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Evaluation of different bioremediation protocols to enhance decomposition of organic polymers in harbour sediments

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Abstract

The response of the microbial community (in term of abundance and enzymatic activity) was investigated to test the effect of different bioremediation protocols to naturally enhance decomposition of organic polymers in harbour sediments (Genoa Harbour, Italy, N–W Mediterranean). Bioremediation techniques tested were bioaugmentation (5 different microorganisms' inocula), biostimulation (air supply), and natural attenuation. The coupling bioaugmentation/biostimulation was also tested. After 60 days, following the bioaugmentation protocol, bacterial densities correlated to the quantities of inocula amended to the boxes, suggesting that allochthonous community was able to survive and multiply. However, while bioaugmentation alone seems not to be able to carry out significative degradation, its coupling with air insufflations produced the best response: here bacterial densities increased, especially in the water (from 2.3×10^7 to 3.50×10^8 cells ml^{-1}), average cell size and enzymatic activities increased, and sedimentary organic matter was significantly depleted (PRT 5-folds reduction, CHO 1.5-folds reduction). The strong coupling observed between the sediment and water compartments together with the greatest microbial response observed in this latter suggest that the sediment–water interface may constitute a key compartment for the occurring of biodegradation processes in organic-rich sediments.

Introduction

In the last decades many efforts have been devoted by the scientific community to the application of bioremediation techniques to efficiently remediate polluted areas. Break-down pathways have been assessed for most contaminants of major environmental concern, from the easily degradable (linear hydrocarbons, domestic effluents, etc.) to the most recalcitrant (PAH, phenol derivates, heavy metals, etc.). Literature reports many examples in which both singular bacterial strains and microbial systems have been successfully utilized to reduce and/or transform selected pollutants in non-toxic molecules in laboratory conditions (Eschenhagen et al. 2003; Gallizia et al. 2003; Harayama et al. 2004; Juhasz & Ravendra 2000; Valls & de Lorenzo 2002; Van Schie & Young 2000; Watanabe 2001). Notwithstanding, results are still contradictory when bioremediation is tested on the field (Zhu et al. 2001). This is largely due to the fact that bioremediation treatments, in order to be effective, need to fulfill some requirements, that can be summarized as (1) presence of a suitable microbial community, with the potential to enzymatically attack the targeted compound/s, (2) presence of energy-rich electron donors, (3) favorable environmental conditions (temperature, pH, redox potential, etc.), and, (4) pollutants (PAH, metals, phenols, etc.) not in concentrations that cause inhibition to microbial metabolism (Alexander 1994; Margesin et al. 2000; Murphy et al. 1999). For these reasons, bioremediation has been identified as a strictly

site-dependent practice (Venosa & Zhu 2003) and, before scaling-up a biological remediation treatment to the field, it is advisable to test – with microcosms – environmental operative conditions as much as possible closer to expected *in-situ* kinetics. This is particularly true for heterogeneous matrixes, such as the submerged mud of Genoa Harbour, constituted by a complex of pollutants and microenvironments (Bertolotto et al. 2003; Gallizia 2000). So, with the final goal of scaling-up a bioremediation treatment in a pilot *in-situ* experiment, a set of microcosms have been set-up, in order to gather information about the response of the microbial community in decomposing heavy organic matter (OM) loads. Three different bioremediation protocols were followed, using weathered non-sterile sediment (to allow competition between the allochthonous and the autochthonous community) and *in situ* Harbour water.

Bioremediation of organic-rich sediments aims at the mobilization and removal of organic macro elements from the accumulation areas (Vezzulli et al. 2004). 'OM' consists of compounds of a high molecular weight and various polymeric structure (Lamy et al. 1999; Münster & Chróst 1990; Unanue et al. 1999): the most representative are proteins, starch, lipids, pectin, cellulose, chitin, nucleic acids or lignin (Arnosti et al. 1998; Martinez & Azam 1993). The 'quality' of sedimentary OM is widely recognized to affect the rate and extent of OM decomposition and re-mineralization (Westrich & Berner 1984). Within the bulk of OM, proteins (PRT) and carbohydrates (CHO) have been identified by several authors as the most bioavailable food source for benthic microbial metabolism (Danovaro et al. 1999; Mayer et al. 1995; Meyer-Reil & Koster 2000): in particular, PRT are more labile if compared to CHO, and are considered the first organic polymers to be degraded for bacterial metabolism, while CHO are more refractory to consumption. In accordance with previous studies (Fabiano et al. 2003; Vezzulli et al. 2003) PRT and CHO concentrations can be utilized as indicators of the biodegradation occurring in organic-rich sediments.

