Title: Ubiquitous and persistent *Proteobacteria* and other Gram-negative bacteria in drinking water

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Abstract

Drinking water comprises a complex microbiota, in part shaped by the disinfection and distribution systems. Gram-negative bacteria, mainly members of the phylum *Proteobacteria*, represent the most frequent bacteria in drinking water, and their ubiquity and physiological versatility raises questions about possible implications in human health. The first step to address this concern is the identification and characterization of such bacteria that is the first objective of this study, aiming at identifying ubiquitous or persistent Gram-negative bacteria, *Proteobacteria* or members of other phyla, isolated from tap water or from its source.

More than 1000 bacterial isolates were characterized and identified, and a selected group (n=68) was further analyzed for the minimum inhibitory concentrations (MIC) to antibiotics (amoxicillin and gentamicin) and metals (copper and arsenite). Total DNA extracts of tap water were examined for the presence of putatively acquired antibiotic resistance or related genes (*intI1*, bla_{TEM}, *qnrS* and *sul1*). The ubiquitous tap water genera comprised *Proteobacteria* of the class *Alpha-* (*Blastomonas*, *Brevundimonas*, *Methylobacterium*, *Sphingobium*, *Sphingomonas*), *Beta-* (*Acidovorax*, *Ralstonia*) and *Gamma-* (*Acinetobacter* and *Pseudomonas*). Persistent species were members of genera such as *Aeromonas*, *Enterobacter* or *Dechloromonas*. *Ralstonia* spp. showed the highest MIC values to gentamicin and *Acinetobacter* spp. to arsenite. The genes *intI1*, bla_{TEM} or *sul1* were detected, at densities lower than 2.3×10^5 copies/L, 2.4×10^4 copies/L and 4.6×10^2 copies/L, respectively, in most tap water samples. The presence of some bacterial groups, in particular of *Beta-* or *Gammaproteobacteria* (e.g. *Ralstonia*, *Acinetobacter*, *Pseudomonas*) in drinking water may deserve attention given their potential as reservoirs or carriers of resistance or as opportunistic pathogens.

Keywords: tap water safety; acquired resistance genes; bacterial diversity; opportunistic pathogens; correlation analysis

1. Introduction

In many countries, tap water is produced from freshwater that undergoes a series of treatment stages necessary to achieve the adequate chemical and microbiological quality and safety (WHO, 2008). Although drinking water microbiota is known to be affected by both treatment and distribution systems (Poitelon et al., 2010; Pinto et al., 2012; Chao et al., 2013; Vaz-Moreira et al., 2013), the tap water reaching the consumer, meeting quality and safety criteria, harbors a wide diversity of bacteria (Vaz-Moreira et al., 2014). Among the most frequent bacterial groups in drinking water are Gram-negative bacteria members of the phylum *Proteobacteria*, mainly of the classes *Alpha-*, *Beta-* and *Gamma-* (Hoefel et al., 2005; Eichler et al., 2006; Poitelon et al., 2009; Kormas et al., 2010; Revetta et al., 2010; Pinto et al., 2012; Vaz-Moreira et al., 2013; Huang et al., 2014; Bai et al., 2015; Jia et al., 2015). In addition to the *Proteobacteria*, members of the phylum *Bacteroidetes* are also among the predominant bacteria in drinking water (Hoefel et al., 2005; Eichler et al., 2006; Henne et al., 2012; Lymperopoulou et al., 2012).

In spite of the importance of this issue, scarce is the recent literature that explores the phylogenetic diversity and physiological characteristics of the predominant bacterial groups occurring in drinking water. Yet, such information would shed additional light on the ecology of tap water bacteria and on their implications on human health. Several recent studies have offered a comprehensive overview of the bacterial diversity in tap water based on next generation sequencing tools (Huang et al., 2009; Chao et al., 2013; Bai et al., 2015; El-Chakhtoura et al., 2015; Jia et al., 2015), however it is increasingly recognized the importance of the use of culture-dependent methods for the characterization of environmental samples (Giovannoni and Stingl, 2007; Alain and Querellou, 2009; Vartoukian et al., 2010). Actually this seems to be a major gap, since the characterization of pure cultures is still the gold standard to boost our understanding of relevant microbial characteristics. Indeed, physiological and structural aspects and the establishment of relationships between the genome and the phenotypic traits, such as stress tolerance, antibiotic resistance or other, are essentially culture-dependent (Zengler et al., 2002; Giovannoni and Stingl, 2007; Vartoukian et al., 2010).

