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Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring

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Marine Pollution Bulletin

Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring --Manuscript Draft--

Manuscript Number:	MPB-D-20-01666R2
Article Type:	Research Paper
Keywords:	Coastal lagoon; Sediment geochemistry; Metal bioaccumulation; biomarkers; Biomonitoring.
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Abstract:	Coastal lagoons are complex environments threatened by natural and anthropogenic stressors. Here, we tested the effectiveness of combining physical, geochemical and chemical measurements with biomarker data obtained in field-exposed marine mussels (<i>Mytilus galloprovincialis</i>) as a biomonitoring strategy for a highly pressured lagoon (Pialassa Baiona, Ravenna, Italy). Data showed a spatial trend of sediment contamination by Hg, Pt, Au, Ag, Mo, Re, Cd, Pd and Zn. Local conditions of high water temperature/low conductivity were detected among selected sites. After a 30-day in situ exposure, Ag and Hg were the most bioaccumulated elements (10 and 5 folds, respectively) in mussels followed by Sb, Al, Ti and Fe. Decreased survival, lysosomal dysfunctions, increased metallothionein content and peroxisome proliferation were observed in mussels in relation to metal spatial distribution and physicochemical fluctuations. Overall, this study provides a further confirmation of the role of biomonitoring to reliably assess the environmental quality of highly pressured lagoons.

Greggio et al. Manuscript Number: **MPB-D-20-01666R1**

Dear Editor

Marine Pollution Bulletin

Dec 28th, 2020

Please find enclosed the revised manuscript authored by Greggio, Capolupo, Donnini, Birke, Fabbri and Dinelli entitled: *Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring*

We are grateful for the confirmed overall appreciation given to our work, and for the pertinent comments we received to improve the manuscript quality.

Almost all suggestions have been accepted and very clear explanations have been provided where necessary. Justification to the use of only inorganic pollutants has been included. Detailed argumentation on the organic pollutants has been provided in order to clarify their role in the work. We do hope more clarity and better focus have been reached.

Specific responses to the Reviewers' comments are provided in a separate file and changes have been highlighted on a second copy of the manuscript for easy of reading.

We do hope the revised manuscript meets the Reviewers' requests and may be considered suitable for publication on Marine Pollution Bulletin

Best Regards,

Your Sincerely

Corresponding Author
Nicolas Greggio

A handwritten signature in black ink, appearing to read 'Nicolas Greggio', with a stylized flourish at the end.

Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring

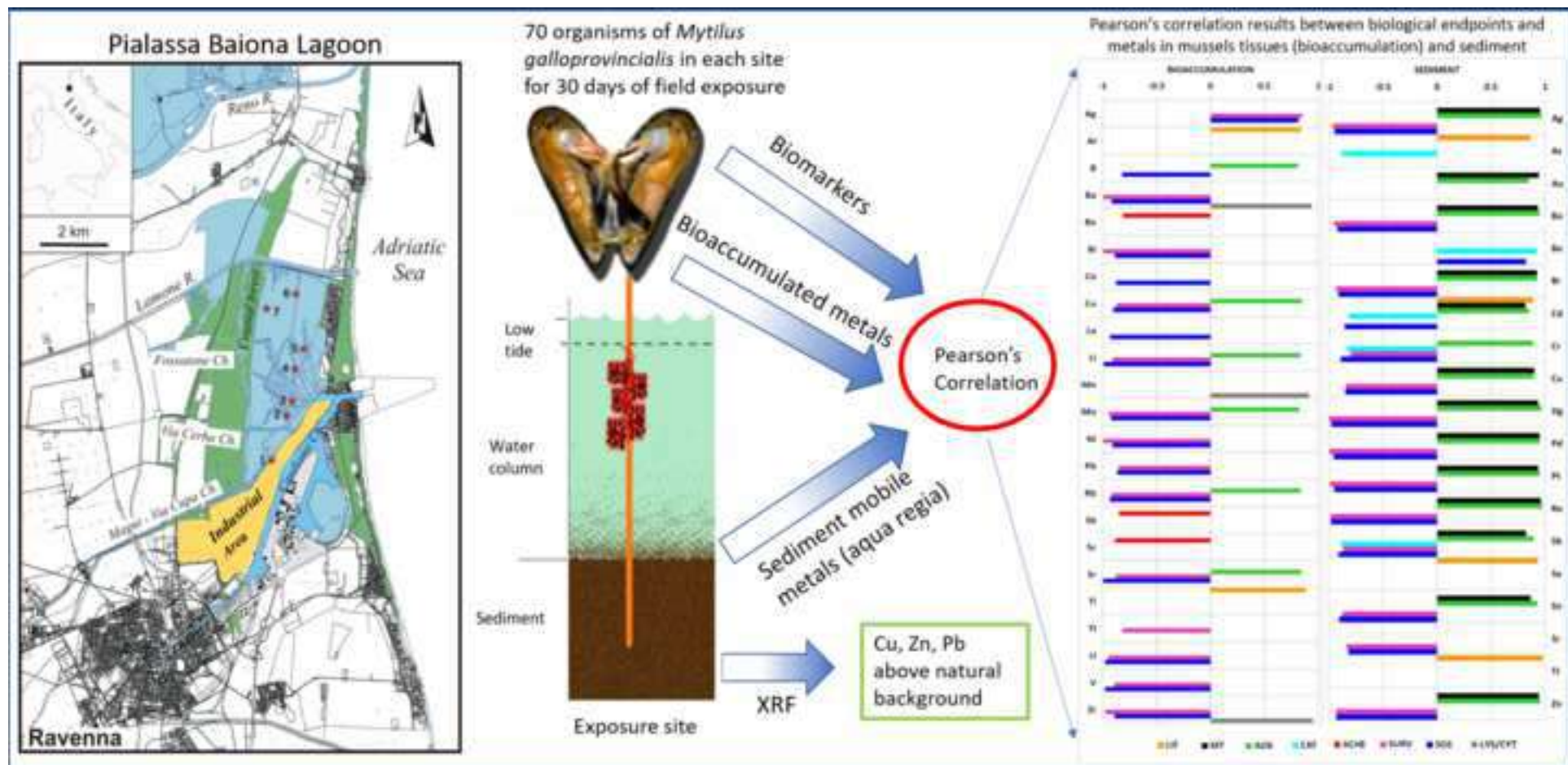
Authors:

Greggio Nicolas, Capolupo Marco, Filippo Donnini, Manfred Birke, Fabbri Elena, Dinelli Enrico

