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"Monitoring of bacterial communities in a phytoremediation plant for the decontamination of polluted marine sediments"

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INDEX

Summary

This thesis was performed within the "Agriport" European Project (Agricultural Reuse of Polluted Dredged Sediments, Eco-innovation EU Project n. ECO/08/239065). The Agriport project aims at developing a new technology for treating polluted sediments dredged from the seabed of a commercial port through phytoremediation. Thanks to plant activities and microorganism metabolism, it is possible to recover dredged saline sediments by decontaminating and conditioning them until the obtainment of an artificially prepared soil that is reusable in the terrestrial environment. This is an important advantage from the environmental point of view, and allows to partially solve one of the main problems of most commercial ports, that is the accumulation, storage and disposal of polluted dredged sediments.

The study was divided into two main parts, involving the study of bacterial community in fresh marine sediments collected in the seabed of an industrial harbor, and the study of bacterial communities involved in the phytoremediation process of marine contaminated sediments.

During the first part of the study, an accurate analysis on bacterial communities composition in fresh marine sediments, directly collected by a scuba diver in five sites of Leghorn harbor was performed. Aim of this first part of the study was to assess the impact of pollution on seabed bacterial diversity, structure and activity in the Port of Leghorn. Seabed sediments of commercial ports are often characterized by high levels of pollution, mainly represented by organic matter, heavy metals and hydrocarbons. One of the main characteristic of harbor sites is the removal of organic matter. Consumption capacity of organic matter is mainly due to the activity of microorganisms. The knowledge of bacterial communities dynamics in fresh marine sediments allowed us to compare the data with the results obtained in the second part of the study, involving bacterial community dynamics in the phytoremediation plant, that were monitored for two years. The phytoremediation plant was made up of a sealed 80 m^3 basin that was filled with a mixture of dredged sediments (75%) and natural soil (25%). It was planted with three plant species, and has been properly cultivated with optimized fertilization and irrigation for two years.

The techniques developed and used in this thesis were Terminal Restriction Fragment Length Polymorphism (T-RFLP) followed by appropriate statistical analysis, and construction of 16S rRNA clone libraries.

Results of the first part of the study, focusing on freshly collected marine sediments, showed that the industrial harbor is mainly contaminated by variable levels of petroleum hydrocarbons and heavy metals, which affect the structure and activity of the bacterial population. A stimulatory effect of nutrients on biological activities and bacterial communities was clearly observed. A

stimulation of bacterial development driven by petroleum hydrocarbon and heavy metals was also detected, although with less evidence. Library data, phylogenetic analysis, and T-RFLP coupled with *in silico* digestion of the obtained sequences, evidenced the dominance of *Proteobacteria* and the high percentage of *Bacteroidetes* in all sites. The approach highlighted similar bacterial community among samples coming from the five sites, suggesting a modest differentiation among bacterial communities of different harbor seabed sediments, and hence the capacity of bacterial communities to adapt to different levels and types of pollution. The second part of the study, focusing on bacterial community dynamics in the phytoremediation plant, highlighted that, at the beginning of the experimentation (construction of the treatment basin), each component used to assemble the phytoremediation treatment basin was characterized by its own bacterial community which was differentiated from the others. Six months after the construction of the treatment basin the original bacterial communities evolved into a single bacterial community, homogeneously distributed in the whole area. The bacterial community got stabilized after one year from the construction of the treatment basin. The phytoremediation process influenced the development of a specific bacterial community of the treatment basin that is completely different from the bacterial communities harbored by the basin at the beginning of the experimentation.

Riassunto

Questa tesi è stata realizzata nell'ambito del progetto europeo "Agriport" (*Agricultural Reuse of Polluted Dredged Sediments*, Eco-innovation EU Project n. ECO/08/239065). Il progetto Agriport mira allo sviluppo di una nuova tecnologia per il trattamento dei sedimenti contaminati dragati dal fondale marino dei porti commerciali attraverso il processo di fitorimediazione. Grazie all'attività delle piante ed al metabolismo microbico, è possibile recuperare i sedimenti salini dragati decontaminandoli fino ad ottenere un suolo preparato artificialmente, che può essere riutilizzabile in ambiente terrestre. Questo rappresenta un vantaggio importante dal punto di vista ambientale e permette la parziale risoluzione di uno dei principali problemi dei porti commerciali che è lo stoccaggio, il trattamento e lo smaltimento dei sedimenti di dragaggio contaminati.

Lo studio è stato suddiviso in due parti principali, riguardanti lo studio delle comunità batteriche nei sedimenti marini freschi raccolti sul fondale di un porto commerciale, e lo studio delle comunità batteriche coinvolte nel processo di fitorimediazione dei sedimenti marini contaminati.

Durante la prima parte dello studio è stata effettuata un'accurata analisi sulla composizione delle comunità batteriche nei sedimenti marini freschi, direttamente prelevati da un sommozzatore in cinque siti del porto di Livorno. Scopo di questa prima parte dello studio è stato di valutare l'impatto dell'inquinamento sulla diversità, sulla struttura e sull'attività dei batteri del fondale del porto di Livorno. I sedimenti marini nei porti commerciali sono spesso caratterizzati da elevati livelli di inquinamento, rappresentati soprattutto da sostanza organica, metalli pesanti ed idrocarburi. Tra le principali caratteristiche dei porti vi è la rimozione della sostanza organica. La capacità di rimozione della sostanza organica è dovuta principalmente all'attività dei microrganismi. La conoscenza delle dinamiche delle comunità batteriche nei sedimenti marini freschi ha permesso il confronto dei dati con i risultati ottenuti nella seconda parte dello studio, che ha coinvolto le dinamiche delle comunità batteriche nell'impianto di fitorimediazione, monitorate per due anni. L'impianto di fitorimediazione è costituito da un bacino di 80 m^3 riempito con una miscela di sedimenti dragati asciutti (75%) e suolo inerte (25%). L'impianto è stato piantumato con tre specie vegetali, ed è stato appropriatamente fertilizzato ed irrigato per due anni. Le tecniche sviluppate ed utilizzate per questa tesi sono state la *Terminal Restriction Fragment Length Polymorphism* (T-RFLP) seguita da analisi statistiche multivariate, e la costruzione di *libraries* di 16S rRNA.

I risultati della prima parte dello studio sui sedimenti marini freschi hanno mostrato che il porto industriale è contaminato soprattutto da livelli variabili di idrocarburi e metalli pesanti, i quali

hanno un effetto sulla struttura e sull'attività delle popolazioni batteriche. È stato chiaramente osservato un effetto stimolatorio dei nutrienti sull'attività biologica e sulle comunità batteriche. Un simile effetto è stato osservato anche da parte degli idrocarburi e dei metalli pesanti, anche se meno evidente. I dati ottenuti dalla costruzione delle *libraries*, dalle analisi filogenetiche e dalla T-RFLP associata alla digestione *in silico* delle sequenze ottenute, hanno evidenziato una dominanza di Proteobatteri ed un'elevata percentuale di batteri appartenenti al *phylum Bacteroidetes* in tutti i siti. L'approccio ha evidenziato comunità batteriche simili tra campioni prelevati nei cinque siti, suggerendo una modesta differenziazione tra comunità batteriche dei sedimenti prelevati in diversi punti del fondale del porto, e dunque la loro capacità di adattarsi a diversi livelli e tipologie di inquinamento. La seconda parte dello studio, focalizzata sulle dinamiche delle comunità batteriche all'interno dell'impianto di fitorimediazione, ha evidenziato che all'inizio della sperimentazione (allestimento dell'impianto sperimentale), ogni componente utilizzata per assemblare l'impianto era caratterizzata da una propria comunità batterica differenziata da tutte le altre. Dopo sei mesi dalla costruzione dell'impianto, le comunità batteriche originali si sono evolute in un'unica comunità batterica distribuita omogeneamente in tutto il bacino di trattamento. La comunità batterica si è stabilizzata dopo un anno dalla costruzione dell'impianto. Il processo di fitorimediazione ha influenzato lo sviluppo di una comunità batterica specifica all'interno dell'impianto di trattamento, la quale si è completamente differenziata dalle comunità batteriche che si trovavano all'interno del bacino all'inizio della sperimentazione.

1 Introduction: an overview on the topic

Human activities impact upon the northern Mediterranean Sea with the consequent severe pollution of the coastal seawater environment. The biotope which is most exposed to the effects of this kind of pollution is the seabed of commercial ports; this is due to the fact that seabed sediments receive and store a wide range of polluting agents derived from commercial, industrial and leisure activities (losses or leaks from the handling of loose or liquid goods, exhausts of combustion for maritime propulsion, unauthorized sea disposal of waste and sewage, residues of paint used on ships and surface treatments).

Once sediments are contaminated, they act as a long-term source of pollutants and they alter the aquatic ecosystem affecting both diversity and function (e.g. Hollert et al., 2003).

The need to safeguard the environment is leading to stricter legislation in northern Mediterranean countries, and requires new technologies, methods and techniques in order to monitor and reclaim the contaminated sites. New methods and techniques are needed more urgently in those sites in which dredging procedures are regularly applied to maintain suitable sea levels for navigation (SedNet, 2004).

Rivers and harbor docks are regularly dredged in order to ensure shipping traffic efficiency. This activity produces large volumes of sediments that show significant levels of contamination mainly due to hydrocarbons and heavy metals, thus hindering their disposal in the sea (Art. 109 of D.lgs 152/06) according to Italian legislature. The contamination of sediments dredged from rivers and coastal harbors represents a quantitative and economical problem (Mulligan et al., 2001, Lors et al., 2004, Meers et al., 2003). It is estimated that every year, in Europe, 100–200 million m^3 of sediments are dredged (Bortone et al., 2004), thus focusing on the increasing need to find locations in which remediation can be performed, and on the choice of measures aimed at minimizing the risk of further contamination of sediments. In Italy, high levels of pollution in many commercial and tourist ports are considered a threat to the stability and functionality of ecosystems (Pellegrini et al., 1999).

The Port of Leghorn, including the sea stretch of its harbor up to the border of the Meloria marine park, is included in the Italian list of 54 nationally relevant polluted sites. Here, the Port Authority has adopted for the management of dredged sediments, the storage of the dredged material in a storage basin obtained from a portion of the sea that was marked off and sealed with a

water-proof liner. After the complete filling of the basin, the Port Authority will provide for the construction of further basins thus expanding its surface land in the sea. The Leghorn Port Authority policy is an economic solution and represents a way to expand the port area, but it can't represent a universal solution for polluted sediments that, in most cases, should be decontaminated.

Looking to international scientific literature, the main processes currently applied to treat contaminated sediments with the aim of reusing them, are sediment washing (a physical-chemical treatment that separates and recovers cleaned sand and gravel), biological decontamination (e.g. land farming), stabilization with ash, lime, silicates or cement, and transformation into reusable soil (Bianchi, 2010). In all cases, the choice of treatment depends on three main parameters: the particle size of the sediments, the type and concentration of the contaminants and the amount of sediments to treat.

Phytoremediation is a technique that was successfully applied for the decontamination of soils in past years (e.g. Weyens et al., 2008; Vangronsveld et al., 2008), and that may also play a central role in the decontamination of polluted sediments dredged from harbors.

This thesis was developed within the framework of the AGRIPORT project (Agricultural Reuse of Polluted Dredged Sediments, Eco-innovation EU Project n. ECO/08/239065) promoting an innovative approach to decontaminate dredging sediments using phyto-treatment. The phytoremediation treatment performed by plants and microorganisms that are present in soil, allows for the transformation of sediments into fertile and nutrient rich soil, and removes salts and pollutants (Bianchi et al., 2010); microorganisms are also very efficient in the degradation of hydrocarbons (Marin et al., 2005). The phytoremediation process decontaminates polluted dredged sediments and turns them into arable land, or soil which is suitable for other uses (landscaping, environmental restoration, gardening, capping of landfills etc.) (Dickinson NM, 2000).

In the phytoremediation process of marine polluted dredged sediments, a key role is attributed to plant species, which must show specific characteristics such as tolerance to a high level of salinity and excessive presence of fines in the composition of such sediments. In order to allow the growth of plants in the phytoremediation process of marine sediments, the dredged material must be mixed with inert material beforehand, such as agronomic soil and/or organic substances, to improve the overall physical and chemical characteristics of the "mixture". The AGRIPORT project intends to demonstrate and commercialize a simple and low cost technology for transforming slightly

polluted saline sediments into soil that can be re-used for agronomic and environmental applications.

1.1 The normative concerning dredged sediments

The main reasons why sediments need to be dredged are the maintenance of the depth in navigation channels, the need to make infrastructural works in port areas, and the requirement to lay cables and pipelines. The management of dredged sediments is very difficult from an environmental, socio economical and legislative point of view.

1.1.1 The European scenario

Among the legislatures of all European countries, there are no specific international or regional conventions on dredged material and there are no specific directives. The conventions that already exist are focused on the prevention of the impact of dumping activities on the marine environment, while the European Community Directives are related to water, soil, waste and landfill. The fact that the legislation on dredged sediments is a patchwork of different regulations makes it very complex and confusing, and liable to incorrect interpretation.

Here follows a list of the main conventions focused on marine environmental pollution matters that have been enacted by the European countries; in some cases, the problem of the management of the contaminated dredged material is faced:

- London Convention (adoption 1972, in force 1975): ***Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter;*** it represents an international regulation where the dumping of hazardous material is prohibited unless they are present as trace contaminants; dumping of other material is permitted with only general or special permits.

- London Protocol (adoption 1996, in force 2006) ***Protocol to the Convention on the Prevention of Marine Pollution by Dumping of Wastes and Other Matter, 1972*****;** it represents an international regulation asserting that all dumping is prohibited (with a few exceptions listed in a "reverse list" and requiring a permit), incineration of wastes and other matter is prohibited at sea, and brought the adoption of the *Dredged Material Assessment Framework* (DMAF), a widely accepted approach to the assessment of suitability of dredged material for sea disposal.

- Barcelona Convention (sign 1976, in force 1978; rev. 1995, in force 2004) Convention for the ***Protection of the Marine Environment and the Coastal Region of the Mediterranean;*** a regional convention to prevent and reduce pollution in the Mediterranean Sea. The "dumping Protocol" prohibited dumping of all waste and matter with exceptions listed in Art. 4.2. Concerning dredged sediments, the Barcelona Convention assesses that dredged material may be dumped at sea but requires a special permit also based on a detailed classification; the main goal is to eliminate pollution to the fullest possible extent and the contracting parties have to carry out measures to ensure the conservation of endangered or threatened species of flora and fauna.

- Water Framework Directive (WFD) (Directive 2000/60/EC of the European Parliament and of the Council of 23rd October 2000): protection of all waters and protection and enhancement of the status of aquatic ecosystems. Concerning dredged sediments: Member States are required by law to submit proposals for quality standards applicable to the concentration of the main substances in surface water, sediments or biota: In Italy, the quality standards for sediments in marine, coastal and transitional water bodies are defined by the Decree n.56/2009 of the Italian Ministry of the Environment.

- Marine Strategy Framework Directive (MSFD) (Directive 2008/56/EC of the European Parliament and of the Council of 17th June 2008): establishes a framework within which Member States shall take the necessary measures to achieve or maintain a good environmental status in the marine environment by the year 2020 at the latest, to protect and preserve the marine environment, prevent its deterioration or restore marine ecosystems where they have been adversely affected.

- Birds and Habitats Directives (Directive 2009/147/EC: Council Directive 79/409/EEC of 2 April 1979 on the conservation of wild birds and amendments; Council Directive 92/43/EEC of 21st May 1992 on the conservation of natural Habitats and of wild fauna): protection of wild bird species and sites of importance for the maintenance of populations of wild birds, protection of natural habitats, wild fauna and flora. Concerning dredged sediments: coastal ports and harbors are often located close to "Natura 2000" sites that are protected; for this reason, dredging and sediment disposal may have a negative impact on the habitats and species. This results in restrictions to dredging permits to undertake works in proximity of the protected areas.

- Waste directive (Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives); dredged sediments are linked to waste because it is not yet clear if they should be considered waste or not waste. Art. 2.3 of the Directive assesses that "Sediments relocated inside surface waters are excluded from the scope of the Waste Directive when they are not hazardous and when they are relocated for the purpose of: managing waters and waterways, preventing floods, mitigating the effects of floods and droughts, land reclamation".

- Landfill Directive (Directive 1999/31/EC of 26th April 1999); it has a limited impact on the disposal of dredged material; it recognizes that the disposal of dredged material along waterways on agricultural land or at suitable subaquatic locations are acceptable solutions, but concentrations of contaminants must remain below certain limits.

1.1.2 The Italian scenario

Italy has a large coastal development and the dredging of sediments from harbors represents an issue of great social and economic relevance. Moreover, coastal areas are rich of valuable and sensitive ecosystems and there are several uses of marine resources to be protected (e.g. aquaculture plants, fisheries, bathing). In most cases, the big port areas are located close to industrial sites, where heavily impacting activities are located. Consequently, the adjacent marine areas are usually heavily contaminated. Italian Government has allocated specific resources for remediation; a list and a brief description of the main actions provided by the Italian Government in the field of management of polluted dredged marine sediments follows:

- **Decree of the Ministry of Environment, 24th January 1996**: describes the procedures to obtain the authorization for dumping into the sea, or into adjacent environments, the materials resulting from the excavation of marine or brackish-water beds or coastal terrains. It also applies to all sediment moving practices in the marine environment.

- **Decree of the Ministry of Environment 5th February 1998:** it is the only existing legislation for inland reuse of dredged material. It is unfortunately only related to inland waters. It states that non hazardous dredging spoil (with specific physical and chemical characteristics) resulting from dredging of lake beds, waterways or irrigation channels or watercourses (inland waters) and from cleaning of water reservoirs, can be recovered after the execution of the leaching test, after drying and, if necessary, sanitation treatment, as

follows: construction of road embankments and pavements; construction of embankments and dams with no direct or indirect contact with the marine environment; profiling of parts of the riverbed morphometry; "environmental restoration" when the material is compatible with the physical, chemical, hydrogeological and morphological area characteristics and concentration of contaminants are compatible with levels of current legislation for contaminated sites.

- **Art. 35 of the Legislative Decree n.152/1999, later regulated by Art. 109 of the Legislative Decree n.152/2006:** it states that the authorization for sea dumping of material dredged from marine or brackish-water beds or coastal terrains can be obtained only when it is demonstrated that it cannot be used, due to technical or economic reasons, for beach nourishment, or recovered or otherwise disposed.

- **Art. 21 of the Law 179/2002:** the Regions are the competent authorities for the release of the authorization for interventions for costal protection (beach nourishment and disposal of material resulting from the excavation of marine or brackish-water beds or coastal terrains into coastal confined disposal facilities or containment basins and structures).

- **Art.1, par. 996 of Law 296/2006 and its implementing decree (Decree of the Italian Ministry of the Environment 7th November 2008):** it has modified Law n. 84 of 28th January 1994 on "reorganization of port legislation" by defining some criteria to perform dredging activities and to manage dredged sediments in port areas located inside the Contaminated Sites of National Relevance.

- **Manual for the handling of marine sediments (2007):** it is a technical document that provides criteria for the whole sediment dredging process, from the characterization to the final sediment destination; criteria are given to identify different sediment quality classes and to select the proper management option depending on the sediment quality class.

- **Ministerial Decree "Ambiente" of the 7/11/2008:** deals with operations of dredging in sites of national interest, considering the entire procedure from the dredging project to the destination of recovered material. Here, criteria and methods are evaluated to classify harbor sediments that have to be excavated and specific techniques for the analysis of sanitary and environmental risk.

- **Art. 48 of Legislative Decree n. 1 of 24/01/2012 as converted into law with amendments by Law n. 27 of 24th March 2012**: it modified the Law n. 84 of 28/01/1994 concerning "Provisions related to dredging" session, which provides criteria to perform dredging activities in port and marine areas and for the management options for dredged sediments. Attention is also given to ports not located within the Contaminated Sites of National Relevance: for these ports the possible management options are summarized, without providing any requirements. Some management options, already adopted occasionally but not yet officially mentioned, are outlined.

- **Art. 24 of the Legislative Decree n. 5 of 9th February 2012 as converted into law by Law n. 35 of 4th April 2012**, which modifies Art. 109 of the Legislative Decree n.152/2006: it established that the competent body to authorize all possible management options for sediments dredged in a port not located inside a Contaminated Sites of National Relevance is the Region (with the exception of sea dumping for sea dumping inside the Italian marine protected areas, whose authorization is still released by the Ministry of the Environment).

In 2012, Law 296/2006 has been also modified, leaving the implementing decree **(Decree of the Italian Ministry of the Environment 7th November 2008)** untouched.

The main objective of port authorities on dredged sediment management is their transformation into a material that can be used for environmental recovery purposes. In this context, the transport of decontaminated sediments from inside the port area to the final location is very important. During the last decades, the normative did not allow this possibility; there was a specific legislature about sediments that exempted them from being considered waste and allowed their transport in a simple and fast way only within the port area. This represented a strong limit, which has been exceeded with the 2012 legislature. From the legislature approved during 2012, it emerged that if dredged sediments located inside a port are already uncontaminated, they can be considered suitable for their transport outside the port; if, conversely, they need to be treated, it is not yet allowed their transport in a simple and fast way outside the port area. In this last case, it would be necessary to consider them as "waste" and the storage basin in the port area where dredged sediments are located, has to be considered an authorized platform for the waste treatment. After the treatment, decontaminated sediments can be transported outside the port area and reused, but just only after a complex bureaucratic procedure that port authorities have to face.

1.2 The problem of dredged sediments

The term sediment refers to a mixture of heterogeneous solid particles left on the bottom of a water body. Each particle may contain different components, depending on its formation conditions (the type of geologic material of origin, alterations due to anthropic activities, duration of alteration processes) but in general, it is always possible to find peptides and ulmic material (Giordano, 2003). A sediment particle usually consists of an inorganic fraction (silicates, carbonates, phosphates, sulphides, manganese and iron oxides) and an organic fraction (living organisms, natural organic substance etc.). The Environmental Protection Agency defines contaminated sediments as "*Aquatic sediments containing chemical substances that exceed geochemical and toxicological measures, or the quality criteria of sediments, or that are considered dangerous for human health and for the environment*". For sediments coming from coastal areas or from harbors, anthropic activities are fundamental for their quality. Moreover, internal harbor structures such as vertical walls, berths and docks, may cause stream decelerations at the harbor entrance, thus causing the formation of zones where the water flux is calm and there are no turbulences in the internal sub areas, with the consequent sedimentation of particles. This causes the periodical necessity to undertake dredging activities (Pellegrini et al, 2002). Because of the huge amount of sediments that are periodically dredged from coastal and harbor areas, the condition and destination of marine sediments has acquired national and international relevance. The awareness of the contamination of dredged marine sediments, and of the necessity to decontaminate them before disposal, has shown a need to research solutions different to those of depositing sediments in the sea. (ICRAM, 2002).

1.2.1 Types and sources of pollutants in marine sediments

Among the main categories of marine pollutants there are aromatic polycyclic hydrocarbons, pesticides, heavy metals and nutrients. The concentration of a pollutant substance is mainly due to the particle dimensions: heavy metals are more concentrated in sediments composed of particles with very low dimensions because of their greater specific available surface. Moreover, clay particles, the main component of sediments, are characterized by many binding sites for heavy metals, making the absorption phenomena easier (Campbell et al., 1988). The causes of pollution of marine sediments in a harbor are mainly due to the variety of activities inside the harbor area. Other causes of pollution must also be addressed in the entry of water from surface water bodies (such as rivers that collect all pollutants from industrialized areas during their course), or for example from pelagic sources. The latter are mainly due to marine traffic, or ships and tankers depositing waste

water into the sea. (Giordano, 2003). Heavy metals have risen in their concentration in a short space of time in big cities and industrialized areas. The main sources of heavy metals pollution in the marine environment are rivers, atmospheric deposits and antrophic contributions. Even if heavy metals are much diluted in the aqueous phase, they can accumulate in the sediments and reach higher concentrations than the upper water column. Among organic compounds in the marine environment, hydrocarbons represent one of the main causes of pollution. Hydrocarbons are divided into aromatic (containing one or more benzene molecule) and aliphatic (divided into alkanes, alkenes and alkynes). The main pollution source for marine water is represented by the washing activity of ships water tanks, and from accidents involving oil tankers (Rowe et al., 1986).

1.2.2 How to treat polluted sediments

The existing techniques of treating sediments derive from the methods used to treat contaminated soil matrices, and can be divided into two main groups:

In situ techniques: removal or stabilization of pollutants in order to avoid environmental risks without moving the material from the original location;

Ex situ techniques: the polluted material is removed from the site and brought to a special treatment plant. Sometimes the treatment plant is constructed close to the site that must be treated, (in this case the technique is named *ex situ-on site*); otherwise, the material which is to be decontaminated is transported to a plant located far from the contaminated site (in this case the technique is named *ex situ-off site*).

In both cases, both chemical methods and biological methods can be chosen (Concas et al., 2006):

***In situ* techniques**	
Chemical methods	**Biological methods**
Soil flushing	Bioventing
Soil venting	Phytoremediation
Mobilization	Use of filamentous fungi
Thermally enhanced recovery	
Electrokinesis	
Vitrification	

***Ex situ* techniques**	
Chemical methods	**Biological methods**
Soil washing	Composting
Thermo-disruption	Landfarming
Mobilization	Bioreactors
Chemical Oxidation	
Electrokinesis	
Thermally enhanced recovery	
Dehalogenation	
Vitrification	

Table1: list of main methods for the reclamation of contaminated sites.

1.2.2.1 Chemical treatments:

Soil flushing: *in situ* washing of soil/sediments through water or solutions able to extract and mobilize the contaminants that are present in the matrices.

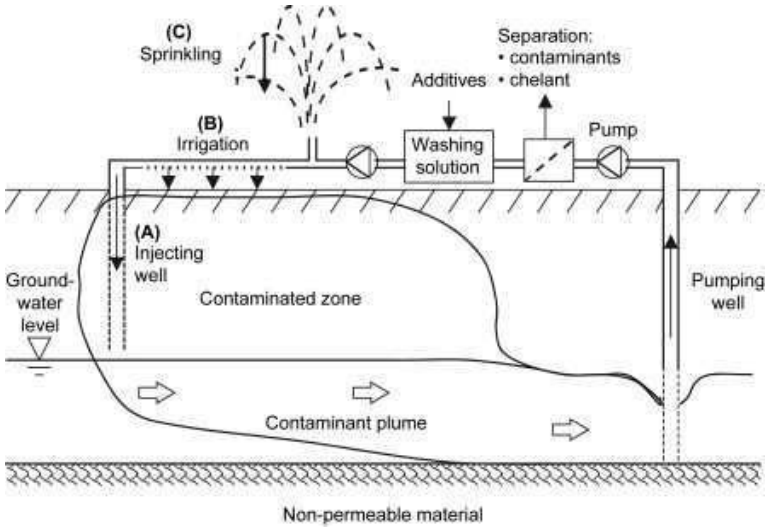

Figure1: soil flushing schematic representation from Lestan et al., 2008.

Soil venting: *in situ* technique used for the removal of volatile organic compounds (VOC) from unsaturated soil/sediments. The technique consists of applying a depression in the contaminated area, in order to make the contaminants volatile and catch them.

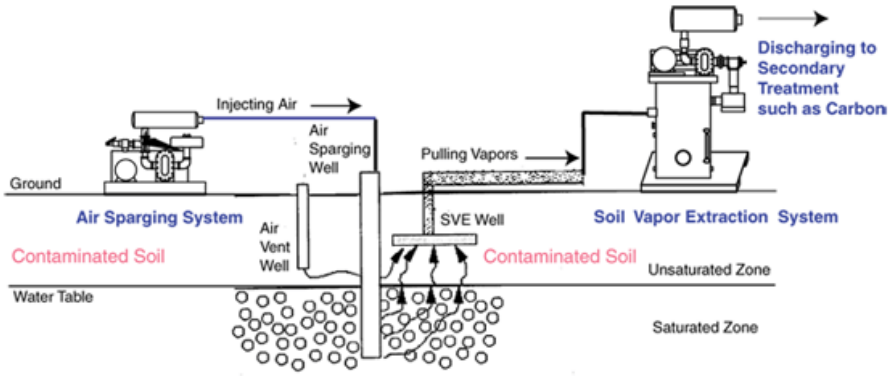

Figure2: Soil venting process example from http://www.fliteway.com/whatissoilremediation.html.

Thermally enhanced recovery: *in situ* increase of the soil/sediment temperature in order to promote volatilization of contaminants and allow their capture with aspiration systems that are analogous to those used for soil venting.

Mobilization (*in situ-ex situ*): inhibition of the motility of the contaminant and reduction of the contact surface between leachate and contaminant.

Vitrification (*in situ-ex situ*): heating of soil/sediment until 1600 –2000 °C is reached in order to fuse the matrix, followed by a rapid cooling. The result is the formation of a crystal able to capture and trap contaminants.

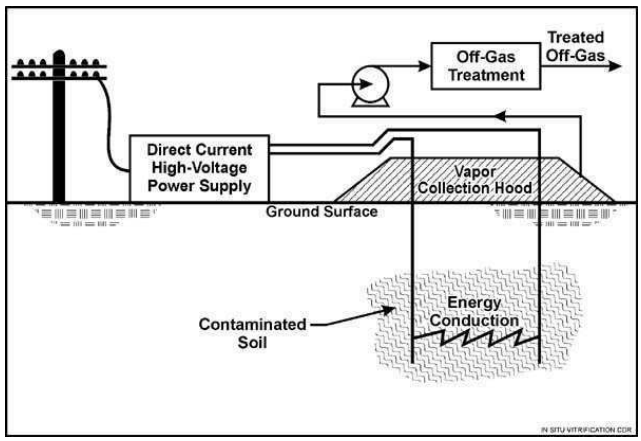

Figure3: vitrification process from https://portal.navfac.navy.mil/portal/page/portal/NAVFAC/NAVFAC_WW_PP/NAVFAC_NFESC_PP/ENVIRONMENTAL/ERB/VITR-INSITU/.

Electrokinesis (*in situ-ex situ*): the passage of the current causes the mobilization of the chemical species with a charge; for example metallic ions, ammonium ions and organic compounds with a positive charge move to the cathode, while chloride fluorine, nitrates ions and organic compounds with negative charge move to the anode.

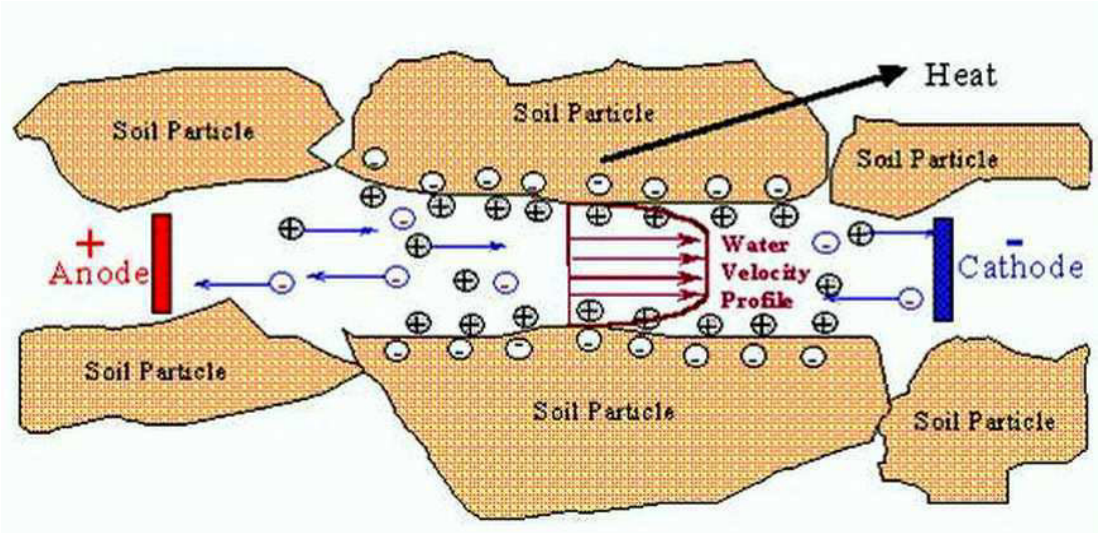

Figure4: electrokinesis process in soil/sediment remediation from http://alianwar.weebly.com/electrokinetic.html.

Soil washing: *ex situ* practice that consists in the same procedure as the previously described soil flushing, but the material is excavated, removed and brought in suitable plants to be treated away from the contaminated site.

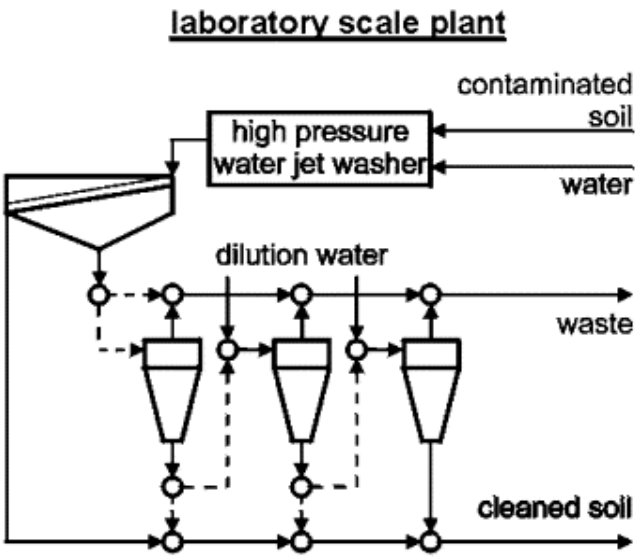

Figure5: example of soil washing plant from Gruhn et al., 2004.

Dehalogenation: *ex situ* practice that consists of a group of chemical reactions that allow contaminated soils/sediments to be treated containing aromatic halogenated compounds; the carbonium-halogen linkage breaks in the molecule.

1.2.2.2 Biological treatments:

Bioventing (*in situ*): it consists of the stimulation of the metabolism of aerobic autochthonous microorganisms that are able to degrade the contaminants present in the matrix using air as a vector to provide oxygen for the microorganisms, especially in the unsaturated part of the matrix.

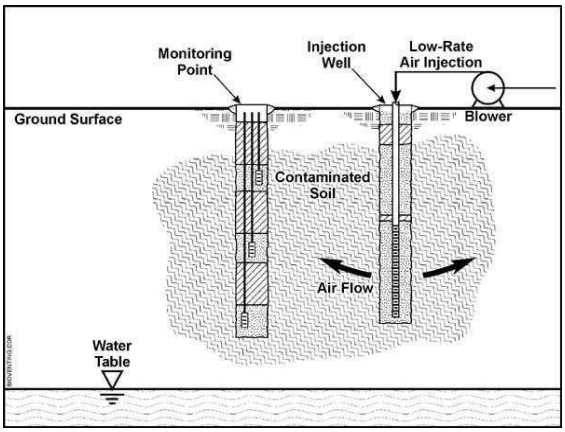

Figure 6: bioventing process scheme from https://portal.navfac.navy.mil/portal/page/portal/NAVFAC/NAVFAC_WW_PP/NAVFAC_NFESC_PP/ENVIRONMENTAL/ERB/BIOVENT/.

Landfarming (*ex situ*): this technique consists of the enhancement of aerobic metabolic processes performed by bacteria (autochthonous or inoculated during the process) trying to guarantee the optimal oxygen exposition and the operational conditions in terms of nutrients, temperature and moisture.

Composting: it is very similar to landfarming but the differences are that oxygen is provided in higher amounts in conjunction with other methods (e.g. blowing), and that the material being treated is accumulated in order to reduce the surface which is to be treated.

Bioreactors: with the use of this technique, the objective is to guarantee and optimize the operational conditions that are necessary for biodegradation of contaminants; the whole process is performed in reactors that allow the complete control of the whole process.

The phytoremediation technique is more closely considered in paragraph 1.3.

1.3 Phytoremediation

The term phytoremediation is used to describe the use of plants to remediate, partially or substantially, a selected contaminant in soil, sludge, sediment, ground, surface water and wastewater. Phytoremediation implies the use of both different biological processes occurring in plants and physical characteristics of plants to make the *in situ* remediation. Processes occur in different degrees and proportions depending on the characteristics of the environment, contaminants and plants, during the whole process. The process can potentially be applied to a huge variety of contaminants, including the most significant, such as petroleum hydrocarbons, chlorinated solvents, metals, radionuclides, nutrients, pentachlorophenol (PCP), and polycyclic aromatic hydrocarbons (PAHs). Phytoremediation includes different methods that lead to different results:

- ❖ Degradation (to disrupt or alter organic contaminants)
  - Rhizodegradation: occurs in the root zone thanks to microorganism activity;
  - Phytodegradation: occurs above or below ground, within the root, stem, or leaves.
- ❖ Accumulation (to contain or remove organic and/or metal contaminants)
  - Phytoextraction: contaminant uptake and accumulation of contaminant to remove it;
  - Rhizofiltration: adsorption of contaminants in plant roots to contain and/or remove them.
- ❖ Dissipation (to remove organic and/or inorganic contaminants and release them into the atmosphere)
  - Phytovolatilization: contaminant uptake and volatilization.
- ❖ Immobilization (to contain organic and/or inorganic contaminants)
  - Hydraulic Control: control of ground-water flow through plant uptake of water;

- Phytostabilization: contaminant immobilization in the soil.

For more details about these different forms of phyoremediation see Pivetz, 2001.

To treat contaminated soil, sediments and sludge, the most suitable methods are phytoextraction, phytostabilization, rhizodegradation, phytodegradation, phytovolatilization, or vegetative cap applications. Phytoremediation gives better results in large areas with a thin surface layer of contaminated soil, within the root depth of the selected plant. To treat contamination at a deeper level, high concentrations of contaminants, and even small volumes of contaminated soil, conventional technologies are recommended.

Phytoremediation technologies show both advantages and disadvantages; the main advantages can be resumed in five points:

- Less impact on the environment;
- Simplest technology if compared with other methods;
- Applicable *in situ* to remediate superficial soil, ground water and surface water bodies;
- No destructive impact on the fertilization of soil and on the structure;
- Prevention of erosion and dust emissions.

The foremost disadvantages of the phytoremediation process are both the limitation in depth due to the fact that roots can reach just superficial levels, and the long time period required for the remediation process, in some cases even decades. Moreover, plant efficiency in performing remediation processes can decrease in winter or if some damage occurs, such as specific plant diseases. It is important to remember that the correct choice of plant species is necessary to avoid an adverse impact on the environment such as the uncontrolled growth of invasive plants with the consequent elimination of local species and negative impacts on animals; the efficiency of the whole process also depends on the choice of plant because the performance of different species varies significantly in the phytoremediation processes, also depending on the kind of contamination, on the nature of soil and on environmental characteristics (e.g. weather conditions, latitude etc.).

1.4 Aim of the study

Bacterial communities in contaminated marine sediments are usually studied to detect particular bacterial groups, such as bacteria which are resistant to heavy metals (Toes et al., 2008), or fecal bacteria (Luna et al., 2012); other studies focus on testing the effect of some contaminants in the composition of bacterial communities (Krumins et al., 2009). The monitoring of bacterial communities in dredged sediments is less common, and only few papers are available in scientific literature (e.g. Baniulyte et al., 2009). When sediments are transferred from an aquatic ecosystem to a terrestrial environment, the equilibrium of the different habitats may change drastically, especially considering the physical and chemical characteristics of the site, and the water content of the soil. This last factor alters the microbial community's composition of the soil (Bossio and Scow, 1998, Drenovsky et al., 2004, Jaatinen et al., 2007, Rees et al., 2006). The bacterial communities which are characteristic of a terrestrial environment are completely different from the ones of the marine sediments (Stephen et al., 1996) and this is why it is necessary to monitor the composition of bacterial communities both in contaminated sea sediments and in sediments placed in a terrestrial environment after dredging.

The aim of this thesis was the monitoring of spatial and temporal variation of the composition of bacterial communities that were present in fresh sea sediments of five sites within the Leghorn harbor area, and in the phytoremediation plant, which was constructed for the AGRIPORT project and filled with sediments dredged from Leghorn harbor seabed. The total duration of the experimentation was three years; it started in May 2009 and ended in May 2012. The molecular techniques used to perform the study were the Terminal Restriction Fragment Length Polymorphism (T-RFLP, Liu et al., 1997) on the 16S rRNA gene, followed by multivariate statistical analysis on obtained data, and the construction of 16S rRNA gene libraries (Amann et al., 1995).

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2 Materials and Methods used to study bacterial communities

2.1 Biomolecular Analysis to study microbial ecology

(van Elsas J.D. and Boersma F.G.H., 2011, Christen, R., 2008)

The analysis of microbial populations in natural habitats is one of the main topics of current research about the functioning of natural ecosystems. Traditional microbiological approaches focus on the analysis of material derived from microbial growth, (e.g., liquid cultures of cells, colonies obtained by plating in solid media). These methods show strong limitations; in fact only a small fraction (~1%) of the microbiota in the environment can be accessed on the basis of cultivation (e.g. Staley & Konopka, 1985, Amann et al., 1995, Hugenholtz et al., 1998, Eilers et al., 2000, Kellenberger 2001, Kennedy et al., 2008). Other methods that derive from microbiology, cellular biochemistry, molecular biology (DNA or RNA-based) and physiology have also been traditionally used and evaluated as much accurate in the aim of understanding the complexity of microbial communities composition in different environments. The majority of currently used methods are based on the isolation of microbial DNA or RNA directly from samples (e.g. Inceoglu et al., 2010). Such approaches should be performed, coupled with the aforementioned cultivation-based approaches, in a polyphasic approach, thus providing for a complete set of information from a single sample. The molecular approaches are divided into three main types; the first one is based on the sequencing of a particular gene obtained through the amplification step of the Polymerase Chain Reaction (PCR), that allows to identify the organisms that were present in the environmental samples, and comparing the obtained sequences with the ones present in public databases; the second kind of approach is the “fingerprinting techniques” that represent the best compromise to compare microbial assemblages and assess their temporal and spatial changes (Casamayor et al., 2002); the last one comprehend the metagenomic approach (not used in this thesis), that is the study of the genomes of microorganisms (as opposed to clonal cultures). The metagenomic technique is to clone DNA in large fragments directly from the microorganism's environment into a culturable host and conduct a sequence-based and functional genomic analysis on it. The choice of the appropriate method depends mainly on the aim of the study and the amount of samples to be processed within a reasonable period of time. A table with information about the main cultivation methods and molecular techniques to study bacterial communities follows (van Elsas J.D. and Boersma F.G.H., 2011), followed by some accurate descriptions of the main approaches used in this thesis.

Method	Advantages	Disadvantages	Major pitfalls	Remarks
Cultivation (plates)	Allows to further analyze colonies including metabolism or whole genome sequence	Low resolution. Morphotypes hard to distinguish.	Only for cultivable microorganisms (~1% of community).	Key support for molecularly-based observations
Nucleic acid extraction	Easy access to genes of organisms	Prone to incomplete and biased sampling	Chemical integrity and purity of DNA may limit analyses	Nucleic acids as the basis of all molecular work: biases need to be reduced
PCR/qPCR	Routine techniques of high sensitivity; used for detection and/or quantification	Several PCR biases and artifacts, including inhibition (Kanagawa, T., 2003)	Only species >0.1-1% abundance are visible	Key method for molecular detection from environmental samples
Fingerprinting: DGGE (Muyzer et al., 1993; Ferris et al., 1996), TGGE, T-RFLP (Liu et al., 1997), SSCP (Orita et al., 1989), RISA (Borneman, J. et al., 1997), LH-PCR (Suzuki et al., 1998)	Easy comparisons between samples, possibility of obtaining different fingerprints from same sample	Only the most represented organisms of a community are identified.	Several pitfalls due to nature of separation techniques	Care to be taken with the interpretations due to biases
Clone libraries (full cycle rRNA approach, Amann et al., 1995)	Easy census of target genes in community; allows diversity estimates	Laborious preparation of sample	Pitfalls due to cloning bias	Nice but limited overview of target gene/organism diversity
Stable isotope probing and BrdU	Gives information on the active community. Relation between structure and function can be elucidated	Problems of opportunists blurring the data	Relies on activity of microorganisms, which can be very low	Widely appreciated method to describe in situ activities
Microarrays (Maskos, U.; and Southern, E.M., 1992).	Currently very high throughput, direct information on sequences. Sensitive	Only chipped genes are found	Problems due to cross-hybridizations with low-homology sequences	Allows high-throughput analyses across habitats
High-throughput sequencing: - pyrosequencing of amplified gene fragments -metagenome -metatranscriptome	All-in-once analysis in high-throughput. High potential for comparative studies	Methods are error-prone and costs are high.	Wrong interpretations due to artifacts/error	Method of choice in many studies. Caution with interpretation of data

Table 2: list and characteristics of the main diffused cultivation dependent and cultivation independent method to study bacterial communities composition. Table modified from van Elsas J.D. and Boersma F.G.H., 2011.

2.1.1 The Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a technique used in molecular biology for the amplification of a single or few copies of DNA targeted sequences in order to generate copies of the selected sequence. The technique was developed by Kary Mullis in the early 80s, who received the Nobel Prize in Chemistry in 1993 together with Michael Smith for the work, that became soon the most commonly and often indispensable technique used in scientific research for a wide variety of applications (e.g. Saiki et al., 1988). The technique is based on the repetition of cycles, alternating heating and cooling to melt DNA and let its enzymatic replication occur. Short DNA fragments named primers, containing sequences complementary to a specific target region, and the DNA polymerase are required as key components of the whole reaction. When DNA replicates, the copies are the templates for replication; in this way, the DNA is exponentially amplified. The PCR standard procedure consists on 20-40 repeated cycles that can be schematized as follows:

- Initialization (for the denaturation of genomic DNA and in case of using a polymerase requiring heat activation by hot start PCR, Sharkey et al., 1994,): heating the reaction to 94°C (or 98°C if an extremely thermostable polymerase is used) for 5-10 minutes;

- Denaturation: heating the reaction to 94–98 °C for 20-30 seconds in order to melt DNA template by disrupting hydrogen bonds between complementary bases; with this procedure it is possible to obtain single-stranded DNA molecules;

- Annealing: temperature is lowered to 45–65 °C for 20-40 seconds to allow annealing of the primers to the single-stranded DNA template;

- Extension/elongation: in this phase the temperature depends on the polymerase used and the duration depends both from the polymerase and the length of the DNA chain that must be amplified; the polymerase synthesizes a new DNA strand complementary to the DNA template trough the addiction of dNTPs (nucleotides containing triphosphate groups) complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the extending DNA strand. As general rule, in optimum temperature conditions the polymerase can add a thousand bases per minute;

- Final elongation: temperature is set on 70-74°C for 5-15 minutes after the last PCR cycle in order to ensure that any remaining single-stranded DNA is fully extended;

- Final hold: the final step at 4-15 °C for an indefinite time may be employed for short-term storage of the reaction.

Results of PCR amplification can be visualized trough electrophoresis on agarose gel and ethidium bromide under UV light.

