Biomonitoring a polluted coastal area (Bay of Muggia, North Adriatic Sea): a five year study using transplanted mussels

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Abstract

Pollution effects at subcellular levels were evaluated by using two lysosomal biomarkers in mussels, *Mytilus galloprovincialis*, deployed periodically over a period of 5 years in an harbor area within the Bay of Muggia (Gulf of Trieste, North Adriatic Sea) influenced by anthropogenic activities. Mussels collected from a clean marine farming plot (sample T0) were transplanted to the harbor site (sample M) for about 12 weeks; at the same time an additional sub-sample was relocated at the farm site as a further control (sample T1). The transplantation experiments were repeated twice yearly for 5 consecutive years, starting from 2009. Two well-established lysosomal biomarkers were evaluated in the cells of the digestive gland, i.e. lysosomal membrane stability and lipofuscin accumulation; body condition index and mortality rate were also assessed. For a better comprehension of the biological parameters various chemical pollutants were determined in sediments and in the whole mussel flesh. Biological results were suitable, as a whole, to discriminate samples between site M and the reference ones. Variations of lysosomal membrane stability and lipofuscin content were mostly related to PAHs and to trace metals, respectively. Our results evidenced the usefulness of active biomonitoring approach in evaluating pollution conditions in marine coastal areas and of lysosomal biomarkers as a rapid screening tool to evidence pollutant effects at least at organism level, stressing their potential use in the context of the European Marine Directive.

Key-words: *Mytilus galloprovincialis,* active biomonitoring, Bay of Muggia, lysosomal biomarkers, sediment pollution, pollutant body burden.

1. Introduction

In the last years, the requirement for an integration between chemical and biological approach in marine monitoring has received increasing attention becoming a crucially important challenge also for management and regulation purposes. In Europe, this issue has been considered in the context of the Water Framework Directive (WFD, Directive 2000/60/EC) and of the Marine Strategy Framework Directive (MSFD, 2008/56/WE) which require to reach the good ecological/environmental status through the periodic assessment of water quality, integrating biological, physico-chemical and hydro-morphological factors. The MSFD has posed additional emphasis on the importance of evaluating the effects of contaminants at various biological levels, including lower levels of organization (Lyons et al., 2010). At organism level, biological effects can be assessed through the so-called biomarker approach, which has shown the potentiality of establishing mechanistic links between molecular/cellular/tissue alterations and environmental pollutants, particularly when confronting the contaminant body burden and the measured biological effects in bioindicator organisms (effect-dose study). Biomarkers evaluated on various sentinel organisms have been recently proposed within the context of the risk assessment processes for both Directives, suggesting their use to establish links between stress and pollution and to develop a cost-effective strategy accessible also by Environmental Agencies (Galloway et al., 2004; Viarengo et al., 2007).

Mussels are widely used as marine biomonitor organisms for being able to accumulate and tolerate high concentrations of xenobiotics and because of the good knowledge of their metabolic pathways, which allows to elucidate mechanistic links between the internal dose of pollutants and the elicited biological effects (Cajaraville et al., 2000; Viarengo et al., 2007a). The active biomonitoring strategy is widely adopted in marine monitoring international programs mainly because it is known

to be suitable to reduce the variability of the results, allowing consequently more significant comparisons between specimens from reference and potentially polluted sites (Viarengo et al., 2007). Also, the active approach is indicated as more suitable to evaluate contaminant bioaccumulation and effects in hard-to-reach areas and for small scale and site-specific studies (Hunt and Slone, 2010).

The lysosomal system of many marine species is known to be particularly sensitive to environmental perturbations and, for this reason, its alterations are widely used as indicators of cellular stress. Lysosomes are membrane-bound organelles, containing various hydrolytic enzymes, which are involved in various cellular processes including digestion, defense, and reproduction (Moore, 1988; Moore et al., 2006). In particular, lysosomes of mollusk digestive cells are involved in the detoxification metabolism, through the sequestration and accumulation of a wide range of chemicals, such as metal ions, PAH and pharmaceuticals (Moore et al., 2006). Exposure to pollutants as well as to other physico-chemical stressors generally gives rise to various lysosomal alterations, namely variation in membrane permeability, increases in lysosomal size and number and content changes, mainly accumulation of lipofuscins and neutral lipids (Marigomez et al. 1996; Viarengo et al. 2007). In particular, the variation in membrane permeability, evaluated through the lysosomal membrane stability test, has been selected as one of the core biomarkers due to its high sensitivity and low-cost, and has been already proposed as one of the biological effects measurement in mussels and fish under the Marine Strategy Framework Directive (Law et al., 2010).

The present study has been conducted with the aim of evaluating possible temporal evolution of the pollution level and its effects in a marine coastal site strongly influenced by anthropogenic activities, the Bay of Muggia (Gulf of Trieste), adopting an active biomonitoring strategy with marine mussels, *Mytilus galloprovincialis*, based on the evaluation of lysosomal membrane stability and lipofuscin accumulation on mussel digestive glands. Moreover, as supporting parameters, the body condition index and the mortality rate have been assessed to characterize the general health

status of mussels (Moschino and Marin, 2006; Viarengo et al., 2007). Lastly, contamination levels (metals, PAHs, PCBs, alkyl-phenols and phthalates) were measured in mussels and sediments to explore links between pollutants and biological responses.

2. Materials and Methods

2.1 Study area, mussel transplantation procedure and sampling design

The Bay of Muggia, located in the Gulf of Trieste (northern Adriatic Sea), is a shallow and narrow environment (8 - 20 m in depth; 7 km long - 4 km wide) which has suffered historical anthropogenic impact because populated for at least the last 2000 years, and because activities such as marble quarrying and oyster culture have been practiced here for centuries. Today, its environmental quality is greatly affected by the presence of the harbor of the city of Trieste, as well as a large industrial district, with an ironmaking plant covering an area of 600,000 m^2, an oil-pipeline terminal, municipal effluents and several other economic activities (Solis-Weiss et al., 2004; Cibic et al., 2008). Waters and sediments are characterized by various inputs of pollutants, which persist in the area due to its morphology and hydrodynamism: waters are indeed protected by three-dam systems, which make them quite still (Adami et al., 2000a). Adami et al. (2000 a, b) reported that the pollution levels of total PAH in the Trieste harbor can be assigned as high and/or very high in comparison to values reported for the other areas of the Mediterranean Sea. Moreover, the same authors found that the highest PCB polluted sediments were those facing the piers in the northern area of the bay. Also the worsening of the benthic environment has been reported (Adami et al., 1997). In particular, the study of Solis-Weiss et al. (2004) described the effects of industrial and urban pollution on the local benthic macrofauna. These latter authors evidenced a gradient of increasing stress when proceeding away from its entrance and towards the sources of pollution,

which can be hierarchically identified as: (1) the reduced hydrodynamism and consequent water stagnation; (2) the high concentrations of heavy metals; and (3) the direct discharge of urban sewage into the bay.