REVISION 2 - RESPONSE TO REVIEWERS

COMMENT	RESPONSE	POSITION
Reviewer #1		
<p>Reviewer #1: The manuscript reports an interesting study on the application of an integrated approach (geochemical and chemical measurements with biomarker data obtained in field-exposed marine mussels) for the evaluation of the environmental quality of a lagoon and despite the necessary amount of information to be reported, the authors have made an great effort to condense all the information.</p> <p>I noted that some recommendations of the other referees were accepted, improving the manuscript. The strong points of this article are: i) the strategy based on the integration of the water physico-chemical parameters and the sediment geochemical data with the measurements of the metal bioaccumulation and biomarkers in mussels. ii) the description and discussion of the results of the various lines of evidence carried out iii) the choice of to determine elements seldom analysed in sediments for environmental aims, and well described in the discussion also linking them with the possible sources of contamination.</p> <p>However, there are some aspects that are missing or need to be more detailed that I would like to highlight</p>	<p>Thanks for the appreciation</p>	
<p>The study is focused on the determination of only metals in sediments and their bioaccumulation in mussels, but in my opinion the reason for this choice should be well explained in the manuscript, having described the lagoon as an environment contaminated by both metals and organic pollutants , and therefore also the latter can contribute to the biological effects on mussels.</p>	<p>A part has been added in the introduction chapter in order to justify the choice of working only with inorganic pollutants. In fact, most studies in the area involved organic pollutants and no complete information exist on inorganic pollutants in sediments and in mussels. Moreover, few information exists on the integration of a complete inorganic pollutants suite and biomarkers .</p>	<p>Introduction chapter Lines 86-101</p>
<p>With regard to organic contaminants, in the final part of the discussion when the overall health status of the mussels of each station is evaluated, the presence of organic xenobiotics (page 23) plays a decisive role in some stations, even if haven't been reported the type of contaminants nor their concentrations. The role of organic contaminants seems a bit weak in this discussion because it relates to trends in measured AOX (Acyl-Co A oxidase), but positive correlated also with metals, and to data on PCB and PAH concentrations measured in previous studies, not reporting however when they were conducted and citing references of some years ago. It is difficult to correlate biomarker outcomes with contaminant concentrations in sediments of</p>	<p>The presence of organic contaminants was not evaluated in this study; we apologize if we did not correctly explain this; perhaps, we did not clarify the principle of the data integration system used to assess the overall mussel health status. The aim of this study was to evaluate the validity of combining geochemical data (i.e. metal concentrations in sediments), metal bioaccumulation and biomarker responses as a suitable tool for coastal lagoon biomonitoring; consequently, the assessment of other organics was not included in the analytical design. However, since we analyzed a wide battery of biomarkers validated in mussels, and since some of these parameters (i.e. AOX) is activated prevalently by POPs exposure, the data integration through the Mussel Expert System (MES) identified the onset of stressful conditions associated to the possible presence of POPs, notably in the sites where mussels showed increase AOX. For these reasons, the plot reporting the MES output (Fig.6) presents symbols corresponding to the "presence of POPs" as suggested by the</p>	<p>Page 10, section 2.6.5., Lines 276-285 and pages 24-25, Lines 604 – 610</p>

which it is not known when and where they were sampled and analyzed.	Expert System output. Moreover, we did not perform any statistical correlation analyses with data from previous studies, but rather pointed out that the stress level identified by the MES in relation to the presence of POPs is coherent with previous reports of PAHs across the lagoon. In line with the Reviewer's comments, we added clarifications about these points in the Materials and Method section (Page 10, section 2.6.5., L276-285) and in Results & Discussion (MES paragraph, page 24-25, L 604 – 610).	
For these reasons, if it is considered important to introduce the role of organic contaminants in the overall assessment then it should review all discussion on a stronger basis and be reported i.e when the sampling was performed, information not present in the materials and methods. This information is not important in the discussion of the data relating to metals since all the measurements were performed on the same samples and in the same period.	Based on the considerations reported in the previous comment, we could not add any method or data on organics, since they were not analysed nor this was included as an objective of this study. However, we acknowledge that POPs, such as PAHs, have previously been reported in the lagoon and that, apart from AOX, some biomarkers of general stress (i.e. LMS, NL and/or LYS/CYT) are known to be modulated in mussels by pollutants of diverse nature, including organic and inorganic compounds, as well as their co-exposure (e.g. Capolupo et al., 2017, in bibliography). Hence, to meet the Reviewer's arguments, we added notions in the Results & Discussion section to address i) the potential relationships between chemical data from this study and those previously reported for other (organic) pollutants y (page 14, L382 – 390) and ii) the possible cumulative effects of metals and organics on the magnitude of biological effects observed in transplanted organisms (page 21, L 534 – 538)	page 14, Lines 382 – 390 and page 21, Lines 534 – 538
Another gap, in my opinion, is not having considered the grain size analysis of the sediments, even if most likely the lagoon sediments collected in the different stations are homogeneous, this information could be important considering how the concentrations of metals are related to the silt/clay fraction.	Authors agree with reviewer and a section has been added at the beginning of the sediment data section. Sediment grain size has been estimated through the major elements geochemical composition as established by Dinelli et al. (2007)	Section 3.2 Page 11 Lines 319- 326
line 30 page 11 replace S3 and S5 at sites 3 and 5	Modified	



Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring

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Highlights

- Total Zn, Cu and Pb in sediments exceed the natural background concentrations;
- Sediments are enriched in mobile Hg, Pt, Au and Ag, with a strong S-N gradient;
- Exposed mussels bioaccumulated Ag (x10) and Hg (x5) compare to reference;
- Sediment concentration of Ag, Au, Ba, Bi, Sb affect mussel physiological parameters;
- Geochemical and physiological endpoints proved suitable for lagoon biomonitoring;

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Abstract:

Coastal lagoons are complex environments threatened by natural and anthropogenic stressors. Here, we tested the effectiveness of combining physical, geochemical and chemical measurements with biomarker data obtained in field-exposed marine mussels (*Mytilus galloprovincialis*) as a biomonitoring strategy for a highly pressured lagoon (Pialassa Baiona, Ravenna, Italy). Data showed a spatial trend of sediment contamination by Hg, Pt, Au, Ag, Mo, Re, Cd, Pd and Zn. Local conditions of high water temperature/low conductivity were detected among selected sites. After a 30-day in situ exposure, Ag and Hg were the most bioaccumulated elements (10 and 5 folds, respectively) in mussels followed by Sb, Al, Ti and Fe. Decreased survival, lysosomal dysfunctions, increased metallothionein content and peroxisome proliferation were observed in mussels in relation to metal spatial distribution and physico-chemical fluctuations. Overall, this study provides a further confirmation of the role of biomonitoring to reliably assess the environmental quality of highly pressured lagoons.

Keywords: Coastal lagoon; Sediment geochemistry; Metal bioaccumulation; Biomarkers; Biomonitoring;

Funding sources

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1 Introduction

Being at the boundary between terrestrial and marine ecosystems coastal lagoons are, by definition, unstable and complex systems morphologically dependent and shaped by natural continental evolution (rivers sediment transport and discharge), sea level variations, coastal dynamics, storminess and local geologic processes (e.g. subsidence) (Bird, 1994; Newton et al., 2014). In natural conditions, coastal lagoons receive fresh and nutrient-rich inputs from the land-side, that combined with shallow, warm and relatively stable water, sustain a complex food chain (from algae, to macro benthos, fish and swamps birds), providing a multitude of life-supporting (Aliaume et al., 2007; Dominik et al., 2014; Petry et al., 2016).