Water disinfection imposes a strong stress on microbial communities (Jia et al., 2015), which allegedly may be associated with the increase of the prevalence of antibiotic resistant bacteria (Xi et al., 2009; Figueira et al., 2012; Shi et al., 2013; Bai et al., 2015) and genes (Xi et al., 2009; Shi et al., 2013; Jia et al., 2015). Previous studies have

shown that the antibiotic resistance patterns of bacteria recovered from tap water are often related to the species rather than to the site of isolation and, therefore, involving mainly intrinsic mechanisms of resistance (Vaz-Moreira et al., 2011; Vaz-Moreira et al., 2012; Narciso-da-Rocha et al., 2013). However, either due to intrinsic or acquired features, antibiotic resistance is always of concern, mainly if it refers to ubiquitous bacteria with high probability of reaching the human body and behave as opportunistic pathogens (Vaz-Moreira et al., 2014; Hugon et al., 2015). Therefore, the identification of the most ubiquitous and persistent bacteria in drinking water may contribute to improve the current knowledge not only on the ecology of bacteria in drinking water systems, but also on the potential human-health risks associated with their presence in tap water.

This study aimed at identifying culturable Gram-negative bacteria from tap water with widespread distribution in tap water and in its source (ubiquitous) or suggested as possible resident bacteria, when were found at different sampling occasions (persistent). Because bacterial resistance to different antimicrobial classes is an issue of concern in surface and tap water (Vaz-Moreira et al., 2011; Vaz-Moreira et al., 2012; Narciso-da-Rocha et al., 2013; Narciso-da-Rocha et al., 2014a; Narciso-da-Rocha and Manaia, 2016), this study also aimed to characterize some of the ubiquitous bacteria in terms of the minimum inhibitory concentration (MIC) to selected antibiotics and heavy metals. In addition, the occurrence of genes commonly associated with acquired antibiotic resistance was surveyed in total DNA extracts of tap water and possible correlations with culturable bacteria were accessed.

2. Materials and Methods

2.1 Samples

Water samples were collected from 11 household taps and at different stages of the drinking water treatment plant - raw surface water, alluvial wells, after ozonation, and after chlorination; and at the bulk supply distribution system, at four sampling points, two before and two after a reservoir and a re-chlorination point (Figure 1). The sampled household taps are located in four distinct municipal distribution networks, in a total area of about 270 km^2, located in the north of Portugal. Samples from the water treatment plant and distribution system were collected in November 2007, December 2008 and September 2009, at the sampling points used for the routine monitoring analyses by water quality control entities. Household tap water samples were collected

in April, July and October 2009. Samples collected after chlorination were neutralized with 0.1 g/L of sodium thiosulfate. All samples were processed within 4 h after collection.

2.2. Microbiological characterization and bacterial isolation

Total heterotrophic bacteria and total cell counts were determined, in triplicate, for all samples, as described before (Vaz-Moreira et al., 2013). Briefly, total cell number was enumerated by fluorescence microscopy after staining with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) as described by Brunk *et al*. (1979). To enumerate and isolate cultivable bacteria, volumes up to 100 mL of water or the respective decimal serial dilutions were filtered through cellulose nitrate membranes (0.45 μm pore size, 47 mm diameter; Albet), which were placed onto the culture media (R2A, Pseudomonas isolation agar, Tergitol-7 agar, mFC, Bile Esculin Agar and Mannitol Salt Agar). Colony forming units were enumerated and representative colonies were selected for isolation and purification according to the following criteria: about 50% of the colonies with a morphotype represented by more than 10 CFU, and all the colonies with a morphotype represented by up to 10 CFU (Vaz-Moreira et al., 2011). Pure cultures of a total of 3157 isolates (1449 from tap water) were preserved and preliminarily characterized for their colony and cellular morphology, Gram-staining reaction, catalase and cytochrome *c* oxidase and Ziehl-Neelsen staining as described by Smibert and Krieg (1994). All isolates observed to stain as Gram-negative (n=1527) were the basis of this study, after the trial of non-repetitive isolates by Random Amplified Polymorphic DNA PCR (RAPD-PCR).

2.3. Gram-negative bacteria typing and identification

All isolates were genotyped based on RAPD-PCR using crude cell extracts and the primer M13 (GAG GGT GGC GGT TCT), as described previously (Ferreira da Silva et al., 2006). This procedure allowed the identification of different RAPD profiles and supported the selection of non-repetitive isolates, i.e. isolated from the same culture and yielding identical genotype. After repetitive isolates removal, was left a group of 1089 that constituted the basis of the results presented in this paper. Most unique or unreliable (e.g. low number of bands) RAPD profiles and at least one isolate of each profile that included more than one isolate, were identified based on the 16S rRNA or *rpoB* (for *Acinetobacter* spp. isolates) gene sequence analysis. Amplicons of the genes 16S rRNA

and *rpoB* were obtained with the primers 27F/1492R and Ac696F/Ac1598R, respectively, as described before (Ferreira da Silva et al., 2007; Narciso-da-Rocha et al., 2013) and sequenced by the Sanger method. In total, at least 75% of isolates of each tap were identified based on the gene sequence analyses. The 16S rRNA gene nucleotide sequences were used to query the EzTaxon library (Chun et al., 2007) and the *rpoB* gene the GenBank database (http://www.ncbi.nlm.nih.gov/). Of the 1089 isolates confirmed as non-repetitive 795 were identified to species level (Table 1).

