Universitat Autònoma de Barcelona

Departament de Genètica i Microbiologia

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MOLECULAR CHARACTERIZATION OF MICROBIAL DIVERSITY IN WASTEWATER TREATMENT AND REUSE

Laura Garrido Molina

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MOLECULAR CHARACTERIZATION OF MICROBIAL DIVERSITY IN WASTEWATER TREATMENT AND REUSE

Memòria de Tesi presentada per obtenir el grau de Doctor en Microbiologia per la Universitat Autònoma de Barcelona, per Laura Garrido Molina.

Vist i plau dels Directors de la Tesi,

Dr. Jordi Mas Gordi

Dra. Olga Sánchez Martínez

A mis padres y a Julio

“The role of the infinitely small in nature is infinitely large”

Louis Pasteur

CONTENTS

SUMMARY

Wastewater generation is an inevitable consequence of human activities. These activities modify the characteristics of the original waters, contaminating them and invalidating subsequent application for other uses. Dumping of raw sewage causes damage, sometimes irreversible, to the environment. On the other hand, the discharge of untreated sewage poses risks to public health. Thus, wastewater treatment becomes an important need in our society. Wastewater treatment plants constitute one of the most important biotechnological processes used worldwide to treat municipal and industrial sewage. From a biological point of view, microorganisms play an important role in wastewater treatment since they are the main responsible of the remove of organic matter and nutrients from this sewage. Furthermore, water reuse is becoming increasingly important as a component of sustainable management of water resources.

In this thesis we investigated the composition of microbial communities in different environments related to wastewater treatment and reuse. First, we studied and compared the microbial communities found in conventional processes with two atypical treatment systems (activated sludge from a seawater-processing EDAR and constructed wetlands). On the other hand, we determined how different was the microbial profile of a treated wastewater *versus* a natural freshwater, and tried to find out an alternative indicator of water quality. Finally, we examined the microbial population associated with the problems arising from water reuse.

Samples of a marine activated sludge were analyzed by using the full-cycle rRNA approach (denaturing gradient gel electrophoresis, clone libraries and *in situ* hybridization), obtaining a picture of the composition of the community completely different from a conventional activated sludge, with particularly a low presence of *Betaproteobacteria*. In addition, detection of the functional gene *amoA* corroborated the presence of ammonia oxidizers, corresponding probably to

different genera from those described in the literature. On the other hand, the microbial communities found in experimental constructed wetlands belonged to species related with organic matter removal. The study of the profiles of these microbial communities allowed determining the effect of the type of plant, hydraulic design and organic matter load of these systems in their composition. Comparing our atypical treatment systems with conventional ones, we can conclude that each system has its specific microbial community, adapted to its environmental conditions, with the same functions being carried out by different groups.

Besides, examination of microbial diversity profiles of treated wastewater from different EDARs and comparison with natural non-contaminated water showed that sewage effluents have a common fingerprint, which differs from natural water. Thus, taking into account the most abundant groups found in these types of waters, we proposed the use of the *Bacteroidetes*, *Gammaproteobacteria* and *Nitrospira* / *Betaproteobacteria* ratio as an alternative indicator of ecological water quality.

Concerning water reuse, we focused on an example of a biofouling problem derived from the use of reclaimed water in a drip irrigation system. In these biofilms the microbial community was mainly composed of thermophilic and sporulated microorganisms, suggesting that high temperatures within the irrigation infrastructure selected these populations. Thus, it seemed that the conditions in the system may be more important than the composition of reclaimed water itself.

RESUM

La generació d'aigua residual és una conseqüència inevitable de les activitats humanes. Aquestes activitats modifiquen les característiques de les aigües de consum, contaminant-les i invalidant la seva posterior aplicació per altres usos. Abocar aquestes aigües sense depurar ocasiona danys, a vegades irreversibles, al medi ambient. D'altra banda, l'abocament d'aigües no depurades suposa riscos per la salut pública. Per aquesta raó, la depuració de les aigües residuals esdevé una necessitat important a la nostra societat. Les estacions depuradores d'aigües residuals (EDARs) constitueixen un dels processos biotecnològics més importants utilitzats arreu del món per depurar aigües residuals municipals i industrials. Des d'un punt de vista biològic, els microorganismes juguen un paper important en la depuració de les aigües residuals ja que són els principals responsables de l'eliminació de la matèria orgànica i dels nutrients d'aquestes aigües. D'altra banda, la reutilització de l'aigua s'està convertint en un aspecte cada vegada més important de la gestió sostenible dels recursos hídrics.

En aquesta tesi s'ha estudiat la composició de les comunitats microbianes en diferents ambients relacionats amb la depuració d'aigües residuals i la seva reutilització. Per un costat, s'han estudiat i comparat les comunitats microbianes trobades en processos convencionals de depuració amb dos sistemes atípics de depuració d'aigua residual (un fang activat provinent d'una EDAR que processa aigua de mar i uns aiguamolls construïts). Per un altre, s'han determinat les diferències en el perfil microbià d'una aigua depurada *versus* una aigua dolça natural, amb la intenció de trobar un indicador alternatiu de qualitat de l'aigua. Finalment, s'ha analitzat la població microbiana associada amb els problemes derivats de la reutilització d'aigua.

Les mostres del fang activat marí van ser analitzades utilitzant una aproximació completa per l'anàlisi de rRNA (electroforesi en gel de gradient desnaturalitzant,

genoteca i hibridació *in situ*), obtenint-se una imatge de la composició de la comunitat completament diferent a la d'un fang activat convencional, amb la particularitat que es va trobar una presència molt baixa del grup *Betaproteobacteria*. A més, la detecció del gen funcional *amoA* va corroborar la presencia d'oxidadors d'amoni, que probablement eren de generes diferents als descrits en la literatura. Per un altra banda, les comunitats microbianes trobades en aiguamolls construïts experimentals pertanyien a espècies relacionades amb l'eliminació de matèria orgànica. L'estudi dels perfils d'aquestes comunitats va permetre determinar l'efecte del tipus de planta, el disseny hidràulic i la càrrega de matèria orgànica d'aquests sistemes en la seva composició. Comparant els nostres sistemes atípics de depuració amb els sistemes convencionals, es va poder concloure que cada sistema té la seva comunitat microbiana específica, adaptada a les seves condicions ambientals, duent a terme les mateixes funcions però amb diferents grups implicats.

Tanmateix, l'examen dels perfils de diversitat microbiana d'aigües depurades provinents de EDARs i la seva comparació amb els perfils d'aigües naturals no contaminades mostrava que els efluents de depuradora tenen una empremta comú, que difereix de la d'una aigua natural. Segons això, tenint en compte els grups taxonòmics trobats més abundants en aquest tipus d'aigües, proposem l'ús de la ratio *Bacteroidetes*, *Gammaproteobacteria* i *Nitrospira* / *Betaproteobacteria* com a indicador alternatiu de qualitat ecològica de l'aigua.

Pel que fa a la reutilització d'aigües, es va estudiar un exemple d'un problema de formació de biofilms derivat de l'ús d'aigua regenerada en un sistema de reg per goteig. En aquests biofilms la comunitat microbiana estava principalment composada per microorganismes termòfils i esporulats, la qual cosa suggereix que les altes temperatures dins de la infrastructura de reg van seleccionar aquestes poblacions. Per tant, sembla ser que les condicions en el sistema de reg poden ser més importants que la composició de la pròpia aigua regenerada.

RESUMEN

La generación de agua residual es una consecuencia inevitable de las actividades humanas. Estas actividades modifican las características de las aguas de consumo, contaminándolas e invalidando su posterior aplicación para otros usos. Verter esta agua sin depurar ocasiona daños, a veces irreversibles, al medio ambiente. Por otro lado, el vertido de aguas no depuradas supone riesgos para la salud pública. Por esta razón, la depuración de las aguas residuales se convierte en una necesidad importante en nuestra sociedad. Las estaciones depuradoras de aguas residuales (EDARs) constituyen uno de los procesos biotecnológicos más importantes utilizados en todo el mundo para depurar aguas residuales municipales e industriales. Desde un punto de vista biológico, los microorganismos juegan un papel importante en la depuración de las aguas residuales ya que son los principales responsables de la eliminación de la materia orgánica y de los nutrientes de estas aguas. Por otra parte, la reutilización del agua se está convirtiendo en un componente cada vez más importante de la gestión sostenible de los recursos hídricos.

En esta tesis se ha estudiado la composición de las comunidades microbianas en diferentes ambientes relacionados con la depuración de aguas residuales y su reutilización. Por una parte, se han estudiado y comparado las comunidades microbianas encontradas en procesos convencionales de depuración con dos sistemas atípicos de depuración de agua residual (un fango activado proveniente de una EDAR que procesa agua de mar y unos humedales construidos). Por otro lado, se han determinado las diferencias en el perfil microbiano de un agua depurada *versus* un agua dulce natural, con la intención de encontrar un indicador alternativo de calidad de agua. Finalmente, se ha analizado la población microbiana asociada con los problemas derivados de la reutilización de agua.

Las muestras del fango activado marino fueron analizadas utilizando una aproximación completa para el análisis de rRNA (electroforesis en gel de gradiente desnaturalizante, genoteca e hibridación *in situ*), obteniéndose una imagen de la composición de la comunidad completamente diferente a la de un fango activado convencional, con la peculiaridad de encontrar una presencia muy baja del grupo *Betaproteobacteria.* Por otra parte, la detección del gen funcional *amoA* corroboró la presencia de oxidadores de amonio, que probablemente eran de géneros diferentes a los descritos en la literatura. Por otro lado, las comunidades microbianas encontradas en humedales construidos experimentales pertenecían a especies relacionadas con la eliminación de materia orgánica. El estudio de los perfiles de estas comunidades permitió determinar el efecto del tipo de planta, el diseño hidráulico y la carga de materia orgánica de estos sistemas en su composición. Comparando nuestros sistemas atípicos de depuración con los sistemas convencionales, se pudo concluir que cada sistema tiene su propia comunidad microbiana específica, adaptada a sus condiciones ambientales, llevando a cabo las mismas funciones pero con diferentes grupos implicados.

Además, el examen de los perfiles de diversidad microbiana de aguas depuradas provenientes de diferentes EDARs y su comparación con los perfiles de aguas naturales no contaminadas mostraba que los efluentes de depuradora tienen un patrón común, que difiere del de un agua natural. Según esto, teniendo en cuenta los grupos taxonómicos más abundantes encontrados en este tipo de aguas, proponemos el uso de la ratio *Bacteroidetes, Gammaproteobacteria* y *Nitrospira / Betaproteobacteria* como indicador alternativo de calidad ecológica del agua.

Por lo que respecta a la reutilización de aguas, se estudió un ejemplo de un problema de formación de biofilms derivado del uso de agua regenerada en un sistema de riego por goteo. En estos biofilms la comunidad microbiana estaba principalmente compuesta por microorganismos termófilos y esporulados, lo que sugiere que las altas temperaturas dentro de la infraestructura de riego

seleccionaron estas poblaciones. Por tanto, parece ser que las condiciones en el sistema de riego pueden ser más importantes que la composición de la propia agua regenerada.

GENERAL INTRODUCTION

WATER AND HUMAN ACTIVITY

Water covers 70.9% of the Earth's surface, and it is vital for all known forms of life. On Earth, 96.5% of the planet's water is found in oceans, 1.7% in groundwater, 1.7% in glaciers and the ice caps of Antarctica and Greenland, a small fraction in other large water bodies, and 0.001% in the air as vapor, clouds and precipitation. Only 2.5% of the Earth's water is represented by freshwater, and 98.8% of that water is found in ice and groundwater. Less than 0.3% of all freshwater is a part of rivers, lakes, and atmosphere (Fig. 1).

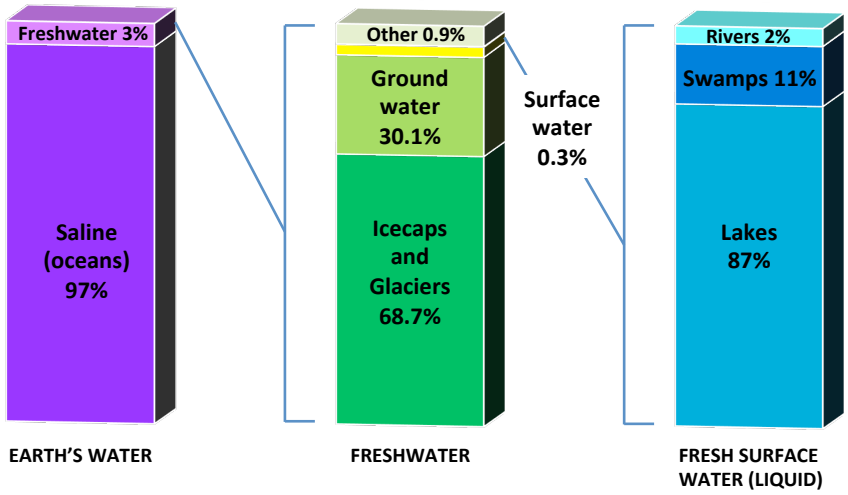

Figure 1. Earth's water distribution

Only a small fraction of freshwater is available to humans, but it is essential for their lifes, and wastewater generation is an inevitable consequence of human activities. These activities modify the characteristics of the original waters, contaminating them and invalidating subsequent application for other uses. Dumping of raw sewage causes damage, sometimes irreversible, to the environment. On the other hand, the discharge of untreated sewage poses risks to public health.

The amount of wastewater generated in built-up urban areas is in direct proportion to the consumption of freshwater supplies, and this consumption is related to the degree of economic and social development, due to the fact that a higher development leads to greater and more diverse use of water in human activities. According to the United Nations Development Programme the minimum water requirement for human health (hygiene, drinking, etc) is 20L per day per person. In a developing country a person consumes 20L/day while in the EU and U.S., consumption ranges between 200 and 600L/day (UNDP, 2006; Morató *et al*., 2009). Between 60 and 80% of the water supply consumed becomes wastewater, thus resulting in a high production, particularly in developed countries. Thus, considering the finite nature of freshwater resources, as well as the increased generation of sewage, wastewater treatment becomes an important need in our society.

In this line, the European Directive 91/271/EEC establishes the necessary measures that member states must adopt to ensure that urban wastewater receives adequate treatment before discharge. The Directive establishes two distinct obligations; first, the "urban agglomerations" shall provide, as appropriate, suitable systems for collecting and conducting wastewater and, secondly, shall supply different wastewater treatments before its discharge to continental or marine environments. In accordance with this Directive, in Spain there are approximately 1888 wastewater treatment plants associated with urban agglomerations (according to the Ministry of Agriculture, Food and Environment, at the date of January 2010).

Wastewater treatment is one of the most important biotechnological processes, which is used worldwide to treat municipal and industrial sewage. The conventional way to carry it out is in a Wastewater Treatment Plant (WWTP, Fig. 2), where a series of physical, chemical and biological processes occur aimed at

reducing the concentration of contaminants and later on discharging the treated effluent, minimizing risks for the environment and the populations.

Figure 2. Picture of Baix Llobregat WWTP (Barcelona)

(http://www.amb.cat/web/emma/aigua/sanejament/depuradores/depuradora_llobregat)

CONVENTIONAL TREATMENTS: WWTPs

The major contaminants found in wastewater are biodegradable organic compounds, volatile organic compounds, recalcitrant xenobiotics, toxic metals, suspended solids, nutrients (nitrogen and phosphorus), and microbial pathogens and parasites. In the beginning, the requirements for treatment plants were the removal of organic matter and suspended solids. Research efforts are now being focused on the removal of nutrients (N, P), odors, volatile organic compounds, metals, and toxic organics after their passage through wastewater treatment plants.

In general, the main objectives of wastewater treatment processes can be summarized in the following items (Bitton, 2005):

- Reduction of the organic content of wastewater (i.e., reduction of BOD).
- Removal/reduction of trace organics that are recalcitrant to biodegradation and may be toxic or carcinogenic.
- Removal/reduction of toxic metals.
- Removal/reduction of nutrients (N,P) to reduce pollution of receiving surface waters or groundwater if the effluents are applied onto land.
- Removal or inactivation of pathogenic microorganisms and parasites.

To target these goals wastewater treatment comprises the following four steps:

1. Preliminary treatment: The objective of this operation is to remove thick objects, sand and grease by physical processes to avoid problems in the later stages of treatment, such as clogging of pipes, valves and pumps, equipment wear, crusting, etc. Basic operations of this stage are:

- Screening: removal of solid small and medium size by bar screens and reduction of suspended solids content through its filtration by means of a thin support provided with passage slots.
- Grit removal: removal of heavy materials larger than 0.2 mm, to prevent sediment in channels and pipes and to protect pumps and other components from abrasion.
- Fat and grease removal: removal of fat and other floating materials lighter than water.

2. **Primary treatment**: In this step sedimentable and floating materials are removed by physical and chemical processes, in order to decrease the BOD and to prepare the water to move to the secondary treatment. The most common primary treatments are:

- Primary sedimentation: removal of sedimentable solids under the action of gravity using primary sedimentation tanks.
- Physico-chemical treatment by coagulation-flocculation processes: increase the reduction of suspended solids by the addition of chemical reagents.

3. Secondary treatment (or Biological treatment): the main objective is to degrade organic matter and thus reduce the BOD using biological treatment processes carried out by microorganisms (mainly bacteria).

Basically there are two biological methods: attached growth systems and

suspended growth systems. In the attached growth systems microorganisms are attached to a stone or plastic surface, forming biofilms that degrade the organic matter when wastewater passes through them. For example, trickling biofilters, biotowers or rotating biological contactors.

The suspended growth systems are the most common. Microorganisms are suspended in wastewater, forming flocs (complex enrichment population) named activated sludge.

There are two types of biological treatment with activated sludge: aerobic and anaerobic.

- Aerobic biological treatment: The main goal is the oxidation of the biodegradable organic matter that occurs in the aeration tank where soluble organic matter is thus converted to new cell mass and flocculation, that is, the separation of the newly formed biomass from the treated effluent. In order to carry out this oxidation a continuous aereation is crucial, since it requires high oxygen consumption (Bitton, 2005). For this reason, one disadvantage of this technology is the large energy consumption associated with this aeration. (Vidal *et al*., 2009; Metcalf and Eddy, 1995).
- Anaerobic biological treatment: in this process, the organic matter is degraded in the absence of oxygen by anaerobic bacteria, producing methane and carbon dioxide. The process is carried out in a completely closed reactor (Metcalf and Eddy, 1995). As an advantage, if this biogas is managed properly, it can be used in generators for electricity production (Navia and Vidal, 2002; Vidal *et al*., 2009). This treatment is usually applied to wastewaters that have a high load of organic matter.

4. **Tertiary or advanced treatment**: It allows obtaining a better quality final effluent that can be reused and can be dumped in areas where the requirements are more demanding. The objective in this step is to remove suspended solids,

residual organic matter, nutrients and pathogens by physical, chemical and biological processes.

To remove particulate and colloidal matter present in the treated effluents physico-chemical treatments (coagulation-flocculation) and subsequent separation steps (decanting, filtration) can be applied.

Nutrient removal (nitrogen and phosphorous) can be accomplished by chemical precipitation or by biological processes. The biological nitrogen removal is performed sequentially under oxic and anoxic conditions, resulting at the end to nitrogen release to the atmosphere in the form of nitrogen gas. The biological phosphorous removal is also performed cycling anaerobic and aerobic conditions, but phosphorous is stored in the microorganisms, which are subsequently extracted as excess sludge. It is possible to combine the two processes for co-disposal of both nutrients.

Disinfection is the process to remove or reduce pathogens. The most common disinfection process is chlorination, but there are others as UV light or ozone.

Finally, the ultimate goal of a wastewater urban station is the evacuation of treated effluents that meet the quality requirements in current regulations with the least possible environmental and economic cost. If they have achieved the degree of treatment required in each case, they can be discharged to the rivers near the treatment plant. Nevertheless, with increasing frequency, the treated effluent is directed to other uses such as reuse.

Microbiology of activated sludge

From a microbiological and biotechnological point of view, knowledge of the microorganisms that comprise activated sludge is very important. During the last decades, many studies have been carried out in order to study the composition of

the microbial communites of activated sludge and their role in the decomposition of organic matter.

Initial studies were based in optical microscopy observations (Eikelboom, 1975; Eikelboom and Buijsen, 1983) giving morphological information, especially of filamentous organisms, or by culture-dependent techniques, which showed that the major genera in the flocs were *Zooglea*, *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, *Achromobacter*, *Corynebacterium*, *Comomonas*, *Brevibacterium*, *Acinetobacter*, *Bacillus* spp., as well as filamentous microorganisms (e.g. *Sphaerotilus* and *Beggiatoa*) (Seiler and Blaim, 1982; Ueda and Earle, 1972; Bitton, 2005).

But later, it became apparent that culture-dependent techniques only detected between 1 and 15% of the microorganisms present, in comparison with the total cell numbers obtained by direct microscopic counts (Wagner *et al*., 1993). The current use of culture-independent techniques, based on the 16S rRNA gene analysis, as clone libraries (Snaidr *et al*., 1997), fingerprinting techniques such as denaturing gradient gel electrophoresis (Boon *et al*., 2002), thermal gradient gel electrophoresis (Eichner e*t al*., 1999) and terminal restriction fragment length polymorphism (T-RFLP) (Saikaly *et al*., 2005), as well as the design of group-specific rRNA-targeted oligonucleotide probes for fluorescence *in situ* hybridization (FISH) (Wagner *et al*., 1993; 1994), allowed a more comprehensive analysis of microbial diversity in wastewater research.

These cultured-independent techniques showed that the Gram negative bacteria constituted the major component of activated sludge flocs (Fig.3), being the dominant group the Beta subclass of *Proteobacteria* (*Comamonas*, *Acidovorax* or *Hydrogenophaga*). The Alpha (e.g. *Sphingomonas*) and Gamma subclasses were also found, as well as groups such as *Bacteroidetes*, the *Nitrospira*-phylum, *Chloroflexi*, *Planctomycetes* and Gram positive bacteria with high G+C DNA content

(*Actinobacteria*).

Some of these microorganisms are responsible of nitrogen and phosphorus removal processes in activated sludge. The mechanisms that perform nitrogen removal in these systems are nitrification and denitrification. Nitrification is the oxidation of ammonia to nitrite and nitrite to nitrate respectively, and it is carried out by nitrifying bacteria. Denitrification is the process that converts the products of nitrification into gaseous nitrogen, and it is performed by denitrifying bacteria. Phosphorous removal is mainly conducted by polyphosphate accumulating organisms (PAOs), which store polyphosphate intracellularly.

In order to detect the microorganisms responsible of these processes, linking diversity with function, FISH with oligonucleotide specific probes directed against rRNA of ammonia, nitrite oxidizers and PAO cluster, or PCRs with specific primers for functional genes (such as the ammonia monooxygenase gene *amoA*) have been utilized (Wagner and Loy, 2002; Wagner *et al*., 2002; Juretschko *et al*., 1998; Crocetti *et al*., 2000; Eschenhagen *et al*. 2003; Sánchez et al. 2011). In these studies the microorganisms identified in nitrification processes were Beta and Gammaproteobacteria ammonia-oxidizers, such as the *Nitrosomonas europaea*/ *Nitrosomonas eutropha*-linage (Beta), the *Nitrosococcus mobilis*-lineage (Beta), the *Nitrosomonas marina* cluster (Beta), and the *Nitrosococcus* group (Gamma). Also *Nitrobacter* and *Nitrospira* have been recognized as nitrite-oxidizers. Besides, several denitrifiers have been identified belonging to the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus*, *Hyphomicrobium*, as well as many members of the betaproteobacterial order Rhodocyclales. On the other hand, members of *Microlunatus* spp. and *Tetrasphaera* spp. (*Actinobacteria*), and uncultured Betaproteobacteria related to Rhodocyclus group (*Candidatus* "Accumulibacter phosphatis") were recognized as PAOs.

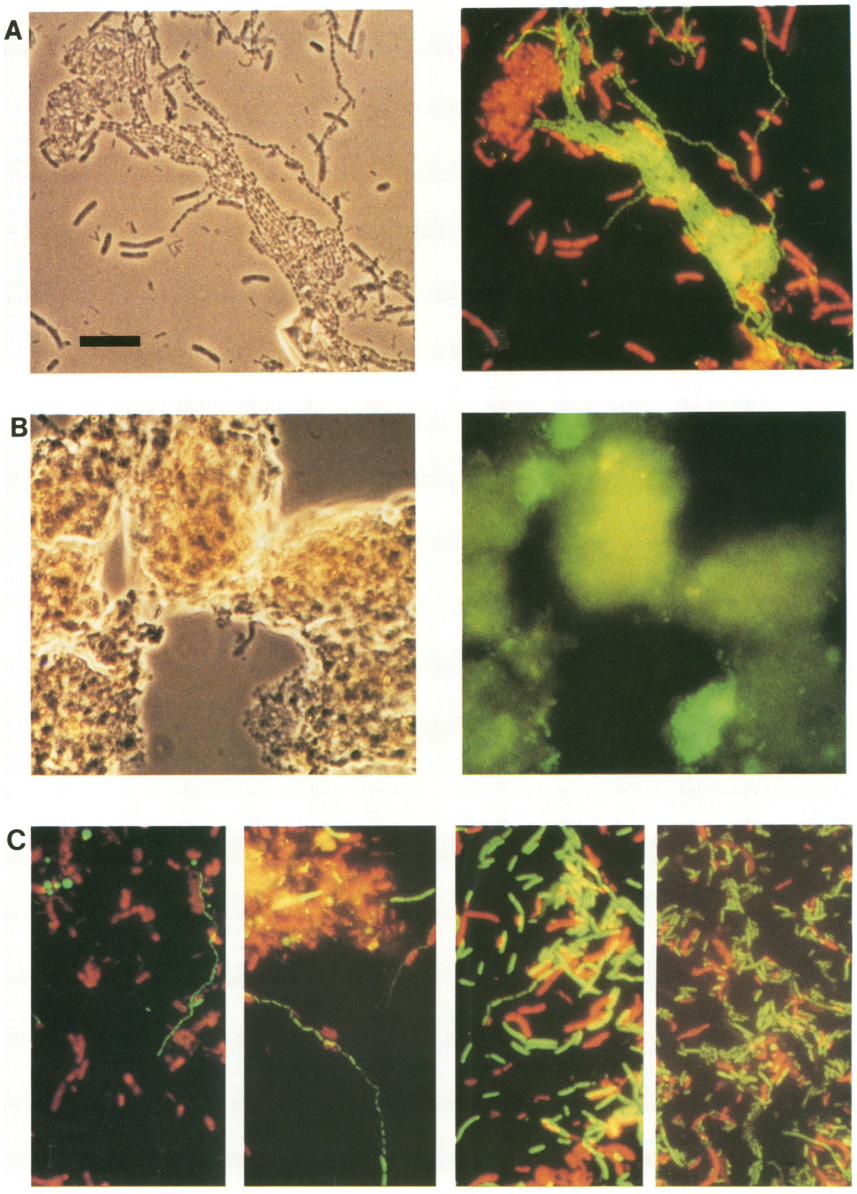

Figure 3. In situ hybridization of activated sludge samples. Phase-contrast (left) and epifluorescence (right) micrographs are shown for identical microscopic fields. (A) Simultaneous hybridization with fluorescein-labeled probe GAM (fluorescein, green) and tetramethylrhodaminelabeled probe BET (rhodamine, red). (B) Hybridization with fluorescein-labeled probe ALF. (C) In situ hybridization of a nutrient-amended activated sludge sample (Wagner *et al.,* 1993).

WASTEWATER TREATMENTS WITH NON-CONVENTIONAL TECHNONOGIES

Non-conventional technologies are those that achieve the elimination of pollutants from wastewater through natural processes and mechanisms that do not require external energy or chemical additives. In these systems a high number of decontamination processes are executed by synergy of different communities of organisms.

In recent decades, non-conventional techniques have been increasingly used due to their design and operation characteristics: the investment cost is often competitive, require little maintenance staff, have no energy cost or the need for pumping head is reduced, and do not generate large amounts of sludge continuously. However, while non-conventional techniques are less energy intensive than conventional processes, they are less compact and require a much higher treatment surface (García and Corzo, 2008).

The processes involved in non-conventional technologies include many of those applied in conventional treatments (sedimentation, filtration, adsorption, chemical precipitation, ion exchange, biological degradation, etc.) along with processes typical of natural treatments (photosynthesis, photooxidation, assimilation by plants, etc.). In other words, the processes underlying the conventional and non-conventional technologies are similar in their basis, but differ in the energy usage. While the conventional process technologies occur sequentially in tanks and reactors with accelerated rates due to the contribution of energy, the non-conventional technologies operate at natural speed (without energy input), developing the processes in a single reactor. This process enables significant energy savings but requires a larger surface area.

Non-conventional technologies are characterized by using natural methods for the oxygenation of the wastewater to be treated; for this reason, the costs associated with this operation are zero or very small. These natural methods of oxygenation

include: photosynthesis (ponds), the diffusion of oxygen by the roots of emergent plants (constructed wetlands) and waterlog-drying cycles (green filters, constructed wetlands and peat filters) (Salas *et al*., 2007).

Under the denomination of non-conventional technologies we can distinguish:

- Wastewater treatment by the use of land, such as surface application systems like green filters. They use an area of land planted with trees, which is then flooded with the wastewater to be treated. Purification is the result of physical, chemical and biological actions (Fahd *et al*., 2007, Salas *et al*., 2007).
- The specific conditions that simulate natural wetlands: Constructed wetlands (CWs) in its various forms, like free flow and subsurface flow CWs (vertical and horizontal). CWs treatment systems are engineered systems that have been designed and created to utilize the natural processes but do so within a more controlled environment (Vymazal 2011).
- Those that try to mimic the natural processes of purification that occur in rivers and lakes, such as stabilisation ponds. This system uses artificial ponds to reproduce the purification processes that occur naturally in rivers and lakes. Wastewater is passed through a series of ponds in series and decreasing depth, in which there are, consecutively, anaerobic and aerobic conditions (Fahd *et al*., 2007; Salas *et al*., 2007).
- Those that are based on the filtration of wastewaters through natural coal, as peat filters. The treatment consists of passing the wastewater through beds using peat as the filtering material. The wastewater is purified through a combination of physical, chemical and biological actions. Finally the treated effluents are collected at the bottom (Fahd *et al*., 2007; Salas *et al*., 2007).

Constructed wetlands

As **Chapter 2** of this thesis refers to the use of experimental CWs and the molecular characterization of the microbial communities involved, an explanation of the different types of CWs used is detailed below.

Constructed wetlands are passive treatment systems consisting of lagoons or shallow channels (usually less than 1 m) planted with characteristic wetland vegetation (aquatic macrophytes) and the decontamination processes are executed simultaneously by physical, chemical and biological components. These wetlands can also be used to restore ecosystems and then treatment can be a secondary objective (García, 2004).

Constructed wetlands may be categorized according to the various design parameters, but the three most important criteria are (Vymazal, 2011; Fig. 4):

- Hydrology: free-water surface flow and subsurface flow
- Type of macrophytic growth: emergent, submerged, free-floating
- Flow path: horizontal and vertical

In free-water surface flow systems water is directly exposed to the atmosphere and preferably flows through the stems and leaves of plants. These types of wetlands can be understood as a modification of the natural lagoons with a depth of the water layer between 0.3 and 0.4 m, and plants. They are usually applied to improve the quality of effluents that have been previously treated in a WWTP.

In subsurface flow wetlands water circulation is underground, through a granular medium in contact with the roots and rhizomes of plants. The depth of the water level is usually between 0.3 and 0.9 m. The biofilm growing attached to the granular medium and the roots and rhizomes of plants has a major role in detoxification processes.

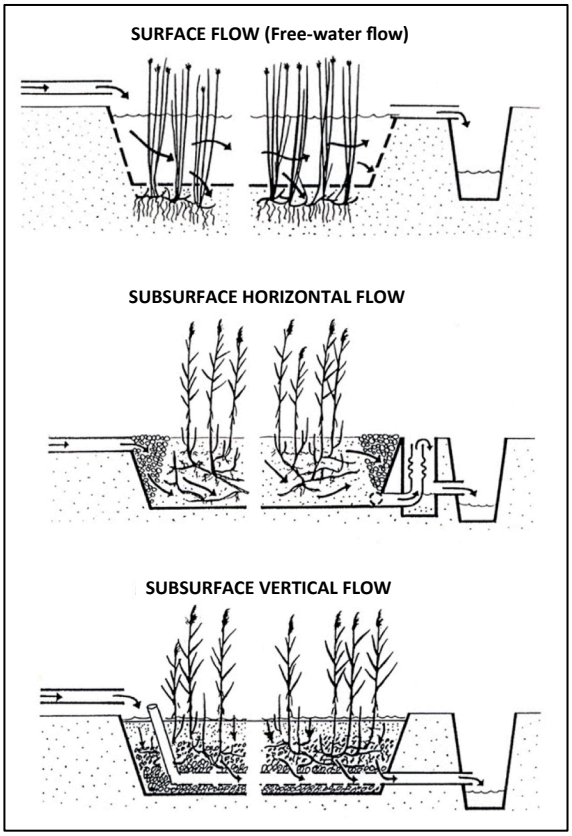

Figure 4. Scheme of flow constructed wetland surface and subsurface horizontal flow and vertical flow (García, 2004)

The main differences between subsurface flow and free-water surface flow systems are the greater treatment capacity of the former (supported higher organic load), low risk of water contact with people and development of insects. Besides, subsurface flow CWs are less useful for environmental restoration projects due to lack of available water surface (García and Corzo, 2008).

WATER DISPOSAL AND REUSE

After some of these wastewater treatments, with conventional or non-conventional techniques, the effluents can be discharged into any aquatic environment, usually the nearest river or sea. These treatments have greatly improved the quality of sewage, but we ignore the environmental impact that may have at the level of microbial communities.

The European Union has developed a guideline, the European Water Framework Directive (WFD, 60EC, 2000), which establishes a framework for community action in the field of water policy. A highlight of this directive is that it expresses the need for Community legislation covering ecological quality and requests proposals to improve the ecological quality of waters. In this particular sense, one of the chapters of this thesis (**Chapter 3**) tries to found an ecological water quality indicator by analyzing microbial community profiles over time.

Taking into account the scarce availability of hydric resources and the large amount of wastewater generated by urban areas, it is important to focus not only in an adequate treatment of these waters to be discharged to nature, but also in its reuse for human purposes.

Water reuse is the beneficial use of treated wastewater for different practices such as crop and garden irrigations or some urban applications. This procedure is becoming increasingly important as a component of sustainable management of water resources, because of the growing lack of freshwater, especially in arid areas, and in wastewater disposal regulations, which rules are being closely revised (Levine and Asano, 2004; Bitton, 2005).

In Spain the Real Decreto 1620/2007 establishes the legal framework for the reuse of treated water. The norm defines the concept of reuse, by introducing the term of **reclaimed water** (municipal wastewater that has gone through various

treatment processes to meet specific water quality criteria with the intent of being used in a beneficial manner) and determining the physicochemical and biological requirements for its application in the various permitted uses. It also establishes the precise quality requirements in each case.