The naturally complicated equilibrium of these environments has often been impacted by strong anthropic development in the last century, especially in those lagoons close to major cities and harbours. With the beginning of demographic boom and of the correlated industrial development, lagoons have been converted into commercial ports or industrial areas and in the worst case as processing water disposal sites (Guerra, 2012; Guerra et al., 2014).

Water and sediment geochemistry of lagoon environments have been widely explored in order to define water status (Mollema et al., 2013; Greggio et al., 2020), sediment provenance and local background concentrations (Migani et al., 2015; Borghesi et al., 2016; Greggio et al., 2018a) or levels of occurring pollutants, notably heavy metals (Nazneen et al., 2019; Saidi et al., 2019), organic compounds (León et al., 2017; Mattes et al., 2018) and emerging contaminants (Pignotti et al., 2017; Pignotti and Dinelli, 2018).

The geochemical characterization of sediments and water is important, but alone often does not allow for an accurate and reliable evaluation of the environmental status of lagoon ecosystems (Carvalho et al., 2014; Dahms, 2014; Moreno-González et al., 2015). Physico-chemical properties and geological background of lagoons can influence the response of exposed organisms to pollutants, inducing potential interactive effects due to changes in bioavailability and toxicity (Migani et al., 2015; Piggott et al., 2015; Borghesi et al., 2016). Chemical assessments fail in detecting biotransformation products, eventually more toxic than the parental compounds (Buryskova et al., 2006; Braga et al., 2018), and in estimating synergistic/antagonistic effects induced on the fitness of the exposed biota (Lari et al., 2017). For these considerations, multidisciplinary applications integrating geological/chemical parameters, pollutants bioaccumulation and biological effects may represent a promising approach to assess the environmental status of highly pressured coastal systems.

In the framework of biomonitoring programs of marine areas, particular attention has recently been addressed to the assessment of biomarkers of environmental stress (Turja et al., 2014; Capolupo et al., 2017; Mansour et al., 2020). Biomarkers are defined as indices of either exposure or effect of pollutants induced at biochemical, cellular and physiological level on exposed organisms (Viarengo et al., 2007a). They have successfully been used in association to bioaccumulation analyses to characterize “sub-organism” toxicological dynamics occurring in highly contaminated environments and provide crucial and early-warning information for planning urgent intervention or following the effects of remediation plans (Donnini et al., 2007; Capolupo et al., 2017). The simultaneous analysis of a wide set of biomarkers offers accurate information on the sub-lethal alterations induced by multiple stressors and may allow to correlate observed effects with specific classes of pollutants (Shaw et al., 2011; Turja et al., 2014). Moreover, biomarkers provide a unique contribution to determine the synergistic effect of pollutant mixtures, even when single contaminants occur at low concentrations. Biomarker-based assessments are thus currently promoted under the current EU legislation for monitoring surveys of marine and transitional environments (2000/60/EC; 2008/56/EC; Piló et al., 2017).

The aim of this work was to evaluate the effectiveness of integrating water physico-chemical data, sediment geochemistry, metal bioaccumulation and biomarker measurements as a biomonitoring strategy for highly contaminated coastal lagoons. The study was conducted in a shallow and brackish basin located along the North-Western coast of the Adriatic Sea (Pialassa Baiona, Ravenna, Italy). In this area, previous works highlighted the presence of organic pollutants in sediments (Trombini et al., 2003; Vassura et al., 2005; Fabbri et al., 2006; Guerra, 2012; Guerra et al., 2014;) and their potential for being bioaccumulated by mussels (Fabbri et al., 2006) and clams (Vassura et al., 2005). Inorganic pollutants were less investigated and the current information is limited to few elements, as Cd, Cu, Hg, Ni, Pb and Zn (Trombini et al., 2003; Matteucci et al., 2005; Guerra et al., 2014). A recent hydrogeochemical study reported water physico-chemical alterations and the abundance of seldom analysed metals (Ag and Sb among others) in surface drainage water flowing into the investigated lagoon (Greggio et al., 2020). Therefore, a wide spectrum of elements was measured in sediments collected from different sites within the lagoon and in adult specimens of the Mediterranean mussel *Mytilus galloprovincialis* transplanted *in situ* at the same locations. Finally, a battery of biological endpoints was measured in mussels, including lysosomal responses, oxidative stress parameters and biomarkers of exposure to specific classes of pollutants.

2 Materials and methods

2.1 Study area and sampling sites

The Pialassa Baiona (Fig. 1) is a brackish lagoon located northward the town of Ravenna (Italy), between 44°27'55.5"N and 44°31'31.3"N and 12°14'9.7"E and 12°16'10"E that cover an area of about 10 km². The lagoon is part of a barrier-lagoon system that characterized the historical evolution of the Adriatic Sea coastline in the area (Amorosi et al., 1999). It is formed by small, shallow ponds (0.5–1 m depth) and deeper artificial channels (2–3 m depth) that permit the incessant water exchange with the Adriatic Sea driven by tides. It is connected to the Adriatic Sea only through the Candiano Channel that also is the Ravenna operative industrial harbour. The Pialassa Baiona is mainly adopted by local citizens as recreational fishing and hunting site and few clam farming activities exist in the northernmost portion.

The lagoon receives freshwaters from the west through three main channels (Fossatone, Cerba and Cupa-Magni channels) which drain agricultural lands and collect effluents from industrial and municipal treatment plants, located in the southernmost part (Fig. 1). The drainage water entering the lagoon are often rich in nutrients and dissolved metals (Mollema et al., 2013; Greggio et al., 2018b). The highest concentration of nutrients, as well as the highest bacteriological content, are observed in the southern part of the lagoon, in proximity of the via Cupa channel (Soprani et al., 1994). In addition, during the 60s and 70s, high quantities of chemical compounds were discharged into the lagoon through the via Cupa and Magni channels, carrying wastewater from the industrial area close to the southern border (Fabbri et al., 1998).

Previous investigations showed that high levels of metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other pollutants occurred in surface, subsurface and suspended sediments with N-S increasing gradient (Trombini et al., 2003; Matteucci et al., 2005; Guerra, 2012; Guerra et al., 2014). On these bases, seven sites were selected within the Pialassa Baiona lagoon in order to cover its overall geochemical complexity and spatial trend of anthropogenic pressures (Fig. 1). All sites (S1 to S7) were investigated for sediment geochemistry, while metal bioaccumulation and biomarker responses were measured on mussels transplanted into six sites (S1, S2, S3, S5, S6 and S7), since *in situ* transplantation into site S4 was not possible for technical reasons. Mussels were exposed into the lagoon for 30 days, which is acknowledged as a suitable time for sessile organisms to bioaccumulate contaminants at environmentally representative levels and to develop physiological responses to “in site” pollution (Viarengo et al., 2007b). Since previous investigations highlighted a lesser incidence of metals and organic

compounds in the north-western part of the lagoon (Trombini et al., 2003; Fabbri et al., 2006; Donnini et al., 2007; Franzellitti et al., 2010), Site 7 (S7), located at about 0.5 km from the north-western border, was defined as internal control site for both geochemical and biological assessments.