2.4. Persistent and ubiquitous Gram-negative taxa

As a way to make the analysis of the results more objective, the genera or species of the isolates were classified as ubiquitous and/or persistent, according to the following criteria: a ubiquitous genus was identified at least in five of the 11 sampled taps or in the three types of water (raw/ozonated, final treated and tap water); a persistent species was identified in the same site in all the three sampling campaigns or in the first and third campaign.

2.5. Determination of MICs to antibiotics and metals in the ubiquitous tap water bacteria

At least 10% of the isolates of each genus classified as ubiquitous (n=9) (*Blastomonas* n=3/26, *Brevundimonas* n=6/34, *Methylobacterium* n=5/17, *Sphingobium* n=5/24, *Sphingomonas* n=10/54, *Acidovorax* n=7/29, *Ralstonia* n=4/34, *Acinetobacter* n=21/155 and *Pseudomonas* n=7/48) were characterized for the MIC to selected antibiotics and metals, based on the microdilution method as described by Andrews (2001). Cultures were tested in 96-well microtiter plates in Mueller-Hinton broth (Oxoid) supplemented with amoxicillin (4-128 mg/L, Sigma), gentamicin (0.5-16 mg/L, Affymetrix), arsenite ($AsNaO_2$; 0.2-10 mM Sigma) or copper ($CuSO_4.H_2O$; 0.5-15 mM Merck), inoculated with a bacterial suspension with an optical density (610 nm) of approximately 0.05, and incubated for 24 h at 30ºC. For copper concentrations higher than 5 mM, the results were confirmed in 0.1 M Tris-buffered Mueller-Hinton broth (Butler et al., 2001). *Escherichia coli* strain ATCC 25922 was used in each plate as a control. The antimicrobials tested were selected based on the fact that the resistance to amoxicillin is frequent in tap water bacteria, often due to intrinsic mechanisms and because in some studies resistance to aminoglycosides was observed in tap water but not in the water source, suggesting a selection effect (Armstrong et al., 1982; Vaz-Moreira et al., 2011;

Vaz-Moreira et al., 2012; Narciso-da-Rocha et al., 2013). The rationale for the selection of the heavy metals arsenite and copper was the common occurrence of arsenic in natural waters (Nordstrom, 2002; Smedley and Kinniburgh, 2002) and the presence of copper in some of the materials used for the water pipes.

2.6. Detection and quantification of genes in total DNA extracts of tap water

The genes 16S rRNA, *intI1*, bla_{TEM}, *sul1* and *qnrS* were quantified in three replicates of total DNA extracts, obtained from 15 L of water, in each sampling date of the 11 household tap water samples (Vaz-Moreira et al., 2013) using quantitative PCR as described before (Moreira et al., 2016).

2.7. Statistical analysis

A Principal Components Analysis (PCA) was performed with the MIC values for the tested isolates belonging to ubiquitous genera. Possible correlations between the diversity of species found and their abundance in tap water, and the total and cultivable bacteria counts (Vaz-Moreira et al., 2013), and the abundance of the selected genes, detected in tap water samples were assessed based on Canonical Correlation Analysis (CCA), using the *log*-transformed values of each variable. For qPCR determinations that were below the limit of quantification (LOQ) but above the limit of detection (LOD) (i.e. based on melting curve analysis, the amplicon corresponded to the targeted gene, and the Ct value was above the minimal concentration in the calibration curve) a value of half the LOQ was used for the matrix construction. The analyses were performed with the CANOCO 5 software (Ter Braak and Šmilauer, 2012).

3. Results

3.1. Phyla and classes bacterial identification

The tap water analyzed in this study undergoes different treatment stages (Figure 1) and for a matter of simplicity, from this point forward "water source" includes raw and ozonated water, "treated water" includes samples collected after chlorination and "tap water" includes household taps. Bacteria staining Gram-negative represented approximately half of the total isolates recovered at the different stages (48%, 1527/3157) (Figure 1). The Gram-negative, non-repetitive isolates, identified in this study (n=795) could be affiliated to 135 species, 61 identified in the water source, 27 in the treated water and 77 in tap water (Table 1, Figure 2). Most of these species (n=118)

belonged to the phylum *Proteobacteria* (classes *Alpha-*, n=36, *Beta-*, n=29 and *Gammaproteobacteria*, n=53), while a minority (n=17) were identified as members of the phyla *Bacteroidetes* (n=16) (classes *Flavobacteriia*, n=11 and *Sphingobacteriia*, n=5) and *Verrucomicrobia* (n=1) (class *Verrucomicrobiae*) (Figure 2). Most *Gammaproteobacteria* (n=36/53), *Betaproteobacteria* (n=15/29) and *Bacteroidetes* (n=6/16) were identified in the water source, in raw water or after ozonation (n=34, 8; n=7, 9; and n=6, 0, respectively). In contrast, *Alphaproteobacteria* were identified mainly in tap water (n=32/36) (Figure 2).