Mussels, *M. galloprovincialis*, were transplanted to one of the three commercial piers opposite the industrial district (Fig. 1). Mussels of homogeneous size (55-65 mm in length) were collected from a clean marine farming area, where they are grown for commercial purposes by a long-line system, and translocated to the harbor site for a period of about 12 weeks (sample M). Biological responses and tissue chemical analyses performed on samples M were compared to those evaluated in organisms before the transplantation period (time 0, T0) to evaluate the effects of the exposure in the harbor site. A further sub-sample was relocated in the farm and collected after 12 weeks simultaneously to sample M (time 1, T1), to better discriminate the natural variability of the responses, since changes in biomarkers are not only related to pollutants, but also to environmental as well as intrinsic biological factors (Bocchetti et al., 2008). The transplantation procedure was performed in each cold (October – January, I survey), and warmer (May – August, II survey) periods over five years (from October 2008 to August 2013). During each survey, water temperature and salinity were recorded with a CTD probe (SeaBird 19 plus).

Sediment were collected once a year in spring at site S (Fig. 1) using a Van Veen grab with a 0.1 m^2 sampling area (lowered at a speed between 1m/s and 1.5 m/s) and sampled at 0-5 cm. After collection, organisms and sediments were rapidly transported to the laboratory in a refrigerated box and processed/stored within few hours according to the different analyses.

2.2 Chemical analyses in mussels and sediments

Pollutants in sediments were determined on samples collected at the harbour site S. Pollutant body burdens were determined in T0 and M samples, and from 2010-I survey onwards also in the T1 sample. Total flesh was dissected from about 50 specimens, pooled, freeze-dried and homogenized, and the wet/dry weight ratio of each sample was then recorded.

Metal concentrations were determined according to EPA 3050B/1996, EPA 3052/1996 and EPA 6010C/2000 methods except for Hg, determined according to IRSA-CNR method Q.64/1988. PAHs were analyzed according to EPA 3545 and EPA 8270C/1996 methods and IRSA-CNR Q.64/1990, method. PCBs were determined according to EPA method 8082A/2007. Phthalates and di-(2-ethylhexyl) phthalate were determined according to the methods proposed by IRSA-CNR Q.64/1998 (Vol. 3.25a), and alkylphenols (di-isobutyl phenol), 4 (para)-Nonylphenol and para-terz-octylphenol, according to EPA 3540, 3630, 8270C/1996.

ΣPAH (i.e. acenaphthene, acenaphthylene, anthracene, benzo(a)antracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo-(ghi)perylene, benzo(k)fluoranthene, chrysene, dibenzo(a,h)antracene, phenanthrene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene, benzene, ethylbenzene, styrene and toluene) and ΣPCB (i.e. 28-CB, 52-CB, 77-CB, 81-CB, 101-CB, 118-CB, 126-CB, 128-CB, 138-CB, 153-CB, 156-CB, 169-CB) were calculated considering the data below the detection limits as equal to half the limit.

2.3 Mussel biological responses

2.3.1 Lysosomal membrane stability

Lysosomal membrane stability (LMS) was determined histochemically on cryostat sections of frozen hepatopancreas (Moore, 1988). Briefly, small portions of 10 digestive glands were fixed in n-hexane pre-cooled in liquid nitrogen, and then sectioned (10 μm) in a MTC SLEE MAINZ Cryotome at a cabinet temperature of −30 °C. The cryostat sections were incubated at 37 °C in 0.1 M citrate buffer containing 2.5% NaCl for various pre-treatment times (0, 2, 5, 10, 15, 20, 25, 30, 35 min) in order to labilise the lysosomal membrane. After this, sections were incubated at 37 °C in 50 mL of 0.1 M citrate buffer with 2.5% NaCl, containing 20 mg naphthol AS-BI-N-acetyl-β-glucosaminide (Sigma) previously dissolved in 2.5 mL 2-methoxyethanol and 3.5 g of polypeptide (Sigma POLYPEP), rinsed at 37 °C in 3% NaCl, treated at room temperature with 1 mg/mL Fast

Violet B in 0.1 M phosphate buffer and fixed for 15 min in Baker's fixative at 4 °C. The time of acid labilisation treatment (labilisation period, LP) required to produce the maximum staining intensity was then assessed under a light microscope as the maximal accumulation of reaction product associated with lysosomes (UNEP/RAMOGE, 1999).

2.3.2 Lipofuscin content

Five digestive glands per site were rapidly fixed in n-hexane pre-cooled in liquid nitrogen and subsequently sectioned (10 µm) in an MTC SLEE MAINZ cryotome. Lipofuscin content was evaluated histochemically according to the Schmorl reaction (Pearse, 1972). A stereological procedure was then adopted to quantify the amount of lipofuscin staining in the digestive gland cells using an automated image analysis system. Images (12-15 per animal) were acquired on a Leica™ DMLB microscopy video camera, and then used to calculate the lipofuscin to digestive cell surface ratio (Lowe et al., 1981). Quantification was performed using Image-Pro Plus software (version 4.0.0.9).

2.3.3 Supporting parameters

The body condition index (CI) was calculated individually in 20 mussels per sample: dry weight of meat (g)*100/dry weight of shell (g) (Walne, 1976). Dry weight was evaluated after meat and shell had been dried separately at 60°C for 48 h. The body condition index was not determined in the first two surveys.

Mussel mortality rate was determined on approximately 100 organisms as the ratio of dead mussels to the total number of specimens in each sample and expressed as percentage.

2.4 Data analysis

To compare the total metal content on the whole, the metal pollution index (MPI) was calculated for both sediments and mussels (Giusti, 2001; Usero et al., 1996):

$$MPI = (M1 \times M2 \times M3 \ldots \times Mn)^{1/n}$$

where Mn is the concentration of metal n expressed in mg kg^{-1} of dry weight.

Labilization times and lipofuscins were compared using Kruskal-Wallis test, mortality rate with G-Test and condition index values with ANOVA test. Normal distribution and homogeneity of variance was established before each statistical analysis. Spearman's correlation coefficients were calculated to investigate the statistical relationships between contaminants in mussel soft tissues and biological response. Finally, Principal Component Analyses (PCA) were performed separately using data matrices made of pollutant body burdens, biomarkers and contaminants in sediments for better comparing sample distribution. Moreover, further PCAs were performed by separating the biological responses of T0, T1 and M samples to highlight possible temporal differences. STATISTICA 6.0 software (StatSoft) was used for all statistical processing.

3. Results

Water temperature and salinity measured during the sampling surveys are shown in Figure 2. An increase of water temperature was observed in the summer surveys since 2011 (i.e., 2011-II, 2012-II and 2013-II), when values were ranging from 24°C to 26°C, far above the ones recorded in the previous surveys (varying from 20°C to 22°C).