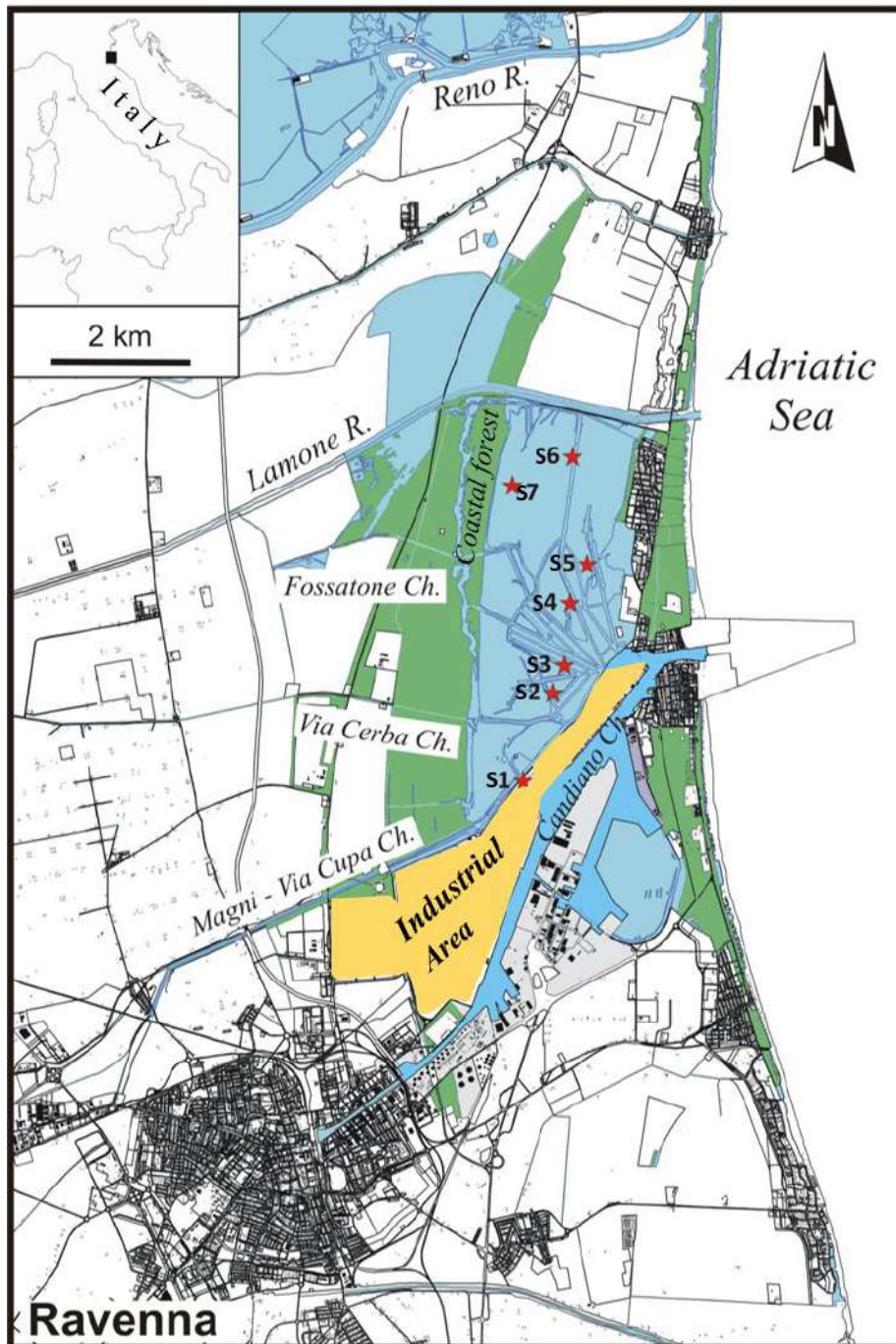


Figure 1. Location map and sampling points (S1 to S7) within the Pialassa Baiona lagoon (Ravenna, Italy). The abbreviations "R." and "Ch." refer to the main rivers and/or channels which supply freshwater to the lagoon. The yellow shape represents the industrial area settled at the south-eastern boundary of the Pialassa Baiona lagoon.

2.2 *Field activities and measurements*

In order to have a better characterization of the environmental conditions influencing the organisms' response, measurements for physico-chemical parameters were conducted at each site. During mussel field exposure (from June 1st to June 30th) six vertical profiles (10 cm resolution) of electrical conductivity, temperature and pH, using a CTD probe were conducted.

2.3 *Sediment geochemistry*

The bottom sediments were sampled at each site with a manual corer using a Plexiglas tube. After the extrusion, the sections 0 - 5 cm was retained for analysis, placed in polyethylene bottles, were stored at -25 °C. For geochemical analysis, sediment aliquots were dried at 60 °C and homogenized by grinding in agate mortar. On the bottom sediments total elements concentration by X-ray fluorescence spectrometry (XRF) and pseudo-total (mobile/available) concentrations by aqua regia digestion (AR) have been performed.

The total metal content of 29 chemical elements (SiO₂, TiO₂, Al₂O₃, Fe₂O₃, MnO, MgO, CaO, Na₂O, K₂O, P₂O₅, S, As, Ba, Ce, Co, Cr, Cu, La, Nb, Ni, Pb, Rb, Sc, Sr, Th, V, Y, Zn, Zr) has been measured by XRF, using a Philips PW 1480 spectrometer equipped with a Rh tube on pressed powder pellets following matrix correction methods suggested by Franzini et al. (1972; 1975) Leoni and Saitta (1976) and Leoni et al. (1982). Thirty international reference materials were used for the instrument calibration, while accuracy was evaluated using 4 international reference samples, namely BR, BCR-1, TB and AGV-1 (Govindaraju, 1984). The average accuracy was higher than 5 % for trace-element determinations. In order to quantify the pseudo total concentration, 15 g of sediment were digested with a modified aqua regia solution of equal parts concentrated HCl, HNO₃ and deionized H₂O for one hour in a heating block of hot water bath at the Bureau Veritas Laboratories in Vancouver (Canada). Fifty-three chemical elements (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hf, Hg, In, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pd, Pt, Rb, Re, S, Sb, Sc, Se, Sn, Sr, Ta, Te, Th, Ti, Tl, U, V, W, Y, Zn, Zr) were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

The volatile content (including humidity, organic matter, water incorporated in the lattice of clay minerals, carbon dioxide in the carbonate minerals) was evaluated by thermal analysis using a Setaram TAG24 double furnace apparatus, with simultaneous registration of thermogravimetric (TG), derivative thermogravimetric (DTG), differential thermal analysis (DTA). A CO₂ atmosphere

was used to increase the temperature of carbonate decomposition and the carbonate content was estimated by quantifying the weight loss at temperature > 700 °C.