More direct evidence of occurring mobilization is given by the rate of extracellular enzymatic activities (Vezzulli et al. 2004). In the marine environment, exo-enzymes are the primary decomposers of the organic macromolecules; they are produced by microorganisms, mainly by bacteria, but also by fungi and some phytoplankton species (Martinez et al. 1996; Meyer-Reil 1991; Romani et al. 1998). In many environments enzymatic activities are considered as the rate limiting step in OM decomposition processes, and thus their detection, via fluorescent substrates, has recognized microbial ecological significance (Hoppe 1991). Furthermore, they are sensitive and respond rapidly to environmental stresses (Dick 1994; Rusch et al. 2003; Yakovchenko et al. 1996) and represent the primary steps for bioremediation of organic rich sediments (Vezzulli et al. 2004). Aim of the present study was to investigate the response of the microbial community (in term of abundance and enzymatic activity) to test the effect of different bioremediation protocols to naturally enhance decomposition of organic polymers in harbour sediments.

Materials and methods

Experimental design, microcosms set-up and samplings

Water and sediment were collected by means of a Van Veen grab with the support of scuba divers in an enclosed basin in the harbour of Genoa (Liguria Region, Italy, North–Western Mediterranean) in November 1999, at a depth of approximately 5 m. After collection, the material was transported to the laboratory, homogenized, and a 20-cm layer of sediment (3 kg wet weight) was placed in 12 square-based boxes (35 cm each side) and submerged under a 20-cm column (12 l) of harbour water (Figure 1). Sediment and water were allowed to settle for 3 days in order to re-establish original condition before the beginning of the experiment (Verrhiest et al. 2002). Four different bioremediation protocols were tested (Table 1): (1) *Natural attenuation*: BLK box was left untreated and monitored for its intrinsic degradation capabilities, in no OM input conditions (enclosed microcosm); (2) *Biostimulation:* S box was continuously aerated by means of air tubes (Penn–Plax silent-air, 5 atm), both oxygenating and stirring the water; (3) *Bioaugmentation*: 5 boxes, designed A1, A2, A3, A4, A5, were added with increasing quantities of a microbial consortium (see below); (4) *Biostimulation + Bioaugmentation*: five boxes, designed S1, S2, S3, S4, S5,

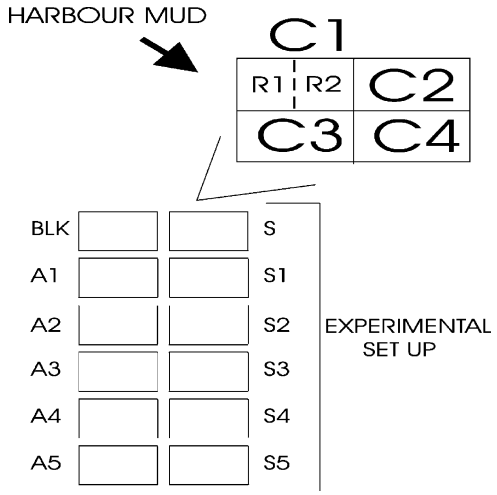

Figure 1. Experimental set up and spatial replication employed in the multifactorial design: 12 boxes (BLK blank, S biostimulation, A1–A5 Bioaugmentation, S1–S5 Biostimulation + Bioaugmentation) × 4 compartments (C1–C4) × 2 replicates (R1–R2).

Table 1. Physical–chemical variables recorded at the water and sediment compartments in the Genoa Harbour

Parameter	Unit	Value
Sedimentary water content	%	62.5
(Silt + clay) fractions	%	78.1
Sediment Eh	mV	−180
Water pH		7.9
Water salinity	‰	36.7
Water temperature	°C	15.3
Dissolved O_2 in water	mg l^{-1}	4.2

were added with increasing quantities of microorganisms and the overlying water continuously aerated by means of air tubes. The microbial consortium utilized in the present research is composed by harmless, widespread, ubiquitous microorganisms with known degrading capabilities, created *ad-hoc* for the present experiment by Idrabel Italia S.p.a. (www.idrabel.it). The product consists of a powder and contains indigenous activated microorganisms, that are inserted or biofixed, through an ion-exchange process, within a protective material support composed by natural components (coccolite, grain size >2 mm). The microbial strains are selected for their capability to

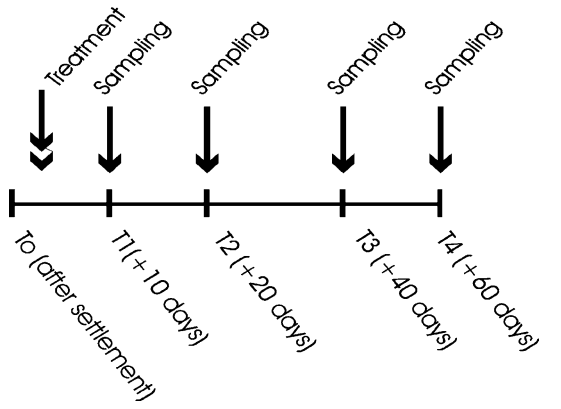

Figure 2. Sampling and temporal replication employed in the multifactorial design (T0–T4).

degrade organic compounds and are formed by *Bacillus subtilis, Bacillus licheniformis, Pseudomonas putida, Lactobacillus helveticus, Lactococcus lactis, Trichoderma reesci, Trichoderma hazonium, Phanerochaete chrysosporium, Nitrosomonas* sp., *Acinetobacter genospecies* and *Arthrobacter* sp.