The categories of water reuse that establishes the Real Decreto 1620/2007 are:

- **Urban use**: as irrigation of private gardens, urban green areas, fire systems, etc.
- **Agricultural irrigation**: that includes different categories, according to various criteria such as whether the product is for human consumption or not.
- **Industrial use**: as process water and cleaning or cooling towers.
- **Recreational use**: as irrigation of golf courses and use for different ornamental water bodies.
- **Environmental use**: as groundwater recharge, irrigation of forest or green areas not accessible to the public and other uses.

Quality criteria for the reuse of water will be different according to use, providing mandatory limits. In compliance with this RD, a series of physicochemical parameters require monitoring. The phosphorous should be controlled for environmental and recreational uses, to recharge aquifers the amount of nitrogen should be checked, and in general, for any use, is important to control suspended soils, turbidity and salinity. This last parameter must be controlled in order to prevent excessive salinization of soils and specially aquifers. For irrigation usages, will be also useful to characterize physicochemical parameters of agronomic interest. Depending on use, regarding to microorganisms and parasites, the limits are established for *Escherichia coli*, intestinal nematode eggs and *Legionella* spp. (See annex).

One important factor to be considered is the ability of microorganisms to grow in deposits and distribution systems, a fact especially enhanced by the high nutrient rates characteristic of reclaimed waters. Consequently, the correct microbiological quality of this water is not guaranteed at the point of use, even though at the point of delivery could be correct.

One of the most frequent microbiological problems in the use of reclaimed waters is biofilm formation (biofouling) on the surfaces exposed to them, such as inner surfaces of distribution ducts, storage deposits and, in general, the submerged elements of installations. Although biofilms do not constitute a health risk by themselves, can cause significant complications. In health terms, biofilms reduce the effectiveness of disinfection treatments, and they act as physicochemical barriers that hinder the action of the treatment against pathogens transmitted by water. Biofilms also allow the growth of certain microorganisms and this fact can distort the validity of their use as contamination indicators. In material terms, biofilms accelerate the corrosion of materials colonized by them and may even lead to total or partial clogging of localized irrigation systems.

AIMS OF THIS THESIS

The main aim of this thesis is the study of the composition of microbial communities in different environments related to wastewater treatment and reuse, in order to improve our knowledge and understanding of wastewater microbiology and contribute to its reutilization.

This global aim has been divided into three specific objectives.

As a first objective, this work attempts to assess to what extent the microbial communities found in different wastewater treatment systems share common features or differ from each other. For that, in **Chapters 1** and **2** we focus on the microbial community composition from two rather unconventional wastewater treatment systems, that is, activated sludge (from a seawater-processing WWTP) and constructed wetlands (a non-conventional treatment) respectively. Particularly, the bacterial and archaeal communities of activated sludge from a seawater-processing wastewater treatment plant have been investigated combining different molecular techniques in **Chapter 1**, while in **Chapter 2** samples from different experimental constructed wetlands have been analyzed in order to determine the effect of plant species, organic matter and hydraulic design on the microbial composition of rhizoplane, gravel biofilm and interstitial water. At the end of thesis, the results are discussed in relation to microbial communities from conventional WWTPs described in the literature.

As a second objective, we question whether waters that leave the treatment plants are assimilable to natural waters, that is, if their microbial profiles differ from those of non-contaminated waters. On the other hand, if these microbial profiles are similar or different depending on the incoming water. **Chapter 3** attempts to answer these questions and it concentrate in the determination of alternative indicators of water quality that go beyond conventional indicator of fecal

contamination by means of microbial diversity profiles.

Finally, as a third objective, we wanted to cover the issue of reuse of this reclaimed water and to determinate to what extent the microbial population associated to problems occurring during water reuse, are conditioned by the microbial community of the incoming water, or by the environmental conditions prevailing in the system. So **Chapter 4** offers an example of reuse of reclaimed water in agricultural irrigation, where clogging problems due to biofilm formation were usual. The analysis of biofilm composition allows drawing some interesting conclusion.

The most appropriate techniques to carry out these studies are molecular techniques based on the 16S rRNA gene since, as it is known, culture-dependent techniques only detect a very low percentage of the microbial community present in natural environments. The main technique used in this thesis was Denaturing Gradient Gel Electrophoresis (DGGE), a fingerprinting method that allows characterization and comparison of many samples at once relatively fast, offering an overview of microbial assemblage dynamics and information of the most abundant microorganisms involved. Furthermore, in **Chapter 1** it has been applied a full-cycle rRNA approach with three molecular tools such as DGGE, clone libraries and fluorescent in situ hybridization (FISH and CARD-FISH) in order to obtain an integrated picture of the diversity of the prokaryotic assemblage.

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Chapter 1

MOLECULAR CHARACTERIZATION OF ACTIVATED SLUDGE FROM A SEAWATER-PROCESSING WASTEWATER TREATMENT PLANT

Published: Microbial Biotechnology

SUMMARY

The prokaryotic community composition of activated sludge from a seawater-processing wastewater treatment plant (Almeria, Spain) was investigated by using the rRNA approach, combining different molecular techniques such as denaturing gradient gel electrophoresis (DGGE), clone libraries and *in situ* hybridization (FISH and CARD-FISH). Most of the sequences retrieved in the DGGE and the clone libraries were similar to uncultured members of different phyla. The most abundant sequence recovered from Bacteria in the clone library corresponded to a bacterium from the *Deinococcus-Thermus* cluster (almost 77% of the clones), and the library included members from other groups such as the *Alpha, Gamma-* and *Delta-* subclasses of *Proteobacteria*, the *Bacteroidetes* and *Firmicutes*. Concerning the archaeal clone library, we basically found sequences related to different orders of methanogenic *Archaea*, in correspondence with the recovered DGGE bands.

Enumeration of DAPI (4', 6-diamidino-2-phenylindole) - stained cells from two different activated sludge samples after a mechanical flocculation disruption revealed a mean cell count of 1.6×10^9 ml^{-1}. Around 94% of DAPI counts (mean value from both samples) hybridized with a *Bacteria* specific probe. *Alphaproteobacteria* were the dominant bacterial group (36% of DAPI counts), while *Beta-*, *Delta-* and *Gammaproteobacteria*, *Bacteroidetes, Actinobacteria* and *Firmicutes* contributed to lower proportions (between 0.5 – 5,7% of DAPI counts). *Archaea* accounted only for 6% of DAPI counts. In addition, specific primers for amplification of the *amoA* (ammonia monooxygenase) gene were used to detect the presence of *Beta*, *Gamma* and archaeal nitrifiers, yielding positive amplifications only for *Betaproteobacteria*. This, together with negative in situ hybridizations with probes for well-known nitrifiying bacteria suggests that nitrification is performed by still undetected microorganisms. In summary, the combination of the three approaches provided different and complementary

pictures of the real assemblage composition and allowed to get closer to the main microorganisms involved in key processes of seawater-processing activated sludge.

INTRODUCTION

Activated sludge systems are one of the most important biotechnological processes in wastewater treatment plants (WWTPs). They consist of a complex mixture of microorganisms able to remove organic substances and nutrient contaminants from municipal or industrial sewage, being thus a crucial tool in environmental protection. For years, researchers have investigated the microbial communities of activated sludge in order to understand their specific biological processes (Amann *et al*., 1998; Wagner *et al*., 2002). Studies of diversity can provide insight on the correlation between microbial composition and ecosystem function, as well as knowledge about temporal and spatial variations in microbial communities. However, the vast majority of bacteria present in activated sludge cannot be isolated by conventional culture-dependent techniques; the percentage of culturable bacteria in comparison with total cell counts is estimated to range between 1 and 15% with optimized media (Wagner *et al*., 1993).

The current use of molecular methods, that do not require the isolation and cultivation of microorganisms, has allowed a more comprehensive analysis of microbial diversity in wastewater research. Sequence analysis of 16S rRNA gene clone libraries (Snaidr *et al*., 1997), fingerprinting techniques such as denaturing gradient gel electrophoresis (Boon *et al*., 2002), thermal gradient gel electrophoresis (Eichner *et al*., 1999), and terminal restriction fragment length polymorphism (T-RFLP) (Saikaly *et al*., 2005), as well as the design of group-specific rRNA-targeted oligonucleotide probes for Fluorescence In Situ Hybridization (FISH) (Wagner *et al.*, 1993, 1994) have greatly expanded our understanding of wastewater microbiology. The cultivation-independent rRNA approach allows to

determine the composition and dynamics of microbial communities in these systems and to identify the microbial key players for the different processes.

Considerable microbial diversity has been detected in WWTPs, including bacteria involved in biological phosphorus removal (Bond *et al*., 1999; Jeon *et al*., 2003; Seviour *et al*., 2003), nitrifiers (Juretschko *et al*., 1998; Coskuner and Curtis, 2002; Otawa *et al.*, 2006), and methanogens (Zheng, 2000). Sequences from *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Actinobacteria* and the *Planctomycetes* were retrieved in significant numbers in different clone libraries (Wagner *et al*., 2002). Nevertheless, all studies of microbial diversity in WWTPs refer to freshwater treatment plants, either domestic or industrial. As far as we know, no studies have been done on WWTPs that utilize seawater for their operation.

On the other hand, only a few works have applied the full-cycle rRNA approach for the study of microbial communities in activated sludges, which includes the establishment of a 16S rRNA gene clone library, the design of a set of clone-specific oligonucleotide probes, and the determination of the abundance of the respective bacterial populations by quantitative FISH (Snaidr *et al*., 1997; Juretschko *et al*., 1998). In this paper, the prokaryotic diversity of a WWTPs from a pharmaceutical industry located in the south of Spain which has the particularity to utilize seawater has been characterized using a polyphasic approach with three molecular tools such as DGGE, clone libraries and FISH. This wastewater treatment plant is in operation since 1998 and today, very few plants of this type are running in the world; its main influent corresponds to intermediate products from amoxicillin synthesis. The use of seawater instead of freshwater responds to the defficiency in hydric resources prevailing in this location, one of the driest areas in Spain. To our knowledge, this is the first study that analyzes an activated sludge with these characteristics.

MATERIAL AND METHODS

Sampling

Samples of aerated mixed activated sludge from a seawater processing wastewater treatment plant located in Almeria (southeast Spain) were collected in December 2007 and November 2008 in a 1 L sterile bottle and stored at 4ºC until processing. The plant treats wastewater from a pharmaceutical industry and the performance of the reactor is constant in function of time, with a continuous entrance of intermediate products from amoxicillin synthesis (between 0-250 mg/L). The mean influent flow of the plant is 300 m^3/h and has a treatment volume of 32000 m^3. Nitrogen and chemical oxygen demand (COD) sludge loads were about 150-170 kg/h and 900-1000 kg/h respectively. Ionic concentrations in the influent were as follows: NH_4^+: 0.6-1.1 g/L, K^+: 121.1 mg/L, Mg^{2+}: 97.3 mg/L, Ca^{2+}: 386.6 mg/L, NO_3^-: 0 g/L, NO_2^-: 0 mg/L, SO_4^{2-}: 4-6.5 g/L and PO_4^{3-}: 3.5-5.4 g/L. In the efluent, the ionic concentrations of nitrogen compounds were: NH_4^+: 0-40 mg/L, NO_3^-: 200-600 mg/L, NO_2^-: 0-20 mg/L, total nitrogen: 100-250 mg/L, being the total nitrogen and COD removals above 80 and 90% respectively.

DNA extraction and PCR amplification

Fifty ml samples were centrifuged and the pellets were stored at –20ºC until use. Upon thawing, community DNA was extracted using the DNA Power Soil kit from MOBIO (12888-50).

Fragments of the bacterial 16S rRNA gene suitable for DGGE analysis were obtained by using the specific primer 358F with a 40-bp GC clamp, and the universal primer 907RM (Sánchez *et al*., 2007). Polymerase chain reaction (PCR) was carried out with a Biometra thermocycler using the following program: initial denaturation at 94ºC for 5 min; 10 touchdown cycles of denaturation (at 94ºC for 1 min), annealing (at

63.5-53.5ºC for 1 min, decreasing 1ºC each cycle), and extension (at 72ºC for 3 min); 20 standard cycles (annealing at 53.5ºC, 1 min) and a final extension at 72ºC for 5 min.

Primers 344f-GC and 915R were used for archaeal 16S rRNA amplification (Casamayor *et al*., 2002). The PCR protocol included an initial denaturation step at 94ºC for 5 min, followed by 20 touchdown cycles of denaturation (at 94ºC for 1 min), annealing (at 71 to 61ºC for 1 min, decreasing 1ºC each cycle), and extension (at 72ºC for 3 min); 20 standard cycles (annealing at 55ºC, 1 min) and a final extension at 72ºC for 5 min.

PCR mixtures for 16S rRNA amplification contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) at a final concentration of 600 µg ml^{-1} was added to minimize the inhibitory effect of humic substances (Kreader, 1996). The volume of reactions was 50 µl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen).

Primers amoA-1F and amoA-2R were used for amplification of ammonia oxidizers of the beta-subclass of *Proteobacteria* (Rotthauwe *et al.,* 1997). The PCR protocol included an initial denaturation step at 94ºC for 5 min, followed by 42 cycles of denaturation (at 94ºC for 60 s), annealing (at 60ºC for 90 s), and extension (at 72ºC for 90 s), and a final step consisting of 90 s at 60ºC and 10 min at 72ºC.

For detection of ammonia oxidizers of the gamma-subclass of *Proteobacteria*, primers amoA-3F and amoB-4R were utilized (Purkhold *et al.,* 2000). Thermal cycling was carried out by an initial denaturation step at 94ºC for 30 s, followed by 35 cycles of denaturation at 94ºC for 15 s, annealing at 48ºC for 20 s, and

elongation at 72ºC for 40 s. Cycling was completed by a final elongation step at 72ºC for 10 min.

The presence of archaeal *amo*A fragments was checked by using the primers Arch-amoAF and Arch-amoAR (Francis *et al.,* 2005) with the following protocol: 95ºC for 4 min, 30 cycles consisting of 94ºC for 30 s, 56ºC for 30 s, and 72 ºC for 60s, and a final step of 72ºC for 10 min.

PCR mixtures for amplification of the *amoA* gene contained 1 µl of template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 1.25 U *Taq* DNA polymerase (Promega) and PCR buffer supplied by the manufacturer. For archaeal *amo*A amplification, BSA at a final concentration of 150 µg ml^{-1} was added. The volume of reactions was 25 µl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen).

DGGE fingerprinting

DGGEs were run in a DCode system (Bio-Rad) as described by Muyzer *et al*. (1998). A 6% polyacrylamide gel with a gradient of 30-70% (*Bacteria*) or 40-80% (*Archaea*) DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). Seven hundred ng of PCR product were loaded for each sample and the gels were run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Gel Doc EQ (Bio-Rad). Prominent bands were excised from the gels, resuspended in milli-q water overnight and reamplified for its sequencing.

Clone libraries

Bacterial 16S rRNA was amplified using universal primers 27F and 1492R (Lane, 1991). Reactions were carried out in an automated thermocycler (Biometra) with the following cycle: an initial denaturation step at 94ºC for 5 min, followed by 30 cycles of 1 min at 94ºC, 1 min at 55ºC and 2 min at 72ºC, and a final extension step of 10 min at 72ºC. Primers 109F and 915R were used for Archaeal amplification (Großkopf *et al*., 1998). The cycle was as follows: 5 min at 94ºC, 38 cycles consisting of primer annealing at 52ºC for 1 min, DNA elongation at 72ºC for 90 s, and denaturation at 94ºC for 1 min, and a final cycle of 52ºC for 1 min and 72ºC for 6 min. PCR mixtures contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer.

PCR products were cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Putative colonies were picked, transferred to a multiwell plate containing Luria-Bertani medium and 7% glicerol, and stored at -80ºC for further amplification and sequencing.

rRNA gene sequencing

Purification of PCR products and sequencing reactions from DGGE bands and clones were performed by Macrogen (South Korea) with primers 907rM (DGGE), 27F (bacterial clone library) and 109F (archaeal clone library). Macrogen utilized the Big Dye Terminator version 3.1 sequencing kit and reactions were run in an automatic ABI 3730XL Analyzer-96 capillary type. Gene sequences were deposited in Genbank under accession numbers FN597722-FN597999 and FN598017-FN598150.

Sequences were subjected to a BLAST search (Altschul *et al*., 1997) to obtain an indication of the phylogenetic affiliation, and to the Bellerophon program (Huber *et al*., 2004) to determine potential chimeric artifacts. Sequences sharing similarities over 98.5% were considered similar phylogenetic entities (OTU). The coverage of the clone libraries was calculated according to the following equation: C=1-(n/N), where *n* is the number of unique clones and *N* is the total number of clones examined. Seventeen sequences (Accession numbers: FN598000-FN598016), corresponding to three OTU from the Deinoccoccus-Thermus group (representative clones: BACDER07_1B5, BACDER07_1G6 and BACDER07_1F5) were fully sequenced using primers 27F and 1492R for probe design purposes.

Phylogenetic analyses

Partial 16S rDNA sequences from each OTU of the archaeal clone library were aligned by using MAFFT version 6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/) with a selection of euryarchaeal sequences from databases (including the closest matches obtained by BLAST search) and three crenarchaeal sequences as an outgroup. Very variable regions of the alignment were automatically removed with Gblocks (Castresana, 2000), using parameters optimized for rDNA alignments (minimum length of a block of 5; allowing gaps in half positions), leaving 695 informative positions. Maximum-likelihood analysis was carried out with PAUP 4.0b10 (Swofford, 2002), with the general time-reversible model assuming a discrete gamma distribution with six rate categories and a proportion of invariable sites. Parameters were estimated from an initial neighbor-joining tree. Bayesian analysis was carried out with MrBayes v3.0B (Huelsenbeck and Ronquist, 2001), using the same model described above but with four rate categories in the gamma distribution. Bayesian posterior probabilities were computed by running 2.000.000 generations by using the program default priors on model parameters. Trees were sampled every 100 generations. 3.000 trees were

discarded as “burn-in” upon examination of the log likelihood curve of the sampled trees, so only the stationary phase was considered in the final tree. Neighbor-joining bootstrap values from 1000 replicates were calculated with PAUP following the same model used for the maximum-likelihood analysis.

***In situ* hybridization**

For separation of the sludge flocs, the original sample was vortexed during 5 min and subsequently diluted, fixed with formaldehyde and filtered on a 0.2 µm pore-size polycarbonate filter. CARD-FISH of prokayotic populations was carried out following the protocol described by Pernthaler *et al*. (2004). Several horseradish peroxidase probes were used to characterize the composition of the prokaryotic assemblage in activated sludge: CREN554 (Massana *et al*., 1997), EURY806 (Teira *et al*., 2004), EUB 338-II-III (Amann *et al*., 1990; Daims *et al*., 1999), ALF968 (Neef, 1997), GAM42a (Manz *et al*., 1992), CF319 (Manz *et al*., 1996), BET42a (Manz *et al*., 1992), DELTA495a (Loy *et al*., 2002), LGC354B (Meier *et al*., 1999) and HGC69a (Roller *et al*., 1994). The EUB antisense probe NON338 (Wallner *et al.*, 1993) was used as a negative control. The probe DT01 (5’-ACCAAGCGCATCACACCG-3’) targeting the clones BACDER07_1B5 and BACDER07_1G6 from the *Deinococcus-Thermus* phylum, was newly designed in this study by using the PROBE_DESIGN tool of the ARB software package (http://www.arb-home.de), and optimized following the protocol described in Pernthaler *et al*. (2001). This probe does not target the clone BACDER07_1F5.

FISH of nitrifying bacteria was carried out following the protocol detailed by Pernthaler *et al.* (2001). The 16S rRNA-targeted oligonucleotide probes used were: NEU, complementary to a signature region of most halophilic and halotolerant ammonia oxidizers, Nso190 and Nso1225, specific for ammonia oxidizers in the beta subclass of *Proteobacteria*, NIT3, complementary to a region of *Nitrobacter* species, and Nsv443, specific for the *Nitrosospira* cluster (Juretschko *et al.*, 1998).

All probes were purchased from Thermo Fisher Scientific (Ulm, Germany). Filters were permeabilized with lysozyme (10 mg ml^{-1}, 37ºC, 1 h) and achromopeptidase (60 U ml^{-1}, 37ºC, 0.5 h) before hybridization. Hybridizations were carried out at 35ºC overnight and specific hybridization conditions were established by addition of formamide to the hybridization buffers (20% formamide for NON338 and EURY806 probes, 30% for Nsv443, 35% for Nso1225, 40% for NEU and NIT3, 45% for ALF968 and LGC354B, 50% for Delta495a and HGC69a, and 55% for the other probes). The optimal hybridization conditions (30% formamide) of the newly designed probe DT01 were experimentally determined. Counterstaining of CARD-FISH preparations was done with DAPI (1 µg ml^{-1}). Free cells and aggregates were counted separately in each field. Also, several transects were inspected and mean numbers of aggregates were calculated. Between 500 and 1000 free DAPI-positive cells were counted manually in a minimum of 10 fields, while several thousands of cells (between 4000 and 10000) were counted in aggregates.

RESULTS

DGGE fingerprinting from seawater-processing activated sludge

The DGGE analysis from the two samples of activated sludge, corresponding to years 2007 and 2008, yielded a total of 20 and 17 different band positions for *Bacteria* and *Archaea* respectively (Fig. 1). Both samples showed virtually the same pattern for both set of primers, although differences in band intensity could be observed in some bands. This finding suggested that the system was rather stable along time. Bands were excised from both gels in order to determine their phylogenetic affiliation, and informative sequences were obtained from 12 (*Bacteria*) and 5 bands (*Archaea*) (Table 1). These bands accounted for 58% (*Bacteria*) and 32% (*Archaea*) of the total mean band intensity and most of them showed similarities with sequences from uncultured clones by BLAST search.

Four bands of bacterial origin affiliated to *Bacteroidetes*, with a contribution of 17% to total mean band intensity. The remaining bacterial bands belonged to different subclasses (*Alpha-*, *Gamma-* and *Delta-*) of the phylum *Proteobacteria* except two bands that affiliated to the *Deinococcus-Thermus* group.

Excision of bands from the archaeal DGGE gel yielded sequences related to methanogenic *Archaea*, although identities were relatively low.

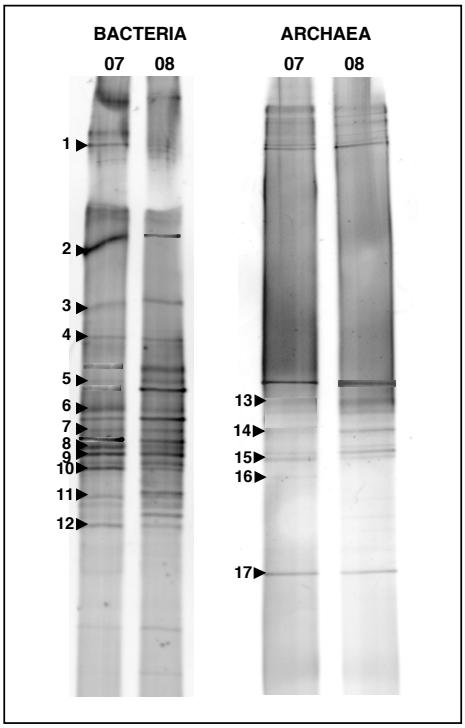

Fig. 1. Negative images of DGGE gels with PCR products amplified with bacterial and archaeal primer sets from samples of activated sludge corresponding to years 2007 and 2008. Bands excised and sequenced are numbered and their affiliations are shown in Table 1.

Table 1. Phylogenetic affiliation of sequences obtained from DGGE bands, with closest uncultured and cultured matches, and relative intensity of the bands.

Band	Closest match	% similarity (nºbases)[a]	Taxonomic group	Accession nº (GenBank)	Cultured closest match (% similarity)	Relative intensity (%)
DER_1	Uncultured *Bacteroidetes /Chlorobi* group clone 3B02-03	92.1 (498)	*Bacteroidetes*	DQ431894	*Marinicola seohaensis* (84.9)	5.6
DER_2	Uncultured *Bacteroidetes* clone ML617.5J-33	93.3 (502)	*Bacteroidetes*	AF507866	*Owenweeksia hongkongensis* (86.5)	24.9
DER_3	Uncultured *Bacteroidetes* clone 02D2Z22	92.8 (482)	*Bacteroidetes*	DQ330313	*Owenweeksia hongkongensis* (88.8)	4.0
DER_4	*Vitellibacter* sp.	77.7 (383)	*Bacteroidetes*	EU642844	The same	0.6
DER_5	Clone nsmp VI41	97.2 (529)	γ-*Proteobacteria*	AB212895	*Luteibactor rhizovicina* (85.8)	5.6
DER_6	Clone nsmp VI41	82.0 (437)	γ-*Proteobacteria*	AB212895	*Aquimonas* sp. (75.7)	2.1
DER_7	Clone Strom2G11	87.2 (449)	α-*Proteobacteria*	EU918039	*Parvibaculum* sp.	7.9
DER_8	*Nitratireductor* sp.	94.4 (487)	α-*Proteobacteria*	EU564843	The same	7.9
DER_9	Clone 101-91	99.8 (539)	δ-*Proteobacteria*	EF157196	*Desulfonatronum cooperativum* (84.1)	2.0
DER_10	Clone nsmo VI20	99.6 (541)	γ-*Proteobacteria*	AB212894	*Frateuria aurantia* (94.1)	2.0
DER_11	Clone OTU_23	85.1 (430)	*Deinococcus-Thermus*	EU083501	Truepera radiovictrix (80.0)	1.5
DER_12	Clone OTU_23	96.1 (493)	*Deinococcus-Thermus*	EU083501	Truepera radiovictrix (89.3)	1.4
DER_13	Clone Hua6-s78	83.7 (385)	*Euryarchaea*	EU481593	*Methanobacterium aarhusense* (78.8)	11.8
DER_14	Clone ss037b	93.8 (379)	*Euryarchaea*	AJ969783	*Aciduliprofundum boonei* (84.2)	7.8
DER_15	Clone ss037b	94.4 (476)	*Euryarchaea*	AJ969783	*Methanobrevibacter* sp. (79.3)	1.3
DER_16	Clone ss037b	95.3 (487)	*Euryarchaea*	AJ969783	*Aciduliprofundum boonei* (79.1)	2.8
DER_17	Clone ss037b	96.3 (494)	*Euryarchaea*	AJ969783	*Methanobrevibacter* sp. (79.7)	26.7

a. Number of bases used to calculate the levels fo sequence similarity

Identification of taxonomic groups by clone libraries

We analysed 278 and 117 clones in bacterial and archaeal clone libraries constructed with a sample from the marine activated sludge. Sequences were grouped in OTUs using a similarity criteria clustering of 98.5% (Stackebrandt and Goebel, 1994). One representative sequence of each OTU is shown in Tables 2 and 3, together with the closest relatives and the frequency of the OTU. Coverage of the libraries was 95.7 and 91.5% respectively, indicating that in both cases this particular approach provided most of the measurable diversity.

Table 2. Phylogenetic affiliation of clones from the bacterial clone library to the closest match and to the closest cultured strain in GenBank. (Bold text represents OUT with the highest percentage of clones).

Representative clone	Closest match (accession number)	% similarity	Cultured closest match (accession number)	% similarity	Nº of clones (%)
α-Proteobacteria					
BACDER07_1C3	Clone DR938CH110701SACH95 (DQ230971)	94.0	*Nitratireductor* sp. (EU564843)	93.4	1 (0.4)
BACDER07_1C12	Clone SC71 (EU735614)	97.8	*Brucella* sp. (DQ167235)	90.2	1 (0.4)
BACDER07_1F8	Clone 81 T12d-oil (FM242433)	95.7	*Subaequorebacter tamlense* (AM293856)	90.1	1 (0.4)
BACDER07_1H9	*Nitratireductor* sp. (EU564843)	94.2	The same	94.2	4 (1.4)
BACDER07_1D12	Clone B1-43 (AM229476)	98.2	*Sneathiella chinensis* (DQ219355)	95.6	1 (0.4)
BACDER07_2B7	Clone CI75cm.2.18 (EF208711)	98.6	*Methylocystis* sp. (AJ868421)	94.7	2 (0.7)
BACDER07_2F8	Clone Strom2G11 (EU918039)	96.3	*Maricaulis* sp. (AJ301666)	90	2 (0.7)
BACDER07_2G10	Clone 256ds10 (AY212705)	98.0	*Sphingomonas* sp. (D16149)	97.1	1 (0.4)
δ-Proteobacteria					
BACDER07_1D11	Clone 101-91 (EF157196)	98.9	*Desulfonatronum cooperativum (AY725424)*	80.7	7 (2.5)
γ-Proteobacteria					
BACDER07_1B8	Clone nsmpVI41 (AB212895)	98.9	*Kangiella koreensis* (AY520560)	85.4	16 (5.8)
BACDER07_1D2	Clone nsmpVI20 (AB212894)	99.6	*Rhodanobacter lindaniclasticus* (DQ507211)	91.6	21 (7.6)
BACDER07_3B12	Clone nsmpVI20 (AB212894)	98.1	*Frateuria aurantia* (AB091195)	90.2	1 (0.4)
Deinococcus-Thermus					
BACDER07_1B5	Clone GZKB22 (AJ853517)	95.8	*Truepera radiovictrix* (DQ022077)	89.3	**212 (76.3)**
BACDER07_1G6	Clone GZKB22 (AJ853517)	94.5	*Truepera radiovictrix* (DQ022077)	89.8	2 (0.7)
BACDER07_1F5	Clone 6 (EU017377)	94.5	*Truepera radiovictrix* (DQ022077)	89.5	1 (0.4)
Bacteroidetes					
BACDER07_1F4	Clone 6 (DQ015772)	96.3	*Lewinella marina* (AB301495)	84.7	1 (0.4)
BACDER07_2D6	Clone Er-LLAYS-51 (EU542514)	97.5	*Owenweeksia hongkongensis* (AB125062)	87.3	1 (0.4)
BACDER07_2H3	Clone HF500_26D14 (EU361310)	95.6	*Sphingobacterium* sp. (AM411964)	90.0	1 (0.4)
Firmicutes					
BACDER07_2F2	Clone p816_b_3.45 (AB305600)	81.7	*Bacillus* sp. (EF422410)	79.0	1 (0.4)
Unclassified bacteria					
BACDER07_3D4	*Denitromonas indolicum* (AY972852)	95.2	The same	95.2	1 (0.4)

Table 3. Phylogenetic affiliation of clones from the archaeal clone library to the closest match and to the closest cultured strain in GenBank. (Bold text represents OUT with the highest percentage of clones)

Representative clone	Closest match (accession number)	% similarity	Cultured closest match (accession number)	% similarity	Nº of clones (%)
Methanosarcinales					
ARCHDER07_1A12	*Methanococcoides* sp. (Y16946)	99.5	The same	99.5	5 (4.3)
ARCHDER07_1C3	Clone Z3-Arc-1 (EU999009)	98.6	*Methanolobus profundi* (AB370245)	97.8	2 (1.7)
ARCHDER07_1D4	Clone TFC20L31Ar (EU362350)	97.0	*Methanosaeta harundinacea* (AY970347)	96.2	2 (1.7)
Methanomicrobiales					
ARCHDER07_2C9	Clone WIP (EF420166)	98.5	*Methanoculleus marisnigri* (CP000562)	97.5	2 (1.7)
ARCHDER07_1B2	Clone GoM-GC234-015R (AY211693)	96.1	*Methanoculleus sp*. (AJ133793)	93.8	6 (5.1)
ARCHDER07_2D6	Clone PMMV-Arc14 (AJ937680)	92.2	*Methanoculleus sp*. (AJ133793*)*	89.8	1 (0.9)
Methanobacteriales					
ARCHDER07_1A2	Clone 4B09 (AY835426)	94.7	*Methanothermus fervidus* (M32222)	81.8	8 (6.8)
ARCHDER07_1B11	Clone ALAS95 (EU616776)	99.2	*Methanobacterium aarhusense (DQ649334)*	84.4	1 (0.9)
DER_1					
ARCHDER07_1D11	Clone ss037b (AJ969783)	97.1	*Methanomethylovorans sp.* (EU544305)	78.0	5 (4.3)
ARCHDER07_1G10	Clone HARR41 (AJ699117)	99.5	*Aciduliprofundum boonei* (DQ451875)	81.2	2 (1.7)
DER_2					
ARCHDER07_2C8	Clone GNA03E09 (EU731492)	94.3	*Methanobrevibacter gottschalkii* (U55239)	70.8	1 (0.9)
ARCHDER07_1C11	Clone MOB4-5 (DQ841225)	91.7	*Methanobrevibacter gottschalkii* (U55239)	70.1	1 (0.9)
ARCHDER07_1E6	Clone GNA03E09 (EU731492)	96.0	*Methanobacterium sp.* (DQ517520)	71.0	3 (2.6)
ARCHDER07_1A5	Clone GNA03E09 (EU731492)	98.2	*Methanococcus infernus* (AF025822)	72.8	**47 (40.2)**
ARCHDER07_1A10	Clone GNA03G10 (EU731491)	94.9	*Methanococcus vulcanus (AF051404)*	71.9	1 (0.9)
ARCHDER07_2D4	Clone CaR3b.h02 (EU244267)	90.1	*Methanobrevibacter sp.* (AJ550156)	70.8	2 (1.7)
ARCHDER07_2A4	Clone CaS1s.h02 (EF014578)	88.9	*Methanococcus infernus* (AF025822)	72.5	1 (0.9)
ARCHDER07_1A4	Clone A21 (EU328111)	88.6	*Methanococcus aeolicus* (CP000743)	72.8	12 (10.3)
ARCHDER07_1D1	Clone KAB187-14 (AB366595)	89.8	*Methanobacterium sp.* (EU366499)	73.8	1 (0.9)
DER_3					
ARCHDER07_1B4	Clone 1ACC-29 (AB175599)	97.2	*Methanothermococcus sp.* (AB175514)	78.0	9 (1.7)
ARCHDER07_1G6	Clone GNA03F04 (EU731409)	95.1	*Methanocaldococcus jannaschii* (L77117)	78.8	1 (0.9)
ARCHDER07_1D10	Clone GNA02E03 (EU731293)	97.4	*Methanococcus aeolicus* (CP000743)	78.0	2 (1.7)
ARCHDER07_2B5	Clone GNA01D07 (EU731138)	91.6	*Methanothermococcus sp.* (AB260046)	77.3	1 (0.9)
ARCHDER07_1A1	Clone ML23_ANME 9 (AY245465)	83.2	*Methanocaldoccus indiensis* (AF547621)	76.1	1 (0.9)

A significant number of clones from both libraries showed similarity to uncultured sequences deposited in GenBank. The most abundant sequence recovered from Bacteria corresponded to a bacterium from the *Deinococcus-Thermus* cluster (almost 77% of the clones) with the same sequence as band DER_12. The library included also members from other groups such as the *Alpha-, Gamma-* and *Delta-*subclasses of *Proteobacteria*, the *Bacteroidetes* and *Firmicutes*. Within the

Proteobacteria, which represented 21% of the clones, members of the *Gammaproteobacteria* predominated (14% of total clones). One clone was affiliated to the unclassified bacterium *Denitromonas indolicum*. Similarities based on sequence comparison of these clones varied between 81.7 and 99.6%. The 16S rRNA similarities were approximately at the species level (≥97%, Stackebrandt and Goebel, 1994) for 19% of the clones, while 79% were similar at the genus level (95-97%). In general, there was agreement between the different sequences retrieved by DGGE and the clone library. Inclusion of all the sequences in a phylogenetic tree indicated that most of the DGGE band sequences corresponded to several of the most abundant clones recovered from the library (tree not shown).