2.4 Mussel collection and handling

Adult specimens of the Mediterranean mussel (*M. galloprovincialis*) with a shell-length of 5 ± 1 cm were collected by professional fishermen of the “Cooperativa CONISUB” (Marina di Ravenna, Italy) along the north-western Adriatic Sea coast in high quality marine areas (Zone A) and immediately transferred to the laboratory in seawater tanks with continuous aeration. Prior to the *in situ* deployment, all animals were acclimatized for 6 days in aquaria containing 60 L of aerated natural seawater (at a ratio of 1 mussel/L) at 16 °C and natural photoperiod, and fed daily on an algal slurry (Coral Diet Filtrator, Xaqua, Italy) after water renewal. Seventy organisms were dissected prior to the *in situ* transplantations (time 0, T0) for bioaccumulation and biological endpoint analyses. Digestive glands and gills were immediately collected, frozen in liquid nitrogen and stored at –80 °C until analysed. At the same time six groups of seventy organisms each were put in fishnet bags, fixed to piles and driven into the sediment at sites S1, S2, S3, S5, S6 and S7 (Fig. 1) at about 50 cm from the bottom to ensure continuous submersion during the whole exposure period. Deployed mussels were regularly controlled at three days intervals and dead mussels were eventually recorded for survival assessment. Since previous investigations identified the Magni channel as a heavily polluted site (Fabbri et al., 2000; 2003; Matteucci et al., 2005), three additional fishnet bags, with seventy organisms each, were transplanted into S1 to execute biomarkers with shorter timestep.

2.5 Metal bioaccumulation analysis

Metal bioaccumulation was evaluated on mussels prior to the *in situ* deployment (Time 0, T0) and after 30 days of field exposure into the lagoon. Metal bioaccumulation from each site was used to calculate the bioaccumulation factor as enrichment/depletion compared to T0. Samples (0.2 g each) were digested in a microwave system with ultrapure HNO₃. The resulting fluid was diluted by adding deionized water to a final volume of 30 mL, at the Department of Environmental Science of the University of Siena. Analytical blanks and certified reference material were included in the analysis, specifically the mussel tissue ERM-CE278 (*Mytilus edulis*) from the Institute for Reference Materials and Measurements, Geel, Belgium.

The samples were analysed by inductively coupled plasma quadrupole mass spectrometry (ICP-QMS) using an Agilent 7500ce instrument at the laboratories of the Federal Institute for Geosciences and Natural Resources in Berlin. Atomic fluorescence spectrometry was used for the determination of mercury (Instrument PSA 10.035 Millennium Merlin 1631). The Table S1 reports the results for the investigated elements in the certified reference material. Additional details can be found in Capolupo et al. (2017).

2.6 *Biological endpoints*

2.6.1 Survival percentage and Stress on Stress

The survival (SURV) was calculated as the percentage of mussels found alive over the exposure period with respect to the initial number of individuals (70 at each site).

For Stress on Stress (SOS) assessment, immediately after collection, 15 mussels per site were subjected to anoxia by air exposure at 15 °C in a humidified chamber. Death symptoms were open valves and absence of muscular activity. The survival was assessed daily, and results expressed as the time at which 100% mortality was achieved (Viarengo et al., 2007a).

2.6.2 Cytochemical parameters

Lysosomal membrane stability (LMS), lysosome to cytoplasm volume ratio (LYS/CYT) and lipofuscin content (LIF) were assessed in the digestive gland from 20 mussels per each site (N = 20). After dissection, glands were mounted on specific aluminium supports for cryostat microtomy (chucks), frozen in N-hexane at -70 °C, and stored at -80 °C. All assessments were performed according to the UNEP/RAMOGGE manual (1999) on 10 µm-thick gland sections obtained using a cryostat (MICROM HM 505 N) at -30 °C. The LMS was measured as the time necessary to produce the maximum staining reaction between the lysosomal hydrolase N-acetyl-β-hexosaminidase and a specific substrate (Capolupo et al., 2016). Briefly, sections were incubated at 37 °C in a shaking water bath with a destabilization buffer (0.1 M Na-citrate, pH 4.5) for different times (0, 3, 5, 10, 15, 20, 30 and 40 min), exposed to the substrate naphtol AS-BI N-acetyl-β-D-glucosaminide (Sigma, N4006) for 20 min and finally stained with the diazonium dye Fast Violet B (Sigma, F1631) (1 mg/ml in 0.1 M phosphate buffer, pH 7.4). For each site, slides not exposed to the destabilization buffer (0 min) were screened for the LYS/CYT assessment, which was determined by evaluating the cytoplasmic and lysosomal areas according to Capolupo et al. (2017). The LIF determination was performed

according to Martin-Diaz et al. (2009). Gland sections were fixed in calcium formol and immersed for 5 min in an aqueous solution of 1 % ferric chloride and 1 % potassium ferricyanide in a 3:1 ratio.

For all lysosomal parameters, tissue sections were assessed under a light microscope (Axioskop 40, Carl Zeiss, Milan, Italy) equipped with a 40X objective and a digital camera (AxioCam MRc, Carl Zeiss, Milan, Italy) as reported by Capolupo et al. (2016). Results were expressed as labilization period for LMS, percentage of lysosomal volume inside digestive cells for LYS/CYT and arbitrary units for LIF.

2.6.3 Enzymatic biomarkers

The acetylcholinesterase activity (AChE) activity was determined in mussel gills as previously reported (Valbonesi et al., 2003). Gills from 5 mussels per site (N = 5) were homogenized in a 0.1 M phosphate buffer, pH 7.4, and centrifuged at 9000 ×g at 4 °C for 30 min. Supernatant aliquots were incubated at 25 °C with 0.5 mM acetylthiocholine iodide (ASCh) and 0.33 mM DTNB (Ellman et al., 1961), and read at 405 nm for 10 min. Results are reported as nmoles/min/mg protein. The sample protein concentration was measured according to Lowry et al. (1951).

For catalase (CAT) assessment, about 50 mg of digestive gland was dissected from 5 mussels per site (N = 5), homogenized in 50 mM potassium-phosphate buffer (pH 7.0, 0.5 mM Na₂EDTA) and centrifuged at 15,000 g at 4 °C. The CAT activity was determined by measuring the time-dependent decrease of absorbance at 240 nm in the presence of 55 mM H₂O₂. Data were expressed as μmol/min/mg of total protein.

The Peroxisomal acyl-CoA oxidase (AOX) activity was determined on five pools of 2 digestive glands per site (N = 5) according to Orbea et al. (2006). Tissues were homogenized in four volumes of TVBE buffer (1 mM sodium bicarbonate, 1 mM EDTA, 0.1 % ethanol and 0.01 % Triton X-100), pH 7.6 and centrifuged at 500 × g for 15 min at 4 °C. Supernatants were diluted 1:10 in TVBE buffer and spectrophotometrically assayed for AOX activity at a wavelength of 502 nm. Data were expressed as milliunits/mg of AOX protein (equivalent to nmol H₂O₂/min x mg protein).