3.2. Ubiquitous and persistent bacterial groups

Ubiquitous bacteria, *i.e.* observed to occur in the source, treated water and in tap water or in at least five of the 11 taps analyzed, were members of genera of the classes *Alpha-*, *Beta-* and *Gammaproteobacteria*. Ubiquitous genera found in the source, treated and tap water were the *Alphaproteobacteria* of the genera *Blastomonas* (*Blastomonas natatoria*) and *Brevundimonas* (*Brevundimonas nasdae*) and the *Gammaproteobacteria* of the genus *Acinetobacter* (*Acinetobacter johnsonii*, *Acinetobacter lwoffii* and *Acinetobacter parvus*) (Figure 2). Tap water ubiquitous genera were the *Alphaproteobacteria Blastomonas*, *Brevundimonas*, *Methylobacterium*, *Sphingobium*, and *Sphingomonas*; the *Betaproteobacteria Acidovorax* and *Ralstonia*; and the *Gammaproteobacteria Acinetobacter* and *Pseudomonas* (Figure 2). Some of the ubiquitous genera comprised species that were identified in at least five taps, as for example, *Blastomonas natatoria*, *Brevundimonas nasdae*, *Sphingobium yanoikuyae*, *Sphingomonas panni*, *Sphingomonas yunnanensis*, *Ralstonia pickettii*, *Acinetobacter beijerinckii*, *Acinetobacter johnsonii* and *Acinetobacter lwoffii*.

Most of the ubiquitous genera, and some of the species, in tap water were observed to be also persistent (Figure 2), i.e. a species isolated from the same site in all the sampling campaigns or at least in the first and last sampling campaigns (which occurred at least six months after the first). The coincidence between ubiquity and persistence for a species may suggest that either there is an environmental reservoir of that microorganism or that it may be able to reside and proliferate in that environment. Species observed to be persistent in the water source (raw/ozonated) were all *Gammaproteobacteria* - *Aeromonas hydrophila* (W3), *Aeromonas veronii* (W1, W3), *Acinetobacter junii* (W1, W2), *Enterobacter ludwigii* (W1), and *Pseudomonas simiae* (W1, W3). In treated water only one species of *Alphaproteobacteria* (*Brevundimonas*

nasdae, W5) was observed to persist. Persistent species in tap water were in general distinct of those detected upstream and were mainly *Alphaproteobacteria - Blastomonas natatoria* (T6, T9), *Sphingomonas dokdonensis* (T2), *Sphingomonas mucosissima* (T2) and *Sphingomonas yunnanensis* (T9). Other tap water persistent bacteria were affiliated to taxa such as *Betaproteobacteria - Acidovorax temperans* (T9) and *Dechloromonas agitata* (T9) and *Gammaproteobacteria - Acinetobacter ursingii* (T10) and *Pseudomonas aeruginosa* (T6) (Figure 2).

3.3. MICs to antibiotics and metals of ubiquitous tap water bacteria

Ubiquity may be associated with the ability of bacteria to cope with adverse conditions. In previous works we observed that aminopenicillins and aminoglycoside resistance is common in tap water bacteria (Vaz-Moreira et al., 2011; Vaz-Moreira et al., 2012; Narciso-da-Rocha et al., 2013) and that metal and metalloid tolerance is also common in these bacteria (unpublished). These were motivations to assess the MIC values for amoxicillin and gentamicin and to copper and arsenite in bacteria belonging to ubiquitous tap water genera (Figure 3, Figure S1).