Sediments collected from site S generally showed slight variations in metal concentrations over the five-study years, as stressed by the calculated MPI values (Table 1). However, in 2012, increased concentrations for most of the analysed metals (As, Cd, Cr, Fe, Pb and Zn) were observed, particularly high for Pb and Zn (104.5 and 188.5, respectively). Micro-organic pollutants exhibited generally a similar behaviour to metals, reaching the highest values in 2012, particularly for PAHs, which had picked at 4.69 μg g^{-1}, for then a sharply decreasing in 2013 (Table 2). PCA performed with sediment contaminant values clearly separated samples collected in 2012 from the others (Fig. 3). Factors 1 and 2 explained over 80% of the variance, with Factor 1 explaining 55% of the total variance characterized by low loadings of the variables As, Cd, Ni, Pb, Zn, PAHs, PCBs, phthalates

and di-(2-ethylhexyl)phthalates) (-0.81, -0.92, -0.73, -0.90, -0.83, -0.90, -0.96, -0.94, -0.87, respectively) and Factor 2 explaining 25% of the total variance characterized by low loadings of the variables Al, Cr and Hg (-0.92, -0.86, -0.70, respectively).

Pollutant body burdens are shown in Tables 3 and 4. In general, metal concentrations were slightly variable both temporally - over the whole study period, and spatially – when comparing the three mussel samples within each survey. Higher metal concentrations were observed in samples M only in 2010-I and 2011-I surveys (Cr, Pb, Al, Fe; MPI: 11.2 and 12.8, respectively). Among the micro-organic pollutants, PAHs reached the highest concentrations in samples M in 2011-I, 2011-II and 2012-I surveys, whereas the other micro-organic contaminants (i.e. phthalates, di-(2-ethylhexyl), alkylphenols (di-isobutyl phenol), 4 (para)-Nonylphenol, para-terz-octylphenol) exhibited low concentrations and were less variables among both samples and surveys.

LP values for each mussel samples at each survey are given in Fig. 4. T0 and T1 mussels generally showed LP values ranging from 20 to 33 min. Lower LP values were observed in T0 samples during the 2012-II and 2013-I surveys (9 min and 14 min, respectively) and in T1 samples during the 2011-II, 2012-II and 2013-II surveys (5 min, 5 min and 9 min, respectively). M mussels showed LP values close to or lower than 10 min in all surveys, except in the first one (23 min). In general, significant differences were detected when comparing T0 or T1 samples with M ones (Table 5). Lipofuscins generally showed higher values in the M samples (Fig. 5). During the 2010 - I survey, particularly higher lipofuscin values were recorded in both T1 and M samples, reaching respectively the values of 0.5 and 1.2 $\mu m^3/ \mu m^3$. Condition index values were generally in the range from 6 to 10, only occasionally higher values were reached (from 11 to 15) in T0 and T1 samples (Fig. 6). Mortality rates recorded in T0 and T1 samples ranged from 1% to 3%, with little exceptions. On the other hand, mortality percentages in M samples were always higher (9% to 21.7%) than in T0 and T1 (Fig. 7, Table 5).

Significant negative correlations were observed between the temporal trend of lysosomal membrane stability with Cd and Pb, and most of the PAH congeners evaluated in mussels tissues, as well as

with total PAHs. Lipofuscin accumulation was instead positively correlated with the total load of metals (MPI) and mortality rate with both MPI and PAHs (Tables 6 and 7).

The PCA performed with biological responses clearly discriminated site M over time (Fig. 8). Factors 1 and 2 explained 66% of the variance. Factor 1 explained 39% of the total variance and it is characterized by low loading of the variable mortality (-0.83) and high loading of the variable LMS (0.76). Factor 2 explained 27% of the total variance and is characterized by high loading of the variable CI (0.94).

4. Discussion

Chemical analyses of the sediments collected in site S indicated that the highest contamination levels were reached in the period 2012 - 2013. In particular, while trace metal concentrations (i.e. As, Cd, Pb, Zn) peaked only in the two 2012 surveys, micro-organic pollutants (PAHs, PCBs and phthalates) were persistently high also in 2013. This observation suggests a transient input of contaminants from point sources, possibly related to the ironwork drainage, in the period 2012 - 2013. In fact, it was reported by the local Harbour Authority that during 2012 summer a spill of dissolved and solid compounds was observed into the water area in the proximity of the plant, to which the concomitant general deterioration of the marine environmental quality was consequently attributed (http://www.greenactiontransnational.org/index.php?option=com_content&view=article&id=292:ferriera-di-servola-i-problemi-della_conversione&catid=36:campagnainquinamento&Itemid=41). The most relevant source of PAHs could be ascribed to combustion products, as highlighted by the anthracene/anthracene + phenanthrene and fluoranthene /fluoranthene + pyrene ratio, which resulted always higher than 0.1 and 0.4–0.5, respectively (Yunker et al., 2002).

Comparing the observed pollutant concentrations with the Sediment Quality Guidelines for marine and estuarine sediments (MacDonalds et al., 1996; Table 4), the threshold effect level (TEL), which

defines the contaminant concentrations above which adverse biological effects could be occasionally associated, was always exceeded by Cu, Hg, Ni and Pb, and only occasionally by As (2010), Zn (2012), PAHs (2011) and PCB (2011, 2012). According to the Sediment Quality Guidelines classification proposed by US EPA (Pekey et al., 2004; Table 4), site S should be consequently regarded as heavily polluted, although occasionally, for As and Pb in the period 2011 – 2013 and for Zn in 2012.

In general, pollutant body burdens showed slight variations over time in mussels from the farming area, and only occasionally higher concentrations of metals and micro-organic pollutants were recorded in transplanted mussels. Although no correlation between contaminants in mussel tissues and sediments has been ever established for both metals and micro-organic pollutants from the contaminated site (Spearman's test never significant, data not reported), the increase of PAHs measured in the sediments from 2011 onward is reflected in the increased body burden observed in the related transplanted mussels. As for metals, it is not remarkable to find a mismatch, particularly when sediment metal contamination is neither high nor completely absent, because trace metals may not be wholly bioavailable (O' Connor et al., 1998; O'Connor and Paul, 2000) and due to metabolic regulation mechanisms involved in the uptake, physiological requirement (i.e. essential metals) and elimination of metals within certain concentrations (Rainbow, 2007).

Among lysosomal biomarkers, the stability of the lysosomal membranes, basically evaluated as variations in permeability, has been proposed as a highly sensitive, low-cost technique for the initial screening in biomonitoring studies to evaluate possible levels of stress syndrome experienced by sentinel organisms (Viarengo et al., 2007). Augmented permeability (or destabilization) of membranes generally precedes lysosomal enlargement provoked by the enhanced catabolic activity and this response has been related to the exposure to a variety of pollutants as well as to environmental stressors (Izagirre et al., 2008; Marigomez et al., 2005; Moore, 1988; Regoli, 1992). Viarengo et al. (2007) proposed three ranges for the labilization times that could be suggestive of different levels of cellular stress conditions in mussels: LP values above 20 minutes should indicate