2.6.4 Metallothionein content (MT)

The metallothionein (MT) content was analysed on five pools of 5 mussel digestive glands (about 1.5 g of tissue) per site according to Viarengo et al. (1997). Final absorbance was measured at 410 nm, using reduced glutathione as reference standard. Data were expressed as μg of MT/mg tissue.

2.6.5 Mussel Expert System

Data from biological responses were integrated using the Mussel Expert System (MES) developed by Dagnino et al. (2007) in a unique and synoptic five-level Health Status Index (HSI) ranging from A (healthy) to E (pathological status). The system classification is based on (i) the number of biomarkers altered in the exposed organisms (significant changes greater than 20 % were utilized to evaluate alterations in the health status of the organisms); (ii) the magnitude of the alteration; (iii) the role of the autophagic process and (iv) the level of biological organization affected (i.e., cell, tissue and organism level). For each biomarker/site, the system calculates an Alteration Factor (AF), which is the ratio between biomarker levels from treatments and control, and an Alteration Level (AL), which defines the magnitude of observed alterations based on the AFs magnitude and the biomarker toxicological profile (i.e. increasing, decreasing, bell-shaped). ALs and AFs are then processed by the system applying a set of rules in the “if...then” form in order to calculate the overall HSI associated to the exposure to each site. The simultaneous alterations of general stress parameters (i.e. lysosomal responses) and biomarkers of exposure to specific classes of compounds (e.g. MT for metals; AOX for POPs) is further computed and eventually recognized by the system as an early warning signal regarding the presence or excess of those pollutants in the monitored sites or tested condition.

2.7 Data analysis

All data were tested for normal distribution using the Shapiro-Wilk test, while the Levene's test was employed to test the equal variance. Significant differences between S1 to S6 compared to the control site S7 were estimated by a One-Way ANOVA (SigmaStat, SPSS) followed by the Duncan's post-hoc test. The evaluation of potential linear relationships between biological endpoints and physico-chemical parameters (including pH, temperature, electrical conductivity, metal concentrations in sediments and mussel tissues) were conducted through the Pearson's correlation analysis. For all performed tests, significance was established at $p < 0.05$. All analyses were performed using the software SigmaPlot 13 (Systat Software Inc. San Jose, CA, USA).

3 Results and discussion

3.1 Physico-chemical data

The vertical profiles of temperature, pH and electrical conductivity (EC) measured during mussels field exposure as well as a graphical representation of the temperature, pH and EC vertical profiles for June 14th are presented respectively in Table S2 and in Figure S1 of supplementary material. The site S1, located in the Magni Channel at the southern side of the lagoon (Fig. 1), is different from the other sites for all monitored parameters. It shows higher water column temperature, reduced EC and a lower pH value, for the entire monitoring period (Table S2). Specifically, S1 shows the lowest EC among the sites with a marked vertical gradient (Fig. S1A) and it is also the site with the highest temperature, up to 32.3 °C. Moreover, it records the highest average profile temperature during the surveying period, at least 2 °C constantly higher compared to all the other sites (Fig. S1B). Site S1 receives freshwater from artificial drainage channel network surrounding the town of Ravenna (Greggio et al., 2018b). Previous investigations also reported water temperature fluctuation wider (10-24 °C) in the southernmost channel with respect to the lagoon average (16-18 °C), which was subjected to variations of about 10 °C within the 24 hours owing to discharges of cooling water from the nearby industrial site (Franzellitti et al., 2010). A thermal vertical gradient is also present in S5 probably associated to local limited water circulation favouring water warming up (Fig. S1A). The pH is the lowest in S1, ranging from 7.2 to 7.8, and with a marked vertical profile. In all the other sites the pH is instead similar and fairly constant around 8. Only at S5 the observed pH values are higher (8.0-8.3) for the entire surveying period (Table S2).

3.2 Sediment data

The results of the geochemical analyses are presented in Table 1. According to the geochemical composition of the major elements (expressed as %), and referencing to the work of Dinelli et al. (2007) which compared geochemical proxies with grain size analysing borehole samples in the same geographic area, the Pialassa Baiona bed sediment samples are classified as fine grained-sediments (with a recalculated $\text{SiO}_2/\text{Al}_2\text{O}_3$ ratio < 3) enriched in carbonates. The sediments are also rich in organic matter (OM in Table 1, ranging from 1.1 to 3.4) as already reported for adjacent coastal lagoons (Migani et al., 2015). The bulk geochemical composition is quite homogeneous among the different sites, with S1 being slightly enriched in carbonates (Table 1).

The geochemical results, in terms of total sediment composition, are comparable with those reported by Donnini et al. (2007) although the number of sites was different. As far as total concentrations are concerned, these are within the range of regional background, as proposed by Migani et al. (2015), taking into consideration Holocene data from boreholes in the area not affected by anthropogenic inputs (Amorosi et al., 2002; Curzi et al., 2006; Dinelli et al., 2012; Greggio et al., 2018a). Except for sites S6 and S7, Zn and Cu concentrations exceed the maximum value observed in the background sediments, respectively of 124 and 41 mg/kg (Table 1). In particular, for S1 site Zn and Cu total concentrations are double respect to background, respectively 316 and 87 mg/kg, in line with concentration detected in the neighbouring Pialassa Piomboni by Pignotti et al. (2018). Pb concentrations in S3 and S5 are 30 mg/kg being slightly higher respect to background level established at 29 mg/kg by Migani et al. (2015), but certainly lower than Pb concentration in the Pialassa Piomboni by Pignotti et al. (2018) where a maximum concentration of 257 mg/kg was measured.

Table 1. Results of the chemical analyses on sediments. The table includes either pseudo total Aqua Regia (AR) and total XRF data. Table includes also the ranges observed in boreholes in the area, considered representative of the natural background (Amorosi et al., 2002; Curzi et al., 2006). Bold elements exceed natural background. AR DL indicates Detection Limit for Aqua Regia extraction.