Concerning the archaeal clone library, we basically found sequences related to different orders of methanogenic *Archaea*, in correspondence with the recovered DGGE bands. In this case, similarities ranged between 83.2 and 99.5%. We paid especial attention to these archaeal sequences, since most of them were only moderately related to known archaea. Phylogenetic analyses were performed by several methods and summarized in a maximum-likelihood tree with Bayesian posterior probabilities and neighbor joining bootstrap values in the relevant nodes (Fig. 2). Based on the tree structure and bootstrap values, a high percentage of archaeal sequences (77%) were grouped into 3 separate clusters, named DER_1, _2 and _3, which formed three independent branches composed exclusively by environmental clones. Other sequences clustered within three major phylogenetic groups of methanogens: Methanosarcinales, Methanomicrobiales and Methanobacteriales, while sequences belonging to Methanococcales and Methanopyrales were not retrieved.

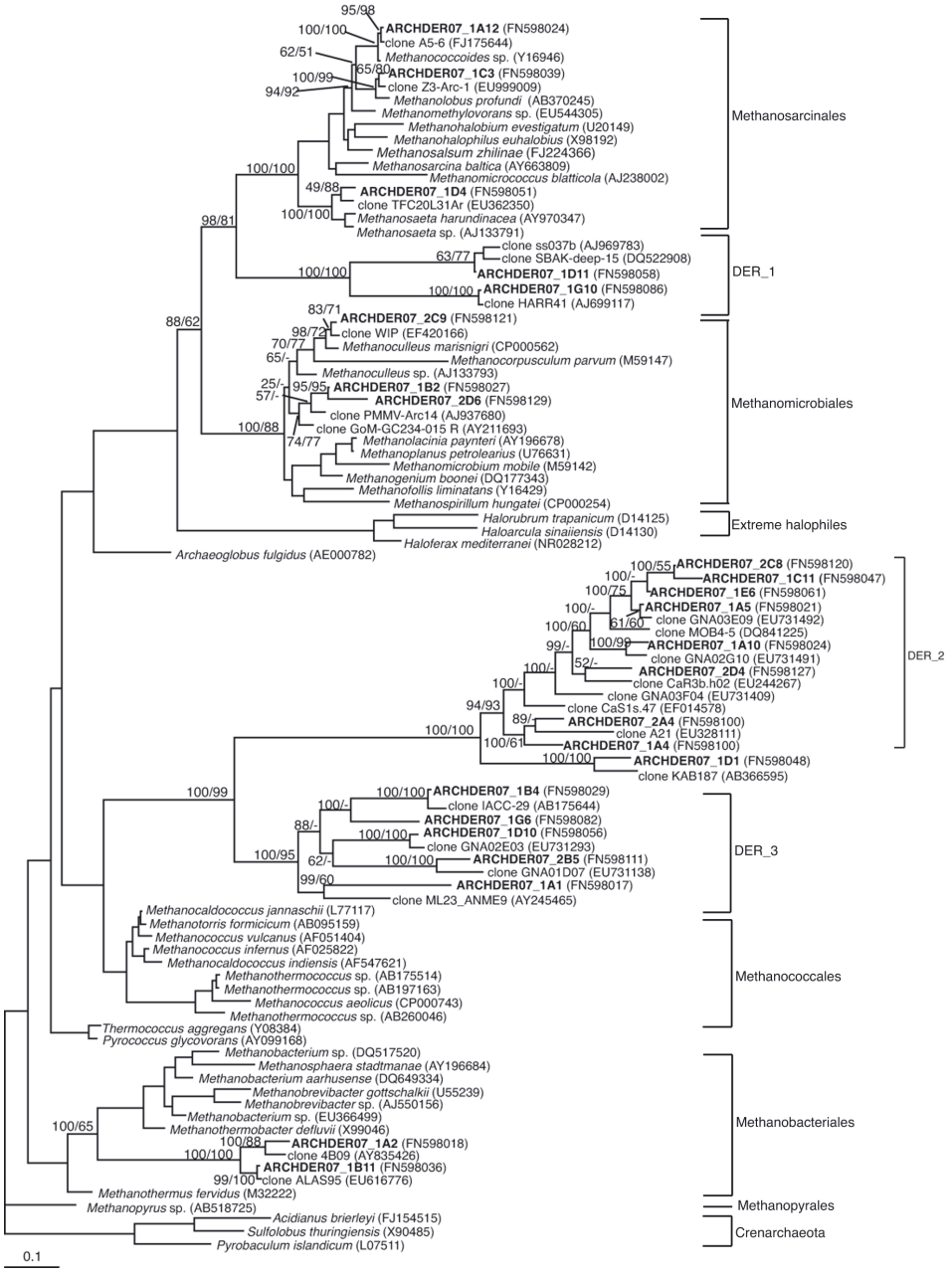

Figure 2. Maximum-likelihood phylogenetic tree with partial 16S rDNA archaeal sequences (695 informative positions). Clones in bold are from this study. Posterior probabilty values and neigbor-joining bootstrap values (1000 replicates) are shown in the relevant nodes. The scale bar indicates 0.1 substitutions per position.

Detection of *amoA* genes

Using specific primers for the amplification of the gene *amoA* from the *Beta*, *Gamma* and archaeal nitrifiers, we were able to confirm the presence of ammonia-oxidizers from the Beta-subclass of *Proteobacteria* (data not shown). The gene *amoA* encodes the catalytic a-subunit of ammonia monooxygenase, the enzyme responsible for catalyzing the rate-limiting step in ammonia oxidation, and it has been used extensively as a molecular marker for cultivation-independent studies of ammonia-oxidizing communities. However, we could not find amplification for *Gamma* and *Archaea* nitrifiers in our samples.

Quantitative analysis of marine activated sludge composition by in situ hybridization

The activated sludge samples from years 2007 and 2008 were also analyzed by DAPI staining and fluorescent in situ hybridization. A sound quantification of activated sludge samples was complicated by the heterogeneous cell distribution caused by the flocculation. Several treatments were tested for cell dispersal, e.g. sonication and vortexing at different times (5, 15, or 30 min, data not shown). No differences in hybridization signals were found in treatments at 5 and 15 min, while the fraction of hybridized cells decreased after sonicating or vortexing samples during 30 min. In any case, microscopic examination clearly showed that there were still aggregates. In order to increase accuracy we counted separately the hybridized free cells and the hybridized cells in aggregates for each probe, evaluating several thousand cells in the case of aggregates. Enumeration of DAPI-stained preparations revealed total cell counts of 1.89×10^9 ml^{-1} (year 2007) and 1.28×10^9 ml^{-1} (year 2008), being 65% (mean value from both years) in the form of aggregates and the rest as free cells. The mean number of cells per aggregate was 444. However, one has to keep in mind that microscopic enumeration of aggregated cell clusters is likely resulting in underestimations, but since this effect

applies equally to DAPI and FISH counts, the conclusions drawn from the different fractions of probe positive-DAPI stained cells remain valid.

The results obtained from CARD-FISH analyses for both years (Fig. 3) indicated that the microbial assemblage had virtually the same composition, and that the percentage of the hybridized groups remained very similar, confirming that the system was stable along time. Hybridization with the universal set of probes EUB (Eub+) detected a mean value from both years of 86% of DAPI-stained free cells and 97% of cells in aggregates (Eub+), while *Archaea* reached only 4 and 3% of mean DAPI counts for free and aggregated cells respectively, indicating that the majority of fixed cells were *Bacteria*. A considerable amount of total Eub+ free cells (54%) were identified with probes for broad phylogenetic groups (*Alpha-, Beta-, Gamma-,* and *Deltaproteobacteria, Bacteroidetes, Firmicutes* and *Actinobacteria*). This value was similar for aggregates (62%). *Alphaproteobacteria* were the dominant bacterial group [27% (free cells) and 44% (aggregates) of mean DAPI counts], while *Gammaproteobacteria* and *Bacteroidetes* contributed to lower and similar proportions in the free-cells fraction (around 8% of mean DAPI counts). These values were lower in the case of aggregated cells (around 4% of mean DAPI counts). Other groups, such as *Beta* and *Deltaproteobacteria,* Firmicutes and Actinobacteria were present even at lower numbers (1-4% of mean DAPI counts in free cells, and 0-1% in aggregates). A specific probe (DT01) corresponding to a sequence that accounted for 77% of the clones in the bacterial clone library was designed in this work. This sequence had approximately 96% similarity with an uncultured bacterium from the *Deinococcus-Thermus* phylum (Table 2). However, CARD-FISH results showed that this microorganism was not particularly abundant (3 and 7% of mean DAPI counts for free and aggregated cells respectively), denoting a large positive bias for this bacterium in the clone library.

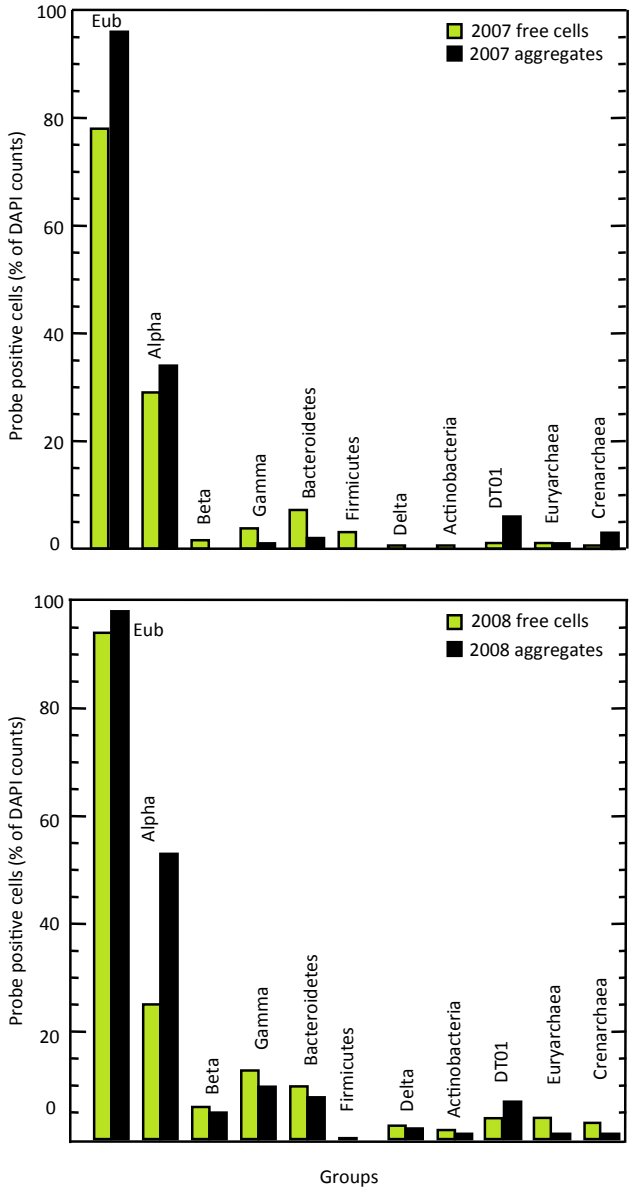

Fig. 3. Proportions of bacterial groups detected by CARD-FISH with HRP probes in free cells and in aggregates from samples of activated sludge corresponding to years 2007 and 2008.

In situ hybridization with a set of well-known hierarchical 16S rRNA-targeted probes for ammonia-oxidizing bacteria (including the genera *Nitrobacter* and most of the betaproteobacterial ammonia-oxidizers, such as members of the genera *Nitrosomonas*, *Nitrosococcus mobilis* and *Nitrosospira*), usually used for activated sludge (Juretschko *et al.,* 1998) showed no signal in our samples, in accordance with the results observed in the DGGE and the bacterial clone library for these particular microorganisms.

Quantitative comparison between DGGE, CARD-FISH and clone libraries

In the case of *Bacteria*, we quantitatively compared the results obtained by the three different molecular methods in order to test the strong and weak points of each approach, and how they affect the overall picture of activated sludge diversity (Fig. 4). For FISH representation, we took into account the contribution of free and aggregated cells for every probe. The most remarkable trend in this figure is the overrepresentation of a sequence corresponding to the *Deinococcus-Thermus* group in the clone library as compared with CARD-FISH and DGGE. *Alphaproteobacteria* and *Bacteroidetes*, by contrast, seemed to be underrepresented in the clone library, while *Alphaproteobacteria* were overrepresented with CARD-FISH. On the other hand, the detection of *Gamma* and *Deltaproteobacteria*, was more proportionate by the three methods. Other groups could only be detected by CARD-FISH, such as *Betaproteobacteria*, and *Actinobacteria*, although at very low relative abundance.

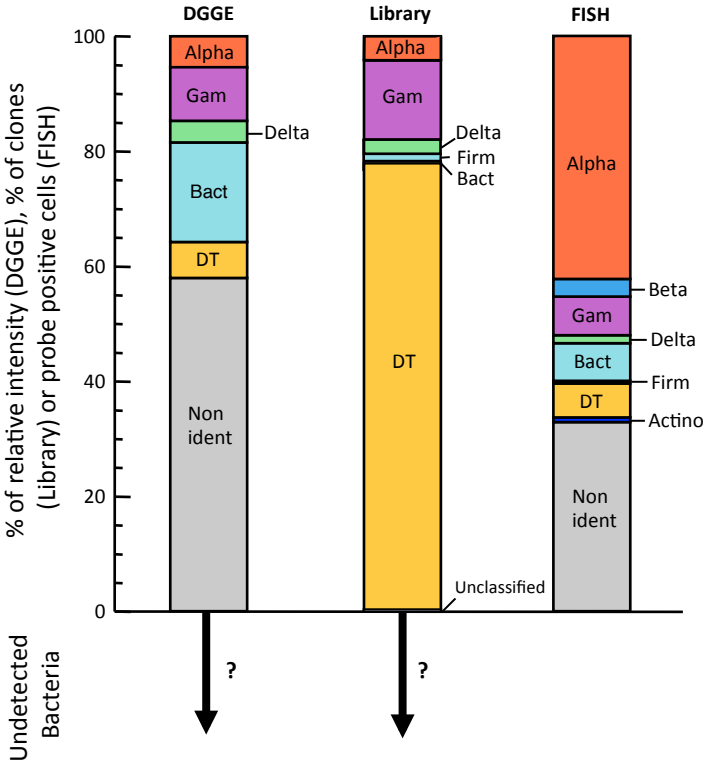

Fig. 4. Percentage of relative intensity of DGGE bands, proportions of clones (Library) and probe positive cells scaled to Eub probes (FISH) affiliated to different phylogenetic groups [*Alphaproteobacteria* (Alpha), *Betaproteobacteria* (Beta), *Gammaproteobacteria* (Gam), *Deltaproteobacteria* (Delta), *Bacteroidetes* (Bact), *Firmicutes* (Firm), *Actinobacteria* and *Deinococcus-Thermus* clone (DT)]. FISH data correspond to the mean of samples from years 2007 and 2008. The arrows with question marks indicate that an unknown proportion of phylogenetic groups cannot be retrieved by the set of primers used in both DGGE and clone libraries.

DISCUSSION

The advent of molecular techniques in the past two decades has provided many insights into the diversity and functions of predominantly uncultured wastewater microorganisms. However, relatively few works have studied activated sludge microorganisms by a full-cycle rRNA approach, and never before the diversity of a

marine activated sludge has been detailed. In our study, the objective was to obtain a comprehensive picture of the diversity of the prokaryotic assemblage in a seawater-processing WWTP, combining and comparing different molecular approaches (DGGE, clone library and FISH).

In this particular WWTP, we predict that the influent composition becomes crucial in order to understand the composition of the microbial community. The presence of amoxicillin, together with a high salt concentration, will surely affect microbial diversity. Actually, concern is growing over environmental contamination with pharmaceuticals because of their widespread use and incomplete removal during wastewater treatment. Thus, Kraigher et al. (2008) investigated the influence of pharmaceutical residues on the structure of activated sludge bacterial communities in wastewater treatment bioreactors and observed a minor but consistent shift in the community structure in bioreactors supplied with pharmaceuticals, as well as a reduction in diversity.

Diversity in seawater-utilizing activated sludge

Different authors have addressed the study of activated sludge diversity either in WWTP or laboratory-scale reactors by means of molecular techniques (Bond *et al*., 1995; Kämpfer *et al*., 1996; Snaidr *et al*., 1997; Christensson *et al*., 1998; Dabert *et al*., 2001; Daims *et al.*, 2001; Liu and Seviour, 2001; Juretschko *et al*., 2002; Eschenhagen *et al*., 2003; Sanapareddy *et al*., 2009). These studies indicated considerable microbial diversity in WWTPs and the dominance of the *Beta* subclass of the class *Proteobacteria*. Apart from the *Proteobacteria*, other groups such as the *Bacteroidetes*, the *Chloroflexi*, the *Actinobacteria* and the *Planctomycetes* could be detected either in clone libraries or using FISH with group-specific probes. The composition of the bacterial community of the seawater activated sludge described here differed strongly from those previously reported, since *Betaproteobacteria* did not seem to be the predominant group. It was even not detected in the clone

library and represented only a 3% of the total hybridized bacteria. This is most likely due to the fact that our WWTP is fed with seawater, which is known to contain very few *Betaproteobacteria* (Rappé and Giovannoni, 2003). In contrast, other subclasses of *Proteobacteria*, such as *Alpha-*, *Gamma-* or *Deltaproteobacteria* were detected with the three methodologies, although in the case of *Alphaproteobacteria*, at different proportions. On the other hand, the *Bacteroidetes* group seemed also to be represented by the three methods.

The most remarkable feature of clone libraries is the severe overrepresentation of one sequence belonging to the *Deinococcus/Thermus* group (around 77% of total bacterial clones). Its closest match in GenBank was an uncultured bacterium, and its closest cultured match was *Truepera radiovictrix*, although at low similarities (less than 90%, Table 1). The phylum *Deinococcus/Thermus* includes extremely radiation resistant bacteria, as well as slightly thermophilic or thermophilic members, and it also comprises a number of environmental 16S rRNA gene sequences, several of which are not closely related to any cultured strains and form distinct lineages. Nevertheless, when comparing to DGGE and CARD-FISH data, this sequence turned out to be less abundant and discrepancies with the clone library become clear.

Most of the sequences retrieved in the bacterial clone library were similar to uncultured members of different phyla, but in some cases, cultured closest matches were related at the genus or even at the species level (Table 1). For example, one clone of *Alphaproteobacteria* (BACDER07_1D12) was similar at the genus level to *Sneathiella chinensis*, a marine chemoheterotrophic bacterium, and another clone (BACDER07_2B7) to *Methylocystis* sp., a methanotrophic bacterium. On the other hand, one clone (BACDER07_2G10) was similar at the species level to *Sphingomonas* sp., a genus recognized by its capability to degrade a wide variety of refractory environmental pollutants and to carry out diverse other biotechnologically useful activities, such as the biosynthesis of valuable

biopolymers (Laskin and White, 1999); Sphingomonads have been identified in situ by FISH in activated sludge samples and turned out to be rather abundant, accounting for about 5-10% of the total cells (Neef *et al*., 1999). Since exopolysaccharides are a significant part of the polymeric extracellular matrix material of flocs, and members of the genus *Sphingomonas* are known to be able to produce slimes and/or capsules, the authors suggested that they could be involved in the formation process of sludge flocs. Finally, another clone was similar at the genus level to *Denitromonas indolicum*, an unclassified bacterium. This genus was found to be able to grow with perchlorate as the sole electron acceptor (Zuo *et al*., 2009).

Concerning the diversity of *Archaea*, less attention has been paid to their role in wastewater treatment processes, since it seems clear that *Bacteria* are responsible for the majority of carbon removal in the activated sludge process (Gray *et al*., 2002). In our samples, *Archaea* represented only a small fraction of total mean DAPI counts from the two samples (4% in free cells and 3% in aggregates). Virtually all sequences retrieved in our archaeal clone library were related to methanogenic bacteria (Fig. 2), consistent with previous reports of the existence of anoxic microenvironments in the flocs, in which methanogens might be active (Schramm *et al*., 1999). All methanogens are strictly anaerobic *Archaea* pertaining to the *Euryarchaeota*. Although they are very diverse phylogenetically, they can only utilize a restricted number of substrates of three major types: CO_2, methyl-group containing compounds, and acetate. Their common habitats include marine and freshwater sediments, flooded soils, human and animal gastrointestinal tracts, termites, anaerobic digestors, landfill, geothermal systems and heartwood of trees. Nevertheless, it has been demonstrated the presence of methanogenic bacteria in aerated activated sludge. Thus, Gray *et al*. (2002) retrieved archaeal 16S rRNA gene sequences related to Methanosarcinales, Methanomicrobiales and Methanobacteriales. However, the relatively low rates of methanogenesis

measured by these authors indicated that, although active, the methanogens played a minor role in carbon turnover in activated sludge.

In our study, the recovered sequences affiliated also within the orders Methanosarcinales, Methanomicrobiales, and Methanobacteriales with the same proportion in the clone library (8% each). However, most of the sequences clustered into novel branches (DER_1, _2 and _3), which were closely related to environmental clones. One of these sequences (representative clone: ARCHDER07_1A5), belonging to DER_2, accounted for 40% of the total clones and was related to a sequence from Guerrero Negro hypersaline microbial mats (Robertson *et al*., 2009).

On the other hand, *Crenarchaeota* were also detected by CARD-FISH (2% of total DAPI counts from free and aggregated cells), but this group was not represented in the archaeal clone library or in the retrieved sequences from the DGGE. PCR bias against *Crenarchaeota* could explain this disagreement.

Linking diversity and function

The functional assignment of detected microorganisms is complicated by the fact that 16S rRNA sequence-based identification does generally not allow to infer their functional properties. Phylogenetically closely related microorganisms may possess different metabolic traits while on the other hand several physiological features like the ability to denitrify are dispersed in different phylogenetic lineages. Therefore, the full-cycle rRNA-approach needs to be supplemented with other techniques that allow a functional assignment of the detected microorganisms. In this particular sense, we included in our study the amplification of the functional gene coding for the active-site polypeptide of ammonia monooxygenase (*amoA*) as a physiological marker, as well as the use of specific rRNA-targeted probes for the

detection of ammonia oxidizers in order to link diversity with function, in particular those aspects referred to nitrogen removal.

In engineered systems such as WWTPs, the coupled nitrification and denitrification processes are considered the major mechanisms of nitrogen removal. The nitrifiers encompass two groups of microorganisms, the ammonia and the nitrite-oxidizing bacteria, which catalyze the oxidation of ammonia to nitrite and of nitrite to nitrate respectively. Many WWTPs harbor diverse *Beta* and *Gammaproteobacteria* ammonia-oxidizers, such as the *Nitrosomonas europaea/Nitrosomonas eutropha*-lineage (*Beta*), the *Nitrosococcus mobilis*-lineage (*Beta*), the *Nitrosomonas marina* cluster (Beta), and the *Nitrosococcus* group (*Gamma)*. Also, heterotrophic microorganisms have been reported to oxidize nitrogen compounds under very specific conditions (Kim *et al*., 2005). In addition, *Nitrobacter* and *Nitrospira* have been recognized as nitrite-oxidizers (Wagner *et al*., 2002). Recently, ammonia-oxidizing organisms belonging to the archaeal domain have also been described (You *et al*., 2009). The denitrification process, i.e., the removal of nitrate to the atmosphere, seems to be mainly done by members of the genera *Alcaligenes*, *Pseudomonas*, *Methylobacterium*, *Bacillus*, *Paracoccus, Hyphomicrobium*, as well as by many members of the betaproteobacterial order Rhodocyclales (Wagner *et al*., 2002; Hosselhoe *et al.,* 2009).

In our study, the seawater-processing WWTP was known to have a nitrogen sludge load of 150-170 kg/h, a nitrification fraction of 98% and a total nitrogen removal over 80% (M.I. Maldonado, personal communication). Thus, nitrification and denitrification are important processes in this system. Although amplification from beta ammonia-oxidizers was detected in the two samples, we have not been able to find sequences corresponding to recognized microorganisms known to catalyze the oxidation of ammonia to nitrite or of nitrite to nitrate in freshwater WWTPs. Sequences from *Betaproteobacteria* (to which a diversity of nitrifiers from freshwater WWTPs belong) have not been recovered from the DGGE gels and the

clone library, although CARD-FISH analyses showed a certain amount of bacteria belonging to this group (3% of total DAPI-stained cells). It is possible that the DNA extraction technique applied was not sufficient rigorous to lyse the cells of ammonia–oxidizers in the activated sludge, or that PCR or cloning biases occurred. However, hybridization with specific probes for beta ammonia-oxidizers showed no signal, suggesting that the microorganisms carrying out this function in our samples could not be assigned to any of the well-known lineages from freshwater activated sludges. Thus, nitrifiers in this specific seawater-processing WWTP correspond to different genera. In fact, it has been shown that several heterotrophic *Bacillus* strains can carry out aerobic nitrification, as well as denitrification (Kim *et al*., 2005), and we have found sequences of *Bacillus* in the clone library (although with a low similarity) and also by culture-dependent techniques (data not shown). Actually, *Bacillus* strains are able to remove nitrogen and phosphorus as well as organic matter. On the other hand, it is also possible that, although being crucial for nitrogen removal, the well-known nitrifiers could not be detected due to their low abundance. Concerning the *Gammaproteobacteria*, no amplification of the *amoA* gene was found and most of the sequences recovered in this study corresponded to unidentified clones.

In contrast, sequences of *Nitratireductor* sp., able to reduce nitrate to nitrite, have been retrieved from the DGGE and the bacterial clone library. However, most candidates for denitrifying bacteria in this work have been found by culture-dependent approaches (data not shown). Thus, members of the genera *Alcaligenes*, *Pseudomonas*, *Bacillus*, *Paracoccus, Halomonas* and *Marinobacter* have been isolated in rich media, although we don't know whether these genera are representative for the *in situ* active denitrifiers of this system.

Recently, it has been shown that autotrophic oxidation of ammonia is not restricted to the domain *Bacteria*. Könneke *et al.* (2005) isolated an ammonia-oxidizing crenarchaeon named *Nitrosopumilus maritimus* able to oxidize ammonia

to nitrite under mesophilic conditions, and Park *et al*. (2006) reported molecular evidence that ammonia-oxidizing archaea occur in activated sludge bioreactors used to remove ammonia from wastewater. However, amplification of the archaeal *amoA* was not found in our samples. Nevertheless, it is important to note that significant diversity exists in each of these functional groups of organisms and that a detailed knowledge of their biology needs to be gained.

Correspondence between DGGE, clone library and FISH

The comparison between clone library, DGGE and FISH results is not straightforward because of the different levels of phylogenetic resolution of each technique. There is general agreement regarding the limitations of each methodology (Amann *et al*., 1995; Wintzingerode *et al*., 1997), but few studies have compared these techniques in activated sludge systems (Snaidr *et al*., 1997; Juretschko *et al*., 2002; Eschenhagen *et al*., 2003), and none has compared them in seawater-processing activated sludge.

DGGE allowed an assessment of the composition of the prokaryotic assemblage of the activated sludge sample with sufficient resolution. However, a failure to obtain sequences from faint bands prevents the use of DGGE for describing bacterial diversity accurately (Sánchez *et al*., 2009). Because not all bands were sequenced, it cannot be discarded that differences between community composition shown by DGGE and by other techniques are due to insufficient sequencing. On the other hand, the clone library provided the highest phylogenetic resolution and a detailed picture of the species within each phylogenetic group. However, PCR bias and the varying copy number of the rRNA operon in different organisms produced severe overestimations (*Deinococcus-Thermus*) or underestimations (*Alphaproteobacteria*) of specific groups compared with the direct quantification obtained by CARD-FISH. On the other hand, the picture of the bacterial assemblage composition provided by CARD-FISH was limited by the number and phylogenetic resolution of the

probes. A substantial proportion of the Eub338-II-III positive cells remained unidentified by the general probes used, while no cell remained undetectable.

Although DGGE is also subject to PCR bias, in our study the group proportion with this technique was more similar to what was found for CARD-FISH than to clone libraries. This discrepancy was also shown by Massana *et al*. (2006), who observed that clone libraries obtained with a primer set amplifying one-third of the 18S rRNA gene from eukaryotes (the set that is regularly used in DGGE studies) provided very good correlation between clonal representation and cell abundance determined by FISH. In contrast, the primer set amplifying the complete 18S rRNA gene gave a very biased view of the phylogenetic groups under study when compared to FISH abundance, with some phylotypes being severely overestimated and others underestimated.

Snaidr *et al*. (1997), however, despite using a primer set which amplified almost-full-length 16S rRNA gene fragments, found a general agreement between clone library and FISH when analyzing the bacterial community structure of activated sludge from a municipal WWTP, although discrepancies became clear when using more specific probes. In their study, almost 20% of DAPI cells remained undetected.

In summary, the combination of the three techniques was very useful for assessing a comprehensive appraisal of prokaryotic diversity, and thus a polyphasic approach is essential to have a complete picture of the prokaryotic assemblage. These methods also showed that this particular activated sludge can contain significant hidden diversity of unknown and uncultured marine-related microorganisms that can contribute to its functioning. Therefore, further attempts to isolate the key microorganisms involved will be essential in order to understand their specific biological processes.

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Chapter 2

MICROBIAL COMMUNITIES IN WETLANDS: THE EFFECT OF PLANT SPECIES, ORGANIC MATTER AND HYDRAULIC DESIGN

To be submitted to: Ecological Engineering

SUMMARY

Experimental constructed wetlands treating diluted wastewater were used to study the effect of plant species (*Typha angustifolia* or *Phragmites australis*), hydraulic design (free-water surface flow or sub-subsurface flow) and organic loading (3 or 9 $gBOD_5/m^2/d$) on the microbial composition of the rhizoplane, gravel biofilm and interstitial water. The analysis of DGGE band patterns showed statistically significant differences in community assemblages between plant species. Hydraulic configuration, and plant presence in a lesser extend, were more important than organic load in shaping microbial communities in the studied wetlands. Distinctive communities were found for roots, gravel biofilm and interstitial water inside the same mesocosm, being differences among these communities higher for *Phragmites* than *Typha* planted tanks. Plants had an effect on all the microbial communities studied into the mesocosms, proving that their influence affect interstitial water and gravel-associated bacteria far beyond their roots. Environmental conditions, mainly redox, are suggested as main driving forces in organizing microbial assemblages in the studied wetlands.

INTRODUCTION

Wetland rhizosphere system is characterized by the presence of oxic-anoxic interfaces in which obligatory aerobic to strictly anaerobic microbes operate in close proximity, thereby facilitating elemental cycling, having a great value for life in our planet (Schlesinger, 2004). Despite the impact of microbes in wetland ecosystems, little is known about the species present and bout their ecology and functioning in situ, especially in the rhizosphere (Bodelier *et al.*, 2006). Former limitations of cultivation dependent techniques on the study of wetland microbiology have been overcome by the development of molecular techniques like FISH (Criado and Bécares, 1999) and specially fingerprinting methods

(Gutknecht *et al.*, 2006). Bodelier (2003) reviewed studies on the microbial ecology of flooded soils and sediments evidencing that detailed studies on the community structure and population dynamics of bacteria in the rhizosphere of wetland plants were extremely scarce. More recently, Gutknecht *et al.*, (2006) revised the molecular approaches in the exploration of rhizosphere in wetland ecosystems. From this last review, research in wetlands has most often been based on processes (e.g methanogenesis) and less often on microbial communities. It has been proved that wetlands plants enhances microbial density and activity in their rhizosphere (Gagnon *et al.*, 2007; Nikolausz *et al.,* 2008,) this mainly due to the fact that plants provide carbon compounds through root exudates and a micro aerobic environment via root oxygen release (e.g. Armstrong and Armstrong 2001; Lu *et al.*, 2006). Microbial densities and activities can also differ depending on the presence of plants or on the species present in the wetland (Angeloni *et al*., 2006; Gagnon *et al.*, 2007; Wang *et al.,* 2008; Ruiz-Rueda *et al.*, 2008; Li *et al*., 2008). A few studies recently found higher functional microbial diversity or higher microbial activity in rhizosphere sediments compared to non-rhizosphere (bulk) sediments (Tam *et al.*, 2001; Vacca *et al*., 2005) indicating some kind of connection between plants and their adjacent microbial communities. The influence of plants on their surrounding environment, clearly evidenced in terrestrial plants (see e.g. Hawkes *et al.,* 2007) seems to be much less clear in aquatic ecosystems, probably due to a potentially more homogeneous environmental conditions in wetlands in comparison with soil.

Polluted areas and constructed wetlands have been the focus of the majority of microbially-based research on plants-bacteria coupling (Gutknecht *et al.*, 2006). Constructed wetlands have been extensively developed in the last decades for the treatment of point and diffuse pollution and their widespread use and research has also improved the knowledge on the functioning of, not only constructed, but also natural wetlands (Reddy and DeLaune, 2008). An important part of the treatment in these systems is attributable to the presence and activity of plants and to the

interactions between plant and bacteria. Nevertheless, until recently the microbial ecology of constructed wetlands has remained relatively uncharacterized (Stottmeister *et al.*, 2003) and there is still little understanding of microbial community structure in constructed wetlands and how they are influenced by plants or other potential factors like hydraulic fluxes or organic matter.

In this study an experimental constructed wetland was built with the objective to stydy, a) the effect of plants on their surrounding rhizosphere by comparing planted and un-planted tanks b) the differences between Typha angustifolia and Phragmites australis with respect their microbial root-associated communities and, c) the effect of hydraulic design (surface vs. sub-surface) and organic loading on the microbial components of planted tanks.

MATERIAL AND METHODS

Experimental pilot plant

The constructed wetland pilot plant was situated in León (north-west of Spain) and received primary settled wastewater from the urban wastewater treatment plant as influent (mean influent BOD_5 of 105 mg O_2/L). Eight mesocosm tanks 1 m^2 surface 0.5 depth were used for the experiment and divided in three sets (Fig.1) . A set of three tanks (tanks numbers 2, 3 and 4) were half-filled with gravel (0.7-15 mm diameter) up to 0.25 m and water up to 0.5 m, there was therefore a free water surface (FWS) layer of 0.25 m above the gravel bed in this set of tanks. Two of the tanks (tanks 2 and 3) were planted with *Typha angustifolia* and the other (tank 4) was unplanted and considered as control for plant effect comparisons. Influent and effluent were surface in tank 2 (i.e. effluent from the surface at the end of the tank), whereas effluent was sub-surface, (i.e. effluent from the bottom at the end of the tank) in tanks 3 and 4. Comparison of tanks 2 and 3 was therefore used to test flow effect (surface *vs.* sub-surface) and comparison of tanks 3 and 4

to test differences between *Typha* planted and unplanted tanks. Another set of three tanks (tanks numbers 6 to 8) were filled up (0.5 m depth) with siliceous gravel, water level being 4 cms below gravel surface (no free-water surface present), flow being sub-surface (SSF tanks) as water was collected from the bottom at the end of the tank. Two tanks (tanks 6 and 7) were planted with *Phragmites australis* and the other tank (tank 8) was left unplanted and considered as control. One of the planted tanks (tank 6) was loaded with 9 g $BOD_5/m^2/d$ whereas both, the other planted tank (tank 7) and control tank (tank 8) were loaded with 3 g $BOD_5/m^2/d$. Comparison of tanks 6 and 7 allowed to check for loading effects while comparison of tank 7 and 8 for *Phragmites* effect. The last set of tanks (tanks 1 and 5) were filled up with 0.25 m water without any gravel inside (hydroponic tanks). Tank 1 was planted with *Typha* and plant 5 with *Phragmites* to compare bacterial community differences among plant species. Plant support in the hydroponic tanks followed previous experiences (Soto *et al.*, 1999), using rolled strips (15 cm diameter, 25 cm length) of garden plastic nets 2 cms pore size to allow both, root support and development. Rolled strips were tided-up to PVC-tube frames to give more stability to the structure when plants reached full development. All tanks received then the same wastewater as influent with a load of 3 g $BOD_5/m^2/d$, with exception of the aforementioned tank 6 which received three times more.