The product, provided as dry matter, becomes active once in contact with moist soil or water. The quantities amended to the boxes were: 9 g of product (boxes A1–S1), 18 g (A2–S2), 45 g (A3–S3), 100 g (A4–S4) and 150 g (boxes A5–S5), the quantity recommended by the purchaser for *in situ* treatments in marine sediments being 2 tonnes hectare^{-1} (corresponding to 18 g in our boxes). Microcosms were maintained at a temperature of 20 ± 2 °C. They were monitored over 60 days and sampled at T = 0 (after sediment settlement and before the beginning of the treatment), T = 1 (+10 days after the treatment), T = 2 (+20 days), T = 3 (+40 days) and T = 4 (+60 days) (Figure 2). Each box was divided into four separate waterproof PVC sectors, in order to maximize independency (Figure 1). In each sector for each sampling operation variables were analyzed in duplicate, both in the water and in sediment (Figure 1). Water was sampled directly, by means of micropipettes or electrodes. Sediment samples were collected by inserting a PVC tube (5 cm i.d., 10 cm height) into the mud. This system allowed the sampling and the measurement of the physical–chemical parameters under undisturbed conditions.

In situ *measurements*

Sedimentary water content was calculated as the difference between wet weight and dry weight after desiccation (60 °C, 48 h). Particle size profiles were determined by dry-sieving (60 °C, 24 h) according to the Udden–Wenthworth φ Classification (Brown & McLachlan 1990), after pre-treating the sediment with H_2O_2 to oxidize the organic fraction. All sedimentary parameters were expressed as a percentage. *In situ* pH, salinity and temperature were recorded with a multiparametric probe (YSI/Grant 3800, Dayton, OH, USA). Redox potential was recorded with a portable eH meter (Hanna Instruments, Ann Arbor, MI, USA) in the top 2-cm of the mud. Dissolved oxygen in the water was calculated with the Wrinkler method, modified by Carpenter (1965).

Biochemical composition of OM

For all biochemical analyses, approximately 0.5 g of mud was used for sediment, and 15 ml of water were filtered on Whatman GF/F filters (0.8 μm nominal pore size) for suspended material. Sedimentary OM concentration was analyzed in the top 2-cm sediment layer of the core by transferring opportune aliquots in Petri dishes. Sediment and filters were stored at −20 °C until analysis. CHO concentrations were calculated according to Dubois et al. (1956) and expressed as glucose equivalents. PRT analyses were carried out following Hartree (1972) and expressed as albumin equivalents. Blanks were obtained using pre-combusted sediment (450 °C, 5 h) and non-utilized filters, respectively for sediment and water analyses. CHO concentrations were converted into C equivalents using 0.40 as conversion factor (Fabiano et al. 1995), and normalized to dry weight after desiccation (60 °C, 24 h) for sediment and per liter for water samples, hereafter reported as mg g^{-1} and mg l^{-1}.

Microbial parameters

Sediment samples for bacterial counting (about 1 m^3) were collected aseptically in the top 2-cm sediment layer of the core, immediately fixed with sterile 0.2 μm pre-filtered, buffered formaldehyde (2% final concentration) and stored at 4 °C until analysis (Hobbie et al. 1977). Samples were then sonicated (3 times, 1 min, Transonic Power 2000 Sonifier, 220 V) and diluted 1500–4000 times according to cellular density with formaldehyde (2% final concentration). Water samples (5 ml aliquots) were immediately fixed with 100 μl pure buffered formaldehyde and stored at 4 °C until analysis. Samples were then sonicated (10 s) and diluted 30–50 times according to cellular density with sterile, 0.2 μm pre-filtered formaldehyde (2% final concentration). Water and sediment subsamples (2 replicates each) were then stained with Acridine Orange (5 mg l^{-1} final concentration) and filtered on Black Nucleopore polycarbonate filters (pore size 0.2 μm) (Hobbie et al. 1977). Thirty randomly chosen fields on each slide were counted with epifluorescence microscopy (Zeiss A-Plan, × 1000). The contribution by different size classes of bacteria to the total density was evaluated by assigning bacteria into different size classes according Palumbo et al. (1984): small size bacteria (SSB, < 0.065 μm^3), medium size bacteria (MSB, 0.065–0.320 μm^3), and large size bacteria (LSB, 0.320–0.780 μm^3). Bacterial abundances were normalized to dry weight after desiccation (60 °C, 24 h) for the sediment and per liter for water samples, hereafter reported as cells g^{-1} and cells l^{-1}. Total bacterial biovolume was estimated by calculating the contribution of different size classes and converted to carbon content (BBM) assuming 310 fg C μm^{-3} (Fry 1990).