		S1		S2		S3		S4		S5		S6		S7		Natural background
		AR DL	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF
Ag	μg/kg	2	991	-	269	-	268	-	173	-	275	-	179	-	62	-
Al	%	0.01	1.39	6.31	1.41	7.02	1.23	6.98	1.10	6.97	1.35	7.09	1.39	7.07	1.41	6.55
As	mg/kg	0.1	7.8	-	5.9	-	6.1	-	5.9	-	7.8	-	6.8	-	6.7	-
Au	μg/kg	0.2	16.2	-	5.4	-	11.1	-	2.6	-	5.1	-	3.4	-	1	-
B	mg/kg	1	25	-	28	-	26	-	24	-	38	-	33	-	22	-
Ba	mg/kg	0.5	61.9	263	43.7	218	41.8	260	42.6	322	40.1	258	38.7	294	36.5	258
Be	mg/kg	0.1	0.4	-	0.7	-	0.7	-	0.8	-	0.5	-	0.6	-	0.6	-
Bi	mg/kg	0.02	0.6	-	0.38	-	0.35	-	0.26	-	0.38	-	0.31	-	0.28	-
Cd	%	0.01	11.27	11.37	7.45	7.51	7.87	7.96	7.34	7.87	6.10	6.65	7.24	7.43	8.61	9.52
Cd	mg/kg	0.01	0.68	-	0.29	-	0.33	-	0.21	-	0.5	-	0.35	-	0.11	-
Ce	mg/kg	0.1	19.4	42	17	55	17.6	71	16.8	52	17.8	64	18.3	42	20.1	58
Co	mg/kg	0.1	10.2	12	10.9	11	10.7	12	9.9	11	11.7	14	11.5	12	10.6	11
Cr	mg/kg	0.5	70.6	131	57.5	103	51.4	132	47.7	131	59.9	138	59.3	130	47	100
Cs	mg/kg	0.02	1.36	-	1.15	-	1.08	-	1.16	-	1.11	-	1.39	-	1.57	-
Cu	mg/kg	0.01	84.1	87	49.1	52	49.5	53	36.7	44	51.1	52	37.5	38	22.4	24
Fe	%	0.01	2.17	3.42	2.46	3.92	2.23	3.86	2.00	3.69	2.54	3.83	2.30	3.64	2.36	3.72
Ga	mg/kg	0.1	4.4	-	4.2	-	4	-	3.9	-	4	-	4.2	-	4.4	-
Ge	mg/kg	0.1	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-
Hf	mg/kg	0.02	0.03	-	0.03	-	0.04	-	0.03	-	0.03	-	0.03	-	0.05	-
Hg	mg/kg	0.005	25.7	-	1.42	-	1.48	-	1.89	-	2.38	-	2.26	-	0.1	-
In	mg/kg	0.02	0.03	-	0.03	-	0.02	-	0.02	-	0.03	-	0.02	-	0.03	-
K	%	0.01	0.33	1.77	0.36	1.98	0.31	2.06	0.28	2.04	0.39	1.97	0.36	2.04	0.35	2.03
La	mg/kg	0.5	8.3	19	7.7	26	7.3	22	6.9	18	8	20	8.5	34	9.5	20
Li	mg/kg	0.1	26.1	-	30.6	-	27.8	-	24.9	-	28.7	-	28	-	28.1	-
Mg	%	0.01	1.37	2.41	1.34	2.38	1.30	2.38	1.22	2.44	1.42	2.54	1.55	2.55	1.58	2.35
Mn	mg/kg	1	531	893	527	865	549	896	513	949	511	801	550	897	644	1396
Mo	mg/kg	0.01	2.67	-	3.33	-	2.61	-	2.28	-	3.34	-	1.57	-	0.54	-
Na	%	0.001	0.85	0.68	0.98	0.69	0.90	0.75	0.65	0.99	1.18	0.67	0.85	0.78	0.42	0.85
Nb	mg/kg	0.02	0.37	15	0.28	13	0.25	13	0.29	14	0.34	16	0.37	10	0.29	13
Ni	mg/kg	0.1	50.9	73	56.5	73	50.7	88	46.3	72	55.3	82	51.5	74	46.4	57
P	%	0.001	0.11	0.10	0.06	0.06	0.06	0.07	0.06	0.06	0.08	0.07	0.07	0.06	0.06	0.06
Pb	mg/kg	0.01	27.92	28	22.71	26	22.14	30	18.16	19	26.22	30	20.71	22	11.99	18

Pd	µg/kg	10	46	-	10	-	12	-	11	-	10	-	<10	-	<10	-	-
Pt	µg/kg	2	37	-	6	-	4	-	4	-	4	-	<2	-	<2	-	-
Rb	mg/kg	0.1	24.9	113	22.2	128	20.8	128	22.4	113	22.3	130	25.1	114	29.8	89	56-200
Re	µg/kg	1	7	-	1	-	2	-	<1	-	2	-	2	-	<1	-	-
S	%	0.02	0.6	-	0.55	-	0.6	-	0.54	-	0.72	-	0.71	-	0.34	-	-
Sb	mg/kg	0.02	0.51	-	0.29	-	0.25	-	0.19	-	0.37	-	0.29	-	0.19	-	-
Sc	mg/kg	0.1	3.4	11	3.6	11	3.4	6	3.2	8	3.7	10	3.7	8	4.1	13	12-21
Se	mg/kg	0.1	0.9	-	0.7	-	0.7	-	0.5	-	1.1	-	0.7	-	0.5	-	-
Si	%	-	-	17.72	-	19.43	-	19.90	-	20.61	-	19.31	-	20.95	-	20.03	14.7-29.3
Sn	mg/kg	0.1	3.2	-	1.8	-	1.5	-	1.3	-	1.9	-	1.5	-	0.8	-	-
Sr	mg/kg	0.5	353	368	274.4	300	224.8	252	218	265	259.1	281	281.6	291	290.5	310	113-442
Th	mg/kg	0.1	3.5	4	4.1	5	3.6	17	3.6	25	4	11	4.2	10	4.7	7	<3-19
Ti	%	0.001	0.009	0.308	0.005	0.328	0.006	0.347	0.008	0.349	0.006	0.324	0.010	0.334	0.015	0.336	0.02-0.58
Tl	mg/kg	0.02	0.26	-	0.17	-	0.21	-	0.19	-	0.27	-	0.2	-	0.14	-	-
U	mg/kg	0.1	1.3	-	1.5	-	1.2	-	1.1	-	1.3	-	1.2	-	1.1	-	-
V	mg/kg	2	32	85	33	85	30	99	26	94	35	113	33	94	31	76	54-169
Y	mg/kg	0.01	10.02	18	9.39	23	9.28	24	8.86	20	9.7	24	9.63	19	10.58	19	18-43
Zn	mg/kg	0.1	306.6	316	129.8	152	133.1	154	101.2	126	146.9	174	109.3	124	58.2	67	43-124
Zr	mg/kg	0.1	1.1	108	1.2	91	1	105	1	124	1.1	86	1.2	92	1.9	106	76-260
H₂O⁻	%	-	-	2.7	-	4.0	-	2.8	-	2.4	-	3.7	-	2.7	-	2.1	-
OM	%	-	-	2.8	-	1.8	-	2.0	-	1.7	-	3.4	-	2.2	-	1.1	-
H₂O⁺	%	-	-	6.0	-	6.5	-	5.4	-	4.4	-	6.5	-	5.8	-	2.3	-
CO₂	%	-	-	11.6	-	8.9	-	9.1	-	9.3	-	8.6	-	7.4	-	12.3	-
LOI	%	-	-	23.1	-	21.2	-	19.3	-	17.8	-	22.3	-	18.1	-	17.8	4.8-29.3