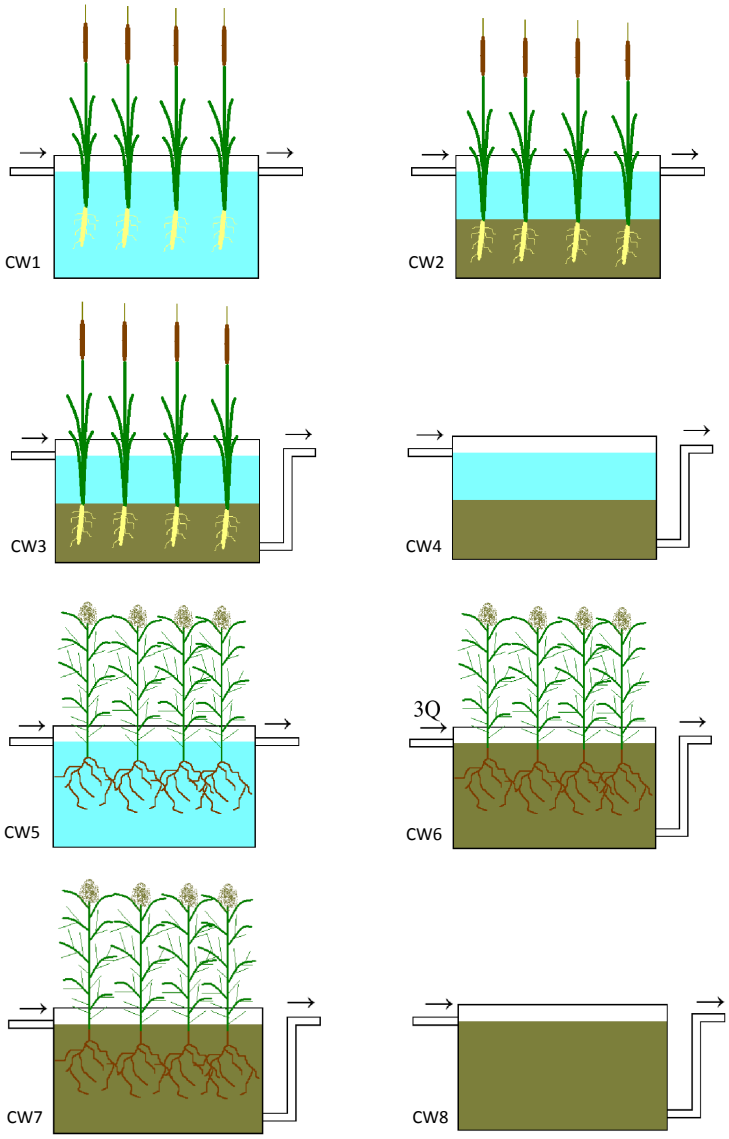

Figure 1. Schematic design characteristics of the CWs.

Collection of samples

Samples were taken in summer 2007 when plants were in their maximum development. Biofilm samples from the gravel and plant roots, and from the interstitial liquid of each of the tanks were simultaneously taken in two zones in the last third of the tanks, close to the final effluent. Bacteria attached to the gravel were dislodged using protocols described by Pierzo *et al.* (1994). About two hundred grams of gravel was firstly washed with sterile tap water to remove all particles loosely attached to the gravel and later with buffered milli-Q water. Sample was divided in tubes with 100 g gravel each and 100 ml buffered milli-Q. Tubes were shaken in an orbital shaker for 15 min at 1000 *rpm* and sonicated later at 3 min interval 1 min rest for four times following previous experiences. Supernatant was centrifugued in 45 ml tubes at 14.000 g for 30 min in a refrigerated (-4ºC) centrifugue. Pellet was preserved at -20ºC until DNA extraction.

Bacterial biofilm from plant roots was extracted using protocols described in Lu *et al*. (2006), Yang and Crowley (2000) and Ibekwe *et al.* (2003). About 25g fresh weight of small active roots were taken form the plants rhizomes. Roots were first washed with sterile tap water and buffered milli-Q water and later homogenized with an Ultra-turrax. Homogenized samples were kept in 30 ml Falcon tubes at -20ºC until DNA extraction. Interstitial water from the gravel bed was sampled with a 100 ML syringe at 15 cm depth from the gravel surface. Samples were centrifuged at 14.000 g for 30 min in a refrigerated (-4ºC) centrifuge and kept at -20ºC until extraction.

DNA extraction

Different kits were utilized for DNA extraction. The DNA Power Soil kit from MOBIO (12888-50) was used for DNA extraction of interstitial liquid and gravel biofilm, while the PowerMax soil kit from MOBIO (12988-10) was utilized for roots. DNA

from the influent was extracted with the UltraClean water DNA kit (MOBIO, 14880-25). DNA integrity was checked by agarose gel electrophoresis, and quantified using a low DNA mass ladder as a standard (Invitrogen).

PCR-DGGE fingerprinting

Fragments of the 16S rRNA gene suitable for DGGE analysis were obtained by using the bacterial specific primer set 358f-907rM (Sánchez *et al.*, 2007). Polimerase chain reaction (PCR) was carried out with a Biometra thermal cycler using the following program: initial denaturation at 94ºC for 5 min; 10 touchdown cycles of denaturation (at 94ºC for 1 min), annealing (at 63.5-53.5ºC for 1 min, decreasing 1ºC each cycle), and extension (at 72ºC for 3 min); 20 standard cycles (annealing at 53.5ºC, 1 min) and a final extension at 72ºC for 5 min. PCR mixtures contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) at a final concentration of 600 $\mu g \cdot ml^{-1}$ was added to minimize the inhibitory effect of humic substances (Kreader, 1996). The volume of reactions was 50 µl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen). The DGGE was run in a DCode system (Bio-Rad) as described by Muyzer *et al.*, (1998). A 6% polyacrylamide gel with a gradient of 30-70% DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). Seven hundred ng of PCR product were loaded for each sample and the gel was run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Gel Doc EQ (Bio-

Rad). Prominent bands were excised from the gels, resuspended in milli-q water overnight and reamplified for its sequencing.

16S rRNA gene sequencing

Purification of PCR products from DGGE bands and sequencing reactions were performed by Macrogen (South Korea) with primer 907rM. They utilized the Big Dye Terminator version 3.1 sequencing kit and reactions were run in an automatic ABI 3730XL Analyzer-96 capillary type. Sequences were subjected to a BLAST search (Altschul *et al.*, 1997) to obtain an indication of the phylogenetic affiliation, and to the Bellerophon program (Huber *et al.*, 2004) to determine potential chimeric artifacts.

Fifty-eight 16S rRNA gene sequences were sent to the EMBL database (http://www. Ebi.ac.uk/embl) and received the following accession numbers: from FM991973 to FM992030.

Quantitative analyses

Digitalized DGGE images were analyzed with Quantity One software (Bio-Rad). Bands occupying the same position in the different lanes of the gels were identified. A matrix was constructed for all lanes, taking into account the presence or absence of the individual bands. This matrix was used to calculate similarity index for presence-absence data using the Past program (Hammer *et al.*, 2001). Raup-Crick index was utilized as this method uses a randomization (Monte Carlo) procedure, comparing the observed number of species occurring in both associations with the distribution of co-occurrences from 200 random replicates. Dendrograms using unweighted-pair group average (UPGMA) given by other three different algorithms (Jaccard, Dice and Simpson) were also compared in order to assess the robustness of the groupings. Statistical differences between groups

were checked with non-parametrics Man-Withney and Kolmogorov-Smirnov test, and with the ANOSIM permutation/randomization test using PRIMER v5 (Clarke and Gorley, 2001). The R statistic produced by this test fluctuates from 0 (no differences) to 1 (perfect separation) with R>0.75 indicating well separated groups, R>0.5 overlapping but clearly different, or barely separated at all when R<0.25. Non-metric multidimensional scaling MDS was also used to check for 2D grouping of sampling points. Raup Crick similarity matrixes from the PAST program (Hammer *et al.*, 2001) were also used as data input for both, ANOSIM and MDS tests. As groups from MDS were mostly the same than those from UPGMA dendrograms only the former will be presented.

RESULTS

Bacterial community composition

Analysis of the bacterial community composition by PCR-DGGE was performed on influent, gravel biofilm, interstitial liquid and root samples collected in summer 2007 for the different experimental wetlands. DGGE was performed independently for each of the environments (roots, gravel and interstitial samples), and also independently for the different hydraulic designs studied: hydroponic, subsurface flow (SSF) and free-water surface flow (FWS). Banding patterns for the 16S rRNA DGGE-PCR amplicons are presented in Fig. 2. The number of bands per lane varied from 10 to 31 (mean values of 20.2, 16.7 and 19.8 for gravel biofilm, roots and interstitial liquid respectively). Some differences could be observed in band position, intensity, and number of bands present in the different samples for each environment, demonstrating that different bacterial communities developed.

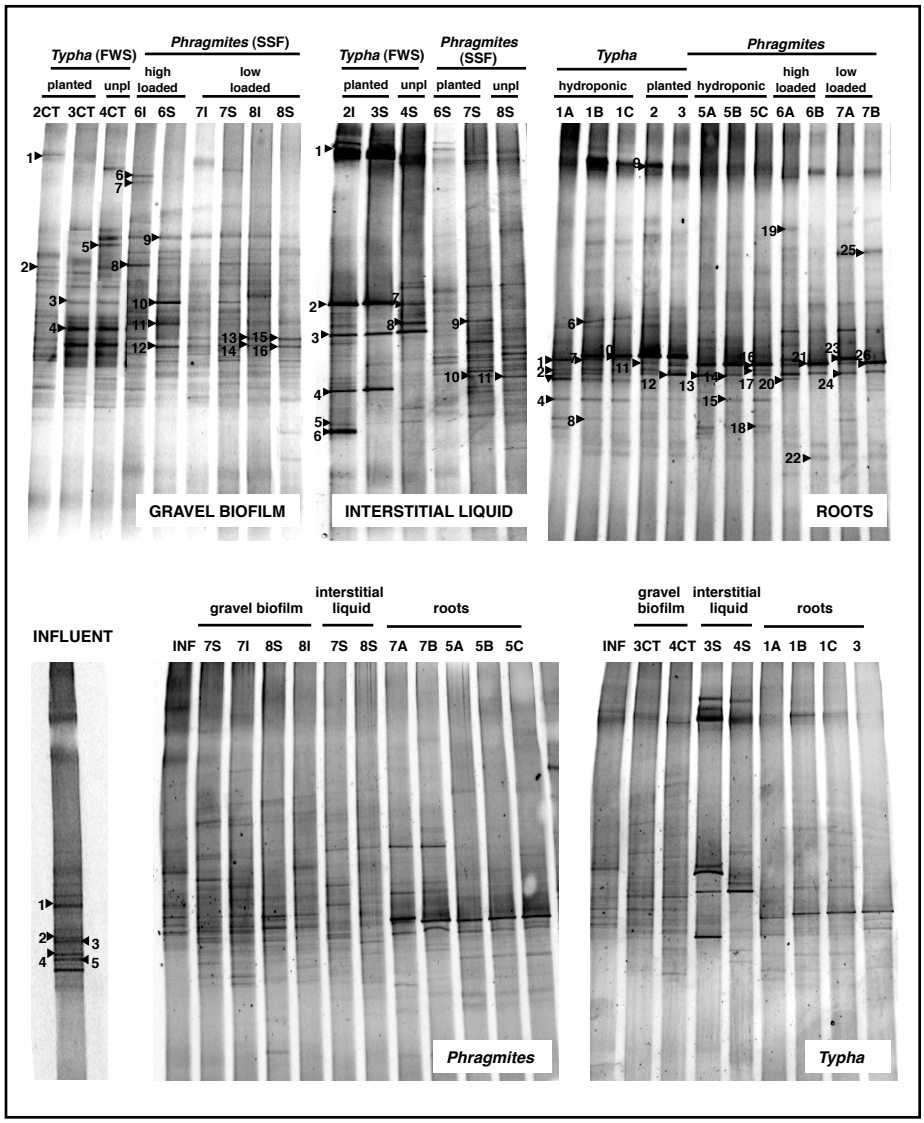

Figure 2. DGGE fingerprints from samples of influent, gravel biofilm, interstitial liquid and roots obtained from the different wetland-tanks. Numbers on the top of lanes refers to the experimental tank, letters A, B anc C refer to replicates inside the same tank (when available); CT: central sampling, S: surface sampling, I: lower part sampling, unpl: unplanted.

Dendrograms and multidimensional scaling (MDS) were applied to discriminate microbial assemblages between the different microenvironments. The dendrograms based on the DGGE banding pattern from roots, gravel biofilm and interstitial water (Fig. S1, supplementary material) separated the samples according to the different wetland design and plant species. Dendrograms were very consistent independently of the algorithm used, being groups fully coincident with those observed in the MDS (Fig. 3).

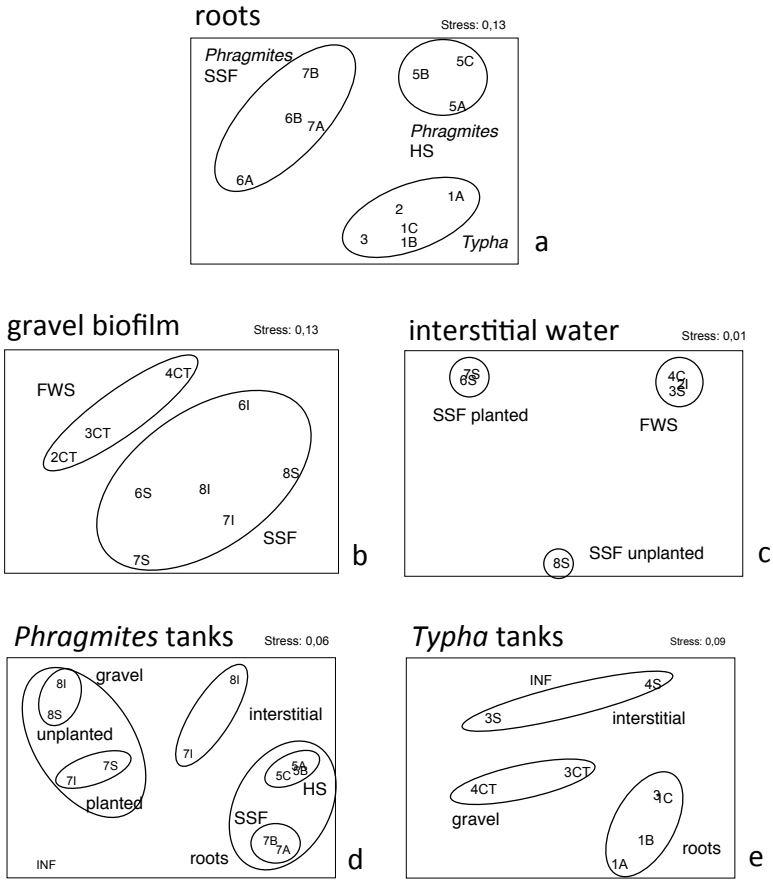

Figure 3. Multidimensional scaling (MDS) analyses generated from the DGGE profiles of the different samples analyzed for each environment (gravel biofilm, interstitial liquid and roots), as well as for each planted tanks (*Phragmites* and *Typha* planted tanks). Type of flow, subsurface (SSF) and free-water (FWS) of planted or un-planted tanks are indicated. Sample number refers to tank type, letters to replicates inside the same tank. Circles refers to main groups in the dendrograms (supplementary material). INF: influent to the tanks.

Samples from roots split in three main clusters or MDS associations according to the different types of constructed wetlands, and on the base of plant type (Fig. 3a). MDS clearly differentiated 3 groups: Samples from *Typha* planted tanks (samples 1A, 1B, 1C, and root samples from tanks 2 and 3), samples from the hydroponic *Phragmites* tank (samples 5A, 5B and 5C), and samples from the gravel (SSF) *Phragmites* planted tanks (samples 6A, 6B, and 7A, 7B). DGGE gel patterns from *Typha* and *Phragmites* roots proved to be statistically different when using one-way ANOSIM test (R = 0.582, P = 0.001). Two-way ANOSIM test also showed differences for plant type (R= 0.864, P=0.007) and also for the type of tank, being the hydroponic tank planted with *Phragmites* (tank 5) statistically different from the gravel planted ones (tanks 6 and 7) (R= 0.663, P=0.009). Bacteria assemblages growing on the roots of *Typha* are therefore statistically different from those growing on *Phragmites* roots. With regard to the hydraulic design of the tank, *Typha* roots harbour the same community composition independently on the type of tank where it is growing as no differences are found between hydroponic (tank 1) and FWS gravel tanks (tanks 2 and 3) (Fig. 3a). On the other hand, bacterial community from *Phragmites* roots growing on the hydroponic tank is statistically different from that growing on SSF gravel tanks, being no statistical differences between high organically loaded (tank 6) and low loaded (tank 7) conditions (K-S test, p=0.73). Microbial community composition on *Phragmites* roots was therefore statistically different depending on the hydraulic growth media (hydroponic or gravel-based) whereas no differences were found for *Typha.*

Within the gravel biofilm (Fig. 3b), samples split into two main groups depending on the type of constructed wetland (SSF or FWS) being these two groups statistically different using ANOSIM (R= 0.519, p=0.029). Statistical differences between plant types (*Typha vs*. *Phragmites*) (R= 0.64, p=0.067), planted *vs*. un-planted (control) tanks (R=0.143, p=0.4), or high *vs*. low loaded tanks (R=0.25, p=0.66) did not show significant differences using ANOSIM. The type of flow (surface in tank 2 *vs*. sub-surface in tank 3) did not influence either bacterial

communities present on the gravel of FWS tanks. Presence (FWS) or absence (SSF) of free water surface is therefore statistically more important than other experimental conditions when comparing bacterial communities growing on gravel biofilm.

The community composition of interstitial liquid was strongly separated (MDS stress: 0, 01) according to wetland design (SSF or FWS) or plant presence (Fig. 3c). MDS clearly separated the un-planted (control) SSF tank (tank 8) from the *Phragmites* SSF planted tanks (tanks 6 and 7) and from the FWS tanks (tanks 2, 3 and 4) being differences statistically significant (R=1, p=0.017). Pairwise test showed significant differences between plant species (*Typha vs*. *Phragmites* tanks, R= 1, p=0.01), and between planted *vs*. control SSF tanks (R=1, P=0.033). These results suggest that interstitial communities are significantly influenced by the type of hydraulic design (SSF or FWS), and by the presence of plants in the case of sub-surface (SSF) systems. Following the pattern previously observed in the gravel biofilm, no differences were found between loading conditions (tank 6 *vs.* 7), neither between *Typha* planted and un-planted tank (tanks 2 and 3 vs. tank 4) nor between surface vs. sub-surface flow *Typha* planted tanks (tanks 2 and 3) with regards to bacterial communities in the interstitial water.

DGGE gels with samples belonging either to the *Typha* planted tanks (tanks 1, 3 and its control tank 4) or to the *Phragmites* planted tanks (tanks 5, 7, and its control tank 8), combining the three different environments and the influent in the same gel were also run (Fig. 2). Both, the dendrograms and MDS obtained from these fingerprints showed that samples separated on the base of the microenvironment (gravel biofilm, interstitial water and roots) (Figs. 3d, e). One-way ANOSIM for SSF tanks (gravel tanks planted with *Phragmites* including unplanted tank 8) (Fig. 3d) showed significant statistical differences among the three environments (gravel, roots and interstitial) (R=0.954, P=0,001). Pairwise tests showed significant differences for all the three combinations, gravel *vs*.

interstitial (R=0.821, p=0.05), gravel vs. roots (R=1, p=0.008), and interstitial vs. roots (R=1, p=0.04). No differences were found between planted and unplanted gravel tanks (tank 7 *vs*. 8) neither between roots from the hydroponic and gravel planted tanks (tank 5 *vs*. 7). Concerning FWS tanks (gravel tanks 2 and 3 planted with *Typha* including control tank 4), differences among the three environments were significant for the global ANOSIM test (R=0.85, p=0.005), being pairwise test almost significant for gravel vs. roots (R=1, p=0.06), interstitial vs. roots (R=0.857, p=0.06) and clearly not significant for gravel vs. interstitial (R=0.25, p=0.33). In general, bacterial communities from roots are different from the other two environments inside the same tank, being differences more evident for *Phragmites* than *Typha* planted tanks.

Analysis of predominant bacterial species by PCR-DGGE

A total of 58 band positions were excised and sequenced in order to determine their phylogenetic affiliation. The closest matches (and percentages of similarity) for the sequences retrieved were determined by a BLAST search (Table 1). The number of bases used to calculate each similarity value is also shown in Table 1 as an indication of the quality of the sequence. Five bands could be retrieved from the DGGE corresponding to the influent. They belonged preferentially to different *Betaproteobacteria*, while one band (band 5I) affiliated with a high similarity (>97%) to *Acinetobacter johnsonii*, a *Gammaproteobacteria*. Bands affiliated to *Betaproteobacteria* constituted a constant in the different environments studied in this work. Thus, some DGGE bands corresponding to different gravel biofilm samples showed similarities higher than 94.4% to uncultured members of this phylogenetic group, while some DGGE bands of samples corresponding to interstitial liquid, significantly affiliated to the freshwater widespread *Polynucleobacter* sp., as well as to uncultured *Betaproteobacteria*. One DGGE band obtained from root samples (band 3R) was affiliated to the recently isolated

Comamonas compostus, although at a low similarity value, and the rest of *Betaproteobacteria* bands from this environment matched well with non-cultured members of this group. Members of uncultured Bacteroidetes were present in all samples of constructed wetlands. Besides, sequences of *Chryseobacterium joostei* were found in gravel biofilm samples (bands 1L and 14L), whereas sequences of *Flavobacterium* sp. were present in interstitial liquid and roots of *Phragmites* (band 13R).

Table 1. Phylogenetic affiliation of sequences obtained from DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Acc nº (GenBank)	Cultured closest match (% similarity)
INFLUENT					
WET-1I	*Acinetobacter johnsonii*	97.6 (532)	γ-*Proteobacteria*	DQ870719	
WET-2I	*Diaphorobacter oryzae*	98.9 (527)	β-*Proteobacteria*	EU342381	
WET-3I	*Acidovorax defluvii*	99.1 (528)	β-*Proteobacteria*	AM943035	
WET-4I	Uncultured β-*proteobacterium*	98.9 (537)	β-*Proteobacteria*	AM940952	*Acidovorax temperans* (98.7)
WET-5I	*Brachymonas denitrificans*	99.3 (540)	β-*Proteobacteria*	EU434449	
GRAVEL BIOFILM					
WET-1B	Uncultured diatom chloroplast	99.4 (521)	*Bacillariophyta*	AY168726	*Phaeodactylum tricurnutum* (97.9)
WET-2B	Uncultured bacterium	97.6 (523)	*Bacteroidetes*	AB237701	*Flexibacter canadensis* (89.9)
WET-3B	Uncultured β-*proteobacterium*	95.6 (517)	β-*Proteobacteria*	AY947965	*Azovibrio* sp. (93.3)
WET-4B	Uncultured bacterium	96.2 (525)	δ-*Proteobacteria*	EU443000	*Desulfobacula toluolica* (94.3)
WET-5B	Uncultured bacterium	97.6 (401)	*Bacteroidetes*	AB237701	*Flexibacter canadensis* (87.8)
WET-6B	*Chryseobacterium joostei*	99.4 (522)	*Bacteroidetes*	EF204455	
WET-7B	*Chryseobacterium joostei*	99.4 (522)	*Bacteroidetes*	EF204455	
WET-8B	Uncultured *Bacteroidetes*	99.6 (534)	*Bacteroidetes*	EF111172	*Alistipes onderdonkii* (85.3)
WET-9B	Uncultured bacterium	98.9 (534)	*Firmicutes*	AY532555	*Erysipelothrix rhusiopathiae* (92.4)
WET-10B	Uncultured bacterium	94.2 (506)	*Bacteroidetes*	AM086159	*Prolixibacter bellariovorans* (90.1)
WET-11B	Uncultured bacterium	99.3 (542)	δ-*Proteobacteria*	EU234252	*Desulfobacter postgateii* (98.9)
WET-12B	Uncultured bacterium	99.3 (541)	β-*Proteobacteria*	AJ318917	*Dechloromonas denitrificans* (96.9)
WET-13B	Uncultured bacterium	94.4 (388)	β-*Proteobacteria*	EU529730	*Rhodocyclus tenuis* (93.4)
WET-14B	Uncultured bacterium	97.1 (529)	β-*Proteobacteria*	AM909879	*Siderooxidans paludicola* (94.5)
WET-15B	Uncultured *Gallionella*	97.2 (529)	β-*Proteobacteria*	AB252929	*Denitratisoma oestradiolicum* (94.5)
WET-16B	Uncultured bacterium	98.9 (539)	β-*Proteobacteria*	AB355063	*Denitratisoma oestradiolicum* (95.6)

a. Number of bases used to calculate the levels of sequence similarity.

Table 1 (continued). Phylogenetic affiliation of sequences obtained from DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Acc nº (GenBank)	Cultured closest match (% similarity)
INTERSTITIAL LIQUID					
WET-1L	*Flavobacterium* sp.	99.8 (533)	*Bacteroidetes*	AB426577	*Flavobacterium succinicans* (98.7)
WET-2L	Uncultured bacterium	98.9 (539)	β-*Proteobacteria*	EU234274	*Azovibrio restrictus* (94.9)
WET-3L	*Polynucleobacter* sp.	99.6 (533)	β-*Proteobacteria*	AB426572	*Polynucleobacter necessarius* (98.7)
WET-4L	Uncultured *Actinobacterium*	100 (526)	*Actinobacteria*	AY948008	*Salinibacterium aquaticus* (98.1)
WET-5L	Uncultured bacterium	98.1 (517)	*Actinobacteria*	AJ863316	*Leifsonia xyli* (97.9)
WET-6L	Uncultured bacterium	98.5 (517)	*Actinobacteria*	AJ863316	*Leifsonia xyli* (98.3)
WET-7L	*Polynucleobacter* sp.	99.4 (541)	β-*Proteobacteria*	AB426572	*Polynucleobacter necessarius* (98.5)
WET-8L	*Polynucleobacter* sp.	100 (542)	β-*Proteobacteria*	AB426572	*Polynucleobacter necessarius* (99.1)
WET-9L	Uncultured bacterium	98.8 (513)	*Firmicutes*	AY754834	*Clostridium botulinum* (90.6)
WET-10L	*Haslea salstonica* chloroplast	93.0 (494)	*Bacillariophyta*	AF514854	
WET-11L	Uncultured bacterium	89.7 (490)	β-*Proteobacteria*	EU133809	*Rubrivirax gelatinosus* (88.8)
ROOTS					
WET-1R	Uncultured bacterium.	98.9 (538)	*Acidobacteria*	EU499471	*Geobacter pickeringii* (83.2)
WET-2R	*Lolium perenne* chloroplast	99.8 (522)	*Spermatophyta*	AM777385	
WET-3R	*Comamonas compostus*	90.1 (491)	β-*Proteobacteria*	EF015884	
WET-4R	Uncultured bacterium	96.9 (402)	*Acidobacteria*	EU499471	*Desulfomonile limimaris* (84.9)
WET-5R	*Lolium perenne* chloroplast	100 (519)	*Spermatophyta*	AM777385	
WET-6R	Uncultured bacterium	93.5 (507)	β-*Proteobacteria*	FJ037637	Candidatus *Nitrotoga arctica* (91.3)
WET-7R	β-*Proteobacterium*	91.9 (487)	β-*Proteobacteria*	AY297807	*Rhodocyclus* sp. (91.1)
WET-8R	Uncultured bacterium	95.2 (511)	*Bacteroidetes*	DQ093919	*Bacteroides* sp. (91.8)
WET-9R	Uncultured bacterium	98.7 (512)	*Firmicutes*	AY754834	*Clostridium botulinum* (90.0)
WET-10R	Uncultured bacterium	89.3 (469)	β-*Proteobacteria*	AY785239	*Rhodocyclus tenuis* (86.9)
WET-11R	Uncultured Rhodocyclaceae	95.1 (504)	β-*Proteobacteria*	EU266786	*Dechloromonas hortensis* (94.3)
WET-12R	Uncultured bacterium	99.4 (540)	β-*Proteobacteria*	EF667706	*Dechloromonas hortensis* (98.7)
WET-13R	*Flavobacterium* sp.	99.8 (533)	*Bacteroidetes*	AB426577	*Flavobacterium succinicans* (98.7)
WET-14R	Uncultured bacterium	98.3 (509)	α-*Proteobacteria*	EU284319	*Rhodobacter blasticus* (97.5)
WET-15R	Uncultured bacterium	99.4 (515)	α-*Proteobacteria*	EU284319	*Rhodobacter blasticus* (97.5)
WET-16R	Uncutlured *Acidobacteria*	98.2 (534)	*Acidobacteria*	DQ676412	*Holophaga fetida* (83.1)
WET-17R	Uncultured bacterium	98.2 (532)	*Acidobacteria*	EU499471	*Desulfofrigus* sp. (82.9)
WET-18R	Uncultured bacterium	98.5 (533)	*Acidobacteria*	EU499471	*Desulfofrigus* sp. (82.7)
WET-19R	Uncultured bacterium	95.2 (499)	α-*Proteobacteria*	AF502221	*Rhizobium giardinii* (90.8)
WET-20R	Uncultured soil bacterium	95.2 (519)	β-*Proteobacteria*	DQ297980	*Methylibium* sp. (94.3)
WET-21R	*Lolium perenne* chloroplast	100 (519)	*Spermatophyta*	AM777385	
WET-22R	*Lolium perenne* chloroplast	99.2 (516)	*Spermatophyta*	AM777385	
WET-23R	Uncultured *Geobacter* sp.	99.3 (542)	δ-*Proteobacteria*	AM159357	*Pelobacter propionicus* (98.7)
WET-24R	Uncultured β-*proteobacterium*	99.1 (540)	β-*Proteobacteria*	AB265946	*Denitratisoma oestradiolicum* (95.4)
WET-25R	*Chlorobium* sp.	98.3 (524)	*Chlorobi*	AB210277	
WET-26R	*Lolium perenne* chloroplast	100 (522)	*Spermatophyta*	AM777385	

a. Number of bases used to calculate the levels of sequence similarity.

Firmicutes (Gram positive bacteria with low G+C content) could also be observed in the three environments studied from artificial wetlands, although closest matches corresponded to uncultured members of this group. On the other hand, sequences from *Deltaproteobacteria* were only present in samples from gravel biofilm and roots, and some taxonomic groups were found exclusively in one of the environments. Thus, uncultured *Actinobacteria* predominated in some samples of interstitial liquid, while groups such as *Acidobacteria*, *Chlorobi* or *Alphaproteobacteria* were specific of roots samples. Sequence identity of most of the bands in Table 1 showed that they were closely associated with different species or bacteria from sludge or wastewater environments.

DISCUSSION

Bacterial assemblage diversity as revealed by DGGE

Excision of prominent DGGE bands and subsequent sequencing allowed characterization of predominant microorganisms from the influent as well as from gravel biofim, interstitial water and roots. Most of these sequences belonged to uncultured microorganisms, while others matched well with cultured bacteria. In general, they were closely associated with different species or bacteria from sludge or wastewater environments. Thus, within the *Beta* and *Gammaproteobacteria* predominating in the influent, the genus *Acinetobacter* (a gammaproteobacterium) and the facultative anaerobe *Brachymonas denitrificans* (a betaproteobacterium) occur in a wide variety of activated sludges in which enhanced biological phosphate removal is observed (Kortstee *et al*., 1994; Ivanov *et al*., 2005). On the other hand, *Acidovorax defluvii* (a betaproteobacterium) was isolated from activated sludge samples from a municipal wastewater treatment plant (Schulze *et al.*, 1999). Furthermore, *Diaphorobacter* sp. is able to perform partial nitrification followed by further aerobic removal of nitrite in high-nitrogen-containing

wastewaters. Overall, ammonia-oxidizing bacteria seem to play an important role in the nitrogen cycle in natural and constructed wetlands.

In contrast, sequences of *Betaproteobacteria*, *Bacteroidetes* and *Firmicutes* could be retrieved from the three environments studied in the different wetlands. Within the *Betaproteobacteria*, most sequences had uncultured closest matches; in these cases, cultured closest matches corresponded mostly to microorganisms related to the nitrogen cycle. For example, several sequences from gravel biofilm and roots were associated to *Denitratisoma oestradiolicum*, a denitrifying bacterium isolated from activated sludge of a wastewater treatment plant (Fahrbach *et al.*, 2006), or to *Dechloromonas denitrificans*, a N_2O-producing facultative aerobe (Horn *et al.*, 2005). On the other hand, samples of interstitial liquid abounded in sequences closely related to *Polynucleobacter* sp., a typical freshwater microorganism detected in acidic, neutral and alkaline habitats located in different climatic zones (Hahn, 2006). A number of sequences belonged to the phylum *Bacteroidetes*. For instance, *Flavobacterium* sp. is also a typical genus, which can be found in activated sludge (Park *et al.*, 2007, Shokrollahzadeh *et al.*, 2008; Yu et *al.*, 2007); it was found in samples of interstitial water and roots.

Other sequences, however, pertained to microorganisms present only in specific environments. Thus, sequences affiliated to uncultured *Deltaproteobacteria* could be detected in gravel biofilm and roots, but were not retrieved in prominent bands of interstitial liquid. Some of these sequences were associated to sulphate-reducing bacteria, a ubiquitous group of microorganisms that can be found in many natural and engineered environments where sulphate is present, such as anaerobic wastewater treatment plants (Ben-Dov *et al.*, 2007; Dar *et al.*, 2005).

