczepanowski R, Krahn I, Bohn N, Puhler A & Schluter A (2007) Novel macrolide
1073 resistance module carried by the IncP-1beta resistance plasmid pRSB111, isolated
1074 from a wastewater treatment plant. *Antimicrob Agents Chemother* **51**: 673-678.
1075 Szczepanowski R, Linke B, Krahn I, Gartemann KH, Gützkow T, Eichler W, Pühler A
1076 & Schlüter A (2009) Detection of 140 clinically relevant antibiotic-resistance genes in
1077 the plasmid metagenome of wastewater treatment plant bacteria showing reduced
1078 susceptibility to selected antibiotics. *Microbiol* **155**: 2306-2319.
1079 Tacão M, Correia A & Henriques I (2012) Resistance to broad-spectrum antibiotics in
1080 aquatic systems: anthropogenic activities modulate the dissemination of *bla*(CTX-M)-
1081 like genes. *Appl Environ Microbiol* **78**: 4134-4140.
1082 Tamames J, Abellan JJ, Pignatelli M, Camacho A & Moya A (2010) Environmental
1083 distribution of prokaryotic taxa. *BMC Microbiol* **10**: 85.
1084 Tamae C, Liu A, Kim K, *et al.* (2008) Determination of antibiotic hypersensitivity
1085 among 4,000 single-gene-knockout mutants of *Escherichia coli*. *J Bacteriol* **190(17)**:
1086 5981–5988.

1087 Tamminen M, Karkman A, Lõhmus A, Muziasari WI, Takasu H, Wada S, Suzuki S &
1088 Virta M (2011) Tetracycline resistance genes persist at aquaculture farms in the
1089 absence of selection pressure. *Environ Sci Technol* **45**: 386–391.
1090 Tanaka MM & Valckenborgh F (2011) Escaping an evolutionary lobster trap: drug
1091 resistance and compensatory mutation in a fluctuating environment. *Evolution* **65(5)**:
1092 1376–1387.
1093 Tao R, Ying GG, Su HC, Zhou HW & Sidhu JP (2010) Detection of antibiotic
1094 resistance and tetracycline resistance genes in *Enterobacteriaceae* isolated from the
1095 Pearl rivers in South China. *Environ Pollut* **158**: 2101-2109.
1096 Taylor NGH, Verner-Jeffreys DW & Baker-Austin C (2011) Aquatic systems:
1097 maintaining, mixing and mobilising antimicrobial resistance? *Trends Ecol Evol* **26(6)**:
1098 278-284.
1099 Tello A, Austin B & Telfer TC (2012) Selective pressure of antibiotic pollution on
1100 bacteria of importance to public health. *Environ Health Perspect* **120**: 1100-1106.
1101 Tenover FC (2006) Mechanisms of antimicrobial resistance in bacteria. *Am J Med*
1102 **119**(6 Suppl 1): S3–S10, discussion S62–S70.
1103 The Human Microbiome Project Consortium (2012) Structure, function and diversity of
1104 the healthy human microbiome. *Nature* **486**: 207-214.
1105 Thomas CM & Nielsen KM (2005) Mechanisms of, and barriers to, horizontal gene
1106 transfer between bacteria. *Nat Rev Microbiol* **3**: 711-721.
1107 Tokajian ST, Hashwa FA, Hancock IC & Zalloua PA (2005) Phylogenetic assessment
1108 of heterotrophic bacteria from a water distribution system using 16S rDNA
1109 sequencing. *Can J Microbiol* **51**: 325-335.