Increased tolerance against each of the tested antimicrobials was, in general, strain dependent, with MIC values of amoxicillin ($MIC_{amoxicillin}$), gentamicin ($MIC_{gentamicin}$), copper (MIC_{Cu}) and arsenite (MIC_{As}) ranging ≤4.0 and >128.0 mg/L, ≤0.5 and >16 mg/L, 2.5 - >15 mM, and ≤0.2 and >10 mM, respectively. A Principal Component Analysis of the data distributed the isolates over axis 1 according to their tolerance to amoxicillin, gentamicin, and copper and over axis 2, based on their tolerance to arsenite (Figure 3). Most of the tested isolates of *Ralstonia* (*R. pickettii*, *R. mannitolilytica*), as well as isolates of *Pseudomonas aeruginosa*, *Pseudomonas chlororaphis*, *Pseudomonas poae*, *Pseudomonas nitroreducens*, *Sphingobium limneticum* and some isolates of *Acinetobacter lwoffii*, *Acinetobacter johnsonii*, and *Acinetobacter tjernbergiae* showed the highest $MIC_{amoxicillin}$ values (≥128 mg/L). Part of these isolates showed also high MIC values to gentamicin ($MIC_{gentamicin}$ > 16 mg/L) and copper (MIC_{Cu} ≥ 10 mM), while others, in particular *Ralstonia pickettii*, combined the highest MIC values of the tested antibiotics ($MIC_{amoxicillin}$ ≥ 128 mg/L and $MIC_{gentamicin}$ > 16 mg/L). One isolate identified as *Acinetobacter ursingii* presented also high $MIC_{gentamicin}$ and $MIC_{amoxicillin}$ values (> 16 mg/L and 64 mg/L, respectively). The combination of high $MIC_{amoxicillin}$ (64-128 mg/L), $MIC_{gentamicin}$ (2-16 mg/L) and MIC_{As} (≥ 5 mM) was observed in particular in *Gammaproteobacteria* identified as *Acinetobacter johnsonii*, *Acinetobacter*

ursingii, *Acinetobacter tjernbergiae* and *Pseudomonas nitroreducens*. The highest MIC_{As} value (≥ 10 mM) was observed not only in the genus *Acinetobacter*, in the species *Acinetobacter pittii*, *Acinetobacter beijerinckii*, *Acinetobacter ursingii*, *Acinetobacter johnsonii*, but also in *Alphaproteobacteria* identified as *Brevundimonas "olei"and Sphingomonas yunnanensis.* Most of these isolates showed low $MIC_{gentamicin}$ values (≤ 6 mg/L) (Figure 3, Figure S1). The combination of different degrees of tolerance to the tested antimicrobials is expressed in the PCA biplot (Figure 3) that suggests the organization of four major groups according to the MIC values.

3.4. Detection and quantification of antibiotic resistance or related genes in tap water

Given their widespread distribution in environmental samples, the genes *intI1*, bla_{TEM}, *sul1* and *qnrS* (Rizzo et al., 2013; Varela et al., 2016) were surveyed in total DNA extracts of tap water. This was an approach to infer about the occurrence of acquired antibiotic resistance or related genes in tap water and their relationship with the tap water cultivable bacterial communities.

In most of the tap water samples, the genes *intI1* and bla_{TEM} were above or close to the limit of quantification (LOQ *intI1* gene = 140 copies/L; LOQ bla_{TEM} gene = 1230 copies/L) (Figure S2.A). The occurrence of gene *intI1* seemed to be variable over time. Except in taps T7 and T8, where it reached the highest abundance *per* volume of water and was detected at all the sampling dates, gene *intI1* was not detected in taps T2, T3, T6, T9, and T10 in at least one of the sampling dates (Figure S2.A).

The gene bla_{TEM} was detected in all the samples and quantified in nine of the eleven taps, but never consecutively over the three sampling dates (Figure S2). The gene *sul1* (LOQ *sul1* gene = 310 copies/L) was above the LOQ only in taps T1 and T8, in one or two of the sampling dates (B or A and B, respectively) although it was detected in all the samples. The *qnrS* gene was not detected in any of the tested samples.

3.5. Relationships between the abundance of cultivable Gram negative bacteria, bacterial counts and genes copy number in tap water

In order to assess possible relationships between the abundance of the cultivable Gram-negative bacterial species and the total and cultivable cell counts and genes copy number in tap water, a Canonical Correlation Analysis was performed. This multivariate analysis showed that about 10% of the observed variance in the abundance of the different cultivable Gram-negative bacteria could be explained based on the total and