Other groups were just detected in one particular environment. Thus, sequences affiliated to *Actinobacteria* could only be retrieved in interstitial water samples, while samples belonging to *Acidobacteria*, *Alphaproteobacteria* and *Chlorobi* were

found in roots. *Actinobacteria* include some of the most common soil life microorganisms, performing an important role in decomposition of organic materials, and thereby playing a vital part in organic matter turnover and carbon cycle. Some studies have reported their ability to degrade environmental pollutants in activated sludge from wastewater treatment (Kim *et al.*, 2007, 2008). *Acidobacteria* was another taxonomic group only observed in root samples. Sequences belonging to this widespread distributed phylum have been found traditionally in soil, aquatic environments and wastewater treatment plants (Ludwig *et al.*, 1997; Juretschko *et al.*, 2002). On the other hand, *Alphaproteobacteria* related to the phoheterotrophic purple nonsulfur bacterium *Rhodobacter blasticus* have also been detected in roots; this microoganism has been isolated mainly from wastewater and polluted environments such as photosynthetic sludge processes (Hiraishi *et al.*, 1995; Okubo *et al.*, 2005). In general, the genus *Rhodobacter* includes species with an extensive range of metabolic capabilities which allow them to survive in a number of varied habitats, thus appearing in all types of aquatic environments. In fact, the group *Alphaproteobacteria* has also been found as attached bacteria from different species of plants in aquatic environments (Crump and Koch, 2008). Sequences belonging to *Chlorobium* sp., a green sulfur bacterium, were also detected only in roots. The ability of this microorganism to use sulfide has been exploited in the past for the treatment of sulfide-containing wastewaters and gas streams (Cork *et al.*, 1983; Kobayashi *et al.*, 1983). Thus, many of the species recovered from DGGE bands in this work have the potential to act as degraders of environmental pollutants in natural and constructed wetlands.

Differences between plant species

Graphical and statistical analysis showed significant differences between *Typha* and *Phragmites* root-associated microorganisms. Our protocol did not differentiate

between endophytic and rhizoplane bacteria (McClung *et al.*, 1983; Chelius and Tripplet, 2001), therefore both root habitats were pooled in the analysis. Plants species in soils clearly influence the microbial communities of their roots, each plant species harbouring a distinctive microbial assemblage (see e.g. Hawkes *et al.*, 2007; Ehrenfeld *et al.*, 2005, for revision). Nevertheless, a small body of research has demonstrated this aspect in the roots of wetland plants as mostly are focused on the rhizosphere and not on the proper roots or their rhizoplanes**.** Abundances of Fe-oxidizing bacteria were different among five wetland species studied by Emerson *et al*. (1999), which isolated different strains in the rhizoplane of different plant species. Bergholz *et al.* (2001) compared the physiological diversity of root-asociated bacteria of *Spartina patens* with results from a previous work studying *S. alterniflora* and *Juncus roemerianus* rhizoplains (Bagwell *et al.*, 1998). Substantial differences among roots communities were explained due to bacterial oxygen preference and therefore to differences in oxygen production by the plant species. The same reasoning was applied by Briones *et al.* (2003) to explain differences in band patters among different rice cultivars. A more evident work on species differences was carried out by Chelius and Lepo (1999), who made RFLP analysis to the rhizosphere of *Spartina alterniflora* and *Sesbania macrocarpa*, revealing differences in the community structure of N2-fixing bacteria. Concerning constructed wetlands, Ruiz-Rueda *et al*. (2008) also found different nitrogen-related microbial communities when comparing *Typha* and *Phragmites* species. In our work, *Typha angustifolia* roots harbour a significantly distinctive community than *Phragmites australis*, with significant variations between hydroponically and gravel grown plants in this last species.

Differences in bacterial assemblages between hydroponically and gravel-filled *Phragmites* tanks could be due to the environmental differences in these tanks. Wirsel *et al*. (2001) found that endophytic fungi biodiversity in *Phragmites* roots changed depending on the type of soil media. In our case, the study of main environmental conditions (redox, pH, or dissolved oxygen; E. Becares in prep.)

showed that pH was the only variable with statistical differences (ANOVA, F= 116, p<0,001) between the hydroponic tank 5 (pH=7) and the gravel planted tanks 6 and 7 (both with a pH= 6.6). Organic loading, another important factor potentially influencing root development and oxygen release due to the toxic effect of low redox conditions (see e.g. Armstrong and Armstrong, 2005) did not affect differences among redox conditions in the experimental tanks probably due to the low loadings applied (redox values of 162mV and 166 mV for high and low loaded tanks, respectively). Growth substrata (i.e. hydroponic or gravel) seemed therefore to have more influence on bacteria assemblages of *Phragmites* roots than the organic load assayed.

The effect of plants and hydraulic design on microbial communities

Hydrology has consistently been proved to be an important control variable in wetland communities (Gutknecht *et al.*, 2006). In our work the influence of hydraulic design on bacterial communities was significant in both, interstitial water and gravel biofilm associated communities when considered separately (Fig. 3b, c) as bands from SSF and FWS systems kept significantly different in both environments. These results agree with Vacca *et al.*, (2005) which found that hydraulic design (horizontal or vertical) produced differences in band patterns in bulk soil of constructed wetlands. Hydraulic patterns were also driving forces shaping bacterial communities in a 0.1 ha constructed wetland (Popko *et al.*, 2006). Hydraulic design mainly affects redox conditions in the water due to a better exchange of oxygen in FWS than SSF systems, FWS having therefore higher redox than SSF systems (Lin *et al.*, 2008; Kadlec and Knight, 1996). The presence of a water layer in FWS wetlands also allows the development of a planktonic and periphytic community in these wetlands which could also influence the microbial community of their sediments and their interstitial water (Reddy and DeLaune, 2008). Studies have revealed that the redox state of the rhizosphere has a

significant effect on the intensity of oxygen release through the roots of helophytes (Wiessner *et al.*, 2002; Armstrong and Armstrong, 2001). In our experiment mean redox conditions were clearly different between FWS and SSF tanks (mean values of 234mV and 137 mV, respectively), with significantly lower values for the unplanted gravel tank 8 (80 mV) than the *Phragmites* planted (tanks 6 and 7, 164 mV mean) (ANOVA, F=23.5, p<0,001). In accordance with the previously mentioned results on bacterial communities in the roots, higher organic loading in SSF tank 6 (9 g $BOD_5/m^2/d$) did not make effect on microbial communities in comparison with low-loaded tank 7 (3 g $BOD_5/m^2/d$). This could be due to the still low loading assayed as 9 g $BOD_5/m^2/d$ is below the recommended design value in some wetland manuals (e.g. Reed, 1990).

Also, no difference was found between gravel and interstitial communities of tank 2 (FWS surface flow) and tank 3 (FWS sub-surface flow). This is in accordance with the absence of differences in redox conditions between tanks 2 (120 ±75 mV) and tank 3 (180±71mV). The effect of redox conditions in shaping the rhizosphere microbial communities has been observed in both, natural and constructed wetlands (Dong *et al.*, 2006; Nikolausz *et al.*, 2008; Wang *et al.*, 2008; Ahn *et al.*, 2008). *Typha* and *Phragmites* are clearly different in root development and oxygen production (Brix, 1990; Reddy *et al.,* 1990; Armstrong *et al.*, 1990), being plausible to think, as it has already shown with other wetland species (Lu *et al.*, 2004; 2006), that these differences could affect the microbial composition of their rhizospheres.

Separate analysis of DGGE band patterns for SSF and FWS tanks significantly differenced three community groups: roots, interstitial and gravel communities (Fig. 3d, e). Differences were, in accordance with previously mentioned results, more significant for SSF tanks than for FWS tanks. Microbial communities are therefore different for the different habitats under the same hydraulic design having plant species a secondary influence on these differences. Differences between planted and non-planted control tanks have been only evident for

Phragmites interstitial water communities but such clear results were not found for *Typha*. These results agree with Vaca *et al*. (2005) who found differences in band patterns between planted and non-planted *Phragmites* tanks and also between the type of soil used for growing. Lower differences among studied habitats for *Typha* tanks could be explained as consequence of a higher influence of this species on their surrounding rhizosphere and to the "planktonic" effect of the free-water environment developed on top of the tanks. In accordance to this hypothesis, redox, dissolved oxygen and pH were significantly higher for *Typha* than *Phragmites* planted tanks (mean redox values of 208±42 mV and 163±49 mV, 1.3±0.1 and 0.46±0.2 mgO_2/L of dissolved oxygen, 7±0.1 and 6.6±0.2 uds pH, for *Typha* and *Phragmites* tanks, respectively).

Comparison of our results with previous works on the same topic is problematic because experiments in constructed wetlands treating wastewater are highly variable in operational conditions (e.g. organic loading, redox values, wastewater characteristics, plant species, plant density). Moreover, constructed wetlands experiments are usually designed to test their efficiency for wastewater treatment, and not specifically to test the effect of plants on wastewater treatment. A detailed look on these papers suggest that either high organic loading, low plant density or inadequate redox conditions for root development could have influenced the absence of plant effects in constructed wetlands (Baptista *et al.*, 2008; Ahn *et al*., 2007; DeJournett *et al*., 2007; Gorra *et al*., 2007), whereas in other works opposed conditions could suggest plant effects on their microbial communities (Wang *et al.*, 2008; Li *et al.*, 2008; Ruiz-Rueda *et al*., 2008; Ibekwe *et al.*, 2007; Vacca *et al.*, 2005; see also Stottmeister *et al*., 2003). This also fits with Ravit *et al.* (2006) suggestions that plant effects on associated microbial communities are less evident in human-disturbed (organically loaded) natural wetlands. Plant effects on their associated bacterial communities could be therefore more evident as physico-chemical conditions in constructed wetlands approach those in natural wetlands.

In our work, plants fully covered all tanks and ground biomass reached high values (mean values of 10 and 4 kg DW/m^2 for *Typha* and *Phragmites* planted tanks, respectively), which maximize oxygen release into the rhizosphere (Wiessner *et al.*, 2002). However, organic load was kept much lower than recommended in constructed wetlands manuals (EPA, 2000). Under these circumstances, we were able to find significant differences between plant species with regard to the microbial community structure of their roots. Hydraulic design of the wetland (surface or sub-surface flow) seems to be more important than plant presence in shaping microbial assemblages of their rhizosphere for *Typha* but not for *Phragmites* planted tanks Microbial communities from roots, gravel biofilm and interstitial water were clearly different inside the same planted tank being these differences more important for *Phragmites* than *Typha* planted mesocosms. This paper gives therefore evidences to support the influence of wetland plant species on their root-attached microbial communities and their effect on their surrounding habitats, and also how this last influence is also driven by the hydraulic patterns of the wetland.

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SUPPLEMENTARY MATERIAL

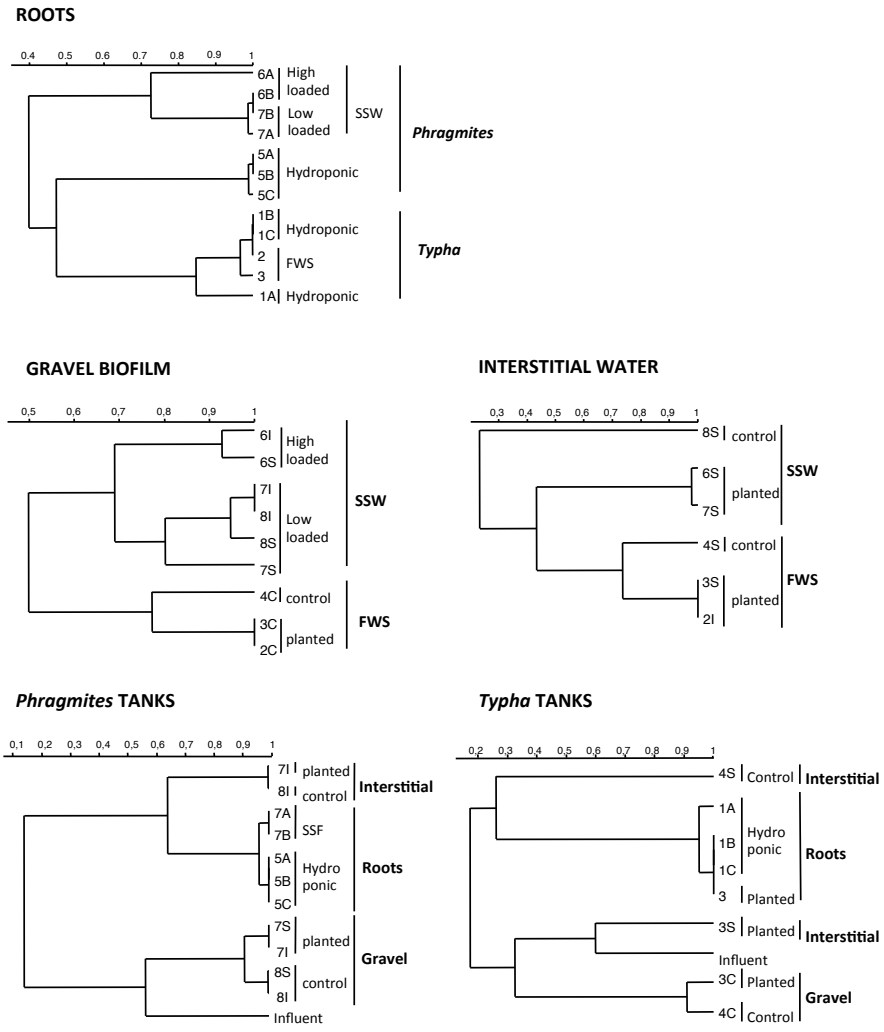

Figure S1. Dendrograms of the bands using similarity Raup-Crick index. Robustness of the associations were confirmed by using other three different algorithms (Jaccard, Dice and Simpson), obtaining the same groups. Dendrograms were carried out individually for root associated bacteria (roots), gravel biofilm and interstitial water. Other two dendrograms were carried out individually for *Phragmites* and *Typha* tanks.

Chapter 3

DYNAMICS OF MICROBIAL DIVERSITY PROFILES IN WATERS OF DIFFERENT QUALITIES. APPROXIMATION TO AN ECOLOGICAL QUALITY INDICATOR

To be submitted to: Water Research

SUMMARY

Over the past two decades, the amount of reclaimed water has increased throughout the world to face the current water shortage, and as a consequence there is an increasing interest to develop good indicators of water quality. Fecal contamination indicators have been typically used but due to their limitations it is important to find alternative indicators of ecological quality. In order to meet this need, in this work the microbial profiles of different wastewater treatment plant effluents, both secondary and tertiary, were studied and compared with water samples from an uncontaminated natural aquifer. Taking into account the most abundant phylogenetic groups found in these water samples, we propose the *Bacteroidetes*, *Gammaproteobacteria* and *Nitrospira* / *Betaproteobacteria* ratio as an alternative indicator since we found a good correlation with water quality, both in our results and when calculated from previous published data.

INTRODUCTION

The increasing demography and the growing water demand for drinking, sanitation, cleaning, production of food and energy, and support of commercial and industrial activities, has lead to the use of reclaimed water in order to face the current water shortage. The amount of reclaimed water, which is derived from treated municipal wastewater, has increased over the past two decades, and it is used for nonpotable applications such as irrigation, cooling water, for industrial processes, and environmental enhancement (Levine and Asano, 2004). Thus, it is important to determine the microbiological quality of these waters for the presence of specific pathogens. During decades, microbial indicators such as total coliforms, fecal coliforms, *Escherichia coli*, and enterococci have been used world wide to evaluate for fecal contamination in water bodies. However, it has been widely demonstrated that these typical indicators do not adequately reflect the

occurrence of pathogens in disinfected wastewater effluents due to their relatively high susceptibility to chemical desinfection and their failure to correlate with protozoan parasites and enteric viruses (Miescier and Cabelli, 1982; Havelaar *et al.,* 1993; Bonadonna *et al.,* 2002). Furthermore, the presence of residual materials and by-products derived from industrial, medical and household usages, has resulted in consistent changes in the characteristics of wastewaters from urban environments and subsequent reclaimed waters, where the prevalence of new contaminants emerges. In this sense, the European Water Framework Directive (WFD, 60EC), which was adopted in 2000, expressed the need to use alternative indicators of ecological water quality, placing aquatic ecology at the base of management decisions.

To that effect, several studies have arisen to determine the health of aquatic ecosystems by bioassessments. Many different indicators have been suggested, including macroinvertebrates such as fish, algae, macrophytes, and microorganisms (Metcalfe, 1989; Barbour *et al*., 1999). For example, the PFU (polyurethane foam units) system, a device colonized by microbial communities in water bodies, has been widely used to evaluate freshwater quality (Cairns *et at.,* 1972; Chung *et al*., 1999; Yu *et al.,* 2005), and systematic studies were undertaken in different countries to determine the simultaneous occurrence and prevalence of various pathogens in natural waters in relation to fecal indicators (Horman *et al*., 2004; Lipp *et al*., 2001; Noble and Fuhrman, 2001; Payment *et al*., 2000; Savichtcheva *et al*., 2007).

It is well-known that bacteria play fundamental roles in nutrient, energy and carbon cycling in aquatic systems (Del Giorgio *et al.,* 1997; Kirchman, 1997; Cotner and Biddanda, 2002), and therefore there is the potential for developing bioassessment tools based on bacterial community composition, bacterial-mediated processes and population dynamics. However, studies dealing with microbial community-based indicators are still scarce. Wu *et al*. (2010) determined

the ratio BBC:A using the relative richness of 4 bacterial classes (*Bacilli*, *Bacteroidetes*, *Clostridia* and *Alphaproteobacteria*) found in two urban watershed systems, representative of the fecal (*Bacilli*, *Bacteriodetes* and *Clostridia*) and the non-fecal (*Alphaproteobacteria*) bacterial community. Despite, an accurate comparison with 124 bacterial communities from other studies strengthened their conclusions, their study was conducted only over a 3-day period.

In this work, we aimed to find an alternative indicator of water quality, which could also offer ecological information while providing insights into the impact of dumping water to a natural environment. For this purpose, we studied the community composition of different types of water: the secondary effluents from two urban Spanish Wastewater Treatment Plants (WWTPs) which underwent primary and secondary (biological) treatments, a tertiary effluent with a higher quality, and a non-contaminated groundwater aquifer as a reference of high quality water. We analyzed the bacterial diversity profiles from samples taken monthly during a year period by using Denaturing Gradient Gel Electrophoresis (DGGE), a molecular technique which allows the comparison of many samples at once. Taking into account the most abundant phylogenetic groups found in our reference contaminated and non-contaminated waters, we propose an alternative indicator of water quality and discuss its reliability in this manuscript. As far as we know, our work represents the first study that approaches an ecological quality indicator examining samples from different water qualities during a long period. In addition, it further provides information into the diversity of microbial communities present in secondary and tertiary effluents of wastewater treatment plants, since up to now, most studies have either focused on the study of activated sludge communities or have reported the presence of conventional fecal microorganisms in these effluents.

MATERIAL AND METHODS

Wastewater treatment plants

Wastewaters correspond to treated effluents from two urban WWTPs, located at El Ejido (southeastern Spain), and El Prat de Llobregat (northeastern Spain). The El Ejido WWTP has a capacity of 64000 habitant-equivalents, and receives 13800 m^3 wastewater on a daily basis from households, industrial activities, greenhouses and from a local hospital. The plant applies a physical pre-treatment to remove coarse solids and greases, primary settling of particulates, and anaerobic-aerobic biological treatment with activated sludge, to remove carbonaceous organic matter and nitrogen. The treated effluent is discharged to the Mediterranean Sea, although a tertiary treatment based on membranes is currently being installed.

The El Prat WWTP (called Baix Llobregat WWTP) is among the largest plants in Spain; it was designed for treating 2 million habitant-equivalents, and a maximum daily flow of 420000 m^3, coming from the Barcelona Metropolitan Area. The plant applies a physical pre-treatment to remove coarse solids and greases, primary settling of particulates, and anaerobic-aerobic biological treatment with activated sludge, to remove carbonaceous organic matter and nitrogen. Phosphorus can additionally be removed by chemical precipitation if needed. Part of the treated effluent at The Baix Llobregat undergoes a reclamation treatment consisting of coagulation-flocculation, settling, filtration and UV disinfection.

Sampling

The secondary and tertiary effluent samples were taken monthly during a one-year period. We took 13 water samples from El Ejido secondary effluent (SE, from March 2007 to May 2008), 10 water samples from Baix Llobregat secondary

effluent (SB, from July 2007 to June 2008) and 12 water samples from Baix Llobregat tertiary effluent (TB, from March 2007 to June 2008).

High quality reference water used in the study corresponded to groundwater from the main Delta del Llobregat aquifer, a strategic water reserve for Barcelona and its Metropolitan Area. The groundwater samples (GW) were taken during a period every other month, with some exceptions. We took a total of 15 water samples from September 2007 to January 2011.

Direct counts and microbiological analyses

From each water sampled, 100 mL were used for analyzing total coliforms and *E. coli*, and 1 mL was used for DAPI counts.

Total counts were performed by epifluorescence microscopy according to Porter and Feig 1980. Cells were fixed with formaldehyde (CH_2O, Sigma) and filtered onto a 0.22 µm GTBP filters (Millipore). Cells were stained with 20 $\mu g.mL^{-1}$ DAPI (4',6-diamidino-2-phenylindole) (Merck) for 5 min and then rinsed in distilled water immediately prior to imaging, and counted by using an Olympus BH Fluorescence Microscope (Olympus).

Total coliforms and *Escherichia coli* were determined using the Colilert™ most probable number method (IDEXX Laboratories Inc), which relies on the presence of b-galactosidase and b-glucuronidase enzymes as described by Niemela *et al.* (2003).

DNA Extraction

About 300 mL of secondary effluents, 1 L of tertiary effluent and 10 L of groundwater were filtered through polycarbonate filters of 0.22 µm of pore size, and filters were stored at –20ºC for further DNA extraction.

Secondary and tertiary effluent and groundwater filtered samples were extracted by using the UltraClean water kit MOBIO ref. 14880-25 according to the manufacturer's protocol. DNA integrity was checked by agarose gel electrophoresis, and quantified using a low DNA mass ladder standard (Invitrogen).

PCR-DGGE fingerprinting

Fragments of the bacterial 16S rRNA gene suitable for DGGE analysis were obtained by using the bacterial specific primer 358F with a 40-bp GC clamp, and the universal primer 907RM (Sánchez *et al.* 2007). Polymerase chain reaction (PCR) was carried out with a Biometra thermocycler using the following program: initial denaturation at 94ºC for 5 min; 10 touchdown cycles of denaturation (at 94ºC for 1 min), annealing (between 63-53 and 65-55ºC for 1 min, decreasing 1ºC each cycle), and extension (at 72ºC for 3 min); 20 standard cycles (annealing at 55.5ºC, 1 min) and a final extension at 72ºC for 5 min. PCR mixures contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U *Taq* DNA polymerase (Invitrogen) and the PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) was added at a final concentration of 600 $\mu g \cdot ml^{-1}$ to minimize the inhibitory effect of humic substances (Kreader, 1996). The volume of reactions was 50 µl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen) using the Quantity One software package (Bio-Rad).

DGGEs were run in a DCode system (Bio-Rad) as described by Muyzer *et al*. (1998). A 6% polyacrylamide gel with a gradient of 30-70% DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). Seven hundred ng of PCR product were loaded for each sample and the gels were run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV light in a Chemi Doc XRS (Bio-Rad). Digitized DGGE images were analyzed using Quantity One from (Bio-Rad). The software performs a density profile along each DGGE lane, detects the bands, and calculates the relative contribution of each band to the total band signal in the lane, after using a rolling disk algorithm correction. A similarity matrix was constructed for each DGGE image based on the relative contribution of each band to the total intensity of the lane. Matrices were imported into the software PRIMER-E (Clarke and Warwick, 2001) and values were square-root normalized prior to calculate similarities in community structure by using the Bray-Curtis index. Average Bray-Curtis values were computed for each matrix as an indicator of variability in community structure over time.

rRNA gene sequencing

Visible bands were excised from the DGGE gels, resuspended in milli-Q water overnight and reamplified for sequencing. Purification of PCR products from DGGE bands and sequencing reactions were performed by Macrogen (South Korea) with primer 907RM. They utilized the Big Dye Terminator version 3.1 sequencing kit and reactions were run in an automatic ABI 3730XL Analyzer-96 capillary type. Gene sequences were deposited in GenBank under accession numbers xx. Sequences were subjected to a BLAST search (Altschul *et al*., 1997) to obtain an indication of the phylogenetic affiliation.

Microbial communities indices

In order to obtain direct descriptors of the diversity of bacterial assemblages, we calculated a widely used diversity index, the Shannon index (*H'*), as explained by Magurran (1988) with the following formula:

$$H' = -\sum_{i=1}^{i=n} p_i \ln p_i$$

where *n* is the number of species in a sample (number of DGGE bands in our study) and p_i the proportion of a certain species (relative band intensity).

Additionally, in order to determine a water quality index we calculated the BGN:β ratio using the relative band intensity corresponding to *Bacteroidetes* (Bac), *Gammaproteobacteria* (GmP), *Nitrospira* (Nit) and *Betaproteobacterial* (BtP) groups in each DGGE lane. The ratio was calculated using the following expression:

BGN:β= Bac + GmP + Nit / BtP

where

Bac= average of the % *Bacteroidetes* (Σ relative band intensity in each sample/number of samples)
GmP= average of the % *Gammaproteobacteria* (Σ relative band intensity in each sample/number of samples)
Nit= average of the % *Nitrospira* (Σ relative band intensity in each sample/number of samples)
BtP= average of the % *Betaproteobacteria* (Σ relative band intensity in each sample/number of samples).

Differences between ratios were tested using a one-way ANOVA with a Tukey-Kramer *post hoc* comparison in R (R Development Core Team, 2008).

RESULTS

Direct counts and microbiological analyses

Total DAPI counts were performed for samples corresponding to the four water qualities over time (Fig.1). In the two secondary effluents (SE and SB) the total cell average was 4.65 x 10^7 and 1.39 x 10^7 cells·ml^{-1} respectively, while in the tertiary effluent (TB) numbers were lower, with an average value of 5 x 10^6 cells·ml^{-1}. Groundwater samples (GW) presented the lowest counts, with an average value of 9.78 x 10^4 cells·ml^{-1}.

Concerning total coliforms and *E. coli*, their presence could only be detected in the two secondary effluents, with an average value of 5.98 x 10^6 MPN · 100 ml^{-1} of total coliforms and 6.4 x 10^5 MPN · 100 ml^{-1} of *E. coli* in SE, and 3.37 x 10^5 MPN · 100 ml^{-1} of total coliforms and 5.35 x 10^4 MPN · 100 ml^{-1} *E. coli* in SB. In TB and GW, coliforms and *E. coli* were below detection limit. In general, the results were fairly constant over time, with little variability in each of the systems sampled.

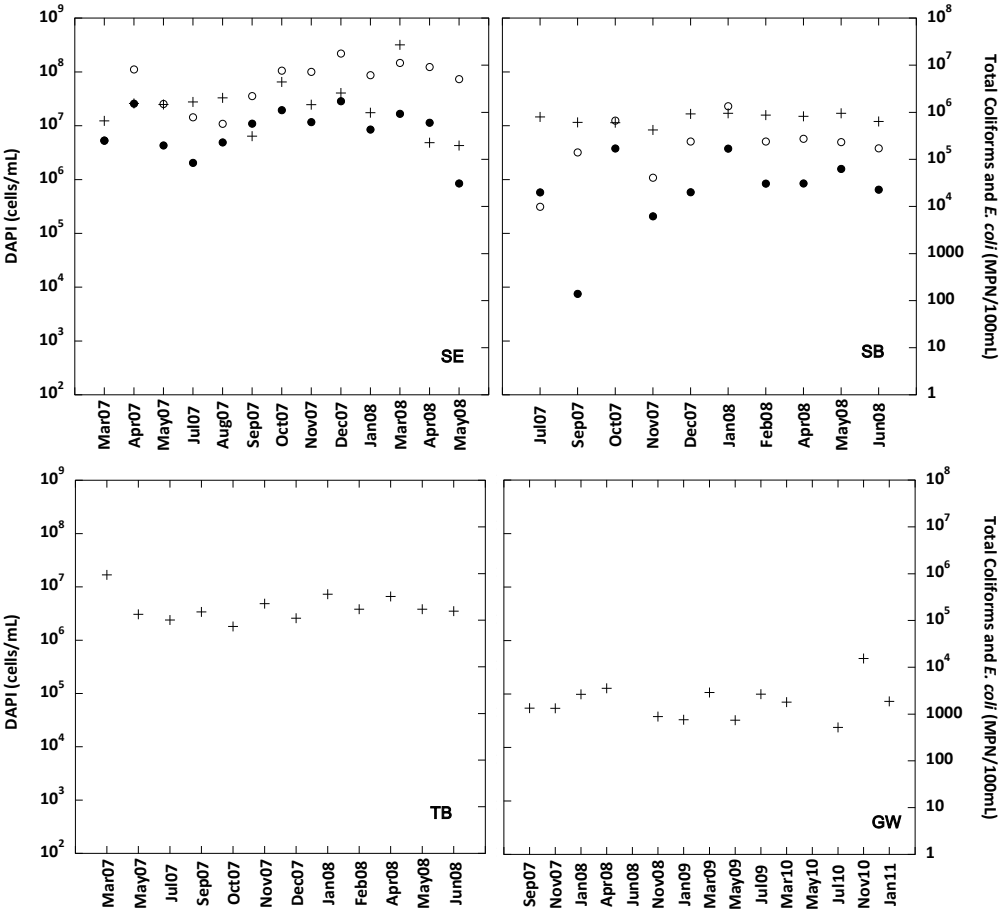

Figure 1. DAPI counts (+), total coliforms (o) and *E. coli*. (.).

Microbial community analyses

Samples from each of the four different water qualities were run in a separated DGGE gel (Fig.2) in order to study the changes that the microbial communities could have underwent throughout time. DGGE analysis gave a total of 279 detectable bands in SE, 402 in SB, 286 in TB and 484 in GW sample. Band number per lane ranged between 14 and 29 (mean 21.5) in SE, between 33 and 47 (mean

40.2) in SB, between 14 and 37 (mean 23.8) in TB and between 27 and 38 (mean 32.3) in GW. We calculated the averaged Bray-Curtis similarity indices for each DGGE gel as an indicator of the temporal variability occurred in the community composition of the four studied systems and found that the average of this similarity index was within the same range for the four waters, being in particular 51.1 (SD=1.26) in SE, 54.5 (SD=2.7) in SB, 53.7 (SD=1.0) in TB and 53.7 (SD=0.9).

Bands were excised from the gels and sequenced to determine their phylogenetic affiliation (Fig. S1, S2, S3, S4 and Tables 1, 2, 3, 4 supplementary material). Informative sequences were obtained from 21 bands in SE, 36 bands in SB, 23 bands in TB and 43 in GW. These bands accounted for 75.0% (SE), 81.3% (SB), 75.2% (TB) and 67.8% (GW) of the total mean band intensity, indicating that most of the bacterial diversity could be retrieved. In general, most of the sequences showed similarity with uncultured clones by BLAST search, although we could determine to which major phylogenetic group they belong to. Taking into account the relative intensity of each group in the different lanes, their contribution (%) could be calculated and summarized in Figure 3 for each gel. A portion of the percentage belonged to unidentified bacteria, due to a failure to obtain sequences from faint bands in the gel and also due to the poor quality of some sequenced bands.

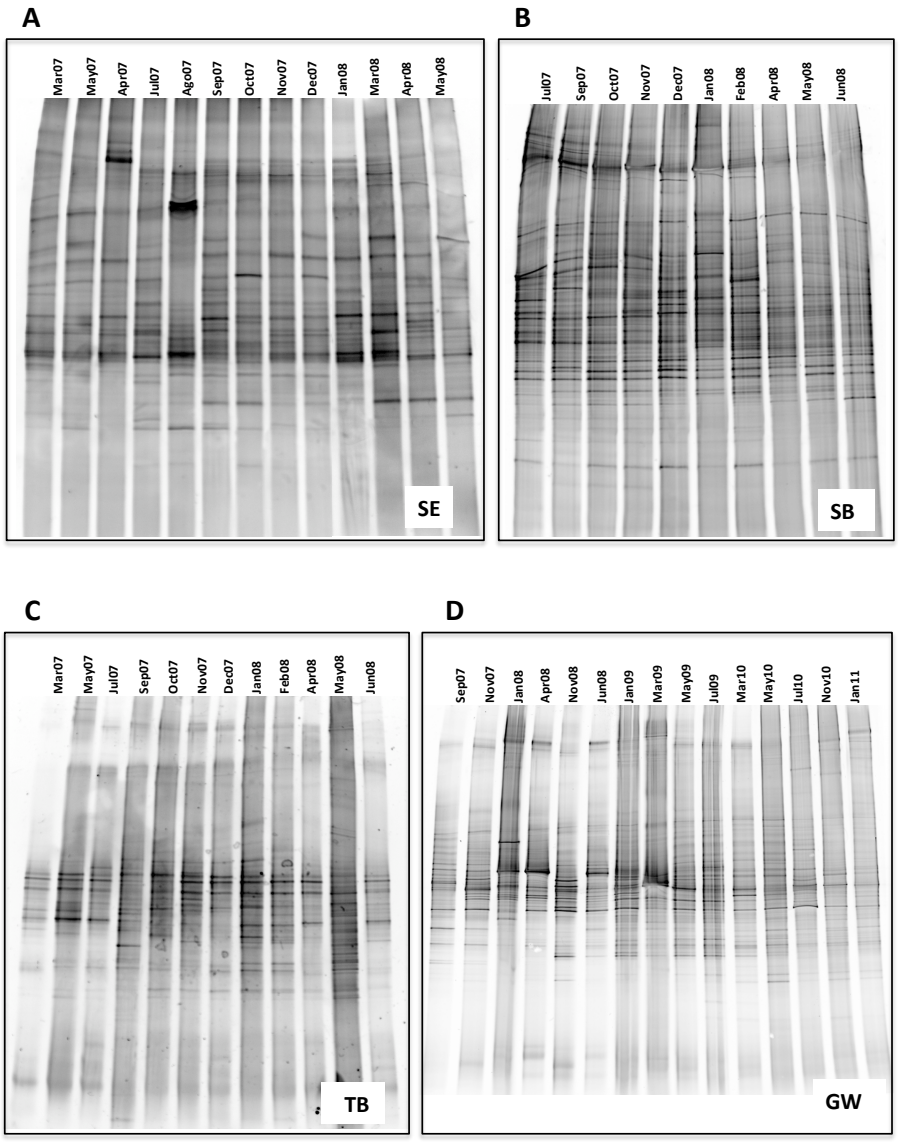

Figure 2. DGGE fingerprints of the four water qualities along time, **(A)** El Ejido secondary effluent (SE), **(B)** Baix Llobregat secondary effluent (SB), **(C)** Baix Llobregat tertiary effluent (TB), **(D)** Groundwater (GW).