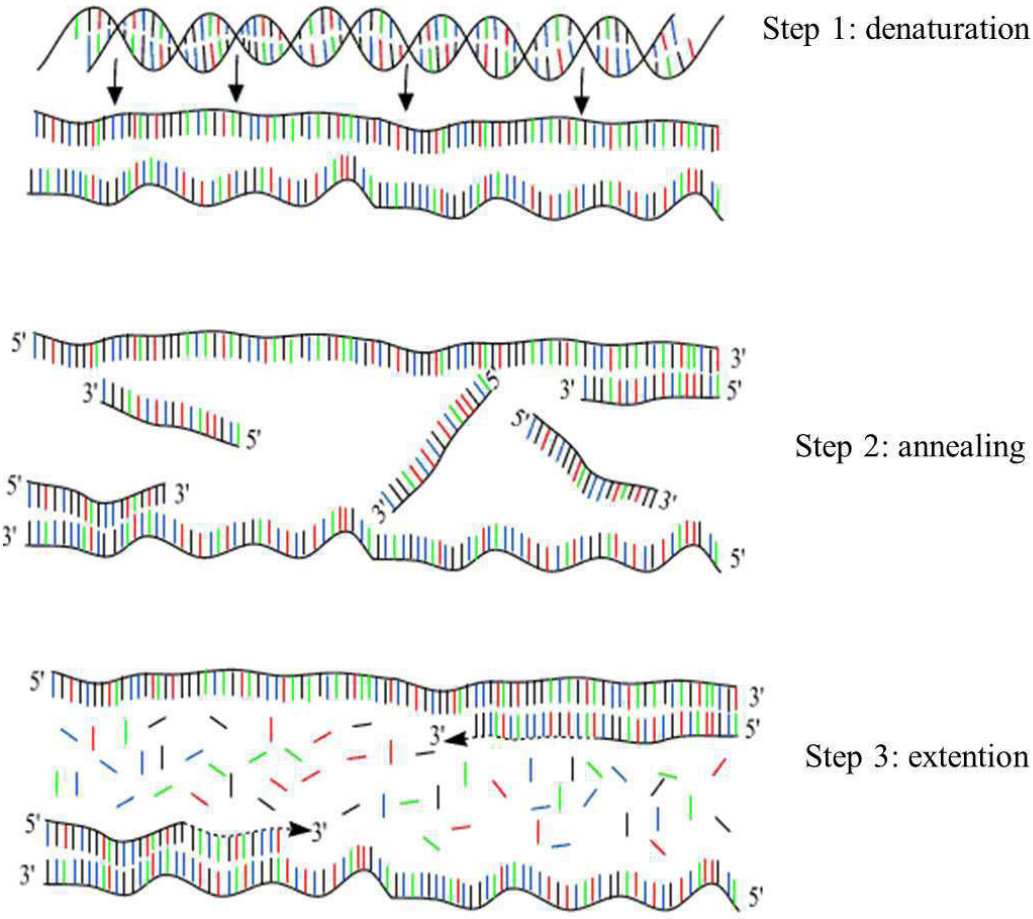

Figure 7: Polymerase Chain reaction Scheme from Andy Vierstraete 1999, modified by Carolina Chiellini.

2.1.2 Full Cycle rRNA approach

The Full Cycle rRNA Approach described by Amann et al., (1995) is a culture independent method commonly used to detect and identify species that are present in complex environmental samples. In the last decades, it was the most used techniques in many different fields of environmental microbiology (e.g. Vannini et al., 2008, Biswas, K. and Turner, S.J. 2012).

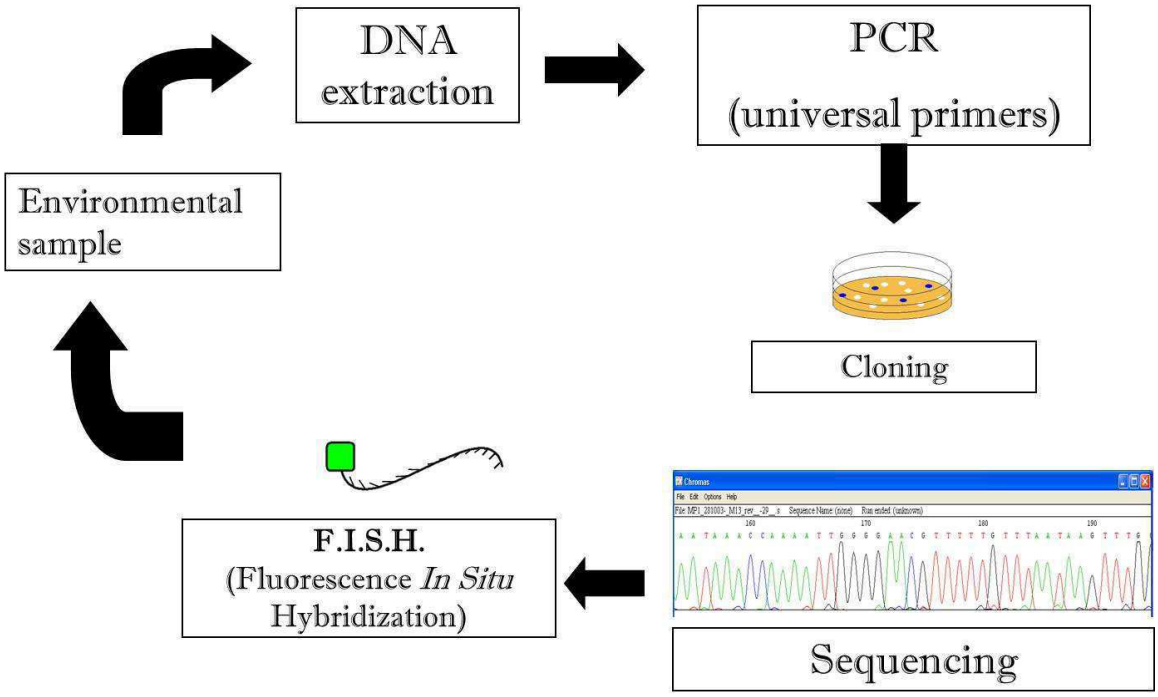

Figure 8: Full Cycle rRNA Approach from Amann et al., 1995, adapted by Carolina Chiellini

The approach starts with the extraction of the whole DNA from a sample, followed by a PCR that amplifies a specific gene from the DNA. The amplified genes in the template belongs to all the organisms that were originally present in the environmental sample; homologous genes belong to different organisms and are all characterized by different sequences. To separate homologous genes, the purified template is cloned into a plasmid vector that is then inserted into chemically competent cells. Each vector receives a single copy of the gene and each cell receives a single plasmid with the inserted gene. Different cells are plated in solid media and then, the grown colonies are put to develop in liquid media. Plasmid DNA is then extracted from each monoclonal liquid culture, and sequencing of the targeted gene is performed. The molecular identification of the organism is obtained through the comparison of the various sequences with all those that are present in public databases. Sometimes, it is not possible to precisely identify an organism at genus or species level because public databases don't contain sequences showing similarities with our sequence. The final step in the Full Cycle rRNA Approach is to perform an experiment of Fluorescence *In Situ* Hybridization (FISH) with specific probes, in order to confirm the results obtained with the sequencing step, and in order to quantify the amount of a certain organisms inside an environmental sample. It is possible to design specific probes for the detected organisms when the sequencing doesn't allow a precise characterization of the organisms and when the taxonomy is not surely

established with the comparison in international databases. In this case the researcher deals with an original description of an organism not yet included in the already available scientific literature.

In this thesis the full cycle rRNA approach was used in a part of the whole research without the final FISH experiment step.

2.1.3 Terminal Restriction fragment Length Polymorphism

Terminal Restriction Fragment Length Polymorphism (T-RFLP, Liu et al., 1997) is a technique commonly used in molecular biology to profile unknown microbial communities. It consists in the amplification of a specific target DNA sequence trough PCR using one or both primers, labeled at the 5' end with a fluorescent molecule. Amplified DNA is then digested with one or more restriction enzymes, resulting in a mixture of fragments of different length with the 5'end labeled. Fragments representative of different organisms are cut in different restriction sites from the endonuclease. The sizes of the different terminal fragments are determined by the fluorescence detector trough capillary electrophoresis performed by a sequencer. The result of capillary electrophoresis is an electropherogram showing peaks in a graph in which the X axis represents the retention time plot that is proportional to the sizes of the fragment expressed in based pairs, and the Y axis represents their fluorescence intensity expressed in fluorescence units. Each peak is commonly considered to be representative of an Operational Taxonomic Unit (OTU, Moesender et al., 2001, Engebretson et al., 2003); the height of each peak is representative of the relative abundance of the sequence in the analyzed sample.

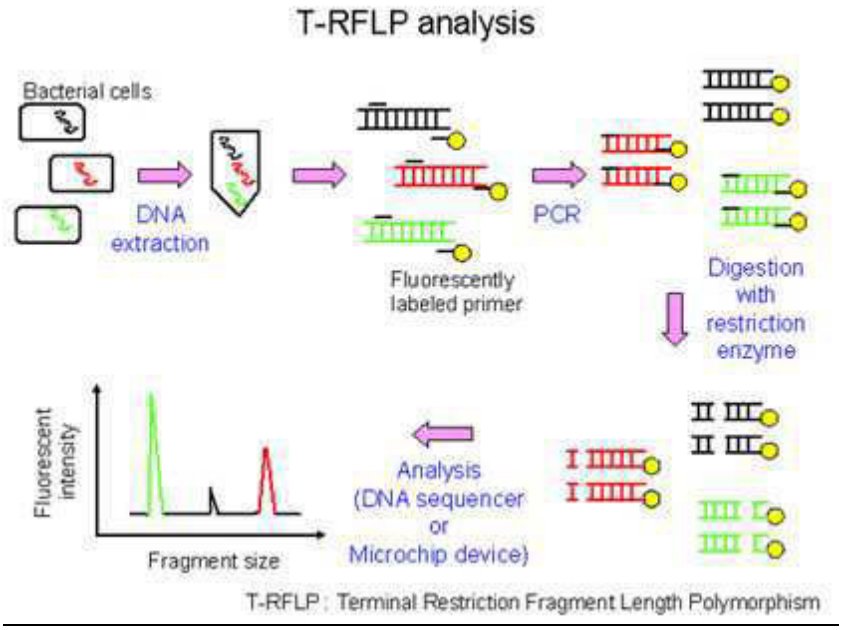

Figure 9: T-RFLP method scheme from http://www.e-cew.co.jp/Microbe-contents/21trflp.html.

2.2 Statistical Analysis applied on data obtained from fingerprint molecular methods

The choice of the best statistical analysis for the interpretation of data obtained with fingerprint methods depends on many different aspects, one of which is the amount of data to compare. The choice of which analysis to use, is influenced by the question that the researchers ask at the beginning of the experiment and by the aim of the whole study. These objectives can be divided into three main categories (Schutte et al., 2008):

- To visualize relationship among fingerprints with Principal Component Analysis (PCA; Clement et al. 1998; LaMontagne et al. 2002; Pereira et al. 2006; Pesaro et al. 2004; Pett-Ridge and Firestone 2005; Schwartz et al. 2007; Wang et al. 2004), Multi-Dimensional Scaling (MDS; Denaro et al. 2005; Pett-Ridge and Firestone 2005; Terahara et al. 2004), self-organizing maps (SOM; Dollhopf et al. 2001), additive main effects and multiplicative interactions (AMMI; Culman et al. 2006);

- To identify significant groups with cluster analysis (Blackwood et al. 2003; Dickie et al. 2002; Fedi et al. 2005; Magalhaes et al. 2008; Moeseneder et al. 1999; Pesaro et al.

2004; Polymenakou et al. 2005; Schwartz et al. 2007; Smalla et al. 2007; Zhou et al. 2007) and model-based approaches (Tang et al. 2007);

- To link differences among the microbial communities to variation observed in the environments sampled using canonical correspondence analysis (CCA; Cao et al. 2006; Grüter et al. 2006; Magalhaes et al. 2008) and redundancy analysis (RDA; Blackwood and Paul 2003).

In this thesis, the statistical analyses used for the interpretation of data are Cluster Analysis, with the aim of identifying the main significant groups among samples, and the Non Metric Multidimensional Scaling, in order to individuate relationship among samples. The Diversity Indexes were also calculated. In next paragraphs will follow their accurate description

2.2.1 Cluster Analysis

Cluster Analysis (CA) is the task of assigning a set of objects into groups called clusters, using the dissimilarities or distances among those objects. It does not distinguish dependent and independent variables and reduces the number of observations or cases trough grouping them in a smaller set of clusters; the result is that objects in the same cluster are more similar to each other than to those in other clusters. Cluster Analysis can be performed with different **algorithms** showing different characteristics, and with different **measures of similarity or distance**. The appropriate algorithm and parameter settings are chosen depending on the setting of individual data. Often it is necessary to modify preprocessing and parameters until the result achieves the desired properties.

There is a great variety of clustering procedures; one of the most used is hierarchical cluster analysis. It is the most common statistical way to find homogeneous clusters of cases based on measured characteristics. Hierarchical clustering procedure considers each case as a separate cluster. Subsequently it combines the cluster sequentially in order to reduce the number of clusters until just one of them is left.

Clustering algorithms can be categorized based on their cluster model. A brief description of the main **algorithms** used for Cluster Analysis is provided (http://www.statsoft.com/textbook/cluster-analysis/?button=1):

Single linkage: the distance between two clusters is determined by the distance of the two closest objects (nearest neighbors) in the different clusters.

Complete linkage: the distances between clusters are determined by the greatest distance between any two objects in the different clusters. Complete linkage is indicated when the objects actually form naturally distinct groups.

Unweighted pair-group average (UPGMA): the distance between two clusters is calculated as the average distance between all pairs of objects in the two different clusters. UPGMA is efficient when objects form natural distinct groups; it also performs well with elongated, chain-type clusters.

Weighted pair-group average (WPGMA): it is identical to UPGMA except that in the computations, the size of the respective clusters (i.e., the number of objects contained in them) is used as a weight. It should be used when the cluster sizes are suspected to be greatly uneven.

Unweighted pair-group centroid (UPGMC): the distance between two clusters is determined as the difference between centroids, where the centroid of a cluster is represented by the average point in the multidimensional space defined by the dimensions.

Weighted pair-group centroid (median, WPGMC): identical to UPGMC but the weighting is introduced into the computations to take into consideration differences in cluster sizes (i.e., the number of objects contained in them). WPGMC is useful in presence of differences in cluster sizes.

Ward's method: It differs from all other methods because it uses the analysis of variance to estimate distances between clusters. Ward's method attempts to minimize the Sum of Squares (SS) of any two (hypothetical) clusters that can be formed at each step. It tends to form small sized clusters.

A list of the main **Distance Measures** used in CA that can be based on a single or multiple dimensions (http://www.statsoft.com/textbook/cluster-analysis/?button=1) will be explained as follow; dimensions represent a rule or condition for grouping objects.

Euclidean distance: The most commonly used, represents the geometric distance in the multidimensional space. It is calculated as:

$$\text{Distance}(x,y) = \{\sum_i (x_i - y_i)^2\}^{1/2}$$

Squared Euclidean distance: it is the square of the standard Euclidean distance, allowing placing progressively greater weight on objects that are further apart. It is calculated as:

Distance (x,y) = $\sum_i (x_i - y_i)^2$

Both Euclidean and Squared Euclidean distances are usually computed from raw data, and not from standardized data. They present many advantages for example, the distance between any two objects is not affected by the addition of new objects to the analysis, which may be outliers. However the distances can be greatly affected by differences in scale among the dimensions from which the distances are computed.

City-block (Manhattan) distance: it is represented by the average difference across dimensions. The city-block distance is calculated as:

Distance (x,y) = $\sum_i |x_i - y_i|$

Chebychev distance: it can be appropriate to define two objects as "different" if they are different on any one of the dimensions. It is calculated as:

Distance (x,y) = Maximum $|x_i - y_i|$

Power distance: it is useful to increase or decrease the progressive weight that is placed on dimensions on which the respective objects are very different. The power distance is calculated as:

Distance (x,y) = $(\sum_i |x_i - y_i|^p)^{1/r}$

where r and p represent user-defined parameters.

Percent disagreement: It is useful if the data for the dimensions included in the analysis are categorical in nature. It is computed as:

Distance (x,y) = (Number of xi ≠yi)/ i

In this thesis, Cluster Analysis is performed with the free statistical software PAST (Paleontological Statistic, Hammer et al., 2001). The three algorithms used in PAST software are:

Paired Group

Single Linkage

Ward's method

The one which was used for the presented analysis is Ward's method, which uses the Euclidean distance measure.

2.2.2 Non-metric Multidimensional Scaling (NMDS)

Non-metric Multidimensional Scaling was first introduced by Shepard R.N. (1962a; 1962b), and has been extended and standardized by Kruskal J.B. (1964a; 1964b). The purpose of this technique is to locate the points in a chosen number of dimensions (usually two), so that the rank order of the distances of the ordination is as close as possible to the rank order of the original distances. Non-metric Multidimensional Scaling is an ordination technique. The main objective of ordination techniques (Goodall, D.W., 1954) is the substitution of the original dimensions with few artificial axis, in order to represent data structure as best as possible (Podani J., 2007). Usually in most ordination methods many axes are calculated, however only few of them are visualized. This creates graphical limitations. MDS gives a different reading because a small number of axes are chosen priory to the analysis and data are fitted to those dimensions. As consequence, no axes of variations are hidden. Another difference is that more ordination methods are analytical and have a unique solution to a set of data, while MDS is a numerical technique that iteratively seeks a solution and stops computation when an acceptable solution has been found, or it stops after some pre-specified number of attempts. This characteristic allows having more than a unique solution with the same set of data using the same methodology. The Third difference is that MDS is not an eigenvalue-eigenvector technique* (such as Principal Components Analysis, PCA, or Correspondence Analysis, CA) and does not ordinate data such as axis 1 explaining the greatest amount of variance, or axis 2 explaining the next greatest amount of variance and so on. An MDS ordination can be rotated inverted or centered to reach the best configuration. Depending on the nature of data, MDS differs from most ordination data because it makes few assumptions, and can be applied for a wide variety of data. MDS also allows the use of any distance measure of the samples without specify any particular measure (e.g., covariance or correlation in PCA). Clarke, K.R. (1993) resumed the main advantages of NMDS as:

- Avoid the assumptions of linear relationships among variables;

- The use of ranked distances linearizes the relationships between distances measured in species and environmental space;
- Allows the use of any distance measure or relativization.

In microbial ecology studies, NMDS is generally used to ordinate similarity data. It represents a simple tool to visualize and interpret data obtained from molecular fingerprint techniques (such as DGGE, T-RFLP, ARISA) concerning the microbial communities composition of samples. NMDS allows the visual interpretation differences and similarities in samples and their comparison in term of spatial and temporal microbial community composition. The first study in which T-RFLP is interpreted trough NMDS statistic is in 2002 from Casamayor et al., and it is followed by lots of similar studies (i. e. Rees et al., 2004, Belila et al., 2011).

In this thesis, NMDS is performed with the free statistical software PAST (Paleontological Statistic, Hammer et al., 2001).

2.2.3 Diversity Indices

In 1972, Whittaker described three different measurements of biodiversity: Alpha, Beta and Gamma diversity. Whittaker's idea was that the total species diversity in an environment (that he called "gamma diversity") is determined by 1) the mean species diversity in sites or habitats in a more local scale (called "alpha diversity") and by 2) the differentiation among those habitats (called "beta diversity").

In synthesis:

Alpha diversity: describes the diversity inside a particular area or ecosystem and it is usually expressed by the number of species included (species richness).

Beta diversity (or species turnover, or differentiation diversity): used to examine the changes in species diversity between the ecosystems, that allows comparing the diversity between ecosystems. It is a measure of how samples are different from each other, and/or how far apart they are on gradients of species composition.

Gamma diversity: it is a measure of the overall diversity for the different ecosystems enclosed in a particular region. Hunter (2002) describes the gamma diversity as "geographic-scale species diversity".

A diversity index is a statistic that measures the biodiversity present in an ecosystem. It gives additional information about community composition than a simply species richness value (the number of species present in a certain environment). Diversity indices provide important information about rarity and commonness of species in a community. The various indices can be used to assess the diversity of any population in which each member belongs to a unique species.

The following list is provided in PAST statistic manual (Hammer et al., 2001) and resumes the most used alpha diversity indices, (for a more detailed explanation, see Harper D.A.T. 1999):

Simpson index: Measures the evenness of the community from 0 to 1.
Dominance: = 1-Simpson index. Ranges from 0 (all taxa are equally present) to 1 (one taxon dominates the community completely).
Shannon index (entropy): Takes into account the number of individuals as well as number of taxa. Varies from 0 for communities with only a single taxon to high values for communities with many taxa, each with few individuals.
Equitability: Shannon diversity divided by the logarithm of number of taxa. This measures the evenness with which individuals are divided among the taxa present.

The PAST statistic module used to calculate beta diversity comprehends 8 measurements, shown in the following table provided directly by the software manual. The eight measurements available are described in Koleff et al. (2003):

Past	Koleff et al.	Equation	Ref.
Whittaker	b_w	$\frac{S}{\overline{\alpha}} - 1$	Whittaker (1960)
Harrison	b_{-1}	$\frac{\frac{S}{\overline{\alpha}} - 1}{N - 1}$	Harrison et al. (1992)
Cody	b_c	$\frac{g(H) + l(H)}{2}$	Cody (1975)
Routledge	b_l	$\log_{10}(T) - \left[\frac{1}{T}\sum_i e_i \log_{10}(e_i)\right] - \left[\frac{1}{T}\sum_i \alpha_i \log_{10}(\alpha_i)\right]$	Routledge (1977)
Wilson-Shmida	b_t	$\frac{g(H) + l(H)}{2\overline{\alpha}}$	Wilson & Shmida (1984)
Mourelle	b_{me}	$\frac{g(H) + l(H)}{2\overline{\alpha}(N - 1)}$	Mourelle & Ezcurra (1997)
Harrison 2	b_{-2}	$\frac{\frac{S}{\alpha_{max}} - 1}{N - 1}$	Harrison et al. (1992)
Williams	b_{-3}	$1 - \frac{\alpha_{max}}{S}$	Williams (1996)

Table 3: PAST statistic manual. Beta diversity measures. *S*: total number of species; α: average number of species; *N*: number of samples; *g*(*H*): total gain of species along gradient (samples ordered along columns); *l*(*H*): total loss of species; e_i: number of samples containing species *i*; *T*: total number of occurrences.

In this thesis, the diversity indices were calculated using two different software, respectively: PAST (Hammer et al., 2001) and MOTHUR (Schloss et al., 2008); see following chapters for a detailed explanation about the calculation procedures.

*Eigenanalysis is the process of finding eigenvectors and eigenvalues. An eigenvector of a square matrix is a non-zero vector that, when multiplied by the matrix, yields a vector that is parallel to the original. Specifically, a non-zero column vector v is a right eigenvector of a matrix A if (and only if) there exists a number λ such that Av = λv. If the vector satisfies vA = λv instead, it is said to be a left eigenvector. The number λ is called the eigenvalue corresponding to that vector. From: http://en.wikipedia.org/wiki/Eigenvalues_and_eigenvectors.

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3 The Agriport project

The AGRIPORT project promotes an innovative approach to decontaminate dredging sediments by a purposely developed phyto-treatment. The action of plants and microorganisms transforms degraded sediments into fertile and nutrient rich soil, while removing salts and polluting substances. This process decontaminates polluted sediments dredged from ports or navigable channels and turns them into arable soil appropriate for other uses such as landscaping, environmental restoration, gardening, capping of landfills, etc. Through a tentative target duration of three years, AGRIPORT focused on optimizing the decontamination method in two pilot applications in the port of Leghorn (Italy) and the agricultural cooperative (kibbutz) Revadim (Israel). A meso-scale experiment was also set up in Pisa (Italy) where the quality of recycled soil was validated and the most appropriate crops for the treated sediment were identified in order to optimize the soil decontamination process. In addition, AGRIPORT fostered the commercialization of the technology and its end product to port authorities, plant nurseries and agronomic businesses in Europe and across the Mediterranean region. The end product is represented by a recycled matrix with natural-soil-like features (techno-soil) that can be used for gardening and forestry, environmental restoration of degraded areas and landscaping as well as agriculture.

The selection of the vegetal species is particularly important since they must be resilient to the difficult conditions of salinity and anoxicity typical of dredged sediments. The growth of such vegetal species is possible thanks to an initial bio-remediation of sediments using inert material, agronomic soil and/or organic substances that improve the physical and chemical characteristics of the sediment.

The plants used in the project are chosen based on their adaptability to several factors such as:

- Physiological capacity to grow on the dredged sediment;
- Potential to stabilize the site, thanks to a fast growth rate and deep rooting features;
- Ability to uptake and remove substantial amounts of metals in plant canopy;
- Adaptability to given climatic conditions;
- Resilience to specific characteristics of selected sediments (granulometric composition, pH, salinity, type and level of contamination).

Based on these factors the following plant species have been used in Leghorn phytoremediation basin:

Paspalum vaginatum: a strong grass species characterized by high salt tolerance (very important feature since salinity is the key problem connected with dredged sea sediments) and ability to grow in difficult environmental conditions;

Tamarix gallica: characterized by the ability to grow on salty soils, wider and deeper root apparatus development and related capability to explore a larger amount of sediment under bioremediation;

Spartium junceum: shrub species characterized by an extensive capacity to adapt to adverse environmental conditions (dry weather conditions, clay soils, etc.).

The AGRIPORT specific objectives are:

1. Development of the use of AGRIPORT phyto-remediation technology as an innovative way to reclaim dredging sediments using plants (phyto-treatment);
2. Optimization of the proposed dredged sediments reclamation process and identification of the most suitable crops to grow on the output material (techno-soil);
3. Contribution to reduce the costs for disposing slightly polluted port sediments and promote commercialization of the phyto-treatment technology and the output material (techno-soil) in gardening, environmental restoration and landscaping.

Three papers have been written about the microbiological part of the study. The first and the second papers focus on the study of bacterial communities in freshly collected polluted sediments of five sites of Leghorn harbor. The five sites were characterized by different kinds and levels of pollution.

In the first paper, published on "Science of the Total Environment" journal, fifteen samples of sediments (three for each of the five sites), were subjected to the following analysis:

-Pollution indicators: total petroleum hydrocarbons, heavy metals (Cd, Cu, Cr, Ni, Pb and Zn);

-Chemical indicators: total organic carbon, total nitrogen, total phosphorus, NO_3^-, Cl^-, SO_4^{2-};

-Biological indicators: enzyme activities and total bacterial count;

- Biomolecular analysis: T-RFLP and derived diversity indices.

The second paper, submitted to "Marine Pollution Bulletin" journal, focuses on the description of bacterial community composition and bacterial species identification in the five sites of Leghorn

harbor, using molecular techniques as 16S rRNA library construction, T-RFLP fingerprinting method and statistical data interpretation.

The third paper, published on "New Biotechnology" journal, describes the bacterial community dynamics in the phytoremediation plant during the three years of experimentation in the treatment basin. The T-RFLP analysis was applied in order to highlight, both temporally and spatially, the evolution of bacterial community structure in the treatment basin.

All papers give an interesting contribute to the study of bacterial community composition, bacterial community dynamics and bacteria identification in marine polluted sediments. The whole study provides original results on decontamination of polluted marine sediments using the phyto-treatment; in fact, this topic is nowadays still poorly developed from a microbiological point of view. Further investigations, and the application of the most modern molecular techniques for the study of bacterial communities, will improve the knowledge on the decontamination dynamics due to bacterial metabolisms.

3.1 Assessment of pollution impact on biological activity and structure of seabed bacterial communities in the Port of Livorno (Italy).

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Assessment of pollution impact on biological activity and structure of seabed bacterial communities in the Port of Livorno (Italy)

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ABSTRACT

The main objective of this study was to assess the impact of pollution on seabed bacterial diversity, structure and activity in the Port of Livorno. Samples of seabed sediments taken from five selected sites within the port were subjected to chemical analyses, enzymatic activity detection, bacterial count and biomolecular analysis. Five different statistics were used to correlate the level of contamination with the detected biological indicators. The results showed that the port is mainly contaminated by variable levels of petroleum hydrocarbons and heavy metals, which affect the structure and activity of the bacterial population. Irrespective of pollution levels, the bacterial diversity did not diverge significantly among the assessed sites and samples, and no dominance was observed. The type of impact of hydrocarbons and heavy metals was controversial, thus enforcing the supposition that the structure of the bacterial community is mainly driven by the levels of nutrients. The combined use of chemical and biological essays resulted in an in-depth observation and analysis of the existing links between pollution macro-indicators and biological response of seabed bacterial communities.

1. Introduction

The northern Mediterranean Sea is impacted by human activities resulting in severe pollution of the coastal seawater environment. The seabed of commercial ports is among the most exposed biotopes to the effects of this kind of pollution, as seabed sediments receive and store a wide range of polluting agents derived from commercial, industrial and leisure activities (losses or leaks from handling of loose or liquid goods, exhausts of combustion for maritime propulsion, unauthorised sea disposal of wastes and sewage, residues of boat painting and surface treatments). After contamination, these sediments act as a long-term source of pollutants, thereby altering the diversity and functionality of the aquatic ecosystem (e.g. Hollert et al., 2003).

The increasing willingness to protect the environment is leading to stricter legislation in northern Mediterranean countries, requiring new methods and techniques to monitor, assess and remediate contaminated sites. These assessment and remediation tools are even more urgent for the numerous sites that undergo regular dredging to maintain suitable sea levels for navigation, as polluted dredged sediments can cause reactivation and spreading of their embedded pollution (SedNet, 2004).

In Europe, 100–200 million m^3 of contaminated sediment are estimated to be dredged yearly (Bortone et al., 2004), pointing out the necessity to identify locations in need of remediation, and to select feasible measures to minimise the risk of further sediment contamination. In Italy, the high pollution levels of many commercial and tourist ports are considered severe threats to ecosystem stability and functionality (Pellegrini et al., 1999). The growing ecological issue of sediment contamination is leading to the need for sediment bioassays to be used in combination with chemical analyses in order to determine and measure the impact of pollution on the seabed ecosystem (Nendza, 2002).

Among the several possible bioassays, the measure of different enzyme activities is a good estimation of overall metabolic processes in the biocenosis of soil (Nannipieri, 1994) and marine sediment ecosystems (Arnosti et al., 2009). Since field and genomic investigations have demonstrated that specific bacteria differ in their biochemical capabilities, the bacterial community composition of a given environment can be considered among the main determinants of its overall enzyme activity (Arnosti et al., 2009). Therefore, enzyme activities, together with total bacterial count and biomolecular characterization, can be used to assess responses to increased pollution by comparison with detected pollution indicators. Indeed, bacterial communities transform nutrients and decompose/detoxificate numerous

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classes of contaminants by producing enzymes that are released into the environment, acting as catalysts of important metabolic functions (Meyer-Reil, 1991; Ceccanti et al., 2006).

Nevertheless, it is nowadays well known that most of the diverse bacterial colonies and biomasses, especially in soils and sediments, are represented by uncultivable bacteria (e.g. Amann et al., 1995; Kennedy et al., 2008). Therefore, a culture-independent approach to the bacterial assessment of seabed sediments is a necessary step to find possible correlations among levels of nutrients and pollutants, bacterial community structure and produced enzymes.

Since the early 1990s, the full cycle rRNA approach (Amann et al., 1995) has been used to study the presence, distribution and phylogenetic position of uncultivable microorganisms. However this approach is expensive, and weak in the investigation of bacteria embedded in sediments or complex matrices, since it involves the Fluorescence in situ Hybridisation (FISH) method, which presents several technical problems that limit its use in these cases.

More recently, Terminal Restriction Fragment Length Polymorphism (T-RFLP) has been proposed to compare, in space and time, different bacterial communities living in soils and sediments (Liu et al., 1997). This technique is cost effective and allows for rapidly comparing a large number of samples.

Few studies are available in the recent literature which have linked pollution indicators to environment perturbations in communities of polychaete (Shen et al., 2010), archaea and bacteria (Cao et al., 2011a). The phylogenetic diversity changes of the dominant genus along the anthropogenic pollution gradient observed in a river mouth have also been studied (Cao et al., 2011b).

In this study, we combined the mentioned techniques with a thorough statistical analysis to assess the impact of commercial and tourist shipping pollution on seabed sediments within the Port of Livorno.

The Livorno Port area, including the sea stretch of its harbour up to the border of the *Meloria* marine park, is included in the Italian list of 54 nationally relevant polluted sites, as most of the main Italian commercial and industrial ports are. The Livorno Port Authority contracted out the environmental site assessment of the sea section of the polluted site. These activities are now nearly completed and include a detailed monitoring of seabed sediments which was used in this study to compare and validate the chemical and biological analyses carried out distinctively for this work.

In this work, fifteen samples of sediments, purposely collected from five different sites of the port seabed (three samples per site), were subjected to the following analyses:

- Pollution indicators: total petroleum hydrocarbons, heavy metals (Cd, Cu, Cr, Ni, Pb and Zn);
- Chemical indicators: total organic carbon, total nitrogen, total phosphorus, NO_3^-, Cl^-, SO_4^{2-};
- Biological indicators: Enzyme activities (arylsulphatase, dehydrogenase, urease, polyphenoloxidase) and total bacterial count;
- Biomolecular analysis: T-RFLP and derived diversity indices.

All of the indicators were assessed and critically compared using five different statistics. The results were then discussed in order to recognise impacts and relations among pollution sources, types and levels, and the corresponding diversity, structure and activity of seabed bacterial communities.

2. Materials and methods

2.1. Seabed sampling sites in the Port of Livorno

Five seabed locations were selected, within the main industrial and shipping channels of the Port of Livorno, in order to obtain representative samples of different port activities, water depths and pollution levels. For each site x, three samples (named x.1, x.2 and x.3) of the top layer of seabed sediment were randomly collected by a scuba diver. The samples were immediately stored at 4 °C and brought within six hours to the microbiological and chemical laboratories, where they were stored at −20 °C for the biomolecular analyses, and left at 4 °C for the biological and chemical analyses.

The five sampling locations are shown in Fig. 1. They can be described as follows:

Site 1: Ferry boat departure area (sandy loam; water depth 11 m);
Site 2: Seldom dredged shipbuilding area (loam; water depth 4 m; last dredging 60 years ago);
Site 3: Container terminal (silt loam; water depth 13 m; located by the open sea mouth of Navicelli canal);
Site 4: Cargo ferry transit (loam; water depth 6 m);
Site 5: Chemical processing and oil refinery terminal (silty clay loam; water depth 9 m; located by the mouth of *Ugione* stream, which crosses the inland industrial area and collects some spare wastewater discharges).

2.2. Analytics

Each of the three sediment samples collected from each site was analysed in triplicate and the means of the three results were used for this study.

2.2.1. Pollution indicators

Total petroleum hydrocarbons (TPH) were determined by the gravimetric method 1664 (U.S. Environmental Protection Agency (EPA), 1983; APHA-American Public Health Association, 1992) using n-pentane (Carlo Erba, Italy) instead of n-hexane, as modified by Ceccanti et al. (2006).

Heavy metals (Cd, Cu, Cr, Ni, Pb and Zn) were determined by atomic absorption spectrometry (Analytikal Jena, Contraa 300, HR-

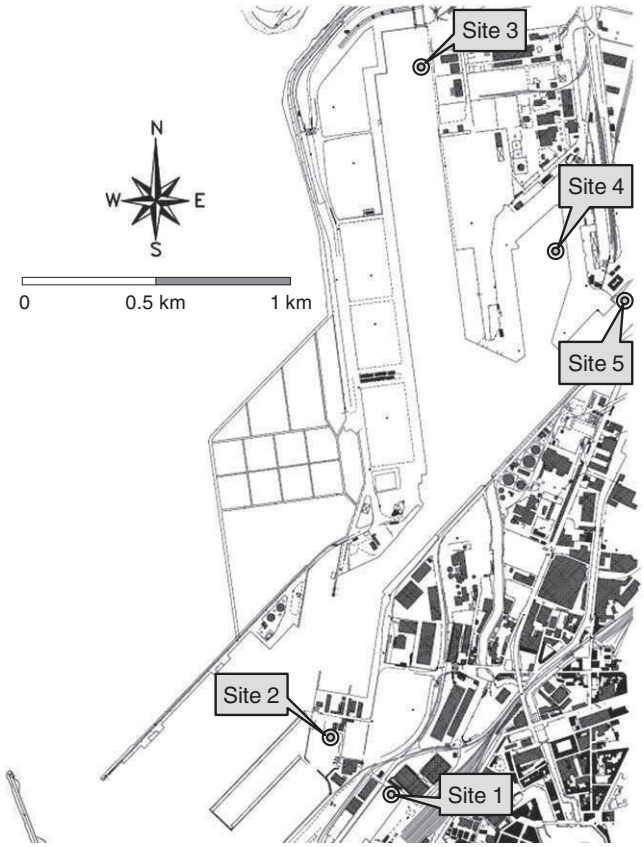

Fig. 1. The five sampling sites in the Port of Livorno.

AAS) after acid digestion using nitric-perchloric acids (Carlo Erba, Italy).

2.2.2. Chemical indicators

Total organic carbon (TOC) and total nitrogen (TN) were determined by dry combustion using a RC-412 multiphase carbon and a FP-528 protein/nitrogen determinator, respectively (LECO Corporation, USA).

NO_3^- was measured in an aqueous extract (1:10, w:v) using selective electrodes (Sevenmulti Mettler Toledo); Cl^- and SO_4^{2-} anions were measured in an aqueous extract (1:10, w:v) using a DIONEX 2000i ion chromatograph (DIONEX Corporation, California, USA), equipped with a Dionex AS4A 4-mm analytical column according to handbook instructions.

Total phosphorus (TP) was determined by a colorimetric method (Murphy and Riley, 1962) after acid digestion using nitric–perchloric acids (Carlo Erba, Italy).

2.2.3. Biological indicators

Four enzyme activities (arylsulphatase, dehydrogenase, urease and polyphenoloxidase) were measured on fresh samples, and the results were expressed as rates of product formation or substrate consumption per unit of dried weight per hour (mg kg_{dw}^{-1} h^{-1}).

Dehydrogenase activity (Dhase) was determined by reduction of 0.4% 2-p-iodo-nitrophenyl-phenyl-tetrazolium chloride to iodo-nitrophenyl formazan (INTF) measured by 490 nm spectrophotometry (Masciandaro et al., 2000). The activity (rate of product formation) was expressed as mg INTF kg_{dw}^{-1} h^{-1}.

Arylsulphatase activity (Asase) was determined using 50 mM potassium p-nitrophenyl sulphate as the substrate, and measuring the produced ρ-nitrophenol (PNP) by spectrophotometry at 398 nm (Tabatabai and Bremner, 1970). The activity (rate of product formation) was expressed as mg PNP kg_{dw}^{-1} h^{-1}.

Polyphenoloxidase activity (PPO) was measured by a slightly modified version of the Allison and Jastrow (2006) method: 0.5 g of fresh sediment was shaken for 30 min with 10 ml of 50 mM Tris buffer pH 7; 1 ml of extract was incubated with 1 ml of 50 mM pyrogallol in water bath at 30 °C for 30 min; after shaking, 3 ml of ascorbic acid 4.2 mM were added and the absorbance of the supernatant was read at 460 nm. The result was read from the standard curve, as a function of the residual content of pyrogallol (PG); the activity (rate of substrate consumption) was expressed as mg PG kg_{dw}^{-1} h^{-1}.

Urease hydrolysing activity was determined following the Nannipieri et al. (1980) method, using urea as the substrate. The activity (rate of substrate consumption) was expressed as mg NH_3 kg_{dw}^{-1} h^{-1}.

All of the substrates for enzyme activities were purchased from Sigma Aldrich, USA.

The total cultivable bacterial population (TBC) was determined by the surface-plate counting procedure (Jayasekara et al., 1998). A preliminary extraction was performed by shaking 1 g of fresh sediment and 9 ml of sterilised water using a vortex for 2 min at room temperature. After decantation of the sediment suspension for 1 min, the supernatant was used for serial dilutions (from 10^{-2} to 10^{-6}), and 0.2 ml of each dilution was spread onto a nutrient agar (PCA) plate to determine TBC. After incubation at 29 °C for 48 h, the colony forming units (CFU) were counted on the plates following Picci and Nannipieri (2002).

2.3. Biomolecular analysis

Total DNA extraction was performed using a Soil master™ DNA extraction kit (Epicentre Biotechnologies). The 16S rRNA genes, which exhibited a total length of about 1550 base pairs (bp), were directly amplified from the extracted DNA using two universal bacterial primers: the 8F primer (5′-AGA GTT TGA T(CT)(AC) TGG CTC AG-3′) labelled with FAM fluorochrome at the 5′ end, and the reverse 1492R primer (5′-GG(AGCT)(AT)AC CTT GTT ACG ACT T-3′).

These two primers were accurately chosen to allow for taxon or strain identification and description of new species (Sacchi et al., 2002): we preliminarily tested, in the NCBI online database, several primers that have been routinely used in similar environmental studies involving bacterial communities (Vannini et al., 2008), and chose those that matched the highest number of bacterial sequences in the tested samples.

A 50 μl PCR was performed for each sample using 0.25 mM deoxynucleoside triphosphates (2.5 mM each), 0.6 pmol/μl primer forward, 0.6 pmol/μl primer reverse, 2.5 μl template DNA and 0.03 μ/μl Taq polymerase (Ex Taq, Takara, Japan).

Thermocycling was performed using a Primus 96 plus thermocycler (MWG Biotech, Germany) at 94 °C for 10 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min with a final extension step at 72 °C for 10 min.

The products of amplification were cleaned using the EuroGold Cycle-Pure Kit (EuroClone®, Italy).

The restrictions were performed independently at 37 °C for 4 h using two enzymes chosen on the basis of prevalent T-RFLP literature (Liu et al., 1997): BsuRI (Fermentas, 0.2 μ/μl final concentration) and RsaI (Fermentas, Canada, 0.2 μ/μl final concentration).

After restriction, DNA was precipitated using sodium acetate and cold 100% ethanol to eliminate salts. For each reaction, a mix with 1.2 μl of loading buffer (GeneScan™ 600 LIZ, Applied Biosystems, USA), a maximum of 5.5 μl of sample and 13.3 μl of deionised formamide (Applichem, Germany) were prepared. The volume of each sample was calculated on the basis of its final concentration after cold ethanol precipitation.

The 600 LIZ fragment size standard was chosen because the adopted BsuRI and RsaI restriction endonucleases recognise and cut target sequences of four bp, obtaining average fragments of $4^4 = 256$ bp. Consequently, most fragments would likely be shorter than 500 bp, and the 600 LIZ standard would be appropriate for their detection. Indeed, the GeneScan™ 500 and 600 LIZ standards, together with 8F and 1492r primers, have been routinely used in several studies adopting similar T-RFLP protocols, despite the different fragment lengths detectable by the chosen primers and standards (e.g. Zhang et al., 2011).

After digestion with restriction endonucleases, each terminal fragment was considered as an Operational Taxonomic Unit (OTU) (Moesender et al., 2001; Engebretson and Moyer, 2003); therefore, the two terms will be used as synonyms henceforth.

Capillary electrophoresis was performed using an Abi Prism 310 Genetic Analyzer (Applied Biosystems, USA); T-RFLP profiles were analysed using GeneScan™ analysis software (Applied Biosystems, USA). Three profiles per sample were compared and fragments visible at least in two profiles out of three were used for a "consensus" profile. The 15 "consensus" profiles were used to assemble a matrix for cluster analysis.

2.4. Diversity indices

To calculate and compare species diversity among samples, we calculated the following indices:

- Simpson (1949) diversity index $Ds = 1 - \Sigma p_i^2$, with p_i representing the population of the i species;
- Shannon (1949) diversity index $H = -\Sigma p_i \ ln \ p_i$, with p_i being the proportion of the i species relative to the total number of species;
- Dominance Index $D = 1 - Ds$, with Ds being the Simpson's diversity index.

The indices were calculated using the T-RFLP profiles obtained with the BsuRI restriction enzyme, which provided a higher number of bands than RsaI. This procedure could slightly underestimate the

real biodiversity, since the same band could theoretically correspond to more than one OTU (Marsh et al., 2000).

The peak heights were standardised by equalising their sums among different runs with their average levels, and recalculating the peak heights in each run. As a final step, peaks showing heights lower than 50 fluorescence units were excluded from the analysis.

2.5. Statistics

For the statistical analysis, the Shannon's and Dominance Indexes were added to the group of biological indicators, and all of the indicators were normalised and autoscaled to obtain a zero mean and a unit standard deviation (Latorre et al., 1999). Five statistical tools were then used to examine and compare the indicators.

The Analysis of Variance (ANOVA) was used to evaluate differences ($P<0.05$) among different sites and different samples of the same site, following Bonferroni's procedure as a *post-hoc* test.

The cross-correlation of indicators was evaluated by calculating correlation coefficients between all couples of indicators. The couples scoring coefficients with magnitude over 0.5 were then subjected to Student's *t*-test with significance levels $P<0.05$ and $P<0.01$. All of the results were assembled in a correlation matrix.

The Principal Components Analysis (PCA) was used to identify patterns or clusters between indicators.

Three runs of Canonical Correlation Analysis (CCA) were used to compare each other the three groups of indicators, and the canonical variate with highest *correlation coefficient* was considered for each run. The canonical scores of the two datasets of each run were calculated and plotted in the canonical variable distribution, and the canonical weight of each variable was used to highlight the level of mutual relationship with each variable of the counterpart group.

The Cluster Analysis (CA) was performed with both Ward's method and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA), to highlight differences in the composition of bacterial communities using both indicators and T-RFLP results. The raw data from T-RFLP analysis were previously transformed as:

$$N_i = (n_i / \sum n_{tot}) \cdot 10000 + 1$$

with *Ni* being the transformed datum; n_i a single raw datum (height of a single peak in the data matrix), and n_{tot} the sum of raw data collected for each sample.

In Ward's method (Ward, 1963), the distances between clusters were evaluated by minimising the sum of squares of each couple of clusters formed at each step. In the UPGMA method (Sokal and Michener, 1958), clusters were joined based on the average distance between all members of two groups.

The CA was performed using PAST software (Hammer et al., 2001), while all of the other statistics were calculated using STATISTICA 6.0 software (StatSoft Inc., Tulsa, Oklahoma, USA).

3. Results and discussion

3.1. Pollution indicators

According to the environmental site assessment, the port seabed is mainly polluted by heavy metals and hydrocarbons. The parameters that most often exceed the contamination thresholds set by the Italian legislation on contaminated sites are six metals (Cd, Cu, Cr, Ni, Pb and Zn), total light (<C12) and total heavy (>C12) hydrocarbons. Table 1 synthesizes the results from the site assessment related to these contaminants, and compares them to some legislative and reference standards.

In this study, the mentioned parameters (with the two hydrocarbons joined in the single TPH value) were elected as pollution indicators. Their concentrations detected in the purposely collected sediment samples are shown in the first section of Table 2. The ANOVA test, shown in the same table, highlighted that almost all of them were homogeneous in sites 3 and 5, and variable in site 1. The most stable metal was Cd, which fluctuated appreciably only in site 1. The most variable metal was Zn, which fluctuated significantly in all sites. The highest ranges of variation were reached by Pb in site 1 and Cu in sites 1 and 3. TPH was quite stable within sites, showing a certain variability only in sites 1 and 3. Among different sites, the most stable parameter was Cd, while the most unstable was Pb. TPH was also quite stable among different sites.

A comparison of the indicators detected at the five sites (Table 2), with the data from the environmental assessment (Table 1), shows ranges close to the means for the six metals, and significantly higher than the means (but significantly lower than the maximums) for hydrocarbons. When compared to the Italian and international references reported in Table 1, the metal concentrations of most samples fall between the two sets of thresholds for civil and industrial contaminated sites. When compared to the reported references for the dredging of sediments, they are generally higher than the thresholds.

Conversely, hydrocarbons exceed all reference standards significantly. Hence, the collected samples can be classified as slightly polluted by heavy metals and significantly polluted by hydrocarbons. In general, these data confirm the significant levels of organic and inorganic contamination that were already highlighted by several studies (e.g. Ausili et al., 1998) as a direct consequence of the various port and shipping activities.

Site 2 emerged as the most polluted by all parameters except Cd and Ni, which reached their maximums in site 5. The pollution of site 2 can be attributed to the long period of accumulation of sediments and to the release of oil products, combustion of fossil fuels, disposal of wastes and loss of residues from ship maintenance and repair, which have been carried out in this area for a long period of time.

Site 3 emerged as the less contaminated by Pb, Cu, Zn and hydrocarbons. This site has been merely used as a container terminal, and no bulk good handling or industrial activities have ever been performed here. Moreover, dredging has been regularly carried out to avoid obstructions to ship traffic.