a good status; LP values from 10 to 20 min and below 10 min should be indicative of stressed and severely stressed conditions, respectively. Mussels sampled in the farm before the transplantation (T0) displayed values above 20 min from the 2009 to the 2012-I surveys, for then decreasing during the 2012-II (9 min) and 2013-I (14 min) surveys. T1 mussels, i.e. samples relocated in the farm for each transplantation period, also exhibited LP values higher than 20 min, except in 2011, 2012 and 2013 warm seasons, when labilization times were below 10 min. The only exception was evidenced during the 2012-II, when even pre-transplanted organisms showed lowered values. A so low condition in animals from the farm could be ascribed to environmental factors such as high water temperature, which were effectively above 24°C in that period. Lysosomal membranes are less stable during the warm season due to the increased lysosomal enzyme activity and autophagy related to increased temperature (Tremblay et al., 1998). The negative effects of thermal stress on lysosomal membrane stability was already been observed in the Mediterranean mussel *M. galloprovincialis* collected from the eastern Adriatic coast by Petrovic et al. (2004). The influence of water temperature on labilization times is also supported by the trend observed in the samples transplanted to the harbour (site M). LP values were generally close to or below the threshold of 10 minutes, except in the first survey. However, during the same warm season surveys in 2011, 2012 and 2013 a marked decrease in LP values was observed, indicating possible additional stressful conditions due to the high temperatures. The low LP values of M samples indicate severely health stressed conditions of mussels through the whole study period and are in agreement with those observed in other harbours along the north-eastern Adriatic coast (Petrovic et al., 2004; Peric et al., 2012). In particular, the temporal trend observed in lysosomal membrane stability in the samples was correlated with most of the analysed PAH congeners in mussels tissues, as well as to total PAHs.

The worst stress condition of mussels from the harbour was also confirmed by lipofuscin accumulation, which were generally higher in these samples during the study period. Lipofuscins are insoluble granules mainly composed of oxidatively modified proteins and lipid degradation

products, along with carbohydrates and metals (Viarengo and Nott, 1993). In particular, lipofuscins may contribute to metal detoxification, trapping inorganic cations such as Cu, Zn, Fe, Mg and Mn (Viarengo and Nott, 1993). A positive correlation was established between lipofuscin and some metals, i.e. Cr, Cu and Fe, as well as with MPI, confirming the role of lipofuscin granules in metal homeostasis. During the 2010-I survey, a particularly higher lipofuscin content was recorded in both T1 and M samples. Although the significant difference between the two samples indicates only in the latter a contamination response, the strong induction observed in this period in comparison to the whole lipofuscin dataset should be related also to other endogenous or/and natural factors. Indeed, fluctuations in lipofuscin content have been ascribed to physiological processes related to spawning and gonad resorption during the reproductive cycle (Petrovic et al., 2004), as well as to the reduction of antioxidant defense system during the winter period (Viarengo et al., 1991).

The condition index values recorded until the 2011-I survey were always below 10, whereas from the II survey of the same year onwards differences were more marked. The condition index is considered an useful ecophysiological signal for the general health state of the organisms under given environmental conditions. Moreover, it has been used to follow seasonal changes in reserve content in bivalve aquaculture, both to designate the quality of marketed products and to characterize the apparent "health" of cultured stocks (Lucas and Beninger, 1985). The decrease in condition index during the summer period, as observed in this study in M mussels during the 2011-II, 2012-II and 2013-II surveys, has already been reported by other authors for the same species (Cravo et al., 2009; Peharda et al., 2007) and might be related to the synergic effect of higher water temperatures and contamination. Similar results were also observed in mortality rate trends, which were generally higher in M samples during the warmest season. These findings supports our hypothesis already discussed about the labilization times observed in T1 and M samples during these three surveys, confirming that summer represents the most stressful situation for these species.

5. Conclusions

The results obtained in the present study stressed the suitability of lysosomal biomarkers, as well as of the other supporting parameters, for monitoring long term pollution trends in coastal sites influenced by anthropogenic activities. The PCA performed with the whole biological data set clearly discriminated the site M over time with respect to the other samples. Lysosomal membrane stability trends were related to the presence of PAHs in the study marine environment, whereas accumulation of lipofuscins was more linked to the presence of trace metals. The results of this study also provide the evidence of the usefulness of the active biomonitoring approach in evaluating long-term pollution trends in marine sites of special interest in the framework of European Directives.

Acknowledgments

We wish to thank Dr Cinzia Comici for her contribution in the sampling activities and for physical data analyses.

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Table 1 – Metal concentrations (μg g^{-1} dw) in sediments from site S and related MPI.

	As	Cd	Cr	Cu	Hg	Ni	Pb	V	Zn	Al	Fe	MPI
2009	3.0	0.08	18.3	28.6	0.64	24.7	55.6	20.1	112	6906	13746	30.8
2010	7.4	0.14	29.3	22.8	0.73	21.0	39.9	32.4	87	8100	12290	35.7
2011	8.9	0.13	17.1	35.6	0.48	36.4	54.0	25.4	92	5020	10170	34.4
2012	13.6	0.31	29.9	32.9	0.51	33.2	104.5	29.5	189	9224	16599	51.1
2013	11.6	0.11	15.9	23.9	0.54	23.4	53.6	18.9	96	4395	10959	31.3

Table 2. Micro-organic pollutant concentrations (µg g^{-1} dw) in sediments from site S.

	ΣPAH	**ΣPCB**	**Phthalates**	**Di-(2-ethylhexyl) phthalate**	**Alkylphenols (di-isobutyl phenol)**	**4 (para)-Nonylphenol**	**Para-terz-octylphenol**
2009	0.15	0.006	0.18	<0.01	<0.01	0.07	0.05
2010	0.55	0.014	0.20	0.02	<0.01	0.06	0.04
2011	3.87	0.028	0.36	0.04	< 0.01	0.05	0.05
2012	4.67	0.030	0.41	0.08	< 0.01	0.03	0.07
2013	2.88	0.017	0.22	0.06	< 0.01	0.02	0.03

Table 3. Metal concentrations (µg g^{-1} dw) in mussel samples collected during the I and II surveys of each year, and related MPI.