Enzymatic activities

Potential enzymatic extracellular activities (α-glucosidase -αG-, β-glucosidase -βG- and leucine aminopeptidase -LEU-) were analysed immediately after retrieval as described by Chrost and Velimirov (1991). Solutions of 4-methylumbellyferone (MUF) and 7-amino-4-methylcoumarin were used as standards. Blanks were obtained using the same procedure on sediment and water samples with no substrate addition. For each sample, kinetics parameters were calculated with Lineweaver–Burk transformation, utilizing 4 concentrations ranging from 25 to 200 μmol l^{-1}, assuming the sedimentary enzymes follow the Michaelis–Menten kinetics (Romani 2000; Williams 1973). Data were expressed as nmol $l^{-1}h^{-1}$ (water) and nmol $mg^{-1}h^{-1}$ (sediment, normalized to dry weight after desiccation (60 °C, 24 h).

Statistical analyses

Multifactorial Analysis of Variance (ANOVA) was used to assess quantitative changes in microbial and biochemical parameters over time within the different treatments. In the analysis two factors were nested ((Compartments, 4 levels, random) plot in (Treatments, 12 levels, fixed)) and one factor was crossed (Time, 5 levels, random). The effect of the bioremediation was then investigated by testing whether the interaction terms (*treatment* × *time*) were statistically significant ($p < 0.05$). To interpret statistically significant interactions effects among the different treatments a Tukey *post-hoc* test was carried out. Prior to the analysis, the homogeneity of variance was tested by Cochran's test and, when necessary, data were appropriately transformed. All statistical analyses were performed using the MATLAB Statistics Toolbox (Version 6.1; The MathWorks).

Results and discussion

Harbour water and sediment

Characteristics of *in situ* water and sediment are listed in Table 1. Harbour ecosystem displayed distrophic conditions, with low concentrations of dissolved oxygen (54% saturation level), anoxic sediments up to the surface layers (Eh −178 mV) and strong OM sedimentary loads (28.7 ± 11.0 and 44.6 ± 3.4 mg g^{-1} for proteins and carbohydrates respectively (Table 2) if compared to similar environments (Manini et al. 2003). The PRT/CHO ratio in Genoa Harbour was sensibly below one (0.64, Table 2), showing that although the organic load is high, its quality is low (Danovaro & Fabiano 1995). This uncoupling seems to be caused by a strong accumulation of the refractory glucidic fraction rather than a protein scarcity (Gallizia 2000).

The patterns of enzyme activity in Harbour sediment (Table 2) show the usual ranking LEU > BG > AG, the ratio being 24/3/1. While BG/AG ratio ($r_{cell/amyl}$) follows values ordinarily found in sediments (Poremba 1995; Wittman et al. 2000; Wobus et al. 2003), glucosidases/LEU is much shifted towards glucidic hydrolysis. In fact, a ratio of 687/3/1 is reported for natural sediments (Poremba 1995), while recorded values are more comparable to those found in water (40/2/1, Mudryk & Skórczewski 2004): the microbial community, in eutrophic environments, displays potential specific pathways, such as the expression of specific exo-enzymatic activities to face and preferentially degrade the most abundant organic fraction. Similar findings were recorded in other organic-rich sediments (Fabiano & Danovaro 1998; Fabiano et al. 1995; Vezzulli et al. 2004).

Finally, the extremely fine texture of the Harbour sediment (silt-clay fractions account for more than 78% of the total sediment fraction, Table 2) has reflections on living communities, because it causes water turbidity, reduces photosynthetic activity, and due to high heterotrophic activity may be subjected to anoxia.

