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UNIVERSIDADE CATÓLICA PORTUGUESA | PORTO

Escola Superior de Biotecnologia

Bacterial diversity and antibiotic resistance from the water source to the tap

Thesis submitted to the Universidade Católica Portuguesa to attain the degree of PhD in Biotechnology with specialization in Microbiology

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Under the supervision of Professor Célia Maria Manaia Rodrigues

Under the co-supervision of Professor Olga Cristina Pastor Nunes

April 2012

‘‘Everything comes from water!

And everything is kept alive by water!’’

J.W. von Goethe, Faust II, 1833

to all the persons who crossed my life

and taught me something

ABSTRACT

Water is one of the most important habitats for bacteria in the environment. The continuous flux in the urban water cycle carries water through many places, dragging bacteria and numerous chemical contaminants. This makes of water one of the most important vehicles, not only for the dissemination of the chemical substances, but also for the dissemination of organisms and, consequently, the respective resistance genes in the environment. The main goal of this study was to investigate if drinking water production and distribution could represent a hotspot for the proliferation, selection or incoming of antibiotic resistant bacteria, and the likelihood of these organisms to reach the final consumer, via tap water. In order to meet this objective, the study was planned aiming the tracking of bacterial communities and individual isolates from the source to the tap.

Firstly, the abundance and diversity of bacteria in raw, treated and final (tap) water was characterized using culture-dependent and culture-independent (16S rRNA-DGGE) approaches. Both approaches showed that the water treatment reduced the bacterial counts, diversity and cultivability, promoting also a shift in the cultivable bacterial community from predominantly Gram-negative to predominately Gram-positive bacteria. Nevertheless, this effect was reverted, and in tap water Gram-negative bacteria became predominant. Moreover, in tap water total and cultivable bacteria counts were higher than in the disinfected water collected from the distribution system. These results suggest the occurrence of bacterial regrowth and/or biofilm formation over the distribution system or at tap level. Although changes in the bacterial community structure over the water circuit were observed, the predominant phylum detected, by 16S rRNA-DGGE, was the same in all the sampling points – *Proteobacteria* (mainly of classes *Alpha*, *Beta* and *Gamma*).

Culture-dependent and culture-independent approaches were compared to assess which groups might be overlooked by cultivation procedures. In order to have a clear evidence of the bacterial groups which could be overlapped using those procedures, culture-dependent and two culture-independent (16S rRNA gene based DGGE and 454 pyrosequencing) methods were compared for their ability to survey the bacterial diversity of a sample. Such a comparison showed that although the different methods detected the same predominant phyla, different bacteria were targeted. Thus, besides the previous expectation that culture-independent methods would detect more bacterial groups than cultivation methods, it was also concluded that both approaches target different bacterial populations.

Based on the study of the bacterial diversity, mainly of cultivable bacteria, and in the literature available, two of the most relevant taxonomic groups detected in drinking waters, due to the widespread distribution and/or abundance, were further studied. Thus, *Sphingomonadaceae* and *Pseudomonas* spp. isolated from the source to the tap were studied for species diversity, intra-species variability and potential to spread antibiotic resistance. Although members of the same species were detected in different sampled sites, the same genotype was never detected in raw water and in tap water. According to these results, the hypothesis that bacteria detected in tap water had origin in the water source had to be rejected. Other hypotheses, namely the occurrence of regrowth in water pipelines or taps or an external contamination downstream the sampled sites in the distribution system, emerged from this study. Additionally, the analysis of the antibiotic resistance profiles confirmed that both *Sphingomonadaceae* and *Pseudomonas* spp. are potential reservoirs of antibiotic resistance. Nevertheless, clear evidences of horizontal gene transfer were not obtained in this study. Indeed, antibiotic resistance patterns were mainly species-, rather than site- or strain-related, suggesting the importance of vertical

resistance transmission in water bacteria. Some antibiotic resistance phenotypes were observed in tap water but not upstream. Examples of this situation were the resistance phenotypes to ampicillin-sulbactam, piperacillin plus tazobactam-pyocyanin, imipenem, ceftazidime, cefepime, gentamicin or tobramycin in *Sphingomonadaceae*, or to streptomycin and rifampicin in *Pseudomonas* spp.

Cultivation-independent methods show invariably that most of the bacteria in a community are unknown, which means that were never cultivated, characterized and integrated in a validly named taxonomic group. Bacterial taxonomy can have a contribution to gradually narrow the tranche corresponding to the unknown bacteria. In this study a new species name *Bacillus purgationiresistens* sp. nov. was proposed, based in a single isolate recovered from treated water.

Drinking water was confirmed as a potential hotspot for the spreading of antibiotic resistant bacteria, with emphasis on the transfer environment-humans.

RESUMO

Título da Tese: "**Diversidade bacteriana e resistência a antibióticos desde a captação da água até à torneira**"

A água é um dos habitats mais importantes para as bactérias no ambiente. O fluxo contínuo da água, nomeadamente ao longo do seu ciclo urbano, faz com que chegue a muitos locais, arrastando microrganismos e inúmeros contaminantes químicos. Isto faz da água um dos veículos mais importantes para a disseminação no ambiente, não só de substâncias químicas, mas também de bactérias e, consequentemente, dos respetivos genes de resistência. O principal objetivo deste estudo foi investigar se a produção e distribuição de água de consumo poderá representar um ponto-chave para a proliferação, seleção e entrada de bactérias resistentes a antibióticos, bem como a probabilidade de estes organismos chegarem até ao consumidor final, através da água da torneira. O estudo foi planeado para atingir esse objectivo, através do rastreio de comunidades bacterianas e de isolados individuais desde a captação até à torneira.

Inicialmente, a abundância e diversidade bacteriana em água não-tratada, tratada e final (torneira) foi caracterizada através do uso de abordagens dependentes e independentes (16S rRNA-DGGE) de cultivo. Ambas as abordagens mostraram que o tratamento da água reduziu as contagens, a cultivabilidade e a diversidade bacteriana, promovendo também a alteração da comunidade bacteriana cultivável de predominantemente bactérias Gram-negativas para predominantemente Gram-positivas. No entanto, este efeito foi revertido, e na água de torneira as bactérias Gram-negativas voltaram a ser predominantes. Adicionalmente, na água de torneira as contagens de microrganismos totais e de bactérias cultiváveis foram mais elevadas do que para a água

tratada recolhida no sistema de distribuição. Estes resultados sugerem a ocorrência de reactivação e crescimento bacteriano e/ou a formação de biofilme ao longo do sistema de distribuição e ao nível das torneiras. Apesar de se terem observado alterações na estrutura da comunidade bacteriana ao longo do circuito da água, o filo detetado como predominante, por 16S rRNA-DGGE, foi o mesmo em todos os pontos de amostragem – *Proteobacteria* (principalmente das classes *Alpha*, *Beta* e *Gamma*).

Abordagens dependentes e independentes de cultivo foram comparadas para avaliar quais os grupos que poderão ser ignorados quando se caracterizam comunidades bacterianas usando métodos de cultivo. De forma a ter uma evidência mais clara dos grupos bacterianos que se sobrepõem usando as duas abordagens, o método dependente e dois independentes de cultivo (DGGE e pirosequenciação 454 com base no gene 16S rRNA) foram comparados quanto à sua capacidade para detectar a diversidade bacteriana de uma amostra de água. Esta comparação mostrou que apesar de os diferentes métodos identificarem o mesmo filo como sendo predominante, as bactérias detetadas eram diferentes. Assim, além da expectativa anterior de que os métodos independentes de cultivo detetassem mais grupos bacterianos do que os dependentes de cultivo, concluiu-se também que as duas abordagens incidem sobre diferentes populações bacterianas.

Com base no estudo da diversidade bacteriana, principalmente das bactérias cultiváveis, e no que se encontra disponível na literatura, dois grupos taxonómicos de grande relevância em água de consumo, devido à sua ampla distribuição e/ou abundância, foram estudados. Assim, *Sphingomonadaceae* e *Pseudomonas* spp., isoladas desde a captação até à torneira foram caracterizadas para a diversidade de espécies, a variabilidade intra-espécie e o potencial para propagar resistências a antibióticos. Apesar de membros da mesma espécie terem sido identificados em diferentes locais, o mesmo genótipo nunca foi detetado na captação ou sistema de distribuição e em água de torneira.

De acordo com estes resultados, a hipótese de que as bactérias detetadas em água de torneira teriam origem na água da captação teve de ser rejeitada. Contudo, este estudo conduz a outras hipóteses, nomeadamente a ocorrência de reactivação e crescimento microbiano nas canalizações ou torneiras, ou de uma contaminação externa, a jusante dos pontos amostrados no sistema de distribuição. Adicionalmente, a análise dos perfis de resistência a antibióticos confirmaram que tanto *Sphingomonadaceae* como *Pseudomonas* spp. são potenciais reservatórios de resistência a antibióticos. No entanto, este estudo não permitiu obter evidências claras da ocorrência de transferência horizontal de genes. Na verdade, os padrões de resistência a antibióticos relacionaram-se principalmente com a espécie e não com o local ou estirpe, sugerindo a importância da transmissão vertical de resistências em bactérias da água. Alguns fenótipos de resistência a antibióticos detectados em água de torneira não foram detetados a montante. São exemplos os fenótipos de resistência a ampicilina-sulbactame, piperacilina e tazobactam-piocianina, imipenemo, ceftazidima, gentamicina ou tobramicina nas *Sphingomonadaceae*, ou a estreptomicina e rifampicina nas *Pseudomonas* spp.

Os métodos independentes de cultivo mostram invariavelmente que a maioria das bactérias de uma comunidade são desconhecidas, o que significa que nunca foram cultivadas, caracterizadas e integradas num grupo taxonómico validamente descrito. A taxonomia bacteriana pode ter um importante contributo para gradualmente se reduzir a parcela correspondente às bactérias desconhecidas. Neste estudo o novo nome *Bacillus purgationiresistens* sp. nov. foi proposto, com base num único isolado recuperado de água tratada.

A água de consumo foi confirmada como potencial ponto-chave para a disseminação de bactérias resistentes a antibióticos, com destaque para a transferência ambiente-humanos.

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Vaz-Moreira, I., Figueira, V., Lopes, A. R., Lobo-da-Cunha, A., Spröer, C., Schumann, P., Nunes, O. C., Manaia, C. M. (2012) *Bacillus purgationiresistans* sp. nov. isolated from a drinking water treatment plant. International Journal of Systematic and Evolutionary Microbiology 62, 71-77. (According to rules of Latin and latinization, the name was corrected to *B. purgationiresistens*)

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Keywords

454 Pyrosequencing

Antibiotic resistance

*atp*D

Bacillus purgationiresistens

Bacterial Diversity

Biofilm

Cultivable bacteria

Culture-dependent methods

Culture-independent methods

Drinking water

Freshwater

*gyr*B

Housekeeping genes

Mineral water

PCR-DGGE

Pseudomonas

*rpo*B

*rpo*D

Sequence type

Sphingomonadaceae

Tap water

Uncultivable bacteria

Water

Water treatment plant

List of Abbreviations

16S rRNA	16S Ribosomal Ribonucleic Acid
A	Aminoglycosides
aac(3)-I	Genes encoding 3-N-aminoglycoside acetyltransferases; confer resistance to aminoglycosides
aac(60)-Ib-cr	Gene encoding an aminoglycoside acetyltransferase; confers reduced susceptibility to ciprofloxacin and norfloxacin
AKN	Amikacin
ANOVA	Analysis of variance
*amp*C	Gene encoding chromosomal β-lactamase; confers resistance to β-lactams
Ap	Amphenicol
*aph*A	Gene encoding acid phosphatase/phosphotransferase; confers resistance to aminoglycosides
APL	Aminophospholipid
APUA	Alliance for the Prudent Use of Antibiotics
ATCC	American Type Culture Collection
atpD	Beta subunit of membrane ATP synthase
B.	*Blastomonas*
Bf	Biofilm
$bla_{(\text{TEM,CTX-M/ GES/OXA/PER/SHV/ TLA/VEB})}$	Genes encoding extended spectrum β-lactamases; confer resistance to β-lactams
$bla_{\text{NDM-1}}$	Gene encoding for the New Delhi metallo-β -lactamase-1; confers resistance to almost all β-lactams
BLAST	Basic Local Alignment Search Tool
cat	Genes encoding chloramphenicol acetyltransferases; confer resistance to chloramphenicol

CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CIP	Ciprofloxacin
cmr	Gene encoding a putative efflux pump; confers resistance to chloramphenicol
COL	Colistin
COST-DARE	European Cooperation in Science and Technology - Detecting Evolutionary hot spots of Antibiotic Resistance in Europe
CP	Cephalothin
Ct	Contact time
CW	Clean Water
D	Dental chairs
DAPI	4',6-diamidino-2-phenylindole
dfr(A12, A17)	Genes encoding dihydrofolate reductases; confers resistance to trimethoprim
DGGE	Denaturing Gradient Gel Electrophoresis
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DPG	Diphosphatidylglycerol
DSM	Deutsch Sammlung von Mikroorganismen (German Colletion of Microorganisms and Cell Cultures
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECDC	European Centre for Disease Prevention and Control
EMBL-EBI	European Molecular Biology Laboratory – European Bioinformatics Institute

erm(A/E)	Genes encoding rRNA methylase; confers resistance to erythromycin
erm(B/C/F)	Genes encoding rRNA methylases; confer cross-resistance to macrolides, lincosamides and streptogramin B
ESAC	European Surveillance of Antimicrobial Consumption
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAM	Ampicillin-sulbactam
FAMEs	Fatty Acid Methyl Esters
FEP	Cefepime
FISH	Fluorescence In Situ Hybridization
*flo*R	Gene encoding an exporter protein that specifically exports amphenicol antibiotics
G	Glycopeptides
G+C	Guanine plus Cytosine
GEN	Gentamicin
GNOxN	Gram-negative Oxidase-negative
GNOxP	Gram-negative Oxidase-positive
GPOxN	Gram-positive Oxidase-negative
GPOxP	Gram-positive Oxidase-positive
*gyr*B	DNA gyrase β subunit
H’	Diversity index
HGT	Horizontal Gene Transfer
IMI	Imipenem
ITS	Intergenic 16S-23S internally transcribed spacer
J	Evenness index
L	beta-Lactam
LRV	Log_{10} Redution Value

M	Mineral water
MAR	Multiple Antibiotic Resistance
Mc	Macrolide
*mec*A	Gene encoding penicillin binding protein 2; confers resistance to penicillins
MEGA	Molecular Evolutionary Genetics Analysis
MEM	Meropenem
MK	Menaquinone
MLST	MultiLocus Sequence Typing
MSA	Mannitol Salt Agar
*msr*A	Gene encoding methionine sulfoxide reductase A; confers resistance to erythromycin
n	Number
N.	*Novosphingobium*
NA	Nalidixic Acid
NARMS	National Antimicrobial Resistance Monitoring System
OTU	Operational Taxonomic Unit
P.	*Pseudomonas*
PCA	Plate Count Agar
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
PFGE	Pulsed Field Gel Electrophoresis
PG	Phosphatidylglycerol
PIA	*Pseudomonas* Isolation Agar
PIC	Piperacillin
PICP	Piperacillin-pyocyanin

PL	Phospholipid
Q	Quinolone
*qep*A	Gene encoding an efflux pump; confers resistance to fluoroquinolone
qnr(D,S,VC)	Genes encoding Qnr proteins, capable of protecting DNA gyrase; confer resistance to quinolone
qPCR	Quantitative real time Polymerase Chain Reaction
R2A	R2 Agar culture medium
RDP	Ribosomal Database Project
*rec*A	Recombinase A
RNA	RiboNucleic Acid
*rpo*B	RNA polymerase beta subunit
*rpo*D	σ^{70} factor
rRNA	Ribosomal RiboNucleic Acid
RT	Resistance Type
S	Sulphonamide
S.	*Sphingomonas*
sat(1-2)	Genes encoding a nourseothricin N-acetyltransferase; confer resistance to aminoglycosides
Sb.	*Sphingobium*
Sp.	*Sphingopyxis*
ST	Sequence Type
STR	Streptomycin
str(A,B)	Genes encoding phosphotransferases; confer resistance to streptomycin
sul(I–II)	Genes encoding a drug-resistant dihydropteroate synthase enzyme required for folate biosynthesis; confer resistance to sulphonamide
T	Domestic tap water

TCC	Ticarcillin-clavulanic acid
TCCP	Ticarcillin-clavulanic acid-pyocyanin
TEM	Transmission Electron Microscopy
TET	Tetracycline
tet(A–D/K/L/Y)	Genes encoding efflux pumps; confer resistance to tetracyclines
tet(M/O/Q/W)	Genes encoding proteins protecting the ribosome from the inhibiting effects of tetracycline
*tet*R	Gene encoding a repressor protein, which regulates the tetracycline efflux system genes
TGGE	Temperature Gradient Gel Electrophoresis
TIC	Ticarcillin
TICP	Ticarcillin-pyocyanin
TLC	Thin Layer Chromatography
TOB	Tobramycin
TSA	Tryptic casein Soy Agar
TSU	Cotrimoxazol
TTC	Tergitol 7-Agar
TZP	Piperacillin plus Tazobactam
TZPP	Piperacillin plus Tazobactam-pyocyanin
USA	United States of America
UV	UltraViolet radiation
UW	Unclean Water
van(A,B)	Genes encoding D-alanine:D-alanine ligases with a broad substrate specificity; confer inducible resistance to the glycopeptides antibiotics, as vancomycin
W	Drinking water treatment plant and distribution system
WGS	Whole Genome Sequence

WHO	World Health Organization
WTP	Water Treatment Plant
WWTP	Waste Water Treatment Plant

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1. Introduction

1.1. The urban water cycle

Water is the most common and important chemical compound on Earth, with an unquestionable importance to all the basic biochemical processes, and therefore, for human health and well-being. The hydrological cycle is often referred to as the water cycle, comprising the storage and circulation of water between the biosphere, atmosphere, lithosphere and the hydrosphere. Due to anthropogenic influences and interventions on the urban areas, a more restricted water cycle was proposed, the so called urban water cycle (Figure 1.1). The urban water cycle combines the hydrological with the human intervened parts of the water cycle. Key components of the urban water cycle are the facilities for water treatment and disinfection, the network of pipelines for drinking water distribution and the waste water municipal collectors. In this cycle, humans are important links, either consuming water for bathing, cleaning, drinking and food preparation or producing waste waters and innumerous anthropogenic substances capable of contaminating the water courses (Figure 1.1).

In the urban water cycle it is possible to recognize two complementary parts – the water destined to human use (for simplicity herein designated clean water, CW in Figure 1.2) and that resultant from human activities (herein designated unclean water, UW in Figure 1.2). CW includes the water source, normally surface water (rivers, lagoons, alluvial wells) or groundwater, treatment facilities where the water is prepared for safe human consumption, and distribution networks which can reach several kilometers (Mitchell *et al.*, 2001; Marsalek *et al.*, 2006; Mitchell and Diaper, 2006). UW includes every type of waste waters, including those produced by houses, industries or hospitals. In

developed regions, before being discharged into the environment, these waste waters undergo treatment in order to remove the excess of organic matter and diverse types of contaminants. With this purpose the effluents are collected into waste water treatment plants (WWTP) prior to its discharge into a natural water course (e.g. a river or a lagoon). Although it is not possible to track the fate of the treated effluents in the environment, a hypothetical contamination of the "clean" part of the water cycle (CW) by these effluents cannot be discarded. For instance, in major rivers, it is possible to observe the discharge of WWTP not only downstream, but also upstream the sites where drinking water treatment plants are located (Sirivedhin and Gray, 2005; Guo and Krasner, 2009).

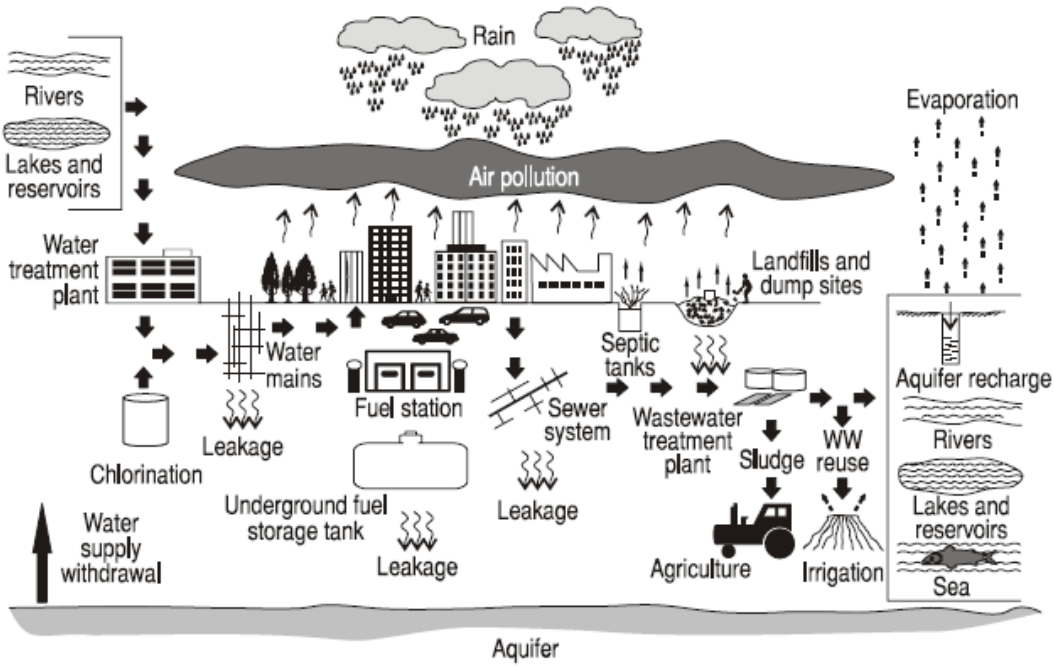

Figure 1.1. The urban water cycle (Marsalek *et al.*, 2006).

The current study is focused on the CW part of an urban water cycle, with major emphasis on final drinking water. By definition, drinking water is suitable for human consumption, washing/showering and domestic food preparation (98/83/EC, 1998; Bartram *et al.*, 2003; WHO, 2008). Factors affecting the drinking water compliance

include chemical and microbiological factors which can affect the health of the consumers, through ingestion, contact or aerosol inhalation (Lee *et al.*, 2002; Reynolds *et al.*, 2008; WHO, 2008). Water quality can be affected at different parts of the water cycle as outlined in Figure 1.1. If it is true that drinking water treatment may largely improve water quality and safety, it is also observed that the treatment efficiency and the quality of the final water depend on the properties of the raw water. Indeed, the contamination of surface waters with fertilizers, pesticides, pharmaceutical products or heavy metals can seriously endanger the final quality of the drinking water (Ritter *et al.*, 2002; WHO, 2008).

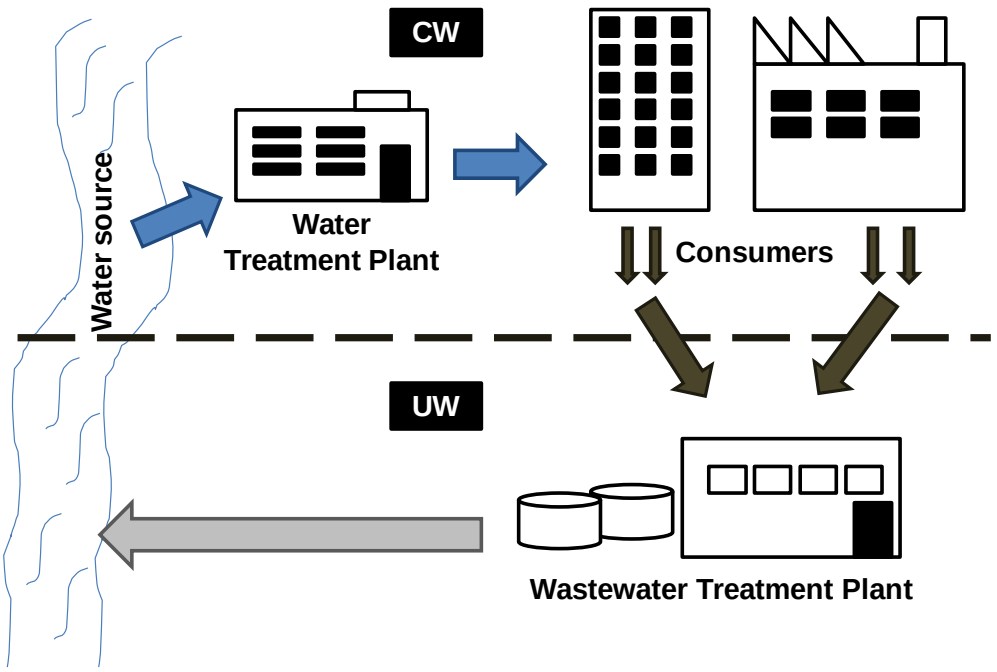

Figure 1.2. The clean (CW) and unclean (UW) water components of the human intervened water cycle.

1.1.1. Water for human consumption

According to the definition of drinking water, the microbiological quality of water is an important aspect regarding its safe use. Indeed, pathogenic bacteria, viruses, protozoa and helminthes, are the most common and widespread health risk associated with drinking water (Lee *et al.*, 2002; Reynolds *et al.*, 2008; WHO, 2008). Given the enormous and unrealistic task that would be the search for all possible pathogens in waters, the detection of indicators is recommended, even though this can represent a shortcoming to assess the microbiological water quality. According to the European (98/83/EC, 1998) and the national (DL306-2007, 2007) legislation, the water supplied for human consumption should be exempt of cultivable *Escherichia coli* and enterococci in 100 mL of water.

Frequently, the elimination or reduction of water biohazards can only be achieved through a combination of treatment processes (Marsalek *et al.*, 2006; WHO, 2008). The destruction and removal of undesired microorganisms involves processes of filtration and disinfection, with chlorine, ozone and UV. Table 1.1 resumes some treatment processes commonly used, individually or in combination, to reduce the microbial loads in waters. Although bacteria are generally regarded as the microbes most sensitive to inactivation by disinfection, some bacterial pathogens are frequently transmitted through water - *Campylobacter jejuni*, *Salmonella*, *Shigella*, *Mycobacterium tuberculosis*, *Vibrio cholerae* and *Helicobacter pylori* (Rusin *et al.*, 1997; Marsalek *et al.*, 2006).

Table 1.1. Treatment processes commonly used individually or in combination to reduce the microbial loads during the production of drinking water (adapted from WHO, 2008).

Treatment process	Range of removal (LRV)	Affected by
Pretreatment		
Roughing filters	0.2 – 2.3	Filter medium and coagulant
Storage reservoirs	0.7 – 2.2	Residence time > 40 days
Bank filtration	2 - > 6	Travel distance; soil type; pumping rate; pH; ionic strength
Coagulation, flocculation and sedimentation		
Conventional clarification	0.2 - 2	Coagulation conditions
Lime softening	1 - 4	pH and settling time
Filtration		
Granular high-rate filtration	0.2 – 4.4	Filter media and coagulation pretreatment
Slow sand filtration	2 - 6	Presence of schmutzdecke; grain size; flow rate; operating conditions (mainly temperature, pH)
Precoat filtration	0.2 -2.3	Chemical pretreatment
Membrane filtration: Microfiltration, Ultrafiltration, Nanofiltration Reverse osmosis	1 - > 7	Membrane pore size; Integrity of filter medium and filter seals; Resistance to chemical and biological degradation
Primary disinfection[a,b]		
Chlorine	2 (Ct_{99} 0.04–0.08 min·mg/l; 5 °C; pH 6-7)	Turbidity and chlorine-demanding solutes;
Chlorine dioxide	2 (Ct_{99} 0.02–0.3 min·mg/l; 15–25 °C; pH 6.5–7)	
Ozone	2 (Ct_{99} 0.02 min·mg/l)	
UV	4 (0.65–230 mJ/cm^2)	Excessive turbidity and certain dissolved species; UV dose and wavelength; exposure time

The minimum and maximum removal rates are indicated as $\log_{10}$ reduction values (LRV) which may be observed under failing and optimal treatment conditions, respectively.
Ct, product or disinfectant concentration and contact time.
[a] Chemical disinfection: Ct values correspond to the required doses to achieve 2 LRV;
[b] UV irradiation: UV dose range corresponds to that required to achieve 4 LRV.

1.1.1. Water distribution

Besides the final quality of the product, water treatment must be also optimized in order to prevent microbial growth, pipes corrosion and the formation of deposits, during storage and distribution. In this respect, the quality of the distribution network, which may include several hundred kilometers of pipes, storage tanks, interconnections and the potential for tampering and vandalism, is also of major relevance. This complex network is full of opportunities for microbial contamination to occur. An intermittent water supply is also critical for microbial contamination, since low water pressure may allow the ingress of contaminants into the system through breaks and joints (Robertson *et al.*, 2003; WHO, 2008).

Disinfection processes such as chlorination, ozonation and UV irradiation reduce significantly the number of bacteria in water. After disinfection, some microorganisms can survive, sometimes as dormant cells and, under favorable conditions, such as the absence of disinfectant residues, can enter regrowth. Under favorable conditions, the planktonic (suspended) surviving bacteria can also form biofilm structures. Biofilms comprise a mixture of microorganisms able to proliferate, often attached to a surface, originating a heterogeneous and discontinuous structure, with a non-uniform distribution over the surface of the materials in contact with water (Batté *et al.*, 2003). The detachment of bacteria from mature biofilms is also know to occur, frequently due to the network pipe walls shearing/erosion, favoring the spreading of biofilm bacteria into the circulating water (Batté *et al.*, 2003). Major factors influencing the bacterial regrowth and biofilm formation are the temperature, nutrients availability, including assimilable organic carbon, the absence of disinfectant residues, hydrodynamic regime (water stagnation or laminar/turbulent flow) and the pipe characteristics (surface, material, etc.) (Camper *et al.*, 1998; Butterfield *et al.*, 2002; Bartram *et al.*, 2003; Chu *et al.*, 2003;

Wijeyekoon *et al.*, 2004; Lehtola *et al.*, 2005; Ndiongue *et al.*, 2005; WHO, 2008; Lautenschlager *et al.*, 2010; Manuel *et al.*, 2010). For instance, some pipe materials can stimulate the bacterial growth by releasing bioavailable forms of iron and phosphorous to the water (Morton *et al.*, 2005), and by contributing to neutralize the disinfectants (Hallam *et al.*, 2002; Lehtola *et al.*, 2005). In contrast, some elements such as copper released from the pipes, showed to slow the biofilm development, presumably due to the toxicity or inhibitory effect on microorganisms (Lehtola *et al.*, 2004; van der Kooij *et al.*, 2005). The biofilms are suspected to be the primary source of microorganisms in water distribution systems fed with treated water and with no pipeline breaches. In this respect, the resilience of biofilms to disinfection is a major limitation. For example, the disinfection with chlorite or chlorine dioxide can reduce the loads of free bacteria, but have little or no effect on the density of biofilm bacteria (Gagnon *et al.*, 2005). In general, multispecies biofilms can have even higher resistance to disinfection than mono-species structures (Berry *et al.*, 2006).

For the reasons presented above, biofilms can potentiate considerably the emergence and persistence of waterborne pathogens (Bartram *et al.*, 2003). Some ubiquitous enterobacteria, such as those of the genera *Citrobacter*, *Enterobacter* and *Klebsiella* are referred to as common biofilm members in drinking water distribution systems (Schwartz *et al.*, 2003; September *et al.*, 2007; WHO, 2008). On the other hand, the low temperatures and nutrients concentration in the distribution systems do not support the growth of bacteria like *E. coli* or enteric pathogens in biofilms (Robertson *et al.*, 2003; Tallon *et al.*, 2005; WHO, 2008). Thus, the presence of *E. coli* in biofilms in the distribution system can be considered an evidence of the occurrence of recent fecal contamination (Robertson *et al.*, 2003; WHO, 2008).

1.2. Drinking water bacterial diversity

From the source to the final consumer, the water bacterial diversity suffers successive alterations (Norton and LeChevallier, 2000; Eichler *et al.*, 2006; Lautenschlager *et al.*, 2010). Therefore, the bacterial diversity in the water that reaches the consumer does not necessarily mirrors the bacterial diversity in the water source. In a pilot study conducted by Norton and LeChevalier (2000) the changes in the bacteriological populations due to water treatment and distribution were evident. These authors showed that although the ozonation process did not alter dramatically the composition of the cultivable bacteria in raw water, the chlorination resulted in a rapid shift from predominately Gram-negative bacteria in the raw water to mostly Gram-positive organisms in the chlorinated water. Nevertheless, downstream the distribution system, the disinfectants relief may enable the Gram-negative bacteria regrowth. In fact, the composition of the final water, after bacterial regrowth, is almost unpredictable. Besides the properties of the water and physicochemical factors, such as total organic content or hydrodynamic regime, also the conditions of the pipes, the range of temperatures, the residence times, among others, may induce changes in the bacterial community (Pepper *et al.*, 2004; Lautenschlager *et al.*, 2010). The wide range of cultivable bacteria frequently found in drinking water include members of the genera *Acinetobacter*, *Actinomycetes*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Citrobacter*, *Comamonas*, *Corynebacterium*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Micrococcus*, *Moraxella*, *Pseudomonas*, *Serratia*, *Sphingomonas*, *Stenotrophomonas*, *Xanthomonas*, atypical *Mycobacterium*, *Bacillus*, *Nocardia*, among others. (Rusin *et al.*, 1997; Norton and LeChevallier, 2000; Szewzyk *et al.*, 2000; Bartram *et al.*, 2003; WHO, 2008). Although occasional episodes of pathogenicity may be associated with some of these bacteria, drinking water ingestion presents a very low risk of promoting gastrointestinal infection in the general population

(WHO, 2008). The calculated risk is less than 1/10 000 for a single exposure to the bacterial agent (Rusin *et al.*, 1997). In spite of such considerations, some drinking water bacteria may be of concern for people under immunosuppression or undergoing antibiotic therapy (Rusin *et al.*, 1997; Norton and LeChevallier, 2000; Bartram *et al.*, 2003; WHO, 2008).

Having in mind that the cultivable bacteria represent a small part of the water microbiota, over the last decade many studies have used culture-independent approaches in order to have a broader perspective of the drinking water bacterial diversity (Farnleitner *et al.*, 2004; Eichler *et al.*, 2006; Wu *et al.*, 2006; Poitelon *et al.*, 2009; Kormas *et al.*, 2010; Lautenschlager *et al.*, 2010). *Proteobacteria* (mainly *Alpha-*, *Beta-* and *Gammaproteobacteria*) were frequently observed as the prevailing phylum in treated drinking waters (Eichler *et al.*, 2006; Poitelon *et al.*, 2009; Kormas *et al.*, 2010; Revetta *et al.*, 2010). Nevertheless, the predominance of other phyla, such as *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes*, and *Planctomycetes* was also reported (Eichler *et al.*, 2006; Revetta *et al.*, 2010). The impact of disinfection processes on the bacterial community diversity was also observed using culture-independent methods (Eichler *et al.*, 2006; Revetta *et al.*, 2010). For instance, Eichler *et al*. (2006) observed that the first steps in the processing of the raw water (i.e. flocculation and sand filtration) did not change the microbiota composition, although chlorination had a significant effect on the bacterial community. After this treatment, phylotypes not detected in previous stages, as for example nitrifying bacteria, were identified. In addition to the reduction of the bacterial counts, water treatment may impose selective pressures capable of selecting bacteria resistant to different types of chemical or physical biocides (e.g. disinfectants, antibiotics, radiation) (Armstrong *et al.*, 1981; Armstrong *et al.*, 1982; Schwartz *et al.*, 2003; Shrivastava *et al.*, 2004; Xi *et al.*, 2009). Hypothetically, the bacteria that can survive the

treatment, will be able to regrow downstream the disinfection points where may contribute to spread and increase antibiotic resistance prevalence. This effect was evidenced by Xi *et al.* (2009) who concluded that the water distribution systems may serve as important reservoirs for the spread of antibiotic resistance.

1.3. Antibiotic resistance in the environment

Most of the antibiotics commercially available nowadays are derivatives of natural compounds produced by bacteria and/or fungi. In nature, it is thought that these microorganisms use the antibiotics, which are secondary metabolites, for microbial cell defense, inhibiting the growth of competitors. However, many bacteria can survive in the presence of natural antimicrobial substances and even benefit from their presence. For example, some bacteria can use antibiotics as biochemical signals, modulators of metabolic activity or even as carbon sources (Davies *et al.*, 2006; Dantas *et al.*, 2008; Martinez, 2009). In other cases, bacteria can tolerate the antibiotics because they have structures similar to the natural substrates and can be inactivated by the bacterial enzymes, leading to a natural form of resistance (Martinez, 2009). These are some evidences that illustrate that antibiotic resistance is a natural property of bacteria, eventually as old as bacteria themselves (Datta and Hughes, 1983; Hughes and Datta, 1983; Aminov, 2010; D'Costa *et al.*, 2011).

Before the introduction of antibiotics in the 1940's, and their increasing use in bacterial infections therapy, the concentrations of these compounds in the environment were low and confined to the site of their production. The increasing use of antibiotics and other substances with antimicrobial activity changed the equilibrium between fully susceptible and resistant bacteria (Larson, 2007; Davies and Davies, 2010). Gradually,

antibiotic resistant bacteria and their specific genetic determinants have reached new habitats, with evident increases on the prevalence of resistance and the extension of the spectrum of antimicrobial substances tolerated (Davies and Davies, 2010). The high prevalence of (multi)-antibiotic resistance has been extensively reported, mainly in clinical environments. Nevertheless, the problem is not limited to the clinical environment, and has been also reported in wild animals, surface waters or agriculture soils, allegedly due to antibiotics use (frequently overuse) and anthropic selective pressures (Literak *et al.*, 2010; Simões *et al.*, 2010; Storteboom *et al.*, 2010; Thaller *et al.*, 2010). Although a relationship between the increase of the antibiotics use and the increase of the antibiotic resistance exists, it was demonstrated that the antibiotic resistance genes already existed in the pre-antibiotic era (Knapp *et al.*, 2010; D'Costa *et al.*, 2011). Nowadays, antibiotic resistance is considered a serious global public health problem, and is receiving the attention of several international health agencies (APUA; CDC; COST-DARE; ECDC; WHO).

1.3.1. Mechanisms of antibiotic resistance acquisition and dissemination

The success of antibiotics as therapeutic agents is due to the capacity of these molecules to interfere with structures and/or functions of the bacterial cell (prokaryotic), which are absent in the host cells (eukaryotic). Antibiotics may interfere with cell wall synthesis, inhibit the protein or nucleic acid synthesis, disrupt the bacterial membrane structure or inhibit a metabolic pathway vital to the cell. In turn, antibiotic resistance mechanisms are related with the ability that bacteria have or may develop to avoid such interferences. Resistance mechanisms are much more diverse than the modes by which a drug can interfere with a cell. These may include the degradation or alteration of the antibiotic by different processes (e.g. hydrolysis, acetylation, phosphorylation,

glycosylation), the removal of the antibiotic from the cell (e. g. efflux pumps), or altered targets for the antimicrobial agent (Mazel and Davies, 1999; Scott, 2005; Tenover, 2006; Manaia *et al.*, 2012)

Some bacteria, given the presence of key genes and/or physiological functions, are intrinsically resistant to one or more classes of antibiotics. This is an ancestral property within a group and thus is common to most or all representatives of a genus or species (EUCAST; Davies and Davies, 2010). In contrast, acquired resistance is observed only in some representatives of a species, in which most of the members are susceptible to that antimicrobial agent (EUCAST). Acquired antibiotic resistance may result from gene mutation or genetic recombination (Martinez and Baquero, 2000; Livermore, 2003; Tenover, 2006; Zhang *et al.*, 2009; Davies and Davies, 2010). Gene mutations occur randomly in the genome, often potentiated by mutagens. Examples of resistance phenotypes emerging by mutation include altered targets for an antimicrobial agent (e.g. quinolones, rifampin, linezolid, clarithromycin, amoxicillin, and streptomycin), limited access of the antimicrobial agent to the intracellular target (e.g. penicillin, cephalosporins, glycopeptides, and tetracyclines), or transformation and further broadening of the range of antimicrobial agents that can be inactivated (e.g. extended spectrum beta-lactamases) (Manaia *et al.*, 2012). Under favorable conditions, the clones harboring the gene mutation may have advantage, achieving higher rates of cell division than the non-mutated cells (higher fitness, i.e., the capacity of an individual to survive and reproduce) and, thus, become dominant. In such a situation the genetic determinant of resistance is disseminated by vertical transmission.

In bacteria, genetic recombination is frequently referred to as horizontal gene transfer (HGT). This process, also named “bacterial sex”, is very common among bacteria and represents a major driving force for bacterial evolution (Ochman *et al.*, 2000;

Wiedenbeck and Cohan, 2011). This form of genetic recombination involves the transfer of genetic material from a donor to a recipient and requires that both share the same space, but not necessarily the same species. HGT can occur by transformation, consisting on the uptake of naked DNA (on plasmids or as linear DNA), released by dead cells; transduction, mediated by bacteriophages; and conjugation, involving cell-to-cell contact through a *pilus* (Davison, 1999; Dröge *et al.*, 1999; Trevors, 1999; Andam *et al.*, 2011; Skippington and Ragan, 2011; Stokes and Gillings, 2011; Manaia *et al.*, 2012). In general, HGT processes are potentiated by genetic elements which facilitate the mobilization and integration of exogenous DNA, either between cells or between chromosomal DNA, and extrachromosomal genetic elements and *vice versa*. Examples of these genetic elements are plasmids, transposons and integrons, in which many of the known antibiotic resistance genes are inserted.

Some studies suggest that the mobile genetic elements are a considerable part of the antibiotic resistome (collection of all the antibiotic resistance genes and their precursors), which means that a high part of the resistome has a high mobility potential (Partridge *et al.*, 2009; Andersson and Hughes, 2010; Parsley *et al.*, 2010). Indeed, Fondi and Fani (2010) concluded that apparent geographical or taxonomic barriers are not a limitation for the occurrence of HGT, as they observed that bacteria phylogenetically unrelated and/or inhabiting distinct environments had similar antibiotic resistance determinants.