		As	Cd	Cr	Cu	Hg	Ni	Pb	V	Zn	Al	Fe	MPI
2009 - I	T0	23.8	0.35	0.9	14.4	0.24	0.9	1.4	0.8	109	52	119	4.6
	T1	-	-	-	-	-	-	-	-	-	-	-	-
	M	23.1	0.41	0.6	6.4	0.07	0.9	0.6	1.7	92	111	167	4.0
2009 - II	T0	11.3	0.22	1.0	2.2	0.11	2.8	2.5	0.6	113	172	172	4.3
	T1	-	-	-	-	-	-	-	-	-	-	-	-
	M	10.8	0.25	1.1	4.6	0.06	4.0	2.7	1.0	129	209	185	4.9
2010 - I	T0	16.0	0.39	0.9	4.0	0.11	1.4	1.4	0.6	118	59	103	3.8
	T1	13.0	0.43	1.0	3.7	0.32	1.3	1.6	1.1	90	127	125	4.6
	M	15.0	0.44	3.3	8.1	0.30	3.2	5.3	3.4	121	1071	899	11.2
2010 - II	T0	12.7	0.17	1.1	3.4	0.11	1.2	0.8	1.0	107	129	97.11	3.5
	T1	5.2	0.33	0.5	2.4	0.08	0.9	1.5	2.8	67	36	43.54	2.7
	M	10.6	0.28	0.9	3.3	0.07	1.0	1.1	3.1	95	151	158.7	4.0
2011 - I	T0	5.2	0.33	0.5	2.4	0.08	0.9	1.5	2.8	67	36	43.54	2.7
	T1	16.9	0.70	1.5	3.1	0.34	3.5	1.7	1.1	102	248	159	6.2
	M	14.4	0.63	4.0	5.1	0.36	4.3	20.8	2.3	155	837	831	12.8
2011 - II	T0	11.5	0.41	2.3	4.3	0.39	1.6	1.7	1.9	93	171	299	6.1
	T1	17.3	0.46	0.3	1.2	0.64	< 0.1	1.6	0.5	116	13	54.8	2.9
	M	18.0	0.77	0.5	2.0	0.73	0.3	2.5	0.8	92	118	57.4	3.9
2012 - I	T0	20.7	0.35	0.2	2.8	< 0.5	1.2	0.7	1.7	30	29	78.6	3.2
	T1	17.6	0.39	0.6	3.0	< 0.5	1.7	1.1	1.2	< 1	80	149.5	3.2
	M	21.1	0.52	1.0	5.8	< 0.5	2.1	3.8	2.3	51	235	472.1	7.8
2012 - II	T0	18.0	0.57	2.0	4.2	< 0.5	< 1	1.7	0.8	100	97	159.6	5.6
	T1	19.2	0.60	0.7	3.4	< 0.5	< 1	1.1	1.6	132	60	99.2	4.9
	M	21.5	0.91	1.0	5.6	< 0.5	< 1	2.3	1.2	171	54	116.8	5.9
2013 - I	T0	18.0	1.00	0.9	0.5	1.03	0.9	1.5	0.9	221	63	175	4.7
	T1	16.4	0.70	1.0	<0.1	0.45	0.3	1.4	0.8	147	68	170	3.9
	M	18.2	0.80	1.7	2.6	0.28	1.7	4.2	2.0	212	229	542	7.8
2013 - II	T0	8.9	0.31	0.7	3.6	0.15	1.6	1.0	0.8	53	39	105	3.1
	T1	9.2	0.35	0.8	3.5	0.08	1.5	0.9	1.2	52.5	35.5	82.4	3.0
	M	11.1	0.43	0.9	4.1	0.11	1.6	1.9	1.3	92.1	54.4	152	4.1

Table 4. Organic pollutant concentrations (µg g^{-1} dw) in mussel samples collected during the I and II surveys of each year.

		ΣPAH	ΣPCB	Phthalates	Di-(2-ethylhexyl) phthalate	Alkylphenols (di-isobutyl phenol)	4 (para)-Nonylphenol	Para-terz-octylphenol
2009 - I	T0	0.30	0.010	<0.1	<0.01	4.69	<0.01	<0.01
	T1	-	-	-	-	-	-	-
	M	0.19	0.016	<0.1	<0.01	0.27	<0.01	<0.01
2009 - II	T0	0.21	0.010	<0.1	<0.01	0.22	<0.01	0.02
	T1	-	-	-	-	-	-	-
	M	0.24	0.012	<0.1	<0.01	0.3	<0.01	0.02
2010 - I	T0	0.26	0.010	<0.1	<0.01	0.3	<0.01	<0.01
	T1	0.18	0.010	<0.1	<0.01	0.24	<0.01	<0.01
	M	0.22	0.012	<0.1	<0.01	0.33	<0.01	<0.01
2010 - II	T0	0.15	0.011	<0.1	<0.01	0.31	<0.01	0.04
	T1	0.16	0.019	<0.1	<0.01	0.19	<0.01	0.02
	M	0.18	0.009	<0.1	0.02	0.06	0.01	0.01
2011 - I	T0	0.16	0.019	<0.1	<0.01	0.19	<0.01	0.02
	T1	0.22	0.015	< 0.1	< 0.01	0.11	< 0.01	< 0.01
	M	1.73	0.015	< 0.1	0.03	0.17	0.02	0.02
2011 - II	T0	0.54	0.013	< 0.1	0.01	0.08	< 0.01	< 0.01
	T1	1.54	0.011	< 0.1	0.03	0.07	0.02	< 0.01
	M	4.72	0.013	< 0.1	0.02	0.11	0.01	0.01
2012 - I	T0	0.23	0.016	< 0.1	< 0.01	0.07	< 0.01	< 0.01
	T1	0.48	0.016	< 0.1	0.02	0.05	< 0.01	0.02
	M	3.04	0.008	< 0.1	0.01	0.08	< 0.01	< 0.01
2012 - II	T0	0.14	0.011	< 0.1	< 0.01	0.03	< 0.01	< 0.01
	T1	0.12	0.010	< 0.1	< 0.01	0.04	< 0.01	< 0.01
	M	0.60	0.014	< 0.1	< 0.01	0.04	< 0.01	< 0.01
2013 - I	T0	0.11	0.008	< 0.01	< 0.01	0.04	< 0.01	< 0.01
	T1	0.14	0.008	< 0.01	< 0.01	0.03	< 0.01	< 0.01
	M	0.38	0.014	< 0.1	< 0.01	0.03	< 0.01	< 0.01
2013 - II	T0	0.14	0.008	< 0.1	< 0.01	0.02	< 0.01	< 0.01
	T1	0.24	0.014	< 0.1	< 0.01	0.04	< 0.01	0.01
	M	0.57	0.015	< 0.1	0.01	0.03	< 0.01	< 0.01

Table 5. Statistical comparison between mussel samples (T0: pre-transplantation; T1: relocated at the farming; M: transplanted at the study site) in the various surveys. LMS and lipofuscin: Kruskal-Wallis; mortality rate: G-test; CI: ANOVA test. * $p<0.05$; ** $p<0.001$; *** $p<0.001$.

	2009		**2010**		**2011**		**2012**		**2013**	
	I	II	I	II	I	II	I	II	I	II
LMS										
T0 vs T1	n.s.	-	n.s.	n.s.	n.s.	***	n.s.	*	*	-
T0 vs M	n.s.	-	*	**	***	***	**	***	n.s.	-
T1 vs M	n.s.	**	*	*	***	*	**	n.s.	**	*
Lipofuscin										
T0 vs T1	n.s.	*	**	n.s.	**	**	n.s.	*	*	n.s.
T0 vs M	**	n.s.	**	n.s.	n.s.	n.s.	*	n.s.	***	**
T1 vs M	*	**	*	n.s.	**	*	*	n.s.	*	*
Mortality rate										
T0 vs T1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	n.s.
T0 vs M	***	**	**	*	*	***	***	***	n.s.	***
T1 vs M	***	**	**	*	n.s.	***	n.s.	***	n.s.	***
CI										
T0 vs T1	-	-	n.s.	n.s.	n.s.	n.s.	***	*	*	n.s.
T0 vs M	-	-	n.s.	n.s.	n.s.	**	**	***	***	***
T1 vs M	-	-	*	n.s.	n.s.	***	***	*	*	***

Table 6. Spearman's correlation coefficients R between biomarkers and metals and PCB concentrations in mussel tissues and related significance (*p < 0.05; **p < 0.01).