cultivable cell counts and gene copy number, with all these variables showing the same pattern of correlation (Figure 4A). Among the bacterial groups significantly correlated with the tested variables were the *Alphaproteobacteria Shingobium amiense*, *Sphingobium limneticum*, *Sphingomonas pituitosa*, the *Betaproteobacteria Dechloromonas agitata*, *Acidovorax soli* and *Pelomonas saccharophila*, the *Gammaproteobacteria Pseudomonas toyotomiensis* and *Pseudoxanthomonas japonensis*, and the *Bacteroidetes Chryseobacterium daeguense* and *Flavobacterium cheonhonense*. The presence of the genes *intI1* and of *sul1*, as well as the total counts of bacteria were the variables with highest correlation with those bacterial groups. In opposition, some bacterial species (*Rhizobium nepotum*, *Sphingobium czehense*, *Sphingopyxis panaciterrae*, *Pseudomonas poae* and *Sphingobacterium caeni*) were negatively correlated with those same variables (Figure 4B). The species positively correlated with the tested variables were mainly associated with tap T8 whereas the negatively correlated were mainly associated with taps T5 and T3. Taps T5 and T3 were those with the lowest abundance of cell counts and gene copy numbers, at least in the first sampling date (A). The organisms associated with these taps were mainly from non-ubiquitous genera, and were most of them sporadically identified in one of that taps: *Rhizobium nepotum, Sphingobium czehense*, *Sphingopyxis panaciterrae*, *Variovorax boronicumulans*, *Pseudomonas poae* and *Sphingobacterium caeni*. In contrast, tap T8 is one of the taps with the highest abundance of cell counts and gene copy numbers (Figure S2). Some of the organisms isolated from this tap were only sporadically detected (e.g. *Pelomonas saccharophila*, *Pseudoxanthomonas japonensis*, *Chryseobacterium daeguense* and *Flavobacterium cheonhonense*) but most of them were members of the ubiquitous genera or persistent species in tap water, such as *Sphingobium* (*Sphingobium amiense*, *Sphingobium limneticum*), *Sphingomonas* (*Sphingomonas pituitosa*), *Acidovorax* (*Acidovorax soli*), *Dechloromonas* (*Dechloromonas agitata*), *Pseudomonas* (*Pseudomonas toyotomiensis*) (Figure 4). With exception of *Dechloromonas agitata*, which was persistent, none of these species was persistent or ubiquitous in taps. In addition, among the isolates tested, only the members of the species *Sphingobium limneticum* were in the group of isolates with the highest amoxicillin and gentamicin MIC values.

4. Discussion

One of the reasons of interest to study Gram-negative bacteria in drinking water was the clear demonstration in various publications that *Proteobacteria* are the most predominant taxa in drinking water bacterial communities (Eichler et al., 2006; Poitelon et al., 2009; Kormas et al., 2010; Lymperopoulou et al., 2012; Pinto et al., 2012; Vaz-Moreira et al., 2013; Huang et al., 2014; Bai et al., 2015; Jia et al., 2015). The focus on cultivable bacteria relied on the fact that it allows the identification into the genus and species level and, simultaneously, offers the possibility to test phenotypic traits such as antimicrobial tolerance.

This study confirmed that Gram-negative bacteria in drinking water were mainly *Proteobacteria*, although represented by different classes in the water source and in tap water. Cultivable *Gammaproteobacteria* predominated in the raw/ozonated water, while *Alphaproteobacteria* prevailed in the tap water. The *Gammaproteobacteria* class, comprises an important group of bacteria frequently associated to human impacted environments (e.g. *Enterobacteriaceae*, *Vibrionaceae*, *Pseudomonadaceae*, and *Xanthomonadaceae*) (Kersters et al., 2006; Williams et al., 2010; Xia et al., 2010; Ye and Zhang, 2012). In contrast, most of the *Alphaproteobacteria* prefer habitats with very low levels of nutrients, a preference that confers an advantage to survive in the tap water environment (Gomila et al., 2005; Chao et al., 2013).

In agreement with the high prevalence of *Alphaproteobacteria* in tap water, a high number of genera from this class was observed to be ubiquitous in tap water (*Blastomonas*, *Brevundimonas*, *Methylobacterium*, *Sphingobium* and *Sphingomonas*). In addition, some *Betaproteobacteria* (*Acidovorax* and *Ralstonia*) and *Gammaproteobacteria* (*Acinetobacter* and *Pseudomonas*) were also ubiquitous. Among these genera, *Sphingomonas*, *Acinetobacter* and *Pseudomonas* are the most frequently recognized as ubiquitous in drinking water (Allen et al., 2004), although all the ubiquitous genera identified have been reported in treated drinking water before (Tokajian et al., 2005; Vaz-Moreira et al., 2014; Jia et al., 2015).

Some species belonging to the ubiquitous bacterial genera were observed to become persistent in some taps (Figure 2). Examples of these species were the *Blastomonas natatoria*, *Sphingomonas dokdonensis*, *Sphingomonas mucosissima*, *Sphingomonas yunnanensis*, *Dechloromonas agitata*, *Acidovorax temperans*, *Acinetobacter ursingii* and *Pseudomonas aeruginosa*. The presence of persistent species was only observed in four taps (T2, T6, T9 and T10), being T9 the tap with the highest number of persistent species. These species or other members of their genera (*Blastomonas*, *Sphingomonas*,

Acidovorax, *Dechloromonas*, *Acinetobacter* and *Pseudomonas*) were previously described as biofilm formers in treated drinking water (Koskinen et al., 2000; Emtiazi et al., 2004; Hong et al., 2010; Schwering et al., 2013; Kelly et al., 2014; Zhu et al., 2014), which may justify their persistence in these taps.