For similar reasons, related to its use as a tourist ferry terminal, site 1 showed the lowest contamination by Cd and Ni and low contamination by Cr, Zn and TPH. Despite the low means, the highest value of Pb was observed in one of the samples from this site.

3.2. Chemical indicators

The parameters shown in the second section of Table 2 were elected as chemical indicators of environmental state. According to the ANOVA test, they were in most cases similar within each site and diverse among different sites, giving statistical significance to the analysis.

The highest presence of C and N in site 2 can be attributed to the same discussed causes of its highest contamination by metals and hydrocarbons. Similarly, site 1 showed the lowest values of all pollution and chemical indicators except TP, confirming its low contamination already observed for metals and TPH.

Phosphorus behaved differently than the other chemical indicators: its maximum was reached in site 1 and its minimums in sites 3 and 4. This can be explained since the most significant fractions of TP in marine sediments have been observed by several authors as being the inorganic Ca-associated apatite form and the residual phosphate form after sequential extraction. Khalil (2007), that observed an overall TP range similar to our study, found that the sum of these two forms accounted for 85–90% of TP in marine sediments. These two forms are not bioavailable, not linked to nutrient balances, and significantly present in many minerals of the earth's crust (Folk, 1974). This last remark can explain the higher TP presence in the

Table 1
Summary of results from the environmental site assessment of the sea section of the polluted Livorno site for six heavy metals and total hydrocarbons, compared with Italian and international reference thresholds.

Values in mg/(kg $_{dry\ weight}$)	Cd	Ni	Pb	Cr	Cu	Zn	TLH	THH	TPH
SCD: mean of 795 samples	0.9	48.1	60.5	62.8	41.5	126.1	6.5	9.8	–
SCD: st. dev. of 795 samples	6.5	20.7	250.9	25.6	75.0	278.3	106.9	32.5	–
SCD: max. of 795 samples	152.9	219.4	4902.3	140.9	1339.8	4360.2	3006.0	540.9	–
CST (green parks/residential sites)	2.0	120.0	100.0	150.0	120.0	150.0	10.0	250.0	–
CST (commercial/industrial sites)	15.0	500.0	1000.0	800.0	600.0	1500.0	50.0	750.0	–
Sediment threshold chemical levels[a]	0.8	75.0	70.0	360.0	52.0	170.0	–	–	–
Harmful aliphatic hydrocarbons[b]	–	–	–	–	–	–	–	–	>10.0
Highly polluted sediments[c]	–	–	–	–	–	–	–	–	>100.0

TLH = total light hydrocarbons (<C12).
THH = total heavy hydrocarbons (>C12).
TPH = total petroleum hydrocarbons.
SCD = seabed concentration data from the phase II environmental assessment of the polluted Livorno site.
CST = contaminated site thresholds (Italian Legislative Decree 152/2006).
[a] Italian Ministry of the Environment, Land and Sea. 2006. Handling of marine sediments guidelines.
[b] UNEP. 1995. Determination of petroleum hydrocarbons in sediments. Ref. methods for marine pollution studies #72.
[c] Goldberg, E.W. 1976. The health of the oceans, UNESCO press, Paris.

Table 2
Detected indicators with ANOVA classification. TPH = total petroleum hydrocarbons; TOC = total organic carbon; TN = total N; TP = total P; TBC = total bacterial count; Asase = arylsulphatase activity; Dhase = dehydrogenase activity; Urease = urease activity; PPOs = polyphenoloxidase activity. All indicators are means of three replicates. The letter-tags represent the results of the ANOVA test, with increasing lowercase letters denoting significantly different increasing values within each site, and increasing capital letters denoting significantly different increasing values among different sites ($P<0.05$, Bonferroni test).

		Sites.Samples														
		1.1	1.2	1.3	2.1	2.2	2.3	3.1	3.2	3.3	4.1	4.2	4.3	5.1	5.2	5.3
Pollution indicators																
Cd	$\frac{mg}{kg_{dw}}$	1.06_b	0.63_a (A)	0.71_a	1.16_a	1.42_a (C)	1.31_a	1.22_a	1.44_a (C)	1.20_a	1.09_a	1.20_a (B)	1.11_a	1.36_a	1.59_a (D)	1.48_a
Ni	$\frac{mg}{kg_{dw}}$	37.7_b	25.2_a (A)	39.6_b	41.0_a	75.4_b (B)	94.9_b	101.0_a	98.4_a (C)	118.0_a	63.1_a	67.0_a (B)	67.4_a	100.0_a	103.0_a (C)	135.0_b
Pb	$\frac{mg}{kg_{dw}}$	33.2_a	271.0_c (C)	104.0_b	249.0_b	221.0_{ab} (D)	171.0_a	47.1_a	36.5_a (A)	37.8_a	58.4_a	54.3_a (B)	90.0_b	62.0_a	65.6_a (B)	67.7_a
Cr	$\frac{mg}{kg_{dw}}$	25.2_b	34.7_c (B)	15.8_a	48.8_b	35.4_{ab} (D)	27.7_a	32.1_a	35.6_a (C)	29.2_a	16.9_a	18.7_a (A)	25.0_b	34.6_a	39.4_a (D)	42.6_a
Cu	$\frac{mg}{kg_{dw}}$	75.0_a	73.5_a (C)	268.0_b	80.5_a	375.0_b (D)	485.0_c	74.4_a	71.5_a (A)	71.4_a	123.0_b	91.3_a (B)	86.4_a	119.0_a	127.0_a (C)	135.0_a
Zn	$\frac{mg}{kg_{dw}}$	313_a	353_{ab} (B)	407_b	308_a	854_b (D)	884_b	257_a	336_b (A)	228_a	568_b	277_a (B)	247_a	500_b	476_b (C)	368_a
TPH	$\frac{mg}{kg_{dw}}$	1433_b	1066_a (B)	1030_a	1915_a	2031_a (D)	1798_a	900_b	766_{ab} (A)	633_a	1765_a	1563_a (C)	1666_a	1266_a	1331_a (B)	1032_a
Chemical indicators																
TOC	$\frac{mg}{kg_{dw}}$	6102_a	8938_a (A)	7720_a	$2.5E4_a$	$3.3E4_a$ (C)	$2.7E4_a$	$2.0E4_a$	$2.6E4_a$ (B)	$2.1E4_a$	$2.0E4_a$	$2.1E4_a$ (B)	$1.7E4_a$	$1.7E4_a$	$2.7E4_a$ (B)	$2.4E4_a$
TN	$\frac{mg}{kg_{dw}}$	197_a	225_a (A)	304_b	2170_b	1580_a (E)	1890_b	1019_a	1325_b (C)	1631_b	730_a	878_{ab} (B)	602_a	1993_b	1660_a (D)	1327_a
TP	$\frac{mg}{kg_{dw}}$	917_a	1024_a (C)	1321_b	696a	759_a (B)	722_a	568_{ab}	651_b (A)	455_a	616_a	504_a (A)	570_a	864_a	724_a (B)	844_a
NO_3^-	$\frac{mg}{kg_{fw}}$	831_b	975_a (B)	903_a	1161_{ab}	1363_b (C)	1262_a	708_b	832_a (B)	770_{ab}	1929_{ab}	2265_a (D)	2097_b	491_b	576_a (A)	534_b
Cl^-	$\frac{mg}{kg_{fw}}$	$1.0E4_a$	$1.2E4_b$ (A)	$1.1E4_a$	$1.7E4_a$	$1.9E4_b$ (B)	$1.8E4_{ab}$	$1.6E4_a$	$1.9E4_b$ (B)	$1.7E4_a$	$1.7E4_c$	$2.0E4_a$ (B)	$1.8E4_b$	$1.7E4_{ab}$	$2.0E4_a$ (B)	$1.9E4_b$
SO_4^{2-}	$\frac{mg}{kg_{fw}}$	3587_a	4211_c (BC)	3899_b	3192_a	3747_b (AB)	3469_{ab}	3417_{ab}	4012_a (B)	3714_b	3848_b	4517_a (C)	4183_{ab}	2807_{ab}	3295_b (A)	3051_a
Biological indicators																
TBC	$\frac{CFU}{g_{dw}}$	$4.7E4_a$	$4.4E4_a$ (A)	$9.8E4_b$	$5.4E6_c$	$3.9E6_b$ (E)	$2.2E6_a$	$5.0E5_a$	$7.7E5_b$ (C)	$1.0E6_c$	$1.6E5_a$	$3.2E5_b$ (B)	$3.9E5_b$	$7.9E5_a$	$9.2E5_a$ (D)	$9.3E5_a$
Asase	$\frac{mg\ PNP}{kg_{dw}h}$	5.31_a	6.01_a (C)	14.14_b	3.28_a	7.89_c (B)	6.39_b	9.59_b	8.58_a (D)	9.50_b	0.37_a	0.63_b (A)	0.74_b	29.82_a	13.44_b (E)	16.64_b
Dhase	$\frac{mg\ INTF}{kg_{dw}h}$	4.50_{ab}	3.81_a (D)	5.68_b	3.22_a	3.22_a (C)	3.95_a	2.11_a	2.98_b (B)	3.06_b	0.91_a	1.15_{ab} (A)	1.48_b	7.25_a	7.23_a (E)	7.79_a
Urease	$\frac{mg\ NH_3}{kg_{dw}h}$	11.7_a	12.3_a (A)	42.1_b	12.8_a	41.9_b (B)	41.7_b	19.2_a	25.3_b (B)	39.0_c	17.2_a	25.0_b (B)	31.7_b	41.8_b	41.6_b (C)	20.7_a
PPOs	$\frac{mg\ PG}{kg_{dw}h}$	6618_a	5459_a (C)	$1.2E4_b$	6266_b	3484_a (B)	$1.2E4_c$	2207_a	7987_c (A)	4425_b	8538_a	6644_a (C)	7640_a	5557_b	2765_a (A)	4607_b

dw = dry weight; fw = fresh sample weight.

most frequently dredged site, since a higher mineral fraction is expected in freshly dredged seabed rocks (Shin et al., 2008).

3.3. Biological indicators

TBC and the four enzyme activities reported in the third section of Table 2 were chosen as biological indicators of bacterial state and biochemical processes in circumstances of environmental alteration (Chander and Brookes, 1993). The ANOVA test showed a generally low statistical significance, with the clearest trends observed for TBC, Dhase and Asase.

The highest TBC values were found in sites 2 and 5, where heavy metal contamination was maximum. This result contrasts with several studies that have reported adverse effects of heavy metals on size, structure and activity of bacterial populations in natural environments such as soil (e.g. Akmal et al., 2005). However, a positive effect of metal stress on bacterial productivity or biomass number can happen in the following cases:

i) a possible stimulation/adaptation of microorganisms to stress conditions caused by pollution (e.g. Masciandaro et al., 1998; Piotrowska-Seget et al., 2005);
ii) the presence of metals in mineral form, chelated to organic matter or in other poorly bioavailable forms that scarcely affect the biota (Sutherland, 2002). Pempkowiak et al. (1999) detected a bioavailable fraction of only 3.7% of heavy metals in marine sediments;
iii) the presence of an altered bacterial community, exhibiting a different response to pollution impact (Shen et al., 2010) which, as it is well known, is poorly represented by the TBC parameter, due to massive presence of uncultivable bacteria.

Dhase, which relates to biochemical oxidation processes in natural environments, has been widely used as an indicator of overall bacterial activity (Masciandaro et al., 2000) and inhibition caused by contaminants (Bartha and Pramer, 1965). The highest Dhase value was detected in site 5, in correspondence of the highest concentrations of Cd, Ni and Cr. Consistently, its minimum was detected in site 4, where all metals were quite low. This circumstance seems to confirm a possible stimulatory effect of metals. The effect of TPH on Dhase seemed minor, as already observed in studies that highlighted the dependency on types of hydrocarbons (Moreno et al., 2009) more than concentrations (Gianfreda et al., 2005).

Asase and Urease are involved in converting organic to inorganic S and N, respectively. They fell in ranges of contaminated soils (Hinojosa et al., 2004), with their maximums in site 5, similarly to TBC and Dhase.

Similarly to Dhase, Asase behaved differently in the most polluted sites 2 and 5, showing a possible inhibition/stimulation by specific contaminants (stimulation by Cd or Ni and/or inhibition by Pb and TPH).

Urease exhibited a high variability within different samples of the same sites, demonstrating sensitivity to short range effects ("hot spots", as suggested by Moreno et al., 2009). It reached its maximum in site 5 similarly to Dhase and Asase but, differently than them, also in sites 1 and 2. A possible explanation is a lower sensitivity to Pb and TPH (which were maximum in those sites), in contrast to what have been often observed for various pollutants (e.g. Gianfreda et al., 2005; Moreno et al., 2009).

PPOs are extracellular enzymes that catalyse the oxidation of phenols and other recalcitrant aromatic compounds (Farnet et al., 2004). Numerous studies have emphasized their sensitivity to several xenobiotics such as aromatic compounds and heavy metals, suggesting their possible use as bioindicators (Floch et al., 2009). Nevertheless, in this study PPOs were minimum in sites 3 and 5, and maximum in sites 1, 2 and 4 with high instability within samples of the same sites and poor correlations with the other indicators.

3.4. Biomolecular analysis and bacterial community diversity

Fig. ESM1 (in the Electronic Supplementary Material) reports the T-RFLP electropherograms obtained by the most significant of the three samples analysed for each of the five sites. Only one electropherogram per site is shown, since the three replicates exhibited homogeneous compositions of bacterial communities (see Fig. 4 and Section 3.5.4 for details). In the five electropherograms, the two restriction enzymes showed peaks ranging in different size intervals: 200–400 bp for BsuRI; 50–200 bp and 400–550 bp for RsaI. This is an interesting finding since each endonuclease gave specific information, and, jointly, they provided a complete dataset to describe the bacterial community composition and distribution. In Fig. ESM1, many peaks were detected in all profiles, representing bacterial OTUs present in all sites (e.g. peaks 253 and 401 for BsuRI and peak 487 for RsaI). However, not all peaks were present in all samples: for example site 4 lacked the peak at size 305 for RsaI, denoting different distributions of some bacterial OTUs in the five sites. Moreover, differences in relative peak heights highlighted the presence of different quantities of specific OTUs: for example, the peak at size 487 for RsaI was less represented in site 4 than in site 5.

To compare bacterial diversity and level of pollution in the five sites, three diversity indices (Shannon, Simpson and Dominance indices) were calculated on the BsuRI standardised T-RFLP data (Table ESM1). They did not diverge significantly among sites and samples, thus suggesting that, despite the different OTUs observed in different sites, the overall diversity was similar.

The Dominance index close to zero for all samples indicates that bacterial communities are not dominated by one or few OTUs, but rather diversified and composed of many different OTUs in low percentages.

Since T-RFLP is only able to measure the more abundant bacteria within a sample, it is worth reminding that the diversity indices based on T-RFLP data only account for the diversity of relatively abundant OTUs.

3.5. Statistical analysis

For the statistical analysis, the biological indicators were integrated with the indices OTUs, D and H (Table ESM1) obtained from the biomolecular analysis, following an approach similar to that used by Cao et al. (2011b) to assess the shift of a bacteria community along an anthropogenic pollution gradient.

3.5.1. Correlation matrix

The cross-correlation coefficients of indicators are reported in Table ESM2. The pollution indicators showed few reciprocal correlations: Ni–Cd and Cu–Zn were the most evident, while TPH correlated appreciably with Pb and Zn. Similar trends have been observed by Ausili et al. (1998). Cr was poorly correlated with all of the other pollution indicators, but exhibited significant correlations with N, sulphates and most biological indicator. A significant correlation with TOC, TN, Cl- and TBC was observed for Ni and Cd.

The positive correlation of TBC with Cd, Ni and Cr seems to confirm the bacterial stimulatory effect of these heavy metals that was discussed in Section 3.3. It can also depend on the high correlation of TBC with TOC, TN and Cl^-, which are, in turn, correlated with Cd, Ni and Cr. This fact suggests a possible common polluting source for metals, nutrients and salinity that can explain the unpredictable dependence of TBC on Cd, Ni and Cr. The stimulatory link with heavy metals is partly confirmed by the three diversity indices, which showed a certain correlation with Cr and Zn.

The positive correlation between TBC and N is well known and suggests a possible relation with the nitrogen cycle, as confirmed by the appreciable correlation of Urease with TOC, TN, Cl^- and TBC.

TOC, TN and Cl^- also showed very high reciprocal correlations, and so did sulphates with nitrates. Similar trends have been often observed in a sea environment (e.g. Thuong et al., 2007). The negative correlation of TP with most of the other parameters confirms the analysis reported in Section 3.2.

Dhase and Asase showed a very high mutual correlation, with similar trends throughout all sites, and a low correlation with all of the pollution indicators. A high negative correlation with nitrates and sulphates was also observed. Since Asase is involved in the conversion of organic S to sulphates, their negative correlation is typically due to a feed-back mechanism driven by the concentration of the final product.

Similar considerations can be expressed for Urease, which exhibited a low positive correlation with all contaminants except Ni and Cu, and a low negative correlation with TP, nitrates and sulphates.

3.5.2. Principal component analysis (PCA)

PCA is a multivariate analysis that aims at identifying possible patterns between variables by reducing the raw dataset to a number of principal components that retain most of the variance of the original data.

The analysis (which is shown in Table ESM3) indicated that 70.35% of the variance was contained in the following three components:

- PC1, which was associated with Cd, Ni, and all of the chemical parameter excepted nitrate and sulphate;
- PC2, which was linked with nitrate, sulphate, Dhase and Asase;
- PC3, which was associated with OTU, Pb, Cu, Zn and TPH.

The biplots of loadings and scores (Fig. 2) provide graphical representations of the links between samples and properties, which identify samples with similar properties as those being graphically close together. In both of the biplots, the replicates of each sample are close together, and hence similar, and sample 1 is discriminated from the other samples for its low content of nearly all the indicators.

In biplot 1, the samples of site 5 are located in the top part where the highest levels of Dhase and Asase are, thus being differentiated along factor 2 from samples of site 4, which are located in the bottom of the plot.

3.5.3. Canonical correlation analysis (CCA)

CCA (Hotelling, 1936) is a multivariate analysis that compares two groups of variables by searching linear combinations of the two datasets which exhibit the highest reciprocal relationships. The results of the three runs performed to mutually compare the three groups of indicators are reported in Fig. 3, which shows the samples as points having the linear combination of their values on the x and y coordinates. The linear combinations use the sets of weights obtained by the corresponding analyses, which are listed in the three columns of Table ESM4. All of the runs obtained very high R coefficients (Table ESM4), denoting significant relations among indicators.

Each plot allows to visually estimate the degree of homogeneity of samples and sites, in relations to the two specific groups of indicators. The following considerations can be drawn:

- chemical vs. biological indicators: the different sites are well differentiated and scaled in the order 4 → 2 → 3 → 1 → 5 with site 4 clearly different than the other sites;
- chemical vs. pollution indicators: site 1 exhibited the lowest values of both groups of indicators and emerged as clearly diverse than the others sites that were poorly differentiated;
- pollution vs. biological indicators: the sites were scarcely differentiated in the order 4 → 1 → 3 → 5 → 2.

The scores reported in the three columns of Table ESM4 allowed us to interpret the behaviours of specific indicators in the three CCAs. Indicators with higher scores have a stronger influence on the counterpart group. Scores with discordant signs in the two groups denote inverted effects. The following considerations can be drawn:

- chemical vs. pollution indicators: the most impacting pollutants are Cd and Ni, while TOC and TN are the most impacted chemical indicators. Since only TP and SO_4 exhibit positive scores, the inverted behaviour of these two indicators is confirmed. All of the other pollution and chemical indicators tend to increase jointly, confirming their possible common origin and showing that all chemical indicators, except TP and SO_4, can themselves indicate pollution rather than environmental quality;
- chemical vs. biological indicators: nitrate is the most related chemical indicators; Asase and Dhase are the most related biological indicators. Their mutual effect is inhibitory as they have opposite sign. All of the other weights have lower magnitudes, denoting less significant mutual impacts. Overall, the mutual impacts of different indicators are either stimulatory (concordant signs) or inhibitory (discordant signs);
- pollution vs. biological indicators: all of the pollution indicators have positive weights, with the highest values for Cr and Cd. TBC and diversity indices are significantly impacted with a positive weight (stimulation). The enzyme activities are moderately stimulated excepted PPOs that are barely inhibited. The weights of the contaminants represent a measure of their relative impact on the microbial activity.

Significantly, the latter analysis contradicted the earlier observations, showing a congruent stimulatory impact of TPH and all metals on all of the biological parameters. The enzymatic activities were similar for Dhase and Asase, lower for Urease and opposite for PPOs., with a congruent general behaviour.

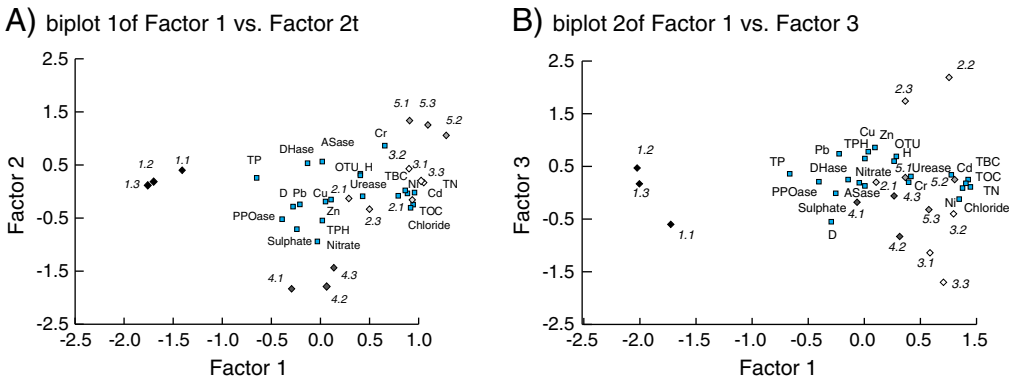

Fig. 2. Principal component analysis: biplots of loadings and scores.

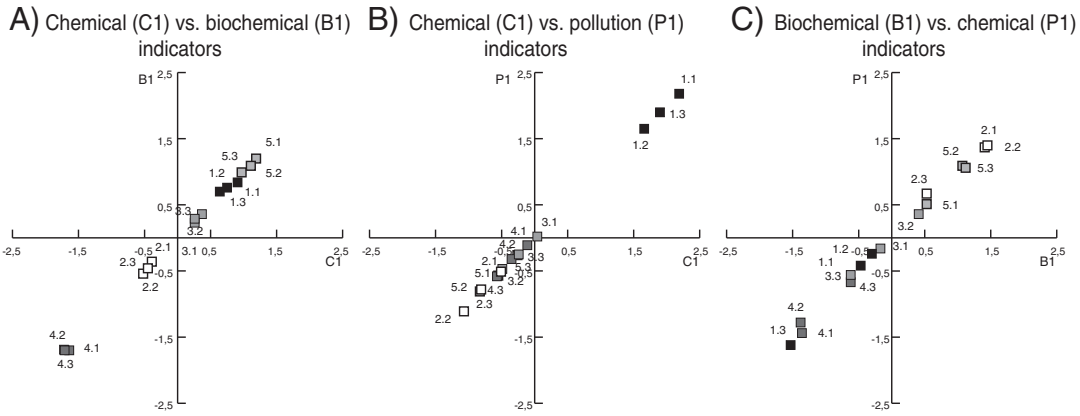

Fig. 3. Canonical correlation analysis score diagrams.

3.5.4. Cluster analysis (CA)

The CA is based on a hierarchical clustering routine that groups the data in dendrograms that highlight their differences and similarities. The two adopted methods gave similar results. Fig. 4 shows the dendrograms obtained with Ward's method, which is considered very efficient, but inclined to create clusters of small size.

The T-RFLP dendrogram (Fig. 4A) shows that the bacterial communities found in the sediments of the five sites are quite different from each other. This is especially true for sites 1 and 5, that are associated with high bootstrap values. Additionally, site 1 is significantly different from the other sites, which are linked together, although with a lower bootstrap support (77). This is congruent with the chemical vs. pollution CCA and both the PCAs, confirming that chemical indicators, and specifically nutrients, are most likely the main determinants of biological activity and bacterial colony structure and distribution.

An additional steady association can be observed in Fig. 4B, showing the CA of indicators. In this dendrogram, sites 3 and 5 exhibit similar properties (83 bootstrap support) in confirmation of what was already observed for all of the PCA and CCA analyses. Moreover, the sites aggregate in two groups, including sites 1 and 4 (58 bootstrap support), and sites 2, 3 and 5 (69 bootstrap support). This trend suggests their dissimilarity on the basis of the considered indicators.

Looking at the ongoing activities, site 1 is among the deepest and most frequently dredged sites, together with sites 3 and 4. Differently than sites 3 and 4, it could have been sporadically polluted by urban-like wastes, since it has been used as a ferry boat terminal. These remarks imply that the biological activity and the bacterial community are likely influenced by type and level of contamination, sea depth and frequency of dredging. A further influence can come from factors that are peculiar to the development of the bacterial community, like the mentioned effect of nutrients, or the presence of dormant microorganisms, as already found for the pollution-driven mineralisation of organic matter in sediments (Hubert et al., 2010).

4. Conclusions

The presented environmental assessment of seabed sediments sampled from five different locations of the Port of Livorno showed that human activities usually performed in a port have produced high quantities of pollutants that have strongly impacted the seabed ecosystem.

A significant pollution from petroleum hydrocarbons and heavy metals was observed, accompanied by increased levels of nutrients and salinity. These parameters, as well as the corresponding biological activities, were homogeneous within each site, while they varied significantly among sites, in dependence of the nature of locally performed activities and the past frequency of dredging.

A similar bacterial diversity was observed in all sites, irrespective of pollution levels.

A stimulatory effect of nutrients on biological activities and bacterial communities was clearly observed. A stimulation of petroleum hydrocarbon and heavy metals was also detected, although with less evidence.

These findings provide useful knowledge for a sound environmental investigation of seabed sediments. The results achieved from this study strongly support the earlier recommendations that an accurate

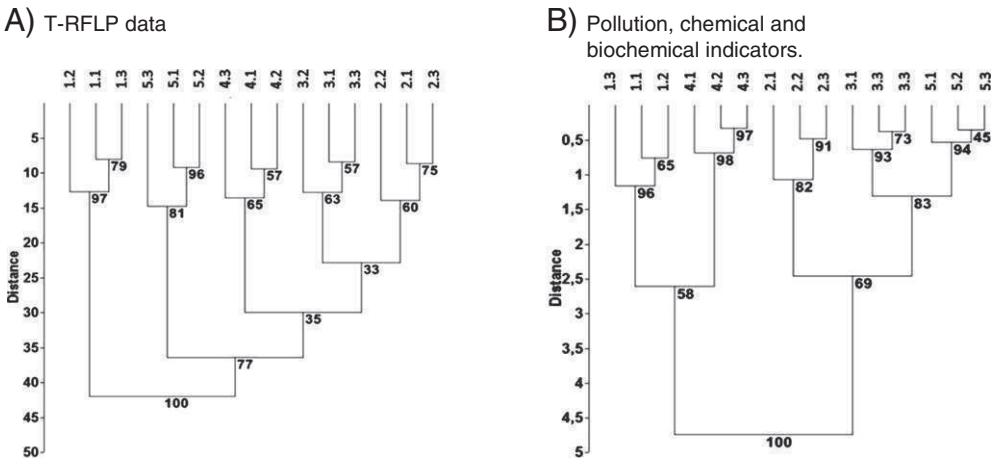

Fig. 4. Dendrograms from the cluster analysis with Ward's method and 500 bootstraps. The vertical axes represent squared Euclidean distances.

sediment assessment should rely on a combination of chemical, biological and biochemical essays for an accurate risk assessment and to wisely link impacts and environmental state to their determining human activities.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.scitotenv.2012.03.033.

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Supplementary material

Figure ESM1. T-RFLP electropherograms of the most significant samples of the five sites. Arrows indicate the positions of four illustrative OTUs, expressed in base pairs from the left size.

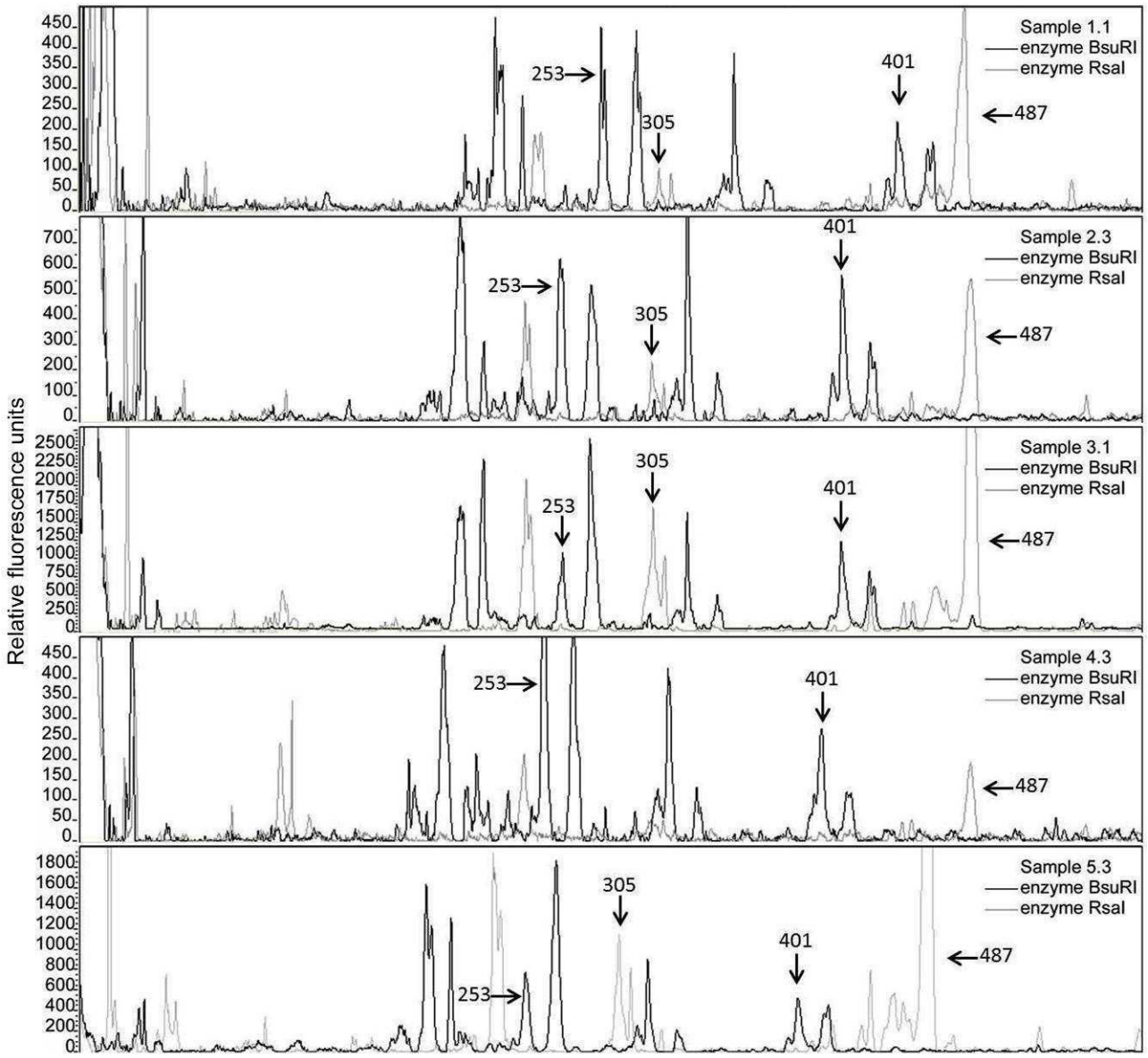

Table ESM1. Diversity indexes: Number of Operational Taxonomic Units (OTUs); Dominance Index (D); Shannon Index (H); Simpson Index (Ds).

Sample	OTUs	D	H	Ds
1.1	29	0.04	3.36	0.96
1.2	34	0.03	3.52	0.97
1.3	24	0.04	3.17	0.96
2.1	29	0.04	3.36	0.96
2.2	43	0.02	3.75	0.98
2.3	35	0.03	3.54	0.97
3.1	30	0.03	3.38	0.97
3.2	40	0.03	3.68	0.97
3.3	24	0.04	3.17	0.96
4.1	28	0.04	3.32	0.96
4.2	24	0.04	3.18	0.96
4.3	32	0.03	3.46	0.97
5.1	34	0.03	3.49	0.97
5.2	34	0.03	3.51	0.97
5.3	29	0.04	3.56	0.96

Table ESM2. Cross-correlation coefficients among indicators. TOC = total organic carbon; TN = total nitrogen; TP = total phosphorus; TBC = total bacterial count; PPOs = polyphenoloxidase activity; Asase = arylsulphatase activity; Dhase = dehydrogenase activity; Urease = urease activity; TPH = total petroleum hydrocarbons; OTUs = number of taxonomic unities; D = dominance index; H = Shannon's diversity index.

	TOC	TN	TP	NO3	Cl	SO4	TBC	PPO	Asase	Dhase	Urease	TPH	Cd	Ni	Pb	Cr	Cu	Zn	OTUs	D	H
TOC	1.00																				
TN	*0.95**	1.00																			
TP	*-0.59**	-0.50	1.00																		
NO3	0.09	-0.22	-0.47	1.00																	
Cl	0.95*	0.89*	*-0.62**	0.15	1.00																
SO4	-0.29	-0.48	-0.30	*0.72**	-0.12	1.00															
TBC	*0.89**	*0.87**	-0.37	-0.06	*0.73**	-0.44	1.00														
PPOs	-0.27	-0.41	0.09	0.43	-0.27	0.31	-0.24	1.00													
Asase	-0.05	0.22	*0.53**	*-0.90**	-0.14	*-0.62**	0.20	-0.33	1.00												
Dhase	-0.13	0.09	*0.76**	*-0.84**	-0.20	*-0.65**	0.12	-0.16	*0.90**	1.00											
Urease	0.42	0.43	-0.12	-0.10	0.46	-0.10	0.40	0.02	0.32	0.24	1.00										
TPH	0.25	0.00	0.05	*0.58**	0.16	-0.01	0.22	0.22	-0.49	-0.25	-0.06	1.00									
Cd	*0.82**	*0.87**	-0.41	-0.22	*0.78**	-0.47	*0.71**	-0.37	0.16	0.10	0.35	0.10	1.00								
Ni	*0.72**	*0.84**	-0.48	-0.34	*0.74**	-0.41	*0.57**	-0.35	0.29	0.12	*0.56**	-0.29	*0.83**	1.00							
Pb	0.08	-0.07	0.32	0.26	-0.03	0.02	0.27	0.13	-0.01	0.11	-0.03	*0.52**	-0.34	-0.46	1.00						
Cr	0.42	*0.56**	0.13	*-0.54**	0.31	*-0.57**	*0.57**	*-0.54**	*0.52**	0.50	-0.13	-0.09	0.44	0.26	0.26	1.00					
Cu	0.23	0.13	0.22	0.11	0.13	-0.13	0.33	0.28	0.19	0.25	*0.58**	0.44	0.10	0.13	0.46	-0.17	1.00				
Zn	0.29	0.21	0.23	0.04	0.19	-0.24	0.30	0.18	0.15	0.22	0.39	*0.52**	0.21	0.10	0.44	0.00	*0.85**	1.00			
OTUs	0.36	0.36	0.02	-0.09	0.31	-0.10	0.37	-0.18	0.26	0.19	0.19	0.22	0.35	0.15	0.32	0.50	0.30	*0.55**	1.00		
D	-0.34	-0.33	0.05	0.04	-0.32	0.02	-0.32	0.34	-0.26	-0.11	-0.38	-0.17	-0.24	-0.18	-0.35	-0.36	-0.34	-0.47	*-0.88**	1.00	
H	0.38	0.40	0.09	-0.19	0.34	-0.22	0.37	-0.21	0.31	0.30	0.10	0.18	0.41	0.23	0.31	*0.61**	0.28	0.51	*0.95**	*-0.78**	1.00

* = p-level <0.05 (Student's t-test)

Table ESM3. Principal component analysis

	Factor 1	Factor 2	Factor 3
Cd	*0.89**	0.18	0.09
Ni	*0.86**	0.25	-0.12
Pb	-0.21	-0.08	*0.74**
Cr	0.41	0.62	0.20
Cu	0.05	-0.01	*0.78**
Zn	0.11	0.03	*0.86**
Total petroleum hydrocarbons	0.02	-0.47	*0.65**
Total organic carbon	*0.94**	-0.09	0.25
Total N	*0.96**	0.20	0.11
Total P	*-0.65**	0.55	0.36
Nitrate	-0.03	*-0.97**	0.19
Chloride	*0.92**	-0.16	0.18
Sulphate	-0.24	*-0.67**	-0.01
Total bacterial count	*0.79**	0.13	0.34
Dehydrogenase activity	-0.13	*0.91**	0.25
Polyphenoloxidase activity	-0.39	-0.44	0.21
Arylsulphatase activity	0.02	*0.95**	0.13
Urease activity	0.43	0.11	0.31
Number of OTUs	0.30	0.21	*0.69**
Dominance index	-0.28	-0.13	-0.55
Shannon's diversity index	0.28	0.21	0.60
Expl.Var	*6.11*	*4.53*	*4.14*
Prp.Totl	*29.08%*	*21.55%*	*19.72%*

Table ESM4. Results of canonical correlation analyses (CCA) among pollution, chemical and biological indicators (first canonical variates)

			Chemical vs. pollution indicators	Biological vs. pollution indicators	Chemical vs. biological indicators
		R	1.00	0.99	0.99
		P	0.00	0.00	0.00
Total petroleum hydrocarbons	-0.23		0.21		
Pollution indicators:		Cd	-0.80*	0.60*	
		Ni	-0.72*	0.34	
		Pb	-0.06	0.37	
		Cr	-0.38	0.90*	
		Cu	-0.21	0.22	
		Zn	-0.27	0.38	
	Extracted variance		*21.15%*	*24.08%*	
Total organic carbon	-0.99*			-0.31	
Chemical indicators:		Total N	-0.93*		-0.03
		Total P	0.61*		0.68*
		Nitrate	-0.12		-0.93*
		Chloride	-0.97*		-0.34
		Sulphate	0.22		-0.51
	Extracted variance		*54.27%*		*30.43%*
Total bacterial count			0.72*	-0.11	
Polyphenoloxidase activity			-0.38	-0.31	
Biological indicators:		Arylsulphatase activity		0.50	0.91*
		Dehydrogenase activity		0.54*	0.89*
		Urease activity		0.10	0.03
		Number of OTUs		0.67	0.12
		Dominance index		-0.41	0.01
Shannon's diversity index			0.65	0.17	
	Extracted variance			*28.52%*	*22.40%*

* values used to interpret the CCA

3.2 Bacterial communities in polluted seabed sediments: a molecular biology assay in Leghorn harbor

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Concept by: All authors.

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Abstract

Seabed sediments of commercial ports are often characterized by high pollution levels. Differences in number and distribution of bacteria in such areas can be related to distribution of pollutants in the port and to the sediment conditions. In this study, the bacterial communities of five sites from Leghorn harbor seabed were characterized and the main bacterial groups identified. T-RFLP was used for all samples; two 16S rRNA libraries and *in silico* digestion of clones were used to identify fingerprint profiles. Library data, phylogenetic analysis, and T-RFLP coupled with *in silico* digestion of the obtained sequences, evidenced the dominance of *Proteobacteria* and the high percentage of *Bacteroidetes* in all sites. The approach highlighted similar bacterial communities between samples coming from the five sites, suggesting a modest differentiation among bacterial communities of different harbor seabed sediments, and hence the capacity of bacterial communities to adapt to different levels and types of pollution.

Key words

Harbor Sediments, SSU rRNA library, *Proteobacteria*, TRFLP, Mothur.

Introduction

Sea stretches of commercial ports are often characterized by high levels of pollution in sediments, low oxygen concentrations in the water column, and low biodiversity of benthic communities (Estacio et al., 1997) that may cause the decrease of fauna in the seabed of these areas (Dhainaut-Courtois et al., 2000). Further reasons of impact on harbor sea stretches often come from high concentrations of organic matter due to eutrophication, time variability of sediment deposition, allochtonous inputs, and low hydrodynamism (Danulat et al., 2002; Guerra-García and García-Gómez, 2005). These reasons demonstrate that the activities conducted in ports, lead to critical conditions in the environments, and the organisms develop several ways to adapt to the alterations of water and the sediments parameters (Cognetti and Maltagliati, 2005). A crucial characteristic of harbor sites is the removal of organic matter. Consumption capacity of organic matter is mainly due to biotic uptake and degradation performed by microorganisms. This process is generally insufficient to maintain the equilibrium of ecosystems; in most cases the presence of detrital organic matter (which increases anoxic conditions), heavy metals, polychlorinated biphenyls (PCBs, Azad et al., 2012), and other toxic compounds plays also a crucial role in negatively impacting the seabed biocenosis (Cornelissen et al., 2008; Kaufman et al., 2004). Although the origin and concentration of pollutants in seabed harbor sediments can be different, many studies demonstrate that in most cases, pollution is mainly due to the presence of hydrocarbons, heavy metals (Buruaem et al., 2012) and organic matter. Possible methods for hindering the problem of contaminated aquatic sediments have been widely discussed (Chapman and Smith, 2012). Polluted seabed sediments can be sanitised via in *situ* actions, which always require an accurate knowledge of the local biocenosis. Moreover, it was demonstrated that bacterial communities have great potential to be used as sensitive indicators of contamination in aquatic sediments (Sun et al., 2012). New and accurate methods to study the composition of microbial communities in harbor seabed sediments are necessary for these reasons. Differences in number and distribution of bacteria, in such a large area as a harbor, can be related to pollutant distribution and general sediment state. Today, Leghorn's harbor is one of the most important ports in the Mediterranean Sea, linked with more than 300 ports worldwide. It is a multipurpose port that can cater for all kinds of vessels and handle all kinds of goods, as well as passenger traffic. It sports a marine area of 1.6 km^2 and a useable land area of 2.6 km^2. The harbor offers 11 km of quays with more than 90 berths, with up to 13 m draughts. The total extension covers one km^2 surface outdoor and 70000 m^2 indoor areas. In the present research, the bacterial community composition of Leghorn harbor seabed sediments was studied with a molecular fingerprint approach, and with the construction of libraries of 16S rRNA gene. The

Terminal Restriction Fragment Length Polymorphism (T-RFLP) approach was used for all samples to identify the community structure. *In silico* digestion of clones retrieved from two clone libraries (one for a less polluted sample and one for a more polluted sample) were used for the identification of bacterial Operational Taxonomic Units (OTUs) in the T-RFLP fingerprint profiles. The aim of the study was to highlight how different conditions of port areas influence the composition of microbial communities and provide hints of dynamics for nutrient removal.

Materials and methods

Description of study area, collection and storage of samples

Figure 1 shows the whole Leghorn's harbor area. The black dots indicate the five sites of sampling; in each site, three replicates were collected for a total of fifteen samples. Sites one and two were chosen for the additional library construction due to their chemical characteristics. According to the site assessment performed by the Port Authority (Iannelli et al., 2012), site one, which is located in the passenger terminal, represents one of the less polluted spots, while site two, which is located inside the "Darsena Calafatari" ship repairing section, presents the highest contamination levels. The latter site in particular, which has never been dredged for 60 years, exhibits high concentrations of heavy metals and hydrocarbons (Iannelli et al., 2012).

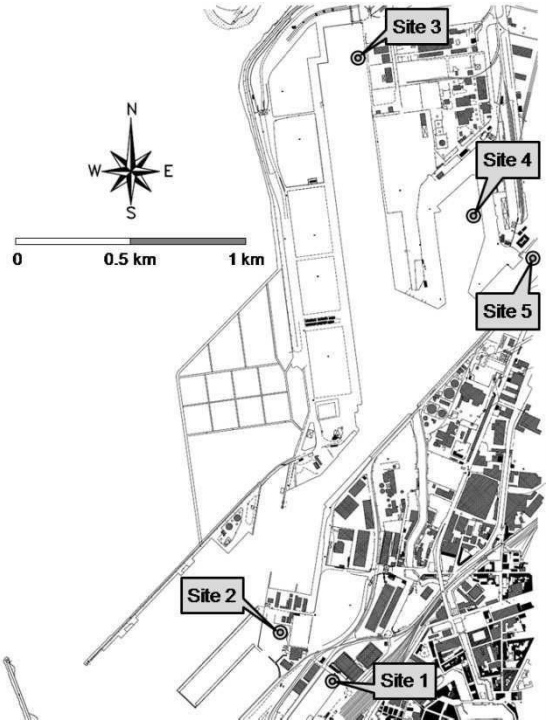


Figure1: representation of the whole Leghorn harbor area. Points represent the sampling sites in the seabed of the area.

The samples analyzed in this work were collected from the seabed of Leghorn harbor on the 3rd of November 2009 by a scuba diver and immediately brought to the laboratory for the extraction of the total DNA. The two samples chosen for the library construction are representative of two different environments in the harbor seabed, the one named 2.1 (site 2 replicate 1) rich in nutrients and characterized by high level of nitrates, sulfates and high concentration of pollution indicators, and the one named 1.2 (site 1 replicate 2) representative of a less polluted environment.

DNA extraction, construction of 16S rRNA gene libraries, sequencing, T-RFLP analysis and *in silico* digestion of sequenced clones

Total DNA extraction was performed on the 15 samples using a Soil master™ DNA extraction kit (Epicentre Biotechnologies, WI U.S.A.). to build the library up (Amann et al., 1995) with samples 1.2 and 2.1, 16S rRNA genes were directly amplified from extracted DNA using universal bacterial primers, 8F (5'-AGA GTT TGA T(CT)(AC) TGG CTC AG-3') and reverse 1492R (5'-GG(AGCT)(AT)AC CTT GTT ACG ACT T-3') (Lane et al., 1991); the amplification products was cloned in a plasmid vector (pCRs2.1-TOPOs, TOPO TA Cloning Kit, Invitrogen, U.K.) and inserted in chemically competent cells (OneShot TOP10, Invitrogen U.K.). The inserted fragments from a representative number of clones were then amplified by control PCR with M13F and M13R universal primers. The inserted genes showing a size similar to the 16S rRNA gene size were directly sequenced with primers M13F and M13R by the Macrogen Inc. sequencing service (South Korea). For T-RFLP analysis, the amplification of the 16S rRNA gene was performed with the same procedures as libraries construction, and primer 8F was labeled with FAM fluorochrome (Applied Biosystem CA, U.S.A.). The template was digested with two different restriction endonucleases: BsuRI (GG^CC, 0.2 u/µl, Fermentas, Canada) and RsaI (GT^AC, 0.2 u/µl, Fermentas). Digested DNA was precipitated with cold ethanol 100% to eliminate salts at 4°C and 10,000 RCF. For each reaction, a mix with 1.2µl of loading buffer (GeneScan™ 600 LIZ, Applied Biosystem), a maximum of 5.5µl of sample (calculated after cold ethanol precipitation on the bases of its final concentration) and 13.3 µl of deionized formamide (Applichem, Germany) was prepared. Capillary electrophoresis was performed with Abi Prism 310 Genetic Analyzer (Applied Biosystem); T-RFLP profiles were analyzed using GeneScan™ analysis software (Applied Biosystem) and the data matrix was transformed for statistics as described in Iannelli et al, (2012). Non Metric Multidimensional Scaling (NMDS) was performed on the whole T-RFLP dataset with the Bray-Curtis coefficient and the Shannon diversity index was also calculated. All statistical analyses were performed using PAST software v.2.15 (PAleontological STatistic, Hammer et al., 2001). Further details on the applied techniques are reported in Chiellini et al, (2012). All sequences obtained from the construction of both libraries were digested *in silico* by searching the restriction site on Arb

database, recognized by restriction enzymes BsuRI and RsaI in order to know the size of the terminal restriction fragments representative of the OTUs. With the *in silico* digestion of retrieved clones, we could correlate the peaks on T-RFLP fingerprints of the 15 samples from Leghorn harbor seabed (3 replicates in 5 different sites) to specific OTUs. The coverage percentages of the peaks corresponding to the recognized OTUs and corresponding bacterial phyla and classes were calculated for each electropherogram.