Natural attenuation protocol

In the natural attenuation protocol no microorganisms or air was supplied (BLK Box). Bacterial density (Table 2) did not undergo any significant change, neither for what concerns TBN ($187.7 \pm 5.7 \times 10^8$ cells g^{-1} at $T = 0$ and $142.7 \pm 1.0 \times 10^8$ cells g^{-1} at $T = 4$) (ANOVA, $p =$ ns) (Figure 3), nor for bacterial size (SSB constituted 43.7% of the total community at $T = 0$ and 50.2% at $T = 4$, while LSB were 13.9% at $T = 0$ and 19.9% at $T = 4$)(Figure 4). Although not significant potential βG exo-enzymatic activity rates shifted from 56.0 to 160.7 nmol mg^{-1} h^{-1}, and αG activity followed a similar trend, increasing from 16.9 to 84.1 nmol mg^{-1} h^{-1} (ANOVA, $p =$ ns). Leucine-aminopeptidase activity, on the other hand, displayed a decrease: V_{max} values were 411.4 nmol mg^{-1} h^{-1} at $T = 0$ and 315.1 nmol mg^{-1} h^{-1} at $T = 4$ (ANOVA, $p =$ ns) (Figure 5). OM turnover in BLK box decreased, both for proteinaceus and glucidic materials (Table 2) the depletion was more important for CHO, that shifted from 14.66 to 2.25 h, (6.5-folds reduction), followed by PRT turnover that underwent a 3.1-folds reduction. The increase of OM turnover rates in sediments during the study period is in relation with the increase of enzymatic activity rates in addition to a lack of continuous organic input as found at *in situ* sediments.

Bacterial density in the water compartment of BLK box were not affected significantly during the experiment: initial TBN was $0.23 \pm 0.01 \times 10^8$ cells ml^{-1} while final TBN was 0.13×10^8

Table 2. Microbiological and biochemical variables recorded at the sediment and water compartment at T = 0 and T = 4 in the experimental microcosms

	T = 0	T = 4										
		BLK	S	A1	A2	A3	A4	A5	S1	S2	S3	S4	S5
Sediment													
Bacterial Biomas (mgC/g)	1.42 ± 0.01	1.07 ± 0.03	1.50 ± 0.28	1.17 ± 0.16	1.67 ± 0.28	2.38 ± 0.86	4.25 ± 0.41	0.64 ± 0.06	1.39 ± 0.1	1.45 ± 0.12	1.78 ± 0.34	2.34 ± 0.33	1.38 ± 0.23
Small size bacteria (SSB) (%)	43.7	50.2	53.4	38.9	48.0	36.7	39.3	32.9	34.2	50.3	54.8	44.4	27.1
Low size bacteria (LSB) (%)	13.9	19.9	17.0	20.0	13.6	17.9	22.9	23.8	24.8	14.5	13.0	12.9	25.0
Frequency of dividing cells (%)	4.8 ± 0.4	7.3 ± 0.3	5.4 ± 0.7	8.2 ± 0.4	5.5 ± 0.4	6.1 ± 0.1	6.5 ± 0.5	6.8 ± 1.2	5.8 ± 0.3	4.6 ± 0.7	4.3 ± 0.4	5.3 ± 0.3	4.1 ± 1.2
Carbohydrate (CHO) (mg/g)	44.6 ± 3.4	34.1 ± 1.9	34.6 ± 0.5	39.2 ± 0.4	43.9 ± 8.2	33.7 ± 1.2	43.6 ± 6.3	26.9 ± 1.1	24.7 ± 4.4	25.7 ± 0.8	35.5 ± 6.9	27.1 ± 5.4	31.2 ± 5.4
Protein (PRT) (mg/g)	28.7 ± 11	7.0 ± 1	10.9 ± 1.9	8.0 ± 1.7	8.2 ± 0.9	6.8 ± 0.9	3.7 ± 0.5	8.2 ± 2.1	5.1 ± 0.8	6.8 ± 0.5	4.8 ± 0.9	6.5 ± 0.6	5.7 ± 1.3
CHO Turnover (h)	14.66	2.25	2.30	0.49	0.59	0.47	0.49	0.56	0.58	0.60	1.12	0.67	1.66
PRT Turnover (h)	0.47	0.15	0.29	0.19	0.18	0.33	0.05	0.13	0.11	0.08	0.10	0.18	0.13
Water													
Carbohydrate (CHO) (mg/l)	0.5 ± 0.1	1.4 ± 0.4	0.7 ± 0.2	1.8 ± 0.6	0.3 ± 0.1	1.4 ± 0.7	0.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	3.6 ± 1	4.1 ± 1.1	0.7 ± 0.2	1.1 ± 0.3
Protein (PRT) (mg/l)	2.5 ± 0.3	3.8 ± 1.2	0.5 ± 0.1	1.0 ± 0.3	1.5 ± 0.5	6.3 ± 1	1.5 ± 0.2	1.0 ± 0.4	2.4 ± 0.7	4.5 ± 1.7	4.8 ± 1.1	4.4 ± 1.2	4.1 ± 1.3
Turnover CHO (h)	150.2	257.1	31.5	189.5	81.4	179.9	27.5	84.5	25.1	103.5	166.8	32.9	22.4
Turnover PRT (h)	6.5	11.6	0.1	2.5	7.5	19.1	2.4	6.1	0.6	0.7	0.9	2.1	0.9