	Al	As	Cd	Cr	Fe	Hg	Ni	Pb	Cu	V	Zn	MPI	ΣPCB
LMS	0.03	-0.16	-0.44*	0.02	-0.06	-0.09	-0.21	-0.47*	-0.05	0.07	-0.29	-0.27	0.13
Lipofuscin	0.35	0.07	0.22	0.41*	0.50**	-0.29	0.23	0.31	0.49**	0.33	0.17	0.44*	-0.14
Mortality	0.54**	0.22	0.35	0.37	0.55**	-0.01	0.30	0.55**	0.31	0.19	0.31	0.50**	0.18
CI	-0.09	0.05	-0.31	-0.14	-0.02	-0.08	0.19	-0.18	0.13	-0.14	-0.43*	-0.16	-0.27

Table 7. Spearman's correlation coefficients R between biomarkers and PAH concentrations in mussel tissues (acenaphthene, acenaphthylene, anthracene, benzo(a)antracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(ghi)perylene, benzo(k)fluoranthene, chrysene, phenanthrene, fluoranthene, fluorene, indeno(1,2,3-cd)pyrene, naphthalene and pyrene) in mussel tissues and related significance (*p < 0.05; **p < 0.01; *** p<0.001).

	Anth	Antl	Atrh	Bz(a)antr	B(a)p	Bz(b)fla	Bz (g,h,i)pyl	Bz(k)fla	Cryh	Phn	Flah	Flrh	Idn(123cd)pyr	Npth	Pyr	**ΣPAH**
LMS	0.07	-0.5**	-0.38	-0.50**	-0.29	-0.60**	-0.68 ***	-0.52**	-0.27	-0.22	-0.16	-0.39*	-0.40*	-0.45*	-0.47*	-0.40*
Lipofuscin	-0.30	-0.01	0.06	-0.05	-0.23	0.10	0.17	-0.06	-0.20	-0.05	-0.26	0.08	-0.25	-0.05	0.15	-0.08
Mortality	-0.21	0.25	0.43*	0.24	0.23	0.33	0.36	0.11	0.20	0.42*	0.32	0.48*	0.04	0.37	0.43*	0.42*
CI	0.37	0.33	0.35	0.07	0.09	0.16	0.10	0.20	0.06	0.17	0.23	0.24	0.001	0.24	0.20	0.20

Table 8. Sediment Quality Guidelines (SQG) for marine sediments. All values are expressed in µg g^{-1}.

	MacDonald et al., 1996		**US EPA**		
	TEL	PEL	Non-polluted	Moderately polluted	Heavily polluted
As	7.24	41.6	<3	3-8	>8
Cd	0.68	4.21	-	-	>6
Cr	52.3	160	<25	25-75	>75
Cu	18.7	108	<25	25-50	>50
Hg	0.13	0.7	-	-	-
Ni	15.9	42.8	-	-	-
Pb	30.2	112	<40	40-60	>60
Zn	124	271	<90	90-200	>200
Fe	-	-	<17000	17000-25000	>25000
ΣPAH	1.684	1.677	-	-	-
ΣPCB	0.0216	0.189	-	-	-

Figure captions

Fig. 1. Study area and location of sampling sites for sediment (S) and mussel transplantation (M) (from Solis-Weiss et al. 2004, modified).

Fig. 2. Temperature and salinity recorded in the sampling surveys in the site S.

Fig. 3. PCA performed with contaminants detected in sediments.

Fig. 4. Lysosomal membrane stability (min) in mussel *M. galloprovincialis* collected at the marine farm (T0 and T1) and transplanted at the study site (M) in the first (I, in black) and second (II, in grey) surveys of each year. Mean ± s.e.; N=10.

Fig. 5. Lipofuscin content (volume density, $\mu m^3/\mu m^3$) in mussel *M. galloprovincialis* collected at the marine farm (T0 and T1) and transplanted at the study site (M) in the first (I, in black) and second (II, in grey) surveys of each year. Mean ± s.e.; N=5.

Fig. 6. Condition index in mussel *M. galloprovincialis* collected at the marine farm (T0 and T1) and transplanted at the study site (M) in the first (I, in black) and second (II, in grey) surveys of each year. Mean ± s.e.; N=20.

Fig. 7. Mortality rate (%) in mussel *M. galloprovincialis* collected at the marine farm (T0 and T1) and transplanted at the study site (M) in the first (I, in black) and second (II, in grey) surveys of each year.

Fig. 8. PCA performed with biological responses detected in *M. galloprovincialis*.

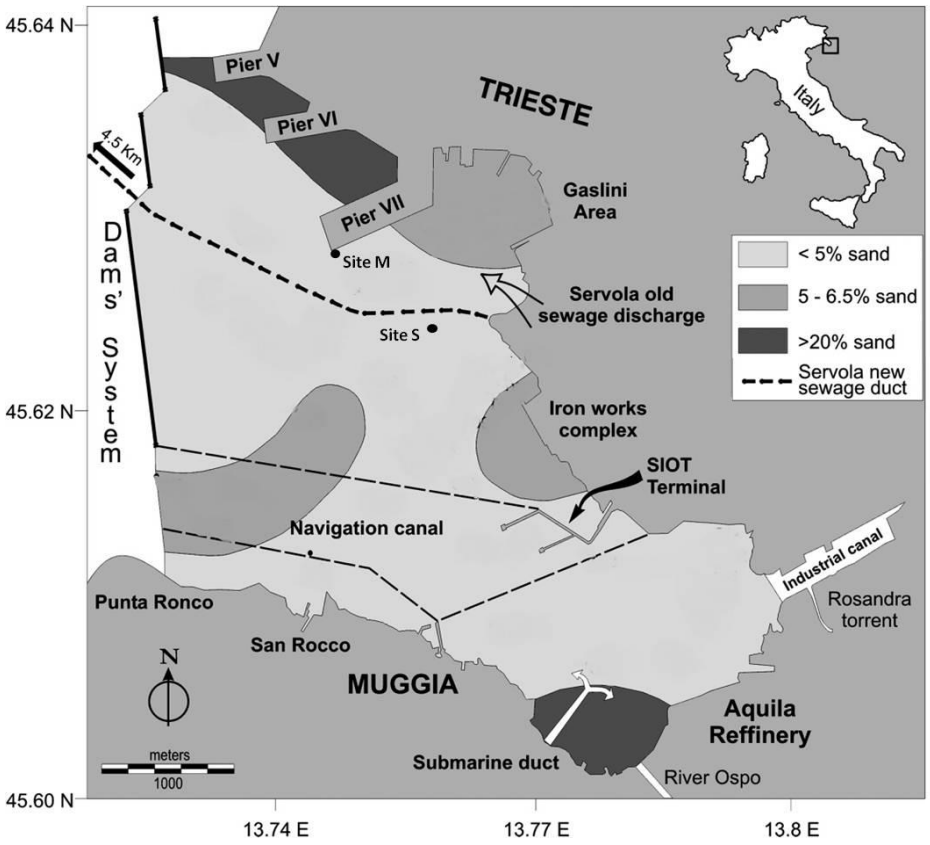

45.64 N
45.62 N
45.60 N
13.74 E
13.77 E
13.8 E
Pier V
Pier VI
Pier VII
TRIESTE
Italy
4.5 Km
Dam's System
Gaslini Area
Site M
Site S
Servola old sewage discharge
< 5% sand
5 - 6.5% sand
>20% sand
Servola new sewage duct
Iron works complex
SIOT Terminal
Navigation canal
Industrial canal
Rosandra torrent
Punta Ronco
San Rocco
MUGGIA
N
meters
1000
Aquila Reffinery
Submarine duct
River Ospo

