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

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Abstract

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], [2], [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 n. ECO/08/239065) promotes an innovative approach to decontaminate dredging sediments by phyto-treatment. Concerning phytoremediation of polluted sediments, many studies that are

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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], [14], [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], [32], [33], [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 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);

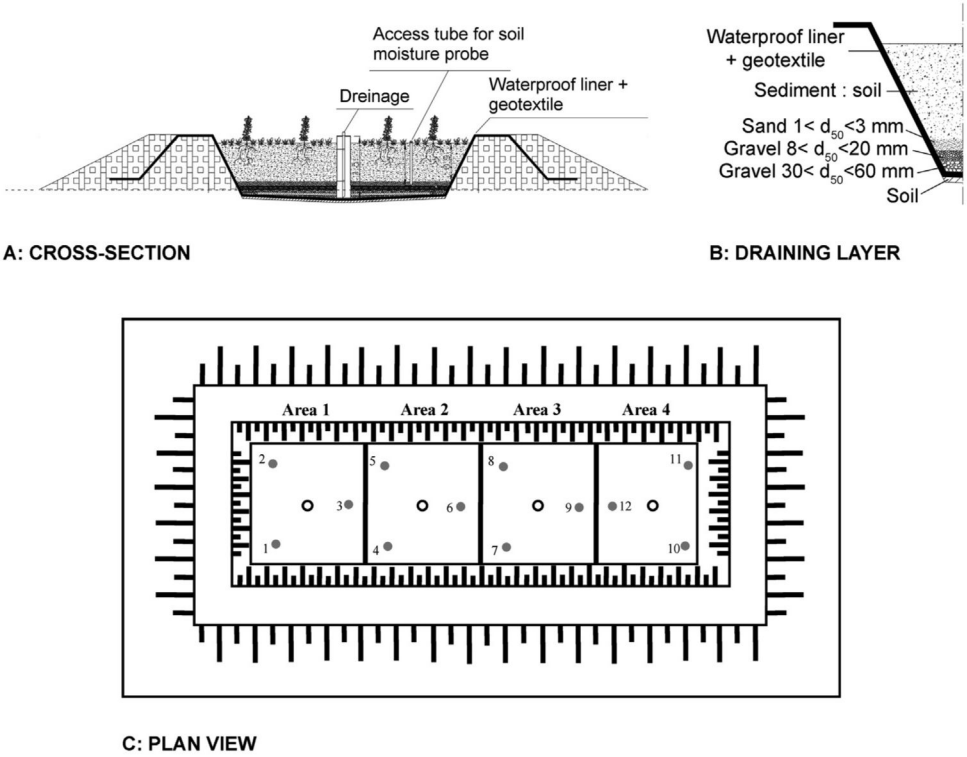

FIGURE 1 (a) Cross-section view of the phytoremediation treatment basin; (b) draining layer; (c) plan view of the phytoremediation treatment basin added with the 12 sampling points that are indicated with numbers.

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

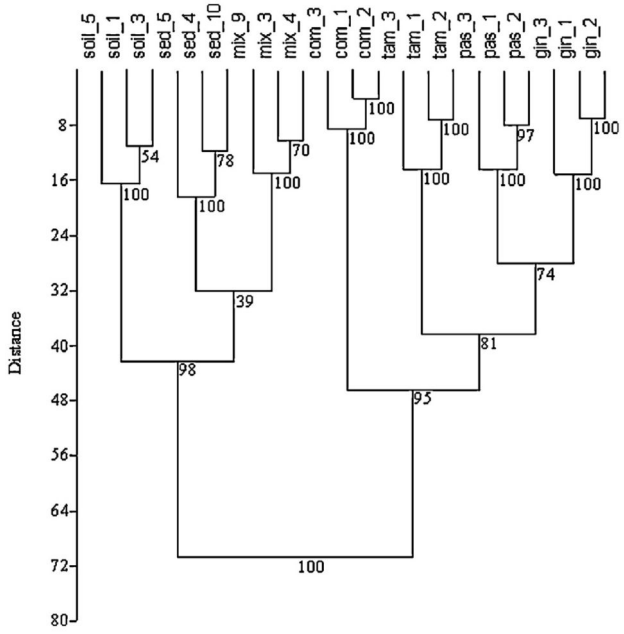

FIGURE 2 Cluster Analysis made on Time Zero samples with Ward's method and 100 bootstraps**.**

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 represents 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.