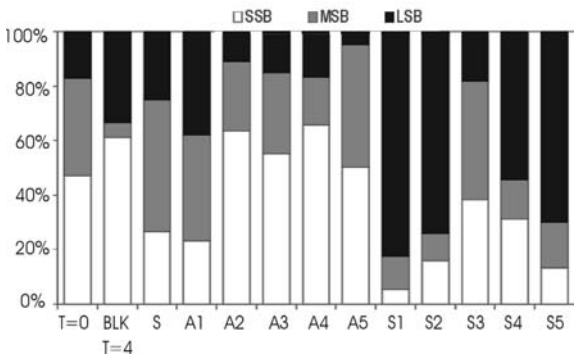

Figure 3. Total bacterial density (*n* cells $\times 10^8$ g^{-1}) in the sediment and water compartments recorded at T = 0 and T = 4 (+60 days after the treatment) in experimental microcosms. Error bars indicate the standard deviation from eight measurements.

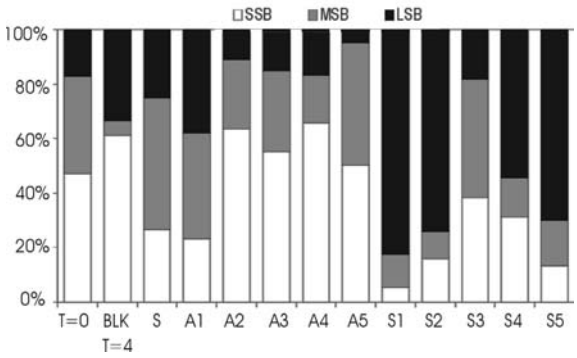

Figure 4. Contribution (%) of different bacterial size classes to the total, in the water compartment of each microcosm, at T = 0 and T = 4 (+60 days after the treatment). Reported are: SSB small size bacteria; MSB medium size bacteria; LSB large size bacteria.

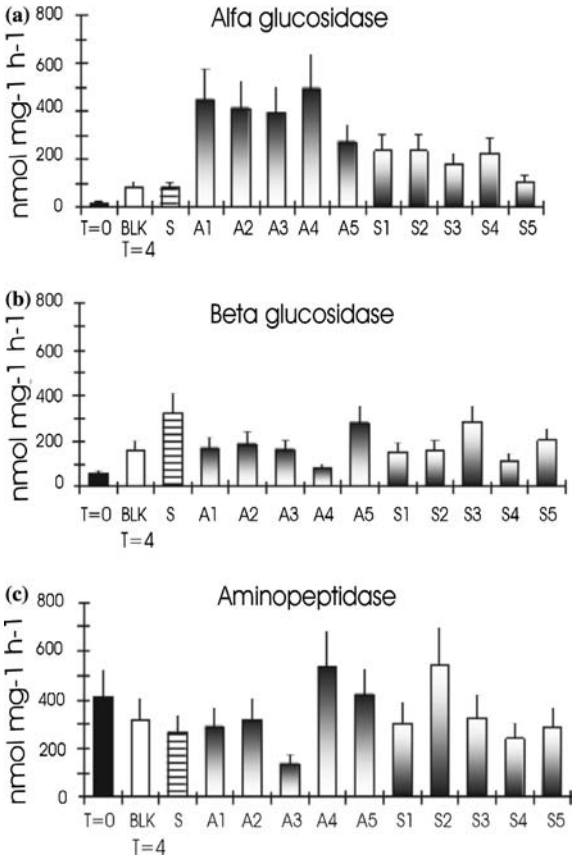

Figure 5. Exo-enzymatic activities rates in the sediment in experimental microcosms, at T = 0 and T = 4 (+60 days after the treatment), expressed as V_{max} maximum velocity of substrate hydrolysis, calculated using Lineweaver–Burk transformation. Error bars indicate the standard deviation from eight measurements.

cells ml^{-1} (Figure 3) (ANOVA, p = ns), the predominant bacterial size found in the community at the end of the experiment being SSB (61.1% of the overall community, Table 2). Exo-enzymatic activities rates in the water compartment of BLK box (Figure 6) at the end of the experiment were comparable or lower to those found at T = 0 (ANOVA, p = ns).