1110 Vaz-Moreira I, Egas C, Nunes OC & Manaia CM (2011a) Culture-dependent and
1111 culture-independent diversity surveys target different bacteria: a case study in a
1112 freshwater sample. *Antonie Van Leeuwenhoek* **100**: 245-257.
1113 Vaz-Moreira I, Nunes OC & Manaia CM (2011b) Diversity and antibiotic resistance
1114 patterns of *Sphingomonadaceae* isolated from drinking water. *Appl Environ Microbiol*
1115 **77**: 5697-5706.
1116 Vaz-Moreira I, Nunes OC & Manaia CM (2012) Diversity and antibiotic resistance in
1117 *Pseudomonas* spp. from drinking water. *Sci Tot Environ* **426**: 366-374.
1118 Vaz-Moreira I, Egas C, Nunes OC & Manaia CM (2013) Bacterial diversity from the
1119 source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-
1120 dependent methods. *FEMS Microbiol Ecol* **83**: 361-374.
1121 Walsh TR, Weeks J, Livermore DM & Toleman MA (2011) Dissemination of NDM-1
1122 positive bacteria in the New Delhi environment and its implications for human health:
1123 an environmental point prevalence study. *Lancet Infect Dis* **11**: 355-362.
1124 Wang X, Hu M, Xia Y, Wen X & Ding K (2012) Pyrosequencing analysis of bacterial
1125 diversity in 14 wastewater treatment systems in China. *Appl Environ Microbiol* **78**:
1126 7042-7047.
1127 WHO & UNICEF (2000) Global water supply and sanitation assessment 2000 Report.
1128 Wiedenbeck J & Cohan FM (2011) Origins of bacterial diversity through horizontal
1129 genetic transfer and adaptation to new ecological niches. *FEMS Microbiol Rev* **35**:
1130 957-976.
1131 Wright GD (2007) The antibiotic resistome: the nexus of chemical and genetic
1132 diversity. *Nat Rev Microbiol* **5**: 175-186.
1133 Wright GD (2010) The antibiotic resistome. *Expert Opin Drug Discov* **5(8)**: 779-788.

1134 Xi C, Zhang Y, Marrs CF, Ye W, Simon C, Foxman B & Nriagu J (2009) Prevalence of
1135 antibiotic resistance in drinking water treatment and distribution systems. *Appl*
1136 *Environ Microbiol* **75**: 5714-5718.
1137 Xia R, Guo X, Zhang Y & Xu H (2010a) *qnr*VC-like gene located in a novel complex
1138 class 1 integron harboring the ISCR1 element in an *Aeromonas punctata* strain from
1139 an aquatic environment in Shandong Province, China. *Antimicrob Agents Chemother*
1140 **54**: 3471-3474.
1141 Xia S, Duan L, Song Y, Li J, Piceno YM, Andersen GL, Alvarez-Cohen L, Moreno-
1142 Andrade I, Huang CL & Hermanowicz SW (2010b) Bacterial community structure in
1143 geographically distributed biological wastewater treatment reactors. *Environ Sci*
1144 *Technol* **44**: 7391-7396.
1145 Xu H, Broersma K, Miao V & Davies J (2011a) Class 1 and class 2 integrons in
1146 multidrug-resistant gram-negative bacteria isolated from the Salmon River, British
1147 Columbia. Can J Microbiol 57: 460-467.
1148 Xu H, Miao V, Kwong W, Xia R & Davies J (2011b) Identification of a novel
1149 fosfomycin resistance gene (*fos*A2) in *Enterobacter cloacae* from the Salmon River,
1150 Canada. *Lett Appl Microbiol* **52**: 427-429.
1151 Yang C, Zhang W, Liu R, Li Q, Li B, Wang S, Song C, Qiao C & Mulchandani A
1152 (2011) Phylogenetic diversity and metabolic potential of activated sludge microbial
1153 communities in full-scale wastewater treatment plants. *Environ Sci Technol* **45**: 7408-
1154 7415.
1155 Ye L & Zhang T (2012) Bacterial communities in different sections of a municipal
1156 wastewater treatment plant revealed by 16S rDNA 454 pyrosequencing. *Appl*
1157 *Microbiol Biotechnol* **97**: 2681-2690.

