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

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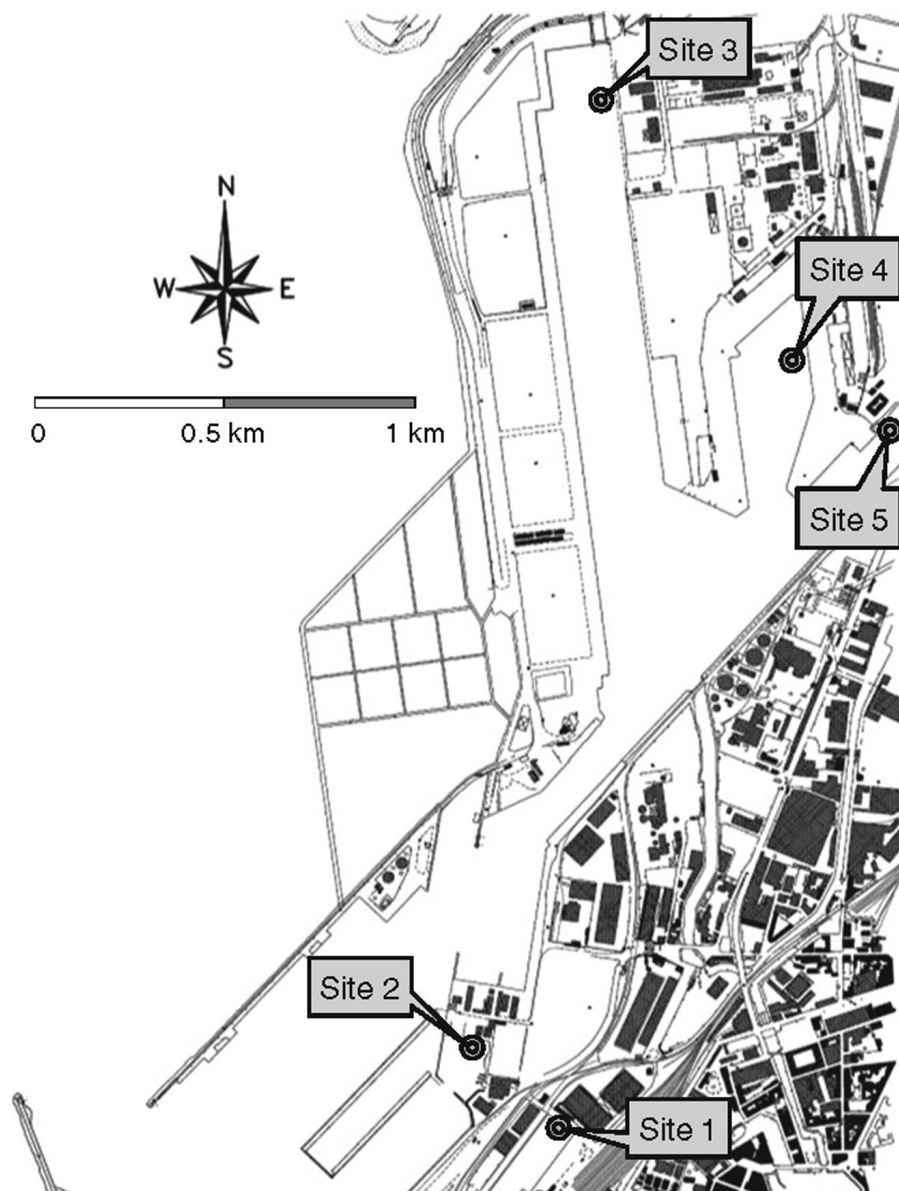

Fig. 1. The five sampling sites in the Port of Livorno.

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:

$$N_i = (n_i / \sum n_{tot}) \cdot 10\,000 + 1$$

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.

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[3]	0.8	75.0	70.0	360.0	52.0	170.0	-	-	-
Harmful aliphatic hydrocarbons[3]	-	-	-	-	-	-	-	-	>10.0
Highly polluted sediments[0]	-	-	-	-	-	-	-	-	>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.

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 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);

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_a	25.2_a (A)	39.6_a	41.0_a	75.4_b (B)	94.9_a	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 (A)	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_a	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)	$9.8E4_c$	$5.4E6_a$	$3.9E6_b$ (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_a	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.

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.

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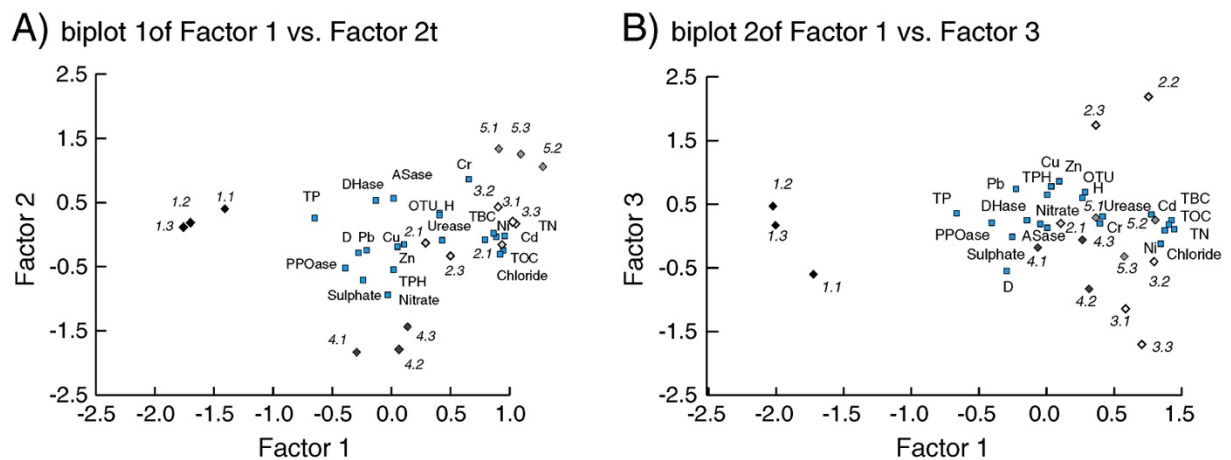
Fig. 2. Principal component analysis: biplots of loadings and scores.

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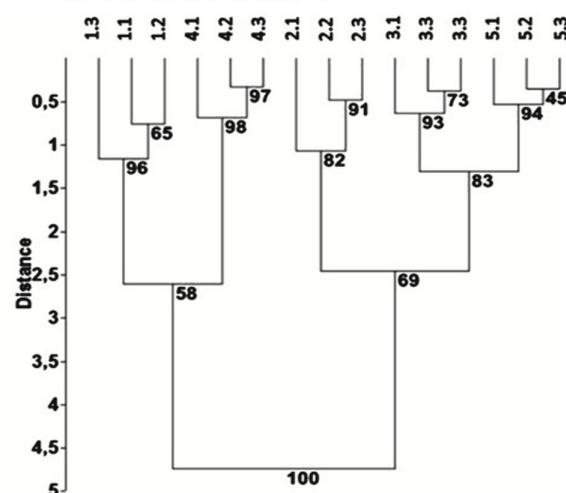
Fig. 4. Dendrograms from the cluster analysis with Ward's method and 500 bootstraps. The vertical axes represent squared Euclidean distances.

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 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.

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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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