Biostimulation

Box S showed, for most of the sedimentary parameters, trends similar to those of BLK box. The hydrolysis of the glucidic component increased not significantly, from 16.9 (T = 0) to 83.5 nmol mg^{-1} h^{-1} ($T = 4$, αG), and from 56.0 (T = 0) to 316.0 nmol mg^{-1} h^{-1} (T = 4, βG, Table 3) (ANOVA, p = ns). Similarly to what found in BLK box, LEU decreased with time of 1.5-folds (Figure 5). Bacterial community density did not record a significant change: from 187.7 ± 5.7 to 213.5 ± 62.9 $\times 10^8$ cells g^{-1} (ANOVA, p = ns) (Figure 3). A significative response, instead, was recorded in the overlying water if compared both at T = 0 and BLK boxes, specifically in the microbial functional parameters (Figure 6): βG activity increased significatively from 20.0 to 117.3 nmol l^{-1} h^{-1} and LEU activity from 2627.7 to 27091.7 nmol l^{-1} h^{-1}: more than a 10-folds increase in the 60-day observation period

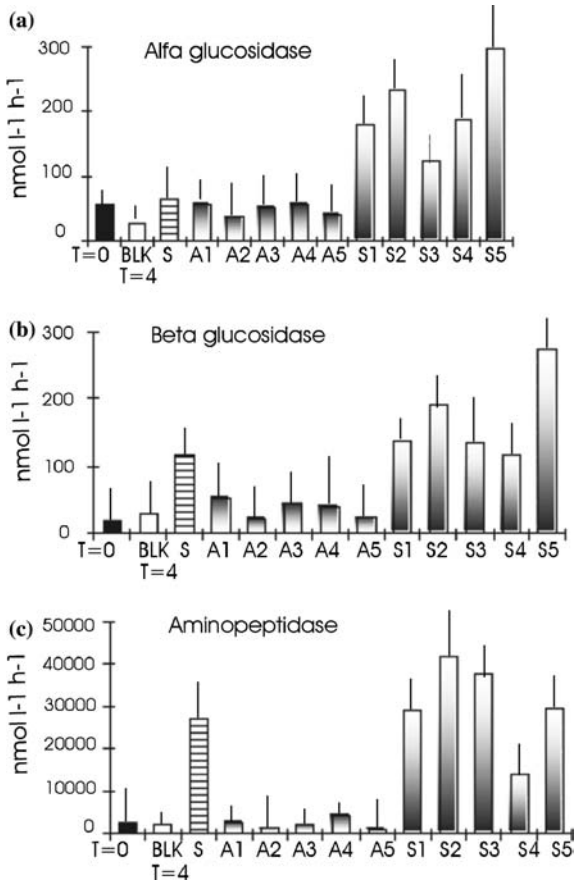

Figure 6. Exo-enzymatic activities rates in the water in experimental microcosms, at T = 0 and T = 4 (+60 days after the treatment), expressed as V_{max} maximum velocity of substrate hydrolysis, calculated using Lineweaver–Burk transformation. Error bars indicate the standard deviation from eight measurements.

(ANOVA, $p < 0.05$). On line with these findings, particulate proteins (Table 2) were significatively depleted in the water, from 2.5 to 0.5 mg l^{-1} (ANOVA, $p < 0.05$), while particulate carbohydrates remained almost unchanged (0.5 mg l^{-1} at T = 0 and 0.7 mg l^{-1} at T = 4) (ANOVA, p = ns). PRT and CHO turnover in the water compartment at S box decreased consistently (Table 2), indicating an efficient degradation of organic polymers when oxic conditions prevailed. Since aeration technique affected directly the water compartment (air tubes were placed in the overlying water), the strong response recorded in this compartment is not surprising: hence, biostimulation helped greatly the autochthonous microbial community in the water compartment, and had a positive effect on the mobilization of OM.

Bioaugmentation

Aim of the bioaugmentation experiment was to detect whether the bio-fixed bacterial matrix had an effect in enhancing OM degradation and cycling, and consequently evaluate the effect of different product quantities on the bioremediation treatment. For this aim, five different boxes, designed from A1 to A5, were set up for the bioaugmentation protocol and amended with increasing bacterial loads. Results from the analyses suggested that the allochthonous community was able to survive and carry out efficient replication. In fact, bacterial counts in the sediment (Figure 3) were correlated to the quantities amended in each box: at T = 4 TBN was $139.0 \pm 17.7 \times 10^8$ cells g^{-1} at box A1, 231.5 ± 46.3 at box A2, 282.89 ± 117.4 at box A3, 493.8 ± 68.7 at box A4, and 70.1 ± 3.9 at box A5. The value recorded at box A5 (more than a half lower of what found in BLK box at T = 4) suggested that quantity of product amended was probably too high and resulted in lower activity and growth of the bacterial community. The same trend can be outlined for functional sediment parameters: αG activity was strongly stimulated: the highest final rate was found at box A4 (496.5 nmol mg^{-1} h) and the lowest at box A5 (268.8 nmol mg^{-1} h). In both case, rates were significantly higher than those found at T = 0. Leucine-aminopeptidase highest value was also found at box A4 (535.9 nmol mg^{-1} h) while in the other boxes the trend was far less clear and interpretable (Figure 5).