Detection of chimeric sequences and phylogenetic analysis

All the retrieved sequences were checked to identify chimeras using the Bellerophon server (Hugenholtz et al. 2003). It was decided to analyze chimeric sequences "cutting" them in proximity of the recombination site identified by Bellerophon software and considering the obtained fragments as independent sequences belonging to different organisms. Fragments were treated in the same way as complete sequences from other screened clones, they were included in 16S rRNA libraries analysis, but they were not included in phylogenetic trees construction, due to their short length. NCBI BLAST analysis (Altschul et al. 1997) was used to determine a preliminary affiliation of clone sequences. After BLAST analysis, sequences were inserted in SILVA 104 database (Pruesse et al. 2007) and aligned using the appropriate tool from the ARB program package (Ludwig et al. 2004). A deeper phylogenetic analysis was performed on *Proteobacteria* phylum, the most represented group in the two libraries, in the 15 T-RFLP profiles, and one of the main groups previously retrieved in marine sediments (e.g. Nithya et al., 2012, Elsabé et al., 2012). Two phylogenetic reconstructions on different subclasses of *Proteobacteria* were performed independently. The analysis focuses in particular on two groups of sequences, one including bacteria belonging to the *Deltaproteobacteria* subclass using *Epsilonproteobacteria* as an outgroup, and the second focused on the *Gammaproteobacteria* group with some sequences of *Alphaproteobacteria* used as an outgroup. Both trees were constructed using Maximum Likelihood algorithm, 100 bootstraps and a filter specifically designed for the selection of sequences in each tree, considering only positions conserved in at least 10% of sequences. A distance matrix constructed using Neighbor Joining algorithm was also calculated and examined for clone sequences of the two samples that clustered together with described and cultivated bacterial species, in order to understand which clades represent the species-level or the genus-level groups. In the construction and interpretation of similarity matrices, we used the 95% limit to represent the threshold for genus definition and the limit of 97% to represents the threshold for OTUs (Stackebrandt et al., 2002).

Statistical analysis of clone libraries

OTUs were identified in each library using MOTHUR software (Schloss et al., 2008). Richness and alpha diversity indices including the Chao1 Estimator (Chao, 1984) and the Shannon index were calculated for each library at different cutoff levels (0.01, 0.03, and 0.05). Chao1 estimates total species richness as: Chao1 = Sobs $+n_1^2 / 2n_2$, where Sobs is the number of observed species, n_1 is the number of singletons (species captured once), and n_2 is the number of doubletons (species captured twice) The Shannon's diversity index (Shannon, 1949) was obtained as $H = -\Sigma p_i \ln p_i$ where p_i is the population of each species i. The resulting product was summed up across species, and multiplied by -1 (Magurran et al., 1998). The MOTHUR software was also applied to calculate the number of OTUs shared among the two different libraries at different cutoff levels. The LIBSHUFF software (Singleton et al., 2009) based on the Jukes-Cantor pairwise distance matrix was also applied to find out similarities in the two libraries, as described in Zhang et al. (2007a).

Results

Construction of 16S rRNA gene libraries and detection of chimeric sequences

Two libraries were constructed on samples 1.2 and 2.1. Sequencing was performed on a total of 194 clones, 101 of which taken from sample 1.2 and 93 from sample 2.1. All obtained sequences were submitted online on DDBJ/EMBL/GenBank databases. They are available under the accession numbers from HE803828 to HE804037. The percentages of detected chimeric sequences were 11.9% in sample 1.2 (12 sequences) and 4.3% in sample 2.1 (4 sequences). All chimeric sequences were composed of partial sequences coming from only two different organisms. Considering fragments constituting chimeras as independent sequences belonging to different organisms, the library from sample 1.2 is composed of a total of 115 sequences, and the library from sample 2.1 of 95 sequences. The affiliation of nucleotide sequences was determined by BLAST analysis. In both libraries, the highest percentage of screened clones emerged as belonging to the *Proteobacteria* phylum (74% to library 1.2 and 73% to library 2.1, Figure 2, upper part).

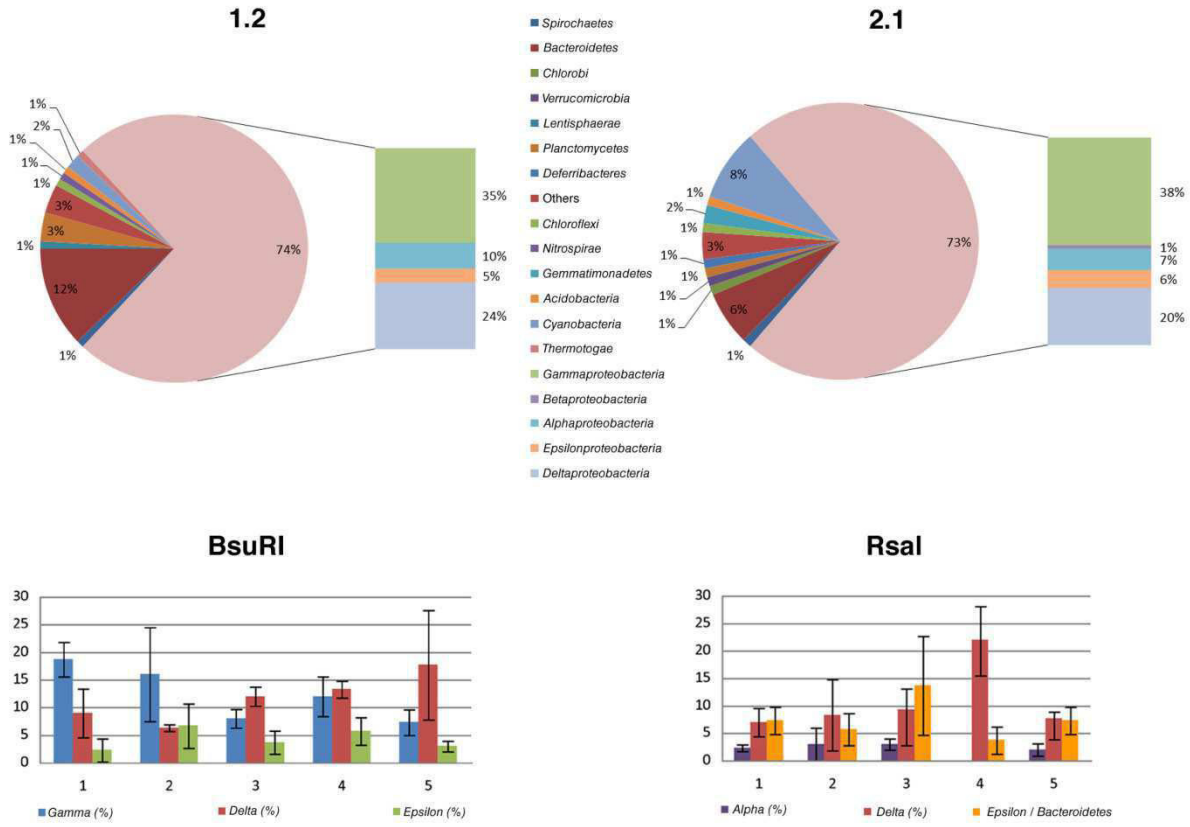


Figure 2: Upper part: comparison among main phyla of bacteria detected in the two clone libraries, the two larger pink sectors represent *Proteobacteria*; lower part: coverage percentages of the main bacterial groups attributed through *in silico* digestion of the 15 T-RFLP elecropherograms. Each of the five sites is represented by the mean of three replicates; standard deviation is shown by the black bar.

Within this group, bacteria belonging to *Alphaproteobacteria*, *Gammaproteobacteria, Deltaproteobacteria* and *Epsilonproteobacteria* classes were present in all libraries, while bacteria belonging to *Betaproteobacteria* class were detected only in library 2.1 with low percent values (1%). In particular, the *Gammaproteobacteria* subclass was the most represented one in both 1.2 and 2.1 libraries (35% and 38% respectively), followed by the *Deltaproteobacteria* subclass (respectively 24% and 20%). The second most represented bacterial taxon detected in both libraries was the *Bacteroidetes* phylum, represented by 12% of the total sequences in library 1.2, and by 6% of the sequences in library 2.1. There are some differences in the distribution of bacterial phyla detected in the two libraries, especially for minor groups such as *Lentisphaerae*, *Nitrospirae* and *Thermotogae* phyla, that were present only in sample 1.2 with percent values lower than 1%, and *Chlorobi*, *Verrucomicrobia*, *Deferribacteres* and *Gemmatimonadetes* phyla, that were detected in sample 2.1 with percentages ranging from 1% to 2%.

T-RFLP analysis

NMDS analysis on T-RFLP data matrix highlights some groups of samples that correspond to the five sampling sites (Figure 3).

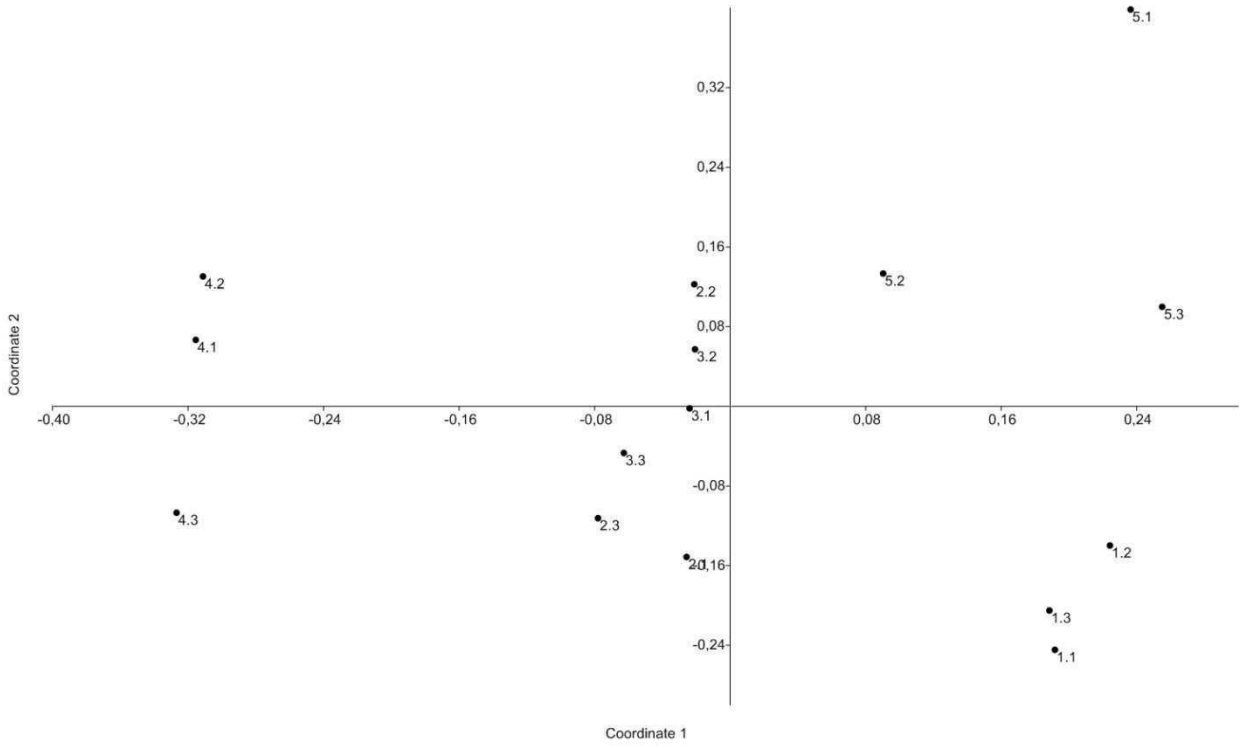


Figure 3: NMDS plot obtained from T-RFLP data analysis.

The grouping of the triplicates is mainly evident for sites 1 (samples 1.1, 1.2 and 1.3) and 4 (samples 4.1, 4.2 and 4.3). Triplicates of sites 2 and 3 group all together in the central part of the plot and triplicates from site 5 (samples 5.1, 5.2 and 5.3) are all located in the second quadrant of the plot, isolated from all the other samples. The Shannon diversity index is calculated from the T-RFLP data matrix ranged from 3.17 to 3.75.

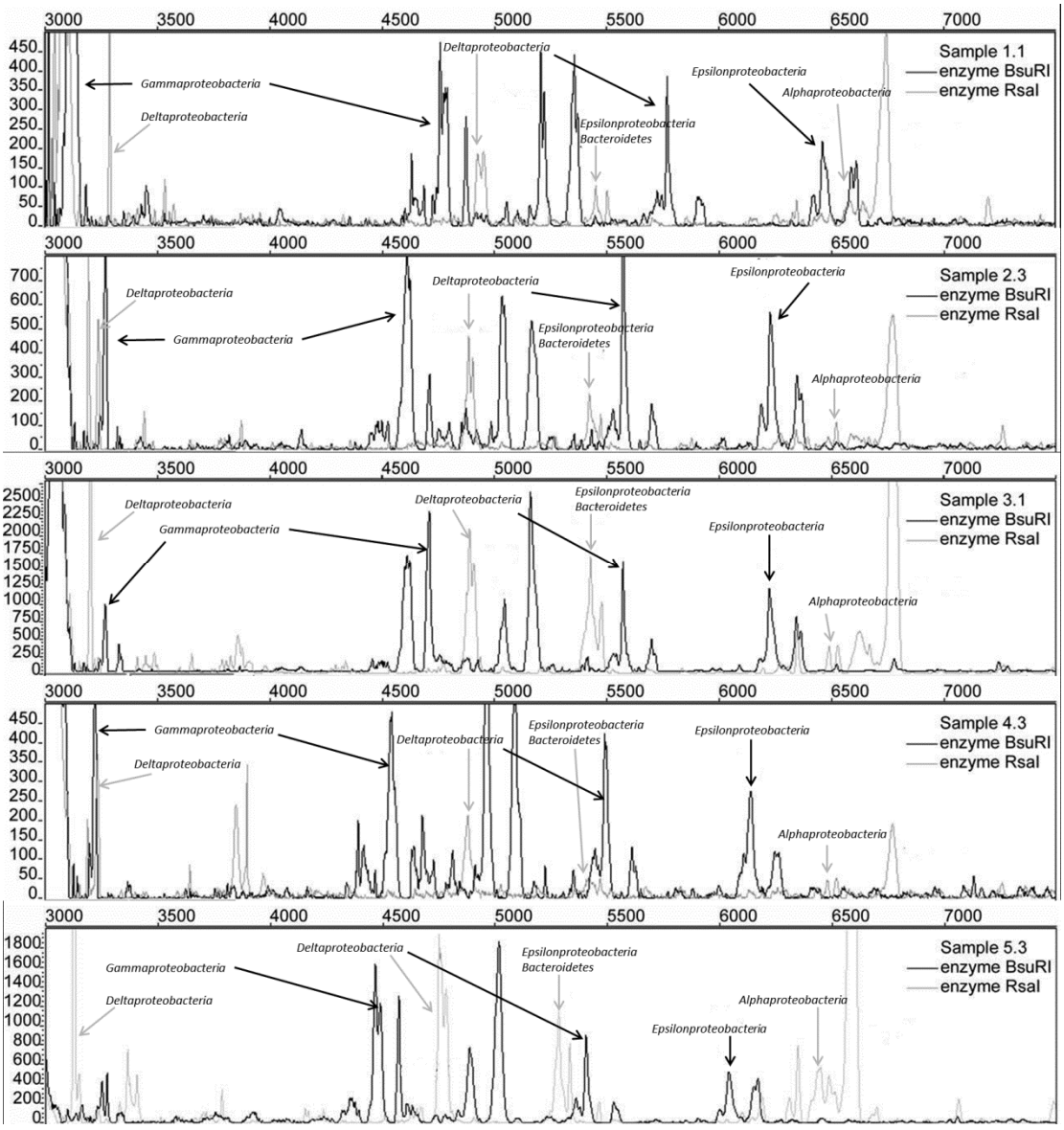


Figure S1: T-RFLP peak attribution after in silico digestion.

Figure S1 (supplementary material) presents some of the results of the attribution of the peaks obtained by *in silico* clone digestion, in comparison with the peaks detected in the microbial communities of the five sampling sites. The coverage percentages of the bacterial groups in T-RFLP profiles are shown in the lower part of Figure 2. Not all the bacterial OTUs detected with the rDNA clone library could be retrieved in the T-RFLP electropherograms of the 15 samples. All OTUs retrieved in the T-RFLP electropherograms through *in silico* digestion belong to subclasses of the *Proteobacteria* phylum, with the only exception of *Betaproteobacteria* class, which has not been detected. The lower part of Figure 2 highlights a dominance of *Gammaproteobacteria* in sites 1 and 2, and a dominance of *Deltaproteobacteria* organisms in sites 3, 4 and 5. The less represented group of *Proteobacteria* is the *Epsilonproteobacteria* in all BsuRI samples. When considering RsaI

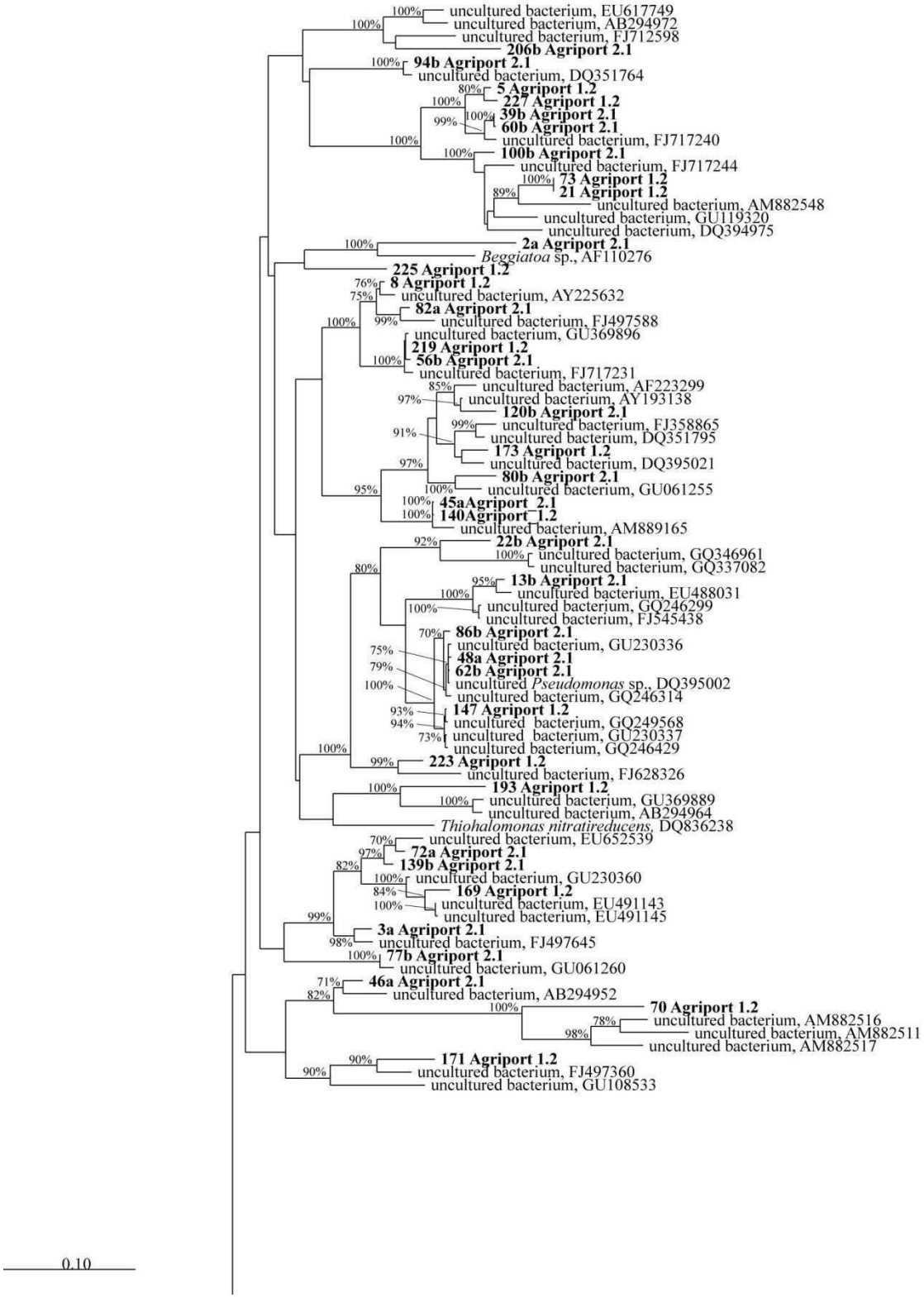
restriction endonuclease, the *Alphaproteobacteria* subclass emerged as the less represented group in each case.

Phylogenetic analysis

In order to assess the relative abundance of *Proteobacteria* related sequences, we focused on the phylogenetic analysis of this group of organisms. In particular, two phylogenetic trees were built: the first one comprised all the retrieved *Deltaproteobacteria* and *Epsilonproteobacteria* clone sequences and their closer relatives (Fig. S2, supplementary material); the second one included all the retrieved *Gammaproteobacteria* and *Alphaproteobacteria* clone sequences and their closer relatives (Fig. S3, supplementary material). The trees were constructed using PHYML with 100 bootstraps (Guindon and Gascuel 2003) from the ARB package. The majority of sequences in the trees derive from studies about marine sediment samples. Some of our clones are closely related to symbiotic bacteria found in marine metazoan bodies (Kurahashi et al., 2007, Kaesler et al., 2008). These sequences belong to the *Gammaproteobacteria* subclass; clone 147b from library 2.1 shows a 99% similarity with the already mentioned *Spongiispira norvegica* (AM117931, Kaesler et al., 2008), while the OTU composed of clones 228, 143, 162, 61 and 160, all coming from library 1.2, show a 98% similarity with *Endozoicomonas elysicola* (AB196667, Kurahashi et al., 2007).


uncultured bacterium, FM179902,
uncultured bacterium, FN396770,
uncultured bacterium, AM040136
164 Agriport 1.2
16 Agriport 1.2
uncultured bacterium, FN421322
19b Agriport 2.1
uncultured bacterium, AB530217
uncultured bacterium, EU617853
uncultured bacterium, GU230394
181b Agriport 2.1
uncultured bacterium, FN396678
uncultured bacterium, AY907763
uncultured bacterium, AJ620500
7 Agriport 1.2
230 Agriport 1.2
uncultured bacterium, DQ394998
uncultured bacterium, GQ259292
194 Agriport 1.2
190b Agriport 2.1
158 Agriport 1.2
uncultured bacterium, EU592471
52b Agriport 2.1
uncultured bacterium, FM242403
uncultured bacterium, DQ351771
Desulfosarcina ovata, Y17286
184b Agriport 2.1
Desulfonema magnum, U45989
159b Agriport 2.1
uncultured bacterium, AY799901
uncultured bacterium, FN429813
61b Agriport 2.1
uncultured bacterium, FJ748772
uncultured bacterium, AM746084
173b Agriport 2.1
uncultured bacterium, GQ246438
uncultured bacterium, DQ395025
uncultured bacterium, EU386051
221 Agriport 1.2
uncultured bacterium, GU302477
uncultured bacterium, Y771932
179 Agriport 1.2
uncultured bacterium, DQ351762
uncultured bacterium, AY771964
uncultured Desulfocapsa sp., DQ831542
188b Agriport 2.1
Desulfobulbus sp., U85473
Desulfobulbus japonicus, AB110549
63 Agriport 1.2
uncultured bacterium, DQ831547
uncultured bacterium, DQ831555
uncultured bacterium, GQ246300
uncultured bacterium, GQ246285
uncultured bacterium, DQ831548
51 Agriport 1.2
uncultured bacterium, FJ416070
uncultured bacterium, AB530214
59b Agriport 2.1
191b Agriport 2.1
188 Agriport 1.2
uncultured bacterium, FJ202627
uncultured bacterium, EF123510
Desulfovibrio sp. r02, AB470955
0.10

Figure S2: Maximum likelihood phylogenetic tree made with PHYML and 100 bootstrap pseudoreplicates. The tree represents the phylogenetic position of characterized *Deltaproteobacteria* and *Epsilonproteobacteria* clone sequences together with closely related sequences present in the database. Characterized sequences are in bold.


uncultured bacterium, EU617749
uncultured bacterium, AB294972
uncultured bacterium, FJ712598
206b Agriport 2.1
94b Agriport 2.1
uncultured bacterium, DQ351764
5 Agriport 1.2
227 Agriport 1.2
39b Agriport 2.1
60b Agriport 2.1
uncultured bacterium, FJ717240
100b Agriport 2.1
uncultured bacterium, FJ717244
73 Agriport 1.2
21 Agriport 1.2
uncultured bacterium, AM882548
uncultured bacterium, GU119320
uncultured bacterium, DQ394975
2a Agriport 2.1
Beggiatoa sp., AF110276
225 Agriport 1.2
8 Agriport 1.2
uncultured bacterium, AY225632
82a Agriport 2.1
uncultured bacterium, FJ497588
uncultured bacterium, GU369896
219 Agriport 1.2
56b Agriport 2.1
uncultured bacterium, FJ717231
uncultured bacterium, AF223299
uncultured bacterium, AY193138
120b Agriport 2.1
uncultured bacterium, FJ358865
uncultured bacterium, DQ351795
173 Agriport 1.2
uncultured bacterium, DQ395021
80b Agriport 2.1
uncultured bacterium, GU061255
45aAgriport_2.1
140Agriport_1.2
uncultured bacterium, AM889165
22b Agriport 2.1
uncultured bacterium, GQ346961
uncultured bacterium, GQ337082
13b Agriport 2.1
uncultured bacterium, EU488031
uncultured bacterium, GQ246299
uncultured bacterium, FJ545438
86b Agriport 2.1
uncultured bacterium, GU230336
48a Agriport 2.1
62b Agriport 2.1
uncultured Pseudomonas sp., DQ395002
uncultured bacterium, GQ246314
147 Agriport 1.2
uncultured bacterium, GQ249568
uncultured bacterium, GU230337
uncultured bacterium, GQ246429
223 Agriport 1.2
uncultured bacterium, FJ628326
193 Agriport 1.2
uncultured bacterium, GU369889
uncultured bacterium, AB294964
Thiohalomonas nitratireducens, DQ836238
uncultured bacterium, EU652539
72a Agriport 2.1
139b Agriport 2.1
uncultured bacterium, GU230360
169 Agriport 1.2
uncultured bacterium, EU491143
uncultured bacterium, EU491145
3a Agriport 2.1
uncultured bacterium, FJ497645
77b Agriport 2.1
uncultured bacterium, GU061260
46a Agriport 2.1
uncultured bacterium, AB294952
70 Agriport 1.2
uncultured bacterium, AM882516
uncultured bacterium, AM882511
uncultured bacterium, AM882517
171 Agriport 1.2
uncultured bacterium, FJ497360
uncultured bacterium, GU108533
0.10

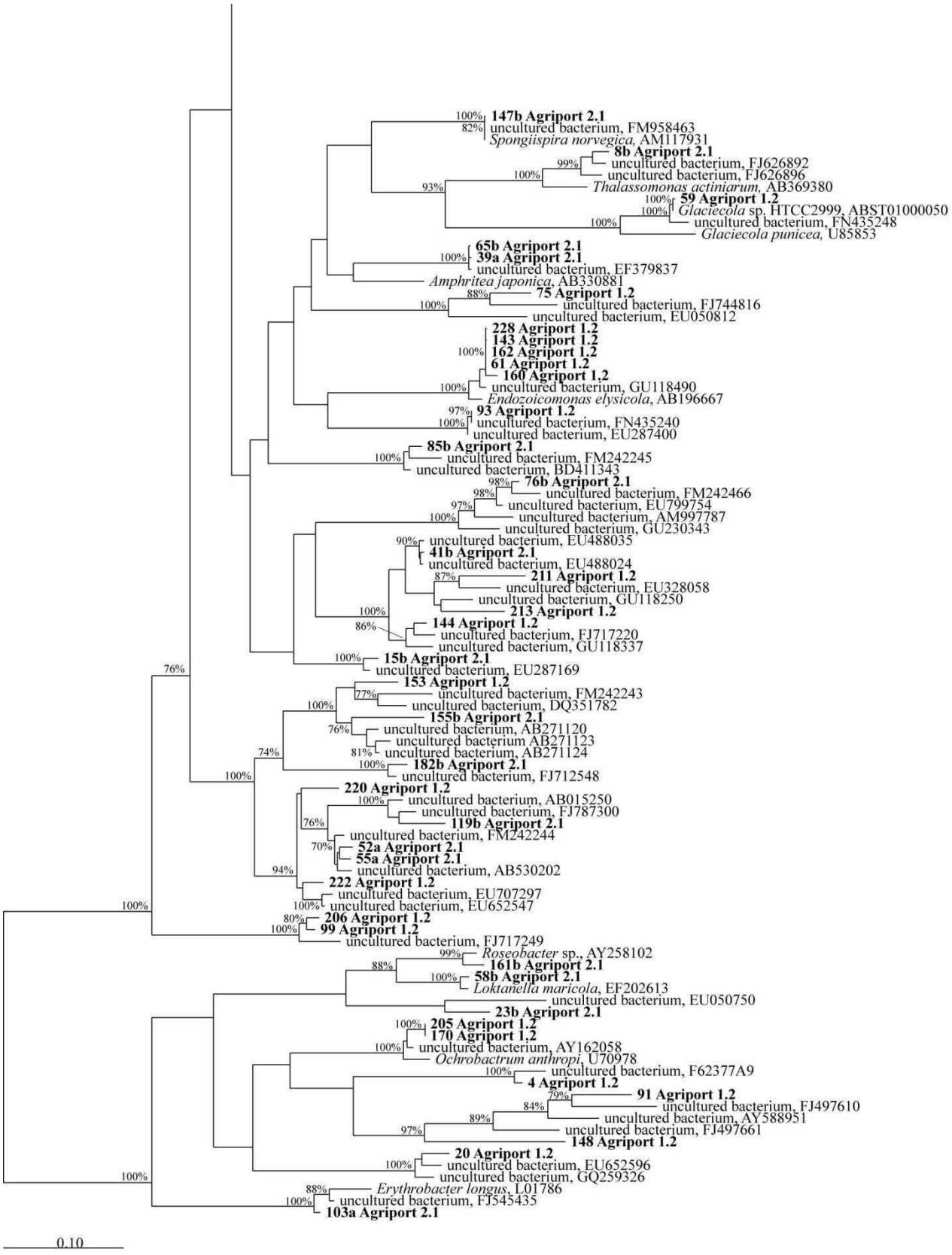


Figure S3: Maximum likelihood phylogenetic tree made with PHYML and 100 bootstrap pseudoreplicates. The tree represents the phylogenetic position of characterized *Alphaproteobacteria* and *Gammaproteobacteria* clone sequences together with closely related sequences present in the database. Characterized sequences are in bold.

All the clones related to the *Deltaproteobacteria* subclass, cluster together with species having a metabolism involving the presence of sulphur (most sulphate reducing microorganisms). The

majority of published sequences that are closely related to our clone sequences belong to uncultured bacteria, with the exception of some *Deltaproteobacteria*, which belong to *Desulfobulbus*, *Desulfosarcina* and *Desulfonema* genera, all providing for the sulfate reduction in the marine environment. The similarity matrix evidenced that, within the *Deltaproteobacteria* subclass, our clone 188b from library 2.1 showed a 95% similarity with the described *Desulfobulbus japonicus* species (AB110549 Suzuki et al., 2007), while the 52b clone from library 2.1 showed a 96% similarity with the described *Desulfosarcina ovata* (Y17286, Harms et al., 1999). The 124b and 130b sequences from library 2.1 and the sequence 184 from library 1.2 are all parts of the same OTU. They cluster together with the described *Epsilonproteobacteria* species *Arcobacter nitrofigilis* (L14627, Wesley et al., 1995), a symbiotic bacteria characterized by a nitrogen fixing metabolism.

Statistical analysis of clone libraries

Table 1 shows the results concerning richness and alpha diversity indices in each of the two constructed libraries at three different cutoff levels. The Shannon diversity index shows similar values in both sampling sites, at all cutoff level. The richness index (Chao 1 estimator) emerged as being higher in sample 1.2 at 0.01 cutoff level, and lower in sample 1.2 at 0.03 and 0.05 cutoff values. The number of detected OTUs emerged as being very similar in both sites. The OTUs at a 0.03 cutoff value are represented by the same number in both cases; library 2.1 at 0.01 and 0.05 cutoff values show less detected OTUs then library 1.2 at the same cutoff levels (Table 1).

Sample	cutoff	Observed richness (OTUs)	Chao 1 estimator	Shannon Index
1.2	0.01	82	667,2	4,36
	0.03	75	258,3	4,24
	0.05	71	193,8	4,17
2.1	0.01	80	620,2	4,33
	0.03	75	306,1	4,24
	0.05	68	239,1	4,07

Table 1: Alpha diversity indices for the two samples at different cutoff values

Table 2 presents the MOTHUR analysis of OTUs that are shared between the two different libraries; at 0.01 cutoff (specie level) there are 7 OTUs shared between site 1.2 and 2.1; at the 0.03 cutoff there are 12 shared OTUs; at 0.05 cutoff the number of shared OTUs, which usually represents the genus level, rises up to 17.

Cutoff	Observed richness shared between 1.2 and 2.1
0.01	7
0.03	12
0.05	17

Table 2: OTUs shared between site 1.2 and 2.1 calculated with MOTHUR software.

Figure 4 presents the results of the LIBSHUFF analysis. The two lines, representing the homologous and heterologous curves, are almost totally overlapping, thus indicating that the samples are similar to each other, as confirmed by the high p value ($p>0.025$ with Bonferroni Correction, as described in Singleton et al., 2001).

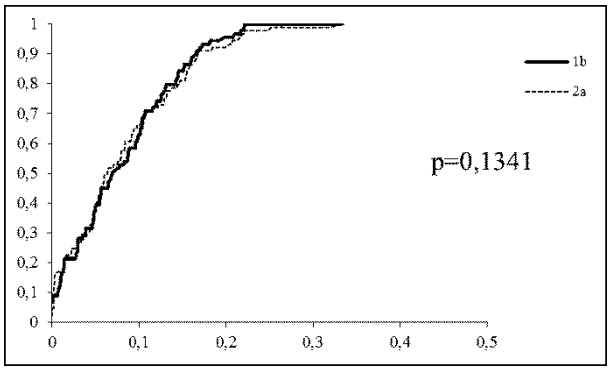


Figure 4: Results of LIBSHUFF comparison between the 1.2 and 2.1 libraries. The solid line indicate the homologous coverage curve $C_X(D)$ and the broken line indicate the heterologous coverage curve $C_{XY}(D)$. The p value is $p>0.025$, thus indicating that the two libraries are similar to each other in OTU composition. The y axis represents the Coverage (C) while the x axis represents the Evolutionary Distance (D).

Discussion

Molecular approach analysis is commonly used in recent literature for the study of bacterial communities in marine sediments (Zeng et al., 2011, Wang et al., 2009, Elsabé et al., 2012, Nithya et al., 2012), and to study the influence of bacterial communities in pollutant removal from marine

sediments (Pringault et al., 2010). The two libraries highlighted a substantial similarity concerning the abundance and diversity of the sequences represented in the two different sites, even if the chemical composition was different (Iannelli et al., 2012). The similarity between the two clone libraries is shown by the alpha diversity indices calculated with MOTHUR software (Table 1). The Shannon Diversity Index calculated for both libraries at different cutoff levels, on the base of the DNA sequences obtained after the library construction, ranged from 4.07 to 4.36. The same Shannon Diversity Index calculated on the base of T-RFLP profiles of the 15 samples ranged from 3.17 to 3.75. This difference can be explained because the diversity in a sample is underestimated when only T-RFLP profiles are used. This approach does not register rare OTUs. The Shannon Diversity Index used in this work should provide more reliable values, although an increase of the sample size of the sequenced clones of each library could probably provide even more accurate values. The construction of the two libraries and the screening of clones coupled with T-RFLP peak identification highlighted the dominance of *Proteobacteria* related microorganisms in all the five sites. There are no significant differences among the coverage percentages of different subclasses of the *Proteobacteria* phylum among the two libraries and the 5 sites; these data are in agreement with previously published studies, where PCR-based techniques on marine sediment samples revealed a dominance of bacteria related to *Proteobacteria* phylum (Zeng et al., 2011, Elsabé et al., 2012) or to *Firmicutes*, *Deltaproteobacteria*, and *Gammaproteobacteria* (Köchling et al., 2011, Castle et al., 2004). In other papers, the analysis of marine sediment showed a dominance of *Actinobacteria* (Nithya et al., 2012). In this study, no significant differences in the coverage percentage of different subclasses of the *Proteobacteria* phylum were observed among the five sites; this agrees with other studies in which different libraries of marine sediments collected in different sites showed similar bacterial compositions (Zeng et al., 2011). The *Bacteroidetes* phylum is the only further phylum with an appreciable presence in all sites, and the percentages of abundance are comparable to those found by other authors (Nithya et al., 2012, Zeng et al., 2011). This result can mean that bacteria belonging to these groups play the main role in nutrient recycling in the harbor seabed ecosystem. Considering the digestion profiles obtained with the BsuRI restriction endonuclease, *Gammaproteobacteria* emerged as being dominant in site 1 and 2, while the sediments were dominated by *Deltaproteobacteria* organisms in sites 3, 4 and 5. Considering the RsaI restriction endonuclease, sites 2, 4 and 5 emerged as being dominated by *Deltaproteobacteria* microorganisms, while sites 1 and 3 were dominated by *Epsilonproteobacteria* and *Bacteroidetes*, two groups of bacteria sharing the same peak interval. Some groups of bacteria could be detected only with one of the two adopted restriction enzymes. For instance, *Alphaproteobacteria* were not detected with BsuRI and *Gammaproteobacteria* were not detected with RsaI. This circumstance can be explained by the fact that the first restriction site in the gene sequences of those bacteria is located outside the

interval 50-500 bp, which is the interval covered by our T-RFLP analysis. In fact, the use of more than one restriction enzyme is a recommended practice in this technique, in order to facilitate the resolution of bacterial populations (Liu et al. 1997; Marsh 1999). The fact that other OTUs identified through clone library construction were not identified in T-RFLP profiles, can be in part explained by their poor representation in the sediment samples, causing the failure of their T-RFLP quantification. In complex, our results are in agreement with results of other authors that examined samples of port sediments. In the study performed by Wu et al., (2011), three different samples of marine sediments were investigated in order to characterize their bacterial communities. In all samples, the dominant phylum was *Proteobacteria* with a presence of 82%, 42% and 42% in the three samples. Zhang W. et al. (2008a) analyzed four sites characterized by different pollution levels in Victoria Harbor (Hong Kong). In all sites, they found the dominance of bacteria belonging to *Proteobacteria* phylum and, especially, belonging to the same subclasses detected in our work. The dominance of *Proteobacteria* was also found in many other studies (e.g. Brown et al., 2009, Schauer et al., 2010, Elsabé et al., 2012). In the paper by Zhang W., et al. (2007), the calculated diversity index did not highlight significant differences among the four analyzed sites; this result is similar to the finding of this work, where the two analyzed libraries highlighted similar Shannon diversity values. Bacteria belonging to the *Alphaproteobacteria* subclass are important for hydrocarbon degradation (Rocchetti et al., 2012). The presence of these bacterial taxa in all five sites confirms a contamination of hydrocarbons in Leghorn seabed sediments (Iannelli et al., 2012). A large portion of the sequences detected in our clone libraries belongs to the *Deltaproteobacteria* subclass. With a deep phylogenetic analysis, we discovered that all these clones are closely related to bacteria characterized by a metabolism involved in the removal of sulphur. This observation is in agreement with other two works performed on marine port sediments (Zhang W. et al. 2008a, b). The percentage of *Deltaproteobacteria* detected in the two different samples is not significantly different (24% library 1.2 and 20% library 2.1) and T-RFLP results suggest that their abundance should be roughly comparable among all the five sites; this result does not agree with the chemical analysis results published in Iannelli et al. (2012), which evidenced that the concentration of SO_4^{2-} in site 2.1 (4399 mg/(kg dw)) was double than in site 1.2 (2321 mg/(kg dw). An interesting finding concerns the *Gammaproteobacteria* subclass, in particular the sequences 228, 143, 162, 61 and 160, from library 1.2, and the sequence 147b from library 2.1. The first group of sequences, all representing the same OTU, shows a 98% similarity with *Endozoicomonas elysicola*, which is a typical symbiont of the *Elysia ornata* slug; conversely, the sequence 147b is 99% similar to the sequence of *Spongiispira norvegica*, a typical symbiont of sponges. This observation possibly suggests that site 1.2 is richer in marine metazoans characterized by a symbiotic association with bacteria than site 2.1. This difference is probably due to the chemical characteristics of the

sediments in Leghorn seabed area. Site 1.2, in fact, emerged as being less polluted than site 2.1, and probably more suitable for being colonized by marine metazoans harboring bacterial symbionts.

Conclusions

In the present study, bacterial communities from five sites of Leghorn harbor seabed were analyzed and identified through T-RFLP analysis, 16S rRNA library construction and *in silico* digestion of retrieved clones. Some considerations were provided about organisms involved in the recycling of organic matter. Although in an earlier study the chemical characterization of the sites evidenced significant differences in the presence of nutrients and pollutants, and the T-RFLP analysis evidenced a heterogeneity among the different sites of the harbour, the present paper highlights a substantial similarity in the composition of the bacterial communities concerning both the diversity indices and the phylogenetic affiliation of bacterial sequences obtained from the molecular screening. Retrieved sequences from the two libraries were more than sufficient to provide generic information on metabolism present in all the 15 seabed sediment samples and to compare them with previous similar studies. On the other side, only a significantly higher sequencing coverage would have probably allowed to distinguish between the samples that, in any case, looked rather similar. The T-RFLP approach proved to be more efficient in highlighting the bacterial community structure in the whole harbour area, whereas the present work helped in clarifying role of specific bacteria present in the studied samples and their related metabolisms.

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3.3 Temporal characterization of bacterial communities in a phytoremediation pilot plant aimed at decontaminating polluted sediments dredged from Leghorn harbor, Italy

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ELSEVIER

Temporal characterization of bacterial communities in a phytoremediation pilot plant aimed at decontaminating polluted sediments dredged from Leghorn harbor, Italy

Research Paper

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The AGRIPORT project (Agricultural Reuse of Polluted Dredged Sediments, Eco-innovation EU Project n. ECO/08/239065) aims at developing a new technology for the treatment of polluted sediments dredged from the seabed of commercial ports through phytoremediation processes. Through plant activities and microorganism metabolisms, it is possible to recover dredged saline sediments by decontaminating them until an artificially prepared soil that is reusable in the terrestrial environment is obtained. This is an important advantage from the environmental point of view, and allows to partially solve one of the main problems of most commercial ports, that is the accumulation, storage and disposal of polluted dredged sediments. Considering that bacteria provide a significant contribution to phytoremediation process. Aim of the present study is the monitoring of temporal variation of microbial communities developing in an experimental phytoremediation plant during the decontamination process. The treatment plant consists of a sealed 80 m^3 basin that is filled with a mixture of dredged sediments (75%) and natural soil (25%). It was planted with three plant species, and has been properly cultivated and fertilized for two years. Terminal Restriction Fragment Length Polymorphism (T-RFLP) on 16S rRNA gene was used to study the composition of bacterial communities at different times and points in the basin. Cluster Analysis (CA) and Non Metric Multidimensional Scaling (NMDS) multivariate statistics were applied for data interpretation. At the onset, the bacterial communities were heterogeneous and discrete, reflecting those inherited from the sediment–soil mixture, from compost and from plant's rhizospheres. The communities' composition rapidly changed to become stabilized after one year.

Introduction

Rivers and harbor docks are regularly dredged to assure shipping traffic efficiency. This activity produces large volumes of sediments that are often contaminated by heavy metals, hydrocarbons and chemical compounds. The contamination of sediments dredged from rivers and coastal harbors represents a severe problem both quantitatively and economically [1–3]. Every year, a huge amount of sediment awaits treatment, however the available conventional options (such as chemical treatments, mobilization, thermal treatment, stabilization or capping) are very costly [4]. In the harbor of Leghorn, the Port Authority adopts, as temporary solution, the storage of the dredged material in a storage basin obtained from an area at the sea that was enclosed by a dock and sealed with a high-density polyethylene waterproof liner. The basin is now full and the construction of more additional basins is planned for future dredging thus expanding the port surface area in the sea. This kind of solution might suit the way to expand the port area, however it cannot represent a universal solution in most other cases, and phytoremediation offers an economically feasible option for the rehabilitation of dredged harbor sediments. The AGRIPORT project (Agricultural Reuse of Polluted Dredged Sediments, Eco-innovation EU Project

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n. ECO/08/239065) promotes an innovative approach to decontaminate dredging sediments by phyto-treatment. Concerning phytoremediation of polluted sediments, many studies that are present in the literature focus on the plant-rhizosphere decontamination activity [5,6]. Several papers focus on the interactions between plants and microorganisms [7,8], as well as on rhizodegradation of organic pollutants [9,10]. Other authors focused their attention on the potential role of ectomycorrhizal associations in rhizosphere remediation of persistent organic pollutants [11,12]. Few authors have described the role of rhizosphere processes in the phytoremediation of inorganic pollutants [13–15]. Studies on bacteria responsible or involved in the phytoremediation process of polluted sediments are instead very specific, focusing on particular aspects of the process, such as heavy metal bacterial resistance [16,17], associations of bacteria to plants that accumulate heavy metals [18], microorganism responses to high hydrocarbon concentrations [19] and to other organic compounds [20]. Few studies on phytoremediation applied to marine polluted dredged sediments are available; among these, the outcomes of the phytoremediation process are different, spacing from a reduction of contaminants that amounts around 90% [21], to a very low [22,23], or even negative [24] remediation result. The most recent and detailed study on the positive effect of phytoremediation in the decontamination of polluted dredged marine sediments is provided by Bianchi *et al*. [25], and highlights interesting results about the change of some main chemical parameters (electrical conductivity, sodium content and nutrients) in dredged sediments subjected to phytoremediation process; the study also highlights that the phytoremediation treatment performed by using plants and microorganisms on marine contaminated sediments, provides for their transformation into fertile and nutrient rich soil, removing salt and degrading pollutants. This process decontaminates polluted dredged sediments and turns them into arable land, or soil appropriate for other uses such as landscaping, environmental restoration, gardening, capping of landfills, among others [26]. A key role in the phytoremediation of contaminated dredged sediments is attributed to the choice of plant species, that resist the high level of salinity and compactness of such sediments. To allow the growth of such vegetal species, the sediments can be previously mixed with inert material, agronomic soil and/or organic substances to improve the overall physical and chemical characteristics of the mixture. The AGRIPORT project objective was to demonstrate and commercialize a simple and low cost technology for transforming slightly polluted saline sediments into soil that can be reused for agronomic and environmental applications.

The study of bacterial communities in marine contaminated sediments is usually carried out to detect particular bacterial groups, such as heavy metal resistant bacteria [27], or fecal bacteria [28], or to test the effect of some contaminants on the composition of bacterial communities [29]. The monitoring of modifications in bacterial community composition in dredged sediments is instead less common, and only few papers are available [30]. The transfer of sediments from an aquatic ecosystem to the terrestrial environment may drastically change the habitats equilibrium, especially considering the physical and chemical characteristics of sediment, its water content and redox. Changes in moisture contents alter the microbial communities composition of soils [31–34]; moreover, microbial communities specific of terrestrial environments are completely different from those found in aquatic sediments [35]. Microbial activity is important for sediment rehabilitation through turnover of organic pollutants and acquisition of soil-like physico-chemical properties. Hence, it is important to monitor the changes in the composition of the bacterial communities in the dredged sediments during their restoration. The aim of the present study is to monitor temporal variation in the composition of bacterial communities under phytoremediation in the reconstructed basin. The molecular technique used in this study is the Terminal Restriction Fragment Length Polymorphism (T-RFLP [36]) on the 16S rRNA gene, followed by multivariate statistical analysis on the data matrix.