A high number of reports and studies have shown that the prevalence of antibiotic resistance, as well as the diversity and distribution of resistance genes has increased over the last decades (EARS-Net; ESAC; NARMS; Houndt and Ochman, 2000; Knapp *et al.*, 2010). In this respect, not only the bacterial pathogens but also the environmental bacteria are important reservoirs of antibiotic resistance (Ash *et al.*, 2002; Ferreira da Silva *et al.*, 2006; Ferreira da Silva *et al.*, 2007; Allen *et al.*, 2010; Figueira *et al.*, 2011a; Figueira *et*

al., 2011b; Figueira *et al.*, 2012). The selective pressures present in the environment (e.g. antibiotics, disinfectants and other antimicrobials, heavy metals, etc.), which supposedly contribute to the increase of the antibiotic resistance, are diverse and act by different mechanisms, most of them still unclear. Some cases of co-selection were reported, for example 1) when the genes specifying the resistant phenotypes for the antibiotic and disinfectant or metal are located together in the same genetic element (co-resistance), or 2) when different antimicrobial agents attack the same target, initiate a common pathway to cell death or share a common route of access to their respective targets (cross-resistance) (Chapman, 2003; Baker-Austin *et al.*, 2006; Martin *et al.*, 2008). Contrary to the initially thought, that in the absence of selective pressures acquired antibiotic resistance genes can be a dead weight for its host, it seems that acquired antibiotic resistance may have a reduced cost for its host and, thus, become stable once acquired (Johnsen *et al.*, 2009; Andersson and Hughes, 2010, 2011). Several factors could contribute to this irreversibility, including the absence of a fitness cost, reduction of the fitness cost, through compensating mutations, and the referred to genetic co-selection between the resistance-conferring gene and another gene under selection (Gullberg *et al.*, 2011). All these factors are supposedly contributing for the continuous increase of the antibiotic resistance dissemination.

1.4. Drinking water as a vehicle of antibiotic resistant bacteria?

Although it is still difficult to establish clear cause-effect relationships, it is widely accepted that chemical pollution, mostly due to anthropic causes, contributes for antibiotic resistance dissemination (McArthur and Tuckfield, 2000; Davies and Davies, 2010; Graham *et al.*, 2011). In this respect, antibiotics seem to be a major, although not

the unique, form of pollution, mainly because it is estimated that about 75 % of the antibiotics consumed by humans and animals are eliminated as active substances, contaminating sewage treatment systems and the respective receptors (Kümmerer and Henninger, 2003; Zhang *et al.*, 2009). The WWTP are the main destination of these substances, where they are only partially eliminated. The antibiotics that are not eliminated during the treatment process pass through the system and may end up in the environment, usually in a water course, entering the urban water cycle. Not surprisingly, residues of antibiotics have been detected worldwide in municipal sewage, hospital effluents, influents and effluents of WWTP, surface water and ground water (Kümmerer, 2004, 2009). In drinking water the detection of antibiotic residues is less frequent. Nevertheless, some recent studies reveal that antibiotics are also present in drinking water, including those submitted to the recommended disinfection process (Ye *et al.*, 2007; Benotti and Snyder, 2009; Touraud *et al.*, 2011).

Once in environment, antibiotics may be eliminated by biotic processes (biodegradation by bacteria and fungi) or by non-biotic processes (sorption, hydrolysis, photolysis, oxidation and reduction), reaching very low concentrations, i.e., sub-inhibitory levels (Halling-Sorensen *et al.*, 1998; Kümmerer, 2009). At sub-inhibitory levels, antibiotics may have a hormetic effect and can promote several alterations in housekeeping functions of the cells. Apparently, some of these alterations may not be associated with antibiotic resistance, but contribute for the perturbation of the microbial community, leading, eventually, to an overall resistance increase (Davies *et al.*, 2006; Fajardo and Martinez, 2008; Yergeau *et al.*, 2010; Graham *et al.*, 2011). Indeed, recent studies suggested that the low antibiotic concentrations found in many natural environments are important for enrichment and maintenance of resistance in bacterial populations (Gullberg *et al.*, 2011).

Bacteria resistant to antibiotics have been extensively found in the aquatic environment, namely in drinking waters (Armstrong *et al.*, 1981; Schwartz *et al.*, 2003; Pavlov *et al.*, 2004; Zhang *et al.*, 2009). Thus, water may be a vehicle, not only for the dissemination of pollutants, but also of bacteria and resistance genes in the environment. Given their ubiquity, bacteria can move between different environmental niches and, like stickers, drive antibiotic resistance determinants from heavily contaminated sites to places in which selective pressures (no matter which they are) may be inexistent or negligible. Some studies have concluded that the treatment of raw water and its subsequent distribution selects for antibiotic-resistant bacteria, increasing phenotypic resistance rates at drinking water sampling points (Kümmerer, 2004; Scoaris *et al.*, 2008; Xi *et al.*, 2009). Although disinfection processes contribute to reduce the number of bacteria, the persistence or re-colonization of antibiotic resistant bacteria in drinking waters is a reality, worsened by the high potential of many bacteria to produce biofilms in pipelines, reservoirs and taps (Schwartz *et al.*, 2003). The risks can be attenuated using expensive treatment systems (e.g. ultrafiltration or reverse osmosis), but still some resistance genes can persist and enter the food chain via drinking water (Bockelmann *et al.*, 2009).

A single gene is often the basic functional unit responsible for resistance to one or more antibiotics. The same antibiotic resistance genes are detected worldwide in hospital and animal husbandry waste waters, sewage, waste water treatment plants, surface water, ground water and drinking water (Table 1.2) (Zhang *et al.*, 2009; Manaia *et al.*, 2012). In respect to drinking water, many are the examples of antibiotic resistance genes found in different world regions, associated with beta-lactam (*amp*C, bla_{TEM} and bla_{SHV}), chloramphenicol (*cat* and *cmr*), sulfonamide (*sul*I and *sul*II), tetracycline (*tet*A, *tet*B and *tet*D), aminoglycosides (*aph*A, *aad*A1, *aad*A2 and *sat*2), trimethoprim (*dfr*A12 and *dfr*A17), erythromycin (*msr*A, *erm*A and *erm*C) and vancomycin (*van*A) resistance

(Schwartz *et al.*, 2003; Cernat *et al.*, 2007; Faria *et al.*, 2009; Xi *et al.*, 2009; Zhang *et al.*, 2009; Figueira *et al.*, 2012; Manaia *et al.*, 2012).

Despite the considerable amount of information published, probably just a little fraction of the resistance genes occurring in waters (and in the environment in general) were characterized till now. One of the reasons is the fact of the antibiotic resistance genes detection is mainly performed by PCR-based approaches and metagenomic analysis, using specific primers, followed by sequence similarity analysis. These procedures limit the detection of resistance genes to those harbored by bacteria which genome is known. Another bias is related with the fact that most of the genes screened were originally described in clinical pathogens, mainly cultivable aerobic bacteria with fast and non-fastidious growth. For these reasons, the current perspective of the antibiotic resistome is still mainly culture-dependent. The simple detection of a gene is not indicative of its expression in its host and in the environment, but it evidences the stability and potential of that gene to spread to other environments or hosts.

1.5. Tools to assess and track bacterial populations in waters

1.5.1. Culture-dependent *vs* culture independent methods

Over the last decades it became evident that only a small fraction of the bacterial diversity is known and that none of the methods to study bacteria in the environment is able to cover the whole community (Muyzer *et al.*, 1993; Amann *et al.*, 1995; Palleroni, 1997; Kemp and Aller, 2004; Sleator *et al.*, 2008; Zinger *et al.*, 2011).

Table 1.2. Examples of antibiotic resistance genes of clinical relevance distributed worldwide in aquatic environments and illustration of some methodological approaches commonly used to detect resistance determinants in the environment (from Manaia *et al.*, 2012)

Gene	Class	Type of water	Biological source	Detection method	Country	Reference
*mec*A	L	Recreational beach and seepage	*Proteus vulgaris, Morganella morganni, Enterococcus faecallis*	Enrichment in oxacillin and polymyxin B supplemented Mueller-Hinton broth / PCR	USA	(Kassem *et al.*, 2008)
*tet*K, *tet*M	Tet	Recreational beach	Methicillin resistant staphylococci	Polymyxin B supplemented *Staphylococcus* agar / PCR	USA	(Soge *et al.*, 2009)
*erm*A, *erm*B, *erm*C	Mc					
bla_{SHV-12}, bla_{TLA-2}, bla_{PER-1}, bla_{PER-6}, bla_{GES-7}, bla_{VEB-1a}	L	River	*Aeromonas* spp.	Isolation on ceftazidime suplemented MacConkey agar / PCR	France	(Girlich *et al.*, 2011)
bla_{NDM-1}	L	Tap water and seepage	*E. coli, K. pneumoniae, Shigella boydii, Aeromonas caviae, S. maltophilia, V. cholerae, Citrobacter freundii, P. aeruginosa*	Vancomycin and cefotaxime or meropenem supplemented Mueller-Hinton agar / PCR / Probes hybridization	India	(Walsh *et al.*, 2011)
*tet*R, *tet*Y	Tet	River	Plasmid pAB5S9 of *Aeromonas bestiarum* clone 5S9	Plasmid sequence analysis	France	(Gordon *et al.*, 2008)
*sul*II	S					
*flo*R	Ap					
*str*A, *str*B	A					
*mec*A	L	River and Waste water	DNA Bacteriophages	qPCR	Spain	(Colomer-Lluch *et al.*, 2011)
bla_{TEM}, bla_{CTX-M9}	L					
*tet*O	Tet	Artificial Ground water	Total DNA	qPCR	Belgium Spain Italy	(Bockelmann *et al.*, 2009)
*erm*B	Mc					
*amp*C, bla_{SHV-5}, *mec*A	L					
*sul*I	S	River	Total DNA	PCR	Australia	(Barker-Reid *et al.*, 2010)
aac(3)-I	A					
*mec*A	L					
*van*A, *van*B	G					
aac(6')-Ib-cr, *qep*A, *qnr*A, *qnr*B, *qnr*S	Q	Wetlands	Total DNA	PCR	USA Mexico	(Cummings *et al.*, 2011)
*tet*L, *tet*M, *tet*O, *tet*Q, *tet*W	Tet	River	Total DNA	qPCR	Cuba	(Graham *et al.*, 2011)
*erm*B, *erm*C, *erm*E, *erm*F	Mc					
bla_{TEM-1}, bla_{CTX-M}, bla_{OXA-1}, bla_{SHV-1}	L					
*sul*II	S	River sediments	Total DNA	Functional metagenomics	India	(Kristiansson *et al.*, 2011)
*qnr*D, *qnr*S, *qnr*VC	Q					
*str*A, *str*B	A					

Classes of antibiotics: L,beta-lactam; Tet,tetracycline; Mc,macrolide; S, sulphonamide; Ap, amphenicol; A, aminoglycosides; G, glycopeptides; Q, quinolone.

The culture-dependent methods allow the characterization of the microbiota that is able to grow in artificial culture media and laboratory conditions. These methods are relatively inexpensive and reproducible, but underestimate the community profile. Indeed, it is estimated that only about 0.01-1 % of the total bacteria in a community is cultivable (Amann *et al.*, 1995; Vartoukian *et al.*, 2010).

Over the last 30 years, the development of molecular biology methods, bioinformatics and the improvement of public databases made decisive contributions for bacterial identification and community structure studies. The use of culture-independent methods permits the analysis of the whole bacterial community and also the detection of non-cultivable bacteria. Non-cultivable bacteria is a vague designation that includes the organisms for which the specific growth requirements are not available, slow-growing microorganisms out-competed in the presence of fast-growing organisms, and injured organisms which cannot stand the stressful conditions imposed by cultivation. Nevertheless, this group of bacteria is believed to have a major relevance in the bacterial communities (Pace, 1997).

Most of the culture-independent methods rely upon nucleic acid amplification and hybridization (Call, 2005; Rudi *et al.*, 2005; Tallon *et al.*, 2005). 16S rRNA gene Denaturing Gradient Gel Electrophoresis (DGGE) or Temperature Gradient Gel Electrophoresis (TGGE), clone libraries, and Fluorescence *in situ* Hybridization (FISH) are among the culture-independent methods most used to explore the bacterial diversity in waters (Amann *et al.*, 2001; Dewettinck *et al.*, 2001; Zwart *et al.*, 2002; Cottrell *et al.*, 2005; Hoefel *et al.*, 2005a; Loy *et al.*, 2005; Bottari *et al.*, 2006; Wu *et al.*, 2006; de Figueiredo *et al.*, 2007; Revetta *et al.*, 2010). More recently, high-throughput sequencing methods such as the 454 pyrosequencing and Illumina's Genome Analyser have been

increasingly used to explore the environmental bacterial diversity (Hong *et al.*, 2010; Roh *et al.*, 2010; Kwon *et al.*, 2011; Widger *et al.*, 2011).

In spite of the supposed high coverage, the culture-independent methods are not free of bias. Examples of possible bias are related with the DNA extraction methods, PCR amplification and the resolution of the analysis technique (V. Wintzingerode *et al.*, 1997; Vallaeys *et al.*, 1997; Muyzer and Smalla, 1998; Farnleitner *et al.*, 2004; Ahmadian *et al.*, 2006; Warnecke and Hugenholtz, 2007; Krause *et al.*, 2008; Zinger *et al.*, 2011). In spite of the relevant improvements over the last years, at this moment, there is no method capable of fully describing the whole bacterial community.

Although the culture-independent methods allow deeper insights into the bacterial communities, culture-dependent approaches are still important since allow the recovery of isolates, required for a comprehensive understanding of biology and ecology of the bacteria (Palleroni, 1997; Cardenas and Tiedje, 2008; Alain and Querellou, 2009). Cultured organisms are needed to characterize phenotypic and metabolic properties, as virulence or antibiotic resistance. In summary, the use of combined approaches of culture-dependent and culture-independent methods may offer a deeper characterization of the bacterial communities (Kisand and Wikner, 2003).

1.5.2. Bacteria identification and typing

Although often ignored, bacterial characterization and identification is one of the major issues in microbiology. A reliable and universal identification system is important to support further studies on the ecology and evolution of bacteria. It is admitted that many identifications of isolates reported in the literature over the past years were incorrect (Bartram *et al.*, 2003). For instance, the identification of bacterial isolates recovered from the environment may have not been always the most correct, since were

often impaired by poor databases. Indeed, until recently, identifications were made employing protocols and databases established mainly based on clinical isolates, which might be inappropriate for environmental strains (Bartram *et al.*, 2003). In the genomic era, DNA-DNA hybridization and 16S rRNA gene sequencing became the gold standard methods for bacterial species determination (Stackebrandt and Goebel, 1994). DNA-DNA hybridization, a requirement for species definition, involves the pairwise comparison of two entire genomes, which is technically challenging, labor-intensive and time-consuming. These constrains associated with the difficulty to establish databases and the low inter-laboratory reproducibility, pushes this method to the background in relation to others (Martens *et al.*, 2008).

With the widespread use and low cost of PCR and DNA sequencing, bacteria identification is increasingly based on gene sequence analyses. The comparison of nucleotide sequences of amplified gene fragments is nowadays routinely used for the identification and differentiation of bacterial species and strains. One of the most used genes for bacteria identification is the 16S rRNA, a molecular chronometer, that allows comparisons between highly divergent bacteria (Weisburg *et al.*, 1991). Its presence in every bacteria and the high degree of conservation allows universal comparisons, whereas the variable regions support the measurement of phylogenetic distances. In spite of those advantages, the comparison of highly related bacteria can be impaired by the small number of informative sites in the 16S rRNA gene sequence. Indeed, in some genera, the 16S rRNA gene variation does not allow confident species delineations, limiting its generalized use for species identification (Fox *et al.*, 1992). Some examples of the limited species resolution are observed within the family *Enterobacteriaceae*, or in the genera *Pseudomonas*, *Ensifer*, *Enterococcus*, among others (Mollet *et al.*, 1997; Yamamoto *et al.*, 2000; Ait Tayeb *et al.*, 2005; Naser *et al.*, 2005; Martens *et al.*, 2008).

An alternative for species or strains differentiation and which also offers a phylogenetic comparative analysis is the MultiLocus Sequence Typing (MLST). By definition, MLST is an unambiguous typing method for the characterization of bacterial isolates using the sequence determination of internal fragments of multiple housekeeping genes (Maiden, 2006). Although the use of seven loci is recommended, currently the most of the published schemes use six to ten loci (Maiden, 2006). The method involves the comparison of highly expressed and highly conserved protein-coding genes, also referred to as housekeeping genes (Maiden *et al.*, 1998; Maiden, 2006; Martens *et al.*, 2008). Those housekeeping genes are characterized by slower rates of evolution than typical protein-coding genes, but faster than rRNA genes, supporting a higher level of resolution (Ait Tayeb *et al.*, 2005). As any other tool used for taxonomic and phylogenetic analyses, loci to use in MLST must fulfill some requisites i) a universal distribution in the target group; ii) a rate of variation commensurable with the level of divergence in the group under study; iii) enough length to contain significant information, without compromising a convenient manipulation and sequencing; iv) the absence of divergent copies of the same gene in a single organism; v) no association with lateral gene transfer processes (Mulet *et al.*, 2010). Examples of housekeeping genes frequently used in multilocus analysis are the RNA polymerase beta subunit (*rpo*B), σ^{70} factor (*rpo*D), DNA gyrase β subunit (*gyr*B), beta subunit of membrane ATP synthase (*atp*D), recombinase A (*rec*A), among others (Mollet *et al.*, 1997; Bennasar *et al.*, 2010). However, the selection of the genes is a critical point of this method, because genes sequences that are highly informative within a genus or family may be useless or even absent in other taxa (Santos and Ochman, 2004). Moreover, unlike for rRNA gene sequence analysis, for which the use of universal primers is possible, the study of housekeeping genes requires the design of specific primers for each genus and most of the times for each species. These

limitations explain the inclusion of less than six genes in some MLST schemes (Baldo *et al.*, 2006; Marcelletti *et al.*, 2010) and are obvious shortcomings of this methodological approach. Another limitation of the MLST is the cost and the time required for the analysis. In the new era of high-throughput sequencing, with the cost of DNA-sequencing continuously decreasing, the whole genome sequence (WGS) appears as a good alternative for bacterial typing. Hopefully, in the next years the WSG may have an important role on the identification of the best loci for the MLST analysis, especially for bacterial groups less studied.

Nowadays, and in spite of the previously mentioned shortcomings, the possibility to make reliable species delineations and to infer intra-species variation make the MLST approach very attractive, with relevant application in molecular epidemiology (www.pubmlst.org; Cooper and Feil, 2004). In this respect, it is particularly important the portability of this method. Based on gene sequence analyses, MLST offers reproducible and unbiased data, which can be shared worldwide. This is an important advantage when compared with other typing methods such as the Pulsed-Field Gel Electrophoresis (PFGE) (Enright and Spratt, 1999; Spratt, 1999).

The first MLST scheme developed was for the human pathogen *Neisseria meningitidis* (Maiden *et al.*, 1998). Nowadays, more than thirty MLST schemes are described for many different pathogenic bacteria, such as *Streptococcus pneumoniae*, *Campylobacter jejuni*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, among other (http://www.mlst.net; www.pubmlst.org; Maiden, 2006). More recently the MLST has been also used for studies with environmental bacteria, such as *Aeromonas* spp., *Lactobacillus sanfranciscensis*, *Pseudomonas* spp., among others (www.pubmlst.org; Bennasar *et al.*, 2010; Picozzi *et al.*, 2010; Martino *et al.*, 2011).

Nevertheless, for these bacteria the MLST schemes proposed by some research groups, are not settled worldwide (Maiden, 2006).

1.6. The hypothesis and objectives of this study

The continuous increase of the antibiotic resistance prevalence, observed not only at clinical level, but also in the environment, along with the fact that Portugal is one of the highest consumers of antibiotics in the European Union, motivated this study.

Three major arguments support the hypothesis behind this study, i) there are evidences that environmental bacteria have an important role on antibiotic resistance dispersion; ii) human activities are associated with the observed increase of antibiotic resistance; iii) water is one of the most important habitats for bacteria in the environment. Through the urban water cycle environmental and human commensal bacteria are in close contact with the myriad of anthropogenic substances and stress factors produced by human activities.

The hypothesis of this study was, thus, if drinking water production and distribution could represent a hotspot for the proliferation, selection or incoming of antibiotic resistant bacteria, which would reach the final consumer.

In order to test this hypothesis, the study was designed aiming the tracking of bacteria from the source to the tap. Specifically, it was intended i) the characterization of the abundance and diversity of bacteria in raw, treated and final (tap) water; ii) the identification of critical points for bacterial selection, proliferation or entrance in the system; iii) inference about the bacterial groups which given their abundance and/or public health relevance could represent relevant harbors of antibiotic resistance in drinking waters.

2. Roadmap for the thesis

Preliminary note: The core of this thesis is composed of five articles, four published in refereed scientific journals and the fifth is in preparation for submission.

The antibiotic resistance is considered nowadays a serious and global problem of public health, with no easy resolution in sight. The continuous increase of the antibiotic resistance prevalence, observed for different taxonomical groups and in a wide range of environments, has motivated the attention of several international health agencies (APUA; CDC; COST-DARE; ECDC; WHO). Although the most critical situations have been reported at clinical level, many evidences suggest that environmental isolates may have an important role on the dispersion of antibiotic resistance (Martinez, 2008). Water is one of the most important habitats for bacteria, with great relevance on the microbial spreading, selection and evolution in the environment. Within the urban water cycle it connects many different places, including waste and drinking water treatment processes. For these reasons, water is regarded as one of the most important vehicles for antibiotic resistance dissemination. The urban water cycle includes many critical zones for bacterial selection and evolution. Among these, water treatment and distribution systems may constitute important habitats for selection and/or dispersion of antibiotic resistant bacteria.

The above mentioned reasons motivated the study of the antibiotic resistance prevalence and dispersion over the urban water cycle. Moreover, Portugal is among the European countries with higher antibiotic consumption and resistance, making this study particularly pertinent. In this study the central hypothesis was that water may vehicle

antibiotic resistant bacteria from the environment to humans. Thus, tap water and the final consumer were the major focus of this study which aimed at tracking antibiotic resistance from the source to the tap. Conscious of the close relationship between bacterial diversity and antibiotic resistance, key sites were characterized using both culture dependent and culture independent methods. Occasionally, samples of drinking water biofilm, cup fillers of dental chairs (Silva *et al.*, 2011) and mineral water (Falcone-Dias *et al.*, in press) were also included in the study.

One of the objectives was to assess the changes in the bacteria community from the source to the tap, inferring about the effect of the water treatment on both total and cultivable bacteria. Eight sites within a drinking water treatment plant and distribution system and 11 household taps were analyzed, over three sampling campaigns. Total DNA for community characterization and more than 3000 bacterial isolates were stored for further studies. The further characterization of these biological materials is included in chapters 5 and 6 of this thesis and in the publications with other co-authors, not included in this manuscript (Faria *et al.*, 2009; Figueira *et al.*, 2011b; Narciso da Rocha *et al.*, submitted for publication).

Chapter 3, *Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA-PCR-DGGE and culture-dependent methods*, describes the variations of total and cultivable bacteria from the source to the tap and compares the bacterial communities over the different sampled sites. Based on the low percentage of cultivability observed in water samples, it was raised the question if culture independent methods could represent a mere extension of the culture dependent approaches or if different population subsets were being investigated. This topic was important, mainly because phenotypic antibiotic resistance patterns were being studied, and is discussed in the Chapter 4, *Culture-dependent and culture-independent diversity surveys target*

different bacteria: a case study in a freshwater sample, which compares the use of cultivation methods, PCR-DGGE and 454 pyrosequencing to make snapshot of the bacterial populations.

Given their abundance and/or persistence in the analyzed samples and in literature reviews, some bacterial groups were studied with more detail for their phylogeny and antibiotic resistance. One of such groups were the family *Sphingomonadaceae*, herein described in Chapter 5, entitled *Diversity and antibiotic resistance patterns of* Sphingomonadaceae *isolates from drinking water*, and the genus *Pseudomonas* presented in Chapter 6, *Diversity and antibiotic resistance in* Pseudomonas *spp. from drinking water*.

The cultivation of bacteria belonging to previously unknown species is a contribution to attenuate the differences between the uncultivable and cultivable fractions of a bacterial community. Often, the isolation of these organisms occurs by chance, based on a single colony detected on a culture medium. A new species, named *Bacillus purgationiresistens*, was described within the scope of this thesis. The type strain was isolated and characterized using a phylogenetic, chemotaxonomic and phenotypic analysis and its description is given in Chapter 7, Bacillus purgationiresistens *sp. nov., isolated from a drinking water treatment plant*.

The results of this study offer an integrated perspective of the bacterial diversity and occurrence and propagation of antibiotic resistance in drinking water. These topics are discussed in Chapter 8.

Sequence of papers in the thesis:

Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA-PCR-DGGE and culture-dependent methods (in preparation for submission).

Vaz-Moreira, I., Egas, C., Nunes, O. C., Manaia, C. M. (2011) Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample. Antonie van Leeuwenhoek 100, 245-257.

Vaz-Moreira, I., Nunes, O. C., Manaia, C. M. (2011) Diversity and antibiotic resistance patterns of *Sphingomonadaceae* isolates from drinking water. Applied and Environmental Microbiology 77(16), 5697–5706.

Vaz-Moreira, I., Nunes, O. C., Manaia, C. M. (2012) Diversity and antibiotic resistance in *Pseudomonas* spp. from drinking water. Science of the Total Environment. doi:10.1016/j.scitotenv.2012.03.046

Vaz-Moreira, I., Figueira, V., Lopes, A. R., Lobo-da-Cunha, A., Spröer, C., Schumann, P., Nunes, O. C., Manaia, C. M. (2012) *Bacillus purgationiresistans* sp. nov. isolated from a drinking water treatment plant. International Journal of Systematic and Evolutionary Microbiology 62, 71-77. (According to rules of Latin and latinization, the name was corrected to *B. purgationiresistens*)

3. Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA-PCR-DGGE and culture-dependent methods

3.1. Abstract

Tap water supplied to consumers must comply with several chemical and microbiological requirements, which imply water treatment, storage and distribution.

This study aimed at evaluating the variations of the bacterial community from the source to the tap, in an attempt to infer about the influence of water treatment and distribution on the bacterial communities. Water samples were collected in three sampling campaigns, from the water treatment plant (4 sites), bulk supply distribution system (4 sites), and from household taps (11 houses) supplied by the same water. The total and cultivable heterotrophic bacteria were enumerated, using 4',6-diamidino-2-phenylindole staining and cultivation on R2A, respectively. Cultivable bacteria, recovered on R2A, *Pseudomonas* isolation agar and Tergitol-7-agar were characterized based on Gram and Ziehl-Neelsen staining, and the cytochrome *c* oxidase testing. Bacterial communities were characterized based on the 16S rRNA-DGGE profiling of the total DNA

Water treatment (filtration, ozonation, coagulation and flocculation, flotation and filtration, and chlorination) imposed a reduction on the counts, diversity and bacterial cultivability, from 0.12 % to less than 0.01%. Chlorination was responsible for the sharpest cultivability reduction and promoted a shift from predominantly Gram-negative to predominately Gram-positive and acid-fast bacteria, although no variation in the bacterial community structure could be detected. Downstream the chlorination stages, in tap water, it was observed an increase of the cultivability and in the proportion of Gram-

negative bacteria. These increases and the observed variance of the bacterial community structure, suggested the occurrence of conditions that favor bacterial (re)-growth in tap water. Based on the 16S rRNA-DGGE analysis members of the classes *Alpha*, *Beta* and *Gamma* of the *Proteobacteria* predominated from the source to the tap, although representatives of the phyla *Actinobacteria*, *Cyanobacteria*, *Planctomycetes* and *Bacteroidetes* are also frequent before water chlorination. This study shows that in spite of the potential of water disinfection to eliminate or inactivate most of the bacteria, at least members of the phylum *Proteobacteria*, such as *Acinetobacter* spp. and *Sphingomonadaceae*, may have a successful colonization of tap water.

3.2. Introduction

Drinking water, commonly produced from natural sources such as surface or ground water, is defined as a water suitable for human consumption, washing/showering, food preparation or other domestic purposes (98/83/EC, 1998; DL306-2007, 2007; WHO, 2008). In order to achieve the standards set by the World Health Organization or by the European Union (98/83/EC, 1998; WHO, 2008), frequently drinking water must undergo a combination of treatment processes. Drinking water treatment aims at assuring the desired chemical and microbiological quality and prevent the occurrence of undesirable transformations during storage and distribution (Marsalek *et al.*, 2006; WHO, 2008). The characteristics of the final product depend on the properties of the water source, on the treatment process, on the nature and extent of the storage and distribution devices, among other factors (Marsalek *et al.*, 2006; WHO, 2008).

Ozonation and chlorination are amongst the processes most frequently used to inactivate water microorganisms. Ozone is considered an excellent disinfectant, able to inactivate resilient pathogens, against which other conventional disinfectants, like chlorine, can fail (von Gunten, 2003). In spite of this, in a pilot study on the bacteriological population changes during potable water treatment and distribution Norton and LeChevalier (2000) did not observe relevant variations on the composition of cultivable raw water bacteria after ozonation. In contrast, water chlorination resulted in a rapid shift in the composition of the population. The strong effect of the chlorine disinfection in the drinking water bacterial diversity was also shown by other authors using real drinking water treatment and distribution systems (Hoefel *et al.*, 2005b; Eichler *et al.*, 2006). For example, Eichler *et al.* (2006), using RNA-based fingerprints analysis, observed that contrary to flocculation and sand filtration, the chlorination strongly affected the bacterial community structure, promoting the growth of nitrifying bacteria.

The combination of different disinfection processes, as ozonation and chlorination, and biomass removal, as flotation, filtration or flocculation, aims at increasing the efficiency of the drinking water treatment and it is expected that different microbial populations may be targeted by each approach. Nevertheless, the efficacy of the drinking water treatment is known to depend on several factors, including the physicochemical properties of the raw water (Norton and LeChevallier, 2000; von Gunten, 2003). In the same way, throughout the storage and distribution system, a myriad of factors can affect the microbial community composition. Among others, is possible to point out the time of water stagnation, the availability of conditions to promote bacterial regrowth (e.g. nutrients, temperature), or the structural and physicochemical properties of the material lining the reservoirs and distribution pipes (Power and Nagy, 1999; Ribas *et al.*, 2000; Niquette *et al.*, 2001; Lautenschlager *et al.*, 2010).

The drinking water bacterial diversity has been extensively studied using culture-independent methods. Members of the phylum *Proteobacteria* are among the most abundant bacterial groups in chlorinated drinking water (Hoefel *et al.*, 2005b; Eichler *et al.*, 2006; Poitelon *et al.*, 2009; Kormas *et al.*, 2010; Revetta *et al.*, 2010). For instance, Poitelon *et al*. (2009), using serial analysis of ribosomal sequence tags, observed the predominance of *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria* in French drinking water samples. Nevertheless, other bacterial phyla have also been frequently detected among the most prevalent bacteria in treated waters. In Germany, Hoefel *et al*. (2005b) and Eichler *et al*. (2006), using fluorescent in situ hybridization and denaturing gradient gel electrophoresis (DGGE) or RNA- and DNA-based 16S rRNA gene fingerprintings, respectively, observed the predominance of *Bacteroidetes*, in addition to *Alpha*- and *Betaproteobacteria*. In Greece, Kormas *et al*. (2010) detected mainly *Mycobacterium*-like bacteria and *Betaproteobacteria*, using 16S rRNA gene cloning and sequencing. Also

using 16S rRNA gene libraries, Revetta *et al*. (2010) detected a high percentage of difficult-to-classify bacterial sequences in USA drinking water samples, although referred also to the presence of *Proteobacteria*, *Cyanobacteria*, *Actinobacteria*, *Bacteroidetes* and *Planctomycetes*.

The diversity patterns as those referred to above, are obviously influenced by the methods used to characterize the communities. Nevertheless, it is estimated that in oligothrophic habitats, like disinfected water, non-cultivable or non-viable bacteria, represent more than 99 % of the environmental bacterial diversity (Amann *et al.*, 1995; Vartoukian *et al.*, 2010). Although part of these bacteria may lack specific growth requirements, other may simply be outcompeted during growth-based experiments. Indeed, some of the prevailing groups found using culture-independent methods are at least members of the same phyla as those retrieved by culture-dependent methods (Hoefel *et al.*, 2005b; Eichler *et al.*, 2006; Kormas *et al.*, 2010; Vaz-Moreira *et al.*, 2011a). Among the most frequent cultivable bacteria found in drinking water are members of the phyla *Proteobacteria* (e.g. genera *Brevundimonas*, *Blastomonas*, *Sphingomonas*, *Novosphingobium*, *Acidovorax*, *Burkholderia*, *Ralstonia*, *Variovorax*, *Aeromonas*, *Pseudomonas*, *Stenotrophomonas*), *Bacteroidetes* (e.g. genus *Flavobacterium*), *Firmicutes* (e.g. genus *Bacillus*) and *Actinobacteria* (e.g. genus *Mycobacterium*) (Zwart *et al.*, 2002; Hoefel *et al.*, 2005a, b; Eichler *et al.*, 2006; Kormas *et al.*, 2010; Revetta *et al.*, 2010). Despite the limitations that are nowadays recognized in the culture-dependent methods, this approach is an invaluable complement in microbiology studies, required for a comprehensive understanding of bacteria biology and ecology (Alain and Querellou, 2009).

This study was designed aiming the comparison of the bacterial populations throughout a drinking water production and distribution system. Based on the hypothesis

that disinfection imposes a bottleneck in the drinking water bacterial diversity, it was intended to assess the variations of the bacterial populations determined using culture-dependent and culture-independent methods. Specifically, it was intended to assess if the bacterial populations reaching the final consumer (taps) had origin in the water source. Given the limited overlapping of bacterial populations observed using culture-dependent and culture-independent methods (Vaz-Moreira *et al.*, 2011a), the use of both approaches was considered necessary in order to attain a comprehensive perspective of the diversity and variation patterns. Moreover, this study was framed in a project assessing the potential of drinking water to contribute for the spreading of antibiotic resistance, in which the study of cultivable bacteria assumes a major relevance. With these objectives, the bacterial populations of 19 sites, from the water source to the tap, were characterized using culture-dependent and culture-independent (16S rRNA-PCR-DGGE) methods.

3.3. Materials and methods

3.3.1. Sampling

Samples were collected in a drinking water treatment plant, downstream in the bulk supply distribution system (from now on referred to as distribution system) and from tap water (Figure 3.1). In this drinking water treatment plant, raw surface water (W1) is pumped up the river basin and alluvial wells (W2). The collected water undergoes sequentially filtration, ozonation (W3), coagulation and flocculation, flotation and filtration, and chlorination (W4). The resultant disinfected water enters a distribution system with 473 Km, and three main reservoirs. Each of these reservoirs supplies different municipal pipeline distribution networks.

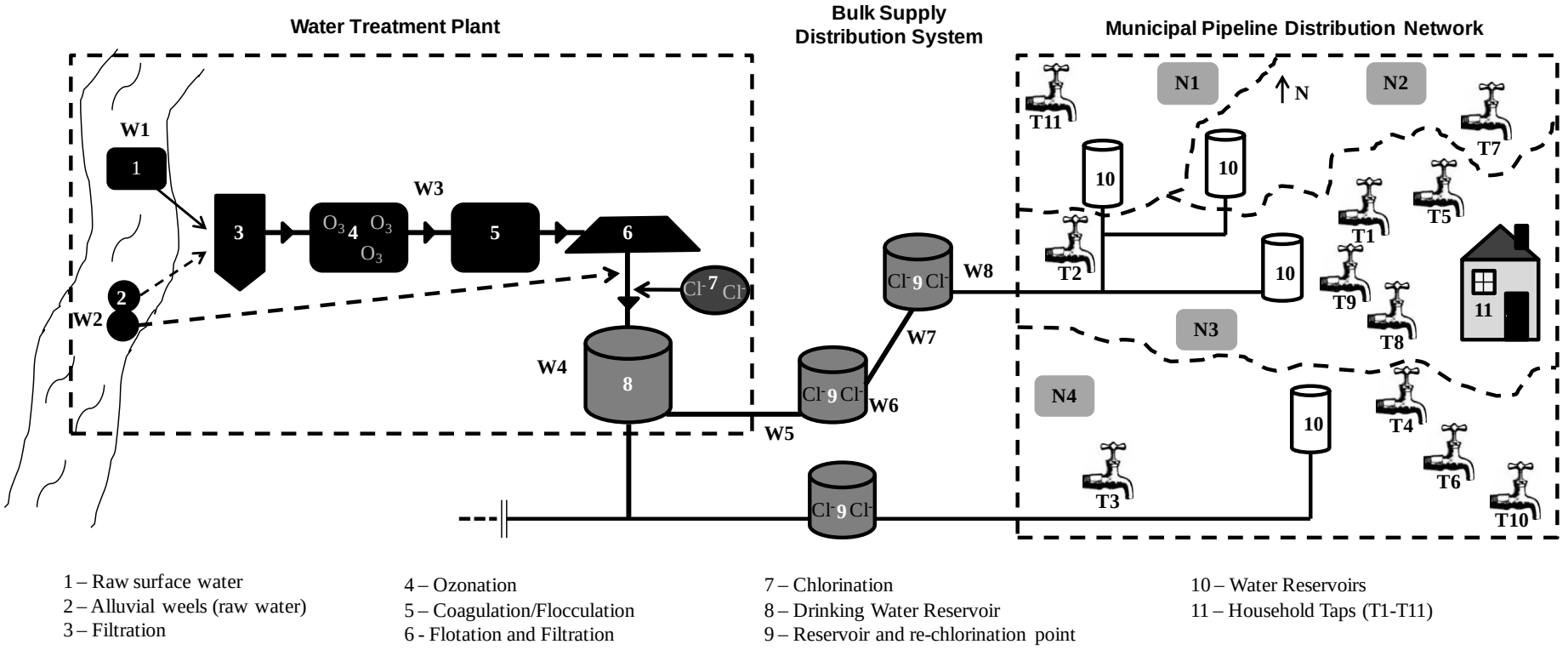

Figure 3.1. Schematic representation process of drinking water production and distribution analysed in this study. Numbers 1-11 indicate water source, disinfection, storage and distribution, and W1-W8 and T1-T11 the sampled sites.
The localization of the water reservoirs (10) is just representative. N1, N2, N3 and N4 represent different municipal distribution networks.

Samples were collected from nineteen sites, eight from the water treatment plant (W1-W4, Figure 3.1) and distribution system (W5-W8, Figure 3.1), and 11 from household taps used 1-4 times a month (T1-T11), at three sampling dates. The sampled household taps are located in four distinct municipal distribution networks (T11 in N1, T7 in N2, T1, T2, T5, T8, T9 in N3, and T3, T4, T6, T10 in N4, as depicted in Figure 3.1), in a total area of about 270 km^2, in buildings recently constructed (T8), with 10-12 years (T1, T3-T4, T6, T10), and more than 20 years (T2, T5, T7, T9, T11) of age. In the water treatment plant and distribution system, samples were collected in November 2007, December 2008, and September 2009, at the sampling points used for the routine monitoring analyses. Tap water samples were collected in April, July and October 2009. Each sample corresponded to volumes up to 45 L (in containers of 5 L each). A composite sample was prepared in the laboratory, by mixing equal volumes of water from each container. In order to neutralize the activity of disinfectants, 0.1 g L^{-1} of sodium thiosulfate was added to all the samples collected downstream the chlorination point. All samples were processed within 4 h after collection.

3.3.2. Microbiological characterization and bacterial isolation

Total cells numbers were determined by fluorescence microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) as described by Brunk *et al*. (1979). Briefly, 1 mL of water was filtered through a 0.22 µm black polycarbonate membrane (Whatman), incubated 15 min with 200 µL of 0.5 mg mL^{-1} DAPI solution in the dark. Cell enumerations were made in triplicate as described previously (Manuel *et al.*, 2007).

Total heterotrophic cultivable bacteria were enumerated on R2A medium (Difco), a non-selective medium recommended for the examination of total heterotrophic bacteria in

potable waters (ISO9308-1, 2000). *Pseudomonas* Isolation Agar (PIA, Difco) recommended for pseudomonads, and Tergitol 7-Agar (TTC, Oxoid) recommended for injured coliforms, were also used for the recovery of cultivable bacteria. Volumes up to 100 mL of water samples or the respective decimal serial dilutions thereof were filtered through cellulose nitrate membranes (0.45 µm pore size, 47 mm diameter, Albet), which were placed onto the culture media and incubated at 30 ºC (R2A and PIA) or at 37 ºC (TTC) up to 7 days. Water dilution and membrane filtration was done in triplicate for every sample. After the incubation period, the number of colony-forming units (CFU) on filtering membranes with up to 80 CFU was registered. In addition, bacteria were isolated from water treatment plant and distribution system samples collected in November 2007 and September 2009 and from all tap water samples, according to the following criterion: about fifty percent 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. The colonies isolated on R2A were purified on the same culture medium, and those isolated on culture media with a higher nutrient content (PIA and TTC) were purified on Plate Count Agar (PCA). Pure cultures were preserved at –80 ºC in nutritive broth supplemented with 15 % (v/v) glycerol. Colony and cellular morphology, Gram-staining reaction, catalase and cytochrome *c* oxidase, and Ziehl-Neelsen staining were characterized for all the isolates as described by Smibert and Krieg (1994). Based on this preliminary characterization, 2690 isolates were divided in five groups: the Gram-negative and oxidase-negative (GNOxN), Gram-negative and oxidase-positive (GNOxP), Gram-positive and oxidase-negative (GPOxN), Gram-positive and oxidase-positive (GPOxP), and the acid-fast bacteria. The proportion of each of these groups was used as the cultivable pattern of each sample.

3.3.3. Extraction of total DNA

For extraction of total DNA, water was filtered through polycarbonate membranes (0.2 µm porosity, Whatman). The water volumes analysed were determined according to preliminary experiments and the objective was to achieve a final DNA concentration of at least 0.2 µg mL^{-1}. These volumes varied between 0.5 and 15 L of water. Each sample was filtered in triplicate. Samples from the sites W4, W6-W8 yielded consistently concentrations of DNA below 0.07 µg mL^{-1}, hampering its inclusion in the DGGE analysis. Total DNA was extracted from the filtering membranes using a commercial kit which proved, based on preliminary assays, to offer good DNA yields and reliable extraction efficiency (PowerSoil™ DNA Isolation kit, MO BIO). DNA extraction was made according to the procedure described by Barreiros *et al*. (2011), with an additional incubation at 65 ºC for 30 min. Three total DNA extracts were obtained for each sampling site and date.