TBN in the sediment was positively correlated with αG activity ($r = 0.76$, $p < 0.05$), and negatively correlated with the protein content of the sediment ($r = -0.94$, $p < 0.01$). Proteins, considered the more labile fraction of OM, underwent a significant depletion in all bioaugmentated boxes (ANOVA, $p < 0.05$) with the exception of A4 box (final PRT concentration was 3.7 ± 0.5 mg g^{-1}, Table 2). In the water compartment, bacterial density was less influenced by the bioaugmentation protocol (Figure 3): TBN increased to a lesser extent, the average being $0.36 \pm 0.04 \times 10^8$ cells ml^{-1} at T = 4 and 0.23 ± 0.01 cells ml^{-1} at T = 0: the contribution of different size classes to the total density in the water compartment showed, as for the sediment, a predominance of

SSB (with the exception of A1), that accounted for 58.7% of the total bacterial number (Table 2). In general in the water compartment bioaugmentation had a negligible effect on OM mobilization and enzymatic activities did not showed any significant increase (Figure 6).

Biostimulation + bioaugmentation

The last series of test (boxes S1–S5) evaluate the combination of air insufflations and microorganisms amendment to naturally enhance decomposition rates. Result from the experiment undoubtly highlights this protocol as the one providing the best response among all the investigated treatments. According to the bioaugmentation experiment, TBN in the sediment (Figure 3) recorded increasing counts from S1 box ($151.8 \pm 1.0 \times 10^8$ cells g^{-1}) to S4 box ($315.6 \pm 90.1 \times 10^8$ cells g^{-1}), while in S5 box the density collapsed (141.9×10^8 cells g^{-1}). OM depletion in sediment resulted significantly higher in these boxes (mean PRT final concentration of 5.8 ± 0.9 mg g^{-1} and mean CHO final concentration of 28.8 ± 4.4 mg g^{-1}) if compared to S (respectively, 10.9 ± 1.9 and 34.6 ± 0.5 mg g^{-1}) and to A1–A5 boxes (Table 2). The greatest response was recorded in the water compartment (Figure 3). Here a striking increase of the bacterial density was recorded. TBN were significantly higher than at $T = 0$ and once again reflected the quantities amended to the boxes: 0.23×10^8 cells ml^{-1} at $T = 0$ and 0.39 ± 0.06, 1.10 ± 0.04, 1.98 ± 0.47, 3.23 ± 0.87, $3.60 \pm 1.06 \times 10^8$ cells ml^{-1} respectively, in boxes S1, S2, S3, S4, S5. In contrast to bioaugmentation highest bacterial loads was recorded at S5 box probably due to air insufflations supporting heterotrophic metabolism at high bacterial density. An increase in bacterial size was recorded and average size fractions were 20.8% SSB, 19.3% MSB and 59.8% LSB at $T = 4$, while they were 56.6%, 43.4% and 20.4% at $T = 0$ respectively. In all S boxes bacteria increased they number (see A1–A5) and size (S1–S5). Substrate hydrolysis in the water compartment (Figure 6) was significantly higher at S1–S5 boxes, if compared to $T = 0$ and the other treatments: αG grew of 3.6-folds on average, βG of 8.6-folds and LEU of 11.6-folds (ANOVA, $p < 0.05$).

Conclusions

In conclusion the biostimulation treatment by water oxygenation via air supply (boxes S–S1–S2–S3–S4–S5) was identified as the best protocol that naturally enhances decomposition of organic polymers in organic-rich harbour sediment. This was supported by the significant increase of enzymatic activities rates both in the water and sediment compartments also correlated with the increase of bacterial cells size and bacterial number in treated sediments. Still much debate remain on the survival of allochthonous microorganisms artificially introduced into the environment (Dejonghe et al. 2001; Liu & Sulfita, 1993), especially in complex and multi-polluted matrix. In our study, the total bacterial counts in bioaugmentated boxes was highly correlated with the quantities of microbial inocula amended to the boxes and although speculative this suggest that allochthonous community was able to survive and multiply. In this context the highest microbial inocula resulted in a collapse of the total bacterial density, probably in relation to the lack of oxygen for heterotrophic metabolism. In fact, while bioaugmentation alone seem not to be able to carry out efficient degradation, its coupling with air insufflation (boxes S1–S5) turned out to produce the best results. A greater response was observed in the water compartment in term of increased mobilisation rates and polymers decomposition if compared to the sediment. This imply a strong coupling between the water and sediment compartments where organic carbon mobilized in this latter may efflux to the water column and fuel the pelagic microbial degradation. A significant benthic–pelagic coupling in term of DOC efflux from marine sediment treated with bio-fixed bacteria consortia was observed by Fabiano et al. (2003) during a microcosm experiment (Fabiano et al. 2003). Based on this consideration the sediment–water interface may thus constitute a key compartment in the biodegradation process of organic-rich sediments.

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