Materials and methods

Description of the phytoremediation plant

The treatment basin was constructed in the new maritime station near the port's Donegani gate from December 2009 to January 2010. The sediment to be used in the installation was taken from the existing confined disposal facility located inside the Leghorn port.

The internal working dimensions of the pyramidal-frustum shaped basin range from 17.96 m × 5.06 m (at the top of the bottom drainage layer) to 18.75 m × 5.85 m (at the surface), with a depth of 0.9 m (excluding the bottom drainage layer, Fig. 1a,b). The working volume of matrix under treatment was about 90 m^3 of sediments mixed with 25% in volume of soil.

After building the containment embankments and leveling off the bottom, the treatment basin was sealed with a polyethylene waterproof liner to prevent the leaching and to allow for the correct inflow-outflow water balance. A geo-textile was laid on the liner to protect it from abrasion.

The basin was divided into four plots (approximately 5 m × 5 m each, Fig. 1c). Three of them were planted with 1140 seedlings each of a salt tolerant grass species (*Paspalum vaginatum*), already used in phytoremediation of marine sediments [37]; 120 one-year-old seedlings of the shrub/tree species *Tamarix gallica* and *Spartium junceum* were additionally planted to each of the plots 3 and 4 respectively, to start the phyto-treatment of sediments (Fig. 1c). A 30-cm draining layer made of gravel and sand was hand laid on the geo-textile before filling it with the sediment mixture. Finally, the matrix was emended by mixing its top 10-cm layer with 4 kg/m^2 of green compost of the same type as the one used to nurse the adopted seedlings.

A system of perforated pipes was included in the draining layer, and the leachate was collected using four wells (one per subarea) equipped with submerged pumps that pumped it to a single tank to be transferred by gully sucker to the nearby treatment basin.

Each subarea was equipped with three access-points for soil-moisture detection by DIVINER 2000 capacitance probe (Sentek, Australia). Each subarea was equipped with independent drip (for shrub species) and sprinkler (for grass species) irrigation fed by tap water.

Collections and storage of samples

Samples were first collected at the time of installation of the phytoremediation treatment basin in February 2010, to analyze the bacterial communities in all the components of the basin at

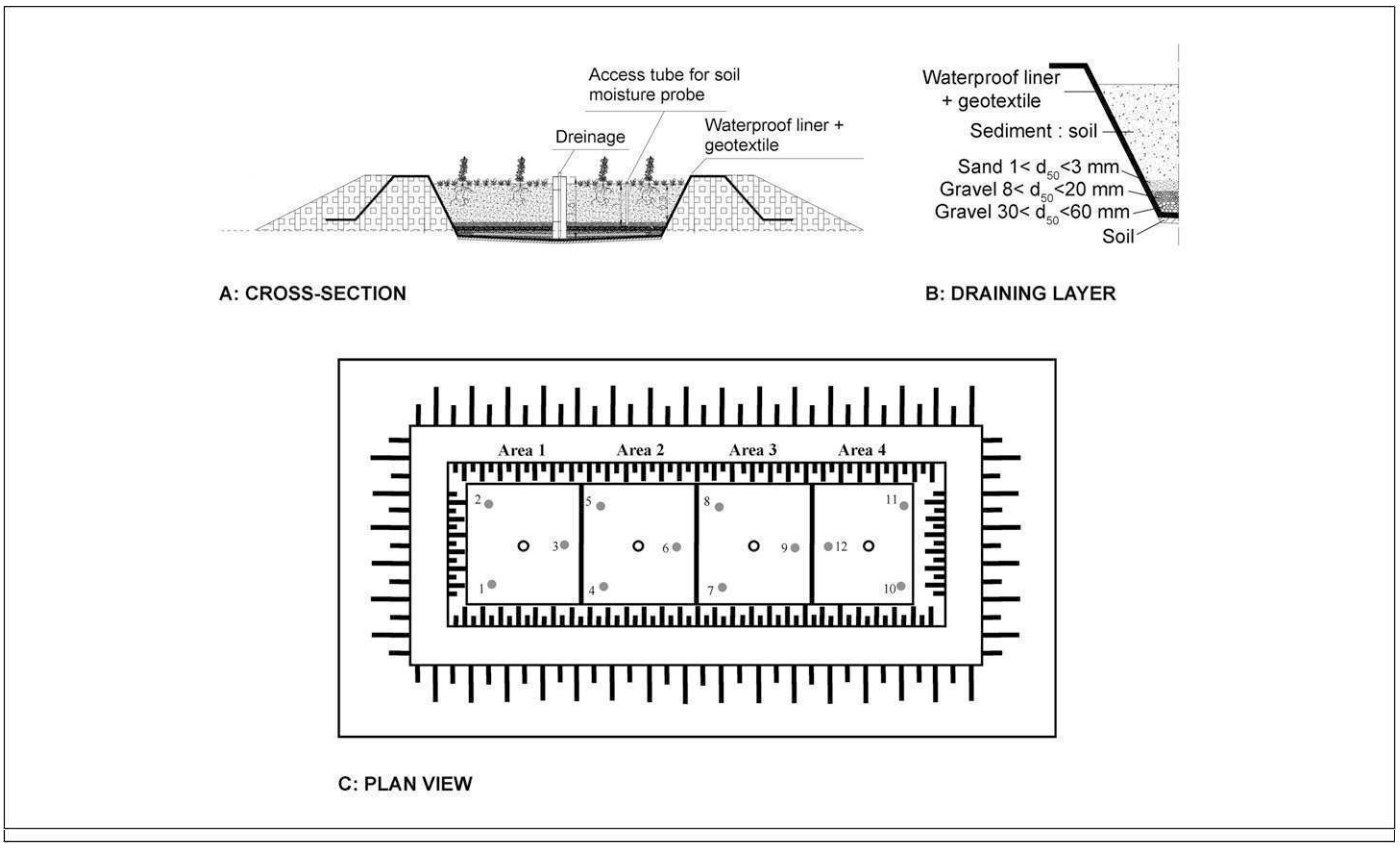

FIGURE 1

(a) Cross-section view of the phytoremediation treatment basin; (b) draining layer; (c) plan view of the phytoremediation treatment basin added with the 12 sampling points that are indicated with numbers.

the onset of the whole experiment. Samples collected during this first phase are named 'Time Zero' and their short names are reported in brackets; they were all collected in triplicates for a total of 21 samples and are constituted by:

- Two-year-old dredged sediments that were stored in the storage basin and were used to fill the phytoremediation plant in a percentage which consisted 75% of the final mixture packed in the treatment basin (sed);
- Natural soil mixed together dried dredged sediments in a percentage of 25% to fill the phytoremediation treatment basin (soil);
- Homogenized mixture of dredged sediments and natural soil (mix);
- Compost that was put on the surface of the filled phytoremediation treatment basin (com);
- Rhizosphere of *P. vaginatum* (pas);
- Rhizosphere of *T. gallica* (tam);
- Rhizosphere of *S. junceum* (gin);

Samples from rhizospheres of the plants, were constituted by 2 cm^3 of soil that was present around plant roots before they were planted in the treatment basin.

After the onset, samples were collected every six months for three years following the scheme that is represented in Fig. 1c.

For each of the four plots, three sampling locations were defined, triplicates were collected near each of the indicated locations, at 0–20 cm depth (Fig. 1c). The triplicates were homogeneously mixed to produce a single sample. As a consequence, a total of 12 samples were collected every six months from the treatment basin for a total of five times (sixty samples in total). Samples were immediately brought to the laboratory and stored at −20 °C for molecular analysis. The total number of analyzed samples from Time Zero to Time Five is 81, and they were collected respectively in February 2010, July 2010, December 2010, June 2011, November 2011 and April 2012.

T-RFLP and statistical analysis

Total DNA extraction, amplification of 16S rRNA gene, T-RFLP protocol and T-RFLP data matrix transformation were performed as described in Iannelli *et al.* [38]. Interpretation of T-RFLP profiles was made using two different statistical methods: Cluster Analysis (CA) and Non Metric Multidimensional Scaling (NMDS [39]) with the Bray–Curtis coefficient. The software used for this part of the study was PAST v.2.15 (PAleontological STatistic [40]). CA was performed using Ward's method and 100 bootstraps as described in Iannelli *et al.* [38]. NMDS is an ordination method that uses an iterative algorithm that takes the multidimensional data of a similarity matrix and presents it in minimal dimensional space (usually two dimensions). The result of MDS ordination is a plot where the position of each point (representing a sample) is determined by its distance from all other points in the analysis. An important component of an MDS plot is a measure of the goodness of fit of the final plot, that is represented by the 'stress' of the plot (*S*). A stress value greater than 0.2 indicates that the plot is close to random, stress less than 0.2 indicates a useful two-dimensional

picture and less than 0.1 corresponds to an ideal ordination with no real prospect of misinterpretation [41]. In the Shepard plot ordination distances are plotted against community dissimilarities, and the fit is shown as a monotone step line; it indicates the quality of the result. Ideally, all points should be placed on a straight ascending line ($x = y$). Shannon's diversity index (H) was calculated on T-RFLP data using PAST software as: $H = -\sum p_i \ln p_i$. It represents the proportion p_i of the species i relative to the total number of species, multiplied by the natural logarithm of the proportion itself. The resulting product is summed up across species, and multiplied by -1 [42]. The Shannon index varies from 0 for communities with only a single taxon, to values that are higher for communities with more taxa, each of which has fewer individuals.

Results

CA made on the 21 samples collected at Time Zero is shown in Fig. 2. It is possible to recognize seven terminal clusters, all supported by high bootstrap values. Each cluster is composed of the three replicates collected for each sample composing the treatment basin at the moment of the onset of the experiment: natural soil, sediments, the mixture of sediments and soil, the compost and the rhizospheres of the three plant species. Another important observation is that soil, sediments and mixture samples cluster all together with 98 bootstrap value support; even the rhizospheres of the three plants cluster together with 81 bootstrap value. The compost samples make a bigger group together with the rhizosphere cluster, supported by a bootstrap value of 95.

CA made separately with Ward's method and 100 bootstraps, on the groups of samples collected at different times after the onset of the experiment (Time One, Two, Three; Four and Five), indicated

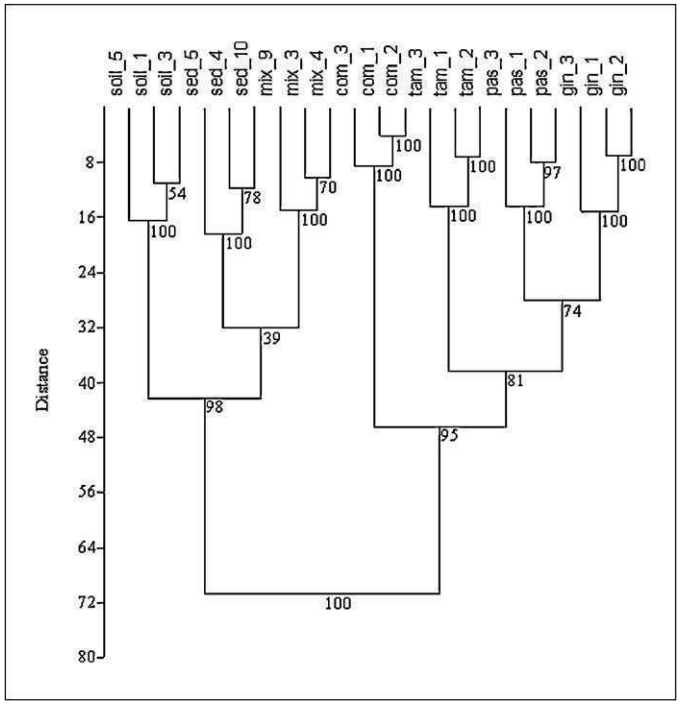

FIGURE 2

Cluster Analysis made on Time Zero samples with Ward's method and 100 bootstraps.

high resemblance among the four plots that comprise the treatment basin (Supplementary Figure S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2012.10.002.

The three samples collected in each of the four plots did not cluster together in the majority of analyses. This indicates that the four plots actually share the same bacterial communities. Figure 3 reperesents NMDS analysis performed on the same dataset represented in CA of Fig. 2. The stress value is 0.1664. Supplementary Figure S2A shows the Shepard plot. In Fig. 3 the difference in composition of bacterial communities occurring between the samples of natural soil, sediment and mixture, and the group of samples including the rhizospheres of the plants is evident. Compost samples are isolated in the plot from all other samples.

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Figure 4 represents the NMDS performed on the complete dataset collected from Time Zero to Time Five. The stress value is 0.1578. Figure S2B represents the corresponding Shepard plot. Samples of Time Zero are divided into two main groups, which appear as differentiated in the previous graph (Fig. 3). The samples of the three filling matrices (soil, sediments and mixture) appear as completely separated from the others, as well as the samples of the rhizospheres of the three plants cluster together with the compost. All samples collected from Time One to Time Five, appear in an intermediate position between the two groups of rhizospheres – compost and treatment basin filling. Samples of Time One (red one) are separated from the others (Time Two to Time Five) which, conversely, appear all grouped together in the middle of the plot.

Figure 5 represents a NMDS analysis made on samples from Time Zero, Time One, Time Two and Time Five. What is worth of notice is that samples of Time One are differentiated both from Time Zero samples and from Time Two and Five samples. Time Two and Time Five samples are instead overlapping in the plot. The accuracy of the analysis is demonstrated by the Shepard plot in Figure S2C, and by the stress value (stress = 0.1484).

Shannon diversity indices were calculated for all samples; for triplicates, the average value of the index has been calculated, to have a single Shannon index value for any kind of sample. Results are shown in Table 1. The highest values were recovered for bacterial community from Time Two to Time Five (values ranging from 4.38 to 4.89). The lowest values were recovered in Time One samples and in Time Zero samples of rhizospheres, compost, and soil (values ranging from 3.48 to 3.98). Original sediments presented an intermediate Shannon diversity index value (4.30) that decreases, after mixing with the soil, to 3.98 value of the mixture.

Discussion

The choice to analyze T-RFLP data with NMDS statistic was made on the basis of the available scientific literature. As described in Rees *et al.* [39], the MDS ordination can be successfully applied to T-RFLP sediment data, obtaining robust analysis results. The choice of Bray–Curtis coefficient also relies on available literature data; in fact, this coefficient has been shown to give high levels of power and robustness [43], and it is a better choice than other coefficients such as the Euclidean [41,44], that, conversely, are not appropriate to determine similarities within T-RFLP data [39]. The Bray–Curtis coefficient has large application in ecological studies;

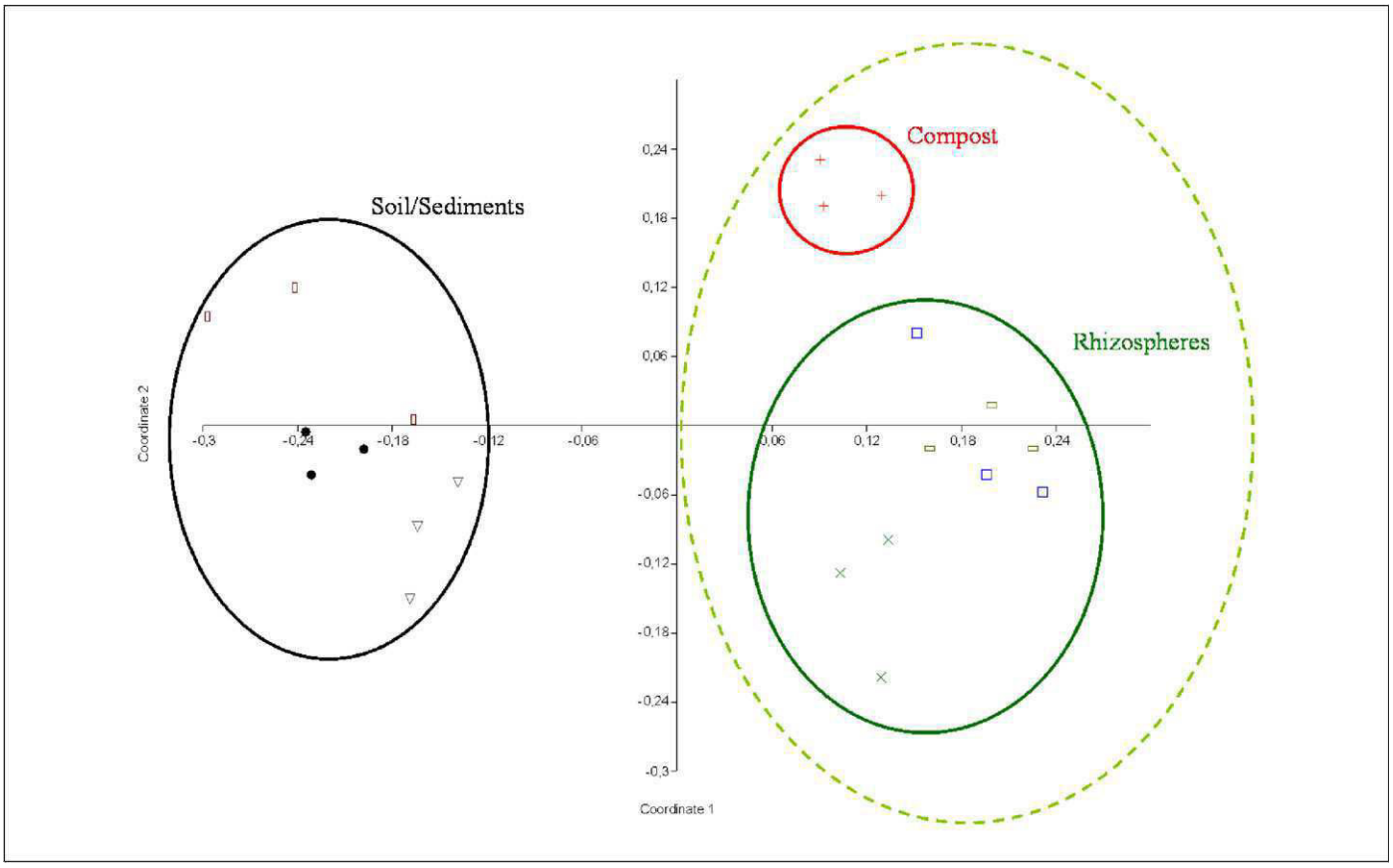

FIGURE 3

NMDS plot on Time Zero Dataset. Red crosses: compost; black solid circles: dredged sediments; brown hollow rectangles: natural soil; gray hollow triangles: mixture; blue hollow squares: *S. junceum*; green hollow rectangles: *P. vaginatum*; green cross: *T. gallica*.

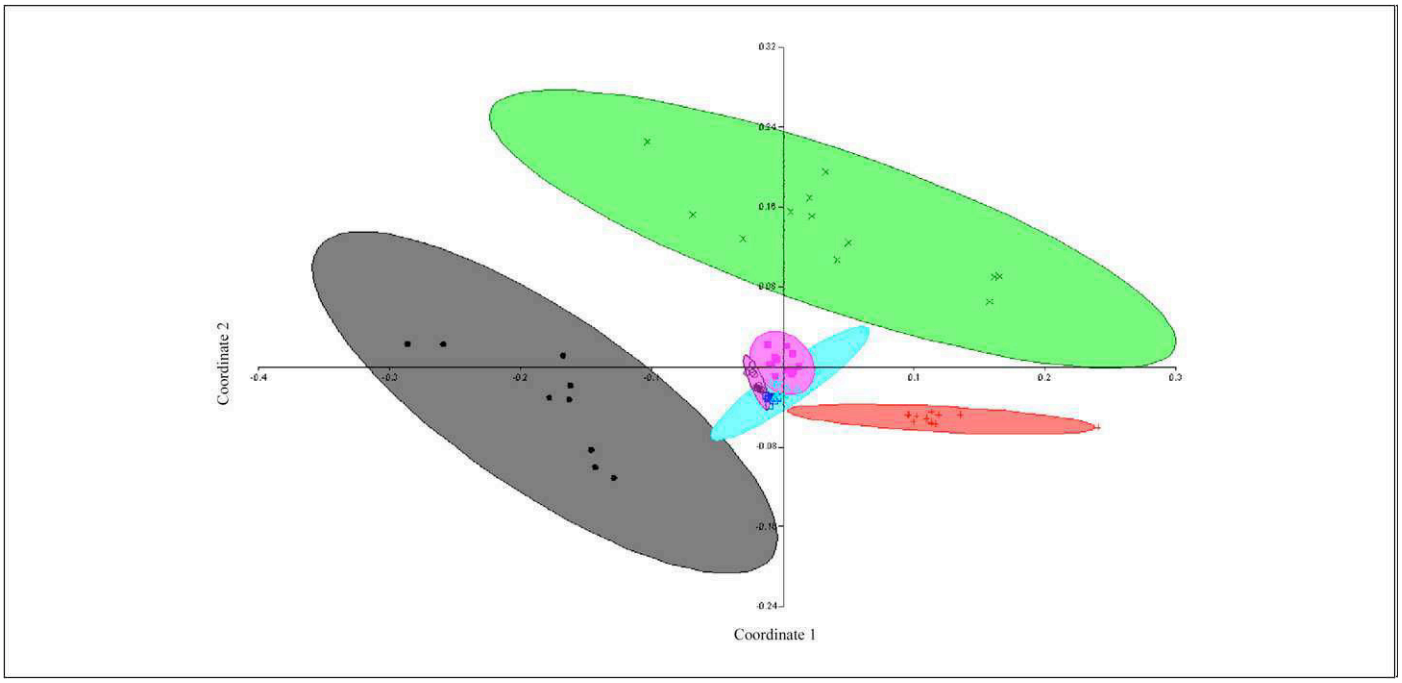

FIGURE 4

NMDS analysis on the whole dataset collected during the experimentation. Black solid circles in gray field: Time Zero plant filling (soil/sediments/mixture); green crosses in green field: Time Zero rhizospheres and compost; red crosses in red field: Time One; blue hollow squares in blue field: Time Two; light blue hollow triangles in light blue field: Time Three; pink solid squares in pink field: Time Four; purple hollow circles in pink field: Time Five. The colored area surrounding the symbols represents the 95% confidence intervals.

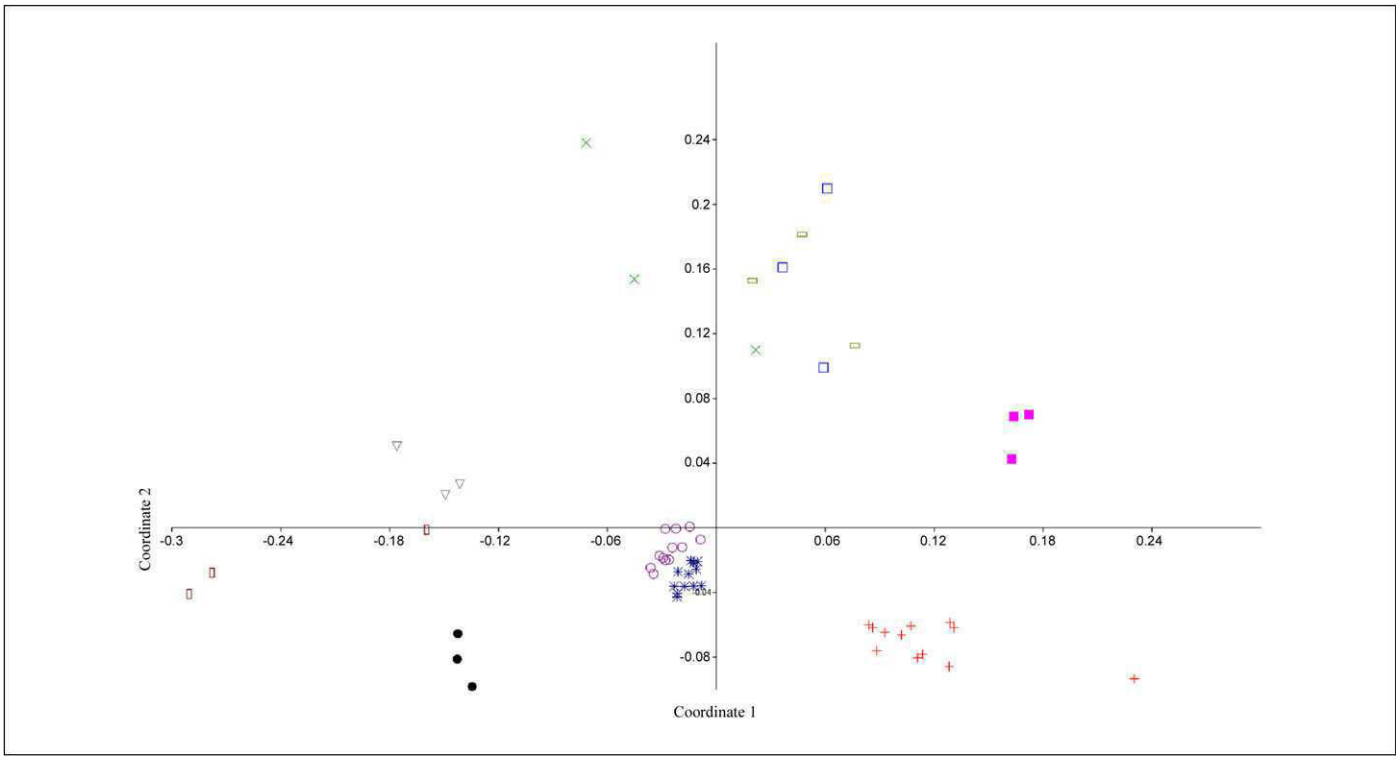

FIGURE 5

NMDS on Time Zero, Time One, Time Two and Time Five samples. Pink solid squares: compost; black solid circles: dredged sediments; brown hollow rectangles: natural soil; gray hollow triangles: mixture; purple hollow circles: Time Five; dark blue asterisks: Time Two; red crosses: Time One; blue hollow squares: *S. junceum*; green hollow rectangles: *P. vaginatum*; green cross: *T. gallica*.

its main property is that identical samples show a 100% similarity value, while samples without any shared species (or T-RFs [45,46]) show a 0% similarity value.

Phytoremediation of contaminated sites using plants is an interesting alternative than commonly used technique, especially in terms of lower costs. Phytoremediation has been mainly applied in contaminated soils, with interesting results concerning removal of heavy metals [47], organic contaminants [48], halogenated compounds as trichloroethene (TCE [49]) and arsenic [50]. As previously mentioned in the introduction section, few studies concerning phytoremediation techniques applied to marine polluted dredged sediments are available in international literature. Among them, no papers on the temporal monitoring of bacterial communities dynamics during the treatment are available. To the best of our knowledge, this is the first paper in which an overview of the bacterial communities harboring a full scale phytoremediation treatment basin for the decontamination of polluted marine sediments is provided. Moreover, this is the first paper providing a two-year monitoring report of bacterial communities in such a system through molecular biology. Starting our consideration from Time Zero, the first result to point out is the homogeneity of the three replicates analyzed from each kind of sample; these data confirm that the bacterial community composition for each kind of sample (e.g. compost, dredged sediments, among others) was homogeneous at the onset of the experiment. At Time Zero, all the components of the phytoremediation basin were characterized by distinct bacterial communities (Fig. 2), and well differentiated from each other (high bootstrap value support). The main clusters that can be recognized in Fig. 2 are those of the rhizosphere, supported by a bootstrap value of 81, and the basin filling (natural soil/dredged sediments) supported by 98 bootstrap value. These data indicate that the bacterial communities of the rhizospheres of the three plants appear similar to each other, as well as the bacterial communities of the two main components of the filling of the basin. Among Time Zero samples, the Shannon diversity index highlights a higher biodiversity in contaminated dredged sediments (4.30) than in not contaminated matrices such as inert soil (3.51), compost (3.48) or the three plants rhizospheres (3.49–3.90). These results, agree with the observation that bacterial

TABLE 1

Shannon index average value calculated with PAST software for each sample.

Sample	Shannon index
Sed	4.30
Soil	3.51
Mix	3.98
Tam	3.49
Gin	3.90
Pas	3.81
Com	3.48
Time One	3.82
Time Two	4.77
Time Three	4.53
Time Four	4.38
Time Five	4.89

diversity can increase in presence of contaminants, probably because bacteria that specifically degrade certain compounds (such as hydrocarbons) are prevalent in contaminated matrices [51,52]. The compost that clusters with the rhizospheres of the plants with a 95 bootstrap value, confirms that the three cultivated plants were previously fertilized with a compost sharing the same microbiological characteristics as the one used to emend the matrix. This scenario represented the starting point of the experimentation in which four different treatments were applied. According to the experimental design, we would have expected the four treated areas to behave differently from each other in bacterial community composition; this was not the case (see supplementary figure S1). Indeed, already at Time One we observed a substantial homogeneity among all 12 samples independently from their origin in the basin; the same is true for subsequent samples. The fact that the four areas behaved homogeneously could be explained by the fact that each area represents an open system in which no physical separations were constructed among each other. Probably, a transfer of microorganisms by passive diffusion through irrigation water and leachate may have occurred. Bacterial motility occurs in many different forms that require various cell appendages [53]. Naturally partial saturated habitats such as soil, limit bacterial motility because aquatic microhabitats are fragmented and connected with liquid films as thin as bacterial cell dimensions, or thinner [54]. Despite these observations, the study of Dechesne *et al.* [55] has demonstrated that under partial hydration conditions, commonly present in many terrestrial bacterial habitats, the thickness and geometry of liquid films control active bacterial motion and dispersal. In our case, this would have determined homogeneity in the bacterial community composition in the whole basin, with no discrimination among the differently treated areas of the treatment basin.

If diversity indices of the mixture filling the treatment basin at the onset of the experimentation are compared to those calculated at the different sampling times, it is possible to highlight that the bacterial diversity significantly increased already at Time Two and reached the maximum at the end of experimentation. These data are in agreement with the results obtained by Tu *et al.* [56], showing that the presence of plants may increase the bacterial diversity. In our case the effect of phytoremediation treatment on bacterial community structure was evident after about one year from the onset of the experimental basin. The most important result that emerged in the present work was that after six months from the beginning of the experimentation (Time One), the bacterial community composition in the phytoremediation basin was not yet stabilized, and that already after one year (Time Two), a defined bacterial community (Figs 4 and 5), developed and remained stable until the end of the experimentation (Time Five). Bacterial community was substantially stable from Time Two to Time Five, the end of the experimentation; this is confirmed by the NMDS plot (Figs 4 and 5 and S3), and from Shannon values.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.nbt.2012.10.002.

Conclusions

In the present study, the bacterial communities harboring a full scale phytoremediation basin for the decontamination of polluted dredged sediments were monitored with molecular techniques during two years of experimentation. The study demonstrated that, at the beginning of the experimentation (construction of the treatment basin), each component used to assemble the phytoremediation treatment basin was characterized by its own bacterial community differentiated from the others. After six months from the construction of the treatment basin (Time One), the original bacterial communities evolved into a single bacterial community, homogeneously distributed in the whole area. The molecular analysis of samples collected from Time Two (one year after the construction of the treatment basin) to Time Five (end point of the experimentation), indicate that the bacterial community got stabilized after one year (Time Two) from the construction of the treatment basin. The phytoremediation process influenced the development of a specific bacterial community of the treatment basin that is completely different from the bacterial communities which harbored the basin at the beginning of the experimentation.

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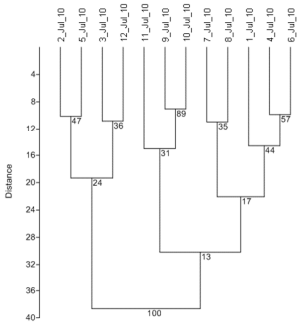
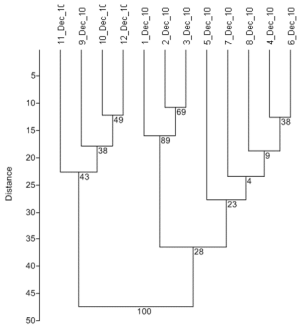
Supplementary material



Time One



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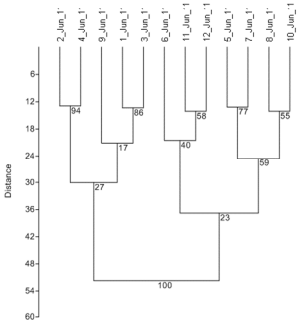

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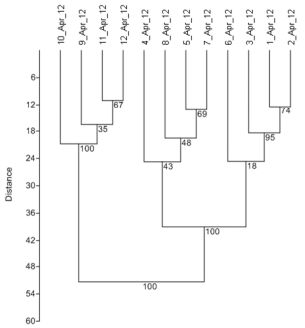
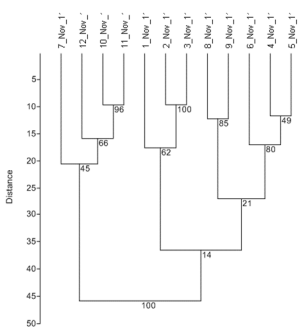


Time Four



Time Five

Figure S1: Cluster Analysis made separately with Ward‘s method and 100 Bootstraps on samples collected at Time One, Two, Three, Four and Five.

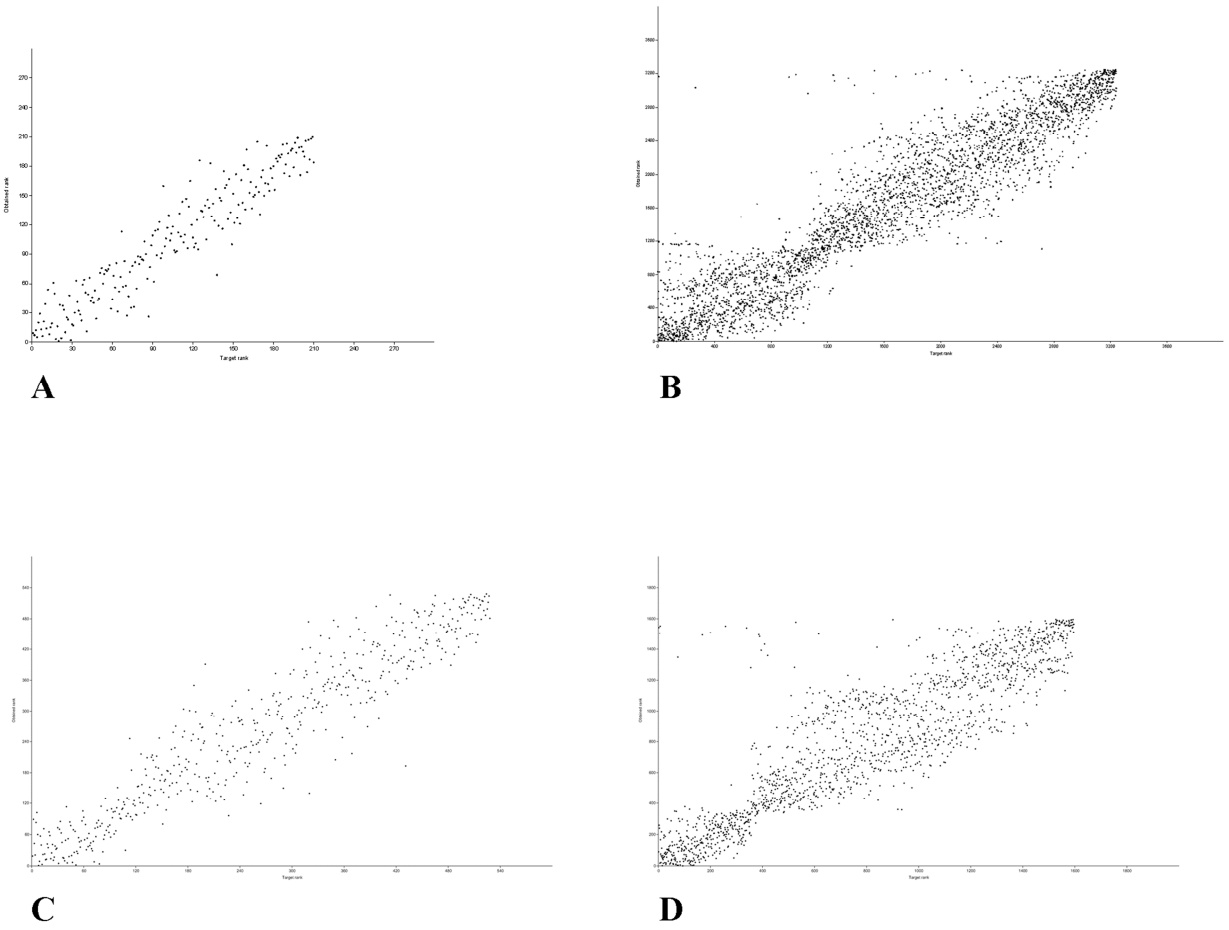


Figure S2: Shepard Plots obtained with NMDS on samples from Time Zero (A), from the complete dataset (B), from samples of Time Zero and Time Five (C) and from samples of Time Zero, Time One, Time Two and Time Five (D).

4 Other studies on bacterial communities composition in environmental matrices performed during the PhD period

Three studies made as side projects during the three years of PhD follow.

The first study concerns the identification of microorganisms responsible for a biofouling phenomenon occurred in a reverse osmosis membrane used in river water purification for drinking purposes. At the moment of the analysis, the reverse osmosis treatment plant was dismissed and only the "old" dried material was available for the analysis. Results highlighted that the biofouling phenomenon was caused by the proliferation of an amoebozoan organism (Lobosea) which was feeding on a bacterial biofilm mainly composed by *Actinobacteria*.

The second study, focuses on the bacterial community characterization in two pilot plants, a conventional activated sludge and a membrane biological reactor. Comparisons and critical discussion on the different results obtained with molecular analysis on both pilot plants are provided.

The third presented study, involves the characterization of bacterial communities detected in a wastewater treatment plant with an additional ozonation treatment. Comparisons among the treated (ozonated) line and the control (untreated) line are provided and discussed.

4.1 Biofouling of reverse osmosis membranes used in river water purification for drinking purposes: analysis of microbial populations

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Biofouling of reverse osmosis membranes used in river water purification for drinking purposes: analysis of microbial populations

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Biofouling in water treatment processes represents one of the most frequent causes of plant performance decline. Investigation of clogged membranes (reverse osmosis membranes, microfiltration membranes and ultrafiltration membranes) is generally performed on fresh membranes. In the present study, a multidisciplinary autopsy of a reverse osmosis membrane (ROM) was conducted. The membrane, which was used in sulfate-rich river water purification for drinking purposes, had become inoperative after 6 months because of biofouling and was later stored for 18 months in dry conditions before analysis. SSU rRNA gene library construction, clone sequencing, T-RFLP, light microscope, and scanning electron microscope (SEM) observations were used to identify the microorganisms present on the membrane and possibly responsible for biofouling at the time of removal. The microorganisms were mainly represented by bacteria belonging to the phylum Actinobacteria and by a single protozoan species belonging to the Lobosea group. The microbiological analysis was interpreted in the context of the treatment plant operations to hypothesize as to the possible mechanisms used by microorganisms to enter the plant and colonize the ROM surface.

Keywords: biofouling; reverse osmosis membrane; 16S rRNA; 18S rRNA; Actinobacteria; Amoebozoa

Introduction

Biofouling is the growth of bacterial biofilms on a surface that can cause a substantial performance decline in a water treatment process (Vrouwenvelder and van der Kooij 2001; Flemming 2002). Water treatment processes that are most at risk are warm-to-high temperature processes (evaporative cooling, steam production, evaporation) and membrane filtration processes such as reverse osmosis (RO), nanofiltration (NF), ultrafiltration (UF) and microfiltration (MF) (Aquafit4use 2010).

At present, the traditional application of RO and NF high density membranes are being extended to the removal of monovalent and divalent ions, respectively, in a variety of industrial and civil uses. In the production of drinking water and ultrapure water the use of RO/NF membranes for the treatment of brackish water is receiving growing interest. Even the most cost-dependent uses, such as irrigation, are now applying RO/NF membranes due to reductions in membrane production costs and energy consumption (Iannelli et al. 2009).

Many strains of bacteria have been isolated from biofouled membranes (Stoodley and Stoodley 2002; Pang and Liu 2007) and identified through common culture methods (Cavicchioli et al. 1999), but it is generally accepted that only a small fraction of prokaryotic biodiversity can be detected by culture (Stackebrandt 2004). During the last few decades, molecular approaches based mainly on the analysis of small subunit (SSU) rRNA genes have been developed in order to fully identify microbial communities (Amann et al. 1995) and these methods have been applied to detect bacteria in drinking water and ultrapure water treatments (Chen et al. 2004; Bohus et al. 2010).

The assessment of a biofouling problem in a membrane filtration plant is usually based on identifying the presence of colonizing microorganisms in the water collected from different process stages, or from the surfaces of membranes suspected of being fouled. This approach is based on a so-called 'autopsy' of membranes, a disruptive technique to analyze foulants by dissecting the membrane (Dudley and Darton 1995; Baker and Dudley 1998). The increasing interest in the membrane-autopsy approach is demonstrated by the extended number of recently published articles on this topic (eg Pontié et al. 2005; Pang and Liu 2007; Vrouwenvelder et al. 2008).

Autopsies are generally conducted on membranes directly collected from pilot (Vrouwenvelder et al.

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1998) or full-scale plants (Butt et al. 1997) and are based on macroscopic observations of fouling layer color and detailed performance-tests extensively applied to samples of the bio-fouled membranes (eg density and chemical composition). In some cases, light microscopy (LM) and scanning electron microscope (SEM) observations are performed (Khan et al. 2011), but these approaches do not always allow the complete identification of the microbial species causing the problem.

Here, the authors report a retrospective study on the possible microbial dynamics leading to biofouling of a surface water treatment plant using reverse osmosis for drinking water production from the analysis of stored membranes.

The water purification plant was located in Poggibonsi (Tuscany, central Italy) and was built in 2007 with the aim of treating the sulfate-rich surface water collected from the Elsa River for the drinking-water needs of the town of Poggibonsi. It is based on RO filtration with MF pretreatment. Most RO membranes become so severely fouled that they are nonfunctional after operation for 6 months.

After removal, the membrane remained in dry conditions for 18 months at a mean temperature of $\sim 10^{\circ}C$, after which the analysis was performed. Through cultivation-independent approaches based on SSU rRNA sequencing, two Operational Taxonomic Units (OTUs) of Eukaryotic organisms, together with 19 Prokaryotic OTUs, were detected from the surface of the membrane despite the age of the samples. The detected OTUs were interpreted as the main cause of biofouling. LM and SEM investigation were also carried out to support the molecular data. It is well known from other research fields such as forensic science and paleoanthropology that DNA stored on dry surfaces for long periods, eg years, decades, and even thousands of years, can still be used for the molecular identifications (Dalton and Kotze 2011). In the present paper it is shown, for the first time, that it might be possible to identify microorganisms present on biofouled membranes many months after they were removed. Although it was not possible to prove that the characterized microbial community was identical to those on fresh membranes, the results suggest that useful information may still be gleaned from 'old samples'.

Materials and methods

The Elsa purification plant in the town of Poggibonsi

The plant under study has a maximum overall production capacity of 120 $m^3\ s^{-1}$, half of which is treated by reverse osmosis, while the other half is subjected only to activated carbon adsorption. The maximum flux that is withdrawn from the Elsa river during production accounts for 230 $m^3\ s^{-1}$, but increases to 280 $m^3\ s^{-1}$ during the washing cycles of the microfiltration stage. The range of variation in the main quality parameters of the surface water being treated by the plant are summarized in Table 1. After a brief preliminary screening and continuous dosing of sodium hypochlorite and hydrochloric acid for Oxidation Reduction Potential (ORP) and pH control, the water collected from the Elsa river is filtered through a submersed hollow-fiber microfiltration membrane (ZeeWeed 500d, Zenon, Canada, nominal pore size 0.04 μm) and then split into two lines. The first line, after de-chlorination by sodium-bisulfite dosing and 5 μm cartridge filtration, undergoes RO filtration by spiral-wound aromatic-polyamide-compound membranes (Vontron ULP 32-8040, China). The second line is subjected to activated carbon adsorption by two parallel columns of coconut-fiber granular activated carbon. After these treatments, the two lines are blended together to obtain the final required sulfate concentration, and the final mix is subjected to chlorine-dioxide dosing for final disinfection and then pumped to the main 'Galloria' tank of the water supply network. A block-diagram of the plant is shown in Figure 1.

The plant entered into standard operation at the beginning of 2008, but, after about 6 months, its RO stage had severe, irreversible fouling problems which caused complete stoppage despite several unsuccessful attempts to solve the problem (Table 2).

According to the description of the episode by the operational staff, the first problem detected was the severe obstruction of the prefiltration cartridges, which occurred immediately after plant startup. An analysis of the reddish cartridge-filter deposition allowed them to attribute this problem to the colloidal deposition of iron which passed the MF stage in the dissolved ferrous form and was then oxidized by the air bubbling from the submerged hollow-fiber MF to the ferric form which precipitated onto the cartridge filters. This problem was solved by introducing a prechlorination stage coupled with pH control by hydrochloric acid, that allowed complete removal of Fe at the MF stage. After this intervention, the plant was described as 'working with normal pressures and flow-rates' for 2 months. After this, the feed pressure of the RO started to increase, the permeate flow decreased significantly, and the chemical-in-place washings were ineffective in restoring the normal flow-rate.

At this point, the specific permeate flow of the RO system decreased to 14.45 $l\ m^{-2}\ h^{-1}$ (63% of the nominal value); the feed-channel pressure dropped as much as 2.2 bar, (more than twice the maximum allowed value), and the NaCl rejection at test conditions was 98.36% (lower than the nominal value of 99.5%) (Table 2).

Table 1. Range of variation in the main quality parameters of the Elsa river raw water treated by the purification plant of Poggibonsi.

Parameters and units of measure	Mean	Max.	SD	Italian law limits		Adopted analysis standards
				Raw water*	Drinking water°	
Turbidity (NTU)	12	100				ISTISAN Rep. 07/31 Met. ISS.BLA.030 Rev. 00
Electr. Conductivity (μS/cm)	1500	2000				APAT - IRSA CNR 29/03 n° 2030
Sodium (mg l^{-1})	44.7	61.4	7.6		200.0	ISTISAN Rep. 07/31 Met. ISS.CBB.038 Rev. 00
Potassium (mg l^{-1})	4.7	7.5	1.7			ISTISAN Rep. 07/31 Met. ISS.CBB.038 Rev. 00
Magnesium (mg l^{-1})	66.4	76.8	11.9			ISTISAN Rep. 07/31 Met. ISS.CBB.038 Rev. 00
Calcium (mg l^{-1})	250.6	282.9	38.2			ISTISAN Rep. 07/31 Met. ISS.CBB.038 Rev. 00
Fluoride (mg l^{-1})	0.8	1.3	0.2		1.5	ISTISAN Rep. 07/31 Met. ISS.CBB.037 Rev. 00
Chloride (mg l^{-1})	65.3	111.9	21.6		200	ISTISAN Rep. 07/31 Met. ISS.CBB.037 Rev. 00
Bromide (mg l^{-1})	0.3	2.5	0.6			ISTISAN Rep. 07/31 Met. ISS.CBB.037 Rev. 00
Nitrate (mg l^{-1})	7.4	16.8	3.1	50	50	ISTISAN Rep. 07/31 Met. ISS.CBB.037 Rev. 00
Phosphate (mg l^{-1})	0.2	0.6	0.2			ISTISAN Rep. 07/31 Met. ISS.CBB.037 Rev. 00
Sulfate (mg l^{-1})	575.3	696.8	136.4	250	250	ISTISAN Rep. 07/31 Met. ISS.CBB.037 Rev. 00
Ammonium (mg l^{-1})	0.4	0.8	0.2	4	0.5	ISTISAN Rep. 07/31 Met. ISS.BHE.019 Rev. 00
Nitrites (mg l^{-1})	0.4	1.3	0.3		0.1	Standard Methods 21st ed. n° 4500
Iron (μg l^{-1})	210.1	880.0	206.5		200	APAT-IRSA-CNR 29/03 n° 3160 (F-AAS)
Manganese (μg l^{-1})	46.9	142.0	32.0		50	APAT-IRSA-CNR 29/03 n° 3190 (F-AAS)

Note: Data provided by the water utility company Acque S.p.A. *Legislative Decree 152/2006 Annex 2 to part III – Tab. 1/A col.A3/I; °Legislative Decree 31/2001 Annex I.