3.3.4. 16S rRNA-DGGE analysis

A 200 bp 16S rRNA gene fragment, corresponding to the region V3, was amplified with the primers 338F-GC-clamp (5'-GACTCCTACGGGAGGCAGCAG-3' with a GC clamp attached) and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer *et al.*, 1993). The amplification was performed in a reaction volume of 50 µL with 1x KCl buffer, 3 mM $MgCl_2$, 0.4 mM dNTP's mix, 5 % DMSO, 1 µM each primer, 3 U of Taq polymerase (Stabvida) and 4 µL of template DNA. The PCR conditions were 5 min at 94 ºC, followed by 35 cycles of 30 s at 94 ºC, 30 s at 55 ºC, 30 s at 72 ºC, and a final extension of 20 min at 72 ºC. A negative control reaction, without template DNA, was carried out simultaneously. The DNA concentration of the PCR products was determined as previously described (Lopes *et al.*, 2011) and approximately 1.2 µg of DNA were loaded

onto a vertical polyacrylamide gel (8 % w/v) with a denaturing gradient ranging from 29 to 59 % (where 100 % denaturing gradient is 7 M urea and 40 % deionized formamide). Electrophoresis was performed in a DCode^{TM} universal mutation detection system (Bio-Rad Laboratories) as described by Barreiros *et al*. (2008). The gel was stained for 15 min with ethidium bromide and the image was acquired with the Molecular Imager Gel Doc XR system (Bio-Rad Laboratories). In order to normalize the DGGE gels, a ruler composed of a set of reference cultures and which profile covered the whole denaturing gradient in use, was introduced in the extremities of each gel. After the visual examination of the patterns, DGGE profiles were compared using the Bionumerics software (version 6.1, Applied Maths). Reference lanes of DGGE markers were used for pattern normalization and to establish inter-gel comparisons, through band-matching. Bands were assigned to classes and compared based on the respective densitometric curves. Tables of band position versus intensity were used for samples comparison.

Bands representative of the various classes were excised and re-amplified with the primers 338F (without the GC clamp) and 518R in a reaction volume of 25 µL under conditions identical to those reported above, differing on the use of 0.2 mM dNTP's mix, 0.3 µM each primer, 0.5 U of Taq polymerase and 1 µL of template DNA. The DGGE band PCR product was cloned with the InsTAclone^{TM} PCR cloning kit (MBI Fermentas), according to the manufacturer's instructions. The clone inserts with the expected size (200 bp) were amplified with the primers 338F-GC-clamp and 518R and analysed by DGGE. DNA inserts matching the original band in the DGGE pattern were sequenced with the primer M13F-pUC (5'-GTTTTCCCAGTCACGAC-3'). Given the fact that some bands corresponded to more than one DNA sequence, up to 8 clones were sequenced. Nucleotide sequences were checked manually for their quality and compared with the

sequences deposited in the GenBank database using the BLAST software (http://www.ncbi.nlm.nih.gov/), in order to infer about their phylogenetic affiliation.

3.3.5. Statistical analyses

Data of total cells and heterotrophic bacteria counts over different sampling dates or sites were compared using analysis of variance (ANOVA) and post hoc Tukey test. The relationship between total and cultivable counts in different types of water was assessed based on a Pearson correlation analysis. The prevalence of different bacterial groups over the sampled sites was compared based on the chi-squared test. A cluster analysis was performed to compare the cultivable bacteria patterns of the different sampling dates of the household taps, using the proximity algorithm of Chebychev with the between groups method for the aggregation criterion. These analyses were supported by the software SPSS 19.0 for Windows.

A table of band position versus band intensity, supplied by the Bionumerics software (version 6.1, Applied Maths), comprising the triplicate DGGE profiles of every sample, was used to analyse possible site and temporal variations of the bacterial community structure, through a Principal Components Analysis (software package CANOCO version 4.5).

The bacterial diversity [$H' = -\sum pi \ln(pi)$] and evenness [$J = H'/\ln(Hmax)$] were estimated using the Shannon's (Shannon and Weaver, 1963) and Pielou's indices (Pielou, 1966), respectively. The indices were calculated based on the DGGE profiles, with the abundance of each operational taxonomic unit (OTU) being estimated on basis of band intensities. These indices were compared using ANOVA and post-hoc Tukey test as described above.

3.4. Results

3.4.1. Total and cultivable heterotrophic bacteria counts

Raw surface water (W1) presented the highest total cell counts, 10^6 cells mL^{-1}. Water ozonation and the subsequent disinfection stages (W3-W8) led to significant ($p<0.05$) reductions of 10-100 times in the total cell counts (Figure 3.2A). These values were maintained in the same order of magnitude in tap water (from 10^4 cells mL^{-1} for T1-T7 and T11, and up to 10^5 cells mL^{-1} for T8-T10) (Figure 3.2B). Total cell counts varied significantly ($p<0.05$) over the three sampling dates (A, B, C) in all the sampled sites of water treatment plant and distribution system (W1-W8), and in most of the taps (T4-T7, and T9-T11).

As observed for total cells counts, the number of total heterotrophic cultivable bacteria had accentuated and significant ($p<0.05$) variations throughout the sampled transect, with the counts decreasing from 10^3 CFU mL^{-1} in surface raw water (W1) to 10^{-1}-10^{-2} CFU mL^{-1} in the distribution system samples (W6-W8) (Figure 3.2A). Comparatively, tap water samples presented a higher number of cultivable bacteria, with values varying from 10^1 up to 10^4 CFU mL^{-1} in taps T8-T9 and T11 (Figure 3.2B). In raw water samples (W1 and W2) heterotrophic counts were stable over time, contrasting with the sites downstream (W3-W8 and T1-T11), in which counts varied significantly ($p<0.05$) (Figure 3.2A, B).

Raw water samples (W1 and W2) presented values of cultivability in the range 0.03-0.18 %, higher than in the distribution system (W6-W8) with values below 0.01 %. Higher values were observed in tap water samples, with percentages of cultivability ranging 0.85 % - 23 %. Although significant positive correlations between the total cell and total heterotrophic counts were observed for all samples, the coefficient of correlation for the raw water (W1 and W2) was higher (0.986, $p<0.001$) than that (0.335, $p=0.016$) of

treated water (W3-W8 and T1-T11). The date of sampling, house localizations or household pipes age did not influence the correlation between the total cell and total heterotrophic counts.

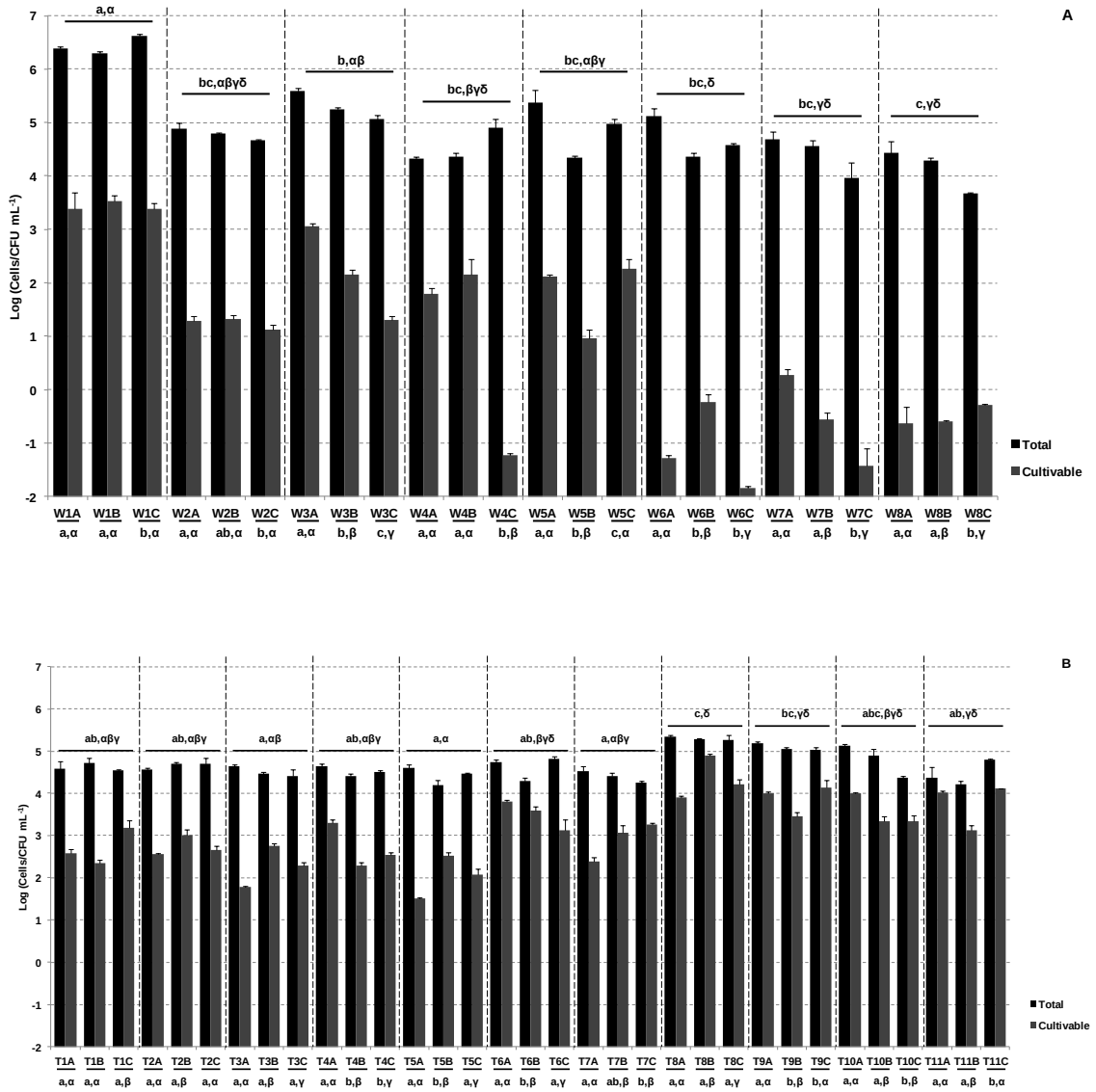

Figure 3.2. Enumeration of the total (black) and cultivable (grey) bacteria over the three sampling dates in the water treatment plant and distribution system (A) and in household tap (B) samples.
A,B,C - 1st, 2nd and 3rd sampling dates, respectively; Significant differences between sampling dates are indicated by letters, below the samples labels and between samples, are indicated by letters above the bars. The Roman letters represent the differences between the total bacteria counts, and Greek letters represent the differences between cultivable bacteria counts.

3.4.2. Diversity of cultivable bacteria

Except the acid-fast bacteria, which could not be isolated from raw surface water (W1), members of the different groups (GNOxN, GNOxP, GPOxN, GPOxP and acid-fast bacteria) were recovered from all types of water (W1-T11) (Figure 3.3A). When comparing the patterns of cultivable bacteria in the distinct types of water some differences were apparent. The raw water from the alluvial wells (W2) presented a lower prevalence of GNOxP and a higher prevalence of GNOxN and acid-fast bacteria ($p<0.05$) than the raw surface water (W1) (Figure 3.3A). The water treatment imposed a significant ($p<0.05$) increase of the Gram-positive (GPOxN and GPOxP) and acid-fast bacteria, and a significant ($p<0.001$) decrease of the Gram-negative bacteria (GNOxN and GNOxP) (Figure 3.3A). Tap water presented a profile of cultivable bacteria identical to that observed for raw surface water (W1) (Figure 3.3A).

As tap water is the one in close contact with humans, a further analysis of the cultivable bacterial diversity over time was performed (Figure 3.3B, C). In general, samples clustered according to the sampling date. For the majority of the analysed taps, from April (sampling date A) to July (sampling date B) it was observed that the percentages of Gram-positive bacteria decreased significantly ($p<0.001$) with a proportional increase of GNOxN bacteria (Figure 3.3B). From July to October (sampling date C), the increase of the GPOxN bacteria was the only significant difference ($p<0.001$) observed. The taps T7 and T8, localized in different municipal distribution networks, and in buildings with distinct ages presented an inverted pattern of cultivable bacteria, with the patterns of October clustering together with those of April of most the taps (Figure 3.3C).

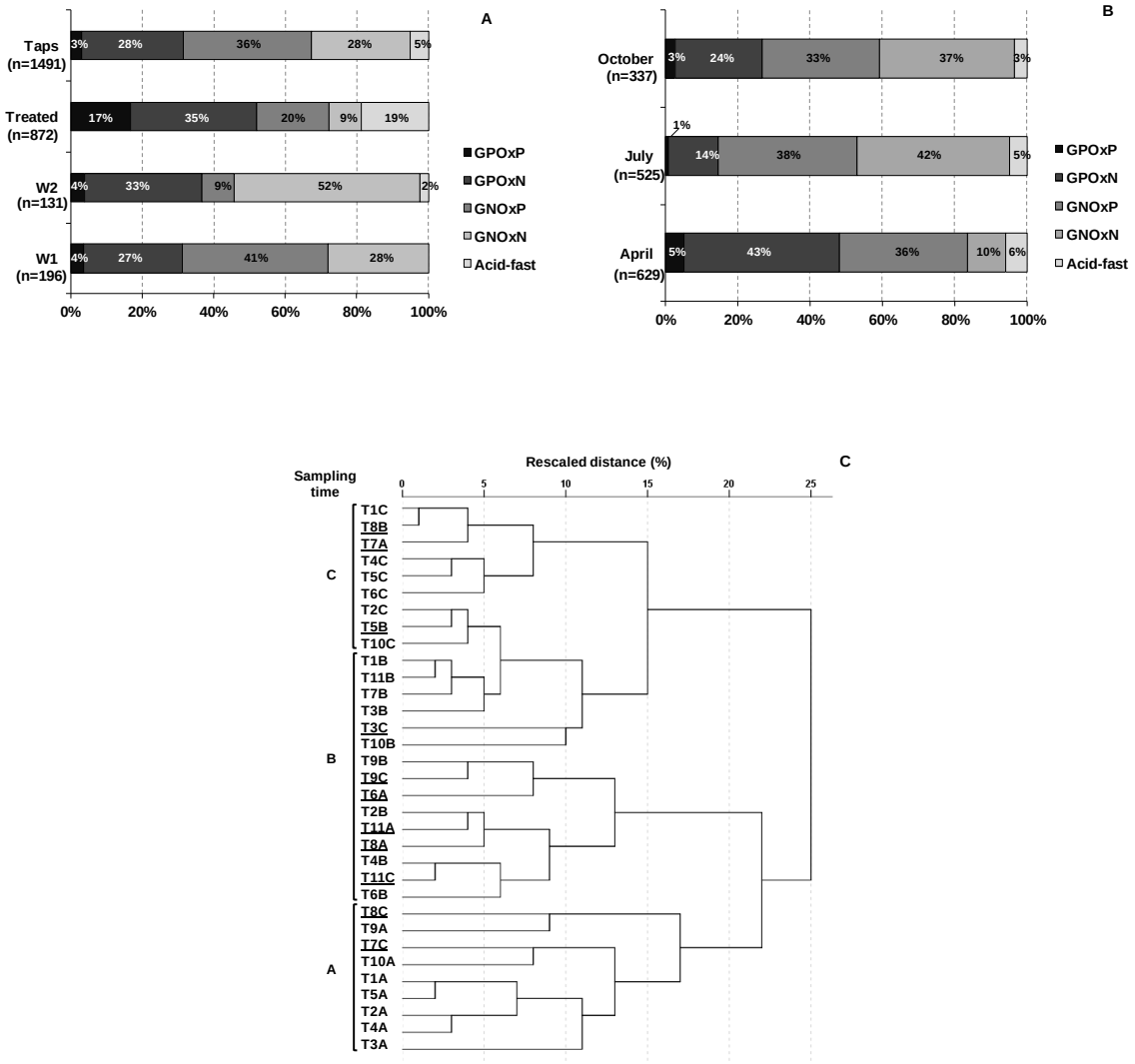

Figure 3.3. Cultivable bacterial diversity in the different types of water (A), and in household taps in the different sampling dates (B). Cluster analysis of the cultivable bacteria patterns of the tap water samples (C).

Raw surface water (W1), Alluvial wells raw water (W2), Treated water samples (W3-W8) and household taps (T1-T11). April corresponds to household taps sampling period A, July to sampling period B, and October to sampling period C.

GNOxN (Gram-negative and oxidase negative); GNOxP (Gram-negative and oxidase positive); GPOxN (Gram-positive and oxidase negative); GPOxP (Gram-positive and oxidase positive).

3.4.3. Bacterial diversity based on 16S rRNA-DGGE

The DGGE profiles of the analysed water samples (W1-W3, W5, T1-T11) contained 7-18 bands and, in total, 45 band classes were observed. In order to have further insights about the taxa prevailing in each sample, 35 representative bands out of the 45 PCR-DGGE band classes detected were excised, cloned and sequenced (supplementary Figure S1 and Table S1). Twenty nine out of the 35 DGGE bands yielded more than one DNA sequence, and the closest neighbors of the nucleotide sequences of 5 out of these 29 bands were members of three distinct phyla, 8 were members of two distinct phyla, and the remaining 16 although yielded more than one DNA sequence were all assigned to the same phylum.

Table 3.1. Phylum affiliation of the closest neighbors of the 16S rRNA gene sequences analysed in the DGGE profiles

Phylum	**W1**	**W2**	**W3**	**W5**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**	**T10**	**T11**
Proteobacteria															
Alpha-proteobacteria															
Beta-Proteobacteria															
Gamma-Proteobacteria															
Delta-Proteobacteria															
Epsilon-Proteobacteria															
Actinobacteria															
Bacteroidetes															
Cyanobacteria															
Planctomycetes															
Aquificae															
Acidobacteria															
Chloroflexi															
Verrucomicrobia															
Firmicutes															
Chlamydiae															
Nitrospirae															

Number of nucleotide fragments per DGGE profile: Black, more than 4; dark grey, 2-4; light grey, 1; white, not detected.

In all the samples, the closest neighbors of the majority of the bands corresponded to organisms of the phylum *Proteobacteria*, mainly *Alpha-*, *Beta-* and, in a lesser extent, *Gammaproteobacteria* (Table 3.1). Additionally, *Actinobacteria*, *Cyanobacteria*, *Planctomycetes* and *Bacteroidetes* were also frequently detected, mainly in raw water (W1, W2) and distribution system samples (W3, W5) (Table 3.1).

DGGE patterns showed that none of the 45 bands was present in all the sampled sites. Nevertheless, 13 (out of the 45) bands were common to raw (W1 and W2) and treated water collected in the distribution system (W5) and 20 were common to raw and tap water. The nucleotide sequences of bands common to all the types of water (raw, water treatment plant distribution system and household taps) were related to family *Sphingomonadaceae* and genera *Bradyrhizobium*, *Methylobacterium*, *Acidovorax*, *Acinetobacter*, among others.

The multivariate analysis of the DGGE profiles showed some variation in the water bacterial community structure throughout the sampled transect. Although the principal components analysis suggested homogeneity among the samples, the first two axes could explain 25.5 % of the bacterial community variation. This analysis evidenced the separation of the bacterial communities of tap water (T1-T11) from those of raw water, treatment plant and distribution system samples (W1-W3, W5) (Figure 3.4A). Bands B1, B3, B7, B9, B14, B20, B23, B30, B33, B34 were the major contributors for the distribution of the DGGE profiles, given they showed the highest Eigenvalues and significant correlation values with axis 1 (Figure 3.4B). Among these, bands B1, B14, B33, related to *Actinobacteria*, *Bacteroidetes* (*Sphingobacteriaceae*), *Betaproteobacteria* (*Ideonella* sp.) and *Deltaproteobacteria* (*Geobacter* sp.), were present only in the raw water, water treatment plant and distribution system samples. Although with slight influence in the separation of the DGGE profiles, bands B5, B12, B36, B44 were also

present only in W1-W3, and W5. These bands corresponded to members of the phyla *Proteobacteria* (*Beta*- division), *Bacteroidetes*, *Cyanobacteria* and *Planctomycetes* (supplementary Table S1). In opposition, bands B23 and B34, related to *Alphaproteobacteria* (genera *Bosea*, *Bradyrhizobium*, and *Nitrobacter*) were present only in tap water DGGE profiles, contributing to their separation from those of raw water and distribution system samples. Other bands detected only in the household taps profiles (B4, B6, B10, B13, B15, B26, B31, B38, B43), were mainly related to members of *Proteobacteria* (supplementary Table S1). Among these bands, B31 and B34 were found, respectively, in all and in 9 out of the total 11 tap water samples analysed. The absence of bands B23 and B34 in T7 and T8 contributed to cluster their DGGE profiles with those of W1-W3 and W5, and for the separation from other tap water profiles, mainly of T2, T10 and T11. On the other hand, the presence of bands B5, B14, B33 and B45, which showed high Eigenvalues and significant correlation values with axis 2, in W1-W3 and W5 but not in T7 and T8, allowed their separation. The fact that the DGGE profiles of T7 and T8 were separated from those of W1-W3 and W5 by principal component 2, indicates small variations among the bacterial community structure of these samples.

In opposition to the cultivable bacteria profiling, for the majority of the samples the DGGE profiles did not cluster by sampling date. Indeed, the cluster analysis of the DGGE profiles revealed also that the samples clustered mainly according to the sampling site (W1-W3, W5 versus T1-T11), irrespective of the sampling date (data not shown). Nevertheless, in the principal component analysis, axis 1 and mainly axis 2 permitted to distinguish slight variations in DGGE profiles over time for the majority of the taps (T2-T3, T5-T10). In this case, and in opposition to the variation observed for the cultivable bacteria profiling, the DGGE profiling variation did not follow a pattern. For T2, T3 and T5 the most distinct bacterial community structure was that of April (sampling date A),

while for T6, T7 and T9 was in July (sampling date B). On the other hand, the most distinct bacterial community structure of T8 and T10 was that of October (sampling date C).

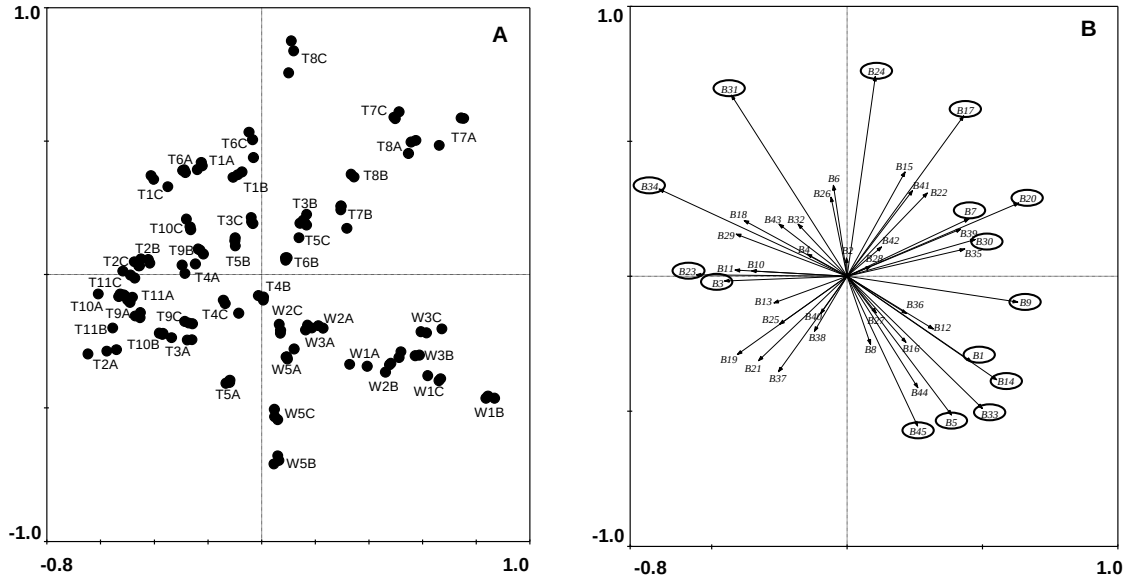

Figure 3.4. Principal components analysis with the PCR-DGGE profiles. A) samples (W1-W3, W5 and T1-T11) distribution and B) PCR-DGGE bands distribution, with the bands presenting the highest Eigenvalues marked with a circle.

The differentiation of the samples could also be inferred from the Shannon (H') diversity index, which ranged between 2.08 in tap T9 and 2.69 in raw surface water (W1) (Table 3.2). The two types of raw water presented diversity indices significantly different (p<0.05), with the water collected in the alluvial wells (W2) presenting a lower bacterial diversity than the raw surface water (W1). In the water treatment plant and distribution system it was observed that the water filtration and disinfection imposed a reduction on the bacterial diversity (H') from 2.69 in raw water to 2.51 after ozonation, and 2.33 after chlorination (Table 3.2). Diversity index (H') values in tap water samples presented wide

variations. The lowest (2.08-2.10) and the highest (2.50-2.53) values were observed in taps T5-6, T9 and T1-T3, T8, respectively (Table 2). Evenness values ranged between 0.29, in T6, T9 and in raw water from alluvial wells, and 0.34, in raw surface water (Table 3.2).

Table 3.2. Mean value and variance analysis of Diversity (H') and Evenness (J) indices calculated on basis of the DGGE profiles.

	H'					J				
Sample	value	a	b	c	d	value	a	b	c	d
W1	2.69				■	0.34				■
W2	2.10	■	■			0.29	■			
W3	2.51		■	■	■	0.32	■	■	■	■
W5	2.33	■	■	■	■	0.31	■	■	■	■
T1	2.53			■	■	0.33		■	■	■
T2	2.53			■	■	0.33		■	■	■
T3	2.50		■	■	■	0.33			■	■
T4	2.47	■	■	■	■	0.32	■	■	■	■
T5	2.19	■	■	■		0.30	■	■	■	■
T6	2.15	■	■	■		0.29	■	■	■	
T7	2.47	■	■	■	■	0.32	■	■	■	■
T8	2.53			■	■	0.33		■	■	■
T9	2.08	■				0.29	■	■		
T10	2.38	■	■	■	■	0.32	■	■	■	■
T11	2.39	■	■	■	■	0.32	■	■	■	■

Note: for the samples W4 and W6-W8, it was not possible to obtain DGGE profiles, and consequently the diversity and evenness indices were not calculated. Significant differences between samples are indicated by letters (a, b, c, d).

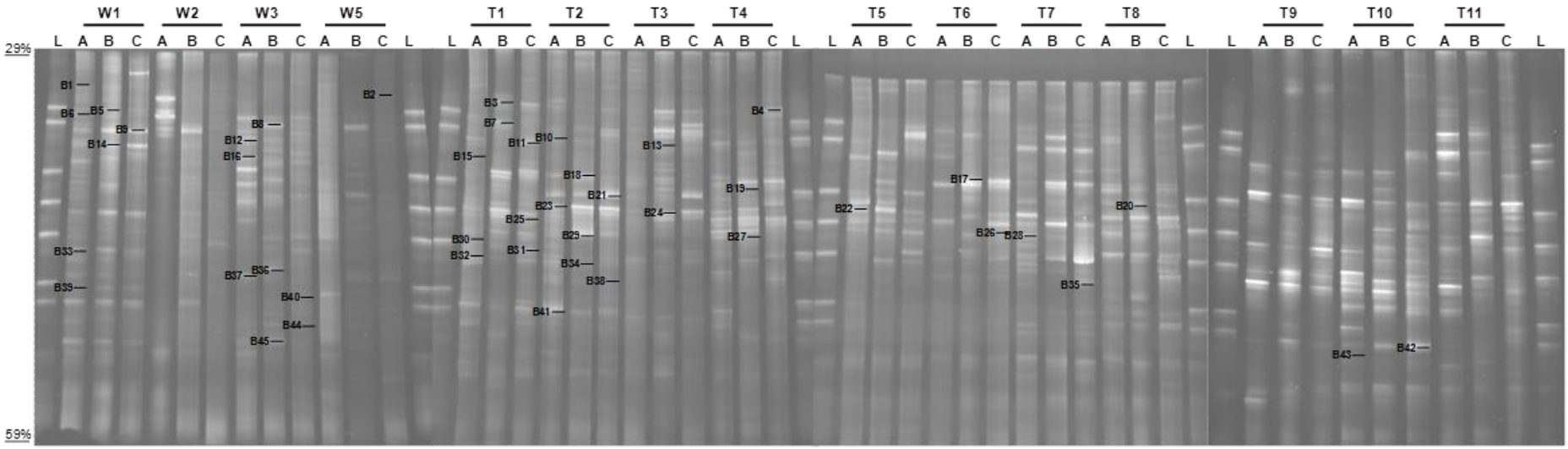

Figure S1. PCR-DGGE gels used for the analysis and band identification

Table S1. PCR-DGGE bands identification

Band class	Eigenvalues		Samples	Band Identification			
	PC1	PC2		Phylum	Closest neighbor	Acc. number	Similarity
B1	0.459	-0.316	W1(A,B,C)	*Bacteroidetes*	unc. *Sphingobacteriaceae*	EU703404	97%
B2	-0.002	0.068	W5C; T6C	Not identified			
B3	-0.453	-0.017	W2(A,C); T1(B,C); T2(A,B,C); T3(A,B,C); T4(A,C); T11(A,B,C)	*Proteobacteria* (*Gamma*)	unc. *Acinetobacter* sp.	GU071279/AM935245	98 – 100%
B4	-0.147	0.083	T1C; T4C;	Not identified			
B5	0.386	-0.514	W1(A,B,C); W2C; W5(B,C)	*Proteobacteria* (*Beta*) *Bacteroidetes* *Cyanobacteria*	unc. *Comamonadaceae* unc. *Sphingobacterium* sp. unc. *Cyanobacterium* sp.	EU641648/FJ916800 FN668086 EU780374	98% 99% 100%
B6	-0.050	0.337	T1A; T3C; T5C; T6A; T7A; T11C	*Proteobacteria* (*Beta*)	unc. *Comamonadaceae*	EU127419	98%
B7	0.451	0.213	W1(A,B,C); W2(A,C); W3(A,B,C); T1B; T2(B,C); T3(A,B,C); T4A; T5C; T6B; T7(A,B,C); T8(A,B,C); T11(A,B)	*Proteobacteria* (*Gamma*) *Proteobacteria* (*Beta*) *Proteobacteria* (*Epsilon*)	unc. *Acinetobacter* sp. *Methylophilus* sp. *Herminiimonas* sp. *unc. Epsilonproteobacterium*	GQ329250/FN794210 EU337121/GU827519 FJ562122 FJ872109 AB512142 GU061286	98-100% 100% 99% 100%
B8	0.087	-0.252	W1A; W2C; W3(B,C); W5(B,C); T2(A,C); T3C; T5A; T8A; T10C	*Proteobacteria* (*Beta*)	*unc. Betaproteobacteria*	AJ231052/CU926747	98%
B9	0.631	-0.096	W1(A,B,C); W2(A,B); W3(A,C); W5A; T3B; T4(A,B,C); T7(A,B,C)	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Gamma*)	unc. *Alphaproteobacterium* unc. *Acinetobacter* sp.	FN665766/GU074262 FJ192809/GU299536/ FM865882	96-99% 98%
B10	-0.352	0.021	T1A; T2(A,B,C); T3A; T5B	*Proteobacteria* (*Gamma*) *Bacteroidetes*	*Acinetobacter* sp. *Flavobacterium* sp.	GU977189 GU596955	100% 98%
B11	-0.414	0.023	W2A; W3A; T1(A,C); T2C; T4B; T5(A,B); T6A; T9A; T10B; T11(A,C)	*Proteobacteria* (*Beta*)	*Acidovorax* sp. unc. *Betaproteobacterium*	FN556569 EF705925	100% 98%
B12	0.318	-0.195	W3(A,B,C)	Not identified			
B13	-0.270	-0.099	T3A; T9(B,C); T10A	Not identified			
B14	0.553	-0.384	W1(A,B,C); W2B; W3B	*Actinobacteria*	unc. *Actinobacterium* sp.	GU074271/GU798095 FN668294/GU323630	98-100%

Table S1. Continued

Band class	Eigenvalues		Samples	Band Identification			
	PC1	PC2		Phylum	Closest neighbor	Acc. number	Similarity
B15	0.214	0.387	T1A; T3(A,B,C); T4C; T7(A,C); T8A	*Proteobacteria* (*Gamma*)	unc. *Acinetobacter* sp.	FJ193190	99%
B16	0.218	-0.245	W1C; W2A; W3(A,C); W5A; T3B; T4C; T10B	*Cyanobacteria*	unc. *Synechococcus* sp.	HM057799/FJ718220 GQ242576	99-100%
B17	0.431	0.594	W1B; W3C; T1(B,C); T3C; T5B; T6(B,C); T7(A,B,C); T8(A,B,C)	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Beta*)	*Altererythrobacter* sp. *Sphingopyxis* sp. unc. *Acidovorax* sp.	GU552682 HM047866 GQ129943/GU294846	97-100% 100% 99-100%
B18	-0.380	0.207	W1A; W2(A,B); W3(A,B); W5A; T1(B,C); T2(A,B); T3(A,B); T4(A,B,C); T6(A,B,C); T7B; T8(A,B,C); T9(A,B,C); T10(A,B,C); T11(A,B,C)	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Beta*) *Verrucomicrobia* *Acidobacteria*	unc. *Rhodobacteraceae* unc. *Sphingomonadaceae* unc. *Comamonadaceae* unc. *Opitutaceae* unc. *Holophaga* sp.	DQ191822 AM940553 FJ946623/EU642217 EF650890 AY509519	97% 99% 98-100% 98% 99%
B19	-0.405	-0.290	W5(B,C); T3A; T4(A,B,C); T5(A,B,C); T9(A,B,C); T11(A,B,C)	*Proteobacteria* (*Beta*) *Clamydiae*	*Acidovorax* sp. unc. *Pelomonas* sp. *Chlamydiales* bacterium	HM027578 HM104442 FJ976098	98-100% 99% 92%
B20	0.634	0.271	W1(A,B); W2(A,B); W3(B,C); T1B; T3B; T6(A,B); T7(A,B); T8(A,B,C)	*Proteobacteria* (*Alpha*) *Cyanobacteria*	unc. *Rhizobiales* unc. *Cyanobacterium* sp.	GU047641 EU751555	99% 95%
B21	-0.327	-0.313	W1(A,B); W2B; W3A; W5A; T2(A,B,C); T3(A,C); T4C; T5(A,B); T10A; T11(A,B,C)	*Proteobacteria* (*Beta*)	unc. *Comamonadaceae*	EU703426/EU642218	98-99%
B22	0.297	0.309	W3(A,B,C); W5(A,B,C); T1A; T4B; T5(A,B,C); T6C; T7(A,C); T8(A,B,C); T9B; T10C; T11C	*Proteobacteria* (*Beta*)	unc. *Curvibacter* sp. unc. *Denitratisoma* sp. *Burkholderia vietnamiensis*	FJ946584 FM175794 FN556562	100% 99% 99%
B23	-0.557	0.006	T1B; T2(A,B,C); T3(A,C); T4A; T6(A,B); T9A; T10B; T11(A,B)	*Proteobacteria* (*Alpha*)	unc. *Alphaproteobacterium* *Bosea* sp.	CU918195/CU919037 AB542375	98% 100%
B24	0.105	0.741	W1(A,C); W2C; W3A; T1(A,B,C); T2(B,C); T3(B,C);T4(A,B,C); T5(B,C); T6(A,C); T7(A,B,C); T8(A,B,C); T9B; T10C	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Beta*) *Proteobacteria* (*Gamma*) *Bacteroidetes*	unc. *Sphingomonas* sp. unc. *Betaproteobacterium* unc. *Gammaproteobacterium* unc. *Bacteroidetes*	GU563739/GQ484402 DQ450772/AY788951 FM253062/AY077611 EF662859 DQ828237	98-100% 98-100% 93-94% 92%

Table S1. Continued

Band class	Eigenvalues		Samples	Band Identification			
	PC1	PC2		Phylum	Closest neighbor	Acc. number	Similarity
B25	-0.249	-0.177	W2C; W5(A,B,C); T1(A,B,C); T2A; T3(A,B); T4(A,B,C); T5A	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Delta*)	unc. *Rhodoplanes* sp. unc. *Deltaproteobacterium*	EU297963 EF188715	98% 96%
B26	-0.059	0.294	T5(A,B,C); T6C; T8(A,B,C); T9C; T11(A,C)	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Beta*)	unc. *Alphaproteobacterium* unc. *Acidovorax* sp.	HM057723 GU294846	100% 100%
B27	0.108	-0.136	W1(A,B,C); W2B; T4(A,B,C); T6B; T7(A,B); T9(A,B,C); T10(A,B,C)	*Proteobacteria* (*Beta*) *Actinobacteria* *Planctomycetes*	*Dechloromonas* sp. unc. *Actinobacterium* unc. *Planctomycetes*	GU557149/GU202936 FN668302/EU703455 AY647321	98-100% 97-99% 90%
B28	0.088	0.036	W3A; T4(A,B); T7A; T9C	Not identified			
B29	-0.409	0.157	W5B; T1(A,B,C); T2(A,B,C); T6B; T8C; T10(A,B,C)	Not identified			
B30	0.474	0.137	W1A; W2(A,B); W3(B,C); W5A; T1A; T5(A,C); T6B; T7A; T8(A,B)	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Beta*) *Proteobacteria* (*Delta*)	*Methylobacterium* sp. unc. *Dechloromonas* sp. unc. *Geobactereaceae*	GU597368/EF520431 FJ610642 EF658025	100% 100% 87%
B31	-0.428	0.671	T1(A,B,C); T2(A,B,C); T3(B,C); T4(A,C); T5(B,C); T6(A,C); T7(A,B,C); T8(B,C); T9(A,B); T10(A,C); T11(A,B,C)	*Proteobacteria* (*Alpha*)	*Bosea* sp. *Methylobacterium tardum*	FN600559 HM069126	100% 97%
B32	-0.181	0.194	W2C; W5(A,C); T1A; T3(A,C); T4B; T7(A,B,C); T8C; T9C; T10(A,B,C); T11(A,B)	*Proteobacteria* (*Alpha*) *Firmicutes*	unc. *Bradyrhizobium* sp. unc. *Firmicutes*	FJ193341/AB480425 GU552899 EU299559	99-100% 97%
B33	0.500	-0.490	W1(A,B,C); W2B; W3C; W5B	*Proteobacteria* (*Beta*) *Proteobacteria* (*Delta*) *Actinobacteria*	*Ideonella* sp. unc. *Geobacter* sp unc. *Actinobacterium* sp.	DQ664241 EF414942 EU640885/EF158352 FJ916753	99% 97% 98-99%
B34	-0.694	0.323	T1(A,B,C); T2(A,B,C); T3B; T4A; T6(A,B,C); T8C; T9(A,B,C); T10(A,B,C); T11(A,B,C)	*Proteobacteria* (*Alpha*)	unc. *Bradyrhizobium* sp. unc. *Nitrobacter*	FJ193114 HM061139	98% 99%
B35	0.435	0.101	W1(A,B); T4C; T7(A,C)	*Chloroflexi*	unc. *Chloroflexi*	EU980305	99%
B36	0.220	-0.138	W3B	Not identified			

Table S1- Continued

Band class	Eigenvalues		Samples	Band Identification			
	PC1	PC2		Phylum	Closest neighbor	Acc. number	Similarity
B37	-0.253	-0.354	W1(A,B); W3A; W5(A,B,C); T2(A,B,C); T5A; T8(A,B); T10(A,B,C); T11(A,B,C)	*Proteobacteria* (*Alpha*) *Actinobacteria* *Cyanobacteria*	*Rhizobiales* bacterium unc. *Actinobacterium* sp. unc. *Cyanobacteria*	GU479717/GU479686 DQ303296/GQ242942 GU074225 CU926221	90-99% 99% 98%
B38	-0.121	-0.204	T3A; T5A	Not identified			
B39	0.420	0.175	W1(A,B,C); W2B; W5A; T6A; T7C; T8(A,B,C);T11C	*Proteobacteria* (*Alpha*) *Proteobacteria* (*Delta*) *Aquificae* *Chloroflexi*	unc. *Alphaproteobacterium* unc. *Deltaproteobacteria* unc. *Aquificae* unc. *Chloroflexi*	FJ916271 CU923178 FN668201 AM935092	99% 100% 98% 96%
B40	-0.098	-0.138	W3C; W5C; T4A; T9B; T10A	Not identified			
B41	0.242	0.317	W1(A,B,C); W2B; T1(A,B,C); T2(A,B,C); T3(A,B,C); T7(A,C); T8(A,B,C)	*Proteobacteria* (*Alpha*) *Actinobacteria* *Aquificae*	unc. *Bradyrhizobium* sp. unc. *Mycobacterium* sp. unc. *Aquificae*	FJ192734 GU433884/GQ203424 EU982466/FJ916496 FN668201	100% 98-99% 97%
B42	0.128	0.109	W3(B,C); T7(A,C); T9B; T10(A,B,C)	*Nitrospirae*	unc. *Nitrospira*	GU047646	100%
B43	-0.251	0.194	T1(A,C); T10A	Not identified			
B44	0.262	-0.413	W3(B,C); W5(B,C)	*Planctomycetes*	unc. *Planctomycetes*	EU980252	98%
B45	0.261	-0.555	W1(A,B,C); W3(A, B); W5(B,C); T2A;	*Proteobacteria* (*Alpha*) *Planctomycetes*	unc. *Rhizobiales* unc. *Planctomycetes*	AM940802 CU925937/EU980252	100% 97-99%

PC, principal component

3.5. Discussion

Although all the bias and limitations associated to DGGE (V. Wintzingerode *et al.*, 1997; Muyzer and Smalla, 1998; Farnleitner *et al.*, 2004), and the fact that this technique retrieves only the most abundant nucleotide sequences that are present in a given sample (Muyzer *et al.*, 1993), the sequencing analysis of the DGGE bands gave a good overview of the type of bacteria present in the samples in study. One of the limitations associated with this method is the fact that different organisms co-migrate in the same band (Vallaeys *et al.*, 1997). In our study 29 out of the 35 DGGE bands analyses yielded more than one DNA sequence suggesting that many 16S rRNA gene fragments of organisms characteristic of aquatic habitats denature at similar conditions, when using the primers 338F-GC-clamp and 518R.

The predominance of members of *Proteobacteria*, in all the types of water analysed was expected, since this phylum have been referred to as predominant in surface and drinking water samples (Zwart *et al.*, 2002; Williams *et al.*, 2004; Hoefel *et al.*, 2005b; Eichler *et al.*, 2006; Kormas *et al.*, 2010; Kahlisch *et al.*, 2012). Also the less frequently detected phyla, are reported also as major (*Actinobacteria*, *Cyanobacteria*, *Bacteroidetes*) or common inhabitants of surface and drinking water (*Planctomycetes*, *Aquificae*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Firmicutes*, *Clamydiae* and *Nitrospirae*) (Hoefel *et al.*, 2005b; Eichler *et al.*, 2006; Baik *et al.*, 2008; Poitelon *et al.*, 2009; Kormas *et al.*, 2010; Revetta *et al.*, 2010; Kwon *et al.*, 2011).

In our study, within *Proteobacteria*, members of classes *Alpha*-, *Beta*- and, in a lesser extent, *Gammaproteobacteria* were predominant, as reported before for drinking and mineral water (Manuel *et al.*, 2010; Falcone-Dias *et al.*, in press). Given this phylum comprises many ubiquitous and opportunistic bacteria, its predominance in drinking water deserves attention. Some recent studies on the diversity and antibiotic resistance of

some *Proteobacteria*, as *Acinetobacter*, *Sphingomonadaceae*, *Pseudomonas*, and others isolated from treated and mineral drinking water, revealed that these bacteria may be important sources of antibiotic resistance (Vaz-Moreira *et al.*, 2011b; Falcone-Dias *et al.*, in press; Vaz-Moreira *et al.*, in press; Narciso da Rocha *et al.*, submitted for publication). Members of *Acinetobacter* and *Sphingomonadaceae* were also detected in our study throughout the sampled transect.