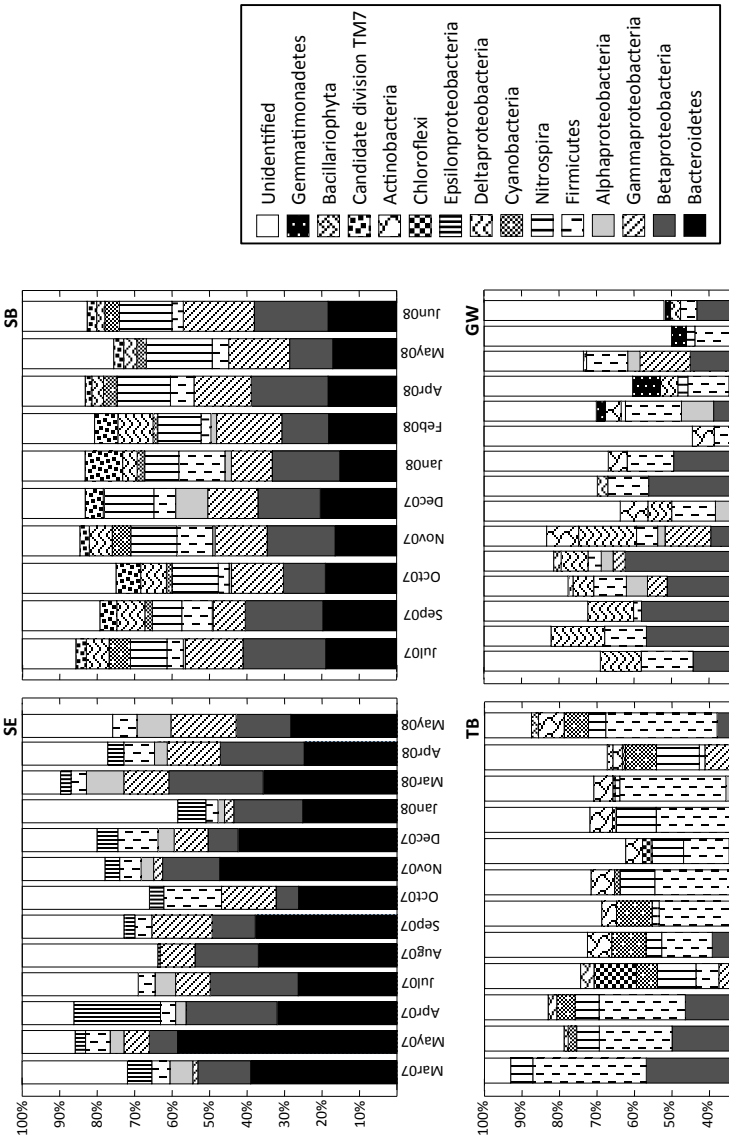

Figure 3. Proportions of bacterial taxonomic groups detected in DGGE bands (percentage of relative intensity).

In SE the most abundant bands belonged to the *Bacteroidetes* and to the *Beta* and *Gammaproteobacteria*, with a contribution of 35.5%, 16.0% and 8.8% to total mean band intensity, respectively. *Firmicutes*, *Epsilon* and *Alphaproteobacteria* were also found. All bands were closely related to uncultured members of these phylogenetic groups and some of these bands appeared with a high frequency (>70% of lanes) and had a high similarity (>95%) with cultured members of genera such as *Bacteroides* and *Arcobacter*. Other bands appeared only in a few lanes, but had also >95% of similarity with cultured members; they belonged to the genera *Acidovorax* and *Hydrogenophaga*.

In SB the main groups belonged to the *Bacteroidetes*, the *Beta-* and *Gammaproteobacteria* and the *Nitrospira*, with a contribution to total mean band intensity of 18.2%, 17.1%, 14.4% and 12.2% respectively. Other minor groups were also found: the *Firmicutes*, *Deltaproteobacteria*, Candidate division TM7, *Cyanobacteria* and *Alphaproteobacteria*. Only one of these bands belonged to a cultured microorganism, *Chryseobacterium*, showing a 98.1% similarity and appeared in all lanes. The remaining bands belonged to uncultured microorganisms, although some of them which appear frequently in the gel (>70% of lanes) had cultured close matches with a high similarity (>95%) to the genera *Bacteroides*, *Thauera*, Candidatus *Accumulibacter*, *Aquabacterium*, Candidatus *Nitrospira* and *Dokdonella*. Also, two bands had a high similarity with *Acidovorax* and *Imtechium*, although they were less frequent (20% and 60%).

In TB the main groups belonged to the *Betaproteobacteria* and the *Firmicutes*, with a contribution of 33.2% and 19.0%, respectively, to total mean band intensity. Other groups were also found: *Nitrospira*, *Actinobacteria*, *Cyanobacteria*, *Bacteroidetes*, *Alpha-* and *Gammaproteobacteria*, *Chloroflexi* and *Bacillariophyta*. All bands were affiliated to uncultured members of these phylogenetic groups, and some of those bands (those wich appear >70% in the gel) had closest cultured matches with a high similarity (>95%) with cultured members of genera such as

Clostridium, *Hydrogenophaga*, *Malikia*, *Rhodoferax* and Candidatus *Nitrospira*. Other bands appeared only in some of the lanes, and were similar to the genera *Acidovorax*, *Imtechium* and *Navicula* (chloroplast).

In GW the main group belonged to *Betaproteobacteria* with a contribution of 37.3% to total mean band intensity. Other groups were also found: *Firmicutes*, *Bacteroidetes*, *Delta-* and *Gammaproteobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Gemmatimonadetes* and *Nitrospira*. Only one of the retrieved bands belonged to a cultured microorganism, *Rhodoferax*, with a 100% similarity and appeared in all lanes. The rest of bands belonged to uncultured microorganisms of the above mentioned phylogenetic groups, and some of these bands had a high similarity (>95%) with cultured members of the genera *Gallionella* and *Sediminibacterium*, which appear with a high frequency in the gel (>70% of lanes). Besides, other bands had a high similarity with *Methylophilus*, *Lutibacter*, *Sideroxydans* and Candidatus *Nitrotoga*, but they were only present in some lanes.

From all the different groups found, the *Bacteroidetes*, *Gammaproteobacteria* and *Nitrospira*, although being present in virtually all the samples, had a higher relative abundance in fecal samples (SE and SB) than in TB and GW samples, while *Betaproteobacteria* had lower richness in contaminated samples. The use of an alternative index taking into account these 4 bacterial groups was therefore further explored as an indicator of water quality.

Microbial communities indices

For the sake of comparison, the Shannon diversity index was calculated from the DGGE fingerprints in each sample (Fig. 4). Shannon diversity indices, which provide information about the richness and the relative abundances of different species, ranged between 2.44 and 3.09 in SE, 3.23 and 3.55 in SB, 2.25 and 3.31 in TB and 2.77 and 3.40 in GW. The results showed that SB corresponded to the most

constant water quality with the highest diversity. Nevertheless, we did not find significant differences between systems.

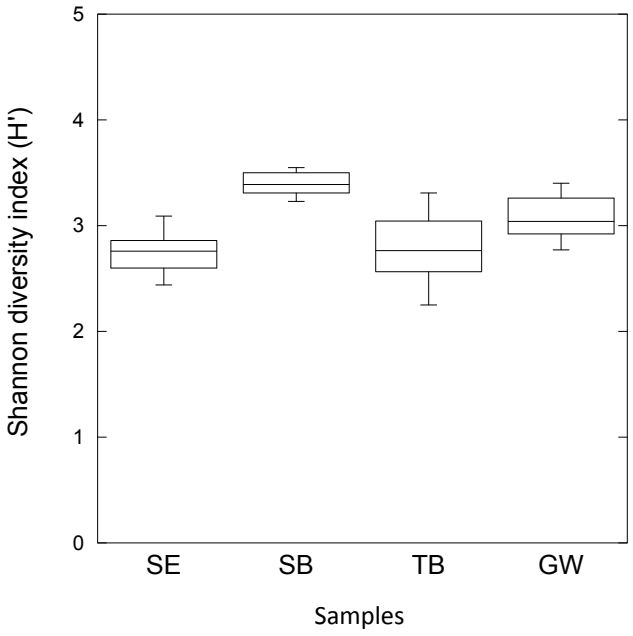

Figure 4. Boxplots of Shannon index of the four water qualities. SE: El Ejido secondary effluent, SB: Baix Llobregat secondary effluent, TB: Baix Llogregat tertiary effluent, GW: groundwater.

On the other hand, we calculated the alternative index BGN:β from the average relative abundance of *Bacteroidetes*, *Gammaproteobacteria* and *Nitrospira* to *Betaproteobacteria* in each DGGE gel. Interestingly, we found significant differences between the mean BGN:β ratios of the four water qualities (one-way ANOVA, $p<0.001$) and a *post hoc* Tukey-Kramer comparison confirmed that ratios for secondary effluent samples were higher than for TB and GW samples (Fig. 5). The secondary effluents ratios were never below 1.3 and the tertiary effluent and groundwater ratios were never over 0.85, which suggests that this alternative index is a good indicator of fecal contamination.

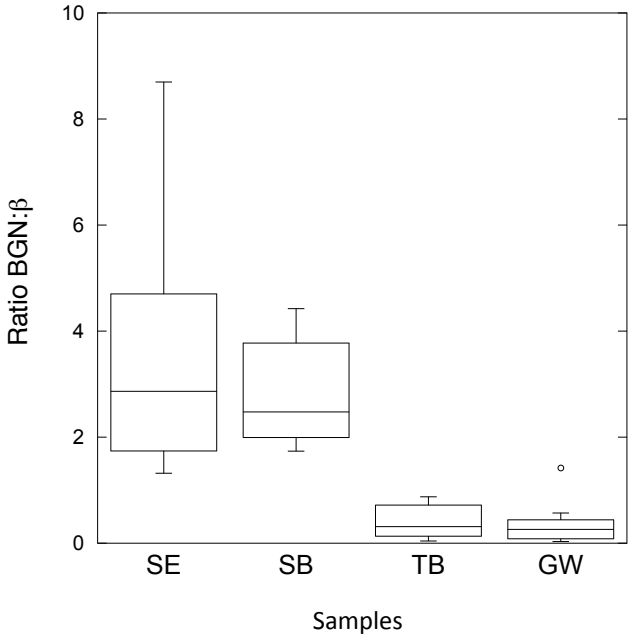

Figure 5. Boxplots of *Bacteroidetes-Gammaproteobacteria-Nitrospira* to *Betaproteobacteria* ratios (BGN:β). SE: El Ejido secondary effluent, SB: Baix Llobregat secondary effluent, TB: Baix Llogregat tertiary effluent, GW: groundwater.

DISCUSSION

One of the goals of this study was the determination of an alternative indicator to test water quality based in the analyses of microbial community profiles over time. Four water types were analyzed in this work, which corresponded to different levels of fecal contamination: two secondary effluents (SE, SB), a tertiary effluent (TB) and groundwater (GW). These different qualities were confirmed by standard microbiological analyses, which showed a lower number of fecal coliforms in TB and GW samples.

We determined the Shannon index as a possible measure of water quality, since changes in microbial composition could reflect variations in biodiversity. Many studies have measured this index in aquatic systems and values typically range between 0.5 and 5, indicating low diversity with values from 0.5 to 2 and high diversity with values from 2 to 5. However, numbers found in freshwater environments, drinking water reservoirs or marine systems are very variable (Lymperopoulou *et al*., 2011; Liu *et al*., 2012; Schauer *et al*., 2000; Vaz-Moreira *et al*., 2011). In this work, calculated Shannon indexes were quite constant over time ranging from 2.25 to 3.55, indicating a high and constant diversity for the four water qualities. These data suggest that the Shannon index does not reflect water quality in terms of fecal contamination and is not therefore a good indicator.

As an alternative, we analyzed the bacterial community structure of these four water types over a long period of time and after examination of their population dynamics, four bacterial groups were chosen for the calculation of the BGN:β ratio (*Bacteroidetes*, *Gammaproteobacteria* and *Nitrospira* to *Betaproteobacteria*), and this index was further explored as a potential indicator of water quality. Concerning these specific microbial groups, DGGE analyses showed that the *Betaproteobacteria* was a common taxonomic subclass found in all systems and it was more abundant in TB and GW systems (as an average, more than 2-fold higher). This subclass of the *Proteobacteria*, all members being Gram negative, consists of several groups of aerobic or facultative bacteria that are often highly versatile in their degradation capacities. They dominate freshwater bacterial communities (Burkert *et al*., 2003; Van der Gucht *et al*., 2005) and may reach high abundance in lakes of diverse trophic status (Glöckner *et al*., 1999; Wu *et al*., 2006). This group is also commonly found in wastewater environments such as activated sludge (Wagner *et al.,* 2002; Yang *et al.,* 2001), seemingly therefore a potential indicator of freshwater quality.

Other bacterial groups, such as *Bacteroidetes* and *Gammaproteobacteria*, although being present in all tested systems, appeared to be more abundant in the secondary effluents, SE and SB. *Bacteroidetes* are commonly recognized as hydrolytic fermentative degraders of polymers mainly in anaerobic habitats, including wastewater environments (Yang *et al.,* 2011) and freshwater and marine sediments (Kirchman, 2002), and *Bacteroides*, a representative genus found in almost all lanes of SE (92.3%) and SB (100%) DGGE gels, is a core component of the human gut microbiota (Costello *et al*., 2009; Eckburg *et al*., 2005; Tap *et al*., 2009). In fact, the *Bacteroides-Prevotella* is one of several non-coliform bacterial groups that has been proposed as an alternative indicator of fecal pollution (Allsop, K., and Stickler, J.D, 1985; Fiksdal *et al.,* 1985; Kreader, 1995), partly because of its abundance in feces. On the other hand, the class *Gammaproteobacteria* includes important groups of microorganisms, such as the Enterobacteriaceae (*Escherichia coli*), Vibrionaceae and Pseudomonadaceae. The phylum *Nitrospira*, a nitrifying bacterial group, was only found in SB and TB effluents, but due to its high abundance and occurrence in SB (it appeared in all DGGE lanes), it was taken into account in the determination of our ecological alternative indicator. Interestingly, the phylum *Firmicutes*, which includes endospore-forming bacteria, appeared as one of the most abundant groups in TB. However we did not consider it in the index since this tertiary effluent had been subjected to UV disinfection, and therefore, sporulated bacteria like *Clostridium* very likely could have been selected.

In general, the alternative index BGN:β seemed to have a good correlation with water quality. Samples TB and GW, representative of higher quality waters, showed a low ratio (never over 0.85), while SB and SB gave a higher ratio (always over 1.3). In order to confirm the reliability of our indicator, we calculated the BGN:β index from published studies which also determined microbial diversity in different systems. For this purpose, we calculated the BGN:β ratios of bacterial communities from 54 mammalian intestines (Ley *et al*., 2008) and the ratio was

very high; 3 sewage-associated samples (Chouari *et al*., 2005; Juretschko *et al.,* 2002; McLellan *et al*., 2010) where the ratio values followed the expected trend of contaminated water (except in nitrifying-denitrifying activated sludge from Juretschko and colleges, where the community had a low ratio due to the prominent presence of nitrifying *Betaproteobacteria*, which dominated the microbial community) , and 20 non-fecal aquatic environments from 6 different studies (Glockner *et al*., 2000; Methé *et al*., 1998; Mosier *et al*., 2006; West *et al*., 2008; Williams *et al*., 2004; Van der Gucht *et al.,* 2005; McLellan *et al*., 2010) where the communities of different freshwater samples, such as rivers and lakes (10 lakes, 3 ponds and 1 river), resulted in very low values. However, samples from marine environments, despite being non-contaminated, had higher BGN:β ratios than the other non-fecal samples although this could be explained because marine bacterial communities are characterized by the low presence of *Betaproteobacteria* (Rappé and Giovannoni, 2003). Thus, with a few exceptions, gut and sewage-associated samples had higher BGN:β ratios that non-fecal samples.

Furthermore, to test whether the observed changes in the BGN:β ratios between water types could be explained by temporal variability within each system, we calculated the average Bray-Curtis coefficient. If no changes in community composition occurred within a system, the average Bray-Curtis similarity index would be 100%. Contrarily, if the community composition changed completely from one sampling time to the next and no single band would be repeated, the average similarity would be 0. We found that the Bray-Curtis indices for the four water quality types were constant (SE=51.1%, SB=54.5%, TB=53.7%, GW=53.7%) (Fig. S5, supplementary material), which indicates that the degree of temporal variability within each system is similar, and therefore, the observed differences in the BGN:β ratios are not the result of temporal variability. This observation thus

strengthens the usefulness of our proposed index as an alternative indicator of water quality.

To sum up, our ratio follows the expected trend when calculated for the four types of water, and this is in agreement with results of microbial analyses of fecal indicators. Furthermore, the fact that our samples were taken over a period of one to three years makes the results representative and reinforces our hypothesis. Finally, in most of the published studies in which the ratio was calculated, it was also correlated with water quality. Thus, the BGN:β ratio seems to work as an alternative indicator of water quality and could provide an approximation of ecological freshwater quality.

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SUPPLEMENTARY MATERIAL

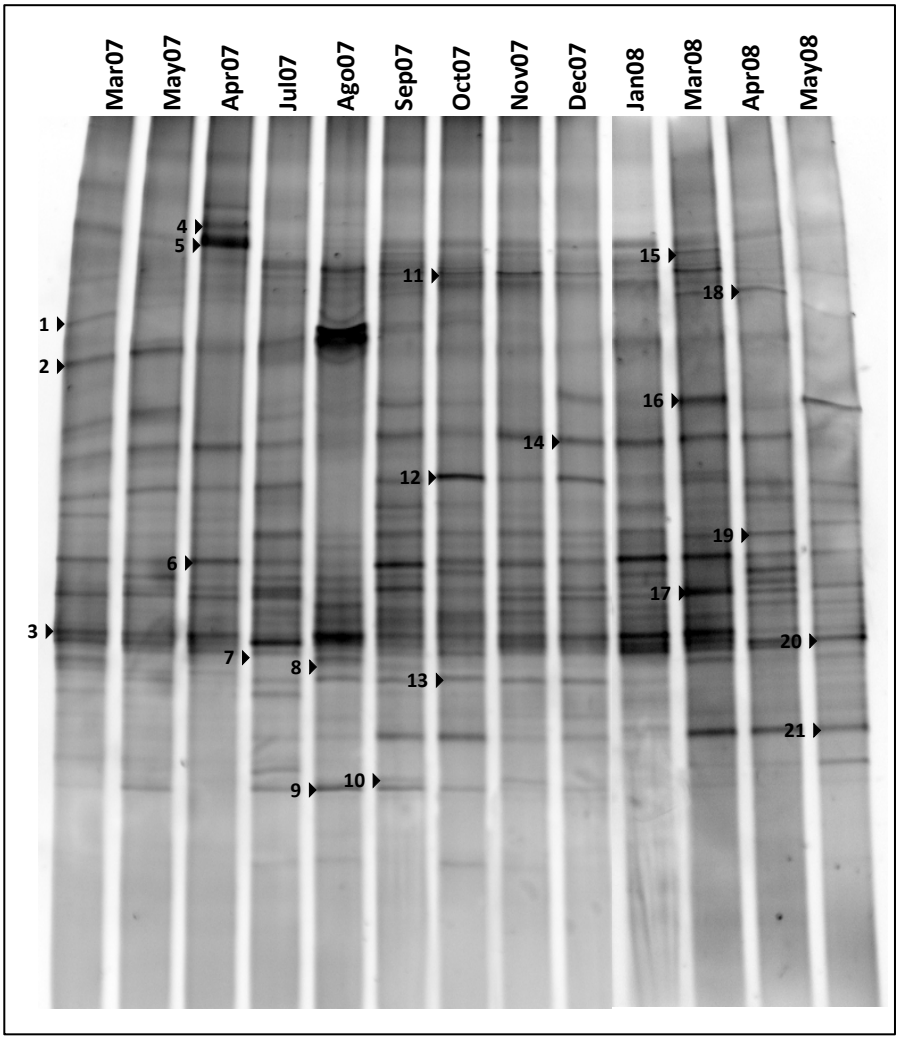

Figure S1. DGGE fingerprints from samples of El Ejido secondary effluent (SE). Bands excised and sequenced are numbered and their affiliations are shown in Table 1.

Table 1. Phylogenetic affiliation of sequences obtained from SE DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Accession nº (GenBank)	Cultured closest match (% similarity)
SE_1	Uncultured bacterium clone 30	94.2 (506)	*Bacteroidetes*	FJ534976	*Bacteroides* sp. XDT-1 (83.1)
SE_2	Uncultured Saprospiraceae bacterium clone Epr65	89.8 (488)	*Bacteroidetes*	EU177705	Candidatus *Aquirestis calciphila* (82.7)
SE_3	Uncultured β-*Proteobacterium* clone D055111B11	99.2 (540)	β-*Proteobacteria*	GU179641	*Acidovorax caeni* (99.2)
SE_4	Unclultured bacterium clone N1512_71	92.8 (477)	ε-*Proteobacteria*	EU104239	*Arcobacter cryaerophilus* (92.8)
SE_5	Unclultured bacterium clone N1512_71	98.0 (506)	ε-*Proteobacteria*	EU104239	*Arcobacter cryaerophilus* (98.0)
SE_6	Uncultured bacterium clone SGUS1002	86.7 (355)	*Bacteroidetes*	FJ202160	*Lewinella coharens* (85.4)
SE_7	Uncultured bacterium isolate DGGE gel band 13	95.9 (522)	β-*Proteobacteria*	EU275391	*Hydrogenophaga* sp. (95.2)
SE_8	Uncultured bacterium clone 197up	84.0 (441)	β-*Proteobacteria*	AY212650	*Acidovorax* sp. (82.5)
SE_9	Uncultured bacterium isolate DGGE gel band 4	92.0 (452)	γ-*Proteobacteria*	EU426848	*Methylocaldum szegediense* (82.8)
SE_10	D7_10	91.9 (446)	*Bacteroidetes*	DQ906118	*Parabacteroides distasonis* (84.4)
SE_11	Uncultured bacterium clone 30	92.9 (498)	*Bacteroidetes*	FJ534976	*Bacteroides* sp. XDT-1 (92.9)
SE_12	Uncultured bacterium clone C1-26	99.1 (529)	*Firmicutes*	HQ453307	*Clostridium* sp. (87.2)
SE_13	Uncultured bacterium clone 31f06	90.1 (469)	*Bacteroidetes*	EF515658	*Prevotella oralis* (86.7)
SE_14	Bacterium enrichment culture clone PA52	99.6 (530)	*Bacteroidetes*	FJ799161	*Bacteroides* sp. (99.4)
SE_15	Uncultured bacterium clone Hel3bh01	84.9 (457)	*Bacteroidetes*	FJ229351	*Riemerella* sp. (84.3)
SE_16	Uncultured bacterium clone:TB127-03	97.5 (525)	*Bacteroidetes*	AB196061	*Lewinella nigricans* (86.3)
SE_17	Uncultured α-*Proteobacterium* clone Ebpr11	89.7 (456)	α-*Proteobacteria*	AF255640	*Rhizobium* sp. (83.0)
SE_18	Uncultured bacterium clone LWS-T4819	95.6 (507)	*Bacteroidetes*	EU546335	*Flexibacter* sp. (89.0)
SE_19	Uncultured bacterium clone: SRRT60	90.7 (470)	*Bacteroidetes*	AB240489	*Prolixibacter bellariivorans* (87.1)
SE_20	Uncultured bacterium clone 52	98.2 (515)	β-*Proteobacteria*	FJ534997	*Acidovorax* sp. (97.5)
SE_21	Uncultured bacterium clone 9_1	96.5 (508)	γ-*Proteobacteria*	EF443088	*Thiodictyon bacillosum* (93.5)

a. Number of bases used to calculate the levels of sequence similarity.

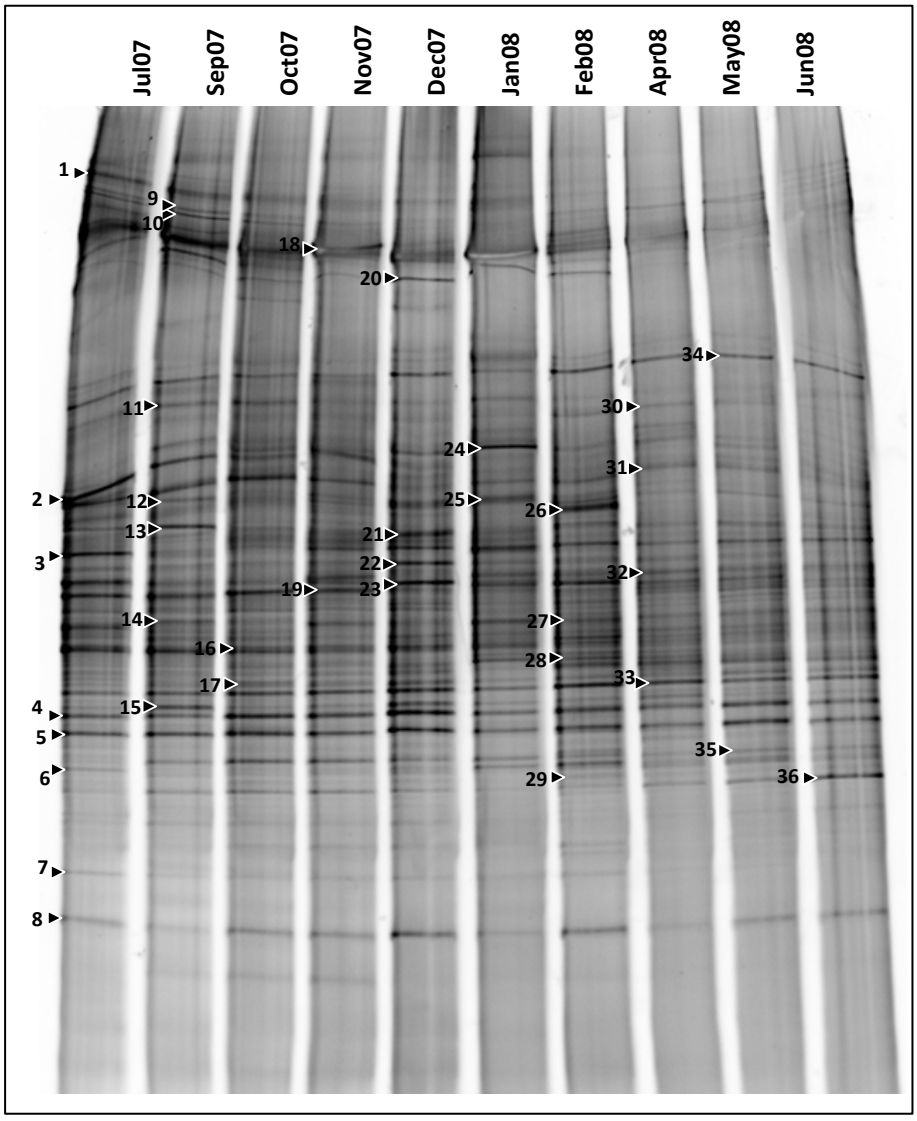

Figure S2. DGGE fingerprints from samples of Baix Llobregat secondary effluent (SB). Bands excised and sequenced are numbered and their affiliations are shown in Table 2.

Table 2. Phylogenetic affiliation of sequences obtained from SE DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Accession nº (GenBank)	Cultured closest match (% similarity)
SB_1	Uncultured bacterium clone SWB0401-02	96.6 (490)	Candidate division TM7	JN398100	TM7 phylum sp. (87.6)
SB_2	Uncultured bacterium clone A08-166-BAC	99.1 (527)	γ-*Proteobacteria*	GQ340280	*Legionella* like amoebal pathogen (90.2)
SB_3	Uncultured bacterium clone ABF-HL4-7	99.0 (509)	β-*Proteobacteria*	HQ403208	*Lutiella* sp. (92.8)
SB_4	Uncultured *Nitrospira* sp. clone 2A-24	100 (514)	*Nitrospira*	JN802219	Candidatus *Nitrospira defluvii* (100)
SB_5	Uncultured *Nitrospira* sp. clone 20	100 (514)	*Nitrospira*	HQ424545	Candidatus *Nitrospira defluvii* (99.5)
SB_6	Uncultured bacterium clone FGL7S_B22	93.4 (492)	γ-*Proteobacteria*	FJ438010	*Methylocaldum* sp. (84.6)
SB_7	Uncultured bacterium clone 67	97.9 (520)	γ-*Proteobacteria*	DQ413125	*Methylocaldum* sp. (88.4)
SB_8	Uncultured bacterium clone PHOS-HE54	100 (520)	γ-*Proteobacteria*	AF314424	*Methylocaldum szegediense* (89.5)
SB_9	Uncultured bacterium clone TB127-25	91.6 (477)	*Firmicutes*	AB196115	*Clostridium* sp. (83.5)
SB_10	Uncultured bacterium clone 124	96.5 (498)	*Firmicutes*	GU123155	*Clostridium* sp. (81.4)
SB_11	Uncultured bacterium clone 0-4-161	98.8 (513)	*Bacteroidetes*	JN609335	*Bacteroides* sp. (98.8)
SB_12	Uncultured bacterium clone F_SBR_30	96.2 (504)	δ-*Proteobacteria*	HQ010805	*Deslulfonatronum cooperativum* (80.3)
SB_13	Uncultured bacterium clone 12up	96.4 (516)	β-*Proteobacteria*	AY212580	Candidatus *Accumulibacter* sp. (95.5)
SB_14	Uncultured *Betaproteobacterium* clone C12-5	96.6 (513)	β-*Proteobacteria*	JN371459	*Acidovorax* sp. (96.5)
SB_15	Uncultured bacterium clone Q7210-HYSO	100 (534)	β-*Proteobacteria*	JN391948	*Thauera* sp. (100)
SB_16	Uncultured Rhodocyclaceae bacterium clone 408	94.9 (507)	β-*Proteobacteria*	FM207908	*Denitratisoma* sp. (93.4)
SB_17	Uncultured bacterium clone RBC2-1	89.6 (413)	*Bacteroidetes*	AB567838	Candidatus *Aquirestis calciphila* (82.4)
SB_18	*Chryseobacterium* sp.	98.1 (515)	*Bacteroidetes*	AB581571	
SB_19	Uncultured bacterium clone QEDN8DD02	99.6 (509)	δ-*Proteobacteria*	CU926467	*Vampirovibrio chlorellavorus* (86.4)
SB_20	Uncultured bacterium clone 0-4-155	93.5 (474)	α-*Proteobacteria*	JN609333	*Gluconacetobacter* sp. (84.5)
SB_21	Uncultured Saprospiraceae bacterium clone Epr.125	96.0 (506)	*Bacteroidetes*	EU177740	*Lewinella nigricans* (86.6)
SB_22	Uncultured bacterium clone AS1_aao40b09.Contig1	96.1 (498)	*Firmicutes*	EU772305	*Clostridium aldrichii* (92.3)
SB_23	Uncultured *Alphaproteobacterium* clone Ebpr11	97.3 (502)	α-*Proteobacteria*	AF255640	*Tristella* sp. (85.6)
SB_24	Uncultured bacterium clone C1-26	99.4 (526)	*Firmicutes*	HQ453307	*Clostridium* sp. (87.4)
SB_25	Uncultured bacterium clone 07Nov4-39	97.8 (485)	Candidate division	AB618449	TM7 phylum sp. (80.6)
SB_26	Uncultured bacterium clone MBR-30_LF_AS78	93.3 (500)	δ-*Proteobacteria*	FM200964	*Myxobacterium* sp. (84.7)
SB_27	Uncultured *Betaproteobacterium* clone DG-KL-C12	96.1 (516)	β-*Proteobacteria*	AB635936	*Aquabacterium commune* (96.1)
SB_28	Uncultured bacterium clone ncd480b04c1	99.1 (534)	β-*Proteobacteria*	HM327703	*Imtechium assamiensis* (99.0)
SB_29	Uncultured bacterium clone ambient_uncontrolled-102	97.4 (527)	γ-*Proteobacteria*	GU454963	*Thiodictyon elegans* (94.3)
SB_30	Uncultured bacterium clone AOA-238	93.1 (497)	*Bacteroidetes*	AB479791	*Flavobacterium* sp. (89.4)
SB_31	Uncultured bacterium clone Q7441-ASSA	99.4 (530)	*Bacteroidetes*	JN391572	*Haliscomenobacter* sp. (87.4)
SB_32	Uncultured bacterium clone S1-24	95.2 (493)	*Cyanobacteria*	JF503019	*Calothrix* sp. (91.2)
SB_33	Uncultured bacterium clone h-29	99.8 (528)	γ-*Proteobacteria*	HQ738462	*Dokdonella* sp. (98.8)
SB_34	Uncultured *Bacteroidetes* bacterium clone 5.17m5	99.6 (530)	*Bacteroidetes*	JN679190	*Flexibacter flexilis* (90.5)
SB_35	Uncultured *Gammaproteobacterium* clone GB198	92.6 (489)	γ-*Proteobacteria*	DQ201873	*Methylocaldum* sp. (86.4)
SB_36	Uncultured bacterium clone NMA7	90.3 (474)	β-*Proteobacteria*	GU183611	*Uliginosibacterium gangwonense* (82.3)

a. Number of bases used to calculate the levels of sequence similarity

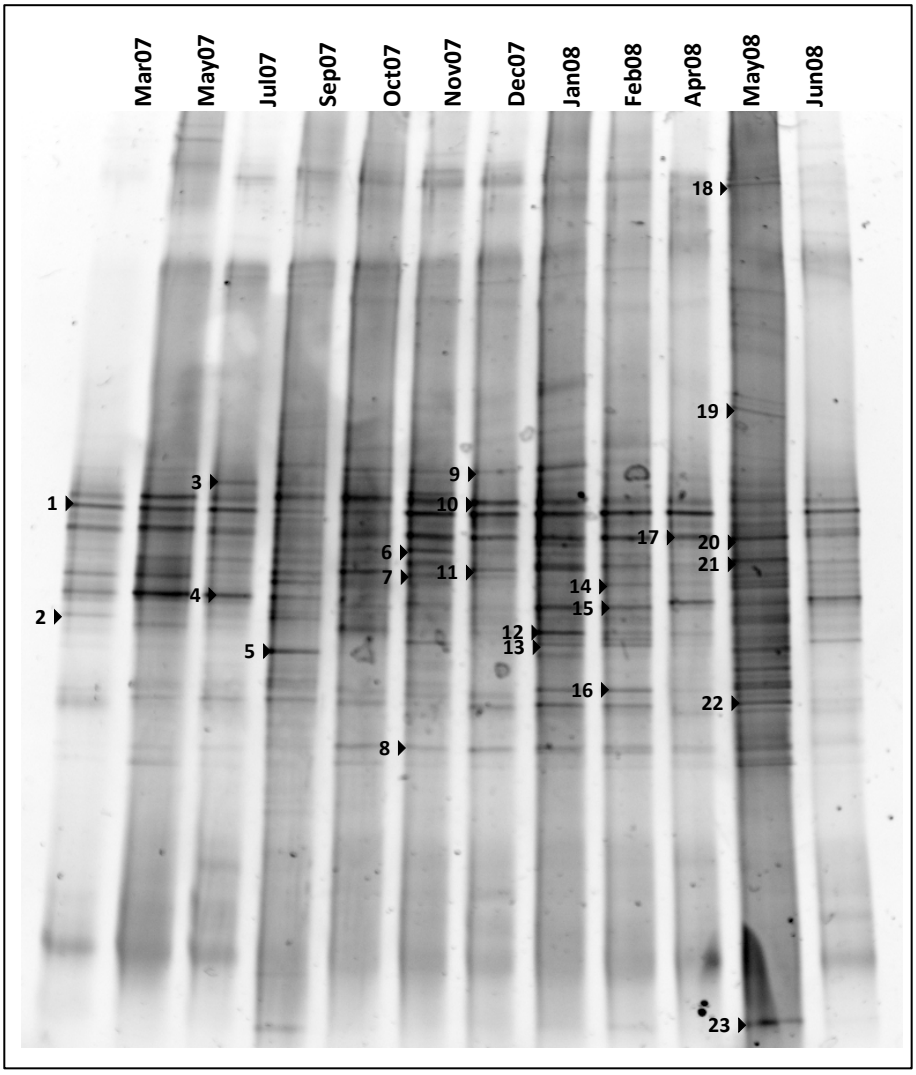

Figure S3. DGGE fingerprints from samples of Baix Llobregat tertiary effluent (TB). Bands excised and sequenced are numbered and their affiliations are shown in Table 3.