A diagnostic autopsy of one of the fouled membranes (the lead module of one of the 40 parallel-mounted six-module vessels of the plant) was immediately performed by the plant operation and maintenance company (Protecno s.r.l. Italy) (Figure 2). The loss-on-ignition test (Pontié et al. 2005) performed during this autopsy revealed that the composition of the fouling matter was mainly (99.55%) organic, with a foulant distribution of 191.6 g m^{-2}. A microscopic biological inspection revealed the presence of several microorganisms that were not identified. Other fouled membranes were removed from the plant and stored in dry conditions. After 18 months, one of these membranes (the lead module of a different six-module vessel) was submitted to the laboratory for molecular microbiological analysis.

Collection and storage of samples

On 18 October 2010, an RO membrane unit was taken from the Elsa purification plant and brought to the laboratory. The membrane unit, after removal of internal water and drying, had been stored for 18 months in a stock room located in the Poggibonsi wastewater plant area, at room temperature. The covering was opened under sterile conditions and the membrane was dissected. Thirty-four membrane squares (between 4 and 9 cm^2) were collected by cutting them from different layers of the membrane, in sterile conditions (under a biological flow cabinet, with a new sterile scalpel for each piece), and separately stored in sterile 1.5 ml tubes. Each membrane square comprised four sub-layers of the RO filtration unit. Fifteen larger circular portions of each membrane layer (10–12 cm in diameter) were also cut and separately stored in 50 ml sterile tubes. All samples were stored at room temperature.

DNA extraction and amplification of 16S and 18S rRNA genes

Total DNA extraction was performed on two small plugs (identified as '32' and '33') using 'Soil master^{TM} DNA extraction kit' (Epicentre Biotechnologies, WI, USA). 16S rRNA and 18S rRNA genes were directly amplified from extracted DNA using primers: 8F 5′-AGA GTT TGA TYM TGG CTC AG-3′ and reverse 1492R 5′-GGN WAC CTT GTT ACG ACT T-3′ (Lane 1991) for prokaryotes that amplify near complete 16S rRNA gene sequences (>1300 bp), and 18S F9 5′-CTG GTT GAT CCT GCC AG-3′ (Medlin et al. 1988) and reverse 18S R1513 Hypo 5′-TGA TCC TTC YGC AGG TTC-3′ (Petroni et al. 2002) for eukaryotes that amplify near complete 18S rRNA gene sequences (>1500 bp).

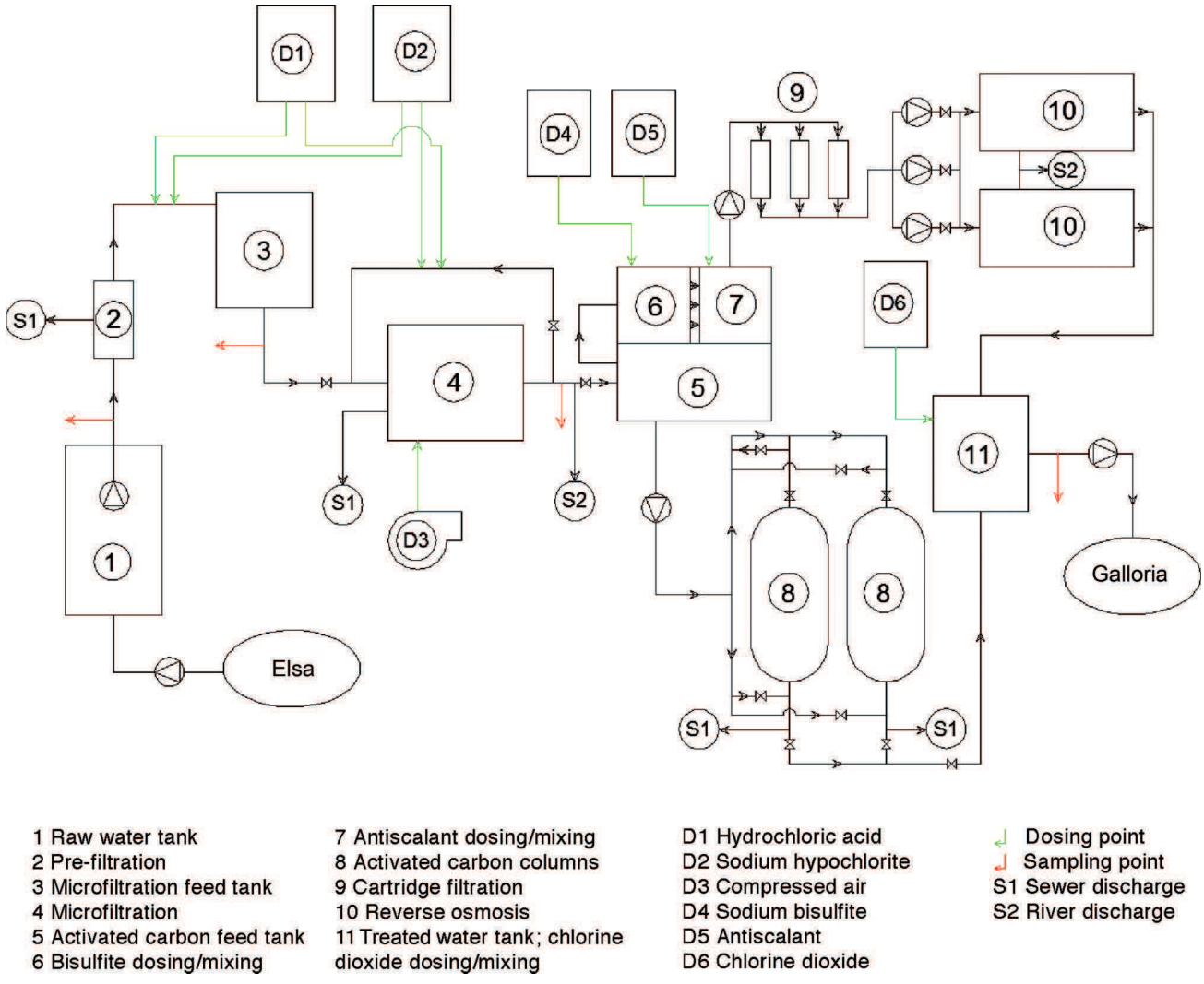

Figure 1. Block diagram of the Elsa purification plant in the town of Poggibonsi. Galloria = a tank in the water supply network.

Table 2. Plant performance data at start-up and moment of sampling.

Value	Date	Feed channel pressure drop (bar)	Normalized flux (l m^{-2} h^{-1})	Salt passage
At start of plant operation	January 2008	0.65	23.0	99.5%
At time of membrane sampling	June 2008	2.2	14.5	98.4%

A 50 μl PCR was performed for each sample using 0.25 mM deoxynucleoside triphosphates (2.5 mM each), 0.6 pmol μl^{-1} of primer forward, 0.6 pmol μl^{-1} of primer reverse, 2.5 μl of template DNA, and 0.03 u μl^{-1} of taq polymerase (Ex Taq, TAKARA, Japan).

Thermocycling was performed using a 'Primus 96 plus' thermocycler (MWG Biotech, Germany) at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 2 min with a final extension step at 72°C for 10 min (Iannelli et al. 2012). Amplicons were purified using the EuroGOLD Cycle-Pure Kit (EuroClone®, Italy).

Characterization of prokaryotic Operational Taxonomic Units (OTUs)

The method described by Amann et al. (1995) was used. The 16S rRNA amplicon from membrane square

Figure 2. Whole leaf view showing foulants. Courtesy of Protecno s.r.l. Italy.

'33' was cloned in a plasmid vector (pCRs2.1-TOPOs, TOPO TA Cloning Kit, Invitrogen, UK) and inserted in chemically competent *E. coli* cells (One Shot TOP10, Invitrogen, UK). Inserted fragments from a representative number of clones were then amplified by PCR with primers M13F (5′-GTAAAACGACGGCCAG-3′, Invitrogen, UK) and M13R (5′-CAGGAAACAG CTATGAC-3′, Invitrogen, UK), included in the Cloning Kit.

The 16S rRNA fragments of the expected size were digested with restriction endonuclease BsuRI (Fermentas®, Canada). The digested fragments were visualized by electrophoresis on 2% agarose gel after ethidium bromide staining. Fragments showing identical electrophoresis pattern were grouped together by Restriction Fragment Length Polymorphism (RFLP) analysis, and each group was characterized by sequencing an appropriate number of cloned inserts. Plasmid DNA was extracted with ZR Plasmid Miniprep™ (Zymo Research, CA, USA), and sequenced with primers T7 (5′-CCCTATAGTGAGTCGTATTA-3′, Invitrogen, UK) and M13R (Invitrogen, UK) by the Macrogen Inc. sequencing service (South Korea).

Characterization of eukaryotic OTUs

RFLP analysis with BsuRI (Fermentas® Canada) on the eukaryotic 18S rRNA amplicon revealed the presence of a dominant pattern. Direct sequencing was performed by Macrogen Inc. sequencing service (South Korea) using the following primers: forward F783 5′-GACGATCAGATACCGTC-3′, reverse R1052 5′-AACTAAGAACGGCCATGCA-3, reverse R536 5′-CTGGAATTACCGCGGCTG-3′ (Rosati et al. 2004), and the primer reverse R536mod 5′-CTGGAATTACCGCGGGTG-3′ specifically designed for the present work. The use of three sequencing primers was necessary to sequence the whole amplicon.

Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis

For T-RFLP analysis (Liu et al. 1997), the 16S rRNA primer 8F was labeled in 5′ with FAM (6-carboxyfluorescein, maximal absorption wavelength: 494 nm, maximal emission wavelength: 518 nm) and the 18S rRNA primer F9 was labeled in 5′ with VIC (Registered Trademark by Applied Biosystem CA, USA, maximal absorption wavelength: 538 nm, maximal emission wavelength: 554 nm). Restrictions of the 16S PCR product were independently obtained by using BsuRI (0.2 u μl^{-1}, Fermentas, Canada) and AluI (0.2 u μl^{-1}, Fermentas, Canada) at 37°C for 4 h. The restriction of eukaryotic 18S PCR was performed using restriction enzyme HpaII (0.2 u μl^{-1}, Fermentas, Canada).

After restriction, DNA was precipitated with cold 100% ethanol to eliminate salts in a 5424 R refrigerated centrifuge (Eppendorf, Italia) at 4°C and 10,000 RCF. For each reaction, a mix with 1.2 μl of loading buffer (GeneScan™ 600 LIZ, Applied Biosystem, CA, USA), a maximum of 5.5 μl of sample (the volume of samples was calculated on the bases of its final concentration after cold ethanol precipitation) and 13.3 μl of deionized formamide (Applichem, Germany) was prepared. Capillary electrophoresis was performed with Abi Prism 310 Genetic Analyzer (Applied Biosystem, CA, USA); T-RFLP profiles were analyzed using GeneScan™ analysis software (Applied Biosystem, CA, USA).

T-RFLP analysis was also performed on sequenced clones to empirically estimate fragment size; these data allowed unambiguous attribution of a species to single peaks derived from the T-RFLP profiling of each analyzed membrane plug.

Phylogenetic analysis

NCBI BLAST analysis (Altschul et al. 1997) was used to determine the affiliation of clone sequences obtained from the sequencing of the library. After BLAST analysis, sequences were inserted in a SILVA 104 ARB database (Pruesse et al. 2007) and aligned using the appropriate tool from the ARB software package (Ludwig et al. 2004). Chimeric sequences were detected using ARB software and visual inspection; they were cut in correspondence of the recombination site and fragments were considered as independent sequences in

the analysis of 16S rRNA gene library data. Chimeric sequences were not included in the phylogenetic analyses due to their shortness.

Phylogenetic analysis was performed comparing all sequences with those of closely related type strains (known gene sequences that identify cultivated and validly described species) present in the SILVA database (Pruesse et al. 2007). When type strains or cultivable organisms were not available, sequences of uncultured bacteria from environmental samples were used in the tree. The root was arbitrarily set to separate Planctomycetales from other organisms. Phylogenetic trees were made using the Maximum Likelihood method with a filter specifically designed in the present study for the selection of sequences used in the trees.

Light microscope observations

Two ml of San Benedetto water (San Benedetto S. p. A. Italy) were added to 1 ml of the stored 34 square pieces (side: $\sim$2 cm) of the RO membrane and kept for 24 h to allow the membrane layer re-hydrate. A Leitz Orthoplan microscope (Ernst Leitz Microscopes, Wetzlar, Germany) equipped for Differential Interference Contrast (DIC) microscopy was used to perform observation of both the re-hydrated membrane pieces and the water over a range of magnifications from 125 to 1250x. Images were captured with a Canon PowerShot S45 digital camera and used to collect measurements with the software GNU Image Manipulation Program (GIMP) 2.6 (http://www.gimp. org/).

Scanning electron microscopy (SEM)

SEM was performed on two of the membrane pieces. An 0.8×0.8 cm square was subsectioned from the larger sample in order to fit on the SEM stubs. The different sub-layers of the RO membrane of each sample were separated and independently mounted on SEM stubs. After gold coating (final gold thickness: 20 nm), the samples were observed using a JEOL JSM-5410 SEM (JEOL Ltd, Tokyo, Japan).

Results

Molecular characterization of the prokaryotic community

Within a total of 55 screened clones having an insert of the expected size, 31 different BsuRI patterns were retrieved. A total of 27 clones, representative of the more abundant patterns, were completely sequenced (Table 3). Accession numbers of newly characterized sequences were from HE575376 to HE575398. After appropriate screening of all sequences with the Bellerophon server (Hugenholtz and Huber 2003), four sequences were determined to be chimeric, one of which comprised fragments deriving from three different organisms. Chimeric sequences were not deposited in the DDBJ/EMBL/GenBank databases. Clones having an identity higher than 97% were grouped together and attributed to the same Operational Taxonomic Unit (OTU).

The majority of analyzed clones (44%) associated with bacteria belonging to the phylum Actinobacteria (Figure 3) and were represented by seven different OTUs belonging to three families: the Microbacteriaceae (28%, three OTUs), the Thermomonosporaceae (12%, one OTU), and the Nocardioidaceae (3%, two OTUs). The seventh OTU was retrieved from a chimeric sequence (1%).

Other microorganisms occurring in the library in high percentages were: the Alphaproteobacteria (17%), represented by four OTUs belonging to the families Sphingomonadaceae (5.25%), Xanthobacteraceae (5.25%), Bradyrhizobiaceae (1.25%, represented by a portion of a chimeric sequence) and the Caulobacteraceae (5.25%); the Planctomycetes (13%) represented by three OTUs; the Firmicutes (9%) represented by two OTUs both belonging to the Clostridiaceae.

The remaining clones were associated with different groups of bacteria, such as the Chloroflexi, Bacteroidetes, and Betaproteobacteria, and were represented by percentages corresponding to 10%.

The results from the analysis of the T-RFLP profiles of two membrane plugs (numbers 33 and 34) (Figure 4) were substantially congruent with each other and with results obtained from 16S rRNA library of membrane plug 33 (Figure 3). The highest percentage of T-RFs was attributed to the Microbacteriaceae (26–36%). An important percentage was also attributed to the Sphingomonadaceae (6–17%). *Thiobacillus* sp. was identified with both enzymes at a low percentage (2–4%).

Considering the two restriction enzymes separately, it was evident that the Sphingomonadaceae were more commonly detected in samples digested with BsuRI (15–17% *vs* 6%). This was also true for Planctomycetacia OTUs 1 and 2 (5–6% *vs* 2–3%).

The Caulobacteraceae group was evident only in the sample digested with AluI and represented only 1% of the total. In the samples digested with the restriction enzyme AluI, the Thermomonosporaceae (Microbacteriaceae) and the Clostridiaceae were represented by the same peak, while in the samples digested with BsuRI, the peaks identifying the two OTUs were clearly separated.

Phylogenetic analysis

In a preliminary phylogenetic analysis the characterized sequences were added, using Interactive

Table 3. 16S rRNA clone library description.

RFLP pattern	N° of Clones	OTU	Name of the sequenced clone	% First described	Affiliation	Classification
A	10	Actino_1	5	97% AY571813	*Frigoribacterium* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
			96	98% AY571813	*Frigoribacterium* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
		Actino_2	78	99% D84638	*Leifsonia* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
			17	99% D84638	*Leifsonia* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
B	4	Actino_3	2	98% AJ717388	*Microcella putealis*	Microbacteriaceae, Actinomycetales, Actinobacteria
			93	99% HQ652551	*Microcella* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
C	4	Actino_4	18	98% AB331652	*Actinomadura bangladeshensis*	Thermomonosporaceae, Actinomycetales, Actinobacteria
D	2		1	100% HQ157187	*Actinomadura* sp.	Thermomonosporaceae, Actinomycetales, Actinobacteria
E	1	Actino_5	77	98% CP000509	*Nocardioides* sp.	Nocardioidaceae, Actinomycetales, Actinobacteria
F	1	Actino_6	92	97% FR733712	*Propionicimonas paludicola*	Nocardioidaceae, Actinomycetales, Actinobacteria
G	3	Alpha_1	25	99% AJ244650	*Brevundimonas*-like sp.	Caulobacteraceae, Caulobacterales, Alphaproteobacteria
H	3	Alpha_2	23	99% EU591707	*Sphingomonas* sp.	Sphingomonadaceae, Sphingomonadales, Alphaproteobacteria
I	2	Alpha_3	3	99% EF592179	*Xanthobacter flavus*	Xanthobacteraceae, Rhizobiales, Alphaproteobacteria
J	1		28	99% EF592179	*Xanthobacter flavus*	Xanthobacteraceae, Rhizobiales, Alphaproteobacteria
K	2	Clos_1	15	97% AJ506120	*Clostridium bowmanii*	Clostridiaceae, Clostridiales, Clostridia
L	1		87	97% AJ506120	*Clostridium bowmanii*	Clostridiaceae, Clostridiales, Clostridia
M	1	Clos_2	10	93% X71853	*Clostridium populeti*	Clostridiaceae, Clostridiales, Clostridia
N	3	Plancto_1	34	88% X81940	*Pirellula* sp.	Planctomycetaceae, Planctomycetales, Planctomicetacia
O	3	Plancto_2	42	90% FJ236059	Uncultured *Pirellula* sp.	Planctomycetaceae, Planctomycetales, Planctomicetacia
P	1	Plancto_3	27	99% X81947	*Pirellula* sp.	Planctomycetaceae, Planctomycetales, Planctomicetacia
Q	2	Beta_1	20	99% DQ390445	*Thiobacillus sajanensis*	Hydrogenophilaceae, Hydrogenophilales, Betaproteobacteria
R	2	Bacte_1	59	95% FJ754321	*Parapedobacter compostus*	Sphingobacteriaceae, Sphingobacteriales, Bacteroidetes
S	1	Chloro_1	13	89% DQ130040	Unc. *Thermomicrobium* sp.	Thermomicrobiaceae, Thermomicrobiales, Chloroflexi
T*	1/3	Clos_1	30_5P	94% GU129927	*Clostridium magnum*	Clostridiaceae, Clostridiales, Clostridia
	1/3	Actino_2	30_mid	99% D84638	*Leifsonia* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
	1/3	Clos_2	30_3P	94% AB573069	*Clostridium aminovalericum*	Clostridiaceae, Clostridiales, Clostridia
U*	1/2	Actino_2	35_5P	96% AM935000	*Leifsonia* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
	1/2	Actino_1	35_3P	98% AY571813	*Frigoribacterium* sp.	Microbacteriaceae, Actinomycetales, Actinobacteria
V*	1/2	Clos_1	86_5P	96% AJ506120	*Clostridium bowmanii*	Clostridiaceae, Clostridiales, Clostridia
	1/2	Alpha_4	86_3P	100% AB099660	*Oligotropha carboxidovorans*	Bradyrhizobiaceae, Rhizobiales, Alphaproteobacteria
W*	1/2	Actino_4	79_5P	99% AB331730	*Actinomadura chokoriensis*	Thermomonosporaceae, Actinomycetales, Actinobacteria
	1/2	Actino_7	79_3P	96% FJ889256	Uncultured Actinomycetales	Actinomycetales, Actinobacteria

Note: *Chimeric sequence.

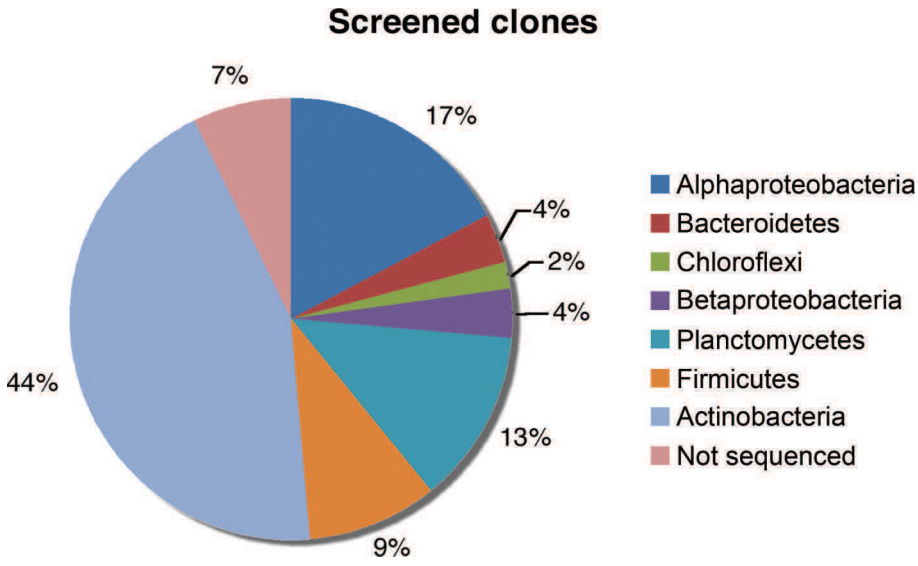

Figure 3. Pie chart representing the different groups of bacteria identified from the membrane by RFLP and sequencing.

Parsimony of Arb software (Ludwig et al. 2004), on a tree containing more than 450,000 bacterial sequences representative of all bacterial taxa (Pruesse et al. 2007). Characterized sequences affiliated to six main bacterial phyla, viz. the Proteobacteria, Bacteroidetes, Chloroflexi, Planctomycetes, Firmicutes, and the Actinobacteria (data not shown). A more refined phylogenetic analysis was performed using PHYML with 100 bootstraps (Guindo and Gascuel 2003) from the Arb package on a total of 87 sequences, including 23 of the clone sequences which had been sequenced, and 60 closely related type strains that were selected on the basis of the preliminary analysis. In four cases no closely related type strains were available and sequences of uncultured bacteria were selected (Figure 5). Accession numbers of newly characterized sequences between HE575376 and HE575398 are shown in Figure 5. Due to the reduced length (ranging from 205 to 1239 bp), chimeric sequences were not deposited in the DDBJ/EMBL/GenBank databases and were not included in the phylogenetic analyses.

All clades corresponding to the six different phyla were supported by high bootstrap values (100%) confirming the stability of nodes and the validity of phylogeny (Figure 5). A large fraction of clone sequences was represented by the Actinomycetales. Clones ROM 5 and ROM 96, were representatives of OTU Actino_1; clones ROM 78 and ROM 17 were representatives of OTU Actino_2; both OTUs were situated in a clade composed of several genera of Microbacteriaceae (e.g. *Rhodoglobus, Leifsonia, Salinibacterium, Cryobacterium, Frondihabitans*, and *Frigoribacterium*) that had tangled phylogenetic relationships. Clones ROM 93 and ROM 2 represent an OTU of organisms affiliated with the genus *Microcella* (family Microbacteriaceae). Clones ROM 1 and ROM 18, representing OTU Actino_4, were clearly members of the genus *Actinomadura* (family Thermomonosporaceae). Two other clones, ROM 92 and ROM 77, branched independently within the family Nocardioidaceae and were associated with the genera *Propionicicella* (Bae et al. 2006) with 59% bootstrap support and *Nocardioides* with 100% bootstrap support, respectively. Alphaproteobacteria were represented by four clones and three OTUs. Clones ROM 28 and ROM 3 were representatives of OTU Alpha_3 and clearly belonged to genus *Xanthobacter*. Clone ROM 23, associated with the genus *Sphingomonas* although with a low bootstrap value (<60%). Clone ROM 25, associated with the genus *Phenylobacterium* and in particular with the species *Phenylobacterium haematophilum* with 100% bootstrap support. Concerning the Betaproteobacteria, clone ROM 20 associated with *Thiobacillus sajanensis*. The only clone representative of the phylum Bacteroidetes was clone ROM 59, which associated with genus *Parapedobacter* (80% bootstrap value). It is worth noting that clone ROM 10 (representative of OTU Clos_2), together with clones ROM 15 and ROM 87 (representing OTU Clos_1), belonged to phylum Firmicutes, and all affiliate with bacteria belonging to genus *Clostridium*, an anaerobe. The three Planctomycetes clones (and OTUs) as well as the single Chloroflexi clone did not match to closely related cultivable organisms. Data concerning the characterized OTUs are summarized in Table 3.

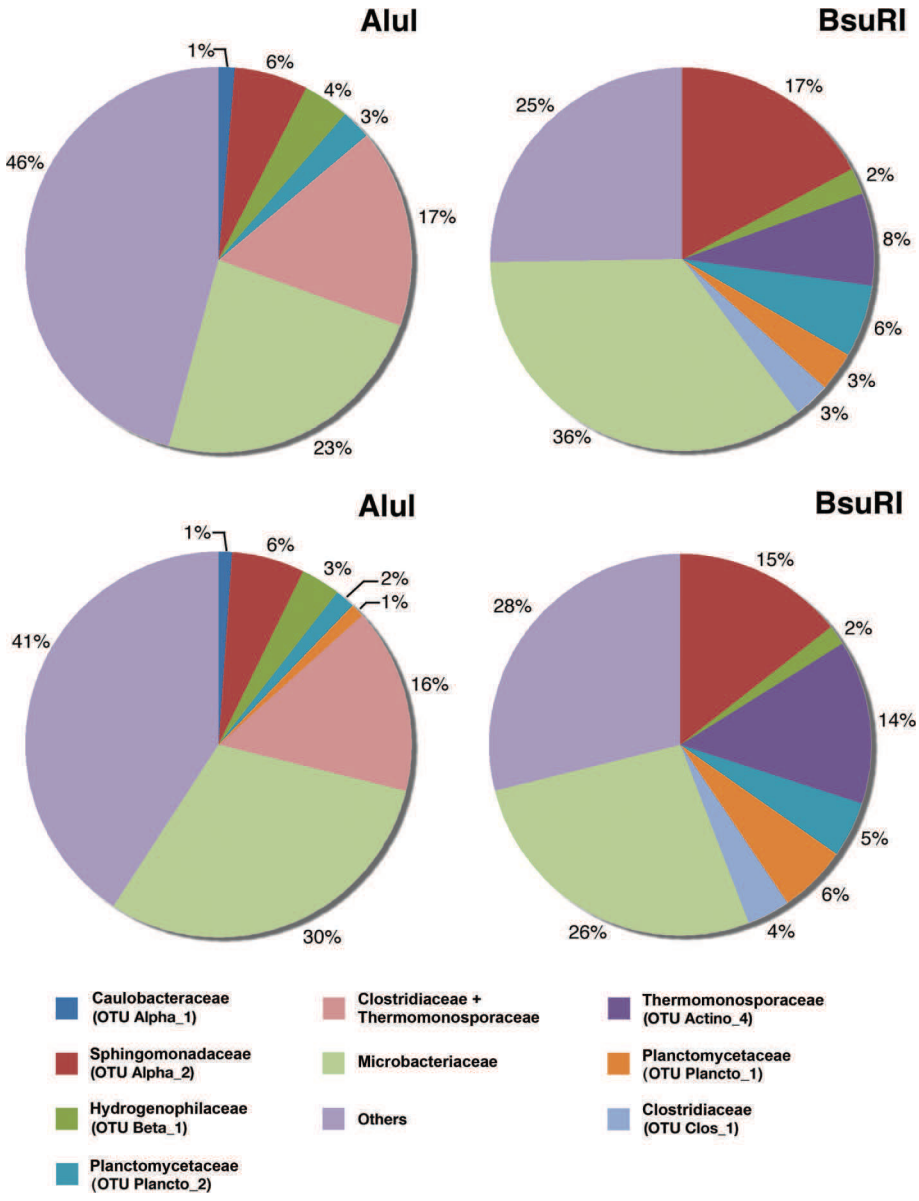

Figure 4. Pie charts representing the quantification of specific sequenced clones in T-RFLP profiles. The upper charts are data for membrane plug 33 and the lower charts are data for membrane plug 34. The charts on the left are based on the BsuRI restriction enzyme, while the pie charts on the right show profile data obtained after digestion with AluI.

Molecular characterization of the eukaryotic community

The three direct sequences of the PCR product of sample plug 33 amplified with universal eukaryotic primers showed in all cases the presence of a dominant sequence. The sequences from primers F783 and R1052 identified the presence of an organism closely related to an unclassified amoebozoan species (99% similarity, Acc. Nr. AB425950, Murase and Frenzel 2008). The sequencing primer R536 produced a sequence (Acc. Number HE575400) that associated with a completely different organism, an uncultured fungus (91% similarity, Acc. Nr.GU919442). Considering that the three sequencing primers used are considered 'universal' for eukaryotes, this result was largely unexpected. A careful reading of the direct sequence obtained with primer R1052, showed an uncommon point mutation in the target region of primer R536 in the amoebozoan organism. In order to sequence the 5′ end of the 18S rRNA gene of the amoebozoan, a new reverse primer called R536mod was designed; with this primer the 18S rRNA sequence of the amoebozoan was completed (Acc. Number HE575399). T-RFLP analysis of the 18S rRNA amplicon digested with restriction enzyme HpaII

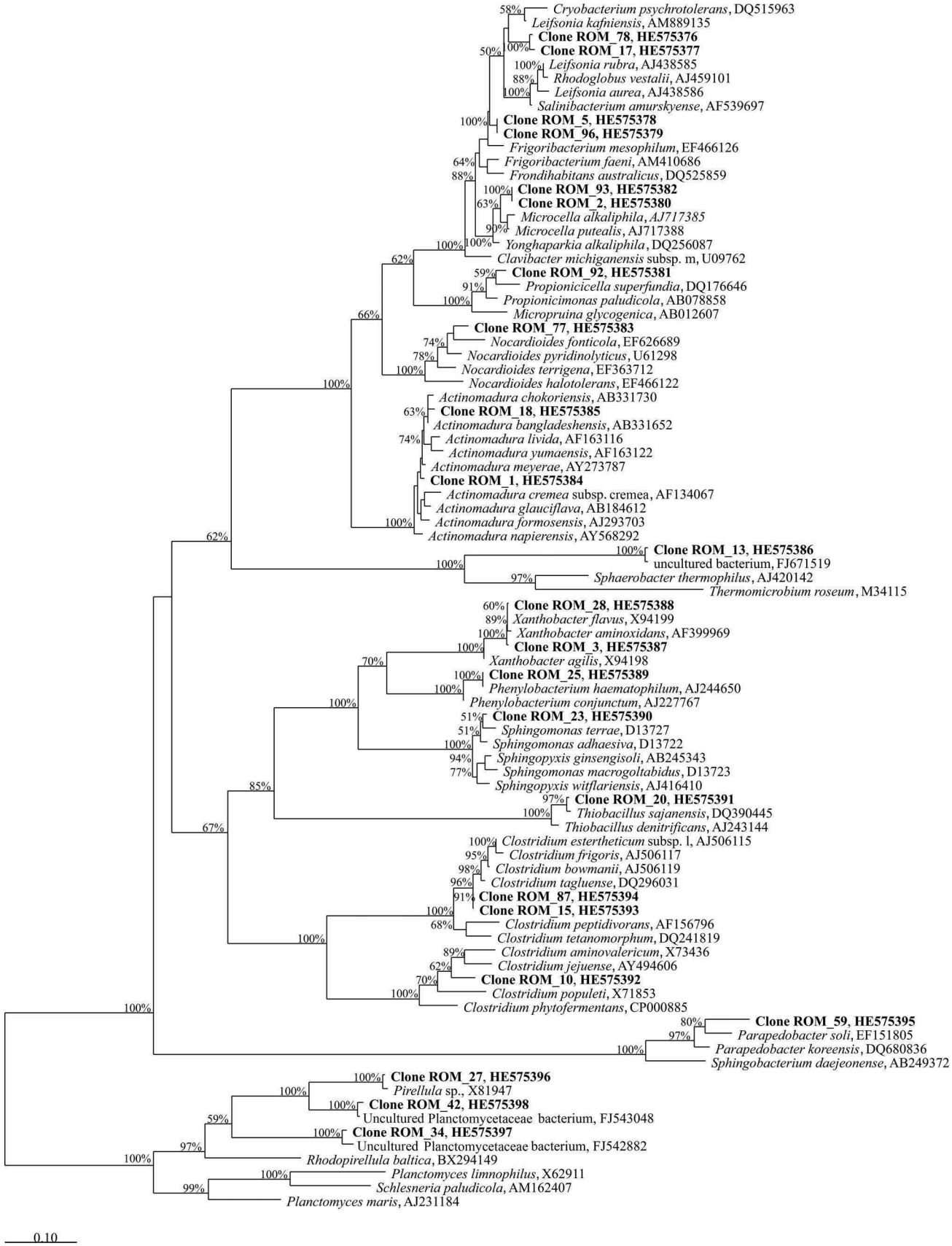

Figure 5. Maximum Likelihood phylogenetic tree of characterized bacterial OTUs and of their closer Type Strains. Numbers indicate bootstrap values out of 100 pseudoreplicates.

confirmed the presence of two main peaks, the dominant (53%) corresponding to the peak expected for the amoebozoan sequence (Figure 6).

Differential interference contrast (DIC) microscopy and scanning electron microscopy

DIC performed after re-hydration of membrane squares revealed only the presence of some brownish to blackish, round bodies, ~19 μm in diameter (mean) (size range: 7.5–24 μm), associated and wrapped up with longer, transparent, branched structures (Figure 7 a, b, c); based on morphology and size these objects were tentatively interpreted as protozoan cysts.

SEM observation of the membrane surface identified various objects which appeared to be of biological origin. These included rare, single, smooth and roundish shaped bodies (possibly cysts) which were ~6 μm in diameter (Figure 8a), relatively abundant straight to spiraled chains with oval bacteria (~0.8 μm in length) scattered over the membrane, (Figure 8b), less abundant chains (between 1.0 and 1.8 μm long) of various bacterial cells with a rough surface and connected to a branched structures (Figure 8c and d) and longer (y ~ 3.5 μm long and 1 μm in width) oval bodies with a chain morphology (Figure 8e, f). In some cases bacterial cells appeared to have collapsed in the centre, possibly representing a dehydration artifact.

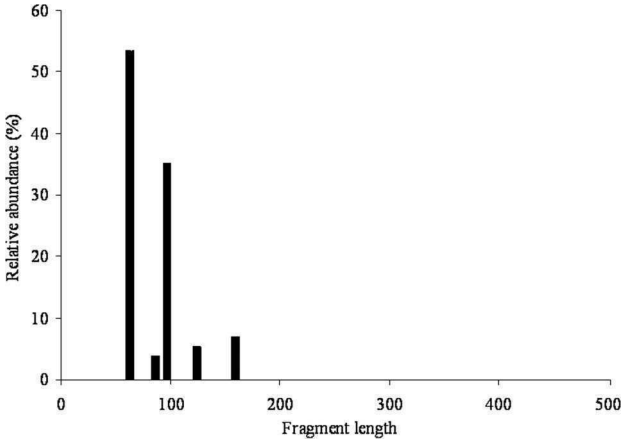

Figure 6. T-RFLP profile of the 18S rRNA amplicon digested with restriction enzyme HpaII. The highest peaks represent the amebozoa (53%) and the fungi (34%) respectively.

Discussion

Molecular characterization of the prokaryotic community

RFLP analysis of the library and sequencing of representative clones suggested that the Actinobacteria was the dominant bacterial group on the membrane (Figure 2). The T-RFLP approach corroborated these data, highlighting the presence of two dominant peaks corresponding to the dominant bacterial OTUs found in the library, such as the Microbacteriaceae and Thermomonosporaceae (Actinobacteria), the Sphingomonadaceae (Alphaproteobacteria), and the Planctomycetaceae (Planctomycetes). The dominance of Actinobacteria in the present study is novel with respect to other studies performed on RO membranes which reported the dominance of Alphaproteobacteria and Betaproteobacteria (eg Pang and Liu 2007, Bereschenko et al. 2008). The data also showed the presence of many lower peaks (data not shown) corresponding to other OTUs detected among the sequenced clones like the Caulobacteraceae, and Hydrogenophilaceae, both members of the phylum Proteobacteria, Clostridiaceae (Firmicutes), and others. Other smaller peaks (data not shown) belonging to various groups of bacteria with a low representation could not be assigned to specific taxa

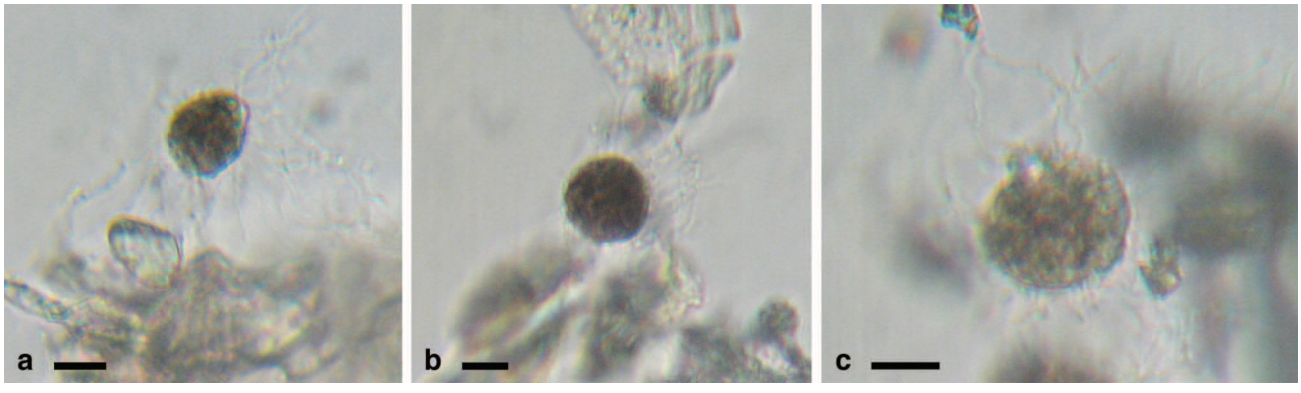

Figure 7. DIC investigation. Three roundish bodies (in a, b and c) interpreted as amebozoan cysts, associated with branched structures. In (b) a separated external layer (likely corresponding to outer cyst wall) is visible. Scale bars = 10 μm.

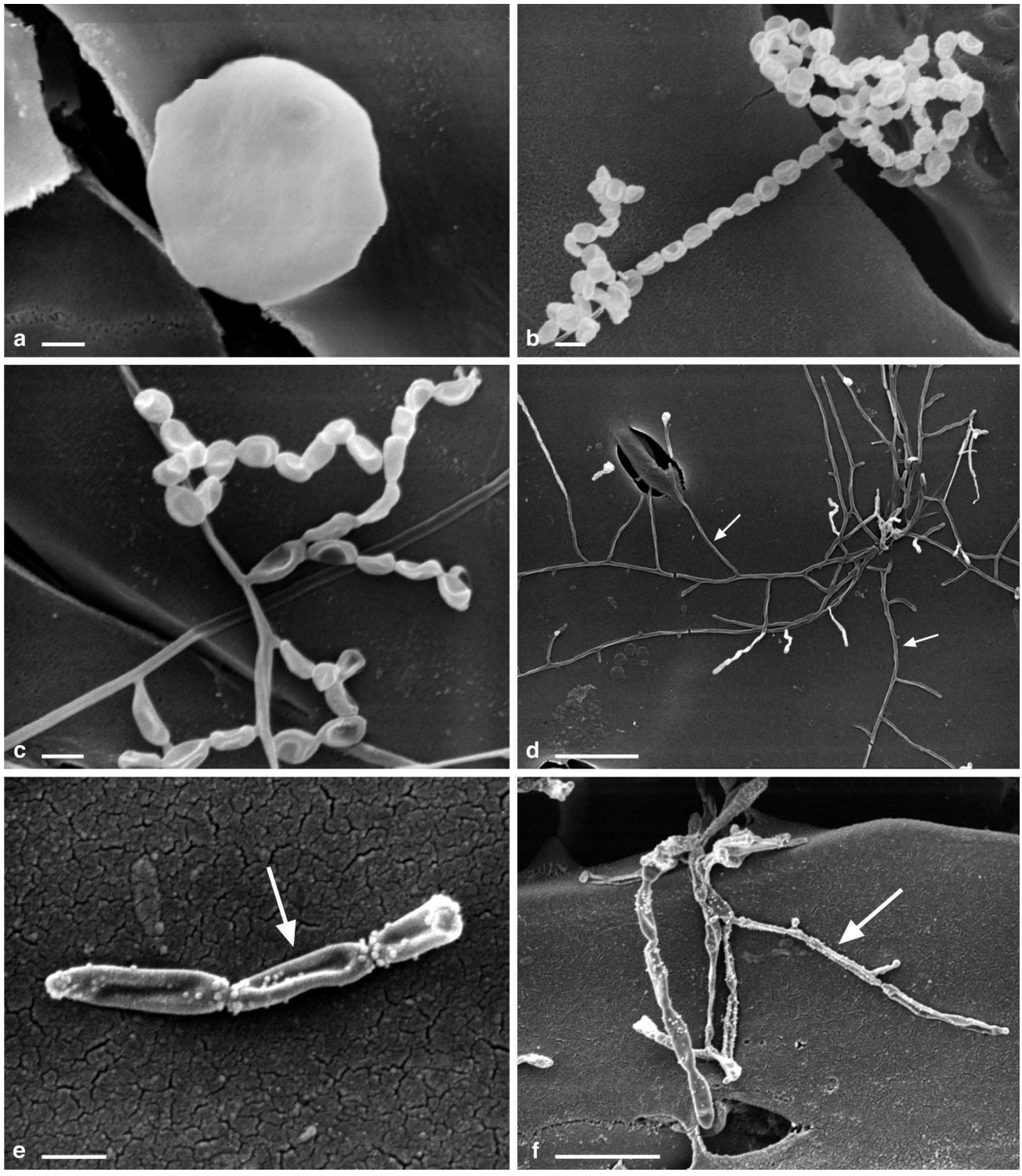

Figure 8. SEM investigation. (a) Single roundish body interpreted as an amebozoan cyst. (b) Chains of connected Actinobacteria-like cells. (c) Actinobacteria-like cells connected to extensively branched structures which were variable in length, and rod shaped structures. (d and f) Rod shaped structures (white arrows) coated with bleb-like structures, and (e) oval, larger bodies in chains (arrow). Scale bars = 1 μm (a–c, e.); 10 μm (d); 5 μm (f).

(indicated as 'others' in Figure 4). Considering the chemical characteristic of incoming water, the presence of sulfate reducing bacteria (SRB) might have been expected as in similar studies, eg Chen et al. (2004). The absence of SRB from the analyzed library can be explained by several possibilities. First, although the

sulfate concentration was high, other conditions might not have been adequate for the proliferation of SRB. Secondly, SRB may have been present at a relatively low concentration and might not have been detected because the number of screened clones in the library was not of sufficient depth. Thirdly SRB DNA might have been preferentially degraded during storage. The presence of Alphaproteobacteria belonging to Sphingomonadaceae and, in particular, to the genus *Sphingomonas*, has been reported as a key element in biofilm formation and biofouling in several cases. *Sphingomonas* species are commonly identified as initial colonizers of RO membranes and, as a consequence, for initiating the biofouling process (eg Chen et al. 2004; Bereschenko et al. 2008, 2010). The presence of Sphingomonadaceae and *Sphingomonas* in the present study suggests that these organisms may have had an early role in the biofouling process in the Elsa plant. SEM observation of the membrane identified bacterial chains (Figure 8b–f) with a morphology consistent with that of Actinobacteria, especially of the genus *Actinomadura* (Kroppenstedt and Goodfellow 2006). In particular, the observed morphologies were similar to *Actinomadura chokoriensis, Actinomadura bangladeshensis* (Ara et al. 2008), and *Actinomadura formosensis* (previously known as *Thermomonospora formosensis*, Hasegawa et al. 1986). The depression that was visible on the surface of the cells may have been artifact caused by the age of the sample or from the SEM critical point drying, which has been shown to produce similar artifacts (Crang and Klomparens 1988). However, generally, the morphological observations were consistent with the species identified using 16S rDNA.

Actinobacteria are widely distributed in both the terrestrial and aquatic ecosystems, especially in soil, where they have an important role in the recycling of refractory biomaterials by decomposition and humus formation (Goodfellow and Williams 1983; Stach and Bull 2005). Their presence on the surface of the membrane could be due to the fact that for maintenance reasons, the microfiltration stage was protractedly run without any preliminary fine screening, possibly causing failure of some of the hollow-fiber membranes. The preliminary chlorination could sporadically allow the passage of some microorganisms either because of the presence of resistant structures such as cysts or spores (Barbeau et al. 1999; Lee et al. 2010), or because of the occurrence of insufficient-dosage episodes due to inaccuracy of the dosing system. The presence of storage tank 7 (Figure 1) between dechlorination (which was required since the RO membranes were not chlorine-tolerant) and reverse osmosis may have provided any microorganisms entering the system with enough retention time to proliferate during periods where chlorine was absent. A second possibility for the introduction of microorganisms into the system might be that during maintenance operations manholes on the top of tank 7 were repeatedly opened. The contamination would have been made more likely by the shape of the manhole covers, which allowed the retention of rainwater which might have subsequently spilled into the tank when opened. The third possibility is that microorganisms were introduced *via* non sterile components such as the membrane modules and membrane filtration installations, the pre-treatment vessels, connecting pipes and air. Moreover, seals like O-rings may bypass some water containing bacteria. Although the authors consider the first and the second possibilities more likely, the chance that contamination occurred *via* alternative and multiple pathways, cannot be excluded.

Regardless of the route of entry, contaminating microorganisms could reach the RO membrane surface because their dimensions allowed their passage through the 5 μm cartridge filtration. The cartridge filtration itself was severely fouled and had required repeated replacements. The water in tank 7 generally presented low nutrient concentrations and Non Purgeable Organic Carbon (NPOC) concentrations ranged during the year from 0.44 to 4.02 mg l^{-1}, suggesting that water in tank 7 would only support the growth of oligotrophic bacteria, thus reducing the diversity of bacteria that can survive and proliferate. Indeed, both RLFP and T-RFLP analyses evidenced the presence of a relatively simple microbial community dominated by only a few OTUs (19 OTUs detected). Bacteria growing in low nutrient conditions are generally characterized by slow growth rates and are generally correlated with a low copy number of rRNA operon per bacterial genome (eg one or two operons). Interestingly, an accurate analysis of genomes of members of the Actinobacteria related to those found in the analyzed membrane (eg *Propionibacterium avidum*, Acc. Nr. NZ_AGBA00000000, *Nocardioides* sp. Acc. Nr. NC_008699, *Clavibacter michiganensis* subsp. *Michiganensis*, Acc. Nr. NC_009480) revealed that these bacteria have just one or two ribosomal operons. The presence of other OTUs related to bacteria such as *Phenylobacterium* suggests that the environment in which the membrane was submerged was nutrient poor. One of the main characteristics of *Phenylobacterium* is its extraordinarily limited nutritional spectrum. *Phenylobacterium* does not use alcohols, amino and carboxylic acids, and ordinary complex media as substrates (Lingens et al. 1985). The authors speculate that the environmental conditions selected for the development of a bacterial biofilm community dominated by bacteria with a low growth rate. The presence of Clostridiaceae, which are

characterized by an obligate anaerobic metabolism, require the prolonged absence of dissolved oxygen. This is consistent with the dosing of sodium bisulfite for the dechlorination stage prior of tank 7. Sodium bisulfite, as a reducing agent, is dosed in excess during dechlorination and likely consumed any dissolved oxygen. It is also reasonable to assume that any anaerobic bacteria in the biofilm which developed after the initial phase would have caused a further local decrease in the dissolved oxygen concentration (Garcia-Ochoa and Gomez 2009). These considerations match the empirical observation reported by the plant-operational staff that the biofouling problems took place a few months after the startup of the plant.