The water treatment imposed a reduction in the number of total and cultivable bacteria, and also in the cultivability. Nevertheless, the bacterial counts and cultivability increased significantly at tap level, for percentages up to 23 %. The comparison of the cultivable bacteria patterns throughout sampled transect permitted also to conclude that the water treatment imposed changes in the proportion of all the groups of cultivable organisms, leading to a transitory reduction of the Gram-negative bacteria and the increase of the Gram-positive and acid-fast bacteria in treated water, which were reverted at the tap water level. However, and despite the reduction in the diversity indices, the cluster and principal components analysis of the DGGE profiles revealed that the water treatment and the distribution system did not impose significant alterations on the bacterial community structure of the raw water. However, variation on the diversity and bacterial community structure at tap level was observed. Nevertheless, such variation was not correlated with the localization of the household taps, and thus, could not be attributed to specific characteristics of each municipal distribution network.

Altogether these results suggest that the water treatment do not completely inactivate the microorganisms of raw water, preferentially the cultivable Gram-negative bacteria. It is possible to hypothesize that disinfection imposed a viable but non-cultivable status, which may be subsequently reversed at the tap level. Additionally, the transitory predominance of cultivable acid-fast, also called *Mycobacterium*-like bacteria,

immediately after treatment is not surprising, since their resistance to chlorine disinfection is known (Torvinen *et al.*, 2004), and their presence in treated water is commonly described (Falkinham *et al.*, 2001; Santos *et al.*, 2005; Kormas *et al.*, 2010; Falkinham, 2011). Torvinen *et al*. (2004) pointed out that water ozonation is a potential enhancer of mycobacterial growth in the distribution system. Indeed, the increase of the water assimilable organic carbon content due to the ability of ozone to degrade organic matter, may promote the development of microorganisms able to survive the stress conditions imposed by the treatment (van der Kooij and Hijnen, 1984; Miettinen *et al.*, 1998).

One of the hypotheses that justify the increase of the total heterotrophic bacterial counts and the proportion of Gram-negative bacteria along with modifications in the bacterial community structure at the tap level, is the bacterial regrowth. Indeed, bacterial regrowth was previously described for Gram-negative bacteria, namely *Pseudomonas* and *Aeromonas* (Ribas *et al.*, 2000). Water stagnation or other factors such as fluctuating temperatures, biofilm formation, composition, diameter and age of pipe materials, and concentration of organic compounds or chlorine has been point out as affecting bacterial regrowth (Niquette *et al.*, 2001; Lee *et al.*, 2007; Lautenschlager *et al.*, 2010). Additionally, the increase of cultivable heterotrophs counts and the proportion of Gram-negative bacteria after chlorination may have occurred in consequence of the ingestion of those bacteria by free-living amoebae (King et al., 1988; Greub and Raoult, 2004). Indeed, some studies revealed that many bacteria, increased their resistance to free chlorine residuals when ingested by free-living amoebae, which work as reservoirs and contribute to the protection, survival and dissemination of these bacteria in water systems (King et al., 1988; Thomas et al., 2008; Loret and Greub, 2010; Thomas et al., 2010). Many of the amoebae-resistant bacteria described are Gram-negative and

Mycobacterium-like bacteria (Thomas *et al.*, 2010). Some of these factors may also explain the weaker correlation between the numbers of total and cultivable bacteria observed for the treated water samples than for the raw water samples, and justify the high variability of the bacterial counts over the sampling dates and among taps. Additionally, the regrowth with consequent restructuration of the microbial population thriving in the household tap water may explain the variation in the bacterial community structure found at this level. In fact, 20 out of 38 bands present in the DGGE profiles of tap water corresponded to organisms thriving in raw water, suggesting that differences on the bacterial community structure of tap water were due to alteration in the abundance (band intensity) of the organisms. The fact that the diversity index values and the bacterial community structure of some taps, as T7 and T8, were close to those of raw and distribution system water, whereas those of other taps were more diverse, supports this hypothesis. On the other hand, the presence of 11 bands exclusively in DGGE profiles of tap water suggests that some organisms may enter into the water distribution network.

Seasonality seemed to affect mainly the cultivable bacterial diversity of tap water, increasing the prevalence of Gram-negative bacteria in July and October, when compared to April. This increase of Gram-negative bacteria may be associated with the increase of the temperature, which is described as a factor that promotes chlorine decay (Powell *et al.*, 2000). Consequently, the synergistic effect of higher temperature values and the presence of lower concentrations of chlorine may have benefited the Gram-negative bacteria regrowth. The un-match between organisms detected with culture-dependent and culture-independent methods (Kisand and Wikner, 2003; Cottrell *et al.*, 2005; Jordan *et al.*, 2009; Vaz-Moreira *et al.*, 2011a) may explain why seasonality did not have a major impact on the bacterial community structure as revealed by the proximity of the DGGE profiles in the principal components analysis biplot. Nevertheless, the variance of the

bacterial community structure among sampling dates and among household taps may reflect variations inherent to the specific conditions of each tap. One example of this specificity is the distinction of taps T7 and T8 from the others. Despite of showing close proximity in cultivable bacteria patterns, diversity index values and bacterial community structure both houses are supplied by different municipal distribution networks and have different ages (1 and 20 years old for T8 and T7, respectively). Beside the factors mentioned above, the specific conditions of each tap (house) may include factors such as the distance between the municipal water reservoir and a given tap, since higher chlorine concentrations (> 0.2 mg L^{-1}) are expected to occur in tap water supplied by a reservoir located nearby than in those more distantly located. All these factors may differentially influence the bacterial community structure of each tap, which supports the need to develop adequate material validation methods, recommendations and spot tests for in-house water facilities (Lautenschlager *et al.*, 2010).

3.6. Conclusions

Even considering all the possible bias or limitations of the methods used, is undeniable that the water treatment imposed an alteration in the composition of the bacterial community. After the treatment, a reduction in the number of total and cultivable bacteria, bacterial cultivability and diversity was observed. The water treatment imposed a clear shift in the composition of the cultivable bacterial population, from predominantly Gram-negative to predominately Gram-positive and acid-fast bacteria. However, strong alterations in the bacterial community structure of treated water were not observed. At tap level, the counts of total and cultivable bacteria increased, the Gram-negative bacteria were the most prevalent, and a wider variation in the bacterial community structure occurred, which suggests the occurrence of bacterial regrowth

and/or biofilm formation at this level. The variation in the diversity and structure of the bacterial communities at the tap level were probably derived from differences in specific conditions prevailing in each household tap.

Proteobacteria, mainly *Alpha-*, *Beta-* and, in a lesser extent, *Gammaproteobacteria*, was the predominant phylum in the analysed water. The ubiquitous and opportunistic character of these bacteria, some of them reported as antibiotic resistance vehicles, allied to the fact that drinking water is in close contact with humans, deserves attention.

4. Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample

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4.1. Abstract

Compared with culture-independent approaches, traditionally used culture-dependent methods have a limited capacity to characterize water microbiota. Nevertheless, for almost a century the latter have been optimized to detect and quantify relevant bacteria. A pertinent question is if culture-independent diversity surveys give merely an extended perspective of the bacterial diversity or if, even with a higher coverage, focus on a different set of organisms. We compared the diversity and phylogeny of bacteria in a freshwater sample recovered by currently used culture-dependent and culture-independent methods (DGGE and 454 pyrosequencing). The culture-dependent diversity survey presented lower coverage than the other methods. However, it allowed bacterial identifications to the species level, in contrast with the other procedures that rarely produced identifications below the order. Although the predominant bacterial phyla detected by both approaches were the same (*Proteobacteria*, *Actinobacteria*, *Bacteroidetes*), sequence similarity analysis showed that, in general, different operational taxonomical units were targeted by each method. The observation that culture-dependent and independent approaches target different organisms has implications for the use of the latter for studies in which taxonomic identification has a predictive value. In comparison to DGGE, 454 pyrosequencing method had a higher capacity to explore the bacterial richness and to detect cultured organisms, being also less laborious.

4.2. Introduction

Bacterial diversity surveys of natural waters are important approaches to assess the ecology and evolution of bacteria, to support management policies or to sustain risk assessment studies. For almost a century, the microbiological quality of waters was based on culture-dependent methods, which have been continuously optimized to detect and quantify the presence of organisms relevant in terms of quality control, public health or risk assessment studies [e.g. (Leclerc, 1994; Leclerc and Moreau, 2002; Mossel and Struijk, 2004)]. The culture-independent methods revealed the immense diversity of uncultured organisms, and thus, highlighted the need to implement complementary approaches for the analysis of water bacterial diversity (Amann *et al.*, 1995; Palleroni, 1997; Hugenholtz, 2002; Kemp and Aller, 2004; Venter *et al.*, 2004; Alain and Querellou, 2009). Several scientific and technological developments, but above all, the inexpensiveness of the nucleic acids sequencing methods, brought obvious improvements to bacterial diversity studies. The use of methods such as 16S rRNA gene clone libraries, fluorescence in situ hybridization (FISH) or denaturing gradient gel electrophoresis (DGGE) are nowadays a common place, and their use to explore the bacterial diversity in waters was exemplified in several publications (Amann *et al.*, 2001; Dewettinck *et al.*, 2001; Zwart *et al.*, 2002; Cottrell *et al.*, 2005; Hoefel *et al.*, 2005a; Loy *et al.*, 2005; Bottari *et al.*, 2006; Wu *et al.*, 2006; de Figueiredo *et al.*, 2007; Revetta *et al.*, 2010). More recently, the potential of the high-throughput 454 pyrosequencing to explore the environmental diversity has been emphasized (Roh *et al.*, 2010). In spite of the scientific and technical advances for bacterial diversity surveys, cultivation methods are still of great importance not only for laboratories equipped for routine monitoring, as those responsible for water quality control, but also for making inferences on the physiological and metabolic properties of the organisms (Palleroni, 1997; Cardenas and Tiedje, 2008).

Un-culturability is a broad sense condition that includes: (i) organisms for which the specific growth requirements (nutritional, temperature, aeration, etc.) are not available; (ii) slow-growing organisms, out-competed in the presence of fast-growing microorganisms and (iii) injured organisms, which cannot stand the stressful conditions imposed by cultivation. These categories, which are not necessarily related with specific taxonomic groups, are estimated to represent about 99% of the environmental bacterial diversity, especially in oligotrophic habitats, as freshwater (Amann *et al.*, 1995; Vartoukian *et al.*, 2010). Based on the analysis of the total DNA of the community, culture-independent methods are supposed to detect a considerable fraction of the uncultivable organisms, eventually in addition to those that can be cultured. Nevertheless it is not self evident that culture-dependent and culture-independent methods overlap on the detection of cultivable organisms. For studies related with risk assessment and public health issues, as for example, the search of virulence or antibiotic resistance traits, often measured in cultivable organisms, it would be important to use culture-independent approaches in complement of culture-dependent methods, as a way to infer the significance of a specific taxonomic group in the whole community. The current work is integrated in a wider study in which different approaches are being used to assess freshwater bacterial diversity. The work reported herein was designed to assess how the bacterial diversity recovered by traditional culture-dependent methods overlapped with that offered by culture-independent approaches (DGGE and 454 pyrosequencing). Specifically, it was intended to: (i) compare the range of bacterial groups and precision of the identification level obtained with each method and (ii) assess if the same organism can be targeted by culture-dependent and culture-independent methods.

For the cultivation-dependent approach was used a set of culture media commonly employed in microbiological water analysis (ISO9308-1, 2000; Eaton *et al.*, 2005) and

the identification of the isolates was based on the 16S rRNA sequence analysis. Culture-independent methods included DGGE and 454 pyrosequencing. DGGE based on the analysis of the 16S rRNA gene sequence has become one of the most popular methods to assess bacterial diversity in environmental samples (Muyzer and Smalla, 1998; Fromin *et al.*, 2002; Haack *et al.*, 2004; de Figueiredo *et al.*, 2007). Even though, some studies demonstrated that bacterial populations revealed by DGGE can represent less than 1% of the total community (Muyzer *et al.*, 1993; Murray *et al.*, 1996). The 454 pyrosequencing is a timely DNA sequencing technique that allows the generation of short reads rapidly and inexpensively, with accuracy and avoiding cloning bias (Ronaghi and Elahi, 2002; Krause *et al.*, 2008). A recently published study evidences the potential of this high-throughput technique to explore the bacterial richness of biofilms of potable waters (Hong *et al.*, 2010).

4.3. Materials and Methods

4.3.1. Sampling

A surface water sample (5 l) was collected in a river (approximately 3 m depth) in the area of the water pumping for a drinking water treatment plant (Faria *et al.*, 2009). The sample was collected in a sterile glass flask, transported to the laboratory, and processed within 4 h, as schematized in Figure 4.1. The physicochemical and microbiological characteristics of the water sample are indicated in Table 4.1.

4.3.2. Microbiological characterization

Total cell number was determined by fluorescence microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Steinheim, Germany) as described

by Brunk et al. (1979). Cell enumerations were made in triplicate as described previously (Manuel *et al.*, 2007; Barreiros *et al.*, 2011).

The diversity of cultivable bacteria was surveyed on three culture media commonly used for water microbiological quality control - R2A (Difco, Le Pont de Claix, France), *Pseudomonas* Isolation Agar (PIA, Difco) and Tergitol 7-Agar (TTC, Oxoid, Hants, UK). Volumes of 1 ml of water or decimal serial dilutions thereof were filtered through cellulose nitrate membranes (0.45 µm pore size, 47 mm diameter, Albet, Barcelona, Spain), which were placed onto the three different culture media and incubated at 30ºC (for R2A and PIA) or 37ºC (for TTC) up to 7 days. All the process, dilutions and filtrations, was done in triplicate.

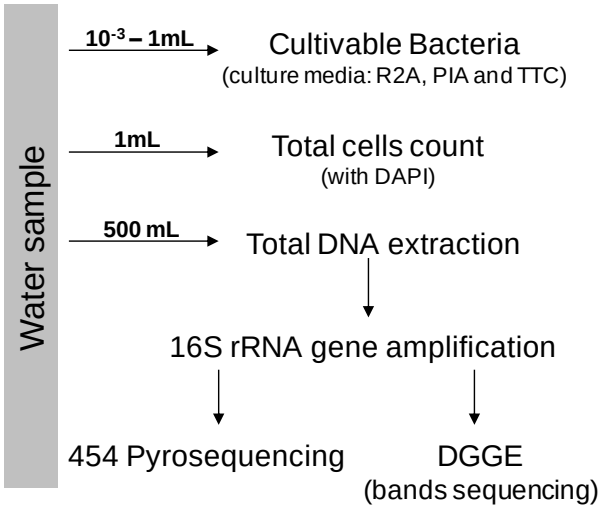

Figure 4.1. Schematic representation of the study methodology. For each approach, the sample was processed in triplicate.

4.3.3. Bacterial isolation and characterization

Bacteria were isolated after the visual examination of the triplicates of culture plates which evidenced a countable number of CFU's. All or half of the colonies were isolated when a morphotype was represented by up to 10 or more CFU's, respectively. The

colonies isolated on R2A were purified on the same medium, and those isolated on more nutritive media (PIA or TTC) were purified on PCA (Plate Count Agar, Pronadisa, Madrid, Spain). Pure cultures were preserved at -80ºC in nutritive broth supplemented with 15% (v/v) glycerol. All the isolates were identified on basis of the 16S rRNA gene sequence analysis, using the 27F and 1492R (Lane, 1991) as described before (Ferreira da Silva *et al.*, 2007).

Table 4.1. Physicochemical and microbiological characterization of the water sample

Physicochemical*		Microbiological	
Chlorides (mg L^{-1} Cl)	20.0	Enumerations (±SD):	
Conductivity, at 25ºC (µS cm^{-1})	338.0	DAPI (Total bacteria, cells mL^{-1})	$4.2x10^6 \pm 3.4x10^5$
Colour (mg L^{-1} Pt-Co)	5.1	R2A (Total heterotrophs, CFU mL^{-1})	$2.5x10^3 \pm 6.1x10^2$
Total hardness (mg L^{-1} $CaCO_3$)	80.0	PIA (*Pseudomonas* spp., CFU mL^{-1})	$5.7x10^2 \pm 4.6x10^1$
Iron (µg L^{-1} Fe)	121.0	TTC (Presumptive coliforms, CFU mL^{-1})	$1.4x10^2 \pm 3.5x10^1$
Nitrates (mg L^{-1} NO3)	<5.0		
pH (Sorensen scale)	7.8	Cultivability (%) [1]	
Total dissolved solids (mg L^{-1})	220.0	R2A	0.059
Total suspended solids (mg L^{-1})	<5.0	PIA	0.014
Temperature *in situ* (ºC)	26.1	TTC	0.003
Turbidity (NTU)	1.7		

*, These parameters were determined in the analytical laboratory of the water treatment plant, according to the recommendations of the drinking water directive (Council Directive 98/83/EC, 1998).

DAPI, 4',6-diamidino-2-phenylindole; R2A, R2A medium; PIA, *Pseudomonas* Isolation Agar; TTC, Tergitol 7-Agar; SD, standard deviation.

[1], cultivability was estimated as the ratio between the CFU mL^{-1} and the total number of cells mL^{-1} determined by DAPI staining.

4.3.4. Total DNA extraction

In preliminary assays, two DNA extraction methods were compared - the PowerSoil™ DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) and freeze-thawing with liquid nitrogen (Kawai *et al.*, 2002; Hoefel *et al.*, 2005a; Wu *et al.*, 2006). The MO BIO kit showed higher efficiency, being selected for further DNA extractions. Four fractions of 0.5 l of water sample were filtered through polycarbonate membranes (0.2 µm porosity, Whatman). DNA extraction was made as described by Barreiros *et al*. (2011), with an additional period of 30 min of incubation at 65ºC. Four DNA extracts were obtained for further analysis.

4.3.5. DGGE analysis

A 16S rRNA gene fragment of 200 bp, corresponding to the region V3, was amplified with the primers 338F-GC-clamp and 518R (Muyzer *et al.*, 1993). The amplification was performed in a reaction volume of 50 µl with 1x KCl buffer, 3 mM $MgCl_2$, 0.4 mM dNTP's mix, 5% DMSO, 1 µM each primer, 3 U of Taq polymerase (Stabvida, Lisbon, Portugal) and 4 µl of template DNA. The PCR conditions were 5 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 30 s at 55ºC, 30 s at 72ºC, and a final extension of 20 min at 72ºC. The DNA concentration of the PCR products was determined as previously described (Lopes *et al.*, 2011). Approximately 1.2 µg of DNA were loaded onto a vertical polyacrylamide gel (8% w/v) with a denaturing gradient ranging from 29 to 59% (where 100% denaturing gradient is 7 M urea and 40% deionized formamide) (DCode™ universal mutation detection system, Bio-Rad Laboratories, Munich, Germany) (Barreiros *et al.*, 2008). DGGE gels were normalized using a ruler composed of a set of reference cultures that had a profile which covered the whole denaturing gradient in use. The DGGE profiles of the four DNA extracts were 100% concordant. Thus, one lane was

selected for further analysis, with the excision and analysis of all bands as described before by Barreiros *et al*. (2011). For bands, sequencing analysis was used the InsTAclone^{TM} PCR cloning kit (MBI Fermentas, Heidelberg, Germany), according to the manufacturer's instructions. DNA inserts of at least three different clones matching the original band in the DGGE pattern were sequenced with the primer M13F-pUC. Nucleotide sequencing and quality checking were performed as described previously (Barreiros *et al.*, 2011).

4.3.6. 454 pyrosequencing

One of the DNA extracts was used for 454 pyrosequencing. The 16S rRNA gene hypervariable V4–V5 region was amplified by PCR using the universal bacterial primers, 520F (50-AYTGGGYDTAAAGNG-30) and 802R (50-TACNVRRGTHTCTAATYC-30) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) fused to the 454 A and B adaptors, respectively. Standard PCR reaction conditions were employed for 50 µl reactions with Fast Start polymerase (Roche, NJ, USA) - 1.8 mM $MgCl_2$, 0.2 µM each primer, 200 mM dNTPs, 5 U of polymerase and 2 µl of template DNA. The PCR conditions were 94ºCfor 3 min, followed by 30 cycles of 94ºCfor 30 s, 44ºC for 45 s and 72ºC for 60 s and a final elongation step at 72ºC for 2 min. The 16S rRNA gene amplicon was sequenced on a 454 Genome Sequencer FLX platform according to standard 454 protocols (Roche - 454 Life Sciences, NJ, USA).

4.3.7. Sequence analysis and phylogenetic classification

A cut-off value of 97% similarity of the 16S rRNA gene sequences was considered to define an operational taxonomic unit (OTU). The 16S rRNA gene sequences obtained from cultivable organisms (read lengths varying from 1357 to 1450 bp) and DGGE bands

(read lengths varying from 164 to 203 bp) were aligned using Clustal W from MEGA 4.0 software (Tamura *et al.*, 2007).

For 454 pyrosequencing, processing of sequencing reads and bacterial taxonomic identification were carried out through an in-house built pipeline (M. Pinheiro and A.C. Gomes, unpublished data). Raw sequencing reads were quality filtered according to the following criteria: (i) exact matches to primer sequences; (ii) sequences with less than two ambiguous bases (Ns), (iii) sequences longer than 100 bp, and (iv) longer sequences trimmed at 250 bp. The sequences were then aligned by making all-against-all possible pairwise sequence alignments with ClustalW, followed by building a pairwise-distance matrix with DNAdist program of the PHYLIP Package, v. 3.69 (Felsenstein, 1993) and finally grouping of identical sequences into OTU (operational taxonomic units) at 97% similarity through MOTHUR (Schloss *et al.*, 2009).

The taxonomical identity of each OTU was assigned through BLAST searches against the Ribosomal Database Project II (Cole *et al.*, 2009), GenBank (www.ncbi.nlm.nih.gov) and SILVA (Pruesse *et al.*, 2007). For dendrogram construction, 16S rRNA gene sequences of the type strain (Euzéby, 1997) of the species observed to represent the closest neighbor of each OTU were included in the sequence based comparative analysis. Dendrogram representations were obtained after pairwise and multiple sequence alignment on basis of the model of Jukes and Cantor (1969) and neighbor-joining method. The phylogeny inference method maximum parsimony was also applied to assess dendrogram reliability and stability. These analyses were performed with the software MEGA 4.0 (Tamura *et al.*, 2007).

4.3.8. Richness, diversity and evenness indices

The diversity [H' = -∑*p*i ln(*p*i)] and evenness [J = H'/ln(Hmax)] were measured using the Shannon's (Shannon and Weaver, 1963) and Pielou's indices (Pielou, 1966), respectively, calculated as described by Wang *et al*. (2008). The OTU, as defined above, was the basis for this calculation. For cultivable bacteria, the abundance of each OUT corresponded to the number of CFU per millilitre. For PCR-DGGE, the abundance of each OTU was estimated on basis of band intensity, measured with the aid of Bionumerics software package version 6.0 (Applied Math, Belgium). When a single band was observed to contain more than one OTU, an equitable distribution of the band intensity was considered. For pyrosequencing the abundance of each OTU corresponded to the number of sequences determined. Non-identified bacteria were excluded from the calculations.

4.4. Results

4.4.1. Cultivable bacteria

Under the conditions used, cultivable bacteria ranged the 10^2-10^3 CFU ml^{-1} and total cells were about 1000 times more abundant. Cultivability ranged 0.003–0.059 %, with the lowest and highest values observed on TTC and R2A, respectively (Table 4.1).

In total, 39 bacterial strains were isolated – 10 from TTC, 14 from PIA and 15 from R2A. Among these, two lost viability after isolation (1 from TTC and 1 from PIA), and four after freezing (2 from R2A, 1 from TTC and 1 from PIA). According to the 16S rRNA gene sequence analysis, TTC, PIA and R2A bacterial isolates were grouped into five, eight and eleven OTU respectively (Figure 4.2a). This observation reflected a lower richness on the culture media TTC and PIA than on R2A and influenced the Shannon's diversity indices, also lower on the two selective culture media (Table 4.2). Presumably,

such a selectivity led to the recovery of organisms of some bacterial genera not detected on R2A (*Ralstonia*, *Chryseobacterium*, *Chitinophaga*, *Bacillus* and *Exiguobacterium* on PIA, and *Delftia* and *Lactococcus* on TTC) (Figure 4.2a). In total, cultivable bacteria were distributed by five phyla (Figure 4.2b). Lower values of diversity and evenness indices were achieved using the culture-dependent survey, than when the culture-independent methods were used (Table 4.2).

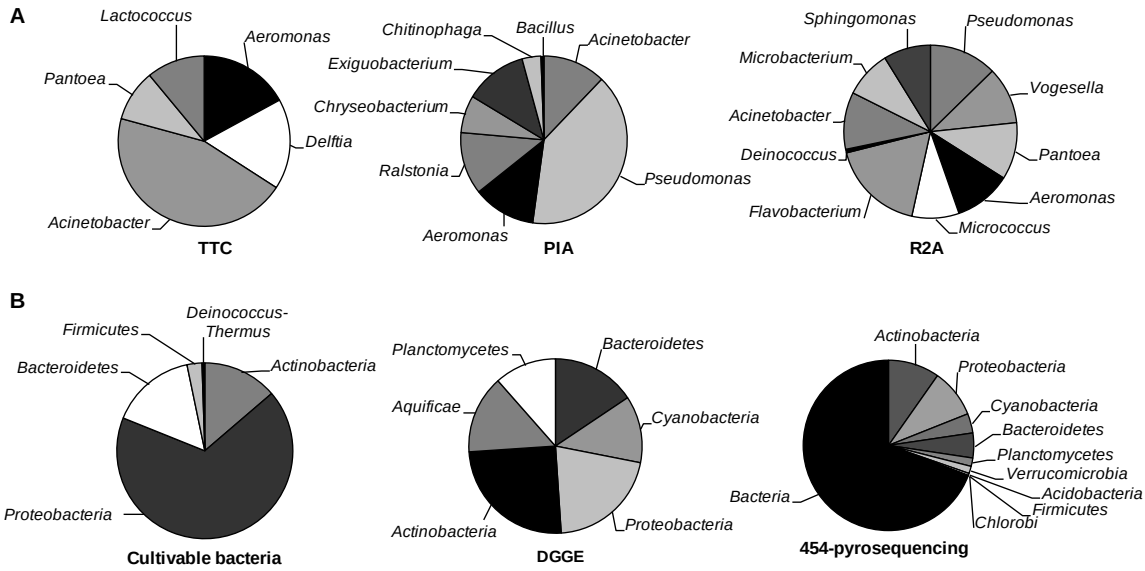

Figure 4.2. A) Bacterial diversity of the cultivable bacteria identified at the genus level, for the three different culture media; B) Bacterial diversity at the phylum level obtained with each method used.

Note: Percentages were estimated as the ratio between the: (i) the number of CFU ml^{-1} of each genus (a) or phylum (b) and the total number of CFU ml^{-1}, for cultivable bacteria; (ii) the intensity of each band and the sum of the intensity of all the bands, for DGGE; (iii) number of nucleotide sequence reads in each phylum and the total number of sequences, for 454 pyrosequencing.

Table 4.2. Shannon's diversity index (H') and Pielou's Evenness index (J) for total and cultivable bacteria

	Phylum		Genus	
	H'	J	H'	J
Cultivable bacteria				
R2A	0.93	0.12	2.20	0.28
PIA	0.71	0.11	1.73	0.27
TTC	0.35	0.07	1.43	0.29
PCR-DGGE	1.75	0.24	-	-
454 Pyrosequencing	1.64[a]	0.25[a]	-	-

[a] Unclassified bacteria (corresponding to H' = 0.26 and J = 0.03) were excluded from this analysis.

4.4.2. Culture-independent methods

DGGE analysis allowed the separation of 11 bands, seven corresponding to unique DNA sequences and four comprising a mixture of two or three DNA sequences. The OTUs identified through this method belonged to six phyla (Figure 4.2b) and only about 50 % could be identified below the phylum level (orders *Rickettsiales*, 2.6 %; *Sphingobacteriales*, 9.7 %; *Actinomycetales*, 2.7 %; *Chroococcales*, 6.3 %). Three of the phyla identified by DGGE (*Cyanobacteria*, *Planctomyces*, *Aquificae*) were not represented among the cultivable bacteria, as expected due to the culture conditions used.

After quality control and filtering, 454 pyrosequencing analysis produced 2776 sequences with good quality (2302 from *Bacteria*, 28 from *Eukarya* and 446 ''unknown''). The ''unknown'' sequences, which did not allow the identification to any validly named taxon, as well those identified as *Eukarya*, were excluded from the analysis. The resultant 2302 sequences identified as members of the domain *Bacteria* were grouped in 348 OTUs, corresponding to a value of bacterial diversity coverage of 62 %. The identification of OTU to at least the phylum level was possible to less than half of the consensus sequences (144 OTU), with 204 identified simply as *Bacteria* (Figure

4.2b). In spite of the observed limitations, the 454 pyrosequencing allowed the identification of nine phyla, 18 orders (*Sphingomonadales*, *Rhodobacterales*, *Rickettsiales*, *Burkholderiales*, *Neisseriales*, *Pseudomonadales*, *Legionellales*, *Chromatiales*, *Methylococcales*, *Pasteurellales*, *Bdellovibrionales*, *Sphingobacteriales*, *Flavobacteriales*, *Cytophagales*, *Nostocales*, *Actinomycetales*, *Solirubrobacterales* and *Verrucomicrobiales*) and 14 genera (*Legionella*, *Polynucleobacter*, *Acidovorax*, *Acinetobacter*, *Novosphingobium*, *Bdellovibrio*, *Vogesella*, *Flavobacterium*, *Rhodobacter*, *Conexibacter*, *Methylobacter*, *Haemophilus*, *Aphanizomenon* and *Caedibacter*). Among the phyla detected by 454 pyrosequencing, but not by DGGE, were the *Firmicutes*, *Chlorobi*, *Verrucomicrobia* and *Acidobacteria*. In contrast, organisms most related to *Aquificae* were detected by DGGE analysis, but not by 454 pyrosequencing. Although the amount of DNA extract used for DGGE was higher than that used for 454 pyrosequencing, it is plausible to admit that this difference is related to the sensitivity or possible bias introduced by the PCR reaction in each method.

4.4.3. Culture-dependent versus DGGE or 454 pyrosequencing

Members of the phyla *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were observed to be predominant in this water sample, irrespective of the method used (Figure 4.2). Nevertheless, when the 16S rRNA gene sequences of the bacterial isolates were compared with those retrieved by each of the methods used, it became clear that different OTUs were being targeted by each method (Figures 4.3 and 4.4). The most evident example of this fact was given by the OTUs of phylum *Proteobacteria*, which through the 16S rRNA gene sequence analysis of cultivable microorganisms comprised mainly *Gammaproteobacteria* (73.2 %) of the genera *Pseudomonas*, *Acinetobacter*, *Aeromonas* and *Pantoea*, through DGGE included the *Alpha* (42.9 %) and *Gamma* (27.6 %) classes,

whereas pyrosequencing revealed the predominance of members of the classes *Alpha* (46.5 %) and *Beta* (37.2 %). Additionally, the comparison of 16S rRNA gene sequences of bacterial isolates with those of DGGE bands demonstrated that, even though members of the same phyla and classes were identified, rarely the sequences clustered together. Sequence similarities were lower than 93 %, indicating that both methods targeted a different set of organisms (Figure 4.3).

The relatedness between the 16S rRNA gene sequences of the cultivable bacteria and through 454 pyrosequencing is shown in Figure 4.4. As observed with DGGE, the 16S rRNA gene sequences from the cultivable bacteria tend to form distinct clusters of the sequences obtained by 454 pyrosequencing. However, in some cases it was possible to observe sequence similarity values higher than 97 % (always lower than 98 %) (grey shadowing in Figure 4.4; members of the family *Commamonadaceae* in the class *Betaproteobacteria*, of the genus *Acinetobacter* in the class *Gammaproteobacteria* and of the genus *Flavobacterium* in the phylum *Bacteroidetes*), suggesting that the same OTU could be detected by cultivation and by 454 pyrosequencing. Among the 454 pyrosequencing nucleotide sequences closely related (>97 %) with cultivable bacteria, only in one case it corresponded to a consensus construct (of 18 nucleotide sequences, 0BQ01AGUSG, within the phylum *Bacteroidetes*, Figure 4.4); all the others corresponded to single nucleotide sequences. This observation excluded the hypothesis that the clustering of sequences from bacterial isolates and from pyrosequencing could be due to the fact that consensus sequences were being used. These results evidence the higher bacterial diversity coverage of 454 pyrosequencing when compared with DGGE.

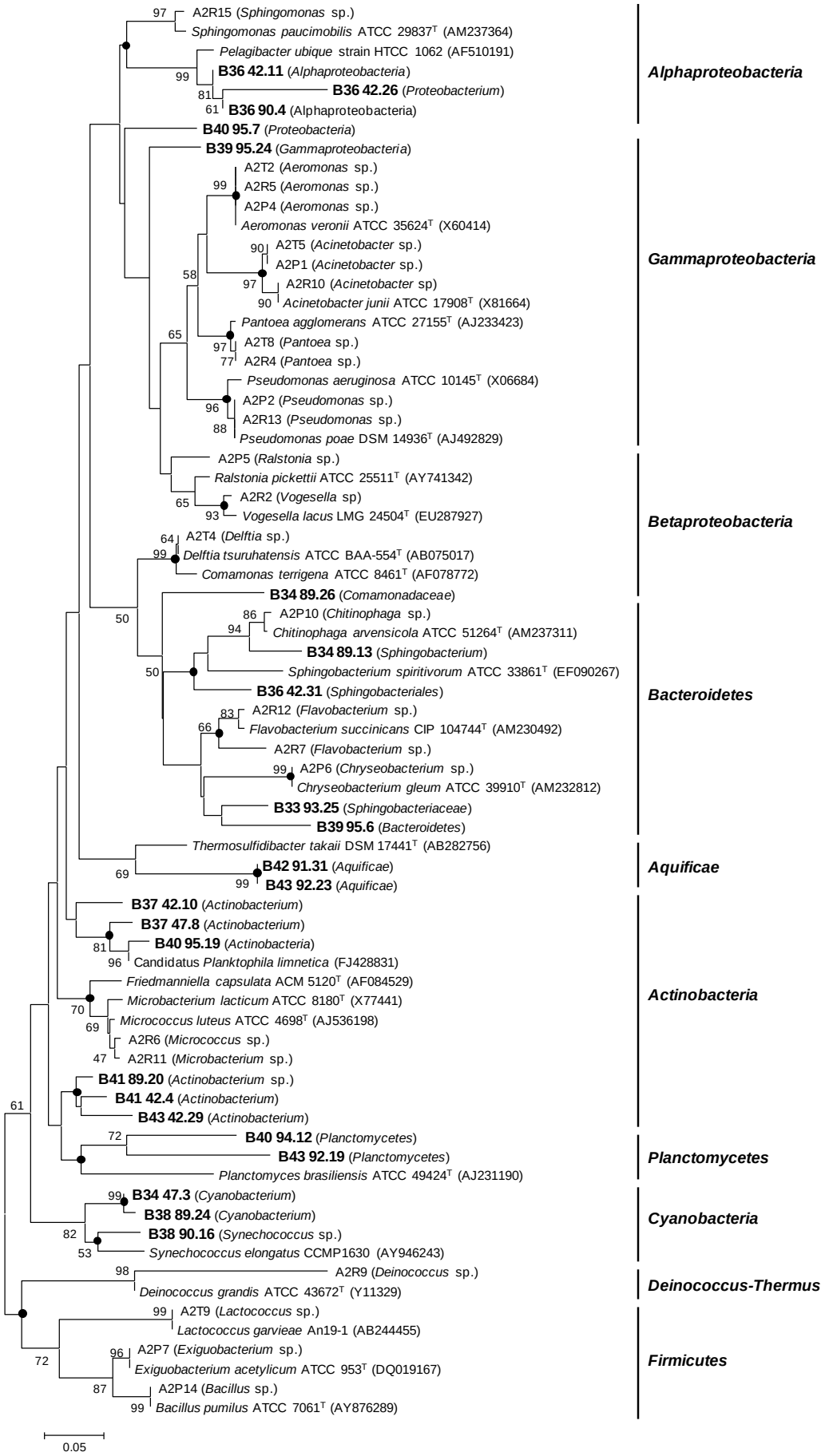
A2R15 (Sphingomonas sp.)
Sphingomonas paucimobilis ATCC 29837T (AM237364)
Pelagibacter ubique strain HTCC 1062 (AF510191)
B36 42.11 (Alphaproteobacteria)
B36 42.26 (Proteobacterium)
B36 90.4 (Alphaproteobacteria)
B40 95.7 (Proteobacteria)
B39 95.24 (Gammaproteobacteria)
A2T2 (Aeromonas sp.)
A2R5 (Aeromonas sp.)
A2P4 (Aeromonas sp.)
Aeromonas veronii ATCC 35624T (X60414)
A2T5 (Acinetobacter sp.)
A2P1 (Acinetobacter sp.)
A2R10 (Acinetobacter sp)
Acinetobacter junii ATCC 17908T (X81664)
Pantoea agglomerans ATCC 27155T (AJ233423)
A2T8 (Pantoea sp.)
A2R4 (Pantoea sp.)
Pseudomonas aeruginosa ATCC 10145T (X06684)
A2P2 (Pseudomonas sp.)
A2R13 (Pseudomonas sp.)
Pseudomonas poae DSM 14936T (AJ492829)
A2P5 (Ralstonia sp.)
Ralstonia pickettii ATCC 25511T (AY741342)
A2R2 (Vogesella sp)
Vogesella lacus LMG 24504T (EU287927)
A2T4 (Delftia sp.)
Delftia tsuruhatensis ATCC BAA-554T (AB075017)
Comamonas terrigena ATCC 8461T (AF078772)
B34 89.26 (Comamonadaceae)
A2P10 (Chitinophaga sp.)
Chitinophaga arvensicola ATCC 51264T (AM237311)
B34 89.13 (Sphingobacterium)
Sphingobacterium spiritivorum ATCC 33861T (EF090267)
B36 42.31 (Sphingobacteriales)
A2R12 (Flavobacterium sp.)
Flavobacterium succinicans CIP 104744T (AM230492)
A2R7 (Flavobacterium sp.)
A2P6 (Chryseobacterium sp.)
Chryseobacterium gleum ATCC 39910T (AM232812)
B33 93.25 (Sphingobacteriaceae)
B39 95.6 (Bacteroidetes)
Thermosulfidibacter takaii DSM 17441T (AB282756)
B42 91.31 (Aquificae)
B43 92.23 (Aquificae)
B37 42.10 (Actinobacterium)
B37 47.8 (Actinobacterium)
B40 95.19 (Actinobacteria)
Candidatus Planktophila limnetica (FJ428831)
Friedmanniella capsulata ACM 5120T (AF084529)
Microbacterium lacticum ATCC 8180T (X77441)
Micrococcus luteus ATCC 4698T (AJ536198)
A2R6 (Micrococcus sp.)
A2R11 (Microbacterium sp.)
B41 89.20 (Actinobacterium sp.)
B41 42.4 (Actinobacterium)
B43 42.29 (Actinobacterium)
B40 94.12 (Planctomycetes)
B43 92.19 (Planctomycetes)
Planctomyces brasiliensis ATCC 49424T (AJ231190)
B34 47.3 (Cyanobacterium)
B38 89.24 (Cyanobacterium)
B38 90.16 (Synechococcus sp.)
Synechococcus elongatus CCMP1630 (AY946243)
A2R9 (Deinococcus sp.)
Deinococcus grandis ATCC 43672T (Y11329)
A2T9 (Lactococcus sp.)
Lactococcus garvieae An19-1 (AB244455)
A2P7 (Exiguobacterium sp.)
Exiguobacterium acetylicum ATCC 953T (DQ019167)
A2P14 (Bacillus sp.)
Bacillus pumilus ATCC 7061T (AY876289)
Alphaproteobacteria
Gammaproteobacteria
Betaproteobacteria
Bacteroidetes
Aquificae
Actinobacteria
Planctomycetes
Cyanobacteria
Deinococcus-Thermus
Firmicutes
0.05

Figure 4.3. Dendrogram constructed on basis of partial 16S rRNA gene sequences (111 bp) of the cultivable bacteria (isolates identified with ‘‘R’’ were isolated from R2A, ‘‘T’’ from TTC and ‘‘P’’ from PIA) and of the DGGE bands (marked in bold in the figure). Some related type species or closest described organisms were added to the dendrogram to validate the taxonomical identifications. The dendrogram was generated using the neighbour-joining method based on the model of Jukes and Cantor and the dark circles indicate branches recovered by the maximum parsimony method. Bootstrap values, generated from 1000 resamplings, at or above 50 % are indicated at the branch points. Bar 1 substitution per 20 nt positions.