Figure 1

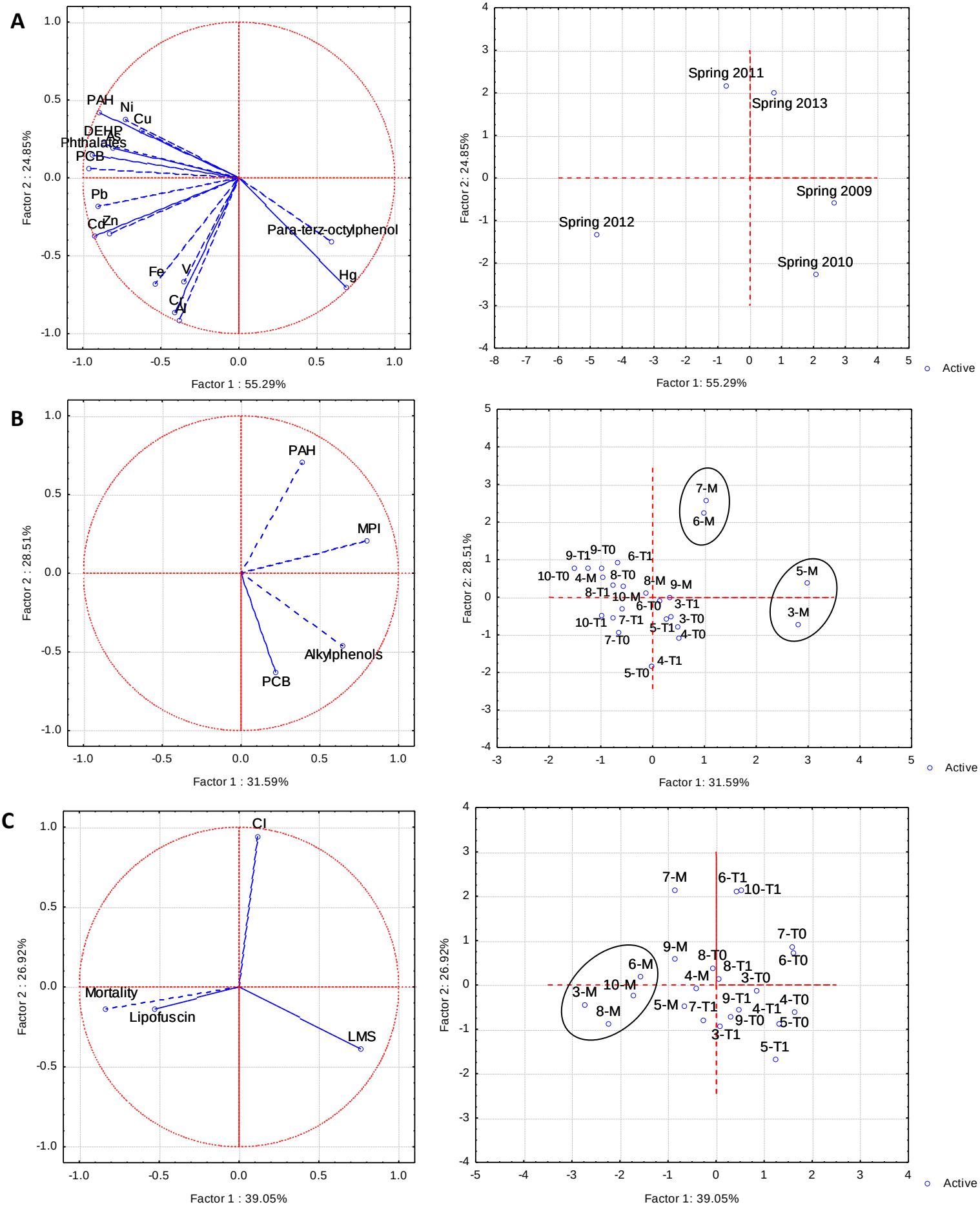
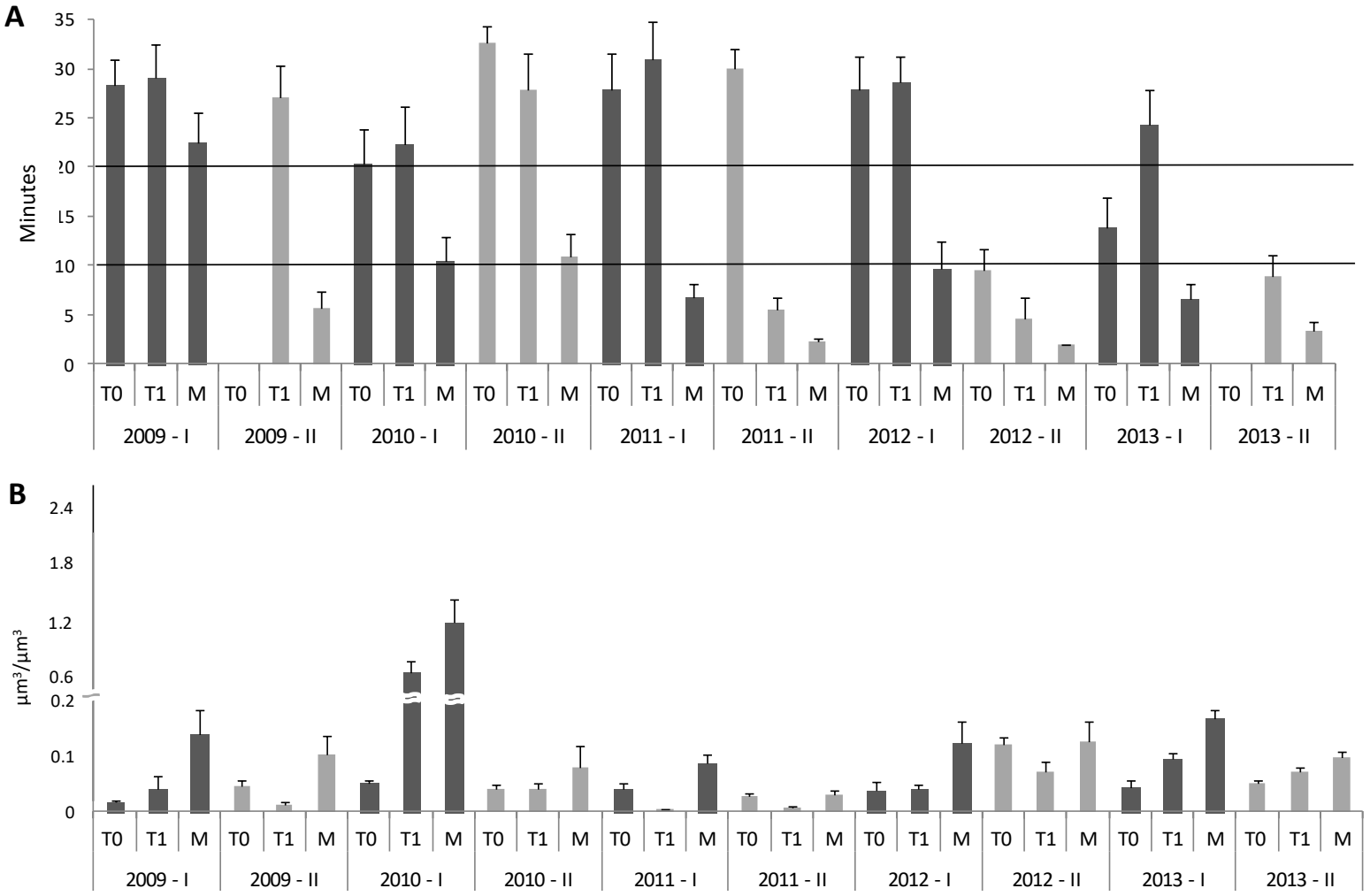
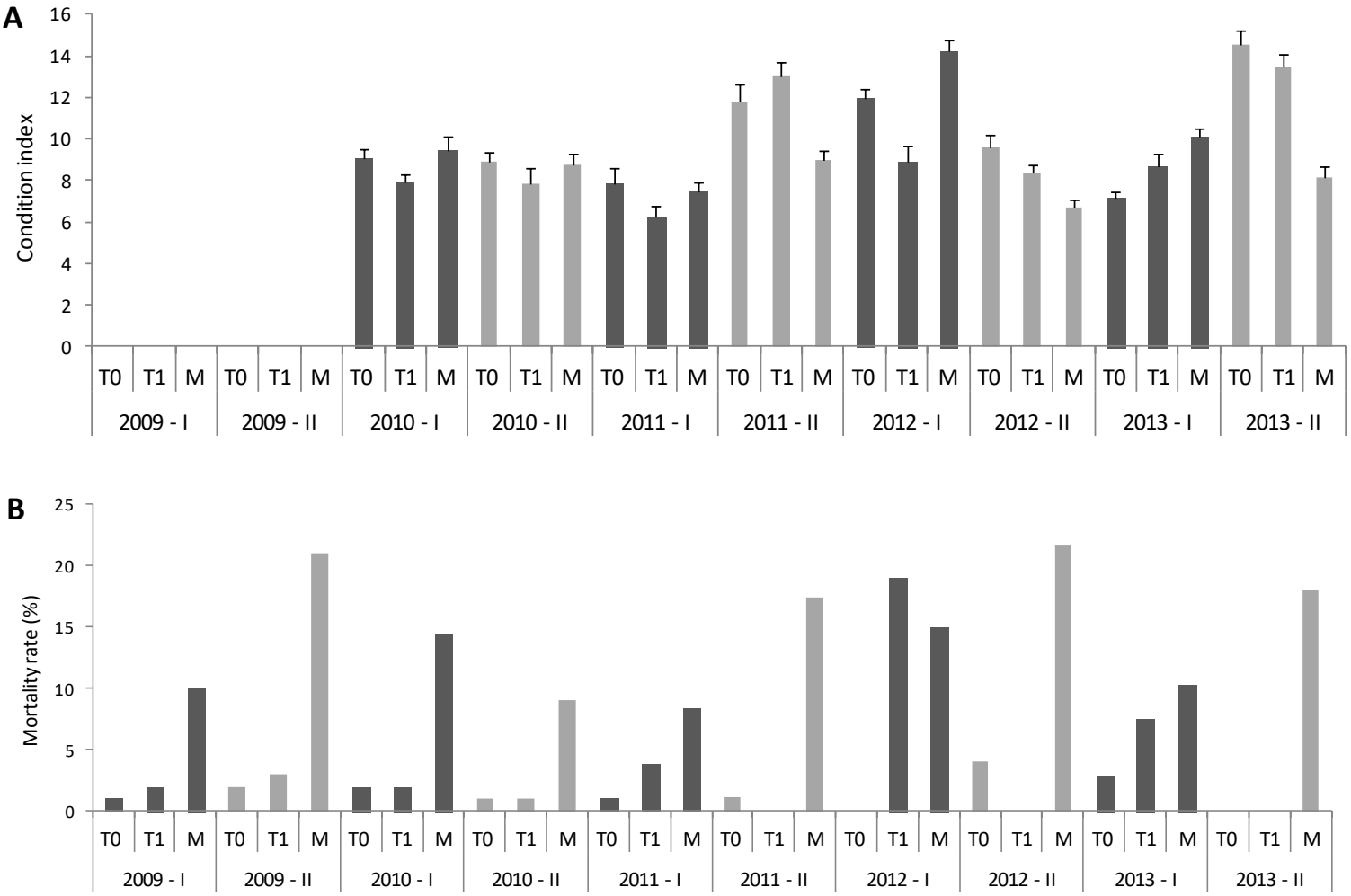