1158 Young VB & Schmidt TM (2004) Antibiotic-associated diarrhea accompanied by large-
1159 scale alterations in the composition of the fecal microbiota. *J Clin Microbiol* **42**: 1203-
1160 1206.
1161 Zeenat A, Hatha AA, Viola L & Vipra K (2009) Bacteriological quality and risk
1162 assessment of the imported and domestic bottled mineral water sold in Fiji. *J Water*
1163 *Health* **7**: 642-649.
1164 Zeng DN, Fan ZY, Chi L, Wang X, Qu WD & Quan ZX (2013) Analysis of the
1165 bacterial communities associated with different drinking water treatment processes.
1166 *World J Microbiol Biotechnol*. DOI 10.1007/s11274-013-1321-5
1167 Zhang T, Zhang XX & Ye L (2011) Plasmid metagenome reveals high levels of
1168 antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS One*
1169 **6**: e26041.
1170 Zhang T, Shao MF & Ye L (2012) 454 pyrosequencing reveals bacterial diversity of
1171 activated sludge from 14 sewage treatment plants. *ISME J* **6**: 1137-1147.
1172 Zhang XX, Zhang T & Fang HH (2009) Antibiotic resistance genes in water
1173 environment. *Appl Microbiol Biotechnol* **82**: 397-414.

1174 Zwart G, Crump BC, van Agterveld MP, Hagen F & Han S-K (2002) Typical
1175 freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton
1176 of lakes and rivers. *Aquat Microb Ecol* **28**: 141–155.

1177

1178

1179

1180 Table S1. Bacterial diversity observed in different types of water, presence in the
1181 human-associated microbiome and occurrence of antibiotic resistance genes already
1182 characterized

1183

1184

1185 Table S2. Examples of antibiotic resistance genes detected in surface water, drinking
1186 water and wastewater

1187

1188 Fig. 1. Dendrogram representations of the bacterial diversity (a. *Proteobacteria* classes
1189 and b. other phyla) observed in different types of water, occurrence in the human-
1190 associated microbiome (H) and previous description of antibiotic resistance genes (R).
1191 The dendrograms were constructed with the iTOL – interactive tree of life (Letunic &
1192 Bork, 2007, 2011), based on the taxon ID codes, corresponding to the identifications
1193 provided in each of the publications cited (see Table S1).

1194 Different phyla or *Proteobacteria* classes (inner circle) are represented by different
1195 colours (when are represented by two or more bacterial genera), and the presence in
1196 different types of water are represented by the outer bars. Types of water: SW, surface
1197 water that includes W (wetlands), R (rivers), L (lakes); MW, mineral drinking water that
1198 also includes spring water; U-DW, untreated drinking water; T-DW, treated drinking
1199 water; Ur-WW, urban domestic wastewater that may also include industrial
1200 wastewaters; A-WW, animal wastewater.

1201

1202 Fig. 2. A domestic wastewater treatment plant (WWTP) discharges about one billion
1203 (10^9) ciprofloxacin resistant coliforms per minute.

1204 Total and ciprofloxacin resistant coliforms (CFU per day) discharged by WWTP in
1205 different countries [WWTP1-WWTP5, Portugal (PT); WWTP6, Poland (PL); WWTP7,
1206 Ireland (IE)], with different sizes (average day flow of 20 000, 32 500, 900, 890, 200,
1207 96 000 and 49 000 m^3, respectively) and treatment processes [activated sludge
1208 (WWTP1 and WWTP6), trickling filter (WWTP2), submerged aerated filter
1209 (WWTP3), aeration lagoon (WWTP4), anaerobic lagoon (WWTP5), unknown
1210 secondary treatment (WWTP7), with bacterial removal rates above of 1.5-4 log(CFU)
1211 (Galvin *et al.*, 2010; Łuczkiewicz *et al.*, 2010; Manaia *et al.*, 2010; Novo & Manaia,
1212 2010).

1213

1214 Fig. 3. Hypothesis about the relationship between environmental and human antibiotic
1215 resistome. A) cycle of known clinically-relevant antibiotic resistance determinants; B)
1216 transfer of antibiotic resistance genetic determinants from clinically-relevant bacteria to
1217 commensal human microbiota; C) transfer of antibiotic resistance genetic determinants
1218 from the natural resistome to clinically-relevant bacteria either thriving in the
1219 environment (C1) or hosted by humans (C2); D) indirect transfer of antibiotic resistance

1220 determinants from the natural resistome to clinically-relevant bacteria via human
1221 microbiome.