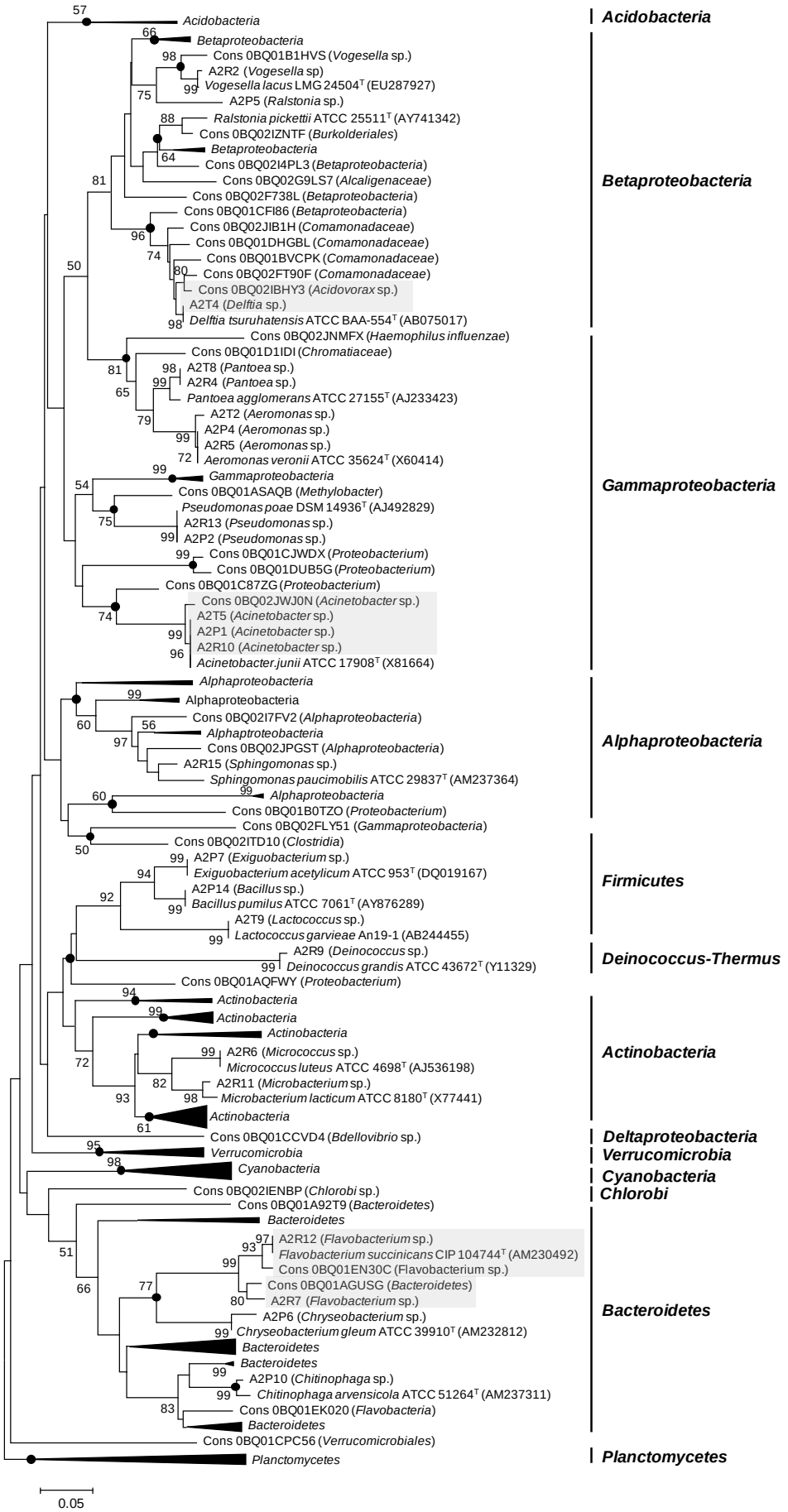
Acidobacteria
Acidobacteria
Betaproteobacteria
Cons 0BQ01B1HVS (Vogesella sp.)
A2R2 (Vogesella sp)
Vogesella lacus LMG 24504T (EU287927)
A2P5 (Ralstonia sp.)
Ralstonia pickettii ATCC 25511T (AY741342)
Cons 0BQ02IZNTF (Burkolderiales)
Betaproteobacteria
Cons 0BQ02I4PL3 (Betaproteobacteria)
Cons 0BQ02G9LS7 (Alcaligenaceae)
Cons 0BQ02F738L (Betaproteobacteria)
Cons 0BQ01CFI86 (Betaproteobacteria)
Cons 0BQ02JIB1H (Comamonadaceae)
Cons 0BQ01DHGBL (Comamonadaceae)
Cons 0BQ01BVCPK (Comamonadaceae)
Cons 0BQ02FT90F (Comamonadaceae)
Cons 0BQ02IBHY3 (Acidovorax sp.)
A2T4 (Delftia sp.)
Delftia tsuruhatensis ATCC BAA-554T (AB075017)
Betaproteobacteria
Cons 0BQ02JNMFX (Haemophilus influenzae)
Cons 0BQ01D1IDI (Chromatiaceae)
A2T8 (Pantoea sp.)
A2R4 (Pantoea sp.)
Pantoea agglomerans ATCC 27155T (AJ233423)
A2T2 (Aeromonas sp.)
A2P4 (Aeromonas sp.)
A2R5 (Aeromonas sp.)
Aeromonas veronii ATCC 35624T (X60414)
Gammaproteobacteria
Cons 0BQ01ASAQB (Methylobacter)
Pseudomonas poae DSM 14936T (AJ492829)
A2R13 (Pseudomonas sp.)
A2P2 (Pseudomonas sp.)
Cons 0BQ01CJWDX (Proteobacterium)
Cons 0BQ01DUB5G (Proteobacterium)
Cons 0BQ01C87ZG (Proteobacterium)
Cons 0BQ02JWJ0N (Acinetobacter sp.)
A2T5 (Acinetobacter sp.)
A2P1 (Acinetobacter sp.)
A2R10 (Acinetobacter sp.)
Acinetobacter junii ATCC 17908T (X81664)
Gammaproteobacteria
Alphaproteobacteria
Alphaproteobacteria
Cons 0BQ02I7FV2 (Alphaproteobacteria)
Alphaptroteobacteria
Cons 0BQ02JPGST (Alphaproteobacteria)
A2R15 (Sphingomonas sp.)
Sphingomonas paucimobilis ATCC 29837T (AM237364)
Alphaproteobacteria
Cons 0BQ01B0TZO (Proteobacterium)
Cons 0BQ02FLY51 (Gammaproteobacteria)
Alphaproteobacteria
Cons 0BQ02ITD10 (Clostridia)
A2P7 (Exiguobacterium sp.)
Exiguobacterium acetylicum ATCC 953T (DQ019167)
A2P14 (Bacillus sp.)
Bacillus pumilus ATCC 7061T (AY876289)
A2T9 (Lactococcus sp.)
Lactococcus garvieae An19-1 (AB244455)
Firmicutes
A2R9 (Deinococcus sp.)
Deinococcus grandis ATCC 43672T (Y11329)
Deinococcus-Thermus
Cons 0BQ01AQFWY (Proteobacterium)
Actinobacteria
Actinobacteria
Actinobacteria
A2R6 (Micrococcus sp.)
Micrococcus luteus ATCC 4698T (AJ536198)
A2R11 (Microbacterium sp.)
Microbacterium lacticum ATCC 8180T (X77441)
Actinobacteria
Actinobacteria
Cons 0BQ01CCVD4 (Bdellovibrio sp.)
Deltaproteobacteria
Verrucomicrobia
Verrucomicrobia
Cyanobacteria
Cyanobacteria
Cons 0BQ02IENBP (Chlorobi sp.)
Chlorobi
Cons 0BQ01A92T9 (Bacteroidetes)
Bacteroidetes
A2R12 (Flavobacterium sp.)
Flavobacterium succinicans CIP 104744T (AM230492)
Cons 0BQ01EN30C (Flavobacterium sp.)
Cons 0BQ01AGUSG (Bacteroidetes)
A2R7 (Flavobacterium sp.)
A2P6 (Chryseobacterium sp.)
Chryseobacterium gleum ATCC 39910T (AM232812)
Bacteroidetes
Bacteroidetes
A2P10 (Chitinophaga sp.)
Chitinophaga arvensicola ATCC 51264T (AM237311)
Cons 0BQ01EK020 (Flavobacteria)
Bacteroidetes
Bacteroidetes
Cons 0BQ01CPC56 (Verrucomicrobiales)
Planctomycetes
Planctomycetes
0.05

Figure 4.4. Dendrogram constructed on basis of partial 16S rRNA gene sequences (205 bp) of the cultivable bacteria (isolates identified with ‘‘R’’ were isolated from R2A, ‘‘T’’ from TTC and ‘‘P’’ from PIA) and the OTU obtained by 454 pyrosequencing.
In order to validate the taxonomical identifications, the 16S rRNA gene sequences of some of closely related type strains were added to the dendrogram. The dendrogram was generated using the neighbour-joining method based on the model of Jukes and Cantor and the dark circles indicate branches recovered by the maximum parsimony method. Bootstrap values, generated from 1000 resamplings, at or above 50 % are indicated at the branch points. Grey shadowing indicates sequences retrieved from the different methods which share at least 97 % similarity. Bar 1 substitution per 20 nt positions.

4.5. Discussion

The rates of cultivability observed confirm that only a small fraction of the bacterial population was recovered on the culture media used. Nevertheless, we admit the overestimation of the uncultivable fraction, given the fact that the method used to quantify the total number of cells (DAPI) neglects the organism viability (Kubista *et al.*, 1987). Bacteria which rendered uncultivable could be injured organisms or members of taxa for which the growth conditions were not gathered. Supposedly, the culture-independent approaches would allow the detection of such taxa. The DGGE method allowed the detection of some taxa not cultivated, namely members of the phyla *Cyanobacteria*, *Planctomyces* and *Aquificae*. In some occasions it was observed the co-migration of DNA fragments with different nucleotide sequence compositions. This effect of co-migration was easily resolved through the analysis of different clones of a single band. Nevertheless, it is a major limitation of this method, mainly when the measurement of bacterial richness and/or diversity, relying on the number/intensity of bands, is the objective (Sekiguchi *et al.*, 2001).

The 454 pyrosequencing analysis allowed the coverage of 62 % of the predicted bacterial diversity, a value which was in the range of others observed for aquatic systems and can be considered representative of the phylotype richness (Kemp and Aller, 2004).

A prominent result of this analysis was the observation of high percentages of unclassified-bacteria. This fact may hint the huge bacterial diversity that presumably exists in a water sample and the potential of 454 pyrosequencing to detect rare organisms in microbial communities (Petrosino *et al.*, 2009). But, most probably, these unknown *Bacteria* result from some drawbacks of this method, namely the occurrence of artifactual sequences and the limitations imposed by the short read lengths (Ahmadian *et al.*, 2006; Warnecke and Hugenholtz, 2007; Krause *et al.*, 2008; Roh *et al.*, 2010). These same drawbacks may be responsible for the lower diversity index value observed for 454 pyrosequencing when compared with the DGGE analysis (Table 4.2). Additionally, another possible bias introduced by this high throughput sequencing method is the preferential amplification of some DNA fragments. This effect may explain the low value of evenness observed for 454 pyrosequencing.

The predominant bacterial phyla in this water sample were *Proteobacteria*, *Actinobacteria* and *Bacteroidetes*, irrespective of the approach used. The members of these phyla are common inhabitants of freshwater, reported using either culture-dependent (high-throughput cultivation method) (Gich *et al.*, 2005), or culture-independent methods [16S rRNA based clonal analyses (Hiorns *et al.*, 1997; Hugenholtz *et al.*, 1998; Zwart *et al.*, 2002) and metagenomic library and FISH analysis (Cottrell *et al.*, 2005). *Cyanobacteria*, *Planctomycetes* and *Verrucomicrobia*, despite of being referred to as common freshwater bacteria (Zwart *et al.*, 2002; Lindstrom *et al.*, 2005), were, as expected, detected only through the culture-independent methods. Members of these groups or of others such as *Acidobacteria*, *Aquificae* and *Deinococcus-Thermus* hardly could be expected with the cultivation conditions used in the current study. *Firmicutes* were minor organisms both in the culture-dependent method and 454 pyrosequencing, suggesting the low abundance of members of this phylum in the sample,

as pointed out before in other freshwater studies (Gich *et al.*, 2005). At a lower taxonomic level, also some of the genera (*Ralstonia*, *Flavobacterium*, *Chitinophaga*, *Micrococcus*, *Synechococcus*) and families (*Sphingobacteriaceae*, *Comamonadaceae*, *Legionellaceae*) detected in this water sample were previously observed in freshwater using 16S rRNA clone libraries (Hiorns *et al.*, 1997; Zwart *et al.*, 2002), reverse line blot hybridization (Lindstrom *et al.*, 2005) or metagenomic and FISH analysis (Cottrell *et al.*, 2005).

The use of different primer sets for the DGGE and 454 pyrosequencing methods (V3 and V4–V5, respectively) was an attempt to compare the methods as they are more frequently used. However, this option limited a straightforward comparison of both culture-independent methods. Nevertheless, the major objective of this study was to infer if culture-dependent and culture-independent methods currently used to survey freshwater microbiota coincided in the detection of cultivable bacteria. Supposedly, through the culture-dependent method only the most abundant organisms or the better adapted to the culture conditions were being screened. Bacterial strains examined in this study were in an abundance of about 10^2–10^3 CFU ml^{-1}, which means that they were effectively isolated from volumes of water of 0.1–0.01 ml. Volumes higher than these corresponded to filtering membranes with ‘‘too much to count’’ CFU, from which bacterial isolation and purification would not be feasible. Through the culture-independent methods, for which total DNA was extracted from a higher volume of water (5000–50,000 times higher), we had anticipated that we would analyse a different fraction of the bacterial population. For this reason and due to the expected higher sensitivity, one would anticipate that the culture-independent methods may target the less abundant organisms. This justifies that some OUT not retrieved by culture-dependent methods were detected using the culture-independent approaches. Nevertheless, the most abundant organisms, namely those retrieved from volumes of 0.1–0.01 ml of the water sample, were also expected to figure

among the taxonomical units detected by the culture-independent methods, but, in fact, this only rarely occurred. A possible explanation is that some of the most abundant organisms (namely some detected by the culture-dependent methods) were probably lessened in favour of others occurring at lower densities, which may gain advantage during crucial stages as the DNA extraction and PCR amplification. This explains why DGGE and pyrosequencing failed to detect all or the majority of nucleotide sequences similar to those of the bacterial isolates. The 454 pyrosequencing, in spite its high coverage, allowed the detection of only four cultivable OTUs, always with sequence similarities lower than 98 %. The inability of the different methods to target the same organisms was previously observed (Kisand and Wikner, 2003; Cottrell *et al.*, 2005; Jordan *et al.*, 2009). For instance, Kisand and Wikner (2003) observed that a culture-dependent method, a 16S rRNA gene clone library and DGGE approaches allowed poor matches at species level for an estuarine bacterioplankton sample. Cottrell *et al*. (2005) through a metagenomic library approach detected some groups of bacteria underrepresented by a PCR-16S rRNA gene clone library in a river water sample. Also Jordan *et al*. (2009) in a study comparing the accuracy of pyrosequencing with culture dependent methods for the identification of isolates from blood culture bottles described that for some isolates no sequence match could be found, or the sequencing reactions repeatedly failed.

Ideally, both approaches, culture-dependent and independent, should be used as complementary, mainly if the objective of the study is related with risk assessment or public health issues. The choice on the culture-independent method to use is also relevant. If time consumption and costs involved versus information given are equated, the DGGE method does not show a worthy cost effectiveness (Table 4.3). In spite of these limitations DGGE is still regarded as an adequate approach to compare microbial

communities and to infer the influence of environmental conditions (Fromin *et al.*, 2002). The 454 pyrosequencing, although more expensive, presented high bacterial richness coverage and offered an efficient way to access the microbial diversity, namely to target some of the cultivable organisms. As a high-throughput approach, 454 pyrosequencing offers a general perspective of the microbial diversity and represent a valuable tool to develop and optimize cultivation methods. In fact, the latter are fundamental when phenotypic information is important, e.g. pathogenicity, antimicrobial resistance, production of novel metabolites and enzymes (Palleroni, 1997; Alain and Querellou, 2009).

Table 4.3. Qualitative analysis of cost-benefits for the three methods in study

		Cultivable bacteria	DGGE	454 Pyrosequencing
Cost	Time consumed	medium	high	medium
	Equipment	low	medium	high
	Reagents	low	high	high
Benefits	Taxonomical accuracy (discriminative power)	high	low	low
	Ease of performance and interpretation	high	medium	low
	Bacterial diversity coverage	low	medium	high

5. Diversity and antibiotic resistance patterns of *Sphingomonadaceae* isolates from drinking water

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5.1. Abstract

Sphingomonadaceae (n = 86) were isolated from a drinking water treatment plant (n = 6), tap water (n = 55), cup fillers for dental chairs (n = 21), and a water demineralization filter (n = 4). The bacterial isolates were identified based on analysis of the 16S rRNA gene sequence, and intraspecies variation was assessed on the basis of atpD gene sequence analysis. The isolates were identified as members of the genera *Sphingomonas* (n = 27), *Sphingobium* (n = 28), *Novosphingobium* (n = 12), *Sphingopyxis* (n = 7), and *Blastomonas* (n = 12). The patterns of susceptibility to five classes of antibiotics were analyzed and compared for the different sites of isolation and taxonomic groups. Colistin resistance was observed to be intrinsic (92 %). The highest antibiotic resistance prevalence values were observed in members of the genera *Sphingomonas* and *Sphingobium* and for beta-lactams, ciprofloxacin, and cotrimoxazol. In tap water and in water from dental chairs, antibiotic resistance was more prevalent than in the other samples, mainly due to the predominance of isolates of the genera *Sphingomonas* and *Sphingobium*. These two genera presented distinct patterns of association with antibiotic resistance, suggesting different paths of resistance development. Antibiotic resistance patterns were often related to the species rather than to the site or strain, suggesting the importance of vertical resistance transmission in these bacteria. This is the first study demonstrating that members of the family *Sphingomonadaceae* are potential reservoirs of antibiotic resistance in drinking water.

5.2. Introduction

At this writing, the family *Sphingomonadaceae*, within the class *Alphaproteobacteria*, comprises 11 genera: *Blastomonas*, *Erythromonas*, *Novosphingobium*, *Sandaracinobacter*, *Sandarakinorhabdus*, *Sphingobium*, *Sphingomonas*, *Sphingopyxis*, *Sphingosinicella*, *Stakelama*, and *Zymomonas* (http://www.bacterio.cict.fr;last full update, 24 November 2010) (Euzéby, 1997). Members of this family are strictly aerobic chemoheterotrophs with a characteristic yellow pigmentation (Yabuuchi and Kosako, 2005). In spite of their frequently observed oligotrophic character, members of this family are widespread in nature, occurring in soils, corals, eutrophic waters, plant surfaces, and clinical samples (White *et al.*, 1996; Cavicchioli *et al.*, 1999; Yabuuchi and Kosako, 2005; Balkwill *et al.*, 2006). The capacity of sphingomonads (a common designation that includes the genera *Novosphingobium*, *Sphingobium*, *Sphingomonas*, and *Sphingopyxis*) to cope with man-made environments is also relevant. For instance, the ability to degrade xenobiotic compounds is one of the most remarkable properties of these bacteria (Balkwill *et al.*, 2006; Stolz, 2009). The capacity to survive in chlorinated waters, allegedly due to the oligotrophic character of these bacteria and their production of biofilms (Koskinen *et al.*, 2000; Furuhata *et al.*, 2007; Hong *et al.*, 2010; Yim *et al.*, 2010), is another demonstration of their plasticity in man-made environments. Sphingomonads are, thus, truly ubiquitous bacteria frequently found in aquatic environments, such as drinking water (bulk water and biofilms formed on pipes, reservoirs, and bathtubs), distilled water, hemodialysis fluids, or supposedly sterile drug solutions (Koskinen *et al.*, 2000; Singh *et al.*, 2003; Gomila *et al.*, 2005; Furuhata *et al.*, 2007; Kilic *et al.*, 2007; Szymanska, 2007; Ryan and Adley, 2010). The fact that sphingomonads are recognized opportunistic pathogens (Charity *et al.*, 2005; Källman *et al.*, 2006; Kilic *et al.*, 2007; Lin *et al.*, 2010) makes such ubiquity potentially

hazardous, mainly in habitats such as tap water. This hazardous potential is exacerbated by the fact that sphingomonads are among the most relevant unappreciated reservoirs of the natural resistome (Dantas *et al.*, 2008). In spite of this, the diversity of *Sphingomonadaceae* in oligotrophic waters and the respective antibiotic resistance patterns are poorly characterized (Koskinen *et al.*, 2000; Furuhata *et al.*, 2007). This study aimed at helping to fill this gap, characterizing the diversity and antibiotic resistance patterns of *Sphingomonadaceae* isolated from drinking water. Bacteria isolated from a water treatment plant (WTP), taps, cup fillers for dental chairs, and a water demineralization filter of the same drinking water network were analyzed. The study was intended to (i) assess the diversity of genera and species in the different sites, (ii) determine whether some genera or species were particularly relevant as antibiotic resistance reservoirs in waters, and (iii) compare the patterns of antibiotic resistance in different genera and sites of isolation, inferring possible modes of resistance dissemination.

5.3. Materials and Methods

5.3.1. Sampling

The bacterial isolates examined in this study were collected from (i) a water treatment plant (both raw and treated water samples), (ii) tap water, (iii) cup fillers for dental chairs, and (iv) biofilm from a water demineralization filter installed in a research laboratory. WTP samples, both raw (groundwater and surface water) and treated (after sand filtration, ozonation, flocculation, activated-carbon treatment, and chlorination), were collected in two sampling periods, in November 2007 and September 2009 (sampling periods A and B, respectively) (see the legend to Figure 5.2). These samples were collected at the sampling points used in routine monitoring analysis in the WTP. Tap water samples were

collected in three sampling periods (April, July, and October 2009 [sampling periods A, B, and C, respectively]) (see the legend to Figure 5.2), from 11 household taps used 1 to 4 times a month and from a tap of a health care unit (T1 to T12 in Figure 2). Forty-five-liter volumes of water were collected from the WTP or taps, using sterile 5-liter containers (these samples were also processed for other analyses; hence the large volume of water sampled). In the laboratory, a composite sample was prepared by mixing equal volumes of water from each container. Samples from the cup fillers of nine dental chairs were collected between February and April 2008 (sampling periods A, B, and C, respectively) (see the legend to Figure 5.2) in a university dental school clinic where the chairs are in use for more than 10 years (Silva *et al.*, 2011). Water was allowed to run for about 1 min before its collection into a 100-ml sterile flask. The biofilm was collected in October 2008 (sampling period A in Fig. 2) from a pleated filter (responsible for the removal of suspended solids) of a laboratory water demineralization system fed with tap water during approximately 4 months of frequent use at a maximal flow rate of 90 liters h^{-1}. The biofilm was collected with a sterile swab and was suspended in 20 ml of sterile saline solution (0.85% [wt/vol] NaCl), and cells were homogenized by vigorous shaking and 15 min of sonication in a water bath. All water and biofilm samples were processed within 4 h after collection. In order to neutralize the activity of disinfectants, 0.1 mg $liter^{-1}$ of sodium thiosulfate was added to the samples of treated water collected in the WTP and from the taps. All the taps, all the dental chairs, and the lab demineralization system were supplied by the same WTP examined in this study.

5.3.2. Bacterial isolation and characterization

Three culture media widely used for microbiological quality control of water were employed: R2A (Difco), *Pseudomonas* isolation agar (PIA; Difco), and Tergitol-7 agar

(TTC; Oxoid). R2A agar is a nonselective medium recommended for the examination of total heterotrophic bacteria; PIA and TTC are recommended for the enumeration of *Pseudomonas* bacteria and presumptive coliforms (ISO9308-1, 2000; Eaton *et al.*, 2005), respectively. One hundred-milliliter volumes of water or of decimal serial dilutions thereof were filtered through cellulose nitrate membranes (pore size, 0.45 µm; diameter, 47 mm; Albet), which were placed on the three different culture media and were incubated at 30°C (for R2A and PIA) or 37°C (for TTC) for as long as 7 days. Decimal serial dilutions and filtrations were carried out in triplicate, and after the incubation period, the number and morphology of CFU on filtering membranes with as many as 80 colonies were registered. About 50 % of the colonies with a morphotype represented by >10 CFU, and all the colonies with a morphotype represented by ≤10 CFU, were isolated. The colonies isolated on R2A were purified on the same medium, and those isolated on culture media with higher nutrient contents (PIA or TTC) were purified on plate count agar (PCA; Pronadisa). Pure cultures were preserved at -80°C in nutritive broth supplemented with 15 % (vol/vol) glycerol. Colony and cellular morphologies, Gram stain reactions, and cytochrome *c* oxidase test results were characterized as described by Smibert and Krieg (1994). After this preliminary characterization, 30 to 40 % of the cultures comprising Gram-negative rods forming yellow colonies on R2A or PCA, isolated from each type of habitat (WTP, taps, cup fillers, or biofilm) in different sampling periods and on different culture media, were selected for further studies.

5.3.3. Bacterial identification and typing

Bacterial isolates were identified to the species level on the basis of analysis of the 16S rRNA gene sequence. The 16S rRNA gene was amplified and sequenced with primers 27F and 1492R (Lane, 1991) according to the method of Vaz-Moreira *et al*.

(2009). The nucleotide sequences were used to query the EzTaxon library (Chun *et al.*, 2007).

In an attempt to assess intraspecies variability, the nucleotide sequence of the housekeeping gene *atpD* (the beta subunit of membrane ATP synthase) was analyzed (Gaunt *et al.*, 2001). *atpD* and 16S rRNA gene sequences were aligned using ClustalW from MEGA software, version 4.0 (Tamura *et al.*, 2007). Nucleotide sequence relatedness was estimated based on the model of Jukes and Cantor (1969), and dendrograms were created using the neighbor-joining method. Other methods, namely, maximum parsimony and maximum likelihood, were used to assess the tree stability, and the branches recovered by these three methods are indicated in Figure 5.3 (black circles). The type species of the closest neighbors were added to the dendrogram in order to allow the identification of the isolates under study. Nonhomologous and ambiguous nucleotide positions were excluded from the calculations, and bootstrap values, generated from 1,000 resamplings, at or above 50 % are indicated at the branch points. A total of 1,229 and 350 nucleotide positions were included in the analysis of the 16S rRNA and *atpD* sequences, respectively. 16S rRNA and *atpD* gene sequences were compared for each pair of isolates belonging to the same species. Strains differing in at least one nucleotide position in any of those gene sequences were classified as belonging to a distinct sequence type (ST).

5.3.4. Antibiotic resistance phenotype

The 86 isolates identified as members of the family *Sphingomonadaceae*—6 from the WTP (3 from raw and 3 from treated water), 4 from the biofilm, 55 from taps, and 21 from the cup fillers - were studied further for their antibiotic resistance phenotypes, determined by using the ATB PSE5 panel (bioMérieux) according to the manufacturer's

instructions. Five classes of antibiotics were tested: beta-lactams, aminoglycosides, a fluoroquinolone, a polymyxin, and a sulfonamide. The beta-lactams tested were ampicillin-sulbactam (FAM) (tested at 8 and 4 mg liter^{-1} and 16 and 8 mg liter^{-1}, respectively), ticarcillin (TIC) (16 mg liter^{-1}), ticarcillin-pyocyanin (TICP) (64mg liter^{-1}), ticarcillin-clavulanic acid (TCC) (16 and 2 mg liter^{-1}, respectively), ticarcillin-clavulanic acid-pyocyanin (TCCP) (64 and 2 mg liter^{-1}, respectively), piperacillin (PIC) (16 mg liter^{-1}), piperacillin-pyocyanin (PICP) (64 mg liter^{-1}), piperacillin plus tazobactam (TZP) (16 and 4 mg liter^{-1}, respectively), piperacillin plus tazobactam-pyocyanin (TZPP) (64 and 4 mg liter^{-1}, respectively), imipenem (IMI) (4 and 8 mg liter^{-1}), meropenem (MEM) (4 and 8 mg liter^{-1}), ceftazidime (CAZ) (8 and 16 mg liter^{-1}), and cefepime (FEP) (8 and 16 mg liter^{-1}). The aminoglycosides tested were amikacin (AKN) (16 and 32 mg liter^{-1}), gentamicin (GEN) (4 and 8 mg liter^{-1}), and tobramycin (TOB) (4 and 8 mg liter^{-1}). Also tested were the fluoroquinolone ciprofloxacin (CIP) (1 and 2 mg liter^{-1}), the polymyxin colistin (COL) (2 mg liter^{-1}), and the sulfonamide cotrimoxazol (TSU) (2 and 38 mg liter^{-1}). Phenotypes were defined as resistant, intermediary, or sensitive according to the manufacturer's instructions.

5.3.5. Statistical analyses

The chi-square test was used to compare the antibiotic resistance prevalences among groups of isolates from the different types of water, or to compare resistant and susceptible bacteria, at a significance level (P) of <0.05. A matrix with nominal variables was constructed based on the resistant, intermediary, and sensitive phenotypes, defined according to the instructions of the manufacturer of the ATB PSE5 panel. In order to establish groups of isolates with similar profiles of resistance to the different antibiotics tested, hierarchical ascendant cluster analysis was carried out using Euclidean distance

and the Ward method for the aggregation criterion. Other methods, namely, between-groups linkage, nearest group, and farthest group, were applied to assess the stability of the dendrogram. To define the groups of resistance profiles that were significantly different, the *R*2 criterion was used, at a significance level (*P*) of <0.05 (see Figure 5.3). The SPSS software package, version 18.0 (SPSS Inc., Chicago, IL), was used for these analyses.

5.3.6. Nucleotide sequence accession numbers

The *atpD* and 16S rRNA gene sequences have been submitted to under accession numbers JF459878 to JF459930 and JF459931 to JF459988, respectively.

5.4. Results

5.4.1. Abundance and diversity of *Sphingomonadaceae*

The cultivable *Sphingomonadaceae* were present at a density of 10^0 CFU ml^{-1} in the raw WTP water and at 10^1 CFU ml^{-1} after treatment. In the taps and cup fillers, the densities of *Sphingomonadaceae* were slightly higher, ranging from 10^1 to 10^3 CFU ml^{-1}. The higher counts of *Sphingomonadaceae* observed in the treated water from the WTP may be due to decreases in the levels of other bacteria, which were eliminated more extensively by the disinfection process. The *Sphingomonadaceae* isolates analyzed in this study were identified as members of the genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, *Sphingopyxis*, and *Blastomonas* (Figure 5.1). Most of the isolates (*n* = 82) presented 16S rRNA gene sequence similarity values higher than 97.4 % with the type strain of a validly named species and were thus considered members of that species. Four strains, three isolated from the health care unit tap water and one from the WTP (raw groundwater), could not be affiliated with any validly named species. In these cases,

16S rRNA gene sequence similarity values lower than 97.0 %, the threshold value recommended for species definition (Stackebrandt and Goebel, 1994), were observed. The closest neighbors were the type strains of the species *Sphingomonas sanxanigenens* (96.3 and 96.9 % similarities), *Sphingomonas changbaiensis* (96.8 %), and *Blastomonas natatoria* (96.3 %).

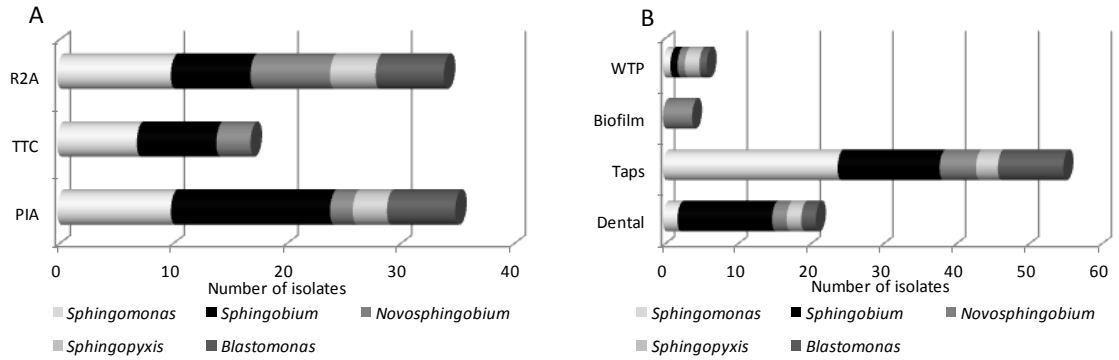

Figure 5.1. Summary of the numbers of isolates examined in this study according to isolation conditions (A) and site (B).

Apparently, the culture media and temperature did not influence the diversity of genera recovered (Figure 5.1A). The only exception was that *Sphingopyxis* spp. and *Blastomonas* spp. were isolated only on R2A and PIA, which were incubated at 30°C, but not on TTC, which was incubated at 37°C.

The distributions of genera were not identical for the different sites of isolation (Figure 5.1B). In this respect it is noteworthy that all five genera identified in this study were represented in the group of six isolates from the WTP. Three isolates, identified as *Sphingomonas melonis*, *Novosphingobium subterraneum*, and *Sphingopyxis ginsengisoli*, were recovered from WTP treated water in 2007. Bacteria isolated in 2009 from raw groundwater and surface water from the WTP were identified as *Sphingopyxis*

taejonensis, *Blastomonas natatoria*, and *Sphingobium yanoikuyae*, respectively. Tap and cup filler water samples also contained isolates belonging to the different genera, although *Sphingomonas* spp. and *Sphingobium* spp., respectively, were prevalent in these samples (Figure 5.1B). The biofilm of the pleated filter was observed to contain only members of the species *Novosphingobium subterraneum.* Considering the whole set of isolates, it was observed that members of the genera *Sphingomonas* and *Sphingobium* were prevalent, constituting 64 % of the total (31.4 and 32.6 %, respectively).

The diversity of *Sphingomonadaceae* species observed in the water samples examined in this study represented only a small part of the validly named species within the different genera (http://www.bacterio.cict.fr) (Euzéby, 1997): only 10 of the 44 in the genus *Sphingomonas* (*Sphingomonas panni*, *Sphingomonas yunnanensis*, *Sphingomonas dokdonensis, Sphingomonas mucosissima*, *S. sanxanigenens*, *Sphingomonas wittichii*, *S. changbaiensis, Sphingomonas koreensis*, *S. melonis*, and *Sphingomonas pituitosa*), 4 of the 24 in the genus *Sphingobium* (*Sphingobium amiense, Sb. yanoikuyae*, *Sphingobium xenophagum*, and *Sphingobium rhizovicinum*), 4 of the 15 in the genus *Sphingopyxis* (*Sp. taejonensis*, *Sphingopyxis witflariensis, Sp. ginsengisoli*, and *Sphingopyxis chilensis*), 3 of the 18 in the genus *Novosphingobium* (*N. subterraneum*, *Novosphingobium aromaticivorans*, and *Novosphingobium panipatense*), and 1 of the 2 in the genus *Blastomonas* (*B. natatoria*) were identified in this study (Figure 5.2).

In an attempt to assess intraspecies variability, the nucleotide sequence of the *atpD* gene was analyzed, as recommended by Gaunt *et al*. (2001). This procedure allowed the differentiation of the 86 isolates into 56 sequence types (Figure 5.2). Of the total, 42 isolates could be distinguished at the strain level. The other 44 isolates shared the same sequence type with at least one other isolate and were divided into 14 STs (with 2 to 6 isolates each) (boldface in Figure 5.2). These 44 isolates were recovered from the biofilm,

cup fillers, and taps and belonged to the genera *Novosphingobium* (all three species), *Sphingobium* (*Sb. amiense*, *Sb. yanoikuyae*, *Sb. xenophagum*), *Sphingomonas* (*S. yunnanensis*, *S. mucosissima*, *S. panni*), *Sphingopyxis* (*Sp. witflariensis*), and *Blastomonas* (*B. natatoria*). Five of the 14 STs comprising more than one isolate (ST8, ST9, ST24, ST25, and ST26) included 12 bacteria from the same site, sampling time, and culture medium, suggesting that they were repetitions (i.e., the same isolate recovered more than once). In other cases, the isolates (n = 5) yielding the same ST were recovered on different culture media, though from the same site and sampling date (ST1 isolates on R2A and TTC; ST46 isolates on TTC and PIA), suggesting that they were possibly clones. The other seven STs common to more than one isolate grouped 27 bacteria isolated from different sites, on different sampling dates, and/or on different culture media (ST7, ST37, and ST53), or isolated from the same site but on different sampling dates and/or on different culture media (ST21, ST23, and ST33). ST43 clustered bacteria of different origins, isolated on different dates and on different culture media. The presence of the same ST at different sites on different dates may suggest a common origin of those bacteria but most probably results from the poor resolution of the method used, since only two gene sequences were examined. Nevertheless, in general, it is possible to conclude that the alleles examined in this study allowed good differentiation of the isolates and showed that the same strain is rarely, if ever, observed in different taps, in different cup fillers, or in samples from the WTP. The impressive diversity of species was also observed at the strain level, evidencing the widespread distribution of members of the family *Sphingomonadaceae* in waters.

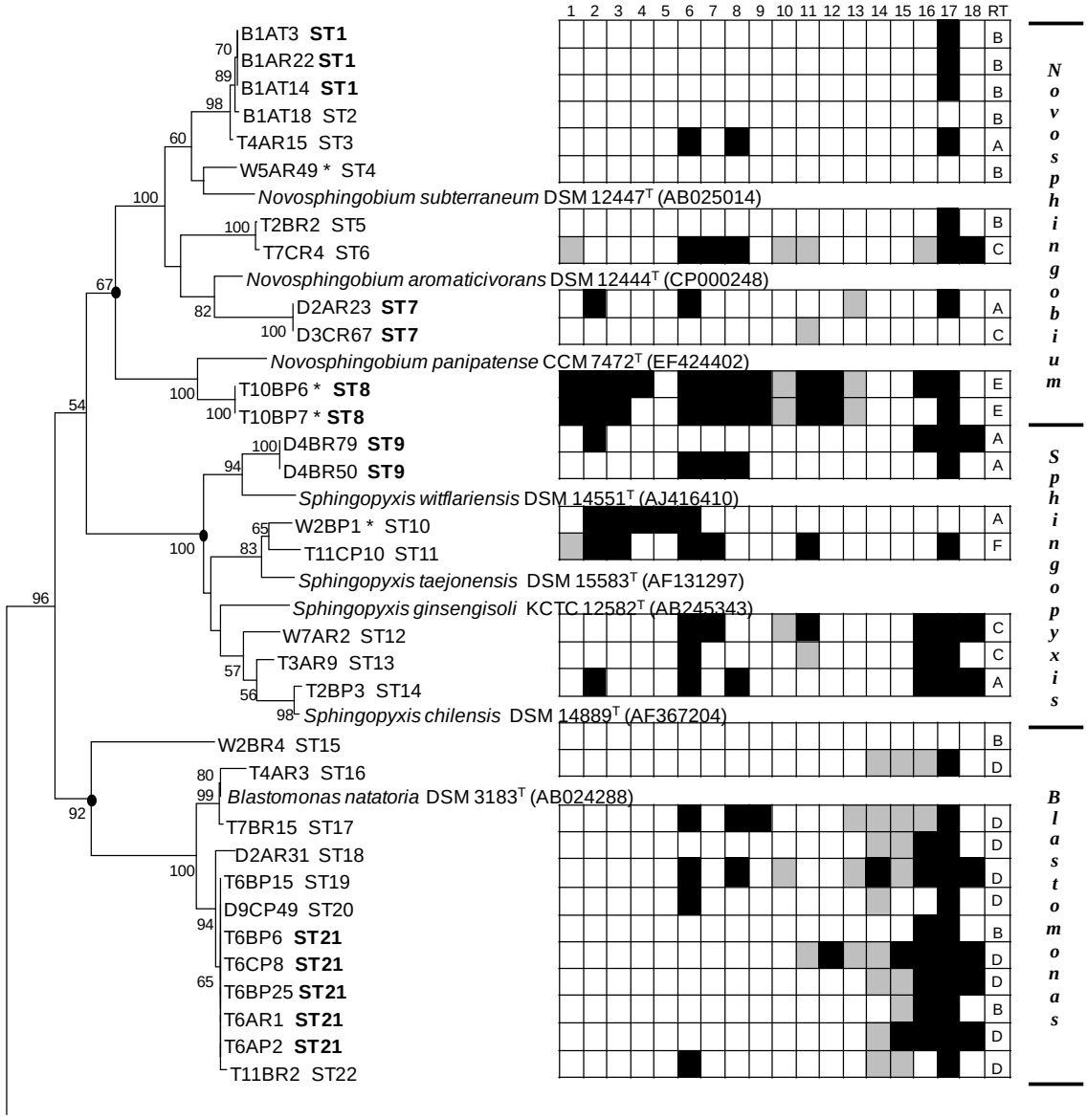

Figure 5.2. (Left) Dendrogram constructed on the basis of 16S rRNA gene sequences (1,229 bp), and (Right) Antibiotic resistance profiles

Notes: (Left) The format of the isolate designations is SxCMn, where "S" stands for the site (W, WTP; T, tap water; D, cup fillers for dental chairs; B, biofilm), "x" for the number of the site, "C" for the sampling period (A, B, or C), "M" for the culture medium (R, R2A; P, *Pseudomonas* isolation agar; T, Tergitol-7 agar), and "n" for the number of the isolate. For the WTP, W1 is the raw surface water, W2 is the raw groundwater, and W3 to W5 are treated-water samples. ST, sequence type. Bar, 1 substitution per 200 nucleotide positions. Strains in which it was not possible to amplify the *atpD* gene are asterisked. (Right) Antibiotic resistance profiles. Black squares indicate resistance; gray squares, an intermediary phenotype; white squares, susceptibility. RT, resistance type. Antibiotics are represented by numbers above the chart as follows: 1, ampicillin-sulbactam; 2, ticarcillin; 3, ticarcillin-pyocyanin; 4, ticarcillin-clavulanic acid; 5, ticarcillin-clavulanic acid-pyocyanin; 6, piperacillin; 7, piperacillin-pyocyanin; 8, piperacillin-tazobactam; 9, piperacillin plus tazobactam-pyocyanin; 10, imipenem; 11, meropenem; 12, ceftazidime; 13, cefepime; 14, gentamicin; 15, tobramycin; 16, ciprofloxacin; 17, colistin; 18, cotrimoxazol.

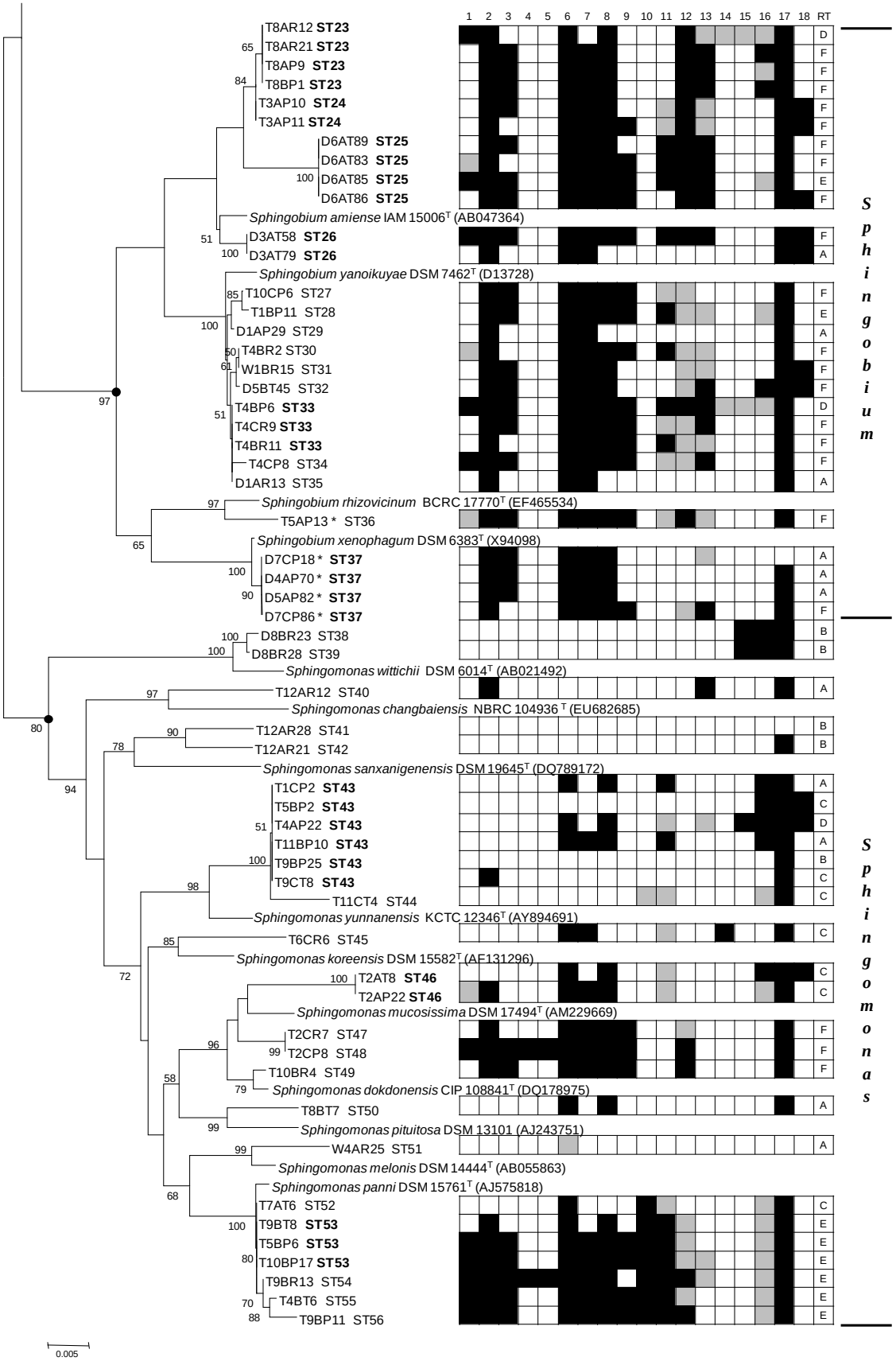

Figure 5.2. *Continued*.