Based on biochemical identification and on 16S rRNA gene cloning and metagenomics analysis, Shi *et al*. (2013) consistently observed that *Proteobacteria* were the antibiotic resistant bacteria predominating in drinking water. Indeed, some *Proteobacteria* bacterial genera, such as *Pseudomonas*, *Acinetobacter*, *Acidovorax* and *Sphingomonas* have been referred to as common examples of antibiotic resistant bacteria thriving in aquatic environments (Papapetropoulou et al., 1994; Furuhata et al., 2006; Vaz-Moreira et al., 2011; Vaz-Moreira et al., 2012; Narciso-da-Rocha et al., 2013; Flores Ribeiro et al., 2014; Narciso-da-Rocha et al., 2014a). These were motivations to assess, in this study, the level of tolerance to some antimicrobials in ubiquitous tap water bacteria. The two underlying hypotheses behind the determination of MIC values were the possibility that 1) similar MIC values are observed within a given taxonomic group and/or 2) some groups of ubiquitous bacteria present a general high tolerance to different classes of antimicrobials. The aim was mainly to assess if irrespective of the taxon, ubiquitous tap water bacteria presented high MIC values or if this was a variable trait that although due to intrinsic mechanisms could vary due to the natural phenotypic variability of individuals within the same taxonomic group. According to the results obtained it is suggested that while in some taxa the ubiquity may be simply due to the natural ecological dissemination of that species, as can be the case of some species with MIC values in general low (e.g. *Methylobacterium* and *Acidovorax*), other may indeed have a selective advantage due to a high resilience potential. Such a selective advantage may explain the ubiquity of some bacteria with the highest MIC values for the antibiotics and metals tested, for example *Ralstonia* spp., *Acinetobacter* spp. or *Pseudomonas* spp. (Supplementary Figure 1), all members of the sub-classes *Beta*- and *Gammaproteobacteria*. Moreover, the combination of high MIC values to different classes of antimicrobials was detected mainly in some isolates of the genera *Pseudomonas*, *Ralstonia*, *Sphingobium* or *Acinetobacter*; and in this last genus it was evidently a property that was not species dependent suggesting possible adaptation processes. These traits may suggest that members of these species have a particular plasticity being able to adapt to different types of environmental stress.

The resistance to chlorine disinfection (Mathieu et al., 2009) described for biofilm bacteria from the *Beta*- and *Gammaproteobacteria* classes, may justify the persistent detection of some bacterial groups such as *Acinetobacter* sp. and *Pseudomonas* sp. in tap water. It would be interesting to explore if there exists a correlation between the increased tolerance to chlorine described by Mathieu *et al*. (2009) and to other antimicrobials (e.g. antibiotics and heavy metals). In this work we observed that some of the isolates belonging to the persistent species in tap water have increased tolerance to arsenite (Supplementary Figure 1), and belong mainly to the genus *Acinetobacter*. Previous studies suggested the importance of the vertical resistance transmission in drinking water bacteria (e.g. *Sphingomonadaceae, Acinetobacter* spp., and *Pseudomonas* spp.) (Vaz-Moreira et al., 2011; Vaz-Moreira et al., 2012; Narciso-da-Rocha et al., 2013; Narciso-da-Rocha et al., 2014a). Although most of the resistance phenotypes observed in culturable bacteria are probably intrinsic, genes associated with acquired antibiotic resistance were detected in some of the tap water samples. Although the genes *sul1*, *intI1* and bla_{TEM} presented quantities *per* mL in the tap water below the average values previously observed for treated wastewater (Narciso-da-Rocha et al., 2014b; Manaia et al., 2016) when the gene abundance was analysed in function of ng of DNA or number of copies of 16S rRNA gene, the prevalence of the gene bla_{TEM}, for some of the taps, was similar or even higher than in treated wastewater (Narciso-da-Rocha et al., 2014b; Manaia et al., 2016) (Figure S2.B and S2.C). These results suggest that also drinking water can be an important vehicle and reservoir of antibiotic resistance.

Although the abundance of cultivable Gram-negative species could explain only a small part of the variability, some correlations were observed between the total cell counts and the quantity of the genes *sul1* and *intI1* and between the cultivable bacteria counts and the genes 16S rRNA and bla_{TEM}. The gene bla_{TEM} followed a pattern that seems to be seasonal, being mainly detected in the second sampling campaign (July) (Figure S2). The ubiquitous genus *Ralstonia* presented the same pattern; nevertheless no correlation was observed between the quantity of the gene bla_{TEM} and the abundance of the *Ralstonia* spp. The quantification of the gene *sul1* only in two of the eleven taps agrees with the observed by Shi *et al*. (2013), that although the genes *sul1* and bla_{TEM} have a high prevalence in drinking water immediately after the chlorination, the gene *sul1* almost disappeared at the tap level.