Table 3. Phylogenetic affiliation of sequences obtained from TE DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Accession nº (Gen Bank)	Cultured closest match (%similarity)
TB_1	Swine fecal bacterium RF1A-Pec1	99.8 (515)	*Firmicutes*	FJ753815	*Clostridium* sp. (99.8)
TB_2	Uncultured Comamonadaceae bacterium LW18m-2-9	96.9 (503)	β-*Proteobacteria*	EU642389	*Hydrogenophaga* sp. (96.9)
TB_3	Uncultured bacterium clone WPUB215	98.4 (511)	*Bacteroidetes*	FJ006842	*Coccinistipes vermicola* (89.8)
TB_4	Uncultured bacterium clone IWENVC15	100 (512)	β-*Proteobacteria*	EU000441	*Acidovorax* sp. (98.4)
TB_5	Uncultured *Chloroflexi* bacterium QEDQ1CA04	98.6 (503)	*Chloroflexi*	CU923706	*Dehalococcoides* sp. (87.7)
TB_6	Uncultured *Betaproteobacteria* clone QEDN7DD06	92.3 (474)	β-*Proteobacteria*	CU926665	*Comamonas* sp. (92.3)
TB_7	Uncultured *Cyanobacteria* bacterium clone QEDN8DD02	94.8 (482)	*Cyanobacteria*	CU926467	*Desulfotomaculum* sp. (83.7)
TB_8	Uncultured bacterium clone PR25	83.3 (360)	*Actinobacteria*	DQ298343	*Amycolatopsis fastidiosa* (82.5)
TB_9	Uncultured bacterium clone AE2_aaa02d07	88.5 (464)	*Bacteroidetes*	EU771211	Bacterium Oil-Tsu-11 (87.3)
TB_10	*Betaproteobacterium* F06002	95.5 (497)	β-*Proteobacteria*	AF236014	*Azospira restricta* (92.5)
TB_11	Uncultured *Alphaproteobacterium* clone Ebpr11	93.1 (477)	α-*Proteobacteria*	AF255640	*Tristrella* sp. (84.0)
TB_12	Uncultured Comamonadaceae bacterium clone GC1m-2-78	98.0 (509)	β-*Proteobacteria*	EU640756	*Malikia spinosa* (98.0)
TB_13	Iron-reducing bacterium enrichment cultured clone FEA_2_B7	99.6 (518)	β-*Proteobacteria*	FJ802332	*Imtechium assamiensis* (99.6)
TB_14	Uncultured bacterium clone 145ds20	95.9 (496)	β-*Proteobacteria*	AY212596	*Rhodoferax ferrireducens* (95.5)
TB_15	Uncultured bacterium clone B49	99.6 (520)	β-*Proteobacteria*	FJ660530	*Aquabacterium* sp. (98.2)
TB_16	Uncultured bacterium clone A208	100 (522)	*Nitrospira*	FJ660604	Candidatus *Nitrospira defluvii* (100)
TB_17	Uncultured *Firmicutes* bacterium clone QEDN5CA05	100 (515)	*Firmicutes*	CU926587	*Clostridium maritimum* (98.0)
TB_18	Chloroplast *Pennate diatom* sp. CCAP 1008/1	99.8 (507)	*Bacillariophyta*	FJ002185	*Navicula* sp. (99.4)
TB_19	Uncultured *Bacteroidetes* bacterium clone QEDN5CB12	96.9 (504)	*Bacteroidetes*	CU925900	*Flexibacter flexilis* (87.0)
TB_20	Uncultured bacterium clone 185up	94.9 (508)	β-*Proteobacteria*	AY212637	*Vogesella* sp. (90.8)
TB_21	Uncultured bacterium clone PR25	93.3 (480)	*Cyanobacteria*	AY328600	*Schizothrix* sp. (83.0)
TB_22	Uncultured bacterium clone MBR-30_LF_BF31	98.8 (515)	*Nitrospira*	FM200870	Candidatus *Nitrospira defluvii* (98.8)
TB_23	Uncultured *bacterium clone G105*	99.8 (517)	γ-*Proteobacteria*	FJ356055	*Methylocaldum szegediense* (89.0)

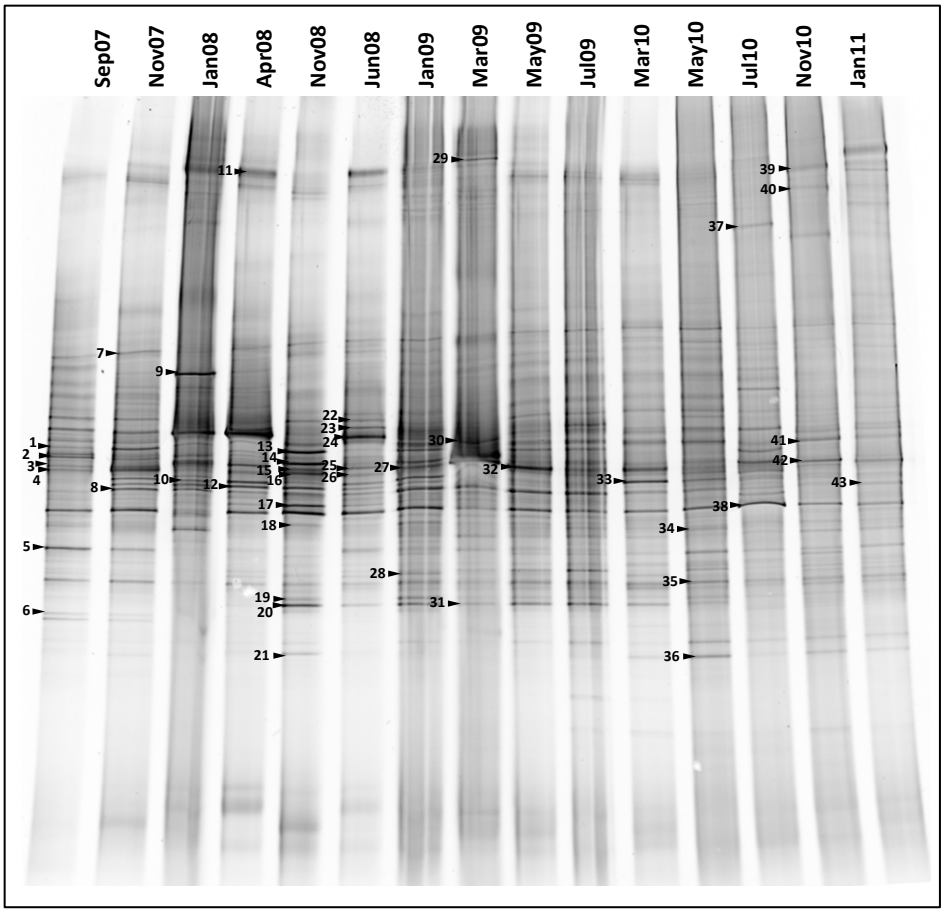

Figure S4. DGGE fingerprints from samples of groundwater (GW). Bands excised and sequenced are numbered and their affiliations are shown in Table 4.

Table 4. Phylogenetic affiliation of sequences obtained from GW DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Accession nº (Gen Bank)	Cultured closest match (% similarity)
GW_1	Uncultured bacterium clone 2C228662	93.3 (491)	β-*Proteobacteria*	EU800531	*Gallionella capsiferriformans* (91.3)
GW_2	Uncultured bacterium clone 2C228662	98.4 (507)	β-*Proteobacteria*	EU800531	*Gallionella capsiferriformans* (95.5)
GW_3	Uncultured bacterium clone 2C228662	93.8 (496)	β-*Proteobacteria*	EU800531	Candidatus *Nitrotoga artica* (92.8)
GW_4	Uncultured bacterium clone 3BH-12A	95.1 (489)	β-*Proteobacteria*	EU937950	*Sideroxidans lithotrophilus* (94.4)
GW_5	Uncultured *Deltaproteobacterium* clone KB47	96.7 (493)	δ-*Proteobacteria*	AB074978	*Desulfovibrio indonesiensis* (93.2)
GW_6	Uncultured *Firmicutes* clone TG-28	88.2(457)	*Firmicutes*	AB451823	*Symbiobacterium* sp. (87.8)
GW_7	Uncultured bacterium clone 081203-OL-PVP22:1-5	90.1 (463)	*Firmicutes*	FJ823208	Candidatus *Desulforudis audaxviator* (79.3)
GW_8	Uncultured *Deltaproteobacterium* clone 49S1_2B_6	92.2 (475)	δ-*Proteobacteria*	DQ837234	*Pelobacter acetylinicus* (88.4)
GW_9	Uncultured bacterium clone PS07AB55	98.1 (504)	β-*Proteobacteria*	HQ271681	*Methylophilus* sp. (97.6)
GW_10	Uncultured bacterium clone TA3_10	99.2 (504)	β-*Proteobacteria*	EU746694	*Gallionella* sp. (97.5)
GW_11	Uncultured bacterium clone 526-70	98.6 (496)	*Bacteroidetes*	EU287370	*Lutibacter* sp. (96.2)
GW_12	Uncultured *Deltaproteobacterium* clone PLMSB-17	83.6 (421)	δ-*Proteobacteria*	AB247834	*Magnetic coccus* (80.1)
GW_13	Uncultured *Gammaproteobacterium* clone G1-79	95.3 (487)	γ-*Proteobacteria*	JN038754	*Acidiferrobacter thiooxidans* (88.6)
GW_14	Uncultured *Gallionella* sp. Clone SB-B-50	98.4 (504)	β-*Proteobacteria*	AB670141	*Gallionella* sp. (96.2)
GW_15	Uncultured bacterium clone B0618R003_E16	88.1 (435)	δ-*Proteobacteria*	AB658547	*Geobacter* sp. (87.5)
GW_16	Uncultured bacterium clone B3_386	90.8 (375)	δ-*Proteobacteria*	HM228726	*Geobacter metallireducens* (89.2)
GW_17	Uncultured denitrifying bacterium clone MBfR_NSP-33	98.0 (510)	β-*Proteobacteria*	JN125789	*Thiobacillus aquaesulis* (92.5)
GW_18	Uncultured *Betaproteobacterium* clone WETLE-7R	90.2 (394)	β-*Proteobacteria*	FM992011	Candidatus *Accumulibacter phosphatis* (88.4)
GW_19	Uncultured Actinomycetales bacterium clone AMAA7	90.2 (450)	*Actinobacteria*	AM935780	*Actinobacterium* sp. (85.6)
GW_20	Uncultured *Actinobacterium* clone ADL-B120	97.9 (478)	*Actinobacteria*	HQ718751	*Actinobacterium* sp. (90.8))
GW_21	Uncultured bacterium clone TSNIR003_P06	94.2 (489)	*Firmicutes*	AB487444	*Symbiobacterium* sp. (89.3)

a. Number of bases used to calculate the levels of sequence similarity.

Table 4. (continued) Phylogenetic affiliation of sequences obtained from GW DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	% similarity (nº bases)[a]	Taxonomic group	Accession nº (Gen Bank)	Cultured closest match (% similarity)
GW_22	Uncultured *Betaproteobacterium* clone 5-8	95.2 (494)	β-*Proteobacteria*	AF351236	*Gallionella capsiferriformans* (94.4)
GW_23	Uncultured *Betaproteobacterium* clone 5-8	94.0 (472)	β-*Proteobacteria*	AF351236	*Gallionella capsiferriformans* (93.3)
GW_24	Uncultured *Betaproteobacterium* clone 5-8	100 (515)	β-*Proteobacteria*	AF351236	*Gallionella capsiferriformans* (93.3)
GW_25	Uncultured bacterium clone SS_LKC22_UB32	91.5 (430)	γ-*Proteobacteria*	AM490765	*Thiothrix* sp. (91.5)
GW_26	Uncultured bacterium clone MIGC06337	90.7 (458)	β-*Proteobacteria*	HM565438	Candidatus *Nitrotoga artica* (90.6)
GW_27	Uncultured bacterium clone B3_166	89.2 (415)	δ-*Proteobacteria*	HM228644	*Pelobacter acetylinicus* (87.4)
GW_28	Uncultured bacterium clone 14s	90.1 (346)	*Firmicutes*	AB154445	*Desulfotomaculum* sp. (89.2)
GW_29	Uncultured *Gallionella* sp. clone HC8	96.4 (503)	β-*Proteobacteria*	FJ391513	*Sideroxydans lithotrophicus* (95.0)
GW_30	Uncultured bacterium clone SAV03601	97.6 (523)	β-*Proteobacteria*	EU542196	*Sideroxidans lithotrophilus* (96.4)
GW_31	Uncultured bacterium clone FinXas1Fe13	97.0 (493)	β-*Proteobacteria*	HE585410	Candidatus *Nitrotoga artica* (96.8)
GW_32	Uncultured bacterium clone SAV03601	97.9 (519)	β-*Proteobacteria*	EU542196	Candidatus *Nitrotoga artica* (97.2)
GW_33	Uncultured bacterium clone Q7641-HYBA	92.4 (462)	α-*Proteobacteria*	JN391746	*Prosthecomicrobium pneumaticum* (85.4)
GW_34	Uncultured bacterium clone PW284	91.5 (460)	*Nitrospira*	GQ402726	Candidatus *Magnetobacterium bavaricum* (85.2)
GW_35	Uncultured bacterium clone AHH11B_159	96.2 (484)	*Firmicutes*	AB588670	*Pelotomaculum thermopropionicum* (87.3)
GW_36	Uncultured bacterium clone B1_58	99.4 (523)	*Gemmatimonadetes*	HM228610	*Gemmatimonas* sp. (87.5)
GW_37	Uncultured bacterium clone ambient_alkaline-56	97.7 (506)	*Firmicutes*	GU455039	*Erysipelothrix* sp. (90.3)
GW_38	*Rhodoferax* sp.	100 (532)	β-*Proteobacteria*	HQ222266	
GW_39	Uncultured bacterium clone C12M13F	96.3 (499)	*Bacteroidetes*	EU796005	*Sediminibacterium salmoneum* (96.2)
GW_40	Uncultured bacterium clone BF2B08	92.9 (481)	*Bacteroidetes*	JN820194	*Haliscomenobacter hydrossis* (92.3)
GW_41	Uncultured *Betaproteobacterium* clone 5-8	97.9 (523)	β-*Proteobacteria*	AF351236	*Gallionella capsiferriformans* (97.5))
GW_42	*Thiothrix nivea*	93.0 (465)	γ-*Proteobacteria*	L40993	
GW_43	Uncultured *Betaproteobacterium* clone WETLE-7R	91.2 (469)	β-*Proteobacteria*	FM992011	Candidatus *Accumulibacter phosphatis* (88.4)

a. Number of bases used to calculate the levels of sequence similarity.

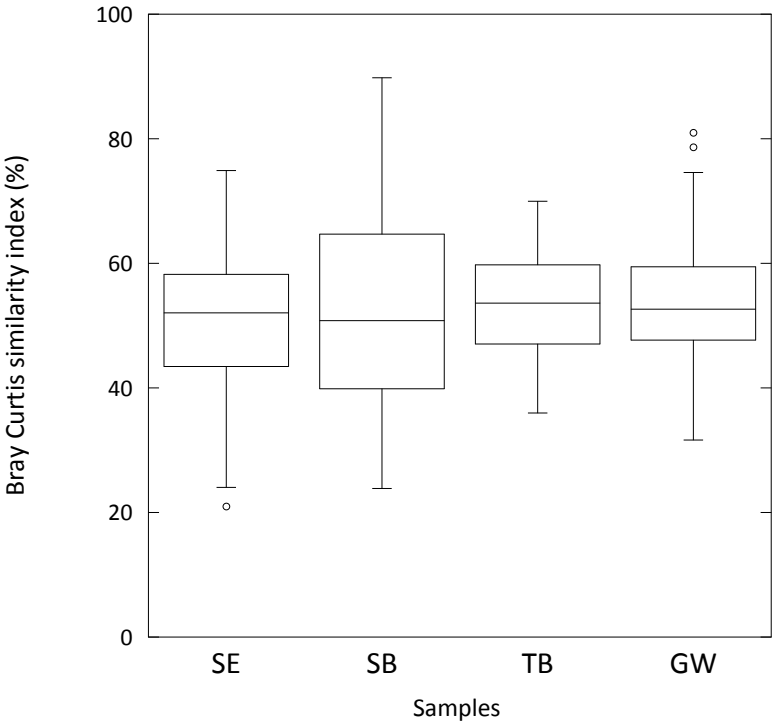

Figure S5. Boxplots of Bray-Curtis similarity index calculed for the four types of water quality. SE: El Ejido secondary effluent, SB: Baix Llobregat secondary effluent, TB: Baix Llogregat tertiary effluent, GW: groundwater.

Chapter 4

PREVALENCE OF THERMOPHILIC MICROORGANISMS IN BIOFILMS FROM GREENHOUSE-ENCLOSED DRIP IRRIGATION SYTEMS

To be submitted to: Microbiology SGM

SUMMARY

The microbial communities in biofilms from drip irrigation systems of an experimental greenhouse in Almería, which used two different qualities of water (treated wastewater and reclaimed water), were analyzed by DGGE (Denaturing Gradient Gel Electrophoresis) and subsequent sequencing of previously amplified 16S rRNA genes. The most remarkable feature of all biofilms from these irrigation systems was that regardless of water origin, sequences belonging to the phylum *Firmicutes* were prevalent and that all sequences recovered had a high similarity to cultured thermophilic organisms. In most cases the cultured organisms were also sporulated, suggesting that specific microbial communities able to grow at high temperatures were selected from the microbiota usually found in the incoming water and became predominant in the biofilms formed in these irrigation systems. As far as we know, this is the first study ever conducted that analyzes a microbial community forming biofilms in drip irrigation systems.

INTRODUCTION

Agricultural practices in Mediterranean countries with low rainfall have progressively shifted towards the use of drip irrigation as a tool to optimize water utilization as well as to improve control of water delivery to the crops.

In recent years, decreasing rainfall coupled to a higher demand of water for urban, industrial and recreational uses, have increased the interest in the use of reclaimed water for irrigation.

Drip irrigation, also called trickle or microirrigation, is particularly suitable for wastewater reuse because it minimizes the health risks to farmers and product consumers due to the little contact with wastewater. Furthermore, it is frequently used in greenhouses due to its high irrigation efficiency (up to 90%). Unfortunately

use of reclaimed water for drip irrigation is often associated to clogging events that impair proper operation of the system. When clogging occurs, more than 90% of the material accumulated has a biological origin. In most cases, the clogging process was usually initiated by bacterial biofilms (biofouling) (Gilbert *et al*., 1981; Taylor *et al*., 1995). Bacteria are able to grow adhered to almost every surface, forming architecturally complex biofilms, in which cells are encased in an extracellular matrix produced by bacteria themselves (Branda *et al.,* 2005; Hall-Stoodley and Stoodley 2009). Thus, assessing the composition of the microbial community constitutes an important issue in order to develop good management strategies for this type of facilities.

Several studies have focused on biofilm formation and clogging during reutilization of wastewater for irrigation purposes (Capra *et al*., 2004; Lubello *et al.,* 2004; Yan *et al*., 2009). Among them, Yan *et al*. (2009) analyzed the matrix structure in biofilms from drip irrigation emitters distributing reclaimed water using scanning electron microscopy (SEM). However, so far, despite their importance in agriculture, there isn't a single publication describing the composition of the microbial communities present in biofilms from drip irrigation systems.

Conventional cultivation-dependent microbiological techniques fail to give an indication of biodiversity, since about 99% of the bacterial cells in biofilms cannot be cultured on standard media (Wimpenny *et al*., 2000). During the last 15 years, molecular techniques based on 16S rRNA have been successfully applied to microbial ecology research, and among these techniques, Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer *et al*., 1998) has proved to be a powerful tool to obtain a fingerprint of natural microbial communities and to identify the more abundant members of the community.

The aim of this work was the molecular characterization of microbial communities forming biofilms in the irrigation systems (drippers and pipes) of an experimental

greenhouse in Almería used to grow a Virginia tobacco crop using two different qualities of water (a treated wastewater and a reclaimed water). The microbiota present in the two types of water employed for irrigation was also analyzed. Previous studies in this experimental greenhouse showed that the irrigation system was prone to develop biofouling and clogging of the drippers (Muñoz *et al*., 2010). We wanted to analyze the composition of these biofilms in order to gain a better understanding of the problem. To our knowledge, this is the first study ever conducted that analyzes a microbial community forming biofilms in irrigation systems. We expect that the results obtained will provide a better understanding of the type of organisms developing biofilms in this particular type of installation and will allow a better assessment of the risk they suppose for the crops involved as well as contribute to develop improved management strategies to avoid or minimize the problems of biofouling observed.

MATERIAL AND METHODS

Description of the experimental greenhouse

The study took place in an experimental greenhouse located at the agricultural research farm belonging to the University of Almería, in southeastern Spain (36°510N, 2°160W and 87 m elevation). The total greenhouse area was 1935 m^2 (43 x45 m), with an available area for cultivation of 1784 m^2.

Virginia tobacco (*Nicotiana tabacum L*.) was grown from October 2007 to June 2008. It was a crop destined to obtain biomass of products with high industry value (Muñoz et al. 2010). Tobacco seedlings were grown over two months by a professional grower (Ejidoplant S.L., Almeria, Spain), and transplanted at the beginning of October into the experimental greenhouse. The plants were grown in the greenhouse for a period of 9 months until harvested. At the end of this period

the faculty was dismantled and we took the opportunity to dissect the irrigation system, taking samples from both drippers and pipes for biofilm analysis.

The plants were irrigated with two qualities of water, which from now on we refer to treated wastewater as **SEW** (Secondary Effluent Water) and to reclaimed water as **TEW** (Tertiary Effluent Water). SEW was the secondary effluent from the municipal wastewater treatment plant of El Ejido (Almería, Spain). This plant treats 13.800 $m^3.day^{-1}$ of urban wastewater. Input wastewater undergoes a physical pre-treatment to remove coarse solids and greases, along with primary settling of particulates. Secondary treatment then takes place with activated sludge, after which the treated wastewater is discharged. The treated wastewater to be reused in the greenhouse was collected after passing through the secondary settling tank, without any further treatment. TEW consisted of the tertiary effluent from the El Toyo Wastewater Treatment Plant. In this plant, primary and secondary treatments are identical to those of El Ejido plus ozone treatment and the addition of rainwater water and desalinated water from the Carboneras desalination plant. TEW is the water normally used in this area for agriculture.

In the greenhouse SEW and TEW distribution and application were separated in two independent irrigation lines. Water was distributed by means of pressure-compensating Autotwin® drippers (Mondragón Soluciones, Albuixech, Spain) located on the soil surface, with a nominal flow of 3 $L.h^{-1}$, placed at a density of 4 units m^{-2}. The material of the drippers was polypropylene and, in the case of the pipes, polyethylene. The crop was typically irrigated once a day, 3-4 times a week, with an average amount of 9 mm water each time.

Sampling

After 9 months of continuous operation of the greenhouse, biofilms from the inner plastic surfaces of both SEW and TEW irrigation lines were processed. The pipes

were cut under sterile conditions and the inner biofilms were scraped by means of a sterilized metal spatula and stored in an eppendorf tube at -20ºC for further analysis. Five pipes sections from the irrigation lines distributing each type of water were sampled. The drippers were dismantled and the inner filter was collected in 15 mL of saline solution (0.9% sodium chloride). The samples were placed in an ultrasonic water bath (Bransonic 5, Branson) for 3 minutes. After sonication, the liquid was centrifuged 5 minutes at 10.000*g* (Eppendorf centrifuge 5804 R) and the resulting pellet was stored at -20ºC for further analysis (Sánchez *et al.,* 2006). In total, five drippers from each irrigation line (TEW and SEW) were sampled and pooled together. By pooling the samples we lost the possibility to compare inter sample variability but as a tradeoff we ensured that enough biomass was present to obtain representative of the entire line.

About 300 mL of each type of water were also filtered through polycarbonate filters of 0.22 µm of pore size and the filters were stored at –20ºC for further DNA extraction.

DNA Extraction

The DNA Power Soil kit MOBIO ref. 12888-50 was used for DNA extraction from biofilms, while the UltraClean water kit MOBIO ref. 14880-25 was utilized for the filters. DNA integrity was checked by agarose gel electrophoresis, and quantified using a low DNA mass ladder as a standard (Invitrogen) using the Quantity One software package (Bio-Rad) for gel documentation and analysis.

PCR-DGGE fingerprinting

Fragments of the bacterial 16S rRNA gene suitable for DGGE analysis were obtained by using the specific primer 358F with a 40-bp GC clamp, and the universal primer 907RM (Sánchez *et al.,* 2007). Polymerase chain reaction (PCR) was carried out with

a Biometra thermocycler using the following program: initial denaturation at 94ºC for 5 min; 10 touchdown cycles of denaturation (at 94ºC for 1 min), annealing (at 65-55ºC for 1 min, decreasing 1ºC each cycle), and extension (at 72ºC for 3 min); 20 standard cycles (annealing at 55.5ºC, 1 min) and a final extension at 72ºC for 5 min.

PCR mixures contained 1-10 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 µM, 1.5 mM $MgCl_2$, each primer at a concentration of 0.3 µM, 2.5 U *Taq* DNA polymerase (Invitrogen) and PCR buffer supplied by the manufacturer. BSA (Bovine Serum Albumin) at a final concentration of 600 $\mu g \cdot ml^{-1}$ was added to minimize the inhibitory effect of humic substances (Kreader, 1996). The volume of reaction was 50 µl. PCR products were verified and quantified by agarose gel electrophoresis with a low DNA mass ladder standard (Invitrogen) using the Quantity One software package (Bio-Rad) for gel documentation and analysis.

DGGEs were run in a DCode system (Bio-Rad) as described by Muyzer *et al*. (1998). A 6% polyacrylamide gel with a gradient of 30-70% DNA-denaturant agent was cast by mixing solutions of 0% and 80% denaturant agent (100% denaturant agent is 7 M urea and 40% deionized formamide). Seven hundred ng of PCR product were loaded for each sample and the gels were run at 100 V for 18 h at 60°C in 1xTAE buffer (40 mM Tris [pH 7.4], 20 mM sodium acetate, 1 mM EDTA). The gel was stained with SybrGold (Molecular Probes) for 45 min, rinsed with 1xTAE buffer, removed from the glass plate to a UV-transparent gel scoop, and visualized with UV in a Gel Doc XRS (Bio-Rad). Digitized DGGE images were analyzed using Quantity One from (Bio-Rad). The software performs a density profile along each DGGE lane, detects the bands, and calculates the relative contribution of each band to the total band signal in the lane, after using a rolling disk algorithm correction. Visible bands were excised from the gels, resuspended in milli-Q water overnight and reamplified for sequencing.

16S rRNA gene sequencing

Purification of PCR products from DGGE bands and sequencing reactions was performed by Macrogen (South Korea) with primer 907RM. They utilized the Big Dye Terminator version 3.1 sequencing kit and reactions were run in an automatic ABI 3730XL Analyzer-96 capillary type. Gene sequences were deposited in GenBank under accession numbers HE573183-HE573228.

Sequences were subjected to a BLAST search (Altschul *et al.*, 1997) to obtain an indication of the phylogenetic affiliation.

RESULTS AND DISCUSSION

The DGGE analysis of four samples of biofilms (each corresponding to 5 pooled samples) from the inner plastic surfaces of the drip irrigation systems (drippers and pipes), which used SEW and TEW, yielded a total of 61 band positions, with an average of 14 to 18 bands per lane (Fig. 1). The fingerprinting obtained showed a limited number of bands, indicating that diversity was low.

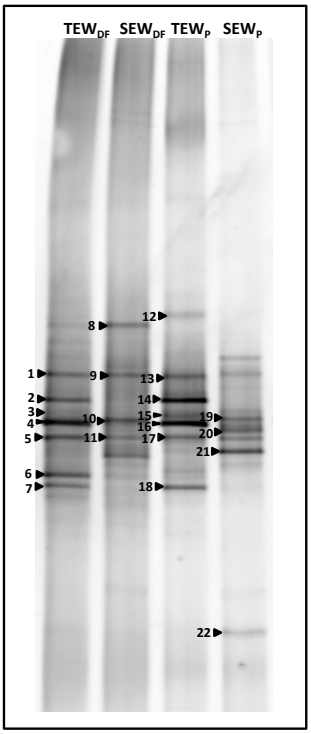

Figure 1. DGGE fingerprints of biofilm samples corresponding to: TEW_{DF} - biofilm from tertiary effluent water dripper filter. SEW_{DF} - biofilm from secondary effluent water dripper filter. TEW_{P} - biofilm from tertiary effluent water pipe. SEW_{P} - biofilm from secondary effluent water pipe.

It can be observed that biofilms from drippers and pipes using TEW presented an almost identical band pattern despite the fact that pipes and drippers were made of different materials (polyethylene and polypropylene respectively) indicating that the type of material used did not have a strong influence on biofilm composition. The band pattern was different in the case of materials exposed to SEW although the existence of coincidences (bands 1 and 9, bands 4 and 10) suggests that, despite the differences observed, some organisms were prevalent in both types of water.

Bands were excised from the gels and sequenced in order to determine their phylogenetic affiliation. Informative sequences were obtained from 22 bands (Table 1). These bands accounted for 63.6% of the total mean band intensity and BLAST analysis showed that most of them showed similarities with sequences from cultured clones, 9.1% at the species level (more than 97% similarity) and 18.2% at the genus level (similarity between 95-97%).

Table 1. Phylogenetic affiliation of sequences obtained from biofilm DGGE bands. Both closest uncultured and cultured matches are shown.

Band	Closest match	% similarity (nºbases)[a]	Taxonomic group	Accession nº (Gen Bank)	Cultured closest match (%similarity)	Relative intensity (%)
1, 9	*Geobacillus* sp.	95.2 (420)	*Firmicutes*	AM749790		8.2, 8.3
2, 14	Uncultured Bacillaceae bacterium clone D6-14	93.4 (495)	*Firmicutes*	AB331459	*Oxalophagus oxalicus* (92.5)	8.5, 11.2
3	*Bacillus thermodenitrificans*	88.5 (362)	*Firmicutes*	Z26928		10.1
4, 10	*Pseudoxanthomonas taiwanensis*	99.6 (526)	γ-*Proteobacteria*	EU438976		19.2, 21.2
5, 17	*Thermomonas haemolytica*	97.4 (484)	γ-*Proteobacteria*	AJ300185		10.2, 8.7
6	*Symbiobacterium thermophilum*	95.8 (460)	*Firmicutes*	AP006848		13.6
7	*Brevibacillus thermoruber*	87.8 (459)	*Firmicutes*	DQ11668		7.3
8	*Ignavibacterium album*	95.5 (490)	*Chlorobi*	AB478415		19.9
11	*Symbiobacterium* sp. KA13	93.1 (493)	*Firmicutes*	AB455239		21.2
12	Uncultured Bacillaceae bacterium clone D6-14	93.1 (486)	*Firmicutes*	AB331459	*Tuberibacillus calidus* (91.4)	7.1
13	Uncultured Bacillaceae bacterium clone D6-14	90.7 (487)	*Firmicutes*	AB331459	*Bacillus* sp. (89.7)	8.3
15	Uncultured synthetic wastewater bacterium tmbr15-26	86.3 (449)	*Firmicutes*	AF309816	*Aneuribacillus* sp. (86.3)	1.9
16	*Bacillus* sp.	86.6 (350)	*Firmicutes*	AY762977		12.5
18	*Brevibacillus* sp.	91.5 (455)	*Firmicutes*	AY397773		10.4
19	Uncultured Firmicutes clone COM-27	81.3 (414)	*Firmicutes*	AB451750	*Tuberibacillus calidus* (81.1)	5.3
20	*Alicyclobacillus macrosporangiidus*	80.2 (344)	*Firmicutes*	AB264025		12.0
21	*Geobacillus stearothermophilus*	97.0 (520)	*Firmicutes*	EU381192		25.7
22	*Thermaerobacter* sp.	86.8 (356)	*Firmicutes*	AB444428		15.2

a. Number of bases used to calculate the levels of sequence similarity.

Three phyla could be retrieved. Seventeen of the total 22 sequences belonged to the phylum *Firmicutes*, with a total contribution of 46% to total mean band intensity, and had a high similarity with cultured microorganisms of the genus *Geobacillus*, *Bacillus*, *Symbiobacterium*, *Brevibacillus*, *Alicyclobacillus* and *Thermoaerobacter.* Four of the remaining sequences belonged to *Gammaproteobacteria* (14.4% of total mean band intensity) and were similar to

Pseudoxantomonas taiwanensis and *Thermomonas haemolytica* (99.6% and 97.4% similarity, respectively) and one sequence was similar to *Ignavibacterium album* (95% similarity) from the *Chlorobi* group.

The most remarkable feature of all biofilms from these irrigation systems, independently of water origin, was the prevalence of sequences belonging to the phylum *Firmicutes*. All the sequences have high similarity to cultured thermophilic organisms. In most cases (with exception of *Thermoaerobacter*), the cultured organisms were also sporulated.

As an example, one of the sequences recovered had a high similarity (97%) to *Geobacillus stearothermophilus*, a Gram positive, chemo-organotrophic, aerobic or facultatively anaerobic, obligately thermophilic bacterium, with an optimum growth temperature of 55-65ºC (Nazina *et al*., 2001).

The four sequences of *Gammaproteobacteria* recovered in this study also belonged to thermophilic microorganisms. Two of them had a high similarity (99.6%) to *Pseudoxanthomonas taiwanensis*, a thermophilic bacterium, isolated from the Chi-ban Hot Springs in Taiwan. This organism is aerobic, Gram negative and has an optimum growth temperature of 50ºC (Chen *et al.,* 2002). The remaining two sequences were highly similar (97.4%) to *Thermomonas haemolytica*, a thermophilic, Gram negative, non-spore-forming bacterium with an optimum growth temperature between 37 and 50ºC. It was isolated from kaolin slurry used in the production of paper (Busse *et al.,* 2002).

Finally, the last sequence obtained had a 95.5% similarity to *Ignavibacterium album*, which is a moderately thermophilic chemoheterotrophic bacterium from the *Chlorobi* group. It was isolated from microbial mats developed in hot spring water streams from Yumata, Japan. Cells of this organism are strictly anaerobic, Gram negative, non-sporulating and grow fermentatively at an optimum temperature of 45ºC (Iino *et al.,* 2009).

We also analyzed the composition of the microbial community present in the incoming water, corresponding to TEW and SEW. The DGGE analysis from the two water samples yielded a total of 56 band positions, 25 from TEW (Fig. 2A) and 31 from SEW (Fig. 2B). In general, the band patterns showed a higher diversity than biofilms with phylogenetic groups typical of aquatic environments. Bands were excised from the gels and sequenced in order to determine their phylogenetic affiliation, and informative sequences were obtained from 15 bands of TEW and 9 bands of SEW (Table 2). These bands accounted for 74.1% of the total mean band intensity in the case of TEW and 42.2% for SEW, and most of them showed similarities with sequences from uncultured clones by BLAST.