5.4.2. Antibiotic resistance phenotypes

Antibiotic resistance patterns were analyzed as a function of the site and conditions of isolation or bacterial genus (Table 5.1). The susceptibility phenotype was determined with respect to 19 antibiotics belonging to five different classes. None of the isolates presented resistance to the aminoglycoside AKN. In contrast, colistin resistance was observed in 91.9 % of the isolates, suggesting that it is an intrinsic phenotype in *Sphingomonadaceae.* On average, simultaneous resistance to antibiotics belonging to three different classes (excluding colistin) was observed in 10.5 % of the isolates. Most of the multiresistance phenotypes included resistance to at least one of the beta-lactams tested. Actually, resistance to beta-lactams, mainly TIC, PIC, PICP, and TZP, was the most prevalent resistance phenotype, with resistance rates above 50 %. For the other classes tested, fluoroquinolone resistance and sulfonamide resistance were the second most prevalent, with rates of 25.6 and 20.9 %, respectively (Table 5.1).

According to our results, the isolation conditions (culture medium/temperature) can have some influence on the resistance phenotypes observed. For instance, isolates recovered on PIA or TTC presented significantly ($P < 0.05$) higher percentages of resistance to the beta-lactams TIC, TICP, PIC, PICP, TZP, and TZPP and the sulfonamide TSU than isolates recovered on the nonselective medium R2A. Among the TTC isolates, a significantly higher percentage of resistance was observed for the aminoglycoside TOB, and resistance to the beta-lactams TCC and TCCP or to the aminoglycoside GEN was not observed (Table 5.1).

Given the low number of isolates from WTP and biofilm samples, it was impossible to compare the resistance prevalence values accurately across all the sites sampled. However, taps and cup fillers could be compared. These sites showed similar percentages of resistance, except for the beta-lactams MEM, TCC, TCCP, and IMI and the

aminoglycoside GEN. For MEM, significantly lower resistance rates were observed in the cup fillers than in the taps, whereas the other resistance phenotypes were not detected in the dental chairs (Table 5.1).

Table 5.1. Percentages and types of antibiotic resistance per culture medium, sampling site, and genus

Culture-medium, sampling site,[a] or genus (no. of isolates)	% of isolates resistant to: Beta-lactams											
	FAM	TIC	TICP	TCC	TCCP	PIC	PICP	TZP	TZPP	IMI	MEM	CAZ
R2A (n=34)	5.9	38.2	14.7	2.9	2.9	55.9	38.2	38.2	17.7	2.9	11.8	11.8
TTC (n=17)	17.7	58.8	35.3	0	0	70.6	47.1	58.8	29.4	17.7	35.3	29.4
PIA (n=35)	20.0	68.6	51.4	8.6	5.7	82.9	65.7	71.4	37.1	8.6	28.6	31.4
WTP (n=6)	0	33.3	33.3	16.7	16.7	50.0	33.3	16.7	0	0	16.7	0
Biofilm (n=4)	0	0	0	0	0	0	0	0	0	0	0	0
Taps (n=55)	18.2	54.6	34.6	5.5	3.6	74.6	50.9	65.5	34.6	12.7	27.3	27.3
Dental cup filler (n=21)	9.5	71.4	38.1	0	0	76.2	66.7	52.4	23.8	0	19.1	23.8
Sphingomonas (n=27)	18.5	44.4	25.9	7.4	7.4	63.0	40.7	55.6	25.9	25.9	29.6	14.8
Sphingobium (n=28)	17.9	100	60.7	0	0	100	96.4	89.3	50.0	0	28.6	46.4
Novosphingobium (n=12)	16.7	25.0	16.7	8.3	0	41.7	25.0	33.3	16.7	0	16.7	16.7
Sphingopyxis (n=7)	0	57.1	28.6	14.3	14.3	85.7	42.9	28.6	0	0	28.6	0
Blastomonas (n=12)	0	0	0	0	0	33.3	0	16.7	8.3	0	0	8.3
Total *Sphingomonadaceae*	14.0	54.7	33.7	4.7	3.5	69.8	51.2	55.8	27.9	8.1	23.3	23.3

[a]WTP, water treatment plant; R2A, R2A medium; TTC, tergitol-7-agar; PIA, *Pseudomonas* isolation agar.
[b]Amikacin resistance was not observed. FAM, ampicillin-sulbactam; TIC, ticarcillin; TICP, ticarcillin-pyocyanin; TCC, ticarcillin-clavulanic acid; TCCP, ticacillin-clavulanic acid-pyocyanin; PIC, piperacillin; PICP, piperacillin-pyocyanin; TZP, piperacillin+tazobactam; TZPP, piperacillin+tazobactam-pyocyanin; IMI, imipenem; MEM, meropenem; CAZ, ceftazidime; FEP, cefepim; GEN, gentamicin; TOB, tobramycin; CIP, ciprofloxacin; COL, colistin; TSU, cotrimoxazol.

Table 5.1. *Continued*

% of isolates resistant to:						Resistance Types (no. of isolates)
Aminoglycoside[b]		Fluoroquinolone	Polymyxin	Sulphonamide	> 3 different classes	
GEN	TOB	(CIP)	(COL)	(TSU)	(multiresistance)	
2.9	5.9	23.5	88.2	11.8	5.9	7A; 9B; 5C; 5D; 1E; 7F
0	22.7	11.8	94.2	29.4	11.8	2A;3B; 4C; 3E; 5F
2.9	8.6	34.3	94.3	25.7	14.3	8A; 2B; 2C;7D;6E; 10F
0	0	16.7	33.3	33.3	16.7	2A;2B; 1C; 1F
0	0	0	75.0	0	0	4B
3.6	3.6	29.1	98.2	20.0	10.9	6A;6B;9C;10D;9E; 15F
0	9.5	23.8	90.5	23.8	9.5	9A; 2B; 1C; 2D; 1E; 6F
3.7	11.1	25.9	92.6	11.1	7.4	5A; 5B; 7C; 1D; 6E; 3F
0	0	10.7	96.4	25.0	3.6	6A; 2D; 2E; 18F
0	0	8.3	75.0	8.3	0	2A; 6B; 2C; 2E
0	0	57.1	85.7	42.9	42.9	4A; 2C; 1F
8.3	16.7	58.3	91.7	33.3	25.0	3B; 9D
2.3	11.0	25.6	91.9	20.9	10.5	17A; 14B; 11C; 12D; 10E; 22F

Comparison of the patterns of antibiotic resistance in the different genera of *Sphingomonadaceae* highlighted some distinctive features. For instance, members of the genus *Sphingomonas* presented the widest range of resistance phenotypes, comprising resistance to 18 of the 19 antibiotics tested. Members of the genus *Sphingobium*, which presented resistance to 13 of the 19 antibiotics tested, yielded significantly higher percentages of resistance to eight beta-lactams (>46 %) than members of the other genera. Members of the genus *Sphingobium* differed from members of the genus *Sphingomonas* by showing no resistance to the beta-lactams TCC, TCCP, and IMI and the aminoglycosides GEN and TOB, and a lower rate of resistance to the fluoroquinolone CIP (Table 5.1).

Differences in antibiotic resistance patterns can result from the ecology and physiology of the bacteria and may suggest distinct modes and mechanisms of resistance acquisition. The antibiotic resistance patterns of isolates belonging to the genera *Sphingomonas* and *Sphingobium* (which had more than 20 isolates and thus supported such an analysis) were compared on the basis of cluster analysis (Figure 5.3). This comparison showed different patterns of antibiotic resistance and of resistance associations in these two genera. In the genus *Sphingomonas*, it was possible to distinguish four significant clusters (S1 to S4). Cluster S3 included the antibiotics for which resistance rates were highest, with prevalences of resistance to PIC, TZP, and COL above 55 %. In contrast, the antibiotics for which resistance rates were lowest (4 to 11 %), comprising beta-lactams (TCC, TCCP, FEP), a sulfonamide (TSU), and aminoglycosides (GEN and TOB), were clustered in S1. Cluster S4 included the antibiotics for which resistance rates ranged from 15 to 44 %: all beta-lactams, including the carbapenem imipenem. Resistance to this carbapenem was observed only in the genus *Sphingomonas* and was associated with CAZ resistance. The other carbapenem tested, MEM, was included in another cluster and was not associated with a phenotype of resistance to any other beta-lactam. In the genus *Sphingobium*, different clusters and patterns of association were observed. In this case, the high-resistance cluster (cluster Sp3, with resistance rates above 46 %) included the beta-lactams TIC, PICP, FEP, and CAZ, in addition to those grouped in the equivalent cluster (S3) of *Sphingomonas* spp. Similarly, the low-resistance cluster (Sp1), with resistance rates ranging 0 to 25 %, included TICP, CIP, and IMI, in addition to the antibiotics observed in the corresponding cluster (S1) of the genus *Sphingomonas*. In contrast to the pattern for the genus *Sphingomonas*, in the genus *Sphingobium* resistance to the carbapenem MEM was

associated with resistance to other beta-lactams. This observation may suggest distinct mechanisms or paths of acquisition of resistance to this carbapenem in the two genera.

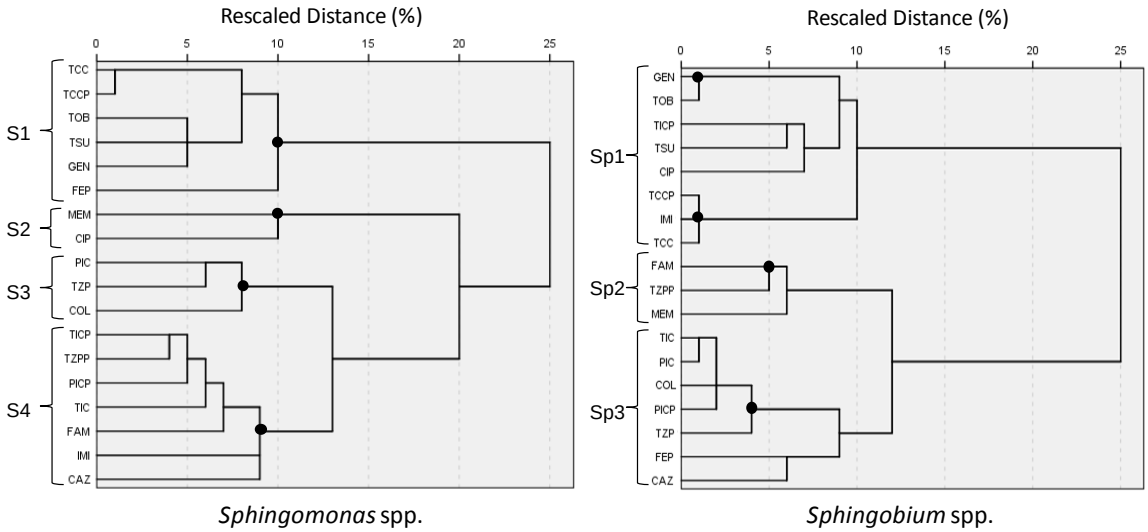

Figure 5.3. Cluster analysis based on the antibiotic resistance profiles observed for *Sphingomonas* and *Sphingobium* spp. using Euclidean distance and the Ward method for the aggregation criterion.

Note: Groups of resistance profiles that differed significantly from each other were determined by the R^2 criterion, at a significance level (P) of <0.05. Black circles indicate branches recovered by other methods.

The distinctive patterns of resistance observed for the genera *Sphingomonas* and *Sphingobium* supported the hypothesis that antibiotic resistance profiles may also differ for different species. Alternatively, it could be hypothesized that the provenience of the isolates was the most important explanatory factor for the different patterns of resistance observed. Two complementary approaches were used to test these hypotheses: (i) cluster analysis of the isolates based on their respective antibiotic resistance patterns and (ii) analysis of the antibiotic resistance patterns as a function of taxonomic classification (on the basis of 16S rRNA and *atpD* gene sequence analyses) and site of isolation (Figure 5.2).

Cluster analysis of the antibiotic resistance patterns led to the definition of six significant clusters, designated resistance type A (RT-A) to RT-F (Figure 5.2 and Table 5.1). The RT-A cluster (*n* = 17) comprised isolates with a core phenotype of resistance to TIC and/or PIC, besides COL. The RT-B cluster (*n* = 14) included mostly isolates susceptible to all antibiotics or resistant only to COL. The RT-C cluster (*n* = 11) consisted of carbapenem-resistant isolates. The RT-D cluster (*n* = 12) comprised isolates with a core phenotype of resistance to CIP and PIC. The RT-E (*n* = 10) and RT-F (*n* = 22) clusters included the isolates with the heaviest multiresistance phenotype, mainly to beta-lactams. RT-E differed from RT-F by including resistance to IMI and CIP.

This analysis showed that most of the time, resistance patterns were similar within the same species, though they often differed among species of the same genus. Within the genus *Sphingomonas*, the majority of species represented by a single isolate (*S. melonis*, *S. pituitosa*, *S. changbaiensis*) were included in RT-A, while those with two isolates were included in RT-B (*S. wittichii*, *S. sanxanigenens*) or RT-C (*S. mucosissima*). All the *S. dokdonensis* isolates belonged to RT-F, while 6 of the 10 *S. panni* isolates clustered in RT-E. In contrast, the *S. yunnanensis* isolates (*n* = 7) were distributed among the RT-A to RT-D clusters. Within the genera *Novosphingobium* and *Sphingopyxis* also, it was possible to observe different resistance patterns for different species. While the majority of the *N. subterraneum* isolates (5 of 6), recovered mainly from the biofilm, belonged to RT-B, both *N. panipatense* isolates, recovered from tap water, were included in RT-E. The four species of the genus *Sphingopyxis* were distributed in three RTs: RT-A (*Sp. witflariensis*, *Sp. taejonensis*, *Sp. chilensis*), RT-C (*Sp. chilensis*, *Sp. ginsengisoli*), and RT-F (*Sp. taejonensis*). In contrast to the other genera, in the genus *Sphingobium* the antibiotic resistance patterns were similar for different species. The majority of isolates in the species *Sb. amiense* (9/12), *Sb. yanoikuyae* (7/11), and *Sb. rhizovicinum* (1/1)

belonged to RT-F. *Sb. xenophagum* (*n* = 4) constituted the only exception, in that three of its members were included in RT-A.

Analysis of the antibiotic resistance patterns as a function of the taxonomic classification (on the basis of 16S rRNA and *atpD* gene sequence analyses) and site of isolation found, in general, similar patterns of antibiotic resistance for members of the same species. Nevertheless, different species of the same genus often exhibited distinct resistance types, and sometimes (in 25 % of the cases) isolates with the same sequence type showed distinct antibiotic resistance types. In some species it was possible to identify a core of resistance phenotypes, present in every isolate, or in most of the isolates, irrespective of the site, conditions of isolation, or sequence type. This was particularly evident in the genus *Sphingomonas*. In this genus, the species *S. dokdonensis* and *S. panni* were characterized by the multiresistance phenotypes RT-F and RT-E, respectively. The same resistance phenotype was common to most, though not all, the species members irrespective of the site of isolation or sequence type. Imipenem resistance was observed only in the species *Sphingomonas panni*, in isolates with different sequence types and sites of isolation. The resistance type observed for *S. dokdonensis* (RT-F) was found in other taxa, predominantly in the genus *Sphingobium* (Figure 5.2). In this genus, *Sb. xenophagum*, with RT-A, differed from the other species by virtue of its general susceptibility to antibiotics. In contrast, *N. panipatense*, characterized by multiresistance phenotypes (RT-E), differed from other *Novosphingobium* species, which were susceptible to most antibiotics (RT-B) (Figure 5.2).

This analysis demonstrated that antibiotic resistance profiles could easily be related to the species, or even the genus, suggesting the relevance of population dynamics for the hypothetical dissemination of resistance. Nevertheless, on rare occasions, different

isolates of the same species, isolated from different sites, yielded distinct antibiotic resistance patterns, suggesting a possible process of resistance acquisition or loss. Examples could be observed in the different genera (Figure 5.2). For instance, within the species *N. subterraneum*, the isolate from the WTP exhibited no resistance phenotype, the biofilm isolates were resistant to COL, and the tap water isolate was resistant to COL, PIC, and TZP. Another example was observed in *Sp. taejonensis*, in which the tap water isolate had resistance to COL, MEM, PICP, and FAM, which was not detected in the WTP strain. In *Sb. yanoikuyae*, two cup filler isolates were susceptible to more antibiotics (RT-A) than the other isolates, isolated from taps, the WTP, and cup fillers. In *S. panni* and *S. yunnanensis* also, distinct patterns could be observed for isolates from different taps. For example, *S. panni* strain T7AT6, isolated from a distinct tap and on a sampling date different from those for all the other *S. panni* isolates, belonged to RT-C, whereas all the other isolates belonged to RT-E. These examples suggest that both vertical and horizontal gene transfer processes are relevant in antibiotic resistance proliferation among *Sphingomonadaceae*.

5.5. Discussion

The presence of members of the family *Sphingomonadaceae* in drinking water and even the observation of higher counts in tap water and in dental chairs than in the WTP can be associated both with resistance to chlorination (Koskinen *et al.*, 2000; Furuhata *et al.*, 2007) and with the capacity to form biofilms (Singh *et al.*, 2003; Hong *et al.*, 2010). Nevertheless, the possibility that external sources of *Sphingomonadaceae*, other than the water supply, exist in the water distribution network cannot be discarded. In this study it was possible to identify 22 species of the family *Sphingomonadaceae.* Among these, to our knowledge, the species *S. koreensis*, *S. pituitosa*, *S. wittichii*, *Sb. xenophagum*, *Sp.*

taejonensis, *Sp. witflariensis*, *N. subterraneum*, and *N. aromaticivorans* were previously referred to as inhabitants of rivers, streams, aquifers, natural mineral water, and tap water (Koskinen *et al.*, 2000; Balkwill *et al.*, 2006; Furuhata *et al.*, 2007). *Blastomonas natatoria* was also referred to as a common inhabitant of disinfected water, namely, chlorinated waters (Balkwill *et al.*, 2006; Furuhata *et al.*, 2007).

The observed levels of antibiotic resistance can be considered high, in particular for colistin and beta-lactams. Although high prevalences of resistance to a polymyxin (polymyxin B) and to beta-lactams were reported previously for clinical isolates of the species *Sphingomonas paucimobilis* (Sader and Jones, 2005), it is relevant that the same findings are extended to other genera and species of the family *Sphingomonadaceae* isolated from water. In general, it was observed that members of the same species shared common antibiotic resistance profiles, suggesting that due to their ecology and physiology, these bacteria tend to acquire and/or develop similar patterns of resistance. Although it was not possible to establish a relationship between the resistance phenotype and the site of isolation, the data analyses demonstrated that multiresistance is widely found in *Sphingomonadaceae* in tap water.

Some of the species that presented the highest rates of (multi)resistance in the current study (RT-E and RT-F) were previously isolated from habitats with human-derived contamination, such as activated sludge (*S. dokdonensis* and *Sp. yanoikuyae*), pet-associated environments (*S. panni* and *N. panipatense*), or potato cultures (*Sp. yanoikuyae*) (Garbeva *et al.*, 2001; Busse *et al.*, 2005; De Gelder *et al.*, 2005; Cardinali-Rezende *et al.*, 2011). On the other hand, some species were distinguished by susceptibility to most of the antibiotics. Some of these species are common inhabitants of environments with low human impact, such as soil (*S. sanxanigenens* and *S. melonis*), sea, river, and lake water and sediments (*S. melonis*, *N. subterraneum*, and *N.*

aromaticivorans), or freshwater biofilms (*S. melonis*). However, *S. melonis* and *N. subterraneum* are also found in polluted environments, such as gasoline-contaminated soils or mines (Fredrickson *et al.*, 1995; Rickard *et al.*, 2004; Boden *et al.*, 2008; Cui *et al.*, 2008; Huang *et al.*, 2009).

Antibiotic resistance in this family of bacteria has not been studied much. Consequently, information on the mechanisms of resistance is scant. Additionally, the complete genome sequences, available for only a few strains, are poorly annotated. These facts represented serious limitations to the detection of antibiotic resistance genes in these bacteria. The current study demonstrates the need for a better understanding of the ecology of antibiotic resistance acquisition and development in these bacteria.

With this study, some major conclusions were reached. Different genera of the *Sphingomonadaceae* were prevalent in distinct parts of the drinking water network. In about one-half of the isolates, the combination of the 16S rRNA and *atpD* housekeeping genes allowed the differentiation of the *Sphingomonadaceae* to the strain level, emphasizing the diversity of these bacteria in drinking water.

No amikacin resistance was observed, although colistin resistance was intrinsic, mainly in tap water and cup filler isolates. Beta-lactam resistance was also observed to be highly prevalent in members of this family. The use of selective culture media favored the growth of multiresistant bacteria. Distinct patterns of resistance association were observed in *Sphingomonas* and *Sphingobium*. Additionally, within each genus, antibiotic resistance patterns were observed to be mainly species related. These observations may suggest distinct mechanisms or paths of resistance acquisition for the different taxa.

Sphingomonadaceae are common inhabitants of drinking water worldwide (Williams *et al.*, 2004; Hong *et al.*, 2010; Li *et al.*, 2010; Revetta *et al.*, 2010) and have a remarkable capacity to cope with stress conditions and to adapt to new habitats (Koskinen *et al.*,

2000; Furuhata *et al.*, 2007; Stolz, 2009; Hong *et al.*, 2010; Yim *et al.*, 2010). The current study provides the first evidence that these bacteria can play an important role as antibiotic resistance reservoirs in drinking water. Further studies on the ecology and genome dynamics (mutation and gene transfer) of these bacteria will make important contributions to the elucidation of their role in the propagation of antibiotic resistance.

6. Diversity and antibiotic resistance in *Pseudomonas* spp. from drinking water

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6.1. Abstract

Pseudomonas spp. are common inhabitants of aquatic environments, including drinking water. Multi-antibiotic resistance in clinical isolates of *P. aeruginosa* is widely reported and deeply characterized. However, the information regarding other species and environmental isolates of this genus is scant. This study was designed based on the hypothesis that members of the genus *Pseudomonas* given their high prevalence, wide distribution in waters and genetic plasticity can be important reservoirs of antibiotic resistance in drinking water. With this aim, the diversity and antibiotic resistance phenotypes of *Pseudomonas* isolated from different drinking water sources were evaluated. The genotypic diversity analyses were based on six housekeeping genes (16S rRNA, *rpo*D, *rpo*B, *gyr*B, *rec*A and ITS) and on pulsed field gel electrophoresis. Susceptibility to 21 antibiotics of eight classes was tested using the ATB PSE EU (08) and disk diffusion methods. *Pseudomonas* spp. were isolated from 14 of the 32 sampled sites. A total of 55 non-repetitive isolates were affiliated to twenty species. Although the same species were isolated from different sampling sites, identical genotypes were never observed in distinct types of water (water treatment plant/distribution system, tap water, cup fillers, biofilm, and mineral water). In general, the prevalence of antibiotic resistance was low and often the resistance patterns were related with the species and/or the strain genotype. Resistance to ticarcillin, ticarcillin with clavulanic acid, fosfomycin and cotrimoxazol were the most prevalent (69–84%). No resistance to piperacillin, levofloxacin, ciprofloxacin, tetracycline, gentamicin, tobramycin, amikacin, imipenem or meropenem was observed. This study demonstrates that *Pseudomonas* spp. are not so widespread in drinking water as commonly assumed. Nevertheless, it suggests that water *Pseudomonas* can spread acquired antibiotic resistance, preferentially via vertical transmission.

6.2. Introduction

The genus *Pseudomonas*, in part due to its long evolutionary history, is one of the most diverse and ecologically significant groups of bacteria on the planet (Spiers *et al.*, 2000; Mena and Gerba, 2009). The prototrophic character and metabolic versatility, the plasticity of the genome and the ability to cope with different forms of stress (physical, chemical and antibacterial compounds) are remarkable characteristics of the members of this genus (Palleroni, 2010). Such characteristics are the driving forces for adaptability, justifying the presence of pseudomonads in all the major natural terrestrial and aquatic environments (Mena and Gerba, 2009; Palleroni, 2010). In particular, drinking water is considered a relevant habitat of *Pseudomonas* spp. (Reitler and Seligmann, 1957; Manaia *et al.*, 1990; Rusin *et al.*, 1997; Jayasekara *et al.*, 1998; Palleroni, 2010). Although its presence in such habitats may result from the colonization of the aquifer, the ability to grow in final drinking water is also supposed to occur. Such expectation led Ribas and colleagues (2000) to propose the use of *Pseudomonas* spp. as indicator of potential regrowth in water distribution systems.

Nevertheless, the widespread distribution of *Pseudomonas* spp. may pose some public health concerns. Indeed, this ubiquitous genus includes species considered opportunistic pathogens that can colonize animals and humans, most of the times due to inadvertent contamination, for instance, via water (Mena and Gerba, 2009). Also the capacity to acquire antibiotic resistance mechanisms has been recognized in literature (Livermore, 2002). In respect to opportunistic pathogens, the species *P. aeruginosa* is by far the most studied in this genus. Members of this species can cause severe infections either in healthy or in immunocompromised individuals (Mena and Gerba, 2009). Although more rarely, other species of the genus, such as *P. fluorescens*, *P. stutzeri* and *P. putida* may

also be associated with human infections (Noble and Overman, 1994; Picot *et al.*, 2001; Carpenter *et al.*, 2008).

Frequently found as nosocomial agent, *P. aeruginosa* meets a large history of multi-resistance acquisition, against most of the last generation antibiotics (EARS-Net; Livermore, 2002; Harada *et al.*, 2012). In addition to a basis of natural resistance conferred by low outer-membrane permeability, inducible beta-lactamases or efflux pumps, members of this species can acquire practically all the known mechanisms of antimicrobial resistance (Hancock, 1998; Strateva and Yordanov, 2009). In contrast, the information on antibiotic resistance in other species of the genus *Pseudomonas* is scant, mainly for environmental isolates. For instance, the few studies found in the literature about diversity and antibiotic resistance phenotypes in *Pseudomonas* from drinking water are mainly focused on mineral water and were published more than 15 years ago (Hernandez Duquino and Rosenberg, 1987; Papapetropoulou *et al.*, 1994). Papapetropoulou *et al*. (1994) observed the occurrence of *Pseudomonas* spp. resistant to different classes of antibiotics but not to the last generation antibiotics, ceftazidime and ciprofloxacin in tap and non-carbonated mineral water in Greece. Approximately in the same period, Massa *et al*. (1995) reported similar results in isolates from non-carbonated mineral water in Italy. Although such studies suggest a low prevalence of resistance, the world scenario on antibiotic resistance changed dramatically over the last decade, supporting the hypothesis that higher and wider resistance phenotypes can be found nowadays in water *Pseudomonas*.

This study was designed with the major objective of assessing the diversity and antibiotic resistance phenotypes in *Pseudomonas* spp. isolated from drinking water, trying to fill in a gap existing in the literature. With this purpose, *Pseudomonas* spp. were screened among the bacterial isolates recovered from bottled mineral waters, and from tap

water collected at household taps, a filter of a domestic water purification system, cup fillers of dental chairs and from the respective drinking water treatment plant and associated distribution system. Specifically, it was intended to compare the isolates identified as *Pseudomonas* in terms of species and strains diversity versus the type of water [drinking water treatment plant and respective distribution system (W), domestic tap water (T), dental chairs (D), biofilm (Bf), and mineral water (M)]. The final aim was to infer about the role of these bacteria as antibiotic resistance reservoirs in drinking water.

6.2. Materials and Methods

6.2.1. Sampling

The isolates examined in the current study were collected in the northern Portugal region, over a period of approximately two years, in which different sites within a drinking water treatment plant (WTP), the respective distribution system and final consumer were sampled (Figueira *et al.*, 2011b; Silva *et al.*, 2011; Vaz-Moreira *et al.*, 2011b). The sampling sites were i) river, at the pumping for the drinking water treatment plant collected at surface and in alluvial wells (W1 and W2, respectively), ii) after the ozonation step at the WTP (W3), iii) at the final treatment step (after sand filtration, ozonation, flocculation, activated-carbon treatment, and chlorination) at the WTP (W4), iv) before and after each of two re-chlorination steps at the main pipeline of the water distribution system (W5-W8), v) household taps (T1-T11), and vi) cup fillers of dental chairs (D1-D9). Additionally, isolates from the biofilm of a domestic system of water filtration (Bf), and from bottled noncarbonated mineral water (M_A, M_B, M_C) of three commercial brands were included. Samples i-iv were collected in November 2007 and in

September 2009 (Figueira *et al.*, 2011b; Vaz-Moreira *et al.*, 2011b). Tap water samples were collected in three sampling periods (April, July and October 2009), from 11 household taps used 1-4 times a month (Vaz-Moreira *et al.*, 2011b). Samples from the cup fillers of nine dental chairs were collected in February, March and April 2008 in a university dental school clinic (Silva *et al.*, 2011). The biofilm was collected in March 2009, from a household tap water purification filter, in use for approximately 3 months. These sampling sites were all served by the same water treatment plant, examined in this study. All water and biofilm samples were processed within 4 h after collection, as described before (Figueira *et al.*, 2011b; Vaz-Moreira *et al.*, 2011b). Isolates from noncarbonated natural mineral bottled water were recovered from three batches of three water brands (two Portuguese and one French) purchased from a retail outlet in Portugal (Falcone-Dias *et al.*, unpublished).

6.2.2. Bacterial isolation and characterization

Given the fact that this work was part of a study on the diversity, distribution and antibiotic resistance of different bacterial groups thriving in drinking waters, bacteria were isolated on five culture media widely used for water microbiological quality control (Figueira *et al.*, 2011b; Silva *et al.*, 2011; Vaz-Moreira *et al.*, 2011b; Falcone-Dias *et al.*, in press; Narciso da Rocha *et al.*, submitted for publication). The culture media used were R2A (Difco), Plate Count Agar (PCA, Pronadisa), *Pseudomonas* Isolation Agar (PIA, Difco), Tergitol 7-Agar (TTC, Oxoid) and m-FC medium (Difco). R2A agar and PCA are non-selective culture media recommended for the examination of total heterotrophic bacteria, PIA is recommended for the enumeration of *Pseudomonas*, and TTC and m-FC are recommended for the enumeration of presumptive coliforms (ISO9308-1, 2000; Eaton *et al.*, 2005). For the mineral water samples were also used the culture media R2A and

PIA supplemented with the antibiotic amoxicillin (32 mg/L) (Falcone-Dias *et al.*, in press). Volumes up to 100 mL of water or of decimal serial dilutions thereof were filtered through cellulose nitrate membranes (0.45 µm pore size, 47 mm diameter, Albet), which were placed onto the different culture media and incubated as described before (Vaz-Moreira *et al.*, 2011b). After the incubation period, triplicates of culture media plates were considered for further isolation according to the following criterion: about fifty percent 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, were isolated. The colonies isolated on R2A were purified on the same culture medium, and those isolated on culture media with a higher nutrient content (PIA, TTC or m-FC) were purified on PCA. Pure cultures were preserved at –80 ºC in nutritive broth supplemented with 15 % (v/v) glycerol. Colony and cellular morphology, Gram-staining reaction and cytochrome *c* oxidase were characterized as described by Smibert and Krieg (1994).

6.2.3. Bacterial identification and typing

Gram-negative rods, catalase- and oxidase-positive were screened using *Pseudomonas* genus-specific primers (Ps-F and Ps-R) (Widmer *et al.*, 1998) targeting the 16S rRNA gene as described by Wesam (2009). After this screening, presumable *Pseudomonas* spp. were identified on basis of nearly complete 16S rRNA gene sequence analysis, using the primers 27F and 1492R (Lane, 1991) (Ferreira da Silva *et al.*, 2007). The nucleotide sequences were used to query the EzTaxon library (Chun *et al.*, 2007).

Intra-species variability was assessed based on the analysis of the nucleotide sequences of the house-keeping genes *rpo*D (σ^{70} factor), *rpo*B (RNA polymerase beta subunit), *gyr*B (DNA gyrase β subunit), and additionally the *rec*A (recombinase A) and ITS (intergenic 16S-23S internally transcribed spacer) genes. The selection of the genes

used in the present study was based on other studies, where they successfully distinguished *Pseudomonas* strains (Guasp *et al.*, 2000; Yamamoto *et al.*, 2000; Hilario *et al.*, 2004; Ait Tayeb *et al.*, 2005; Mulet *et al.*, 2009; Mulet *et al.*, 2010). For *rpo*D gene amplification, two pairs of primers were used (70F/70R or PsEG30F/PsEG790R, Table 6.1), 70F/70R for a normal amplification of the gene and PsEG30F/PsEG790R for a Nested-PCR amplification of the samples that did not give a visible amplification with the pair 70F/70R. The *gyr*B gene was amplified with the primers APrU/UP-1E; the isolates for which no PCR product was obtained were assayed with the primers gBMM1F/gBMM725R. The gene segments were amplified in a reaction mixture of 50 µL, with 1x Taq polymerase buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1µM/0.2µM/0.4µM of each primer (Table 6.1; for *rpo*D/*rpo*B/*gyr*B, respectively), 1.25 U of Taq polymerase (Fermentas) and 2 µL of DNA or first PCR product in the case of the Nested-PCR. The amplification reactions were performed with an initial denaturation step at 94ºC for 2 min, followed by 30 cycles at 94ºC for 1 min, the respective annealing temperature (Table 6.1) for 1 min, 72ºC for 2 min, and a final extension step at 72ºC for 2 min. PCR products were sequenced with the primers 27F and 1492R for total 16S rRNA gene, 70Fs or PsEG30F for *rpo*D, LAPS for *rpo*B and M13 or gBMM1F for *gyr*B (Table 6.1). The partial sequences of the genes *rec*A and ITS, were analysed for isolates that could not be distinguished based on the comparison of the nucleotide sequences of the genes 16S rRNA, *rpo*D, *rpo*B and *gyr*B, i.e. when those sequences did not differ in a single nucleotide position. The genes *rec*A and ITS were amplified as described by Hilario *et al*. (2004) and Guasp *et al.* (2000), respectively.

Table 6.1. Primers used in the study of *Pseudomonas* spp. diversity

Gene	Primer	Sequence	Fragment (bp)	Annealing temperature	Reference
16S rRNA	27F	GAGTTTGATCCTGGCTCAG	1465	55 ºC	Lane, 1991
	1492R	TACCTTGTTACGACTT			
Pseudomonas identification (16S rRNA)	Ps-F	GGTCTGAGAGGATGATCAGT	986	50ºC	Widmer *et al.*, 1998
	Ps-R	TTAGCTCCACCTCGCGGC			
*rpo*D	70F	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT	800	59ºC	Yamamoto *et al.*, 2000
	70R	ATAGAAATAACCAGACGTAAGTTNGCYTCNACCATYTCYTTYTT			
	70Fs	ACGACTGACCCGGTACGCATGTA			
	PsEG30F	ATYGAAATCGCCAARCG	760	51ºC	Mullet *et al.*, 2009
	PsEG790R	CGGTTGATKTCCTTGA			
*rpo*B	LAPS	TGGCCGAGAACCAGTTCCGCGT	1229	59ºC	Ait Tayeb *et al.*, 2005
	LAPS27	CGGCTTCGTCCAGCTTGTTCAG			
*gyr*B	APrU	TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	966	55ºC	Yamamoto *et al.*, 2000
	UP-1E	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA			
	M13	CAGGAAACAGCTATGACC			
	gBMM1F	GTGTCGGTKGTRAACGCCC	966	55ºC	Mullet *et al.*, 2010
	gBMM725R	GCYTCRTTSGGRTTYTCCAGCAGG			
*rec*A	recA_Ps fw	TCSGGYAARACCACSCTGAC	600	55ºC	Hilario *et al.*, 2004
	recA_Ps rev	RTACCAGGCRCCGGACTTCT			
ITS	16F945	GGGCCCGCACAAGCGGTGG	1300-1500	55ºC	Guasp *et al.*, 2000
	23R458	CTTTCCCTCACGGTAC			
	rrn16S	GAAGTCGTAACAAGG			
	rrn23S	CAAGGCATCCACCGT			

The nucleotide sequences were aligned using Clustal W from MEGA 5.0 software (Tamura et al., 2011). A total of 1227, 568, 742, 667, 553 and 808 nucleotide positions were included in the analyses of the 16S rRNA, *rpo*D, *rpo*B, *gyr*B, *rec*A and ITS gene sequences, respectively. The gene sequences were compared for each pair of isolates belonging to the same species, as identified based on the 16S rRNA gene sequence analysis and EzTaxon library (Chun *et al.*, 2007). Strains differing at least in a nucleotide position in any of those gene sequences were classified as belonging to a distinct sequence type (ST). Nucleotide sequences relatedness was estimated based on the model of Jukes and Cantor (1969). For a matter of simplicity, a single dendrogram was constructed based on concatenated sequences of the genes 16S rRNA, *rpo*D, *rpo*B and *gyr*B. Type strains of species closest to the isolates under study were included in the dendrogram. Dendrograms were created using different methods, such as neighbor-joining, maximum parsimony and maximum likelihood, in order to assess the tree stability. Non-homologous and ambiguous nucleotide positions were excluded from the calculations, and bootstrap values, generated from 1000 re-samplings, at or above 50 % were indicated at the branch points.

Pulsed field gel electrophoresis (PFGE) was used in an attempt to differentiate the isolates that, on basis of the analysis of the six housekeeping genes referred to above, yielded the same sequence type. PFGE was performed as described by Almeida *et al*. (2010) using the enzyme *Xba*I (Fermentas). The electrophoresis conditions were changed to pulse times ramped from 4 to 40 s over 22 h at 6 Vcm^{-1} and 14ºC. *Salmonella enterica* serotype Braenderup H9812 was used as ladder. Restriction fragments were visually compared and interpreted. Isolates with identical nucleotide sequence types and PFGE profiles were considered to represent the same strain.

Representative sequences of each sequence type for 16S rRNA, *rpo*D, *rpo*B and *gyr*B genes were deposited in the GenBank with the accession numbers JQ317787-JQ317814, JQ317815-JQ317842, JQ317844-JQ317871 and JQ317872-JQ317899, respectively. The partial *rpo*B gene sequence of the type strain *P. arsenicoxydans* CCUG 58201^{T}, not available in public databases, was determined in this study and deposited in GenBank (accession number JQ317843).

6.2.4. Characterization of antibiotic resistance phenotypes

The antibiotic resistance phenotypes of a total of 138 isolates identified as *Pseudomonas* spp. were characterized, using the ATB PSE EU (08) (BioMérieux) panel, following the manufacturer instructions, and the agar diffusion method. The latter method was used to test the antibiotics nalidixic acid (NA, 30 µg); cephalothin (CP, 30 µg); tetracycline (TET, 30 µg) and streptomycin (STR, 10 µg), not included in the ATB panel. Phenotypes were classified as resistant, intermediary, or susceptible according to the manufacturer's instructions for the antibiotics tested with the ATB panel, and using the following criteria for the antibiotics testes by disk diffusion: NA: R≤13, I = 14–18, S≥19; CP: R≤14, I = 15–20, S≥21; TET: R≤11, I = 12–14, S≥15; and STR: R≤11, I = 12–14, S≥15. The strains *Escherichia coli* ATCC 25922 and *P. aeruginosa* DSM 1117 (=ATCC 27853) were included as quality controls in the disk diffusion assays. An isolate was classified as multiple antibiotic resistant (MAR) when presented resistance to antibiotics belonging to three or more classes. The index MARn was used to indicate the number of classes to which resistance was observed.

6.2.5. Diversity and evenness indices and statistical analyses

The strains isolated from the same sampling point, in the same date and on the same culture medium, which presented the same sequence type, PFGE pattern and antibiotic resistance profile, were considered repetitions and were excluded from further data analysis. The diversity [$H_0 = -\sum pi \ln(pi)$] and evenness [$J = H_0/\ln(H_{max})$] were measured using the Shannon's (Shannon and Weaver, 1963) and Pielou's indices (Pielou, 1966), respectively. The indices were calculated based on the sequence type diversity, excluding the repetitions (determined as explained above). In order to compare the antibiotic resistance profiles of isolates from different sites, date and species, hierarchical ascendant cluster analysis was carried out. A matrix with nominal variables was constructed based on the resistant, intermediary, and susceptible phenotypes, defined as referred to above. Significant clusters obtained on basis of the Square Euclidean distance and the Ward method led to the definition of resistance types (RT), as described by Vaz-Moreira *et al*. (2011b). The SPSS software package, version 19.0 (SPSS Inc., Chicago, IL), was used for these analyses.

6.3. Results

6.3.1. Isolation and identification

The isolates were confirmed as *Pseudomonas* and identified to the species level based on the 16S rRNA gene sequence analysis and EzTaxon library (Chun *et al.*, 2007). From a collection of more than 3500 isolates, 138 *Pseudomonas* spp. were identified, comprising isolates from the WTP (n=26) and distribution system (n=6), from household taps (n=63), from dental chairs (n=1), from biofilm (n=24) and from bottled mineral

water (n=18) (Table 6.2). Specifically, members of this genus were isolated from raw surface water

Table 6.2. Counts of total heterotrophic bacteria on R2A medium, and proportion of *Pseudomonas* spp. isolated for each of the sampling points.