5. Conclusions

This study revealed a higher diversity of *Gammaproteobacteria* in the raw water than in taps, where was found a higher diversity of *Alphaproteobacteria*. Some of the persistent and ubiquitous bacterial groups often coincided, suggesting that members of these groups have increased capacity to survive the water treatment or to colonize treated water environments. Among the ubiquitous bacteria in tap water, the *Ralstonia* spp. and *Acinetobacter* spp. showed the highest MIC values for gentamicin and arsenite, respectively. *Acinetobacter johnsonii* was one of the species with most variable MIC values, suggesting a high potential of adaptation. The abundance of isolates affiliated to the genera *Acidovorax*, *Pelomonas*, *Dechloromonas*, *Pseudomonas*, *Pseudoxanthomonas*, *Sphingomonas*, *Sphingobium* (*Beta-*, *Gamma-* and *Alphaproteobacteria*), *Flavobacterium* and *Chryseobacterium* (*Bacteroidetes*) correlated positively with the abundance of the genes *sul1* and *intI1*or with the total cell counts. Some of those bacterial groups were not among the most abundant in tap water, neither those with the highest MIC for the antimicrobials tested.

These results suggest that the complex microbiota of tap water may hide populations with potential to serve as carriers of genes such as *sul1* and *intI1*, hypothetical proxies of acquired antibiotic resistance, and to adapt to some antimicrobial agents, as metals.

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Table 1. Counts of bacterial isolates and species identified per sample location

Sample		No. Gram-negative isolates (%)	No. non-repetitive Gram-negative isolates	No. of isolates identified (%)	No. of identified species
Water source	W1	193 (59.2)	155	97 (62.6)	43
	W2	139 (54.3)	61	50 (82.0)	9
	W3	156 (68.1)	87	57 (65.5)	17
	Total	**488 (60.2)**	**303**	**204 (67.3)**	**61**
Treated water	W4	6 (3.0)	6	1 (16.7)	1
	W5	102 (36.2)	74	45 (60.8)	22
	W6	23 (17.2)	22	5 (22.7)	4
	W7	8 (7.8)	4	3 (75.0)	2
	W8	9 (5.1)	9	0	0
	Total	**148 (16.5)**	**115**	**54 (47.0)**	**27**
Tap water	T1	73 (59.4)	58	44 (75.9)	15
	T2	69 (60.0)	55	45 (81.8)	15
	T3	68 (61.8)	42	33 (78.6)	9
	T4	73 (57.0)	47	40 (85.1)	12
	T5	91 (61.5)	65	54 (83.1)	18
	T6	100 (72.5)	71	64 (90.1)	12
	T7	87 (64.9)	72	56 (77.8)	19
	T8	78 (55.3)	57	44 (77.2)	13
	T9	67 (46.2)	54	42 (77.8)	14
	T10	95 (66.9)	72	55 (76.4)	17
	T11	90 (72.0)	78	60 (76.9)	23
	Total	**891 (61.5)**	**671**	**537 (80.0)**	**77**
Total		**1527 (48.4)**	**1089**	**795 (73.0)**	**135**

Figure 1. Schematic representation of the process of drinking water production and distribution analyzed in this study. Samples were collected at the water source: raw surface water (W1), alluvial wells (W2) and after filtration and ozonation (W3); treated water: at the end of the treatment process (W4) and at the distribution system before (W5, W7) and after (W6, W8) two reservoir and distribution points (1); and from eleven household taps (T1-T11).
(2) water reservoirs. This figure was adapted from Vaz-Moreira *et al*., 2013

Figure 2. Dendrogram representation of the drinking water bacterial diversity. Different Gram-negative bacteria phyla or *Proteobacteria* classes (inner circle) are represented by different colors, and the presence in different types of water (raw/ozonated water, treated water and tap water) are represented by the outer bars. The clades of the tap water ubiquitous genera and the persistent species are highlighted in red, with the indication of the locals where the persistence was observed. The taps where the ubiquitous species were observed are indicated next to the green bar. The dendrogram was constructed with the iTOL – interactive tree of life (Letunic and Bork, 2007; 2011), based on the taxon ID codes.

Figure 3. Principal Components Analysis (PCA) of the Minimum inhibitory concentration (MIC) values for some isolates belonging to the ubiquitous bacterial genera in tap water. AML, amoxicillin; GEN, gentamicin; As^{3+}, arsenite; Cu^{2+}, copper.

The correspondence between the codes in the figures and the species names is in Table S1.

Figure 4. Canonical correlation analysis (CCA) biplots of the relationship between abundance of the cultivable bacterial species and the total cells and cultivable bacteria counts *per* mL and the quantification of genes *per* mL of sample (16S rRNA, *sul1*, *intI1* and bla_{TEM}). A) correlation between samples and variables and B) correlation between species and variables.

The correspondence between the codes in the figures and the species names is in Table S1.