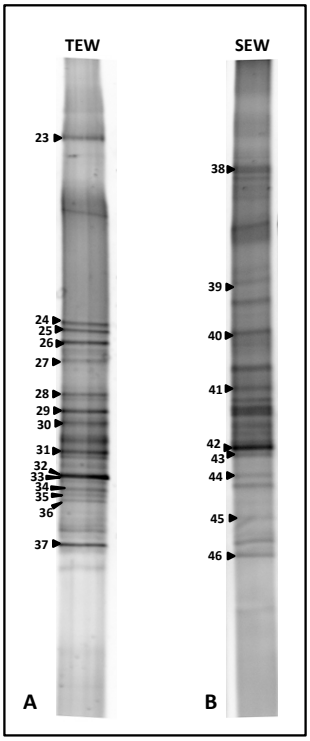

Figure 2. DGGE fingerprints of the two types of incoming water **(A):** tertiary effluent water (TEW), **(B):** secondary effluent water (SEW).

Table 2. Phylogenetic affiliation of sequences obtained from tertiary effluent water (TEW) and secondary effluent water (SEW) DGGE bands, with closest uncultured and cultured matches.

Band	Closest match	%similarity (nº bases)[a]	Taxonomic group	Accession nº (Gen Bank)	Cultured closest match (% similarity)	Relative intensity (%)
23	Clone nbw691c02c1	99.6 (520)	*Bacteroidetes*	GQ116309	*Flavobacterium sasangense* (98.5)	11.2
24	Uncultured *Bacteroidetes* bacterium clone Gap-1-42	98.8 (513)	*Bacteroidetes*	EU642075	*Flexibacter* sp. (87.8)	3.4
25	Clone 1013-1-CG43	99.8 (517)	*Firmicutes*	AY532555	*Erysipelothrix rhusiopathie* (93.5)	3.8
26	Uncultured *Anaerophaga* sp. Clone MDAF17	99.6 (523)	*Firmicutes*	EU214543	*Bacillus* sp. (98.8)	6.4
27	Clone Mar_CL_100633_OTU-56	98.5 (516)	*Firmicutes*	EU808593	*Clostridium* sp. (95.6)	2.7
28	Uncultured *Hongiella* sp. Clone XZTSH58	90.5 (468)	*Bacteroidetes*	EU703374	*Algoriphagus aquatilis* (87.1)	4.1
29	Clone 12up	90.5 (485)	β-*Proteobacteria*	AY212580	*Propionivibrio* sp. (88.4)	5.6
30	*Pseudomonas pseudoalcaligenes*	96.2 (513)	γ-*Proteobacteria*	EU780001		4.5
31	*Pseudomonas putida*	96.4 (507)	γ-*Proteobacteria*	EF011017		5.1
32	Uncultured *Betaproteobacteria* bacterium QEDN10BE03	98.6 (527)	β-*Proteobacteria*	CU925683	*Dechloromonas* sp. (98.1)	12.6
33	Clone SH3-3-118	97.7 (523)	β-*Proteobacteria*	FJ830747	Rhodocyclaceace bacterium enrichment clone (96.6)	12.6
34	Clone IWENVC15	95.9 (512)	β-*Proteobacteria*	EU000441	*Acidovorax* sp. (95.7)	2.4
35	Uncultured Burkholderiales bacterium clone Gap-2-44	94.2 (502)	β-*Proteobacteria*	EU642184	*Acidovorax konjaci* (93.4)	1.1
36	Uncultured Comamonadaceae bacterium clone	97.2 (513)	β-*Proteobacteria*	EU642195	*Acidovorax* sp. (96.6)	1.2
37	Clone BR1F12	98.8 (523)	*Firmicutes*	FJ916817	*Moorella* sp. (81.2)	9.9
38	Clone 30	92.9 (498)	*Bacteroidetes*	FJ534976	*Bacteroides* sp. XDT-1 (92.9)	4.8
39	Clone PA52	99.6 (530)	*Bacteroidetes*	FJ799161	*Bacteroides* sp. (99.4)	0.9
40	Clone AE2_aaa02d07	88.6 (476)	*Bacteroidetes*	EU771211	Bacterium Oil-Tsu-11 (86.2)	4.4
41	Clone SGUS1002	86.7 (355)	*Bacteroidetes*	FJ202160	*Lewinella coharens* (85.4)	3.4
42	Uncultured beta proteobacterium clone K13	86.6 (468)	β-*Proteobacteria*	AM940953	*Diaphorobacter* sp. (86.5)	17.2
43	Uncultured bacterium isolate DGGE gel band 13	95.9 (522)	β-*Proteobacteria*	EU275391	*Hydrogenophaga* sp. (95.2)	2.3
44	Clone 31f06	90.1 (469)	*Bacteroidetes*	EF515658	*Prevotella oralis* (86.7)	1.8
45	Clone 9_1	96.5 (508)	γ-*Proteobacteria*	EF443088	*Thiodictyon bacillosum* (93.5)	2.6
46	Uncultured bacterium isolate DGGE gel band 4	92.0 (452)	γ-*Proteobacteria*	EU426848	*Methylocaldum szegediense* (82.8)	4.9

a. Number of bases used to calculate the levels of sequence similarity.

The retrieved bands corresponded to *Bacteroidetes* (with a total contribution of 18.7% to total mean band intensity in TEW and 15.3% in SEW), *Betaproteobacteria* (22.9% in TEW and 19.5% in SEW) and *Gammaproteobacteria* (9.6% in TEW and 7.5% in SEW). *Firmicutes* (22.8% contribution) could only be retrieved from TEW. Most of these sequences belonged to uncultured microorganisms. The similarities ranged between 86.6 and 99.8%.

Only two bands from TEW corresponded to cultured microorganisms, *Pseudomonas pseudoalcaligenes*, which is an aerobic, Gram negative soil bacterium that was first isolated from swimming pool water. It is able to use cyanide as a nitrogen source, and as a result it may be used for bioremediation (Huertas *et al.,* 2006), and *Pseudomonas putida*, a metabolically versatile Gram negative saprophytic soil bacterium with potential new applications in agriculture, biocatalysis, bioremediation and bioplastic production (Nelson *et al.,* 2002). In contrast to what we found when analyzing the biofilms, thermophilic microorganisms were not prevalent in the incoming SEW or TEW although, of course, our results do not allow us to discard their presence at low concentrations.

Overall, the results show that a microbial community able to grow and survive at high temperatures develops and becomes predominant in the inner surfaces of this irrigation system. The fact that these organisms were not present at high amounts in the incoming water suggests that conditions within the irrigation infrastructure specifically high temperatures, selected these populations. Although we have no data on the actual temperatures within the pipes and drippers, the temperatures recorded daily inside the greenhouse during the 9 months of operation indicate maximum temperature between 40 and 46 ºC. The temperature in the tubes and drippers could be even higher due to their dark color. Finally, the irrigation lines operated only once a day (3-4 times a week) and at very low flows (3 L h^{-1}). This means that during most of the time water did not circulate and refrigeration due to external cold water entering the system did not occur.

From a point of view of microbiological risk for the irrigated crops, the prevalence of thermophilic microorganisms suggests that conditions inside the irrigation system are far from friendly for usual pathogenic organisms that thrive well in the mesophilic range, therefore their presence in the biofilms is unlikely. On the other hand, the presence of sporulated thermophiles might constitute a problem when

attempting to control biofilm formation through the action of biocides since endospores are highly resistant to the action of disinfectants.

In conclusion, this work has shown that the microbial community in this drip irrigation system is mainly composed of thermophilic microorganisms. The presence and the type of thermophilic organisms found does not seem to be affected by the type of water used, as the thermophilic/thermoresistant trait is present in biofilms exposed to secondary and tertiary water alike. Of course, we are aware that the type of microbial community found might differ under other environmental conditions, since in other irrigation systems different factors could be involved in the selection of biofilm communities.

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SYNTHESIS AND DISCUSSION

The main goal of this thesis was the study of the composition of microbial communities in different environments related to wastewater treatment and reuse, in order to improve our knowledge and understanding of the microbiology of wastewater and contribute to its reutilization.

As stated in the introduction, this global aim was divided into three specific objectives, the first devoted to compare microbial communities found in conventional processes with two atypical treatment systems studied in **Chapters 1 and 2**. The second, dedicated to compare the microbial diversity profile of treated wastewaters *versus* natural freshwaters (**Chapter 3**). Finally, the third objective addressed the reuse of water analyzing the possible influence of the type of reuse operations on the microbial composition of the associated microbiota (**Chapter 4**).

To address the first objective, one of the atypical environments that we chose was a seawater-processing activated sludge (**Chapter 1**). Although there are many studies concerning microbial diversity in freshwater activated sludges, no studies have been done on WWTPs that utilize seawater for their operation. A full-cycle rRNA approach, combining and comparing different molecular methodologies (DGGE, clone library and CARD-FISH), was applied to obtain a comprehensive picture of the diversity of the prokaryotic assemblage (*Bacteria* and *Archaea*) in this special activated sludge. Different bacterial groups were found by means of the three molecular methods employed, such as *Alpha-*, *Gamma-*, *Delta-* and *Betaproteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes* and *Actinobacteria*. Most techniques detected many groups, but depending on the technique used, the proportions differed, showing that the three methods are complementary (see Figure 4, Chapter 1, p.47). Some of the sequences retrieved were similar at the genus level to marine chemoheterotrophic bacteria and methanotrophic bacteria, as well as to genus capable to degrade a wide variety of refractory environmental pollutants or genus known to produce slimes and/or capsules, which could be involved in the formation process of sludge flocs.

Few *Archaea* were found, and most of them were methanogenic *Euryarchaeota*, which are strictly anaerobic. This suggests that these organisms are active within anoxic microenvironments in activated sludge flocs (Schramm *et al.,* 1999). *Crenarchaeota* were also detected but only by CARD-FISH. Low archaeal diversity was expected, as it seems clear that *Bacteria* are responsible for most of the carbon removal in activated sludge processes (Gray *et al*., 2002).

Furthermore, in order to link diversity and function, we confirmed the presence of ammonia-oxidizers by amplification of the functional gene coding for the active-site polypeptide of ammonia monooxygenase (*amo*A) from *Betaproteobacteria*. Although we also tried to amplify *amo*A genes from *Gammaproteobacteria* and *Archaea*, they were not detected. Even though amplification from beta ammonia-oxidizers was observed, we were not able to find sequences corresponding to recognized microorganisms known to catalyze the oxidation of ammonia to nitrite or nitrite to nitrate in freshwater WWTPs. It is possible that nitrifiers in this specific seawater-processing WWTP belonged to different genera. However, we retrieved sequences of an *Alphaproteobacteria* (*Nitratireductor* sp.), able to reduce nitrate to nitrite. Therefore, we could see that functions related to nitrogen removal were carried out, but perhaps the microorganisms responsible were slightly different due to the peculiar characteristics of the sludge.

Concerning the different methodologies employed, our results showed that the group ratios found with DGGE were more similar to CARD-FISH than to clone libraries. Although this was not expected, other studies have described similar results in this regard (Massana *et al*., 2006). In comparison with other studies carried out in conventional activated sludge, the composition of the bacterial community of the seawater-activated sludge described here differed strongly, since *Betaproteobacteria* did not seem to be the predominant group, and instead, other subclasses of *Proteobacteria* such as *Alphaproteobacteria* were detected in a higher proportion. This is most likely due to the fact that seawater is known to contain very few *Beta*- and

more *Alphaproteobacteria* (Rappé and Giovannoni, 2003; Barberán and Casamayor, 2010).

The second type of environment related to wastewater treatment was an experimental constructed wetland, in which we analyzed the composition of the microbial community by means of molecular methods in order to determine the effect of plant species, organic matter and hydraulic design (**Chapter 2**). We characterized the microbial assemblages of gravel biofilm, interstitial liquid and root samples in different experimental CWs, since it is known that microorganisms play an important role in this type of environments that treat wastewater through natural processes.

On the one hand, the results obtained allowed to characterize the main microorganisms involved in these treatment processes. The taxonomic groups retrieved from all samples were *Betaproteobacteria*, *Bacteroidetes* and *Firmicutes*, while *Deltaproteobacteria* could be detected in gravel biofilms and roots, but were not recovered in prominent bands of interstitial liquid, where the *Actinobacteria* group predominated. Sequences affiliated to *Acidobacteria*, *Alphaproteobacteria* and *Chlorobi* were found only in roots. The sequences of these taxonomic groups with high similarity to cultured microorganisms belonged to species with the potential to act as degraders of environmental pollutants in natural and constructed wetlands.

On the other hand, analyses of band patterns allowed to obtain dendrograms and to carry out multidimensional scaling (MDS), which were applied to discriminate microbial assemblages in the different microenvironments. These analyses showed that the type of plant used (*Typha* or *Phragmites*) in the different constructed wetlands affected the microbial community that developed in their roots. This could be explained by differences in oxygen production and transport by the plant species to the root environment, as *Phragmites* has the ability to supply oxygen to structures in the root (Briones *et al*., 2003; Chelius and Lepo, 1999). Also, we could see that the hydraulic design [free-water surface (FWS) or subsurface flow (SSF)] influenced the

microbial community, in agreement with other studies (Vacca *et al*., 2005; Popko *et al.,* 2006). The possible reason could be that hydraulic design had effects on the redox conditions in the water due to differences in oxygen exchange between FWS and SSF systems, FWS having better aeration and therefore a higher redox than SSF systems (Lin *et al*., 2008; Kadlec and Knight, 1996). These results were corroborated with an average redox potential clearly different in the two hydraulic designs. Finally, no differences were found between the tanks that had different load of organic matter, because, despite the fact that increasing the organic load decreases the redox potential and it could affect the microbial communities, there were no differences between the redox potentials, probably due to the fact that the loadings applied were low.

In summary, different conclusions can be inferred from the results obtained in the two atypical treatment systems studied (seawater-processing WWTP and experimental constructed wetlands) when comparing with data from conventional wastewater treatment plants. First of all, in our activated sludge system as well as in experimental CWs, we found typical activated sludge microorganisms, such as ***Sphingomonas*** (an *Alphaproteobacteria* capable to degrade a wide variety of refractory environmental pollutants, which could be involved in the formation process of sludge flocs), ***Denitratisoma oestradiolicum*** (a denitrifying *Betaproteobacteria*) and ***Flavobacterium*** (a *Bacteroidetes* typically found in activated sludge). Besides these genera, we found other microorganisms specific of our treatments, such as ***Sneathiella chinensis*** (a marine chemoheterotrophic *Alphaproteobacteria*), ***Methylocystis*** (a methanotrophic *Alphaproteobacteria*) and ***Nitratireductor*** (an *Alphaproteobacteria* able to reduce nitrate to nitrite). In our experimental CWs we retrieved sequences from ***Dechloromonas denitrificans*** (a N_2O-producing *Betaproteobacteria*), ***Rhodobacter blasticus*** (an *Alphaproteobacteria* isolated mainly from wastewater and polluted environments) and ***Chlorobium*** sp. (a green sulfur bacterium able to use sulfide). Even though these bacteria may not be typical of activated sludges, they are all involved in the degradation of pollutants and are usually present in wastewater environments. In

fact, the common characteristic of all treatment systems is that most of these bacteria are involved in the degradation of organic matter and some are concerned with the nitrogen cycle. Therefore, the microbial communities responsible of wastewater treatments belonged to different species depending on the type of treatment, but despite differences found in the species composition, they were very similar from a functional perspective. The same physiologic groups developed in both systems, and aerobic, anaerobic, chemotrophic and heterotrophic microorganisms were recovered.

In order to tackle the second main objective, we questioned whether the microbial profiles of waters that leave the treatment plants differed from those of non-contaminated waters and if these microbial profiles were affected by the type of incoming wastewater. Thus, in **Chapter 3** we studied different WWTP effluents, both secondary and tertiary effluents, and we compared them with water samples from an uncontaminated natural aquifer. We analyzed standard fecal indicators using conventional techniques and the bacterial diversity profiles over time (during one-three year period) using DGGE. The presence of *E. coli* was only detected in secondary effluents, confirming worse quality than tertiary effluents and groundwater. Regarding the microbial profiles of the four types of water quality, we considered the most abundant phylogenetic groups found in our reference contaminated and non-contaminated waters, proposing an alternative indicator of water quality, with the purpose of meeting the need to find an indicator of ecological quality. We observed that the taxonomic group *Betaproteobacteria* could be found in each of the water qualities, but the interesting thing was that the proportion was higher as the water quality increased (Figure 3 of Chapter 3, p. 115). We also detected that secondary effluents had a higher proportion of *Bacteroidetes* and *Gammaproteobacteria*, which let us think that this trend could be characteristic of waters coming from wastewater or treated wastewater environments. Furthermore, the *Nitrospira* group, typical from WWTPs environments, was mainly present in the secondary effluents of one of the WWTP analyzed.

Taking into consideration these microbial diversity profiles, we chose the BGN:β ratio (*Bacteroidetes*, *Gammaproteobacteria* and *Nitrospira* to *Betaproteobacteria*) and proposed it as a potential indicator of water quality. This alternative index seemed to have a good correlation with water quality, since representative samples of high quality waters always showed a lower ratio (never over 0.85) than representative samples of poor quality waters (always over 1.3) (Figure 5, Chapter 3, p. 119). The Bray-Curtis coefficient allowed to discard that the observed differences in the BGN:β ratios were due to the temporal variability of the samples (Figure S5, Chapter 3, p. 139). This observation thus strengthened the usefulness of our proposed index as an alternative indicator of water quality. With the aim of confirming the reliability of our indicator, we calculated our ratio with data from published studies which provided microbial diversity data in different systems and, as a rule, gut and sewage-associated samples had higher BGN:β ratios that non-fecal samples.

Finally, we addressed the third objective of this thesis, that is, to cover the issue of the reuse of reclaimed water. We wanted to determine to what extent the microbial population associated to problems occurring during water reuse were conditioned by the microbial community of the incoming water, or by the environmental conditions prevailing in the system. We had the opportunity to study the microbial communities related to an environment where the use of reclaimed water generated different problems (**Chapter 4**): the drip irrigation systems of an experimental greenhouse, which used two qualities of reclaimed water and the irrigation system was prone to develop biofouling and clogging of the drippers. We analyzed the microbial community of the biofilms formed in the different irrigation systems (drippers and pipes) and also in the two types of water employed for irrigation, in order to gain a better understanding of the type of organisms developing biofilms in this particular installation. This study has allowed a better assessment of the risk they suppose for the crops involved and it has contributed to develop improved management strategies to avoid or minimize the problems of biofouling observed.

Our results showed that, independently of the type of irrigation water (secondary or tertiary effluent water) or the type of irrigation system (dripper or pipe), most of the sequenced bands of the organisms found in these biofilms were *Firmicutes*, although *Gammaproteobacteria* and *Chlorobi* were also found in some of samples. The most remarkable feature was that all *Firmicutes* sequences had high similarity to cultured thermophilic organisms. In most cases, the cultured organisms were also sporulated. The *Gammaproteobacteria* and *Chlorobi* sequences also belonged to thermophilic organisms. In contrast, in the incoming water (secondary and tertiary effluents) thermophilic microorganisms were not prevalent, suggesting that conditions within the irrigation infrastructure, specifically high temperatures, selected these populations in biofilms.

From a point of view of the microbiological risk for the irrigated crops, the fact that most of microorganisms present in this irrigation systems were thermophilic, suggest that it is very unlikely to find usual pathogenic organisms, since they thrive well in the mesophilic range. Furthermore, the presence of sporulated thermophiles could be a problem with the use of biocides to control biofilm formation, because endospores are highly resistant to the action of disinfectants.

In general, it appeared that the biofilm microbial community developed in irrigation systems could be more influenced by environmental conditions than the type of irrigation water.

To conclude this general discussion, we would like to summarize the main contributions. Answering the question about to what extent microbial communities found in different wastewater treatment systems share common features or differ from each other (**Chapter 1 and 2**), we have observed that each system has its specific microbial community, adapted to its environmental conditions, with the same functions being carried out by different groups.

Concerning the second objective, we wanted to know whether treated WWTPs effluents were assimilable to natural water and according to their microbial profiles if they followed a constant pattern or were different depending on the incoming water. Although these effluents can be different according to their origin and their treatment, in **Chapter 3** we saw that these different water types have a common footprint. Wastewater and thus WWTP effluents have a different microbial profile from natural waters. In our study, the proportions of *Bacteroidetes*, *Gammaproteobacteria* and *Nitrospira* taxonomic groups were higher in this type of effluents than in natural waters, such as a groundwater, which on the contrary, there were a very high proportion of *Betaproteobacteria*.

Finally, to address the ultimate aim, in **Chapter 4** of the thesis we have seen that in the reutilization of treated wastewater or reclaimed water, conditions in the system may be more important than treated or reclaimed water itself, that is, although the water has some special features, with more organic matter or different microbial profile, specific conditions in the reutilization systems will determine the characteristics of the microbial community. Our study of biofilms in drip irrigation systems constitutes a prime example in which despite the type of water used, environmental conditions (specifically temperature) allow only the development of thermotolerant and sporulated microorganisms.

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CONCLUSIONS

1. DGGE, clone libraries and CARD-FISH are complementary techniques that allow obtaining a comprehensive picture of the diversity of the prokaryotic assemblage (*Bacteria* and *Archaea*) of an activated sludge; using these techniques we found *Alpha-*, *Gamma-*, *Delta-* and *Betaproteobacteria*, *Bacteroidetes*, *Deinococcus-Thermus*, *Firmicutes* and *Actinobacteria*, as well as *Euryarchaeota* and *Crenarchaeota* in our samples.

2. The prokaryotic community of an activated sludge from a seawater-processing wastewater treatment plant had different proportions of taxonomic groups when compared to conventional activated sludges, particularly a low presence of *Betaproteobacteria* probably due to high salt concentration.

3. In our activated sludge there were *Betaproteobacteria* ammonia-oxidizers detected by amplification of amoA gene, which probably belonged to different genera than ammonia-oxidizers of freshwater WWTPs.

4. Taxonomic groups retrieved from the samples of different experimental constructed wetlands belonged to species with the potential to act as degraders of environmental pollutants in natural and constructed wetlands.

5. Hydraulic design influenced the microbial community developed in constructed wetlands since it had effects on the redox conditions in the water. The type of plant also affected the microbial community due to differences in oxygen production and transport by the plant species. However, different load of organic matter did not seem to affect the microbial community.

6. Comparison of microbial communities found in conventional wastewater processes with two atypical treatment systems (seawater-processing activated sludge and an experimental constructed wetland), allowed to conclude that some microorganisms were common to all systems, while others were specific of each treatment. This suggests that in different wastewater treatment processes the same functions are being carried out but by different bacterial groups.

7. The microbial diversity profiles of treated wastewaters share some common features constituting a differentiated fingerprint that allows distinguishing them from natural waters.

8. This thesis proposes the use of the ratio *Bacteroidetes*, *Gammaproteobacteria*, *Nitrospira* / *Betaproteobacteria* (BGN:β) as an alternative indicator of ecological water quality. Secondary effluents of wastewater treatment plants presented higher ratios than tertiary effluents or groundwater.

9. The microbial community of biofilms developed in a drip irrigation system of a greenhouse operating with treated wastewater and reclaimed water, was dominated by thermophilic and sporulated microorganisms, suggesting that high temperatures within the irrigation infrastructure selected these populations.

10. The microbial community from a biofilm developed in drip irrigation systems using two types of water quality, seemed to be more influenced by environmental conditions than by the type of irrigation water.

ANNEX

ANNEX I. Quality criteria for reuse of water according to their uses (Real Decreto 1620/2007).

ANEXO I.A: CRITERIOS DE CALIDAD PARA LA REUTILIZACIÓN DE LAS AGUAS SEGÚN SUS USOS

CALIDAD REQUERIDA

USO DEL AGUA PREVISTO	VALOR MÁXIMO ADMISIBLE (VMA)				
	NEMATODOS INTESTINALES[1]	*ESCHERICHIA COLI*	SÓLIDOS EN SUSPENSIÓN	TURBIDEZ	OTROS CRITERIOS
1.- USOS URBANOS					
CALIDAD 1.1: RESIDENCIAL[2] a) Riego de jardines privados.[3] b) Descarga de aparatos sanitarios.[3]	1 huevo/10 L	0 (UFC[4] /100 mL)	10 mg/L	2 UNT[5]	OTROS CONTAMINANTES[6] contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas[7] deberá asegurarse el respeto de las NCAs.[8] *Legionella spp.* 100 UFC/L (si existe riesgo de aerosolización)
CALIDAD 1.2: SERVICIOS a) Riego de zonas verdes urbanas (parques, campos deportivos y similares).[9] b) Baldeo de calles.[9] c) Sistemas contra incendios.[9] d) Lavado industrial de vehículos.[9]	1 huevo/10 L	200 UFC/100 mL	20 mg/L	10 UNT	

[1] Considerar en todos los grupos de calidad al menos los géneros: *Ancylostoma, Trichuris y Ascaris*.

[2] Deben someterse a controles que aseguren el correcto mantenimiento de las instalaciones.

[3] Su autorización estará condicionada a la obligatoriedad de la presencia doble circuito señalizado en todos sus tramos hasta el punto de uso

[4] Unidades Formadoras de Colonias.

[5] Unidades Nefelométricas de Turbiedad.

[6] ver el Anexo II del RD 849/1986, de 11 de abril.

[7] ver Anexo IV del RD 907/2007, de 6 de julio.

[8] Norma de calidad ambiental ver el artículo 245.5.a del RD 849/1986, de 11 de abril, modificado por el RD 606/2003 de 23 de mayo.

[9] Cuando exista un uso con posibilidad de aerosolización del agua, es imprescindible seguir las condiciones de uso que señale, para cada caso, la autoridad sanitaria, sin las cuales, esos usos no serán autorizados

USO DEL AGUA PREVISTO	VALOR MÁXIMO ADMISIBLE (VMA)				
	NEMATODOS INTESTINALES	*ESCHERICHIA COLI*	SÓLIDOS EN SUSPENSIÓN	TURBIDEZ	OTROS CRITERIOS
2.- USOS AGRÍCOLAS[1]					
CALIDAD 2.1[2] a) Riego de cultivos con sistema de aplicación del agua que permita el contacto directo del agua regenerada con las partes comestibles para alimentación humana en fresco.	1 huevo/10 L	100 UFC/100 mL Teniendo en cuenta un plan de muestreo a 3 clases[3] con los siguientes valores: n = 10 m = 100 UFC/100 mL M = 1.000 UFC/100 mL c = 3	20 mg/L	10 UNT	OTROS CONTAMINANTES contenidos en la autorización de vertido de aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs. *Legionella spp.* 1.000 UFC/L (si existe riesgo de aerosolización) Es obligatorio llevar a cabo la detección de patógenos Presencia/Ausencia (Salmonella, etc.) cuando se repita habitualmente que c=3 para M=1.000

[1] Características del agua regenerada que requieren información adicional: Conductividad 3,0 dS/m ; Relación de Adsorción de Sodio (RAS): 6 meq/L; Boro: 0,5 mg/L; Arsénico: 0,1 mg/L; Berilio: 0,1 mg/L; Cadmio: 0,01 mg/L; Cobalto: 0,05 mg/L; Cromo: 0,1 mg/L; Cobre: 0,2 mg/L; Manganeso: 0,2 mg/L; Molibdeno: 0,01 mg/L; Níquel: 0,2 mg/L; Selenio : 0,02 mg/L; Vanadio: 0,1 mg/L..
Para el cálculo de RAS se utilizará la fórmula:

$$RAS(meq/L) = \frac{[Na]}{\sqrt{\frac{[Ca]+[Mg]}{2}}}$$

[2] Cuando exista un uso con posibilidad de aerosolización del agua, es imprescindible seguir las condiciones de uso que señale, para cada caso, la autoridad sanitaria, sin las cuales, esos usos no serán autorizados

[3] Siendo n: nº de unidades de la muestra; m: valor límite admisible para el recuento de bacterias; M: valor máximo permitido para el recuento de bacterias; c: número máximo de unidades de muestra cuyo número de bacterias se sitúa entre m y M.

USO DEL AGUA PREVISTO	VALOR MÁXIMO ADMISIBLE (VMA)				
	NEMATODOS INTESTINALES	*ESCHERICHIA COLI*	SÓLIDOS EN SUSPENSIÓN	TURBIDEZ	OTROS CRITERIOS
CALIDAD 2.2 a) Riego de productos para consumo humano con sistema de aplicación de agua que no evita el contacto directo del agua regenerada con las partes comestibles, pero el consumo no es en fresco sino con un tratamiento industrial posterior. b) Riego de pastos para consumo de animales productores de leche o carne. c) Acuicultura.	1 huevo/10 L	1.000 UFC/100 mL Teniendo en cuenta un plan de muestreo a 3 clases[1] con los siguientes valores: n = 10 m = 1.000 UFC/100 mL M = 10.000 UFC/100 mL c = 3	35 mg/L	No se fija límite	OTROS CONTAMINANTES contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs. *Taenia saginata y Taenia solium:* 1 huevo/L (si se riegan pastos para consumo de animales productores de carne) Es obligatorio llevar a cabo detección de patógenos Presencia/Ausencia (*Salmonella*, etc.) cuando se repita habitualmente que c=3 para M=10.000
CALIDAD 2.3 a) Riego localizado de cultivos leñosos que impida el contacto del agua regenerada con los frutos consumidos en la alimentación humana. b) Riego de cultivos de flores ornamentales, viveros, invernaderos sin contacto directo del agua regenerada con las producciones. c) Riego de cultivos industriales no alimentarios, viveros, forrajes ensilados, cereales y semillas oleaginosas.	1 huevo/10 L	10.000 UFC/100 mL	35 mg/L	No se fija límite	OTROS CONTAMINANTES contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs. *Legionella spp.* 100 UFC/L

[1] Siendo n: nº de unidades de la muestra; m: valor límite admisible para el recuento de bacterias; M: valor máximo permitido para el recuento de bacterias; c: número máximo de unidades de muestra cuyo número de bacterias se sitúa entre m y M.

USO DEL AGUA PREVISTO	VALOR MÁXIMO ADMISIBLE (VMA)				
	NEMATODOS INTESTINALES	*ESCHERICHIA COLI*	SÓLIDOS EN SUSPENSIÓN	TURBIDEZ	OTROS CRITERIOS
3.- USOS INDUSTRIALES					
CALIDAD 3.1[1] a) Aguas de proceso y limpieza excepto en la industria alimentaria. b) Otros usos industriales.	No se fija límite	10.000 UFC/100 mL	35 mg/L	15 UNT	OTROS CONTAMINANTES contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs *Legionella spp.:* 100 UFC/L
c) Aguas de proceso y limpieza para uso en la industria alimentaria	1 huevo/10 L	1.000 UFC/100 mL Teniendo en cuenta un plan de muestreo a 3 clases[2] con los siguientes valores: n = 10 m = 1.000 UFC/100 mL M = 10.000 UFC/100 mL c = 3	35 mg/L	No se fija límite	OTROS CONTAMINANTES contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs. *Legionella spp.*: 100 UFC/L Es obligatorio llevar a cabo detección de patógenos Presencia/Ausencia (*Salmonella*, etc.) cuando se repita habitualmente que c=3 para M=10.000
CALIDAD 3.2 a) Torres de refrigeración y condensadores evaporativos.	**1 huevo/10 L**	Ausencia UFC/100 mL	5 mg/L	1 UNT	*Legionella spp*: Ausencia UFC/L Para su autorización se requerirá: - La aprobación, por la autoridad sanitaria, del Programa especifico de control de las instalaciones contemplado en el Real Decreto 865/2003, de 4 de julio, por el que se establecen los criterios higiénico-sanitarios para la prevención y control de la legionelosis. - Uso exclusivamente industrial y en localizaciones que no estén ubicadas en zonas urbanas ni cerca de lugares con actividad pública o comercial.

[1] Cuando exista un uso con posibilidad de aerosolización del agua, es imprescindible seguir las condiciones de uso que señale, para cada caso, la autoridad sanitaria, sin las cuales, esos usos no serán autorizados

[2] Siendo n: nº de unidades de la muestra; m: valor límite admisible para el recuento de bacterias; M: valor máximo permitido para el recuento de bacterias; c: número máximo de unidades de muestra cuyo número de bacterias se sitúa entre m y M.

USO DEL AGUA PREVISTO	VALOR MÁXIMO ADMISIBLE (VMA)				
	NEMATODOS INTESTINALES	*ESCHERICHIA COLI*	SÓLIDOS EN SUSPENSIÓN	TURBIDEZ	OTROS CRITERIOS
4.- USOS RECREATIVOS					
CALIDAD 4.1[1] a) Riego de campos de golf.	1 huevo/10 L	200 UFC/100 mL	20 mg/L	10 UNT	OTROS CONTAMINANTES contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs. Si el riego se aplica directamente a la zona del suelo (goteo, microaspersión) se fijan los criterios del grupo de Calidad 2.3 *Legionella spp*. 100 UFC/L (si existe riesgo de aerosolización)
CALIDAD 4.2 a) Estanques, masas de agua y caudales circulantes ornamentales, en los que está impedido el acceso del público al agua.	No se fija límite	10.000 UFC/100 mL	35 mg/L	No se fija límite	OTROS CONTAMINANTES contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs. P_T : 2 mg P/L (en agua estancada)

[1] Cuando exista un uso con posibilidad de aerosolización del agua, es imprescindible seguir las condiciones de uso que señale, para cada caso, la autoridad sanitaria, sin las cuales, esos usos no serán autorizados

USO DEL AGUA PREVISTO	VALOR MÁXIMO ADMISIBLE (VMA)				
	NEMATODOS INTESTINALES	*ESCHERICHIA COLI*	SÓLIDOS EN SUSPENSIÓN	TURBIDEZ	OTROS CRITERIOS
5.- USOS AMBIENTALES					
CALIDAD 5.1 a) Recarga de acuíferos por percolación localizada a través del terreno.	No se fija límite	1.000 UFC/100 mL	35 mg/L	No se fija límite	N_T [1]: 10 mg N/L NO_3 : 25 mg NO_3/L Art. 257 a 259 del RD 849/1986
CALIDAD 5.2 a) Recarga de acuíferos por inyección directa.	1 huevo/10 L	0 UFC/100 mL	10 mg/L	2 UNT	
CALIDAD 5.3 a) Riego de bosques, zonas verdes y de otro tipo no accesibles al público. b) Silvicultura.	No se fija límite	No se fija límite	35 mg/L	No se fija límite	OTROS CONTAMINANTES contenidos en la autorización de vertido aguas residuales: se deberá limitar la entrada de estos contaminantes al medio ambiente. En el caso de que se trate de sustancias peligrosas deberá asegurarse el respeto de las NCAs.
CALIDAD 5.4 a) Otros usos ambientales (mantenimiento de humedales, caudales mínimos y similares).	La calidad mínima requerida se estudiará caso por caso				

[1] Nitrógeno total, suma del nitrógeno inorgánico y orgánico presente en la muestra