Local	Total heterotrophs counts (CFU mL^{-1})	% of *Pseudomonas* spp. isolated (nº *Pseudomonas*/total nº of isolates)
Water treatment plant and distribution system		
W1	$2.5x10^{3} – 3.6x10^{3}$	5.1 (17/334)
W2	$1.4x10^{1} – 2.1x10^{1}$	0 (0/259)
W3	$2.0x10^{1} – 1.2x10^{3}$	3.8 (9/236)
W4	$5.9x10^{-2} – 1.7x10^{2}$	0 (0/203)
W5	$9.7x10^{0} – 2.0x10^{2}$	1.0 (3/289)
W6	$1.5x10^{-2} – 6.0x10^{-1}$	0 (0/135)
W7	$4.4x10^{-2} – 1.9x10^{0}$	2.9 (3/105)
W8	$2.3x10^{-1} – 5.1x10^{-1}$	0 (0/177)
Tap water		
T1	$2.3x10^{2} – 1.6x10^{3}$	0 (0/126)
T2	$3.6x10^{2} – 1.1x10^{3}$	0.8 (1/122)
T3	$6.0x10^{1} – 5.7x10^{2}$	0 (0/115)
T4	$2.0x10^{2} – 2.0x10^{3}$	0 (0/133)
T5	$3.3x10^{1} – 3.3x10^{2}$	0.7 (1/149)
T6	$1.5x10^{3} – 6.3x10^{3}$	11.5 (16/139)
T7	$2.5x10^{2} – 1.8x10^{3}$	0 (0/139)
T8	$7.9x10^{3} – 7.7x10^{4}$	12.3 (18/146)
T9	$2.9x10^{3} – 1.4x10^{4}$	0 (0/148)
T10	$2.2x10^{3} – 9.9x10^{3}$	5.4 (8/149)
T11	$1.4x10^{3} – 1.3x10^{4}$	16.0 (20/125)
Dental chairs	$2.0x10^{-1} – 2.0x10^{1}$	6.3 (1/16) (D8)
(D1-D9)		0 (0/3-21)* (D1-D7, D9)
Biofilm	$1.3x10^{4}$	92.3 (24/26)
Mineral water		
M_A	$4.4x10^{1} – 1.2x10^{2}$	12.8 (12/94)
M_B	$2.5x10^{2} – 7.7x10^{3}$	0 (0/95)
M_C	$2.8x10^{1} – 5.8x10^{2}$	10.0 (5/50)

The CFU counts on the other culture media were lower or in the same order of magnitude, as the observed for the R2A medium.
* the number of total isolates recovered for the cup fillers of the dental chairs D1-D7 and D9, ranged between 3 and 21.

(W1), after the ozonation process (W3), from treated water samples (before re-chlorination steps, W5 and W7), from six out of the eleven household taps (T2, T5, T6, T8, T10 and T11), from one of the cup filler of dental chairs (D8), from the biofilm (Bf), and from two of the three mineral waters (M_A and M_C). From the other sampled sites, no

successful isolation of *Pseudomonas* was achieved. These sites included the alluvial wells (W2), the points located immediately after the chlorination steps in the WTP (W4) and distribution system (W6, W8), five out of the eleven taps (T1, T3-4, T7 and T9), eight of the nine cup fillers of dental chairs (D1-D7, D9), and one of the three mineral water brands (M_B). The samples in which *Pseudomonas* were not detected presented total heterotrophic counts ranging from $1.5x10^{-2}$ CFU mL^{-1} to $1.4x10^{4}$ CFU mL^{-1} (Table 6.2). Noteworthy, in all these samples the counts were in the same order of magnitude as those observed for the samples from which *Pseudomonas* were isolated ($4.4x10^{-2}$ - $7.7x10^{4}$ CFU mL^{-1}). In mineral waters the recovery of *Pseudomonas* was mainly observed on culture media supplemented with amoxicillin. Indeed, only from a single sample (M_A) were recovered three isolates on PIA without amoxicillin.

Among the 138 isolates, 83 were considered repetitions and were not included in further analysis. The resultant collection of 55 isolates comprised 13 from WTP, four from the distribution system, 20 from taps, one from dental chairs, three from biofilm, and 14 from mineral water.

According to the EzTaxon library (Chun *et al.*, 2007), the isolates examined in this study were affiliated to twenty species (Table 6.3). Based on the analysis of the other housekeeping genes (*rpo*B, *rpo*D and *gyr*B), the identifications achieved were, in general, coincident with those supported by the 16S rRNA gene sequence analysis. Exceptions were observed for the species *P. jessenii* and *P. toyotomiensis*, which closest neighbors based on the sequence analysis of the other three genes studied were members of the species *P. moraviensis* and *P. alcaliphila*, respectively. For *P. koreensis*, the closest neighbor based on *rpo*B and *rpo*D genes sequence analysis was *P. moraviensis*, while for *P. peli*, based on *rpo*D gene sequence, was *P. anguilliseptica*. The closest neighbors of

Table 6.3. Sequence types obtained for the different housekeeping genes and the combination of them (ST_{final}) for the *Pseudomonas* spp. recovered from the different general types of water (W, drinking water treatment plant and respective distribution system; T, domestic tap water; D, dental chairs; Bf, biofilm; M, mineral water) for the different sampling points and sampling dates or batches (A, B and C).

Species	No. isolates	General type of water														Sequence types				
		W				T						D8	Bf	M		ST_{16S}	ST_{rpoD}	ST_{rpoB}	ST_{gyrB}	ST_{final}
		W1	W3	W5	W7	T2	T5	T6	T8	T10	T11			MA	MC					
P. aeruginosa	1												A			1	1	1	1	ST1
	6							A,B,C								1	2	2	2	ST2
P. alcaligenes	1	A														2	3	3	3	ST3
P. argentinensis	1	A														3	4	4	4	ST4
P. arsenicoxydans	2													B		4	5	5	5	ST5
P. chlororaphis	3									A	A					5	6	6	6	ST6
P. frederiksbergensis	1														B	6	7	7	7	ST7
P. jessenii	1			A												7	8	8	8	ST8
P. koreensis	1				A											8	9	9	9	ST9
P. mandelii	1														B	9	10	10	10	ST10
	1														B	9	11	11	11	ST11
P. moraviensis	3													A,C		10	12	12	12	ST12
	2													B		11	13	13	13	ST13
P. nitroreducens	6					B				A	A					12	14	14	14	ST14
P. peli	1	A														13	15	15	15	ST15
P. poae	1						A									14	16	16	16	ST16
P. rhodesiae	1			A												15	17	17	17	ST17
P. simiae	8	A,B	A,B													16	18	18	18	ST18
P. stutzeri	1												A			17	19	19	19	ST19
	1											A				17	20	19	20	ST20
P. thivervalensis	1														B	18	21	20	21	ST21
P. toyotomiensis	1	A														19	22	21	22	ST22
	4								A	A	A					20	23	22	23	ST23
P. veronii	1	A														21	24	23	24	ST24
	1			A												22	25	24	25	ST25
	2													A		23	26	25	26	ST26
	1													B		23	27	26	27	ST27
P. xanthomarina	1												A			24	28	27	28	ST28

Notes: Species identifications are based on 16S rRNA gene sequence analysis and EzTaxon database. The number of isolates does not include the replicates. In grey are marked the isolates not differentiated by the gene sequences.

Local of isolation: W1, raw surface water; W3, after ozonation; W5 and W7, treated water before the first and second re-chlorination steps, respectively; T2, T5, T6, T8, T10 and T11, domestic tap water samples; D8, cup filler of a dental chair; Bf, biofilm; M_A and M_C, mineral water samples.

the isolates identified as *P. argentinensis*, *P. poae*, *P. veronii*, *P. xanthomarina*, *P. arsenicoxydans* and *P. thivervalensis* differed for each of the four genes (16S rRNA, *rpo*D, *rpo*B, *gyr*B). As a summary, Figure 6.1 shows the relatedness of the isolates with the closest type strains, based on the comparison of the concatenated gene sequences (16S rRNA, *rpo*D, *rpo*B, *gyr*B).

The majority of the 55 non-repetitive isolates belonged to the species *P. aeruginosa*, *P. nitroreducens*, *P. toyotomiensis* and *P. simiae*. The three first species were mainly found in taps (*P. nitroreducens* - T2, T10, T11; *P. aeruginosa* -T6 and Bf; *P. toyotomiensis* - T8, T10, T11, and W1), while *P. simiae* was isolated preferentially from the WTP (W1 and W3) (Table 6.3).

6.3.2. Diversity of *Pseudomonas* species over the sampled sites

The patterns and richness of *Pseudomonas* species differed over the sampled sites. The highest species richness was observed before the drinking water treatment (W1), with six species detected in raw surface water (*P. alcaligenes*, *P. argentinensis*, *P. peli*, *P. simiae*, P. *toyotomiensis* and *P. veronii*) (Table 6.2 and 6.3). Among these species, only *P. simiae* was also found after ozonation (W3). Downstream the water treatment, in the distribution system (W5), members of species *P. jessenii*, *P. rhodesiae* and *P. veronii* were isolated (Table 6.2 and 6.3). Downstream this point, after the first re-chlorination point, but before the second (W7), only *P. koreensis* was isolated. In this sampling point (W7) the density of total heterotrophs was about 1000 times lower than in W5 (Table 6.2 and 6.3). In water samples from household taps, considerably higher counts of total heterotrophs as well as a distinct pattern of species were observed. *P. aeruginosa, P. chlororaphis*, *P. nitroreducens*, *P. poae* and *P. toyotomiensis* prevailed at this stage. From most of the taps it was isolated a single *Pseudomonas* species. The exceptions were taps

T10 and T11 (Table 6.3). *P. aeruginosa* was isolated only from tap T6, and in the three sampling dates (Table 6.3). Additionally, *P. aeruginosa* was isolated from the biofilm sample, from which were isolated also bacteria affiliated to *P. stutzeri* and *P. xanthomarina*. The mineral waters from which it was possible to isolate *Pseudomonas* yielded mainly the species *P. arsenicoxydans*, *P. moraviensis* and *P. veronii* (from M_A), and *P. frederiksbergensis*, *P. mandelii* and *P. thivervalensis* (from M_C), most of these not observed in the other types of water.

The distinctive patterns of species according to the type of water were, in fact, a general trend in this study. Indeed, only the species *P. aeruginosa*, *P. stutzeri*, *P. toyotomiensis* and *P. veronii* were isolated from more than one general type of water (W, T, D, Bf or M). Additionally, although found in a single general type of water, members of the species *P. chlororaphis*, *P. nitroreducens* and *P. simiae* were isolated from more than one sampled site, i.e. different taps or different points of the WTP or distribution system (Table 6.3). The other species (half of the total) were represented by a single isolate (Table 6.3).

Housekeeping genes sequence analysis was used to track members of the same species isolated from different locations. This analysis allowed the differentiation of 28 sequence types (ST) (Table 6.3). The 16S rRNA gene sequence analysis led to the definition of 24 ST, presenting lower resolution than *rpo*B, *rpo*D and *gyr*B gene sequences. The ribosomal RNA gene sequence did not allow the differentiation of strains within *P. aeruginosa*, *P. mandelli* and *P. stutzeri* (in grey in Table 6.3). The *rpo*B gene allowed the differentiation of 27 ST, and the *rpo*D and *gyr*B permitted to distinguish 28 ST each (Table 6.3). The partial sequences of the genes *rec*A and ITS did not bring additional resolution, confirming the ST determined with the four genes. The ability of the 16S rRNA, *rpo*B, *rpo*D and *gyr*B genes sequence analysis to differentiate the isolates

under study was confirmed by PFGE. Isolates from the same species (*P. aeruginosa*, *P. mandelii*, *P. moraviensis*, *P. stutzeri*, *P. toyotomiensis* and *P. veronii*) but with different ST presented different PFGE profiles (data not shown). In contrast, identical PFGE profiles were obtained for isolates with the same ST based on the analysis of the six housekeeping genes.

By the use of the multi-locus sequence analysis it was possible to demonstrate that members of the same species found in different general types of water, corresponded to distinct strains (Table 6.3). Nevertheless, the same ST, presumably representing the same strain, was recovered from different locations of the same general type of water (Table 6.3). For example, isolates with the same ST were recovered from different taps (*P. chlororaphis*, *P. nitroreducens*, *P. toyotomiensis*), or from two different points of the WTP (*P. simiae*). Additionally, the same ST was isolated from the same location in more than one sampling date (*P. aeruginosa*, *P. moraviensis* and *P. simiae*). The PFGE typing confirmed these findings, with the isolates with the same ST but recovered from different sample locations (ST6, ST14, ST18, ST23) or from the same location in different campaigns (ST2, ST12, ST18) presenting the same pulsotype.

The indices of diversity, calculated based on the assumption that each ST represents a distinct operational taxonomic unit, confirmed the analysis based on species richness. According to the data obtained, water treatment and distribution imposed a ST diversity reduction, maintained also in the tap water (Table 6.4). Curiously, mineral waters, which are not subjected to any kind of disinfection, presented diversity indices similar to those observed in raw water, 1.56 for M_A and 1.39 for M_C (Table 6.4).

6.3.3. Antibiotic resistance in *Pseudomonas* species

The analysis of the antibiotic resistance phenotypes revealed high percentages of resistance to some beta-lactams, with more than 80 % of the isolates resistant to cephalothin (100 %), ticarcillin (84 %), and ticarcillin with clavulanic acid (80 %). Regarding other beta-lactams, resistance to the cephalosporins ceftazidime and cefepim was lower (18 and 2 %, respectively), and no piperacillin, piperacillin plus tazobactam, imipenem and meropenem resistance was observed. High antibiotic resistance prevalence values were also observed for the sulphonamide cotrimoxazol (78 %) and for the epoxide fosfomycin (69 %). Although resistance to the quinolone nalidixic acid (36 %) and to the aminoglycoside streptomycin (18 %) was observed, no resistance phenotypes were observed for other antibiotic of the same classes, *i.e.*, the fluoroquinolones levofloxacin and ciprofloxacin and the aminoglycosides gentamicin, tobramycin, and amikacin. No resistance phenotypes were also observed for tetracycline.

The antibiotic resistance prevalence values in the isolates recovered from the WTP and distribution system were different ($p<0.05$) from those recovered from taps. Resistance to streptomycin and rifampicin, which was absent in WTP and distribution system isolates, reached percentages of 45 % and 30 % in tap water isolates, respectively. Also the prevalence of resistance to fosfomycin and nalidixic acid was significantly ($p<0.005$) higher in tap water isolates than in WTP and distribution system isolates (90 and 41 % for fosfomycin; 80 and 24 % for nalidixic acid, respectively). In contrast, resistance to ceftazidime, that presented a prevalence of 53 % in WTP and distribution system, was not detected in tap water isolates (Figure 6.1).

The absence of resistance to nalidixic acid, streptomycin and rifampicin differentiated the mineral water isolates from those of tap water, in which, resistance percentages above the 30 % were observed for these antibiotics. In contrast, the prevalence of cotrimoxazol

and colistin resistance was higher in mineral water (100 and 36 %, respectively) than in tap water isolates (70 and 5 %, respectively). Curiously, although resistant to cephalothin, ticarcillin, and ticarcillin with clavulanic acid, none of the mineral water isolates was resistant to any of the other beta-lactams tested (Figure 6.1). Unlike the majority of the others, biofilm isolates were susceptible to ticarcillin and ticarcillin with clavulanic acid, two of the most prevalent resistance phenotypes in the isolates studied. In contrast, all the biofilm isolates were resistant to fosfomycin (Figure 6.1).

Table 6.4. Sequence type diversity and evenness indices for the samples from the different sampling sites (W, drinking water treatment plant and respective distribution system; T, domestic tap water; Bf, biofilm; M, mineral water).

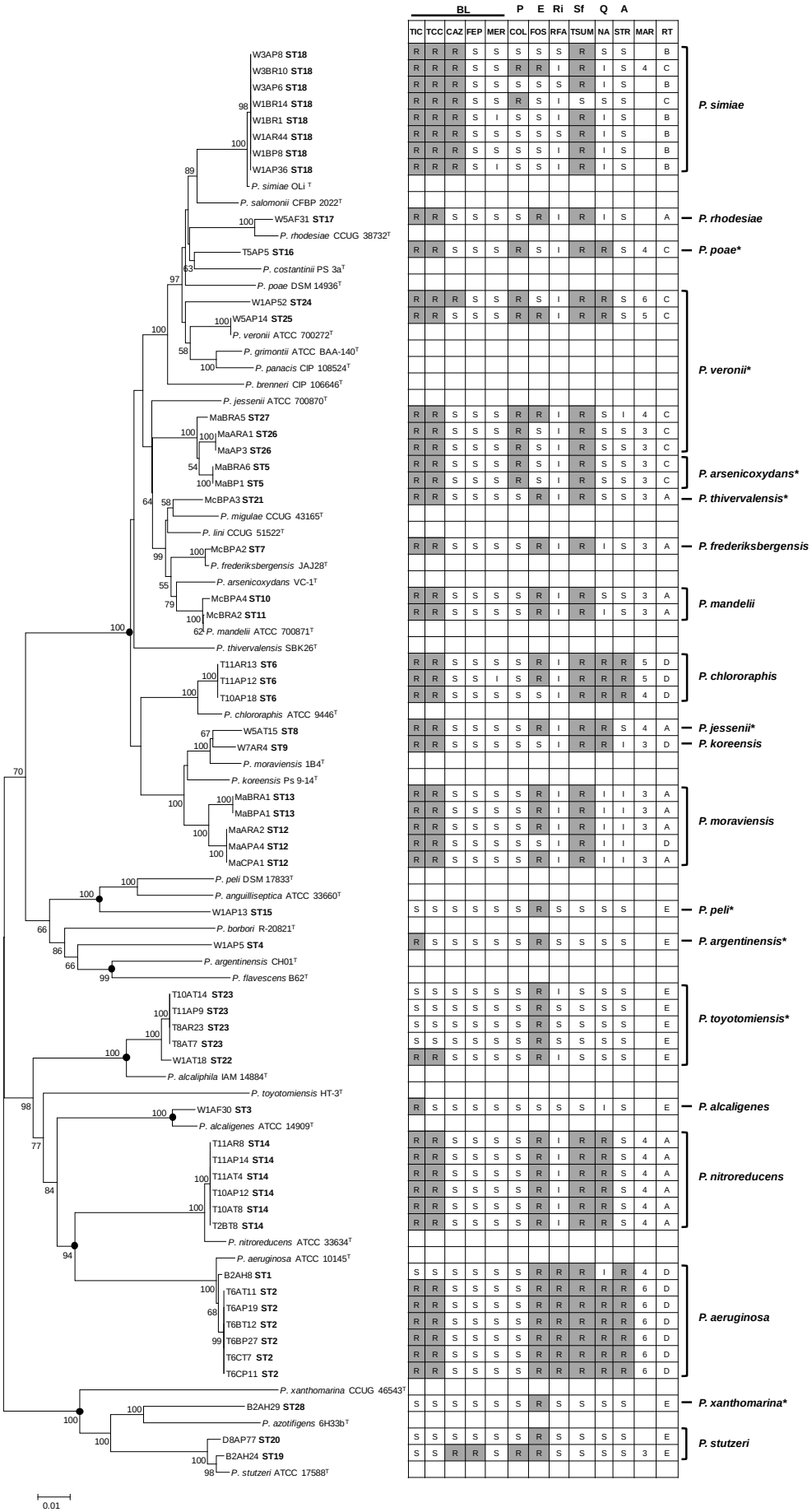
Samples	Diversity	Evenness
W		
W1	1.50	0.65
W5	1.10	1.00
T		
T10	1.04	0.75
T11	1.01	0.56
Bf	1.10	1.00
M		
M_A	1.56	0.68
M_C	1.39	1.00

The locations where only one species was detected are not represented in this table. These samples were: W3 and W7, after ozonation and before the second re-chlorination, respectively; T2, T5, T6 and T8, domestic tap water samples; and D8, cup filler dental chairs.

Multiple antibiotic resistance (MAR, resistant to three or more classes of antibiotics) - was observed in more than half of the isolates (Figure 6.1). The occurrence of MAR phenotypes was mainly related with the taxonomic affiliation of the organisms. The species *P. chlororaphis* and *P. aeruginosa*, presented the highest multi-resistant indices, with resistance to five or more classes of antibiotics. Contrary to the observation of a

species-related phenotype of resistance, the *P. veronii* recovered from the water treatment plant and the *P. aeruginosa* recovered from the biofilm, presented respectively a higher and lower MAR index than the observed for the other isolates of the same species (Figure 6.1). The *P. veronii* isolated from the water treatment plant presented resistance phenotypes to five or six of the eight classes tested, contrary to the three or four observed for the mineral water isolates of the same species (Figure 6.1). In the case of the *P. aeruginosa*, the isolates recovered from the biofilm presented MAR to four distinct classes of antibiotics, instead of the six observed for the tap water isolates (Figure 6.1).

The comparison of the isolates based on the respective resistance profiles led to the definition of five significant clusters, here designated as resistance types (RT) (Figure 6.1). The RT-A was characterized by a core resistance phenotype of ticarcillin, ticarcillin plus clavulanic acid, fosfomycin, cotrimoxazol, and intermediary phenotype to rifampicin (Figure 6.1). Variable phenotypes in RT-A were the resistance to nalidixic acid and streptomycin. RT-A cluster comprised 16 isolates, including all the *P. nitroreducens*. The RT-B grouped six isolates identified as *P. simiae*, recovered from the WTP (raw surface water and after the ozonation step), which core resistance profile differed from RT-A, because included ceftazidime but not fosfomycin. The RT-C, grouped 10 isolates mainly from the species *P. veronii*, with a core resistance to ticarcillin, ticarcillin plus clavulanic acid, colistin and intermediary phenotype to rifampicin (Figure 6.1). Resistance to cotrimoxazol, ceftazidime, fosfomycin and nalidixic acid were variable traits in RT-C. The RT-D grouped 12 isolates, mainly from the species *P. aeruginosa* and *P. chlororaphis*, with a core resistance phenotype to cotrimoxazol, but also a high prevalence of resistance and intermediary phenotypes to fosfomycin, rifampicin, nalidixic acid , streptomycin , ticarcillin and ticarcillin plus clavulanic acid. The ST1 of



	BL					P	E	Ri	Sf	Q	A			
	TIC	TCC	CAZ	FEP	MER	COL	FOS	RFA	TSUM	NA	STR	MAR	RT	
W3AP8 **ST18**	R	R	R	S	S	S	S	S	R	S	S		B	
W3BR10 **ST18**	R	R	R	S	S	R	R	I	R	I	S	4	C	
W3AP6 **ST18**	R	R	R	S	S	S	S	S	R	I	S		B	
W1BR14 **ST18**	R	R	R	S	S	R	S	I	S	S	S		C	***P. simiae***
W1BR1 **ST18**	R	R	R	S	I	S	S	I	R	I	S		B	
W1AR44 **ST18**	R	R	R	S	S	S	S	S	R	I	S		B	
W1BP8 **ST18**	R	R	R	S	S	S	S	I	R	I	S		B	
W1AP36 **ST18**	R	R	R	S	I	S	S	I	R	I	S		B	
P. simiae OLi T														
P. salomonii CFBP 2022T														
W5AF31 **ST17**	R	R	S	S	S	S	R	I	R	I	S		A	***P. rhodesiae***
P. rhodesiae CCUG 38732T														
T5AP5 **ST16**	R	R	S	S	S	R	S	I	R	R	S	4	C	***P. poae****
P. costantinii PS 3aT														
P. poae DSM 14936T														
W1AP52 **ST24**	R	R	R	S	S	R	S	I	R	R	S	6	C	
W5AP14 **ST25**	R	R	S	S	S	R	R	I	R	R	S	5	C	
P. veronii ATCC 700272T														
P. grimontii ATCC BAA-140T														
P. panacis CIP 108524T														***P. veronii****
P. brenneri CIP 106646T														
P. jessenii ATCC 700870T														
MaBRA5 **ST27**	R	R	S	S	S	R	R	I	R	S	I	4	C	
MaARA1 **ST26**	R	R	S	S	S	R	S	I	R	S	S	3	C	
MaAP3 **ST26**	R	R	S	S	S	R	S	I	R	S	S	3	C	
MaBRA6 **ST5**	R	R	S	S	S	R	S	I	R	S	S	3	C	***P. arsenicoxydans****
MaBP1 **ST5**	R	R	S	S	S	R	S	I	R	S	S	3	C	
McBPA3 **ST21**	R	R	S	S	S	S	R	I	R	S	S	3	A	***P. thivervalensis****
P. migulae CCUG 43165T														
P. lini CCUG 51522T														
McBPA2 **ST7**	R	R	S	S	S	S	R	I	R	I	S	3	A	***P. frederiksbergensis***
P. frederiksbergensis JAJ28T														
P. arsenicoxydans VC-1T														
McBPA4 **ST10**	R	R	S	S	S	S	R	I	R	S	S	3	A	***P. mandelii***
McBRA2 **ST11**	R	R	S	S	S	S	R	I	R	I	S	3	A	
P. mandelii ATCC 700871T														
P. thivervalensis SBK26T														
T11AR13 **ST6**	R	R	S	S	S	S	R	I	R	R	R	5	D	
T11AP12 **ST6**	R	R	S	S	I	S	R	I	R	R	R	5	D	***P. chlororaphis***
T10AP18 **ST6**	R	R	S	S	S	S	S	I	R	R	R	4	D	
P. chlororaphis ATCC 9446T														
W5AT15 **ST8**	R	R	S	S	S	S	R	I	R	R	S	4	A	***P. jessenii****
W7AR4 **ST9**	R	R	S	S	S	S	S	I	R	R	I	3	D	***P. koreensis****
P. moraviensis 1B4T														
P. koreensis Ps 9-14T														
MaBRA1 **ST13**	R	R	S	S	S	S	R	I	R	I	I	3	A	
MaBPA1 **ST13**	R	R	S	S	S	S	R	I	R	I	I	3	A	
MaARA2 **ST12**	R	R	S	S	S	S	R	I	R	I	I	3	A	***P. moraviensis***
MaAPA4 **ST12**	R	R	S	S	S	S	S	I	R	I	I		D	
MaCPA1 **ST12**	R	R	S	S	S	S	R	I	R	I	I	3	A	
P. peli DSM 17833T														
P. anguilliseptica ATCC 33660T														
W1AP13 **ST15**	S	S	S	S	S	S	R	S	S	S	S		E	***P. peli****
P. borbori R-20821T														
W1AP5 **ST4**	R	S	S	S	S	S	R	S	S	S	S		E	***P. argentinensis****
P. argentinensis CH01T														
P. flavescens B62T														
T10AT14 **ST23**	S	S	S	S	S	S	R	I	S	S	S		E	
T11AP9 **ST23**	S	S	S	S	S	S	R	S	S	S	S		E	
T8AR23 **ST23**	S	S	S	S	S	S	R	S	S	S	S		E	***P. toyotomiensis****
T8AT7 **ST23**	S	S	S	S	S	S	R	S	S	S	S		E	
W1AT18 **ST22**	R	R	S	S	S	S	R	I	S	S	S		E	
P. alcaliphila IAM 14884T														
P. toyotomiensis HT-3T														
W1AF30 **ST3**	R	S	S	S	S	S	S	S	S	I	S		E	***P. alcaligenes****
P. alcaligenes ATCC 14909T														
T11AR8 **ST14**	R	R	S	S	S	S	R	I	R	R	S	4	A	
T11AP14 **ST14**	R	R	S	S	S	S	R	I	R	R	S	4	A	
T11AT4 **ST14**	R	R	S	S	S	S	R	I	R	R	S	4	A	***P. nitroreducens***
T10AP12 **ST14**	R	R	S	S	S	S	R	I	R	R	S	4	A	
T10AT8 **ST14**	R	R	S	S	S	S	R	I	R	R	S	4	A	
T2BT8 **ST14**	R	R	S	S	S	S	R	I	R	R	S	4	A	
P. nitroreducens ATCC 33634T														
P. aeruginosa ATCC 10145T														
B2AH8 **ST1**	S	S	S	S	S	S	R	R	R	I	R	4	D	
T6AT11 **ST2**	R	R	S	S	S	S	R	R	R	R	R	6	D	
T6AP19 **ST2**	R	R	S	S	S	S	R	R	R	R	R	6	D	
T6BT12 **ST2**	R	R	S	S	S	S	R	R	R	R	R	6	D	***P. aeruginosa***
T6BP27 **ST2**	R	R	S	S	S	S	R	R	R	R	R	6	D	
T6CT7 **ST2**	R	R	S	S	S	S	R	R	R	R	R	6	D	
T6CP11 **ST2**	R	R	S	S	S	S	R	R	R	R	R	6	D	
P. xanthomarina CCUG 46543T														
B2AH29 **ST28**	S	S	S	S	S	S	R	S	S	S	S		E	***P. xanthomarina****
P. azotifigens 6H33bT														
D8AP77 **ST20**	S	S	S	S	S	S	R	S	S	S	S		E	***P. stutzeri***
B2AH24 **ST19**	S	S	R	R	S	R	R	S	S	S	S	3	E	
P. stutzeri ATCC 17588T														

Figure 6.1. Dendrogram constructed on basis of 16S rRNA, *rpo*D, *rpo*B and *gyr*B concatenated gene sequences (1227+568+742+667 bp, respectively).
The isolates designation is SxCMn, with "S" as the site (W, water treatment plant and distribution system; T, tap water; D, cup filler of dental chairs; B, biofilm; MA and MC, mineral water), "x" as the number of the site, "C" the sampling campaign (A, B or C), M as the culture media (R, R2A; H, Plate Count Agar; P, Pseudomonas Isolation agar; T, Tergitol 7-agar; F, mFC; RA, R2A with ampicillin; PA, PIA with ampicillin), and "n" as the number of the isolate. Bar, 1 substitution per 100 nt positions. The antibiotic resistance profiles: R, resistant; I, intermediary; S, susceptible phenotype. Classes of antibiotics: BL, beta-lactam; P, polymyxin; E, epoxide; Ri, rifampicin; Sf, sulphonamide; Q, quinolone, and A, aminoglycoside. MAR, resistance to three or more classes of antibiotics (number of resistances to different classes of antibiotics); and RT, resistance type.

* Species that gave non-concordant identifications by the partial sequence analysis of the different genes (16S rRNA, *rpo*D, *rpo*B and *gyr*B).

Nalidixic acid (NA, 30 µg) and streptomycin (STR, 10 µg) phenotypes were determined by disk diffusion. With the ATB PSE EU (08): ticarcillin (TIC, 16 mg L^{-1}); ticarcillin-clavulanic acid (TCC, 16/2 mg L^{-1}); ceftazidime (CAZ, 4 and 8 mg L^{-1}); cefepime (FEP, 4 and 8 mg L^{-1}), meropenem (MER, 2 and 8 mg L^{-1}), colistin (COL, 2 mg L^{-1}), fosfomycin (FOS, 32 mg L^{-1}), rifampicin (RFA, 4 and 16 mg L^{-1}), and cotrimoxazol (TSUM, 4/76 mg L^{-1}).

Intrinsic resistance was observed to cephalothin (30 µg) and no resistance phenotypes to tetracycline (30 µg) by disk diffusion. Piperacillin (16 mg L^{-1}), piperacillin plus tazobactam (16/4 mg L^{-1}), imipenem (4 and 8 mg L^{-1}), levofloxacin (1 and 2 mg L^{-1}), ciprofloxacin (0.5 - 2 mg L^{-1}), gentamicin (GEN, 4 mg L^{-1}), tobramycin (TOB, 4 mg L^{-1}), and amikacin (AKN, 8 and 16 mg L^{-1}) phenotypes were always susceptible in the ATB gallery.

P. aeruginosa, recovered from the biofilm, was the only isolate from this cluster without resistance to any beta-lactam. The RT-E grouped 11 isolates susceptible to cotrimoxazol, a differentiating characteristic of this cluster. Resistance to ticarcillin and ticarcillin plus clavulanic acid, ceftazidime, cefepime fosfomycin and colistin was variable in this group.

Strains with identical ST and distinct antibiotic resistance phenotypes were rarely observed and such differences were related with the loss or gain of resistance to colistin or fosfomycin. This was observed in isolates identified as *P. simiae* (ST18), *P. moraviensis* (ST12) and *P. chlororaphis* (ST6) (Figure 6.1). For this reason ST18 and ST12 were split into distinct resistance types (RT-C and RT-B, and RT-A and RT-B, respectively).

6.4. Discussion

The first motivation for this study was the gap found in the literature about the *Pseudomonas* species diversity and respective antibiotic resistance phenotypes in drinking water. One of the aims was to determine the origin of the bacteria isolated from the end points of the drinking water treatment/distribution system and to compare the cultivable *Pseudomonas* of the drinking water network with that found in mineral water. Considering that the analysis based on the comparison of the partial sequences of housekeeping genes is considered one of the best approaches to differentiate strains at the genotype level (Mulet *et al.*, 2010), in the current study, the genes 16S rRNA, *rpo*D, *rpo*B and *gyr*B were used to track *Pseudomonas* spp. in different water samples. Previous studies reporting a high resolution of *Pseudomonas* spp. based on these *loci* (Yamamoto *et al.*, 2000; Ait Tayeb *et al.*, 2005; Mulet *et al.*, 2009; Bennasar *et al.*, 2010; Mulet *et al.*, 2010) supported the decision on use of these housekeeping genes to fulfill the purpose of

the current study. In agreement with previous reports, in the current work the genes *rpo*D and *gyr*B presented the highest resolution, followed by the *rpo*B and 16S rRNA *loci* (Table 6.3). Mulet *et al*. (2010) inferring the phylogeny of *Pseudomonas* isolates, including 107 type strains, reached identical conclusions. Based on such results, those authors proposed that the use of 16S rRNA, *gyr*B and *rpo*D concatenated gene sequences supports a reliable phylogenetic analysis of *Pseudomonas*. As suggested by Mullet *et al*. (2010), in the current study the partial *rpo*B gene sequence was included to improve the resolution, although in most of the cases it proved useless in such respect. The same was concluded for *rec*A and ITS gene sequences which did not bring additional discrimination. The resolving power of the multi-locus approach used in the current study was confirmed by PFGE, bringing additional robustness to the analysis made.

Considering the threat source-to-tap, a total of 29 sites were sampled, from raw surface water to the final consumer (household taps, cup filler of dental chairs and biofilm). Curiously, *Pseudomonas* could not be isolated from 17 of those sampling sites although it was possible to isolate other bacteria. Indeed, from those sites, were recovered other *Proteobacteria* such as members of the genera *Acinetobacter*, *Sphingomonas*, *Sphingobium*, *Ralstonia*, among others (Silva *et al.*, 2011; Vaz-Moreira *et al.*, 2011b; Falcone-Dias *et al.*, in press; Narciso da Rocha *et al.*, submitted for publication). Noting also the scarcity of *Pseudomonas* in some drinking water samples, Ribas *et al.* (2000) associated the decrease in the number of these bacteria with the increase of chlorine concentration. Based on these findings, the authors proposed the use of *Pseudomonas* as indicators of microbial regrowth in water distribution systems. Given that in the current study *Pseudomonas* were not detected in samples colonized by other *Proteobacteria*, it is suggested that this bacterial genus may be not the most representative indicator.

Based on the physicochemical characteristics of the water samples analyzed in this study, for a matter of results organization, distinct categories of general types of water were defined (W, T, D, Bf and M). Overall, it was observed that distinct *Pseudomonas* species colonized each of these general types of water. Additionally, when the same species was found in more than one type of water, the isolates were represented by different sequence types (Table 6.3). Although a larger number of isolates would be necessary to consolidate this conclusion, the data suggest that the major source of *Pseudomonas* in tap water is not the water source. This conclusion leads to the hypothesis that re-colonization by *Pseudomonas* may occur downstream the water treatment plant. Indeed, this same conclusion is supported by the fact that the same ST and PFGE profile was isolated from different taps, some of them in houses up to 25 km apart, although fed by the same main pipeline water reservoir. Nevertheless, the re-colonization is not the only possible explanation. Indeed, some *Pseudomonas* spp. may have not been isolated in the upstream sampling points due to their low numbers and/or viability. For instance, the production of bacteriocins by related bacteria or even by *Pseudomonas* spp. is a possible explanation (Parret and De Mot, 2002). Also the occurrence of regrowth downstream the chlorination can be hypothesized (Ribas *et al.*, 2000). Indeed, in tap water, chlorine concentrations may decrease to levels that support regrowth. In particular, the ingestion by free-living amoebae may aid these bacteria to overcome chlorine stress (King *et al.*, 1988; Greub and Raoult, 2004). Indeed, the role of free-living amoebae as reservoirs and protectors of bacteria, including *Pseudomonas,* in water systems, is documented and may contribute to increase the survival and dissemination (King *et al.*, 1988; Thomas *et al.*, 2008; Loret and Greub, 2010; Thomas *et al.*, 2010).

Although the species pattern in each type of water may be related with the biotic and abiotic characteristics of that habitat, the isolation conditions may introduce some bias.

For example, the use of culture media supplemented with the antibiotic amoxicillin is the most plausible explanation for the detection of some of the species only in mineral water. Indeed, *P. moraviensis*, *P. frederiksbergensis*, *P. mandelii*, and *P. thivervalensis* were only isolated on culture media supplemented with the antibiotic amoxicillin, but not on the same culture medium without antibiotic. Thus, the presence of a selective pressure (amoxicillin) may have contributed to recover species that would be outcompeted in the absence of the antibiotics.

Although the percentages of resistance to some antibiotics (cephalothin, ticarcillin, ticarcillin with clavulanic acid) were above 80 % and, thus, indicative of a possible natural phenotype, antibiotic resistance prevalence in the *Pseudomonas* analyzed in the current study was, in general, low. This is in agreement with Ruiz *et al*. (2004) who observed a higher antibiotic susceptibility in environmental than in clinical *P. aeruginosa*. In turn, these findings contrast with what was observed, for instance, in sphingomonads (Vaz-Moreira *et al.*, 2011b). In spite of the low antibiotic resistance prevalence in the water *Pseudomonas* examined in this study, the comparison of those values in the different general types of water may suggest the influence of the environmental conditions on the selection or development of given resistance types. The clearest examples of this situation were for the antibiotics nalidixic acid and streptomycin which resistance was significantly higher in tap water (Figure 6.1). Although the resistance acquisition by horizontal gene transfer can never be disregarded, the importance of the vertical resistance transmission (through the line of descendants) is evidenced by the results obtained. Indeed, the resistance patterns differences coincided mainly with the *Pseudomonas* species detected in each general type of water (Figure 6.1). For example, the higher percentages of resistance to streptomycin, rifampicin, fosfomycin and nalidixic acid in tap water than in WTP and distribution system were related with the

predominance of *P. chlororaphis*, *P. toyotomiensis*, *P. nitroreducens* and *P. aeruginosa* in tap water (Figure 6.1). On the other hand, the absence of ceftazidime resistance in tap water may be related with the absence of *P. simiae*, since ceftazidime resistance was predominantly observed in this species, which was not isolated from tap water. Also the significantly higher (p<0.05) colistin resistance in mineral water than in tap water may be related with the *P. veronii* and *P. arsenicoxydans* isolates since colistin resistance was characteristic of these species, which were recovered from mineral water but not from tap water. Thus, given the fact that each type of water presents a distinct species profile and each species may show a distinct antibiotic resistance profile, the contrasts observed on antibiotic resistance profiles in each general type of water may be due to the ecology of *Pseudomonas* in water habitats. This same conclusion was evidenced in previous studies with other bacterial groups. Figueira *et al*. (2011b) observed for *Aeromonas* recovered from a drinking and waste water treatment plant that the antimicrobial resistance patterns were primarily function of the prevailing species. Similar results were obtained by Vaz-Moreira *et al*. (2011b) in a study with *Sphingomonadaceae* recovered from the same drinking water network.

The final aim of this study was to infer about the role of these bacteria as antibiotic resistance reservoirs in drinking water. In general, the results put in evidence the importance of vertical transmission on the spreading of antibiotic resistance in aquatic environments. Indeed, if horizontal gene transfer was involved, resistance patterns would be associated with sites (general types of water). In contrast, in case of vertical transmission, the resistance patterns would be species-related, as indeed was observed. Thus, some environmental factors may favor the development of given species/phenotypes, promoting a vertical transmission of antibiotic resistance phenotypes. Additionally, the occurrence of some antibiotic resistance phenotypes exclusively in tap

water hints the entrance of resistant bacteria at an uncertain stage of the threat source-to-tap. Nevertheless, the meaning of these findings to assume tap water as a possible route of antibiotic resistance transmission to the final consumer is not clear at the moment.

6.5. Conclusions

This study contributed for the knowledge of the *Pseudomonas* spp. diversity and antibiotic resistance phenotypes in drinking water. The main conclusions achieved were:

- From more than half of the sites analysed it was not possible to isolate *Pseudomonas* spp., although other *Proteobacteria* were successfully cultured from the same samples.
- In general, each type of water was characterized by a distinct profile of *Pseudomonas* species and the same genotype was never found in different types of water.
- The *Pseudomonas* detected in the water supplied to the consumer (taps, dental chairs and biofilm) were not the same detected in the water source.
- It was presumed that external sources of contamination, regrowth or amoebae protection may explain the emergence of different sequence types after disinfection relief.
- Antibiotic resistance in water *Pseudomonas* presented low prevalence values and was mainly species-related.

7. *Bacillus purgationiresistens* sp. nov., isolated from a drinking water treatment plant

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7.1. Abstract

A Gram-positive, aerobic, non-motile, endospore-forming rod, designated DS22^T, was isolated from a drinking-water treatment plant. Cells were catalase- and oxidase-positive. Growth occurred at 15–37 ºC, at pH 7–10 and with <8 % (w/v) NaCl (optimum growth: 30 ºC, pH 7–8 and 1–3 % NaCl). The major respiratory quinone was menaquinone 7, the G+C content of the genomic DNA was 36.5 mol% and the cell wall contained meso-diaminopimelic acid. On the basis of 16S rRNA gene sequence analysis, strain DS22^T was a member of the genus *Bacillus*. Its closest phylogenetic neighbours were *Bacillus horneckiae* NRRL B-59162^T (98.5 % 16S rRNA gene sequence similarity), *Bacillus oceanisediminis* H2^T (97.9 %), *Bacillus infantis* SMC 4352-1^T (97.4 %), *Bacillus firmus* IAM 12464^T (96.8 %) and *Bacillus muralis* LMG 20238^T (96.8 %). DNA–DNA hybridization, and biochemical and physiological characterization allowed the differentiation of strain DS22^T from its closest phylogenetic neighbours. The data supports the proposal of a novel species, *Bacillus purgationiresistens* sp. nov.; the type strain is DS22^T (=DSM 23494^T=NRRL B-59432^T=LMG 25783^T).