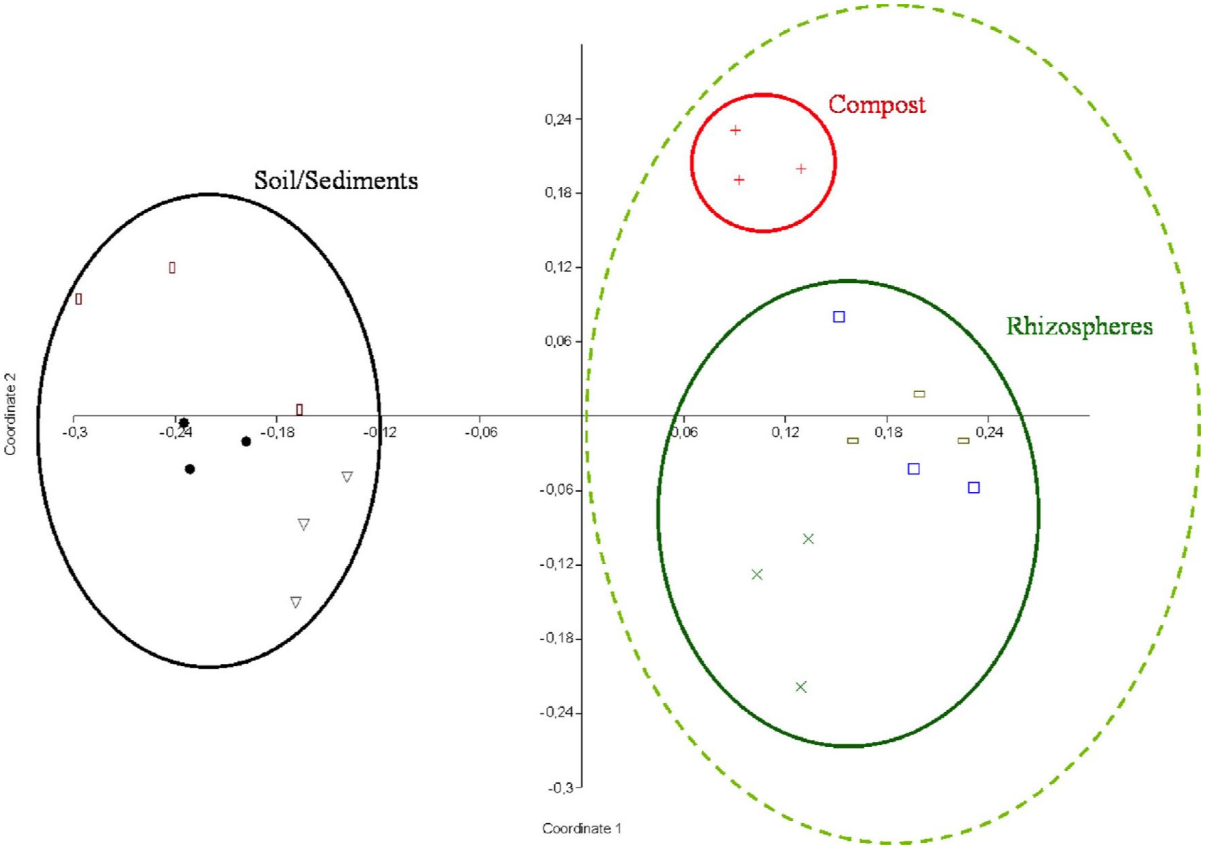

FIGURE 3 NMDS plot on Time Zero Dataset. Red crosses: compost; black solid circles: dredged sediments; brown hollow rectangles: natural soil; gray hollow triangles: mixture; blue hollow squares: *S. junceum;* green hollow rectangles: *P. vaginatum,* green cross: *T. gallica.*

Figure 4 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.