A
PAH
Ni
Cu
DEHP
As
Phthalates
PCB
Pb
Zn
Cd
Para-terz-octylphenol
Fe
V
Hg
Cr
Al
Factor 2 : 24.85%
Factor 1 : 55.29%
Spring 2011
Spring 2013
Spring 2009
Spring 2012
Spring 2010
Factor 2: 24.85%
Factor 1: 55.29%
Active
B
PAH
MPI
Alkylphenols
PCB
Factor 2 : 28.51%
Factor 1 : 31.59%
7-M
6-M
9-T1
9-T0
6-T1
10-T0
4-M
8-T0
8-M
9-M
8-T1
10-M
6-T0
3-T1
10-T1
7-T1
3-T0
5-T1
4-T0
7-T0
4-T1
5-T0
5-M
3-M
Factor 2: 28.51%
Factor 1: 31.59%
Active
C
CI
Mortality
Lipofuscin
LMS
Factor 2 : 26.92%
Factor 1 : 39.05%
7-M
6-T1
10-T1
7-T0
9-M
8-T0
6-M
8-T1
6-T0
4-M
3-T0
3-M
10-M
9-T1
4-T0
5-M
7-T1
4-T1
8-M
9-T0
5-T0
3-T1
5-T1
Factor 2: 26.92%
Factor 1: 39.05%
Active

Figure 2


A
Minutes
35
30
25
20
15
10
5
0
T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M
2009 - I
2009 - II
2010 - I
2010 - II
2011 - I
2011 - II
2012 - I
2012 - II
2013 - I
2013 - II

B
µm³/µm³
2.4
1.8
1.2
0.6
0.2
0.1
0
T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M
2009 - I
2009 - II
2010 - I
2010 - II
2011 - I
2011 - II
2012 - I
2012 - II
2013 - I
2013 - II

Figure 3


A
Condition index
16
14
12
10
8
6
4
2
0
T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M
2009 - I
2009 - II
2010 - I
2010 - II
2011 - I
2011 - II
2012 - I
2012 - II
2013 - I
2013 - II

B
Mortality rate (%)
25
20
15
10
5
0
T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M T0 T1 M
2009 - I
2009 - II
2010 - I
2010 - II
2011 - I
2011 - II
2012 - I
2012 - II
2013 - I
2013 - II

Figure 4