7.2. Introduction

Gram-positive endospore-forming bacteria of the genus *Bacillus* are widespread in nature and can be found in a large variety of environments, such as terrestrial and aquatic habitats, clinical samples and even spacecraft assembly facilities. Such ubiquity is also reflected in the phenotypic and phylogenetic diversity of this genus, which currently includes more than 150 species (Logan *et al.*, 2009).

In many world regions, drinking-water treatment involves the disinfection processes of ozonation and chlorination. Such treatments are known to impose dramatic changes in the water's bacterial population, markedly by a shift to Gram-positive bacteria, namely endospore formers of the genus *Bacillus* (Norton and LeChevallier, 2000).

7.3. Materials and Methods

A single strain, designated DS22^T, was isolated from a drinking water treatment plant located in northern Portugal. In this plant, the water is collected from a river basin and treated by initial filtration, ozonation and treatment with activated carbon followed by a final disinfection with chlorine. According to our data (unpublished), this treatment reduces the number of total cells by 99 % and the number of cultivable bacterial counts by about 98 %. After membrane filtration of 1 L water from the final reservoir, strain DS22^T was isolated on mannitol salt agar (MSA; Pronadisa). The isolate was purified by subcultivation on plate count agar (PCA; Pronadisa), which contains (L^{-1}): 5 g tryptone, 2.5 g yeast extract, 1 g glucose, 15 g agar. Cultures were incubated at 30 ºC. Strain DS22^T was preserved at -80 ºC in nutrient broth with 15% (v/v) glycerol.

Colony and cell morphology, Gram-staining, cytochrome *c* oxidase and catalase tests, endospore production, motility and casein hydrolysis were analysed according to the methodologies of Murray *et al*. (1994) and Smibert and Krieg (1994). Additional

phenotypic characterization was based on methods described previously (Vaz-Moreira *et al.*, 2007b; Vaz-Moreira *et al.*, 2010). Conditions for growth were tested at 6–40 ºC, with 0.1–10.0 % (w/v) NaCl and at pH 5.0–10.5. Biochemical and nutritional tests were performed using the API 20NE, API ZYM and API 50CH systems inoculated with API 50 CHB/E medium (bioMérieux), according to the manufacturer's instructions. Additionally, the assimilation of L-alanine, L-histidine, lactic acid, L-proline, propionic acid and L-serine was tested in mineral medium B supplemented with 4 mM $(NH_4)_2SO_4$ and 40 µg vitamins and nitrogenated bases l^{-1} and 5 mg amino acids l^{-1} (Vaz-Moreira *et al.*, 2007a). The Voges–Proskauer test was assayed in methyl red and Voges–Proskauer media (Oxoid) at 30 ºC for 48 h. Hydrolysis of Tween 80 and starch was tested as described by Tiago *et al*. (2004). The ability to grow on MSA and *Bacillus cereus* agar (Bio-Rad) at 30 ºC was tested after 4 days of incubation. Lecithinase activity was observed on *B. cereus* agar supplemented with egg yolk (Merck) by the formation of a translucent halo. Production of gas from glucose was tested in glucose broth (Pronadisa). Phenotypic tests, for which results may vary between different laboratories, were assayed in parallel with the reference strains *Bacillus oceanisediminis* H2^T, *Bacillus horneckiae* NRRL B-59162^T, *Bacillus muralis* DSM 16288^T, *Bacillus firmus* DSM 12^T and *Bacillus infantis* DSM 19098^T.

Endospores were observed by transmission electron microscopy. Bacteria were fixed for 4 h at 4 ºC in 2.5 % glutaraldehyde and 4 % formaldehyde (obtained from hydrolysis of para-formaldehyde) diluted with 0.1 M cacodylate buffer (pH 7.4). After washing in the same buffer, bacteria were post-fixed overnight in 2 % OsO_4 buffered with cacodylate, washed in buffer, treated with 1 % uranyl acetate for 1 h, dehydrated in increasing concentrations of ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100CXII transmission

electron microscope (60 kV). The G+C content of the genomic DNA and the respiratory quinones were analysed as described previously (Vaz-Moreira *et al.*, 2007b) using the methods of Mesbah *et al*. (1989) and Tindall (1989), respectively. The polar lipid composition was determined as described previously (Manaia *et al.*, 2004). Fatty acid methyl esters (FAMEs) were analysed using cells prepared for 24 h on tryptic casein soy agar (TSA; Pronadisa) at 28 ºC. All six strains (the isolate and the five reference strains) grew as expected and provided sufficient cells of comparable physiological age from the third streak on TSA. Cell harvesting and FAME preparation were performed as described by Kuykendall *et al*. (1988). The separation, identification and quantification of the individual FAMEs were done using the Sherlock Microbial Identification System version 4.6 (MIDI). FAMEs were extracted and analysed twice. Diaminopimelic acid isomers in whole-cell hydrolysates (4 M HCl, 100 ºC, 16 h) were analysed by TLC on cellulose plates using described solvent systems (Rhuland *et al.*, 1955; Schleifer and Kandler, 1972).

The nucleotide sequence of the 16S rRNA gene was determined after PCR amplification of total DNA extracts as described elsewhere (Ferreira da Silva *et al.*, 2007). The 16S rRNA gene sequence was compared with others available in the public databases using the FASTA package from EMBL-EBI (http://www.ebi.ac.uk). Phylogenetic analysis was conducted using MEGA version 4.0.2 (Tamura *et al.*, 2007). Sequence relatedness was estimated using the model of Jukes and Cantor (1969) and dendrograms were created using the neighbour-joining method. Tree stability was assessed by also constructing trees with the maximum-parsimony and maximum-likelihood methods. Non-homologous and ambiguous nucleotide positions were excluded from the calculations and a total of 1149 nt positions were included in the analysis. For spectroscopic DNA–DNA hybridization, cells were disrupted using a French pressure cell

(Thermo Spectronic) and DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al*. (1977). DNA–DNA hybridization was carried out as described by De Ley *et al*. (1970) under consideration of the modifications described by Huß *et al*. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6x6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian).

7.4. Results and Discussion

On TSA after 48 h at 30 ºC, strain DS22^T formed white, slightly convex colonies with irregular margins (approximately 2 mm in diameter). Growth on other media such as PCA, R2A agar (Difco) and *B. cereus* agar was slightly slower. Even though strain DS22^T was isolated on MSA, growth was not observed on this medium. This could have been because of different culture conditions, as this study isolated the strain with the membrane-filtration method, which avoids direct contact between cells and the medium, or because the NaCl concentration in MSA (7.5 %) is close to the upper limit for growth of strain DS22^T. Strain DS22^T formed subterminal endospores in a non-swollen sporangium (Figure 7.1).

The G+C content of the genomic DNA of strain DS22T was determined to be 36.5±0.12 mol% (Table 7.1). Strain DS22T had the respiratory quinone menaquinone 7 (MK-7) and the cell wall contained meso-diaminopimelic acid. The major cellular fatty acids were iso-C15:0, C16:1ω7c alcohol, anteiso-C15:0 and iso-C14:0 (Table 7.2). The polar lipid analysis showed the predominance of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (Figure 7.2), which are characteristic of the genus Bacillus (Kampfer *et al.*, 2006; Vaishampayan *et al.*, 2010; Zhang *et al.*, 2010). The unidentified phospholipids found in strain DS22T are also present in *B. horneckiae*

NRRL B-59162T (Vaishampayan *et al.*, 2010) and included three phospholipids and two aminophospholipids. One of the aminophospholipids has also been found in *B. subtilis* DSM 10^T (Kampfer *et al.*, 2006). However, the glycolipid β-gentiobiosyldiacylglycerol, which is present in *B. subtilis* DSM 10^T (Kampfer *et al.*, 2006), was not detected in strain $DS22^T$.

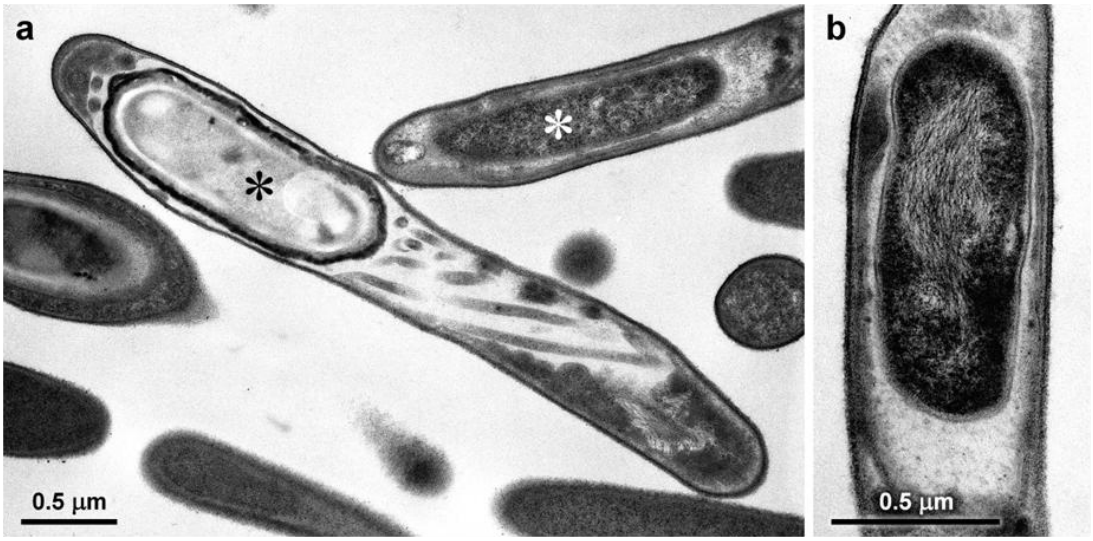

Figure 7.1. Transmission electron micrographs of cells of strain $DS22^T$. (a) Cells after growth for 2 days at 30 ºC on nutrient agar, showing cell morphology and endospore positions (asterisks). (b) Detail of an endospore. Bars, 0.5 mm.

The chemotaxonomic characterization of strain $DS22^T$ was confirmed by the results of the 16S rRNA gene sequence analysis. In the neighbour-joining tree, strain $DS22^T$ was placed in a cluster within the genus *Bacillus* (Figure 7.3). Strain $DS22^T$ was most closely related to *B. horneckiae* NRRL B-59162^T (98.5 % 16S rRNA gene sequence similarity), *B. oceanisediminis* $H2^T$ (97.9 %), *B. infantis* SMC 4352-1^T (97.4 %), *B. firmus* IAM 12464^T (96.8 %) and *B. muralis* LMG 20238^T (96.8 %).

Table 7.1. Distinctive characteristics of strain DS22^T and its closest phylogenetic neighbours

Strains: 1, *Bacillus purgationiresistens* sp. nov. DS22^T; 2, *B. horneckiae* NRRL B-59162^T; 3, *B. oceanisediminis* H2^T; 4, *B. muralis* DSM 16288^T; 5, *B. firmus* DSM 12^T; 6, *B. infantis* DSM 19098^T. Data are from this study unless otherwise indicated. +, Positive; W, weakly positive; 2, negative; ND, no data available.

	1	**2**	**3**	**4**	**5**	**6**
Colony pigmentation	White	White	White	Pink	White	Pink
Growth on/at:						
Mannitol Salt Agar	-	+	+	-	-	+
40 ºC	-	+	+	+	+	+
9 % NaCl	-	+	+	-	-	+
Nitrate reduction	-	+	+	-*	+	-
Cytochrome *c* oxidase	+	-	+	+	-	-
Hydrolysis:						
Starch	-	-	+	-*	-*	+
Aesculin	-	-	-	+	-	+^{w}
Assimilation:						
D-Glucose	-	-	+	+	+	+
L-Arabinose	-	-	-	+	-	-
D- Mannose	-	-	-	+	-	-
D-Mannitol	-	-	-	+	+	+
N-Acetylglucosamine	-	-	+	+	+	+^{w}
D-Maltose	-	+	+	+	+	+
Potassium Gluconate	-	+	+	+	-	+
Adipate	-	+	-	-	-	-
Malate	-	+	+	-	+^{w}	+
Citrate	-	+	+*	+	-	-
L-Alanine	-	+	+	+	-	-
L-Histidine	-	+	+	+	-	-
Lactic Acid	-	-	-*	+	+	+
L-Proline	-	+	-	+	+	-
Propionic Acid	-	-	-	+	-	-
L-Serine	-	+	+	+	+	-
Enzymes produced:						
Alkaline phosphatase	-	+	+	+	-	-
Leucine arylamidase	+	+	+	+	+	-
Trypsin	-	-	-	-	-	+
α-chymotrypsin	+	+	+	+	-	+^{w}
Acid phosphatase	-	+	-	-	-	-
β-galactosidase	-	-	-*	+	-	+
α-glucosidase	-	-	+	+	+	+
DNA G+C content (mol%)†‡	36.5	35.6 (Tm)[a]	44.8 (Tm)[b]	n.a.	46.1-47.4[d]	40.8 (Tm)[e]
Isolation Source‡	Treated water	Clean room[a]	Sediment[b]	Mural painting[c]	Soil[d]	Sepsis[e]

*Differs from the original description.

†DNA G+C values for columns 1 and 5 were determined by HPLC; values for columns 2, 3 and 6 were determined by thermal denaturation.

‡Data were taken from: *a*, Vaishampayan *et al*. (2010); *b*, Zhang *et al*. (2010); *c*, Heyrman *et al*. (2005); *d*, Sneath (1986); *e*, Ko *et al*. (2006).

Table 7.2. Cellular fatty acid compositions of strain DS22^T and its closest phylogenetic neighbours

Strains 1, *Bacillus purgationiresistens* sp. nov. DS22^T; 2, *B. horneckiae* NRRL B-59162^T; 3, *B. oceanisediminis* H2^T; 4, *B. muralis* DSM 16288^T; 5, *B. firmus* DSM 12^T; 6, *B. infantis* DSM 19098^T. All data were taken from this study.
Cells were cultivated on TSA at 28 ºC for 24 h. -, Not detected.

Fatty acid (%)	**1**	**2**	**3**	**4**	**5**	**6**
Saturated straight-chain						
$C_{14:0}$	0.9	0.7	6.4	3.9	13.4	1.7
$C_{15:0}$	-	-		-	-	-
$C_{16:0}$	0.5	0.8	13.5	4.5	15.7	0.9
$C_{16:0}$ N alcohol	-	-	-	-	0.3	-
Unsaturated straight-chain						
$C_{16:1}$ ω5*c*	-	-	1.4	-	3.7	-
$C_{16:1}$ ω11*c*	2.5	2.1	13.1	9.4	14.6	1.5
$C_{16:1}$ ω7*c* alcohol	15.2	9.8	3.2	1.9	1.2	1.5
Summed feature 3*	-	-	1.4	-	1.2	-
Saturated branched-chain						
iso-$C_{13:0}$	0.2	-	-	0.3	-	0.2
iso-$C_{14:0}$	9.2	4.1	3.9	3.8	2.3	1.3
iso-$C_{15:0}$	41.9	51.1	27.7	27.7	23.9	49.2
iso-$C_{16:0}$	6.7	5.9	4.1	1.1	1.7	1.3
iso-$C_{17:0}$	1.1	3.2	1.6	1.2	0.9	1.4
anteiso-$C_{13:0}$	-	-		0.3	-	0.1
anteiso-$C_{15:0}$	14.2	13.5	15.8	42.1	16.5	29.9
anteiso-$C_{17:0}$	2.3	3.3	3.4	1.0	2.5	5.4
Unsaturated branched-chain						
iso-$C_{15:1}$$\omega$9*c*	-	-	-	0.5	-	0.5
iso-$C_{17:1}$$\omega$10*c*	1.8	2.7	0.8	1.5	0.4	1.3
Summed feature 4*	3.5	2.9	2.0	0.9	1.5	3.7

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}$$\omega$7c and/or $C_{16:1}$$\omega$6c. Summed feature 4 consisted of iso-$C_{17:1}$ and/or anteiso-$C_{17:1}$.

Strain DS22^T was compared with the reference strains with respect to phenotypic properties (Table 7.1) and cellular fatty acid composition (Table 7.2), which allowed the observation of distinctive features. Strain DS22^T could be distinguished phenotypically from all of the reference strains by its inability to grow at 40 ºC and assimilate any of the tested carbon sources, from *B. muralis* DSM 16288^T and *B. infantis* DSM 19098^T by colony colour, from *B. horneckiae* NRRL B-59162^T, *B. oceanisediminis* H2^T and *B. firmus* DSM 12^T by its inability to reduce nitrate, and from *B. horneckiae* NRRL B-

59162^T by its inability to produce acid from API 50CH carbon sources. Strain DS22^T could also be distinguished from the reference strains on the basis of differences in the fatty acid composition, mainly because of the proportions of $C_{16:1}\omega7c$ alcohol, iso-$C_{14:0}$ and iso- $C_{15:0}$. The phenotypic and fatty acid data suggested that strain DS22^T represented a distinct novel species.

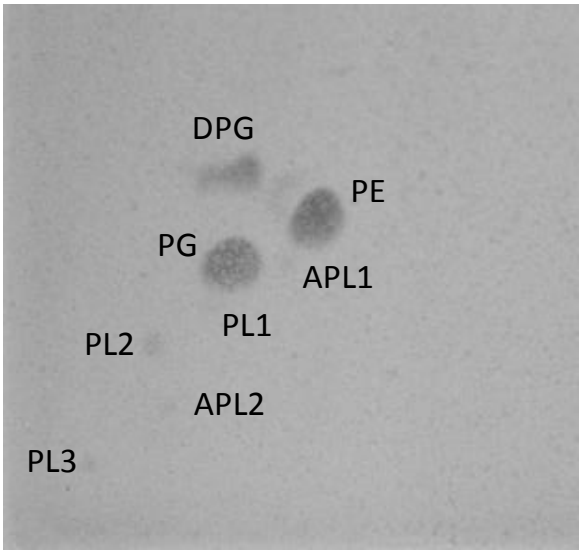

Figure 7.2. Polar lipid profile of strain DS22^T after separation by two dimensional TLC, spraying with 50 % (v/v) aqueous sulfuric acid and charring at 160 ºC for 25 min. APL, Unknown aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unknown phospholipid.

Low DNA–DNA relatedness was observed between strain DS22^T and its closest neighbours: 13.6 and 7.6 % with *B. oceanisediminis* H2^T, 32.3 and 30.0 % with *B. horneckiae* NRRL B-59162^T, 17.5 and 12.9 % with *B. muralis* DSM 16288^T, 13.8 and 13.7 % with *B. infantis* DSM 19098^T, and 16.6 and 8.1 % with *B. firmus* DSM 12^T. These values were clearly below the threshold of 70 % DNA–DNA relatedness recommended for the definition of bacterial species (Wayne *et al.*, 1987).

On the basis of the differentiation of strain DS22^T from its closest phylogenetic neighbours by 16S rRNA gene sequence analysis, DNA–DNA relatedness, cellular fatty acids and physiological characters, strain DS22^T is proposed to represent a novel species, *Bacillus purgationiresistens* sp. nov.

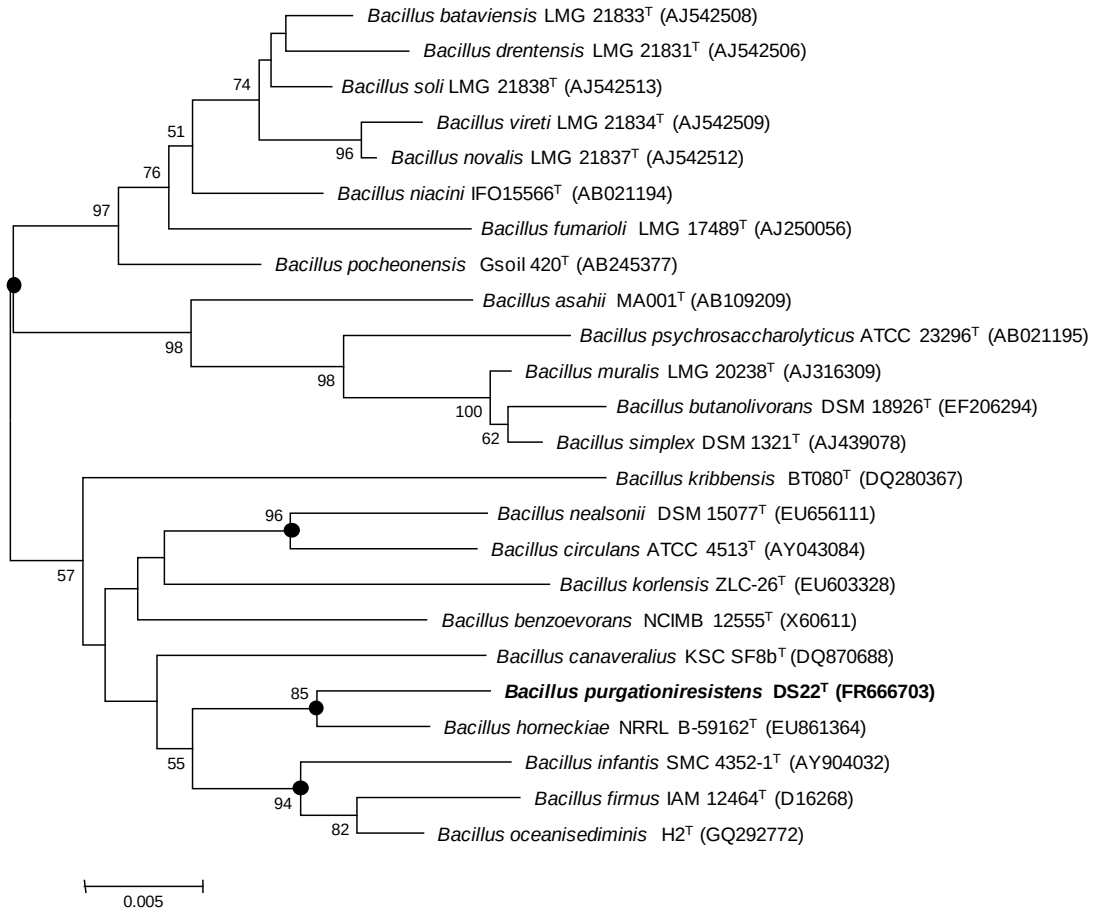

Figure 7.3. Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences, showing the relationships of strain DS22^T with members of the genus *Bacillus*. Bootstrap values (≥50 %) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. Bar, 1 substitution per 200 nt.

7.5. Description of *Bacillus purgationiresistens* sp. nov.

Bacillus purgationiresistens (pur.ga.ti.o.ni.re.sis'tans. L. n. *purgatio -onis* a cleansing, purification; L. part. adj. *resistens* resisting; N.L. part. adj. *purgationiresistens* resisting cleansing, purification).

Colonies are white and slightly convex (~2 mm diameter) with irregular edges on TSA after 48 h at 30 ºC. Forms pink colonies on *B. cereus* agar. Rods (3.5 mm long and 0.5 mm wide in very young cultures) are non-motile, aerobic and Gram-positive with subterminal endospores in a non-swollen sporangium. Catalase- and cytochrome *c* oxidase-positive. Grows at 15–37 ºC, at pH 7–10 and with , 8 % NaCl (optimum growth at about 30 ºC, pH 7–8 and 1–3 % NaCl). Does not grow on MSA. Nitrate is not reduced. Citrate is not used. H_2S, indole and acetoin are not produced. No fermentation or gas production from D-glucose. Gelatin, Tween 80 and casein are hydrolysed, but starch and aesculin are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and lecithinase are produced, but arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase, acid phosphatase, alkaline phosphatase, lipase (C14), cystine arylamidase, trypsin, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, N-acetyl-b-glucosaminidase, α-mannosidase and α-fucosidase are not produced. Acid is not produced from any of the carbon sources in the API 50CH system. None of the carbon sources in the API 20E and API 20NE systems are oxidized or assimilated; also, L-alanine, L-histidine, lactic acid, L-proline, propionic acid and L-serine tested in mineral medium are not assimilated. The predominant cellular fatty acids are iso-$C_{15:0}$, $C_{16:1}\omega7c$ alcohol, anteiso-$C_{15:0}$ and iso-$C_{14:0}$ and the major respiratory quinone is MK-7 (100 %). The predominant polar lipids are phosphatidylethanolamine,

phosphatidylglycerol and diphosphatidylglycerol. The peptidoglycan contains mesodiaminopimelic acid.

The type strain is DS22^{T} (=DSM 23494^{T}=NRRL B-59432^{T}=LMG 25783^{T}), isolated from water of the final reservoir of a drinking-water treatment plant. The DNA G+C content of the type strain is 36.5 mol%.

8. General Discussion

Antibiotic resistance determinants have an ancient presence in the environment, even in places with minimal human activities impact (D'Costa *et al.*, 2011). Although antibiotic resistance is recognized as a natural phenomenon, antibiotic resistance genes present in the environment have increased and diversified over the last years (Davies and Davies, 2010; Knapp *et al.*, 2010). In part, it is believed that such a transformation results from selective pressures imposed by the clinical use of antibiotics (since the 1940's). Nowadays, not only antibiotics but also antibiotic resistance genes and resistant bacteria are recognized environmental pollutants (Kümmerer, 2004; Pruden *et al.*, 2006; Martinez, 2009). In consequence, different entities are focused on the assessment of the risks associated with the increase of these forms of pollution and in which way this may affect the environmental equilibrium and especially the human health.

Based on the argument that many of the antibiotics are produced by environmental microorganisms, some authors believe that antibiotic resistance genes of clinical relevance have an environmental origin (Alonso *et al.*, 2001; Martinez, 2008; Cantón, 2009; Allen *et al.*, 2010; Wright, 2010; Lupo *et al.*, 2012). These considerations led to the proposal of the concept of environmental antibiotic resistome (D'Costa *et al.*, 2006), which includes the whole set of antibiotic resistance genes. In areas colonized by humans, the environmental antibiotic resistome is composed of both naturally existing and human-activities-derived antibiotic resistance genes (Wright, 2010; Lupo *et al.*, 2012). Undoubtedly, it is hard, if possible, to separate both components of the environmental antibiotic resistome. Nevertheless, it is possible to identify relevant environmental hotspots for antibiotic resistance emergence and dissemination. Good examples are

municipal and hospital waste waters, drinking and recreational waters, food products and urban wild animals (Ferreira da Silva *et al.*, 2006; Ferreira da Silva *et al.*, 2007; Kassem *et al.*, 2008; Poeta *et al.*, 2008; Faria *et al.*, 2009; Soge *et al.*, 2009; Literak *et al.*, 2010; Novo and Manaia, 2010; Simões *et al.*, 2010). Water plays a particularly important role as source and vehicle of dissemination of antibiotic resistant bacteria. Treated waste waters are discharged into natural water courses, usually surface waters. Untreated surface waters are used to irrigate agriculture soils, vegetable products, and feed and/or raise (aquaculture) animals. Surface and ground waters are also used to produce drinking water.

Beside these multiple activities referred to above, water is also the residence and landing area for numerous urban and wild animals. Therefore, water is: i) colonized by a myriad of microorganisms, of human, other animals, and environmental origin, including antibiotic resistant bacteria (Armstrong *et al.*, 1981; Schwartz *et al.*, 2003; Pavlov *et al.*, 2004; Zhang *et al.*, 2009); ii) unconfined, meaning that it is in constant movement (in the urban water cycle) and can reach many different places (Marsalek *et al.*, 2006); iii) a place where many pollutants, mainly due to human contamination, can exert selective pressures (Hernandez *et al.*, 1998; Beaber *et al.*, 2004; Baquero *et al.*, 2008). All these characteristics make of water a hot-spot for antibiotic resistance propagation. In terms of human health, the contamination of drinking water is one of the major concerns. Nevertheless, up to now did not exist clear evidences that the drinking water may represent a source of resistance to humans. Human gut microbiome studies reveal an impressive diversity of known and unknown resistance genes that may constitute a substantial reservoir of antibiotic resistance genes to pathogens (Sommer *et al.*, 2009). The ingestion of bacteria dwelling in water, with unknown resistance genes, may be part of the explanation for their occurrence in the human gut, and the urban water cycle may

have an important role in this process. It is important, however, to emphasize that ingestion is not the only route for water bacteria transmission. Its use for hygienic purposes or the recreational contact with water may constitute other routes. Indeed, numerous opportunistic infections originated by water bacteria are reported in the scientific literature (Rusin *et al.*, 1997; Leclerc *et al.*, 2002; Nwachcuku and Gerba, 2004; Brunkard *et al.*, 2011). The impressive diversity of water bacteria and the close contact with humans, give strong support to the hypothesis that water may represent a relevant source of antibiotic resistant bacteria to humans.

The study of the variations of total and cultivable bacterial communities over the part of the urban water cycle under study (from raw drinking water up to the household taps) was thus considered a priority (chapter 3). *Proteobacteria* (mainly *Alpha-*, *Beta-* and *Gammaproteobacteria*) was the predominant phylum in all the sampled transect, as revealed by PCR-DGGE band sequence analysis. *Proteobacteria* were previously referred to as predominant in drinking water samples (Williams *et al.*, 2004; Hoefel *et al.*, 2005b; Eichler *et al.*, 2006; Poitelon *et al.*, 2009; Kahlisch *et al.*, 2010; Manuel *et al.*, 2010; Revetta *et al.*, 2010; Kahlisch *et al.*, 2012; Lymperopoulou *et al.*, 2012). Using culture-independent methods it was observed that drinking water treatment did not influence the total bacterial community structure, although affected the pattern of cultivable bacteria and cultivability rates. Gram-negative bacteria, the major targets of disinfection, presumably due to their cell structure and physiology (Norton and LeChevallier, 2000), were, nevertheless, predominant in tap water. The data gathered in this study did not indicate if this re-emergence of Gram-negative bacteria in tap water was due to the regrowth of inactive or dormant bacteria, as has been suggested by some authors (Niquette *et al.*, 2001; Xi *et al.*, 2009; Lautenschlager *et al.*, 2010), or to successive portals of entry of these bacteria in the distribution system. Indeed, some bacterial groups

not detected at the water source or distribution system (e.g. *Bosea* sp., *Nitrobacter* sp.) were detected downstream at tap level. None of the variables location, age, and municipal network supplying system was shown to influence the variations in density, cultivable pattern and the bacterial community structure at the tap level. The myriad of factors that may influence the bacterial diversity in tap water hamper the definition of prediction indicators. Factors such as biofilm formation and/or the ingestion of bacteria by free-living amoebae as well as chlorine concentration, pipes materials and age, periods of water stagnation, and temperature can influence the development of bulk water bacteria (Bartram *et al.*, 2003; Chu *et al.*, 2003; Greub and Raoult, 2004; Lehtola *et al.*, 2004; Wijeyekoon *et al.*, 2004; Ndiongue *et al.*, 2005; Thomas *et al.*, 2008; Kormas *et al.*, 2010; Lautenschlager *et al.*, 2010; Loret and Greub, 2010), with consequent bacterial community restructuration, particularly after chlorination and at the tap level. These findings support the need to develop adequate material validation methods, recommendations and spot tests for in-house water facilities (Lautenschlager *et al.*, 2010). In spite of the conclusion that each tap may prefigure a specific case, the general predominance of Gram-negative bacteria, most of them *Proteobacteria* of the divisions *Alpha-*, *Beta-* and *Gamma-*, in tap water leaves an important message – these bacteria may be important sources of antimicrobial resistance.

The use of culture-dependent and culture-independent methods to characterize microbial communities became a common place. In the present study it was important to understand how cultivable bacteria, those that could be characterized for their antibiotic resistance phenotypes, mirrored the total bacteria in the community. This type of analysis would permit to assess which were the bacterial groups being disregarded when using cultivable-dependent methods. Indeed, despite the glimpse given by the comparison of the cultivable patterns with the DGGE band sequence profiling, it was not known if, and

at what extent, cultivable and total bacterial populations coincided. This rationale motivated the comparison of a bacterial community based on16S rRNA gene sequence-454-pyrosequencing and 16S rRNA-DGGE analyses, and cultivable bacteria (chapter 4). Although the same predominant bacterial phyla were detected with the different approaches, not surprisingly the culture-dependent method allowed a lower coverage of the bacterial diversity than the culture-independent methods. Some phyla, including the *Cyanobacteria, Planctomycetes, Verrucomicrobia, Acidobacteria, Aquificae, Firmicutes* were overlooked by culture dependent methods. But, what was surprising and meaningful in this context was that in general, different operational taxonomic units were targeted by each method. Even when members of the same phyla were targeted by both approaches (culture-dependent and independent), distinct bacteria were identified. The almost total absence of coincidence between culture-dependent and culture-independent bacterial surveys, suggests that continuous efforts to isolate drinking water microbiota members should not be regarded as an outdated aim.

The approach used in this study to assess the relevance of tap water as antibiotic resistance supplier or reservoir was the tracking of bacteria that being prevalent in tap water were also detected upstream (in the treatment plant and distribution system). Among these were detected members of the genera *Pseudomonas, Acinetobacter*, *Ralstonia*, *Mycobacterium*-like, *Flavobacterium*, *Sphingomonas, Sphingobium, Methylobacterium* and *Lysinibacillus*, genera commonly described as present in drinking water (Kuhn *et al.*, 1997; Koskinen *et al.*, 2000; Norton and LeChevallier, 2000; Leclerc *et al.*, 2001; Biscardi *et al.*, 2002; Hoefel *et al.*, 2005b; Furuhata *et al.*, 2007; Kampfer *et al.*, 2008; Pablos *et al.*, 2009; Palleroni, 2010). The aim was to assess the potential of these bacteria to disseminate antibiotic resistance via drinking water and to assess whether the disinfection, storage and distribution could represent critical points for

resistance emergence or proliferation. Based on these criteria two groups, belonging to the most predominant phylum, *Proteobacteria*, were examined in this study – *Sphingomonadaceae* (chapter 5) and *Pseudomonas* spp. (chapter 6).

The water treatment process used to achieve drinking water conditions of salubrity (ozonation, chlorination, and others) are supposed to impose selective pressures that may increase the percentage of antibiotic resistance, through the selection of the antibiotic resistant bacteria (Armstrong *et al.*, 1981; Armstrong *et al.*, 1982; Xi *et al.*, 2009). Nevertheless, the good practices recommend that drinking water must be produced with suitable chemical and microbiological quality, ensuring the absence of fecal contamination and a low density of microorganisms (WHO, 2008). However, nothing is stated about antibiotic resistance. It is assumed that in these conditions the risks are reduced, because the antibiotic resistant bacteria, if present, would be with high probability, in low numbers. Nevertheless, this study (chapter 5 and 6) and others (Armstrong *et al.*, 1981; Armstrong *et al.*, 1982; Shehabi *et al.*, 2006; Pathak and Gopal, 2008; Xi *et al.*, 2009; Figueira *et al.*, 2012), showed that antibiotic resistant bacteria colonize treated drinking water, and the abundance is not as low as though before. Consequently, the hypothesis that tap water can act as an antibiotic resistance reservoir, and represent an important mode of transmission of antibiotic resistant bacteria to humans should not be discarded (Faria *et al.*, 2009; Xi *et al.*, 2009; Figueira *et al.*, 2012; Narciso da Rocha *et al.*, submitted for publication). In fact, from the source to the taps, it was observed the presence of antibiotic resistant bacteria of different taxonomical groups, such as *Sphingomonadaceae*, *Brevudimonas*, *Ralstonia*, *Cupriavidus*, *Achromobacter*, *Pseudomonas*, *Acinetobacter*, *Staphylococcus*, among others (Faria *et al.*, 2009; Narciso da Rocha *et al.*, submitted for publication; our studies, unpublished). In contrast, *Aeromonas* spp., with high prevalence of resistance to ticarcillin, cephalothin and

streptomycin, were only observed in the raw surface water and after the ozonation, being not detected after the chlorination (Figueira *et al.*, 2011b). This suggest that the capacity to act as a reservoir of antibiotic resistance results from a complex interplay of factors, including the ecology and physiology of bacteria and the whole set of abiotic conditions. In fact, among the bacteria recovered from tap water, a wide array of antibiotic resistance patterns was observed and often it was genus- or species-related, which may suggest distinct mechanisms or paths of resistance acquisition for the different taxa and also the importance of vertical resistance transmission in water bacteria. For instance, compared to the *Sphingomonadaceae*, a group of bacteria shown to be among the dominant from the raw up to the tap water, the *Pseudomonas* and *Acinetobacter* spp. presented lower values of antibiotic resistance prevalence. It is also noteworthy that in these three bacterial groups (*Pseudomonas*, *Acinetobacter* and *Sphingomonadaceae*) some antibiotic resistance phenotypes were significantly more prevalent or detected only at tap level.

Some bacterial species and antibiotic resistance phenotypes detected in tap water, were not observed in the water treatment plant. This suggests that these bacteria may have been "hidden" during the treatment process (for example by the amoebae ingestion), and entered in the circuit along the distribution system or at household level, or that the acquisition of resistance may have occurred after the water disinfection. This, along with the detection of different ST at the water source and in tap water, leads to the conclusion that the water source is not the direct supplier of the antibiotic resistance detected in tap water. Nevertheless, these bacteria may be important for human health. First, because being resistant to antibiotics, these bacteria have an increased potential as opportunistic pathogens, mainly for immunocompromised people. Second, because these bacteria are potential reservoirs of antibiotic resistance.

Most of the bacteria that colonize drinking water, for instance pseudomonads (non-*P. aeruginosa*), *Acinetobacter* spp. (non-*Acinetobacter baumannii*) or sphingomonads, have not been intensively studied in what respects antibiotic resistance genes and acquisition mechanisms. Additionally, most of the studies on antibiotic resistance in drinking water have been performed with resource to cultivation techniques (Armstrong *et al.*, 1981; Pavlov *et al.*, 2004; Xi *et al.*, 2009). Although the search of antibiotic resistance genes in the total DNA could offer a culture-independent survey of the resistome in drinking water, such an analysis would be biased by the search of already known resistance genes (Manaia *et al.*, 2012). The detection of resistance phenotypes in bacteria which are not normally studied under this perspective may offer new insights for the search of novel genetic determinants of resistance. The functional metagenomic studies can bring some important avenues to study the antibiotic resistome in drinking water, since it overcomes the limitations of the methods based on culturing and amplification (Berry *et al.*, 2006; Gilbert *et al.*, 2011; Schmieder and Edwards, 2012). This approach was already used for the analysis of the resistome of soils, being observed a greater genetic diversity than the previously accounted for, and also a higher diversity than can be surveyed by a culture-independent method (Riesenfeld *et al.*, 2004).

9. Main Conclusions

The main conclusions of this work can be summarized as follows:

- The culture-dependent and culture-independent methods target different sets of organisms, which makes their combined use an advantage for analyses of bacterial diversity;
- The changes in the bacterial community imposed by the drinking water treatment were detected mainly with the culture-dependent approach, which evidenced a clear shift in the composition of the bacterial cultivable population, from Gram-negative to Gram-positive, including acid-fast, bacteria;
- The reduction of the counts of total and cultivable heterotrophic bacteria, and of the bacterial cultivability and diversity, imposed by the water treatment, were reverted at the tap water level. This, and the fact that, in general, the bacteria detected downstream the water treatment had no apparent origin in the water source, suggests the existence of external sources of contamination, or low densities in raw water and subsequent bacterial regrowth and/or biofilm formation after the disinfection relief;
- Variations in the diversity and structure of the bacterial communities of tap water samples were observed, probably due to differences in the specific conditions of each household tap. This result evidences the need for the development of methods to monitor tap water quality;
- *Proteobacteria* (mainly of the classes *Alpha*, *Beta* and, in a lesser extent, *Gammaproteobacteria*) was the predominant phylum from the water source to the tap. *Actinobacteria*, *Cyanobacteria*, *Planctomycetes* and *Bacteroidetes* were also frequent before water chlorination;

- *Sphingomonadaceae* and *Acinetobacter* are widespread along the drinking water circuit, from the source to the tap. However, other genera with a generally assumed widespread occurrence in waters, such as *Aeromonas*, were rarely found or absent in tap water;
- Some of the *Proteobacteria*, present all over the water circuit, as *Pseudomonas*, *Acinetobacter*, and mainly *Sphingomonadaceae*, proved to be potential reservoirs of antibiotic resistance;
- Some resistance phenotypes, not detected in the water source or distribution system were detected in tap water. Examples are resistance to ampicillin-sulbactam, piperacillin plus tazobactam-pyocyanin, imipenem, ceftazidime, cefepime, gentamicin or tobramycin in *Sphingomonadaceae*, or to streptomycin and rifampicin in *Pseudomonas* spp.;
- The antibiotic resistance patterns were mainly species related rather than with the site of isolation or the strain, suggesting the importance of vertical resistance transmission in water bacteria;
- Drinking water disinfection, mainly chlorination, can originate an extremely harmful environment to which only some bacteria can survive. Habitats like this may hide bacteria still belonging to the immense group of the unknown. *Bacillus purgationiresistens* sp. nov. was one of such examples.

In summary, it is possible to conclude that drinking water may be an important hotspot for the proliferation of antibiotic resistant bacteria, which can be transmitted to the final consumer.

10. Proposals for Future Work

The present study brought some glimpses on bacterial diversity and antibiotic resistance prevalence in drinking water. The hypothesis about the role of these bacteria as potential vehicles for dissemination of antibiotic resistance is now more robust. Some other questions that should be addressed in future studies come out in parallel with conclusion:

- The role of other bacteria cultivated from drinking water such as *Mycobacterium*-like, *Burkholderia*, *Ralstonia* and *Methylobacterium* (data not shown) as vehicles of resistance dissemination needs to be assessed.
- It is important to close the urban water cycle, tracking antibiotic resistant bacterial isolates of taxonomic groups abundant in different types of water, including drinking water (e.g. *Shingomonadaceae*). Such analysis should include the inflow of waste water treatment plants, effluents and the receptor water body, and drinking raw and tap water. The results obtained would shad some light on the possibility that waste waters may constitute as a vehicle of antibiotic resistance dissemination from the humans to the environment and from environment to humans.
- Study the potential of the uncultivable fraction of the drinking water microbiota as reservoir of antibiotics resistance. The study of the antibiotic resistance gene expression in the whole bacterial community may constitute an important indication of the active resistome in environments submitted to selective pressures, such as the treated waters.

- Study some resistance mechanisms poorly characterized, mainly for some of the environmental isolates recovered from drinking water that could act as opportunistic pathogens, as for example some members of the *Sphingomonadaceae* family. Even for the species not recognized as opportunistic pathogens, this study could be of great importance since these organisms may work as antibiotic resistance vehicles in the environment. The complete genome sequence and annotation for some of these organisms should also be important, since in many cases only few strains are poorly annotated up to now.
- Functional metagenomic studies can also bring important insights into the microbiomes and antibiotic resistomes variability for the different water bodies along the urban water circuit, e.g. drinking water, waste water and freshwater.

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