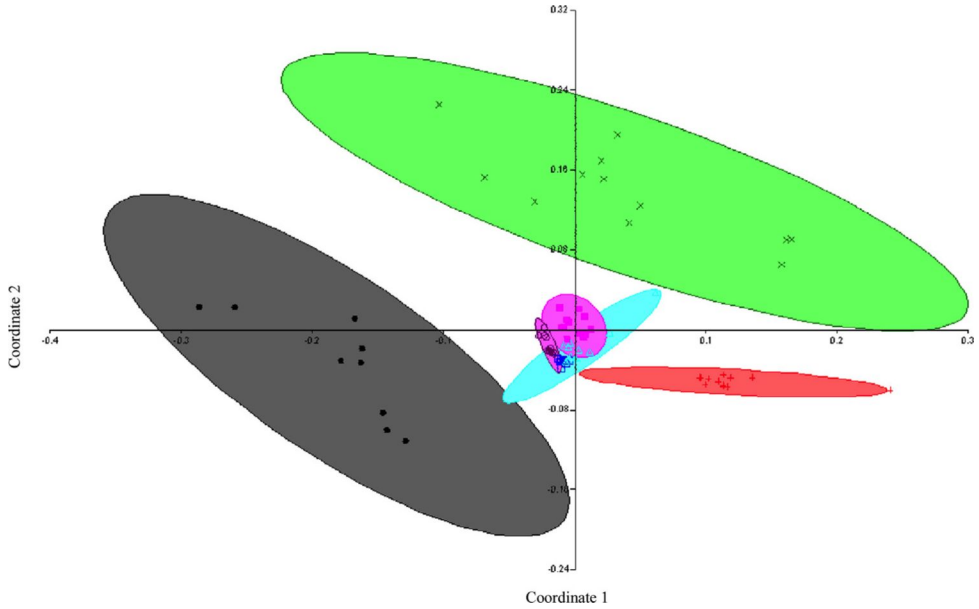

FIGURE 4 NMDS analysis on the whole dataset collected during the experimentation. Black solid circles in gray field: Time Zero plant filling (soil/sediments/mixture); green crosses in green field: Time Zero rhizospheres and compost; red crosses in red field: Time One; blue hollow squares in blue field: 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.

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; 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.

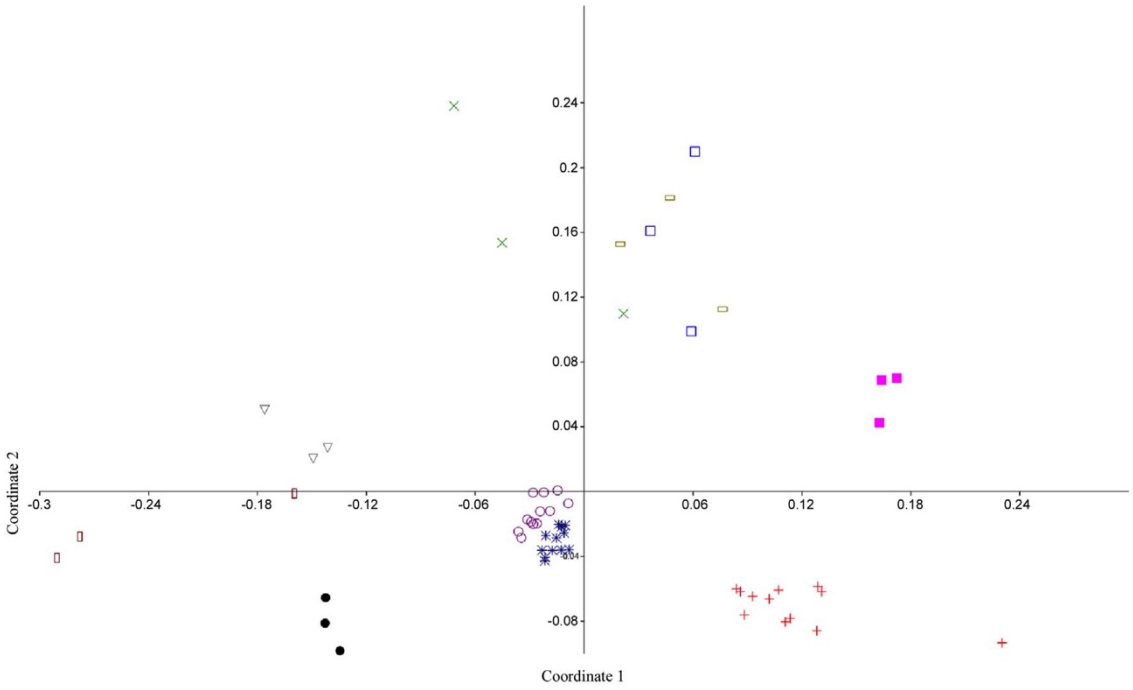

FIGURE 5 NMDS on Time Zero, Time One, Time Two and Time Five samples. Pink solid squares: compost; black solid circles: dredged sediments; brown hollow rectangles: natural soil; gray hollow triangles: mixture; purple hollow circles: Time Five; dark blue asterisks: Time Two; red crosses: Time One; blue hollow squares: *S. junceum;* green hollow rectangles: *P. vaginatum,* green cross: *T. gallica.*

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 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].

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

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 (Figure 4, Figure 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 (Figure 4, Figure 5 and S3), and from Shannon values.

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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