The molecular results of the eukaryotic microbial community on the RO membrane showed the dominance of a single OTU having a significant similarity (>99%) with NCBI sequence AB425950, retrieved from an Amoebozoa (strain Mb_5C) found in a rice field soil in Vercelli (Italy), an environment characterized by low oxygen concentrations. Unfortunately, an exact identification of this organism was not available in the original paper (Murase and Frenzel 2008). It was likely that the amoebozoan cysts reached the RO membrane either through damaged microfiltration membranes or through the inspection manholes and through tank 7. This hypothesis is supported by DIC investigation that showed the presence of roundish bodies which were interpreted as protozoan cysts. It should be noted that the reduced size of similar structures in SEM observations were expected due to the fixation and dehydration steps (see Jerome et al. 1993; Caron and Schnetzer 2007). Based on size and morphology these structures were interpreted as cysts of representatives of the phylum Amoebozoa (for a review on cysts in Gymnamoebae, see Smirnov and Brown 2004). It is interesting to note that Amoebozoa strain Mb_5C is able to produce cysts (Jun Murase personal communication). On the basis of Murase and Frenzel's study (2008) on strain Mb_5C, the authors hypothesize that the amoebozoan might have fed by grazing on a variety of biofilm forming bacteria. The second, but less dominant eukaryote organism associated with the membrane was identified as a fungus. Fungi are saprobic organisms present in wide range of environments including freshwater. Considering these observations as a whole, the authors hypothesize that the Amoebozoan proliferated on the membrane by grazing on the bacterial biofilm, eventually causing the complete blockage of the RO process.

Conclusions

In the present study a retrospective analysis of prokaryotic and eukaryotic microorganisms possibly involved in biofouling was performed on a dried RO membrane collected from a plant 18 months after removal. Although there are several recent reports on the molecular microbiological characterization of freshly collected biofouled membranes, this is the first time that an autopsy was made on 'old material'. It is not possible to exclude the possibility that the long storage period altered the original microbial composition and it is certainly good practice to work with freshly collected material where possible. Nevertheless, the results obtained from the stored membranes are in substantial agreement with similar studies. It is possible that the isolation of the membrane in its original cover and under dry condition may have limited microbial contamination during storage. However, to validate the approach of using nucleic acid based community analysis and microscopic examination of stored membranes to retrospectively diagnose the role of microbial fouling in reduced performance of RO plants it is necessary to perform repeated sampling during storage, starting with freshly removed membranes.

Acknowledgments

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4.2 Characterization and comparison of bacterial communities selected in conventional activated sludge and membrane bioreactor pilot plants: a focus on *Nitrospira* and *Planctomycetes* bacterial *phyla*.

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Abstract

A pilot scale membrane bioreactor (MBR) and a conventional activated sludge system (CAS) were in parallel operated to investigate the impact of the separation technology on the structure and functionality of the selected microbial community. Microbial communities as well as nitrogen removal efficiency of the biomass were characterized. Kinetics and microbial community structure turned out to be correlated. The impact of the separation technology on selective conditions and, in particular, the higher variability of solid separation efficiency in CAS with respect to MBR pilot plant possibly represented the main factor influencing the selection of bacterial communities.

Concerning nitrifiers, bacteria of the genus *Nitrospira* were predominant in the MBR. This was in accordance with kinetics of nitrite oxidizing bacteria that suggested the presence of k-strategists, while r-strategists were selected in the CAS plant, possibly due to the presence of transient higher concentrations of nitrite (in the range of 0.05-0.18 and of 0.05-4.4 mg NO_2^--N L^{-1} in the MBR and CAS effluents, respectively). An unexpectedly high presence of bacteria belonging to two specific phylogenetic clades of *Planctomycetes* was found in both reactors.

Keywords

Membrane Biological Reactor (MBR), Conventional Activated Sludge (CAS), k-strategist, nitrification, *Planctomycetes, Nitrospira.*

Introduction

The main advantage of membrane bioreactors (MBR) with respect to conventional activated sludge (CAS) is the possibility of accurately setting the desired solids retention time (SRT) and of avoiding the separation problems typically occurring during settling in conventional activated sludge systems. In order to compare these technologies several factors must be taken into account; among them, filtration process, energy consumption, and effluent quality have been mainly investigated in the past. MBR came out to offer better performances in terms of effluent quality [7, 20, 50], while CAS technology allows significantly lower energy consumption [18, 19], even though MBRs still need to be optimized from this point of view [15]. However, for a complete comparison, a deeper understanding of the effect of selective pressures on microbial community structure and functionality is still needed.

Several studies related to biomass selection have been carried out comparing MBR and CAS systems in order to evaluate their efficiency in removing various pollutants (e.g. [9, 14, 20, 44]).

Fewer, partially controversial studies [17, 37, 41], focused on the comparison between kinetics of the biomasses selected by MBR and CAS, mainly in relation to nitrifying biomass.

A lower apparent half-saturation constant for oxygen of nitrifiers was found in MBR due to the smaller size of flocks and, thus, to low mass transfer limitations [11, 35]. In the same study, half saturation constants for ammonia were found to be similar.

A negligible difference was found between decay coefficients [34] of ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) in parallel MBR and CAS; uncertain is the difference for what concerns the maximum specific growth rates of both AOB and NOB [17, 37]. However, the variability of settling characteristics of the mixed liquor, typical of full scale CAS plants, often produces fluctuations of SRT and, as a consequence, of ammonia and nitrite concentrations in the nitrification tank. In accordance with the resource-ratio theory [10], the abundance of substrates can select for fast growth rate (r-strategist) bacteria, while a low concentration of substrate can select for bacteria characterized by a high affinity (K-strategist), that is a low half-saturation constant for respective substrates. According to resource-ratio theory, in the transient higher ammonia and nitrite concentrations that occurred in the CAS plant, r-strategists can be more competitive [16], while k-strategists could be favoured when constantly low concentrations of both substrates are present, such as in MBR.

Despite this intriguing aspects, only a few studies has been undertaken in order to compare differences in microbial community structure in the two processes, most of them being limited to the detection of the presence of main bacterial phylogenetic groups performed by Fluorescence *In Situ* Hybridization (FISH) methods (see for example [4, 37]). Only in two papers [26, 47] a deeper characterization of the microbial community has been performed and used for a comparative analysis between CAS and MBR processes. In both cases the studied plants were fed with particularly selected wastewaters: the wastewater coming from petroleum refineries [47] and a synthetic ammonia-bearing inorganic wastewater [26]. Therefore, in these systems, the correlation between microbial community structure and functionality still need to be deeply and comparatively investigated, especially in relation to nitrogen removal dynamics.

In this paper we present an extensive characterization of the biomass, both from a biomolecular and a kinetic point of view, selected in two pilot plants (CAS and MBR) fed with municipal wastewater, designed with the same process configuration (predenitrification-nitrification-separation) and operating in parallel. The community structure was characterized by mean of molecular, cultivation independent techniques such as 16S rRNA gene library construction, and FISH. An accurate phylogenetic analysis was also performed on two of the main bacterial *phyla*.

Aims of the study were to investigate and compare the effect of CAS and MBR processes in shaping the structure of the microbial community and to verify if the two different environments truly select for specific nitrifying microbial components, differing in kinetic features and taxonomical identity.

Material and methods

Set up and monitoring of the pilot plants

The pilot plants (schematic in Figure 1) were fed with the same municipal wastewater and inoculated with the same activated sludge collected from a wastewater treatment plant where mixed industrial and domestic wastewater are treated. Influent wastewater was directly derived from the sewer system and used as feeding without any storage. The operating conditions were identical (HRT =18 h; T = 18 °C; DO = 2 mg L^{-1}) between the two reactors; the SRT was maintained at about 20 d in both plants, however, phenomena of poor settleability of the CAS mixed liquor occurred immediately after the start up (Period I; from t = 0 d to t = 80 d), with the consequence of partial sludge losses and temporarily reduced SRT. Then, the settleability of sludge and the concentration of the mixed liquor suspend solids (MLTSS) remained stable until the end of the experiment (Period II – from t = 80 d to t = 240 d).

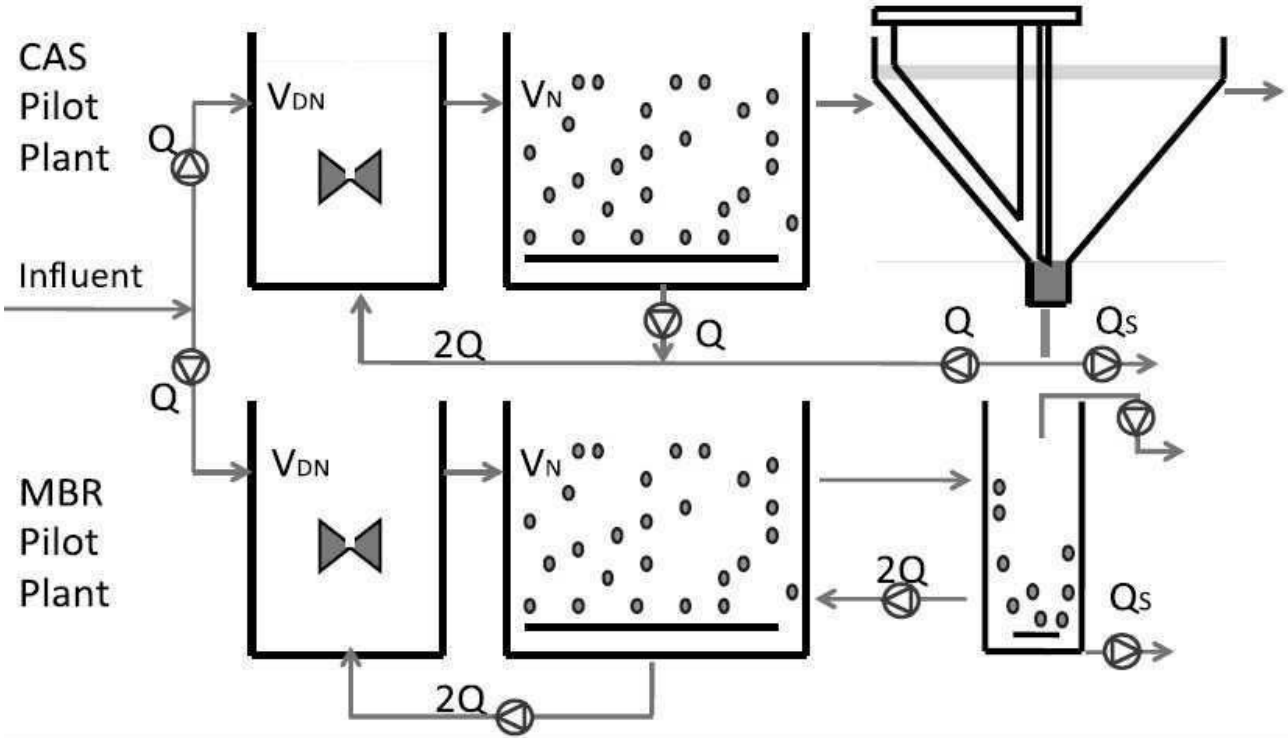


Fig. 1 - Schematic of the MBR and the CAS pilot plants.

Samples of influent, effluent and mixed liquor were analyzed twice a week for chemical characterization. Hach COD digestion vials were used to measure COD and TKN. Dissolved phosphate and ammonium were measured using a Lachat Instrument Quik Chem 8500, following the Quik Chem orthophosphate method 10-115-01-1-O, and Quik Chem ammonia method 10-107-06-1-I. Nitrate and nitrite were analyzed by flow injection analysis (LACHAT Quickchem 8500). DO was measured with an Hach-Lange DO meter.

Titrimetric and respirometric batch tests (pH-stat, DO-stat) were carried out on inoculated biomass and on mixed liquor samples collected from the CAS and MBR plants when steady state conditions were reached; the main objective of these tests was to monitor the maximum specific growth rates of AOB ($\mu_{max,AOB}$) and NOB ($\mu_{max,NOB}$). For kinetic tests, an open respirometer/titrimeter was used (static liquid-flowing gas); the control unit (named MARTINA and produced by Spes Srl, Italy) allowed executing OUR-test and the pH-stat, DO-stat tests in two (2 L) plexi-glass reactors simultaneously. Respirometric and titrimetric techniques and related calibration procedure are

described in detail in Munz et al. [37], while an Activated Sludge Model (ASM) with two-steps nitrification denitrification processes was used as reference [38].

Sampling and sample storage for biomolecular characterization.

Samples were collected from the aerobic tanks of CAS and MBR plants at day 240 (end of Period II) and immediately fixed before storage; for DNA extraction, two aliquots (14 ml each) of cell suspension for each sample were harvested and centrifuged (10 min at 5000g). Samples were then mechanically homogenized on ice by a potter blender for 30 minutes and centrifuged at 5000g for 10 min. The pellets were re-suspended in 500 µl saline EDTA (0.15M NaCl, 0.01 M EDTA, pH 8) and stored at –20°C. For FISH experiments, aliquots of 1.5 ml were harvested by centrifugation at 5000g for 5 min. Pellets were fixed with 4% (w/v) paraformaldehyde in PBS at room temperature for 15 min, washed with PBS and stored in PBS: ethanol 1:1 at –20°C.

DNA extraction, construction of 16S rRNA gene libraries and restriction fragment length polymorphism (RFLP) screening.

Total DNA extraction from all the stored samples was performed with the same protocol, namely a chloroform-isoamilic alcohol method. Extracted DNA was diluted 1:200 in distilled water and used for the amplification step. The 16S rRNA genes were amplified using two different couples of bacterial universal primers: forward 8F (5'-AGRGTTYGATYMTGGCTCAG-3' [24]) together with reverse R1492, slightly modified from Lane et al., [24], (5'-GGNWACCTTGTTACGACTT-3') and forward 8F together with UNI-b-rev (5'-GACGGGCGGTGTGTRCAA-3' [3]). Four different libraries were constructed with PCR products obtained with the first (libraries A and B) and second (libraries C and D) primer combination, two for the samples from CAS pilot plant (libraries A and C) and two for the samples from the MBR pilot plant (libraries B and D). Products from the amplification steps were cloned in a plasmid vector (pCR®2.1-TOPO, TOPO TA Cloning® Kit, Invitrogen U.K.) and inserted in chemically competent cells (One Shot TOP10, Invitrogen

U.K). Inserted fragments from a representative number of clones were then amplified by control PCR with primers M13F and M13R, provided with the TOPO TA Cloning® Kit. The 16S rRNA gene-sized fragments were digested with restriction endonuclease BsuRI (0.2 u/µl, Fermentas®, Canada). Digested fragments were visualized by electrophoresis on 2% agarose gel and subsequent ethidium bromide staining. Fragments showing an identical electrophoresis pattern were grouped together by RFLP analysis, and each group was characterized by sequencing an appropriate number of cloned inserts. Plasmid DNA was extracted with EuroGold Plasmid miniprep kit® (EuroClone®, Italy), and sequenced with primers M13R and T7pro by the Macrogen Inc. sequencing service (South Korea).

Detection of chimeric sequences and phylogenetic analysis.

All sequences were checked for the presence of chimeras using Bellerophon software [22]. Chimeric sequences were included in the analysis by "splitting" them at the recombination site identified by the software and considering the obtained fragments as independent sequences belonging to different organisms. This allowed to retrieve information on the composition of microbial communities also from chimeric sequences and, additionally, to partially tackle the problem of these experimental artefacts. Fragments were treated in the same way as all the complete sequences from the other screened clones, and included in 16S rRNA libraries analysis; they were not considered for phylogenetic tree construction due to their short length. NCBI BLAST analysis [1] was used to determine the affiliation of clone sequences. After BLAST analysis, sequences were inserted in SILVA 104 database [42] and aligned using the appropriate tool from the ARB program package [30]. The number of OTUs was estimated considering a threshold similarity value of 99%, using the appropriate tool from the ARB program package [30]. As several clone sequences were retrieved from Nitrite-Oxidizing Bacteria (NOB) belonging to the *phylum Nitrospira* (see below), a deeper phylogenetic analysis was performed on this group in order to precisely defining phylogenetic and systematic placement of the *Nitrospira* detected in the two

reactors. Sequences were also retrieved from organisms belonging to a poorly known clade comprised in the *phylum Planctomycetes* (see below); such organisms were then shown by FISH to represent a conspicuous fraction of the biomass (see below): for this reason a deeper phylogenetic analysis was performed on this group, too. Three different algorithms from the ARB package were used for tree construction. The PHYML tool was applied for maximum likelihood reconstruction, the PHILIP tool was used for maximum parsimony and the DNA DIST without correction was applied in neighbour joining analysis. Obtained topologies were then compared to recognize stable nodes. The trees showing the phylogeny of *Nitrospira phylum* bacteria were constructed on a carefully chosen selection of sequences comprising all our *Nitrospira* clones together with 50 almost complete sequences belonging to the type strains of each of the four species of the genus and other closely related sequences belonging to representatives of the *phylum Nitrospira*. Phylogenetic analysis was done using three different filters: filter bacteria (retaining conserved positions in 50% of sequences belonging to Eubacteria), filter Nitrospira_0 (considering conserved positions in all sequences belonging to *Nitrospira phylum*) and filter Nitrospira_30 (retaining conserved positions in 30% of sequences belonging to *Nitrospira phylum*). Trees showing the phylogeny of *Planctomycetes phylum* bacteria were constructed on a carefully chosen selection of sequences comprising all of our *Planctomycetes* clones together with 116 almost complete sequences comprehensive of 9 type strains and closely related sequences representative of many groups of the *Planctomycete phylum*. Phylogenetic analysis was done in this case using the filter Planctomycetes_5 which retains conserved positions in the 5% of the selection of sequences chosen for tree construction.

A distance matrix was also calculated with the appropriate tool from the ARB program package and examined for the analysed group of sequences, in order to understand which clades represent species-level or genus-level groups.

Diversity indices.

Simpson's diversity index and Shannon's diversity index were calculated on the data obtained from libraries screening, respectively as:

$Ds = 1 - \Sigma p_i^2$;

$H = -\Sigma p_i \ln p_i$

Simpson's index (Ds) represents the probability that two randomly selected individuals drawn from a population p_i belong to the same species i; it measures the "evenness" of a community ranging from 0 to 1. Shannon's diversity index (H) represents the proportion p_i of the species i relative to the total number of species, multiplied by the natural logarithm of the proportion itself. The resulting product is summed up across species, and multiplied by -1 [31].

The Shannon index varies from 0 for communities with only a single taxon, to values that are higher for communities with more taxa, with fewer individuals each.

The Dominance Index (D = 1-Ds) was also calculated; it ranges from 0 (when all taxa are equally present) to 1 (when a single taxon dominates the community completely).

Fluorescence *in situ* hybridizations.

In order to optimize the visualization by conventional epifluorescence microscope, the protocol was implemented with mechanical disruption of flocks. For this purpose, the samples were treated with two passages trough syringe needle before the hybridization step. For FISH experiments, samples were fixed with paraformaldehyde 4% (v/w) in PBS, dehydrated using ethanol and resuspended in PBS. Ten microliters of each sample were filtered on Nucleopore polycarbonate filters (0.2 micrometers pore size). FISH was then performed on filters following the protocol by Manz et al. [33]. Probes were synthesized and labelled with Cy3 and fluorescein by Eurofins MWG Operon (Ebersberg, Germany); hybridization buffer contained a percentage of formamide (FA) for each oligonucleotide probe according to the author's suggestions or according to information available from ProbeBase [29]. Probes targeting some of the most commonly retrieved bacterial *phyla*, classes of the *phylum Proteobacteria*, and the *phylum Planctomycetes* were used. As we were

particularly interested in detecting the presence of AOB and/or NOB bacteria, also probes targeting *Nitrospira* bacteria and betaproteobacterial AOB were employed. Table 1 contains the list of all the probes used for FISH experiments. The samples were observed with a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) equipped for epifluorescence.

Probe	**Sequence**	**Specificity**	**Reference**
Eub 338 I	5'-GCT GCC TCC CGT AGG AGT-3'	Most *Bacteria*	[2]
Eub 338 II	5'-GCA GCC ACC CGT AGG TGT-3'	*Planctomycetales*	[12]
Eub 338 III	5'- GCT GCC ACC CGT AGG TGT -3'	*Verrucomicrobiales*	[12]
Eub 338 IV	5'-GCA GCC TCC CGT AGG AGT-3'	Anammox, *Isosphaera*, other *Planctomycetes*	[44]
b-AO233	5'- AGC TAA TCA GRC ATC GG -3'	Betaproteobacterial ammonia-oxidizing bacteria	[46]
NTSPA714	5'- CCT TCG CCA CCG GCC TTC -3'	Phylum *Nitrospira*, not *Thermodesulfovibrio islandicus*	[27]
Alfa 19	5'-CGT TCG (CT)TC TGA GCC AG-3'	*Alphaproteobacteria*, some *Deltaproteobacteria*, *Spirochaetes*	[32]
Pla46	5'- GAC TTG CAT GCC TAA TCC -3'	Fam. *Planctomycetaceae*	[38]
Delta495a	5'- AGT TAG CCG GTG CTT CCT -3'	*Deltaproteobacteria*, most *Gemmatimonadetes*	[26]
Bet 42a	5'-GCC TTC CCA CTT CGT TT-3'	*Betaproteobacteria*	[32]
Gam 42a	5'-GCC TTC CCA CAT CGT TT-3'	*Gammaproteobacteria*	[32]

Table 1 - List of probes used for *in situ* hybridization experiments.

Cell count.

After the two steps of hybridization and washing, filters were put on a clean slide and mounted with Slow Fade Light Antifade Kit (Molecular Probe, Invitrogen) containing 4',6-diamidino-2-phenylindole (DAPI). Three slides were prepared for each sample and 10 randomly chosen fields were considered for counting on each slide. The total number of cells in each observation was determined by visualization of DAPI staining, while the number of cells belonging to a specific bacterial group was assessed by the fluorescent signal of each group-specific oligonucleotide probe. Relative abundance of each specific group of bacteria was then expressed as a percentage on the total number of cells evidenced by DAPI staining.

Results

Pilot plant monitoring

Table 2 shows the results of chemical characteristic monitoring of the influent and of the effluents during Period I and Period II. On the whole, COD removal rates are in a typical range for the treatment (CAS or MBR) of domestic wastewater.

During Period I (from t = 1 d to t = 80 d), a stable and complete nitrification was obtained only with MBR plant, while the lower SRT in the CAS pilot plant did not allow a complete nitrification to be reached. On the contrary, during period II in both plants a stable and complete nitrification occurred.

The results of pilot plant monitoring and of respirometric-titrimetric batch tests allowed us to estimate the maximum specific growth rates ($\mu_{max,AOB}$ and $\mu_{max,NOB}$ for AOB and NOB respectively) during Period II, when steady state conditions were reached in both plants. The estimated $\mu_{max,AOB}$ of the CAS biomass during Period II turned out to be higher than the $\mu_{max,AOB}$ of the MBR biomass (0.86±0.10 vs 0.46±0.13 respectively) with a statistical significance of 99% (Student's t-test); moreover, it turned out to be higher than the $\mu_{max,AOB}$ of the inoculums. Similar results were found for what concerns NOB, where the $\mu_{max,NOB}$ of CAS and MBR plant were respectively 0.68±0.14 and 0.48±0.08.

Parameter	**Units**	**Influent**	**MBR Effluent Period I**	**MBR Effluent Period II**	**CAS Effluent Period I**	**CAS Effluent Period II**
COD	mg L^{-1}	257±54	18±5	17±4	65±21	36±12
TSS	mg L^{-1}	91±36	< 5	< 5	37±15	10±6
NH_4^+	mg N L^{-1}	35±7.7	0.7±0.5	0.4±0.2	13±7.1	0.8±0.4
NO_2^-	mg N L^{-1}	-	0.1±0.05	< 0.1	2.2±0.7	0.3±0.2
NO_3^-	mg N L^{-1}	-	12.2±3.1	12.4±2.8	7±2.4	11.0±3.1
TN	mg N L^{-1}	44.1±9.2	14.2±3.4	14±2.9	26±3.8	13.2±3.2
pH	-	7.5±0.1	7.6±0.1	7.6±0.1	7.6±0.1	7.5±0.1

Table 2 - Characteristics of the influent and of the effluents of both CAS and MBR pilot plants. Mean values and standard deviation during Period I and Period II.

Construction of 16S rRNA gene libraries, RFLP screening and detection of chimeric sequences.

Two libraries were constructed from CAS pilot plant (libraries A and C) and two from MBR pilot plant (B and D). RFLP screening was performed on a total of 333 clones, 130 from CAS and 203 from MBR pilot plant. After digestion with restriction endonuclease BsuRI, a representative number of clones from each digestion pattern was sequenced. 105 sequences of bacteria were obtained from libraries A and C (CAS) and 142 from libraries B and D (MBR). The EMBL accession numbers of rRNA gene sequences determined in this study are HE646287-HE646350, HE647647-HE647685, HE649968-HE650103 and HE964760-HE964768. Among all the screened clones for both plants, 32 were identified as chimeric sequences (9.6%). The ratio between the number of detected OTUs and the number of screened clones was 0.62 (library A), 0.38 (library B), 0.55 (library C), 0.64 (library D). Distribution of screened clones among different phyla in the libraries is shown in figure 2. Libraries A and C, obtained from samples coming from CAS pilot plant, show high percentages of clones associated with *Planctomycetes phylum* (20% library A and 18.35% library C), which are less represented in libraries B and D from MBR pilot plant (5% library B and 8% library D, Figure 2). *Phylum Nitrospira* is much more represented in MBR libraries B (15%) and D (10%) than in CAS libraries A and C (3% and 4%, respectively). Other *phyla* of bacteria are represented in all libraries with small differences in quantitative distribution among the two pilot plants (figure 2): libraries from CAS pilot plant contain higher percentages of microorganisms belonging to *Chlorobi* and *Verrucomicrobia phyla*, while libraries from MBR pilot plant are characterized by higher percentage of clones associated to *Acidobacteria phylum*. It is worthy of notice that both BLAST and phylogenetic analysis (see section 3.5) revealed that bacteria belonging to *Nitrospira phylum* were different in CAS and MBR pilot plants. While MBR plant harboured bacteria belonging to *N. moscoviensis* clade (having 99% similarity with sequences of *Nitrospira* cf. *moscoviensis* AF155152 and AF155153 according to BLAST), in CAS pilot plant they belong to "*Candidatus*

Nitrospira defluvii" clade (99% similarity with sequence of "*Candidatus* Nitrospira defluvii" EU559167 according to BLAST). Considering possible differences connected with the two couples of primers used, the most relevant data concern *Proteobacteria phylum*. In libraries A and B sequences related to microorganisms belonging to *Proteobacteria phylum* are clearly higher than in libraries C and D (A: 45.71%, B: 48.00% vs C: 22.44%, D: 38.79%). Finally, considering differences among distribution of chimeric sequences in the libraries and their affiliation with different bacterial taxa, a relevant datum concerns *phylum Proteobacteria* detected in library C: the percentage of sequences related to this group is lower when chimeric sequences are included in the analysis (22.4% vs. 34.3%). On the contrary, in the same library, sequences related to *Firmicutes phylum* are more abundant considering chimeric sequences (8.2% vs. 2.9%). The presence of sequences related to *Verrucomicrobia phylum* is only observed when chimeric sequences are included in library C (6.1% vs 0%).

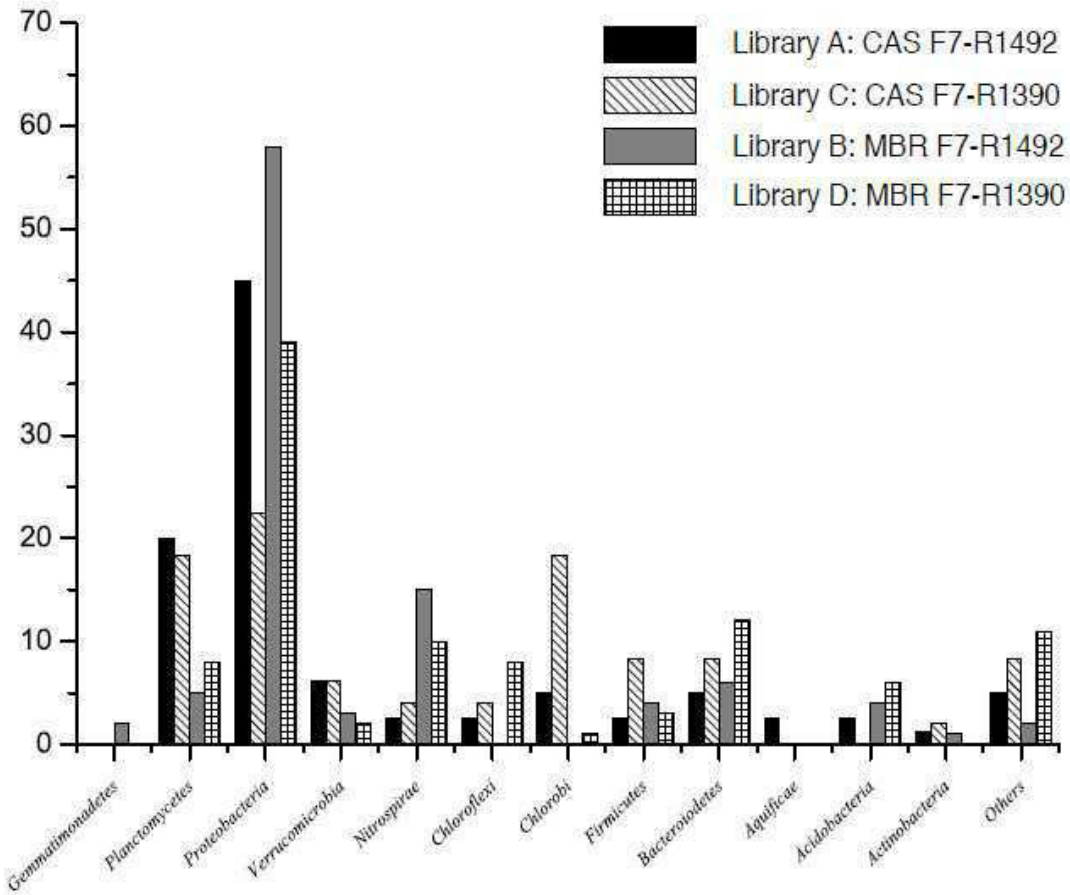


Fig. 2: Relative abundance of screened clones belonging to different bacterial phyla.

Diversity indices.

Table 3 shows diversity indices calculated on library data. The Shannon index is quite similar in both samples ranging from 4.15 in CAS sample to 4.18 in MBR sample; the Simpson index ranges from 0.97 to 0.98 respectively for MBR and CAS pilot plants; the Dominance index ranges from 0.02 to 0.03 respectively in CAS and MBR plants.

	OTUs	**1-Ds**	**H**	**Ds**
CAS	81	0.02	4.15	0.98
MBR	102	0.03	4.18	0.97

Table 3 - Diversity indices. Number of Operational Taxonomic Units (OTUs); Dominance Index (1-Ds); Shannon Index (H); Simpson Index (Ds)

Fluorescence *in situ* hybridizations.

Fluorescence *in situ* hybridization was used to confirm and quantify the presence of different groups of bacteria detected with the screening of clone libraries. Figure 3 represents relative abundance on total DAPI-stained cells of bacterial groups targeted by probes listed in table 1. It is worthy of notice that probe NTSPA714, targeting for bacteria belonging to *Nitrospira phylum*, gives positive signal in samples from both pilot plants, but the percentage is definitely higher in MBR pilot plant (24%) than in CAS (5%). Probe b-AO233 targeting for *Betaproteobacteria*-Ammonium Oxidizers, revealed the presence of this group of microorganisms in both pilot plants although percentages are low (4.8% for CAS plant and 5.6% for MBR plant). Probe Eub338 IV, specifically designed for Anammox bacteria and *Isosphaera genus* [46], showed positive signal in both samples with definitely high percentages (26.87% in CAS pilot plant and 29.41% in MBR pilot plant). All the other probes targeting the major groups of bacteria gave a positive signal in samples from both pilot plants with similar percentages between sample from CAS and sample from MBR.

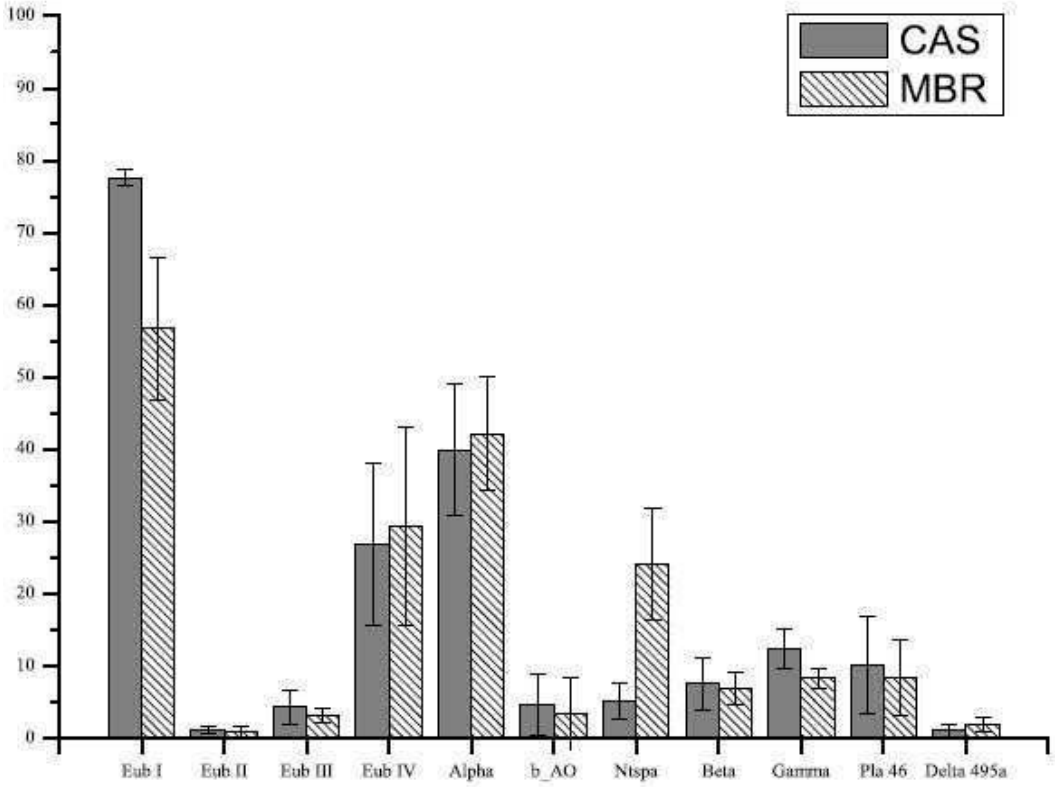


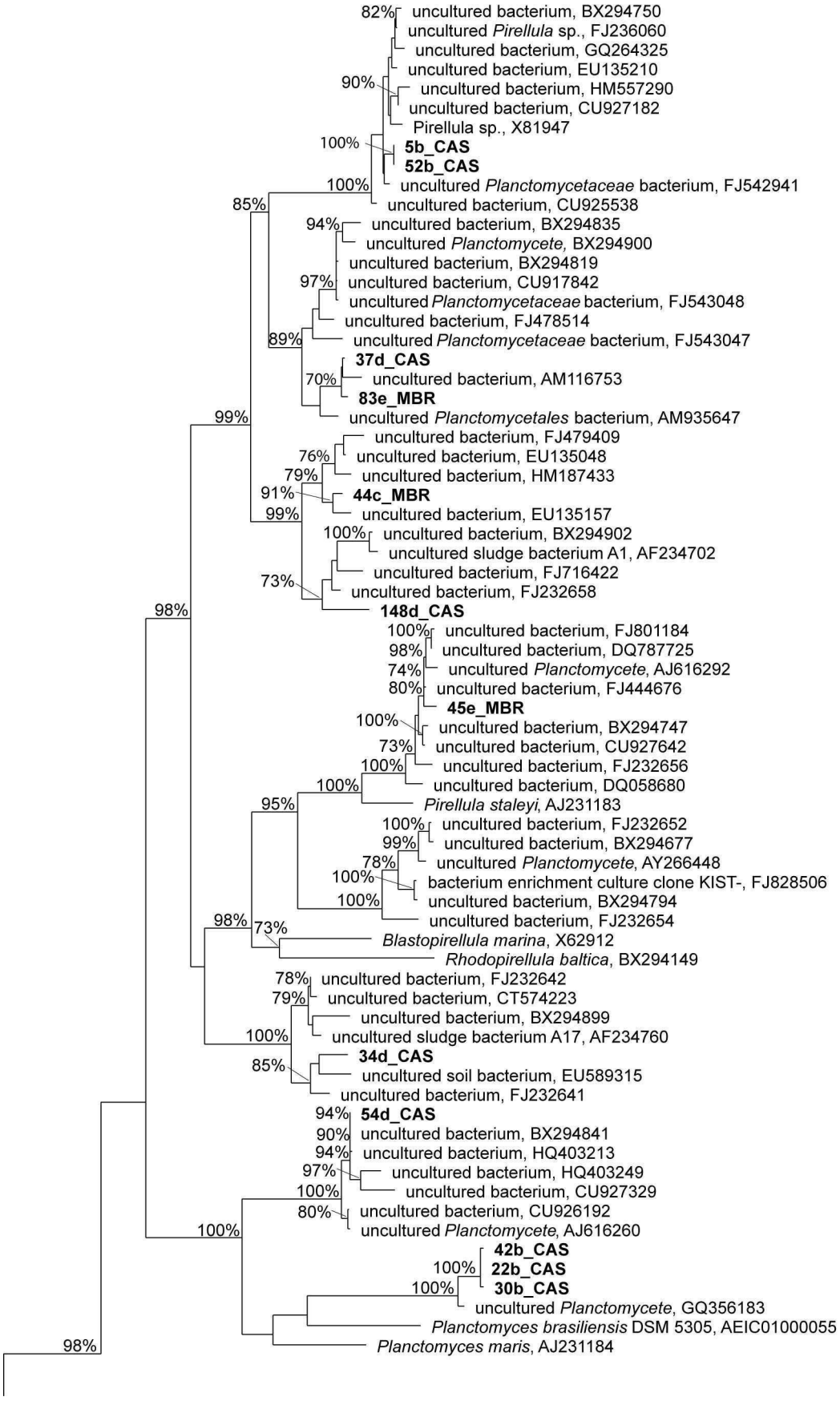
Fig. 3: Relative abundance on total DAPI-stained cells of bacterial groups targeted by probes Eub 338 I, Eub 338 II, Eub338 III, Eub 338 IV, ALF1b , b-AO233, NTSPA714, BET42a, GAM42a, Pla46 and DELTA 495a.

Phylogenetic analysis.

In every obtained topology, with all algorithms applied and with the different filters, the clones coming from MBR pilot plant are all grouped into the *N. moscoviensis* clade, while all clones from CAS pilot plant cluster within the "*Candidatus* Nitrospira defluvii" clade (Figure 4). Both these clades are well supported by bootstrap analysis (always > 95%). The association between these two clades is always strongly supported too (>96%). The position of "*Candidatus* N. Bockiana" (acc. EU084879) and of the clade including *N. marina* and associated sequences is far from being resolved within the genus. Similarity data indicate that CAS clone sequences share 98.8-99.9% similarity values with the sequence of "*Candidatus* Nitrospira defluvii". The same values are lower (96.5-97.2%) between MBR clone sequences and the sequence of *N. moscoviensis* type strain.

In all the topologies obtained with the three algorithms the associations among sequences belonging to a specific *Planctomycetes* clade are always well supported with bootstrap values above 70% (see figure 5). The similarity matrix (data not shown) provides further information about the relationships among sequences within each clade. The obtained clone sequences belong to clades comprising sequences that share minimum similarity values of 95%, 97%, or even 98%. Nevertheless, only the sequence of clone 45e_MBR belongs to a phylogenetic clade which comprises a validated species (*Pirellula staleyi*, AJ231183, [21]).

Fig. 4: Phylogenetic tree showing relationships among considered groups of *Nitrospira* phylum obtained with maximum likelihood algorithm, filter Nitrospire_30 and 100 bootstrap values. Bar, 10% estimated sequence divergence.


82% uncultured bacterium, BX294750
uncultured *Pirellula* sp., FJ236060
uncultured bacterium, GQ264325
uncultured bacterium, EU135210
90% uncultured bacterium, HM557290
uncultured bacterium, CU927182
Pirellula sp., X81947
100% **5b_CAS**
52b_CAS
100% uncultured *Planctomycetaceae* bacterium, FJ542941
85% uncultured bacterium, CU925538
94% uncultured bacterium, BX294835
uncultured *Planctomycete,* BX294900
uncultured bacterium, BX294819
97% uncultured bacterium, CU917842
uncultured *Planctomycetaceae* bacterium, FJ543048
uncultured bacterium, FJ478514
89% uncultured *Planctomycetaceae* bacterium, FJ543047
37d_CAS
70% uncultured bacterium, AM116753
83e_MBR
99% uncultured *Planctomycetales* bacterium, AM935647
uncultured bacterium, FJ479409
76% uncultured bacterium, EU135048
79% uncultured bacterium, HM187433
91% **44c_MBR**
99% uncultured bacterium, EU135157
100% uncultured bacterium, BX294902
uncultured sludge bacterium A1, AF234702
uncultured bacterium, FJ716422
73% uncultured bacterium, FJ232658
98% **148d_CAS**
100% uncultured bacterium, FJ801184
98% uncultured bacterium, DQ787725
74% uncultured *Planctomycete*, AJ616292
80% uncultured bacterium, FJ444676
45e_MBR
100% uncultured bacterium, BX294747
73% uncultured bacterium, CU927642
100% uncultured bacterium, FJ232656
100% uncultured bacterium, DQ058680
95% *Pirellula staleyi*, AJ231183
100% uncultured bacterium, FJ232652
99% uncultured bacterium, BX294677
78% uncultured *Planctomycete*, AY266448
100% bacterium enrichment culture clone KIST-, FJ828506
100% uncultured bacterium, BX294794
uncultured bacterium, FJ232654
98% 73% *Blastopirellula marina*, X62912
Rhodopirellula baltica, BX294149
78% uncultured bacterium, FJ232642
79% uncultured bacterium, CT574223
uncultured bacterium, BX294899
100% uncultured sludge bacterium A17, AF234760
34d_CAS
85% uncultured soil bacterium, EU589315
uncultured bacterium, FJ232641
94% **54d_CAS**
90% uncultured bacterium, BX294841
94% uncultured bacterium, HQ403213
97% uncultured bacterium, HQ403249
100% uncultured bacterium, CU927329
80% uncultured bacterium, CU926192
100% uncultured *Planctomycete*, AJ616260
42b_CAS
100% **22b_CAS**
100% **30b_CAS**
uncultured *Planctomycete*, GQ356183
Planctomyces brasiliensis DSM 5305, AEIC01000055
98% *Planctomyces maris*, AJ231184

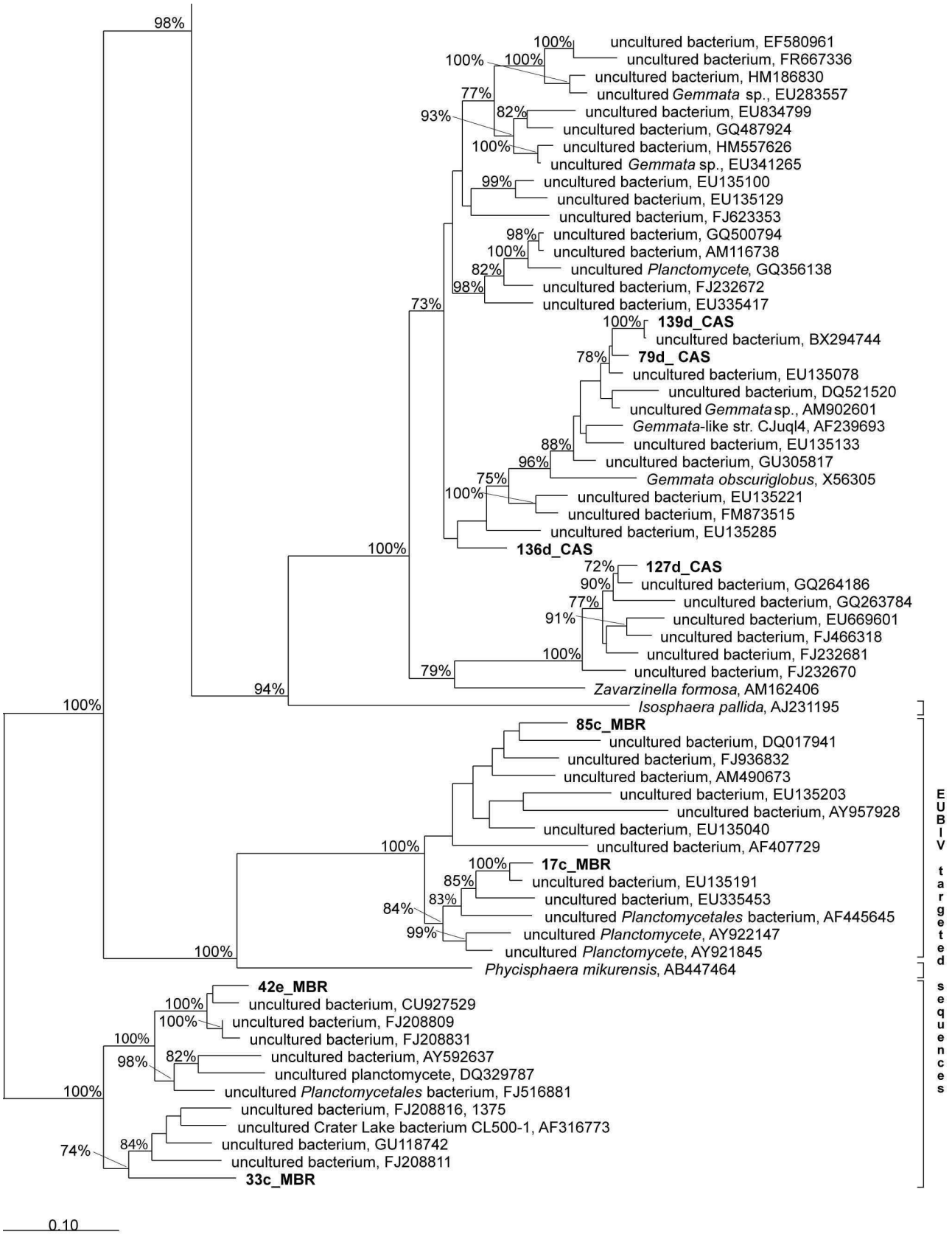


Fig. 5: Phylogenetic tree showing relationships among considered groups of Planctomycetes phylum obtained with maximum likelihood algorithm, filter Planctomycetes_5 and 100 bootstrap values. Bar, 10% estimated sequence divergence.

Discussion

The results obtained from the present study highlight the presence of heterogeneous microbial communities in both CAS and MBR reactors: almost all main groups of bacteria have been detected in all four libraries, and each of these groups was represented by a high number of OTUs. The presence of a high biodiversity level in both plants is also confirmed by the values obtained for diversity indices. These results were confirmed also by FISH experiments, in which the presence of most of the main groups of bacteria has been demonstrated in all samples (Figure 3).

It has to be mentioned that some differences between the two libraries constructed from the same sample and with the same DNA extraction emerged, concerning both MBR and CAS pilot plants. This was probably due to the two different couples of PCR primers used for the amplification step.

Considering differences between biomasses of CAS and MBR reactors, library construction results indicate that some *phyla* are more represented in one of the two pilot plants (Figure 2). Hence, although a high degree of heterogeneity, some relevant differences in the microbial community composition between the two reactors are evident even at higher taxa level. Even at the OTU level, despite the differences in biomass composition, the high degree of biodiversity in both microbial communities is evident, as clearly demonstrated by the values of the calculated indexes (Table 3). Retrieved data indicate that the different environmental selective pressure did not result, either in CAS or in MBR reactor, in the establishment of a biomass dominated by a few microbial components: both processes maintain a highly diversified and highly-structured microbial community.

One of the most interesting data of the present study concerns bacteria belonging to the *phylum Nitrospira* (nitrite oxidizing bacteria). The presence of *Nitrospira* bacteria have been highlighted by library construction both in the sample from CAS pilot plant and in the sample from MBR pilot plant, with clones having comparable percentages in each of the two libraries for each sample. The same data indicate a higher abundance of *Nitrospira* bacteria in the MBR plant (Figure 2). Considering that the screening of libraries only supplies an indirect estimation of the real situation,

FISH experiments confirmed the higher relative abundance of microorganisms belonging to *Nitrospira phylum* in the sample collected from MBR pilot plant with respect to the one of CAS pilot plant (Figure 3). As bacteria belonging to the *Nitrospira* genus are k-strategist bacteria [23], it is not surprising to find that they are selectively advantaged in MBR environmental conditions, where nitrite concentration was always very low. The preferential selection of k-strategist NOB in MBR is also in agreement with data collected from pilot plant monitoring through chemical analyses, since nitrite concentration was always very low in MBR effluent. Indeed, such data can be explained by assuming that the difference either in the separation technology or in the environmental conditions, caused a diversification of the biomass in terms of kinetic parameters. During the start-up, the instability of settling characteristics of mixed liquor in the CAS plant possibly caused the wash out of slow growing nitrifiers (k-strategists); on the contrary, in the MBR plant k-strategist nitrifiers experienced a more favourable environment, due to high SRT and stable operation conditions during the whole experiment. This is also confirmed by the fact that, while in CAS plant the concentrations of ammonia and nitrite reached values higher than typical half saturation constants of AOB and NOB (0.2 – 3 mg N L^{-1} for their respective substrate [16, 39], in the MBR plant the concentration of both ammonia and nitrite always remained very low.

From clone sequence analysis we found the presence of two different *Nitrospira* species, one for each pilot plant. Both sequence similarity data and phylogenetic analysis show that "*Candidatus* Nitrospira defluvii" is present in the CAS reactor. On the other side, in the MBR reactor different *Nitrospira*-like microorganisms are present. These microorganisms could probably represent a yet-undescribed species of the genus, strictly related to *N. moscoviensis*. As already stated in the past by Daims et al. [13], the genus *Nitrospira* is probably highly diversified. It is known from literature that *N. moscoviensis* and "*Candidatus* N. defluvii" are characterized by different metabolic needs [32]. "*Candidatus* N. defluvii" is selectively advantaged in the presence of high concentration of nitrite, while, at lower nitrite concentration, *N. moscoviensis* is prevalent [32]. As nitrite

concentration was higher in CAS pilot plant than in MBR during the start-up period, this could have influenced a positive selection of "*Candidatus* N. defluvii" in the former reactor.

Despite the use of different reverse primers for the construction of libraries (in order to enlarge the range of detectable bacterial species), there was no evidence from library screening of sequences belonging to bacteria phylogenetically related to the commonly described AOB; it is important to highlight that all primers have been tested in order to check their ability to match the sequences of AOB present in the on line databases. On the contrary, FISH results obtained using b-AO233 probe, specific for Ammonium Oxidizing *Betaproteobacteria,* demonstrated the presence of this group of bacteria in both samples through the detection of positive signals (Figure 3). Ammonium oxidation occurred in both plants, but AOB bacteria were not detected by library screening; they only came out in small amount by FISH counting. It is therefore possible to hypothesize that the oxidation of ammonium could be also performed by microorganisms belonging to AOB taxa other than the ones traditionally described in the literature [24].

FISH results indicate bacteria targeted by probes ALF1b and Eub338 IV as the most represented groups in the two reactors. A conspicuous presence of *Alphaproteobacteria* is not surprising, as prokaryotes of this highly diversified class are well-known to have often a high abundance in all kinds of environmental samples, included wastewater treatment plants (see for [5, 6, 45]). On the other hand, the conspicuous relative abundance of bacteria targeted by probe Eub338 IV in both plants constitutes an unexpected result. Representatives of the phylogenetic groups of *Planctomycetes* have been detected in previous studies on both CAS [51] and MBR [52]; usually their amount turned out to be between 5 and 8 % of the active biomass, while a higher percentage was found only when referring to loosely attached fraction of activated sludge bacteria [36]. Within this group, bacteria labelled by probe Eub338 IV (but not labeled by Pla46) showed a very high relative abundance in both the examined reactors, as emerged from FISH data (27% and 29% in CAS and MBR, respectively, see figure 3). Although this probe was mainly designed for the detection of Anammox bacteria and *Isosphaera* [46], all sequences belonging to our outgroup clade,

to the clade associated to *Phycisphaera mikurensis*, and the sequence of *P. mikurensis* itself are targeted by this probe (Figure 5). The high abundance of bacteria belonging to these clades in the studied systems was shown by FISH experiments, but it was not confirmed by library screening data. Indeed, clones whose sequence is targeted by probe Eub338 IV do not represent a high percentage of the total. These apparently conflicting results could be explained if we hypothesize that such bacteria possess a low number of gene copies or that their DNA was extracted and amplified with a particularly low efficiency. From phylogenetic analysis made on the *Planctomycetes phylum*, it was possible to identify the groups of this *phylum* in which our clone sequences are comprised. The majority of sequences related to our clones have been published in papers aiming at the identification of microorganisms in wastewater treatment plants [8, 43].

According to recently re-defined notes on 16S rRNA gene sequence-based taxonomy [49], both phylogenetic analyses and sequence similarity values indicate that only clone 45e_MBR represents a bacterium reasonably considered as belonging to a validated genus (*Pirellula*). All the other *Planctomycetes* sequences detected in the two samples, and, among them, the ones targeted by probe Eub338 IV, cannot be associated to any described species (or genus) and show high levels of similarities with uncultured organisms, whose metabolic skills are completely unknown. At present, it is not possible to infer the ecological relevance, if any, of these microbial components. Anyway, considering also that such microorganisms have been several times detected in waste water treatment plants, their role in activated sludge processes would deserve a deeper investigation. The presence of *Planctomycetes* bacteria in waste water processes has been mainly investigated with respect to studies focusing on Anammox processes, and most of times by the use of probe Pla46 [40]. Our data show that a conspicuous and well-represented fraction of *Planctomycetes* bacteria, commonly present in waste water treatment plants, are not detected by this probe, and that the use of probe Eub338IV should be also re-evaluated for this purpose.

Conclusions.

Data collected allowed an extensive characterization and comparison of the microbial communities selected in a MBR and a CAS pilot plant; both plants were fed with the same domestic wastewater, inoculated with the same biomass and operated in parallel. A rich and heterogeneous community, comprising representatives of most of the main bacterial *phyla*, was selected in both plants. Nevertheless, significant differences, even at higher taxa level, were evidenced between the two communities. Bacteria belonging to the *Nitrospira phylum* (and genus), typically recognized as k-strategist, have been demonstrated to be more abundant in the MBR plant. The instability of the solid-liquid separation during the start-up in the CAS plant was hypothesised to be the main difference in selective pressure that possibly caused NOBs diversification between MBR and CAS. Indeed, the operating conditions characterizing each of the two plants selected different *Nitrospira* species, one of them probably representing a new species.

The results of kinetic tests allowed us to differentiate between fast and slow growing bacteria in CAS and MBR respectively, and to consistently correlate the functionality and the structure of the microbial communities with the operational regimes at least for what concerns NOB.

Finally, an unexpectedly high relative abundance of bacteria belonging to poorly known phylogenetic groups of the *phylum Planctomycetes* was found, suggesting the possibility that they could play a key-role in the metabolic and ecological dynamics of these reactors.

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4.3 Effects of ozonation for excess sludge reduction on the bacterial communities composition in a full scale activated sludge plant for domestic wastewater treatment

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Abstract

The activated sludge process is the most widely diffused system to treat wastewater for controlling the discharge of pollutants into the environment. Microorganisms are responsible for the removal of organic matter, nitrogen, phosphorous and a large quantity of emerging contaminants. The environmental conditions of biological reactors significantly affect the ecology of the microbial community and, therefore, the performance of the treatment process.

In the last years, ozone has been used to reduce excess sludge production by wastewater treatment plants, whose disposal represents one of the most relevant operational costs. The ozonation process, in the aerobic digestion tank or in the return activated sludge line has demonstrated to be a viable method to allow a consistent reduction of excess sludge.

This study was carried out in a full scale plant treating municipal wastewater in two parallel lines, one of which was ozonated in the digestion tank and the other one was used as control. Samples were collected from the digestion tanks of both lines and the bacterial communities were compared in order to assess differences related to the ozonation treatment. The complete dataset was analyzed with Terminal Restriction Fragment Length Polymorphism (T-RFLP) analysis on 16S rRNA gene. Differences between bacterial communities of the treated and the untreated line appeared starting two weeks after the beginning of the treatment. Results demonstrate that ozonation treatment significantly affected the activated sludge in wastewater treatment plant.

Key words

Ozonation, wastewater treatment, bacteria, T-RFLP, NMDS, 16S rRNA

Introduction

Biological treatments are well known practices in order to remove pollutants from civil and industrial wastewater using microorganisms metabolism. The biological processes are based on the transformation of dissolved and suspended nutrients into new biomass (sludge), residual soluble and gaseous end products. Excess sludge has to be regularly removed and treated before final disposal or reuse. Costs for treatment and disposal of the excess sludge are very high for Waste Water Treatment Plant (WWTP), representing more than 50% of the total operating costs (Low and Chase, 1999; Zhang et al., 2009), so, there is the necessity to develop new strategies to reduce the excess

sludge (e.g. Ødegaard 2004; Wei et al., 2003). One of the most studied and viable method to reduce the amount of excess sludge is the ozonation (Yasui et al., 1996; Müller, 2000; Caffaz et al., 2005). Ozone (O_3) is a strong oxidant (E = 2.07 V) commonly used in tertiary treatment of industrial wastewater (e.g. for oxidation of recalcitrant compounds) as well as in drinking water treatment for disinfection (Paraskeva and Graham, 2002) and/or organic compounds oxidation. At low dosage, ozone destroys the cell wall of bacteria that are in suspension or that are on the surface of flocks, causing their lysis (e.g. Guo et al., 2007; Kepp et al., 2000; Liang et al., 2006; Saby et al., 2002; Wei and Liu, 2006). At higher dosage, ozone causes the destruction of flocks, acting directly on the bacterial Extracellular Polymeric Matrices (EPM) that plays a crucial role in bacteria aggregation (Foladori et al., 2010). Moreover, ozone contributes to the oxidation of the organic material dissolved after bacterial cell lysis, and improves sludge sedimentation (Kamiya e Hirotsuji, 1998), allowing the control of bulking and foaming phenomena (Caravelli et al., 2006). So far, two approaches have been proposed for excess sludge reduction through ozonation: the first one is the reduction of bacterial growth, and the second one is the induction of bacterial cell lysis processes (e.g. Liang et al., 2006; Saby et al., 2002). Ozonation treatment can be applied in different points of the treatment train, such as in the sludge recirculation line, or in the sludge digestion tank (Chu et al., 2009). Despite the effect of ozone treatment for the reduction of biological sludge has been already widely demonstrated in literature, no studies have been yet performed on the monitoring effects of ozonation on bacterial communities present in the WWTP.

Aim of this study is to assess the effect of ozonation treatment for excess sludge reduction on bacterial community in a sludge digestion tank of a full scale WWTPwhen applied in the recirculation loop of a full-scale aerobic digestion unit in presence and in absence of the ozonation treatment. The study was carried out in the full scale municipal WWTP located in Sabaudia (LT, Italy) and managed by Acqualatina S.p.A. The technique chosen for the study is the Terminal Restriction Fragment Length Polymorphism analysis (T-RFLP).

Materials and methods

Description of the study area

The municipal WWTP managed by Acqualatina S.p.A. and located in Sabaudia (LT, Italy), is a conventional activated sludge plant consisting of pre-treatments (fine bar screen, sand removal and degrease), pre-denitrification, oxidation-nitrification, secondary settling, aerobic digestion, sludge thickening and belt-press dryer. Disinfection occurs by chlorination just in case of emergency. The plant, divided into two parallel trains, respectively characterized by 10,000 Population Equivalent (PE) in "Line 1" and 20,000 PE in "Line 2", receives wastewater from the touristic area of Sabaudia, whose population varies from a minimum of 10,000 inhabitants during the winter season, to a maximum of 30,000 inhabitants during summer. The touristic nature of the area is responsible for wide variations in the influent flow rate, ranging from 3,500-4,500 m^3/d during winter season, to 6,000 m^3/d during summer season.

The treatment of excess sludge consists of aerobic digestion, sludge thickening and dewatering with a belt-press. The ozonation treatment was performed in the aerobic digestion tank of Line 2, characterized by a total volume of 990 m^3 and a mean cell residence time (MCRT) of 14 d. About 100 m^3/d of Waste Activated Sludge (WAS) is fed to the tank, with a total suspended solids (TSS) concentration of approximately 7000 mg/L. A 155 kW compressor provide air for the aeration of both the secondary activated sludge and aerobic digester of line 2. Airflow to the digester is distributed through a fine bubble diffuser system and controlled to maintain dissolved oxygen (DO) of about 1 mg/L. Nevertheless, operation of the blower is not continuous because controlled on the basis of the oxygen requirements in the activated sludge oxidation tank. This determines periodical lack of available oxygen thus reducing the digestion efficiency.

Ozonation treatment

The ozonation system was performed trough the Praxair's Lyso™ process composed of: (a) ozone supply with a Wedeco ozone generator; (b) Lyso™ centrifugal pump (70 m^3/h) for sludge drawing from the digestion tank and recirculation to the same unit after ozonation; (c) Venturi-tube to provide contact between ozone and sludge; (d) a stainless pipe as contactor between ozone and sludge; (e) liquid oxygen storage system; (f) ozone destructor; (g) piping.

The dosage of the ozone treatment during the experimental period is provided in Table 1.

Time	**O_3 flow rate**	**O_3 specific dosage**
	(gO_3/h)	(gO_3/kgTSS_0)
February-March (Period I, duration 37 d)	60	0.3
April-June (Period II, duration 82 d)	500	2.3
July-August (Period III, duration 60 d)	670	2.1

Table 1: ozone dosage occurred during the experimental period.

In this paper we refer the ozone dosage to either the Total Suspended Solids (TSS) concentration in the WAS, which corresponds to the content in the influent to the digester unit (TSS_{in}), or to the TSS concentration measured inside the digester unit (TSS_0). For this reason TSS concentration variation could determine increase or decrease in specific dosage concentration even if the applied ozone flow rate was the same.

Collection of samples

Samples were collected from February 2010 to July 2010 in the aerobic sludge digestion tank both in 10,000 PE line, representing the negative control with no ozone treatment, and in 20,000 PE line, that was the ozone treated line. Samples were collected four times:

- 22nd February 2010
- 9th March 2010
- 25th May 2010
- 20th July 2010

Figure 1 represents the scheme followed to collect samples in the two tanks; four samples were collected each time from the control line, and six samples from the line with ozone treatment, for a total of forty samples during the whole study period. Samples were collected at 1 meter depth in sterile plastic bottles (2 liters capacity).

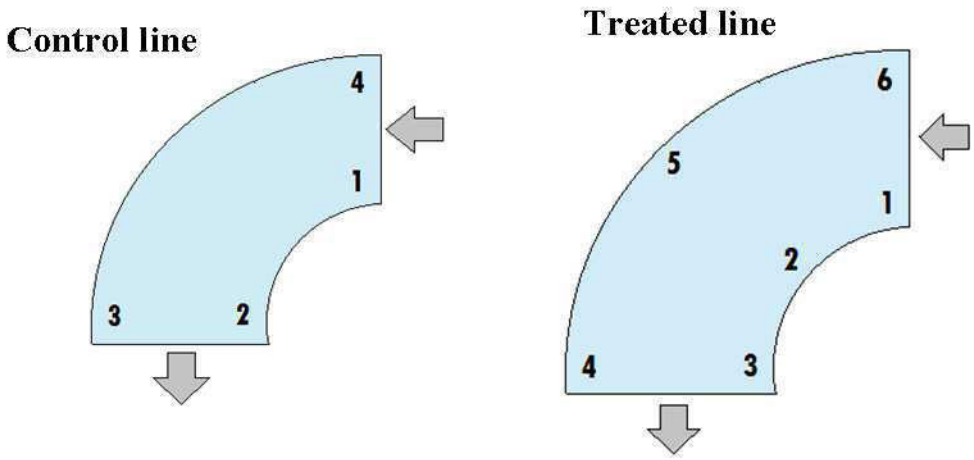

Figure 1: sample collection scheme in the two tanks.

DNA extraction and T-RFLP analysis

Total DNA extraction was performed on all samples using a Soil master™ DNA extraction kit (Epicentre Biotechnologies, WI, U.S.A.). 16S rRNA genes were directly amplified from extracted DNA, using PCR conditions reported in Chiellini et al., (2013a). Universal bacterial primers were used: 8F (5'-AGA GTT TGA T(CT)(AC) TGG CTC AG-3') and reverse 1492R (5'-

GG(AGCT)(AT)AC CTT GTT ACG ACT T-3') (Lane et al., 1991). Primer 8F was labeled with two different fluorochromes (Applied Biosystem CA, U.S.A.): FAM fluorochrome and VIC fluorochrome. In the first case, the template was digested with restriction endonuclease BsuRI (GG^CC, 0.2 u/µl, Fermentas, Canada) and in the second case with restriction endonuclease RsaI (GT^AC, 0.2 u/µl, Fermentas,). The use of more than one restriction endonuclease in T-RFLP approach is recommended for a better resolution of the experiment (Liu et al., 1997). After restriction, DNA was precipitated with cold 100% ethanol to eliminate salts in a 5424 R refrigerated centrifuge (Eppendorf, Italia) at 4°C and 10,000 RCF. For each reaction, a mix with 1.2µl of loading buffer (GeneScan™ 600 LIZ, Applied Biosystem), a maximum of 5.5µl of sample (the volume of samples was calculated on the bases of its final concentration after cold ethanol precipitation) and 13.3 µl of deionized formamide (Applichem, Germany) was prepared. For further details about the experimental procedure see Chiellini et al. (2013b). Capillary electrophoresis was performed with Abi Prism 310 Genetic Analyzer (Applied Biosystem); T-RFLP profiles were analyzed using GeneScan™ analysis software (Applied Biosystem). T-RFLP data matrix was transformed for statistical analysis as described in Iannelli et al., 2012.

Statistical analysis on T-RFLP dataset

All statistical analyses showed in this paper have been performed using PAST software v.2.15 (PAleontological STatistic, Hammer et al., 2001). A Cluster Analysis (CA) using Ward's method and 100 bootstrap value was performed in order to evaluate which groups of samples clustered together. Non Metric Multidimensional Scaling (NMDS) was also performed on the whole dataset using the Bray-Curtis coefficient. This analysis was chosen because it is the most indicate for non homogeneous data (Rees et al., 2004). The quality of the dataset was assessed trough the Shepard plot (where ideally, all points should be placed on a straight ascending line, x=y) and the stress value calculation. A stress value greater than 0.2 indicates that the plot is close to random, stress less than 0.2 indicates a useful 2 dimensional picture and less than 0.1 corresponds to an ideal ordination

with no real prospect of misinterpretation (Clarke 1993). Three different diversity indices were also calculated in order to compare species diversity among samples on the normalized T-RFLP data matrix, containing profiles from both the restriction enzymes, and assuming that each T-RF corresponds to one Operational Taxonomic Unit (OTU) (Engebretson et al., 2003):

- Simpson's (1949) diversity index Ds = 1 - Σpi, with pi representing the population of the i species;
- Shannon's (1941) diversity index H = -Σpi ln pi, with pi being the proportion of the i species relative to the total number of species;
- Dominance Index D = 1-Ds, with Ds being the Simpson's diversity index.

The Simpson's index (*Ds*) gives the probability that two randomly selected individuals drawn from a population p_i belong to the same species *i*. It measures the "evenness" of a community ranging from *0* to *1*.

The Shannon index varies from *0* for communities with only a single taxon, to values that are higher for communities with more taxa, each of which has fewer individuals.

The Dominance Index (*D = 1-Ds*) was also calculated; it ranges from *0* (when all taxa are equally present) to *1* (reached when a single taxon dominates the community completely).

Results

Ozonation effect on sludge reduction

Profiles of TSS and VSS measured in the influent and effluent of the Line 2 digester as a function of time and of the applied ozone dosages are showed in Figure 2.

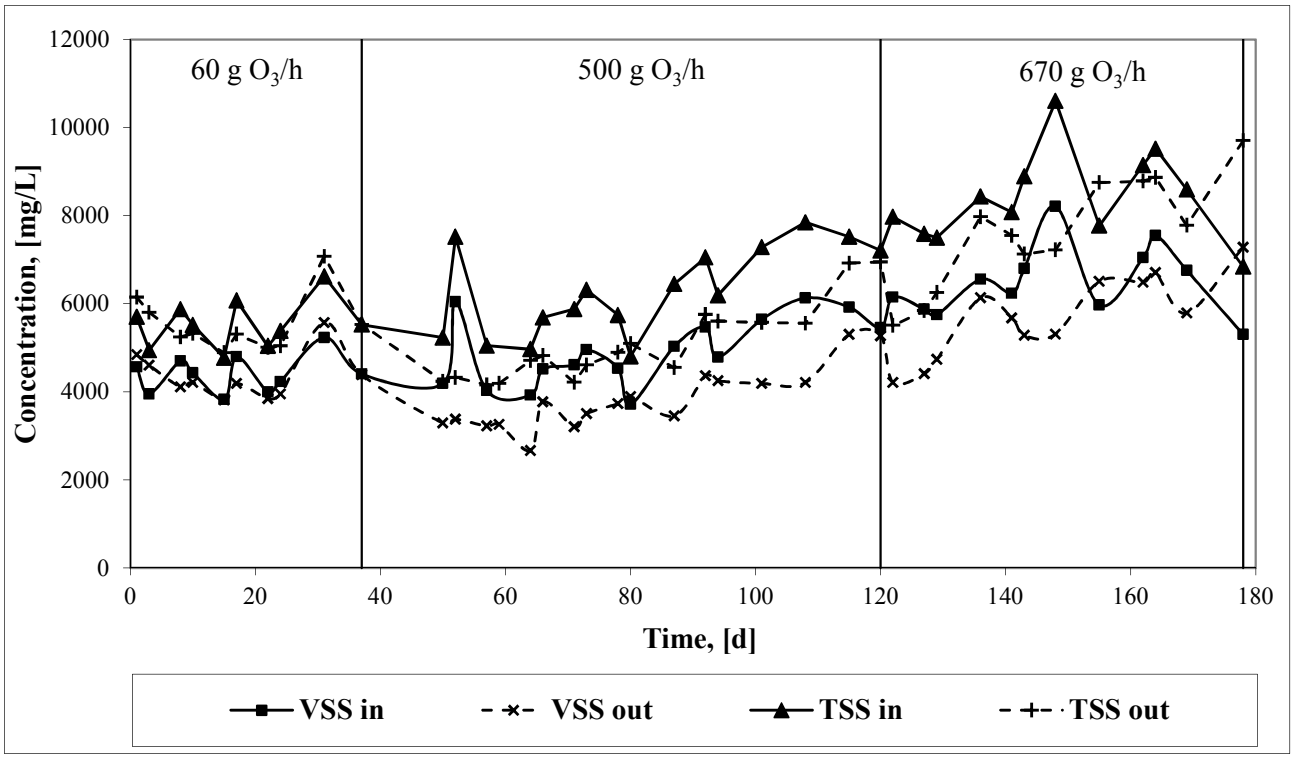

Fig. 2–Change in the TSS and VSS concentration in the digester inlet and outlet at different ozone dosages.

Concentration of both TSS and VSS concentrations in the influent of the line 2 digestion tank was fairly constant during the first and second periods of operation, while a significant increase was registered in the summer period due to the higher influent organic load. The VSS/TSS ratio in the outlet from the ozonated digestion tank, decreased continuously from about 0.79 to about 0.74.

For both TSS and VSS, reduction efficiency measured in the ozonated digester was very low in the first period, and then increased appreciably when the ozone dosage was increased.

Table 2 reports average TSS and VSS removal (indicated as Δ) measured in both the digesters during the experimental periods. It can be noted that in the Line 1 digester (control), the percentage reductions were always very low.

Period	Line 1 (control)		Line 2 (ozonated)	
	ΔTSS (%)	ΔVSS (%)	ΔTSS (%)	ΔVSS (%)

1	0	0	0.2	1.4
2	0	0	19.5	22.6
3	1.1	1.4	9.5	12.4

Table 2-Average monthly TSS and VSS percentage reductions in the digesters of Line 1 (control) and Line 2 (ozonated).

The low values observed during the first period are still higher than the efficiency measured in the Line 1 in the same period: in this case, the specific ozone dosage was too low to determine a significant improvement of cell oxidation process, but was capable of providing the additional oxygen required to promote sludge stabilization.

In the third operational period, the value decreased.

Cluster analysis

Cluster Analysis made with Ward's method and 100 bootstrap on the whole T-RFLP dataset is shown in Figure 3. The replicates of samples collected in the same tank during each sampling time, are associated together with high bootstrap value support (84-100) in all cases. Samples of the treated line collected in February, before the onset of the ozonation treatment, are associated with samples of the control line (not treated) collected in the same sampling date, with 100 bootstrap support. This clade, composed by samples of control and treatment of February, associates with samples collected in the control line in March (88 bootstrap support). Samples collected in the treated line in March, are instead different from the control line collected at the same time, and they are placed in a basal position in the clade, with 78 bootstrap support. Samples collected in May, show a strong association between the control line and the treated one (100 bootstrap) while the association between the control and the treated line of July, is supported by a low bootstrap value (71).

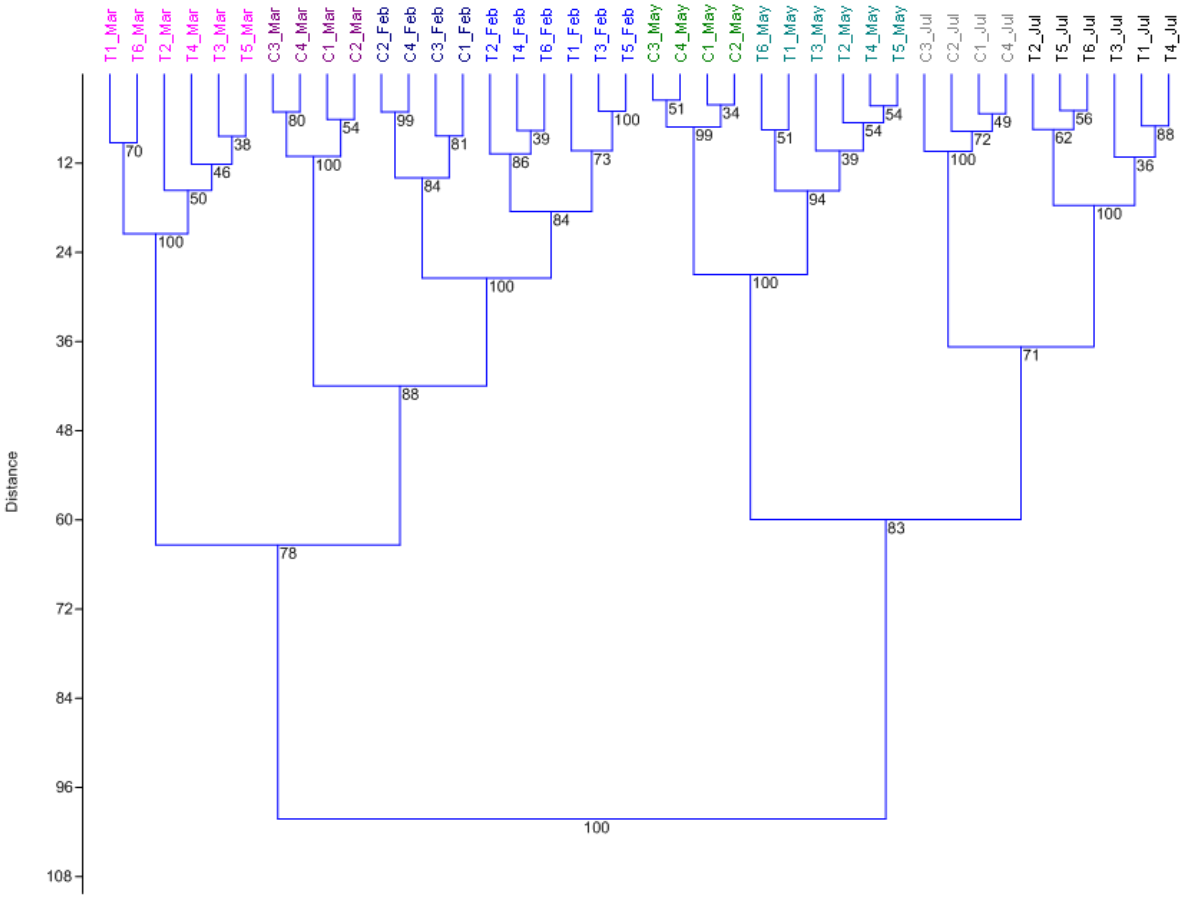

Figure 3: Cluster Analysis made with Ward's Method and 100 bootstrap on the whole T-RFLP dataset.

Figure 4 shows the NMDS plot based on Bray-Curtis similarity measure on T-RFLP dataset. In order to discriminate between samples collected in both tanks and among samples collected in different sampling dates, different colors have been used. The stress level is 0.1089. Figure S1 (Supplementary material) Shows the Shepard Plot which indicates the quality of the result. Samples collected in February (blue hollow squares for treatment and blue asterisk for control) are associated to each other, and they are closely related to samples collected in the control line of March (purple hollow circles). Samples collected in May, are all similar between control and treated lines (green hollow ovals for treatment and light green crosses for control). July control samples (gray hollow triangles) are placed close to May samples. March treatment samples (pink closed squares) are

separated to all other samples in the left side of the plot, as well as July treatment samples (black closed circles) that are placed in the right side of the plot.

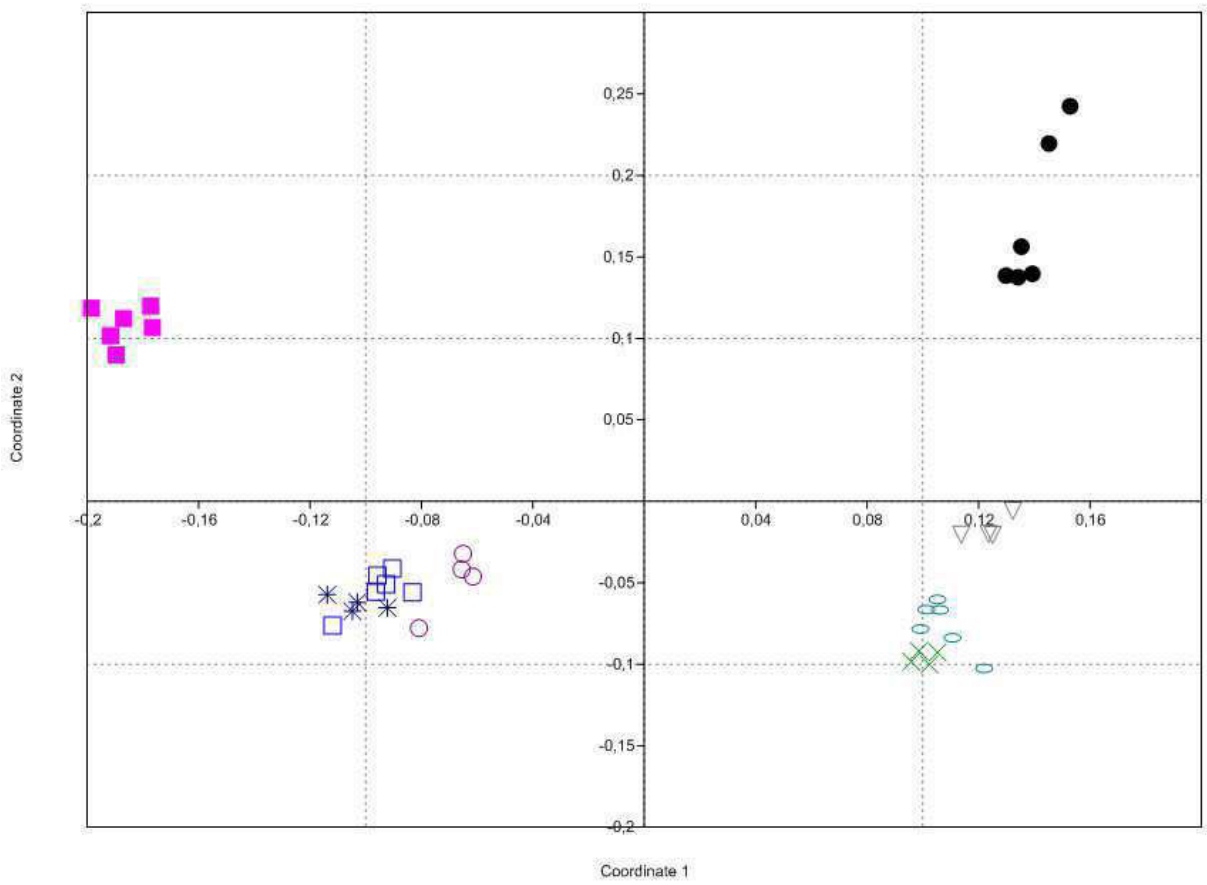

Figure 4: NMDS scaling plot based on Bray-Curtis similarities of the T-RFLP data plot on T-RFLP dataset: blue hollow squares: February Treatment; blue asterisk: February Control; pink closed squares: March Treatment; purple hollow circles: March Control; green hollow ovals: May Treatment; light green crosses: May Control; black closed circles; July Treatment; gray hollow triangle: July Control.

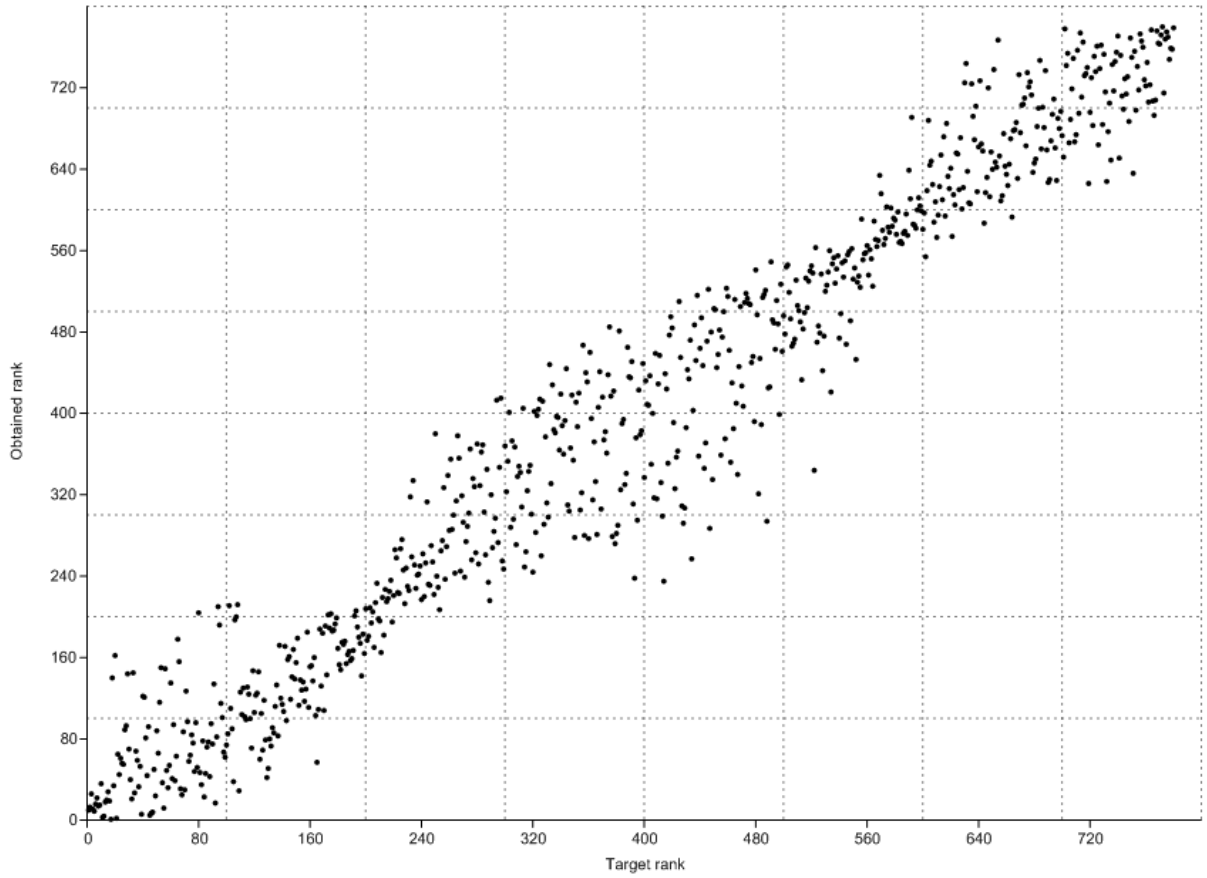

Figure S1: Shepard Plot demonstrating the quality of T-RFLP results.

Table 3 resumes the Diversity indices calculated for all samples. In the complex, the values of the indices are homogeneous among the replicates of each tank in each sampling time, for this reason, the average values with the standard deviation have been calculated for each group of samples. Values are also similar considering all samples collected during the whole experimentation time, with the exception of samples collected in the treated tank of the last sampling time (July 2010), that show a significant difference in the values of all three indices. In fact, Simpson and Shannon diversity indices show lower values in treated samples of July 2010, while Dominance diversity index presents higher values in the same samples.

	Simpson Ds	Dominance D= 1-Ds	Shannon_H
T_Feb	0,981±0.001	0.019±0.001	4,337±0.052
C_Feb	0,980±0.002	0.020±0.002	4,230±0.085
T_Mar	0,980±0.003	0,020±0.003	4,404±0.089
C_Mar	0,979±0.001	0,021±0.001	4,209±0.018
T_May	0,979±0.001	0,021±0.001	4,229±0.063
C_May	0,981±0.001	0,019±0.001	4,338±0.022
T_Jul	0,897±0.023	0,104±0.023	3,335±0.053
C_Jul	0,974±0.001	0,026±0.001	4,119±0.030

Table 3: Diversity indices calculated as average and standard deviation for each group of samples collected in different sampling date: Ds= Simpson diversity index; D= Dominance diversity index; H= Shannon diversity index.

Discussion

The effect of ozone on bacterial cells has been carefully tested from many authors during the past decades. In details, the ozone treatment may facilitate the protein denaturation and the decrease of enzymatic activities (Hinze et al. 1987; Mehlman and Borek 1987; Takamoto et al. 1992; Komanapalli and Lau 1996); it causes the degradation of the unsaturated lipids that are present on the bacterial cell envelop (Guzel-Seydim et al., 2004), and as a consequence, it increases the cytoplasmatic membrane permeability thus causing the release of cytoplasmatic compounds such as ATP, proteins and nucleic acids (Hinze et al. 1987; Komanapalli and Lau 1996; Thanomsub et al. 2002). The application of ozonation treatment in WWTPs has been widely explored focusing on the sludge reduction efficiency, or to test the efficiency of microorganisms degradation (e.g. Reungoat et al., 2012, Di Iaconi C., 2012). To the authors' best knowledge, no data are available about the effect of ozonation on bacterial communities composition in WWTPs. This paper represents the first study in which a molecular fingerprint technique is used to highlight changes in wastewater

bacterial community structure after the ozonation treatment; indeed an effect of ozone treatment on the bacterial community structure has been observed during the experimentation.

The continuous decrease of the VSS/TSS ratio in the outlet from the ozonated digestion tank (Figure 2) can be regarded as an increase of the concentration of the inorganic part of sludge likely due to the release of inorganics from cell lysis into the medium as a consequence of ozone oxidation. In fact, this phenomena was not observed in the control digestion tank. The fact that for both TSS and VSS, reduction efficiency measured in the ozonated digester was very low in the first period and increased appreciably when the ozone dosage was increased (Figure 2), might be attributed to either the biological stabilization process taking place in the digester and/or to the disintegration effect by ozonation. However, by comparing these data with the TSS and VSS variations in the Line 1 digester, which was operated without ozonation, the effect due to ozone oxidation appears evident. Concerning the average monthly TSS and VSS percentage reductions in the digesters of Line 1 and Line 2 (Table 2), the zero values reported for the first two periods of operation indicate that the solid concentration in the effluent from the digester of Line 1 was approximately equal to that measured in the influent. The lack of an efficient sludge stabilization was likely due to a limited availability of oxygen supply in the tank. The more significant abatement of TSS and VSS observed in the Line 2 digester was likely due to the disintegration effect caused by ozonation. This is confirmed by the higher reduction measured as the ozone dosage increased: particularly, a linear correlation was found between TSS removals and the applied specific ozone dosages. The oxidation treatment gave thus an important contribution to the sludge reduction achieved in the stabilization tank. Decay of some biomass determined the release of compounds which were then partly used for the synthesis process by other microorganisms. Salsabil et al. (2010) observed similar VSS removal yields due to disintegration (about 20%) at 0.1 gO3/gTSS0 in lab-scale batch conditions. At full-scale, Sievers et al. (2004) measured a mass reduction of 20-35% for the aerobic stabilization with a specific ozone consumption of about 0.05 kgO_3 per kg TSS to be treated. It is worth noting that all these reduction efficiency values were

achieved at significantly higher dosages than those used in this study. The decrease of the values in the third operational period (Table 2), might indicate that the higher TSS concentration in the digester determined a lower specific oxidant dosage, which limited the ozone disintegration effect.

Focusing on the biomolecular analysis performed on bacterial community, it is clear from Cluster Analysis (Figure 3) and from NMDS (Figure 4) that replicates collected in each tank are homogeneous at each sampling time, indeed they always cluster together supported by bootstrap values >80 (Figure 3). This result is in agreement with previously published studies testing the homogeneity of bacterial community in replicates collected from the same tank (Wittebolle et al., 2009). From Cluster Analysis it is also evident that in samples collected at the onset of the experimentation in February, when the ozonation treatment was not yet active, the association between samples collected from the two different tanks is well supported (100 bootstraps); these samples are all grouped together also in NMDS plot (Figure 4) thus demonstrating that at the onset of the experimentation, the bacterial community composition of the two tanks was substantially similar. The Shepard plot (Figure S1) and the value of the stress level (0.1089) support the robustness of our NMDS analysis on T-RFLP dataset, as recommended by Clarke (1993).

The samples collected in March, after few weeks of treatment, are different in the two tanks (Figures 3 and 4). Probably, after two weeks of treatment, the bacterial community composition has been already modified by the effect of ozonation. March control samples associates with February control samples, both in CA (Figure 3) and in NMDS (Figure 4). This demonstrates that the bacterial community of the control line remains substantially stable during the first months of experimentation, while the bacterial community of the treated line, starts to differentiate, as it is visualized in NMDS plot (Figure 4). In May, after almost three months from the starting of the treatment (period 2), the two tanks appear similar concerning the bacterial communities composition (Figures 3 and 4). The last sampling date (July, period 3 of ozone treatment) situation is similar to the one of March, highlighting a differentiation among treated and control samples especially in NMDS (Figure 4). The calculation of diversity indices on molecular data obtained for

bacterial community in wastewater treatment plants is a commonly used approach (Hu et al., 2012). In the present study, data concerning diversity indices (Table 3) highlight significant differences that are present in samples collected in the treated tank in July 2010. The lower values of Shannon index indicates that the treated July 2010 samples present a lower number of bacterial taxa. At the same time, higher values of Dominance index (and, as direct consequence, lower values of Simpson index) indicate that these samples show a lower number of taxa that are dominant respect to all other samples, which instead present more bacterial taxa with comparable richness. This is in agreement with the consideration that the ozonation treatment affects the bacterial community composition, probably reducing the number of taxa, allowing the development of lower species number that probably are more resistant to the treatment or exhibit a higher division rate. On the contrary, control and treated samples collected in May 2010 are substantially similar in all performed analyses.

Considering NMDS and diversity indices data (Figures 3 and 4, Table3) in correlation with the three periods of ozonation treatment (Table 1), it is worthy of notice that during period 1 of treatment, in which the ozone dosage was the lowest, some differences in bacterial community composition between treated and control lines were highlighted (samples collected in March); during period 2, in which the ozone dosage was the highest, bacterial community composition did not show significant differences between treated and control lines. During the third period, when the ozone treatment was decreased, differences between control and treated bacterial communities were again evidenced as observed in period 1. Probably, the lower ozone dosage, damaged only few bacterial species and it influences selectively the composition of bacterial community in the treated line; on the contrary, highest dosages of ozone destroys all bacterial cells that are present in the treated tank, and for this reason it does not contribute to the selection of a peculiar bacterial community. From a practical point of view, the lower ozone dosages of period 1 significantly influence the bacterial community composition but didn’t induce a reduction in sludge production. On the contrary high ozone dosages (period 2) seem to have a less strong effect on the bacterial community composition, but can

positively affect the reduction of sludge. At period 3 of ozone treatment, it's possible to observe an intermediate situation. From the point of view of the management of the plant, the results shown in this paper suggest that the effect on sludge production should be obtained through a massive release of nutrients caused by the bacterial cell lysis occurred after the ozonation process and not through the selection of more "efficient" bacterial communities.

Conclusions

In this paper, the bacterial community composition of a full scale plant for domestic wastewater treatment was studied during an ozonation treatment for the reduction of excess sludge.

The ozonation treatment was able to significantly reduce excess sludge production when ozone specific dosage was in the range 2.1-2.3 $gO_3/kgTSS_0$ while negligible reduction was observed in case of the lowest specific ozone dose experienced in this study (0.3 $gO_3/kgTSS_0$).

Samples from two different tanks (control and treated) were compared in order to verify the homogeneity of replicate samples in each tank, the homogeneity of samples between control and treated tanks before the starting of the ozonation process, and the effect of ozonation treatment on bacterial communities during the whole study period. What the study highlighted was homogeneity in composition of bacterial community among replicates collected in each tank during the whole experimental period, and a homogeneity between the control and the treated tank at the moment of the onset of the study, before the dosage of ozone. A differentiation of treated and control samples was highlighted in March 2010 samples with multivariate statistical analysis, and a complete differentiation in the composition of bacterial communities in the treated and control line was also evidenced in July 2010, at the end of the experimentation, both with multivariate statistics than with diversity indices, thus indicating that low or moderate ozonation levels produced an overall effect, probably reducing the number of taxa present in the samples, and allowing the development of few dominant species. On the contrary, high level of ozonation apparently did not influence the composition of the bacterial community.

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