As regards the Aqua Regia extraction, data showed the presence of elements seldom analysed in sediments of the area. The most striking features are the differences observed between S1 and all the other sites for a number of elements, in particular Ag, As, Au, Bi, Cd, Cr, Cu, Hg, Pb, Pd, Pt, Re, Zn (Table 1). According to Donnini et al. (2007), S7 should be considered as unpolluted site and if its results are taken as reference, a strong enrichment is clear for several elements (Fig. 2). Mercury, which is a well-known pollutant in the area (Miserocchi et al., 1993; Fabbri et al., 1998; Fabbri et al., 2001a, b; Trombini et al., 2003; Matteucci et al., 2005; Guerra et al., 2007; Covelli et al., 2011; Guerra, 2012; Dominik et al., 2014; Borghesi et al., 2016), is the element with the largest enrichment (up to 250 times compared to S7) and reaches relatively high concentrations in the Magni Channel site (S1, up to 25 mg/kg). Mercury dynamics in the lagoon is complex and although the maxima in concentrations are not at the surface, the element is heavily mobilized at the water/sediment interface in the southern part of the lagoon (Covelli et al., 2011). Borghesi et al. (2016) highlighted similar behaviour among Hg and Ag, Au, Cd, Cu and Zn for six different coastal lagoons in the same Region due to metal affinity for the fine fraction of the sediment.

Notable enrichments are then evident in Figure 2 for valuable metals like Pt, Au, Ag, Re and Pd, which showed substantial increase (up to 10-fold) compared to S7 and to median values reported by Cicchella et al. (2015), that represent a useful reference for metal concentrations in mineral matrix obtained with the same analytical technique and at the same laboratory. In particular, Platinum and Palladium are of recent technogenic use and their anthropogenic fluxes greatly exceed the natural ones (Ruchter and Surer, 2015; McGillicuddy et al., 2017). Platinum and Palladium are largely used in automobile catalytic converters (Bossi and Gediga, 2017) and their

dispersion increases particularly around urban areas in roadside soils (Ravindra et al., 2004; Ruchter and Surer, 2015; Leopold et al., 2018; Zuzolo et al., 2018), but also in marine areas close to urban settings (Cobelo-García et al., 2011; Abdou et al., 2016).

The Silver concentration reaches a peak at S1 with 0.99 mg/kg, at least four times more than the other sites in the Pialassa Baiona and ten times more than other coastal lagoons in the same Region as resulted by Borghesi et al. (2016). The Ag enrichment is controlled by many factors such as pH, OM, as well as the source material. At typical environmental pH values, Ag is adsorbed onto Fe hydroxides, in preference to Cu and Zn (Lottermoser et al., 1999). In the near Venice lagoon, Giusti and Zang (2002) measured Ag sediment concentrations 5-6 fold higher than this study, justifying the abundance with both famous glassmaking firms in Murano island and the large volume of untreated sewage sludges discharged into the lagoon from the nearby Marghera industrial area.

Moderate enrichments (four times compared to S7) are shown by Mo, Cd, Zn, Sn and Cu that can be more directly linked to industrial effluents as reported by Giusti and Zang (2002) in Venice lagoon and Borghesi et al. (2016) for the same Region. Enrichments in all these elements also characterize the other sites, although at an order of magnitude lower than S1 (around 2 times). Many of the metals detected in sediments show a decreasing concentration with increasing distance from the industrial area located in the southern part of the lagoon. This suggests that industrial discharge effluents play a major role in the lagoon contamination compared to effluents from urban and agricultural activities. In facts, the distribution of some elements, including Zn, Cr, Cu, Mo, Ag, and Hg, follow trends similar to those reported for other organic pollutants of industrial origin in the same area (Vassura et al., 2005; Fabbri et al., 2006). Vassura et al. (2005), for instance, detected a clear South-North increasing pattern of prioritized PAHs, with sediments from the Magni channel area (the present S1) showing concentrations of pyrene, fluoranthene, anthracene and phenanthrene up to 10,000 times higher compared to the northern sites.

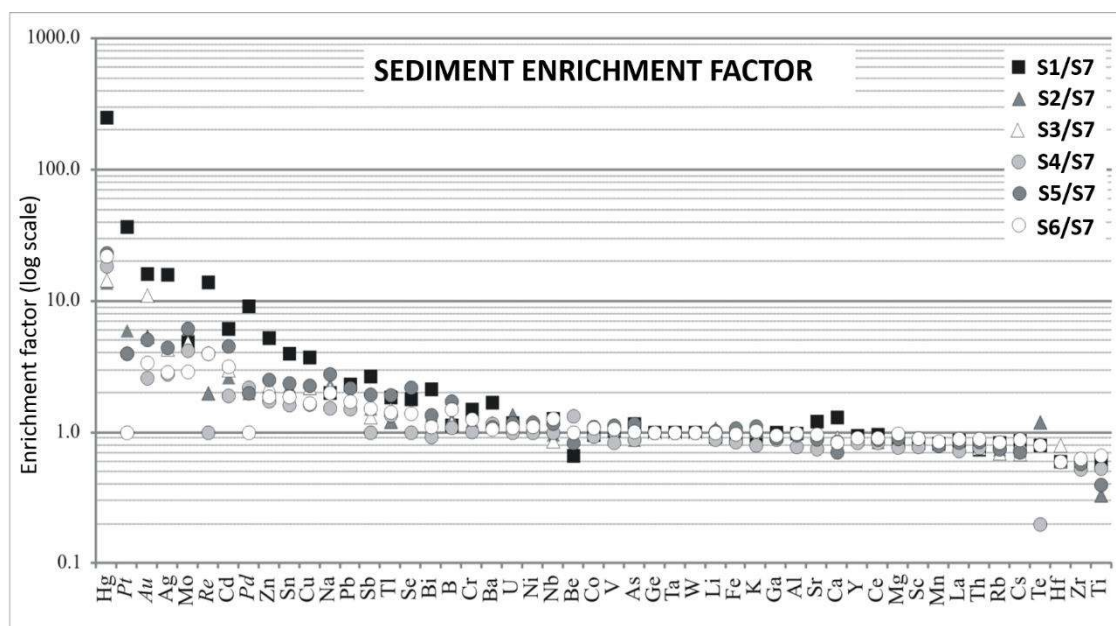


Figure 2. Metal enrichment in sediments from selected sites within the Pialassa Baiona lagoon. Data are normalized on Site 7 (S7), which was selected as internal reference based on previous literature (Trombini et al., 2003; Fabbri et al., 2006; Donnini et al., 2007; Franzellitti et al., 2010). Elements in italics (*Pt*, *Au*, *Re*, *Pd*) were not investigated in the mussel tissues.

3.3 Metal bioaccumulation

Results of the chemical analyses on mussel tissues are presented in Table 2. Data were obtained for all sites except S1, which was characterized by a high mortality rate (more details in section 3.4). T0 data, originating from mussels growing in the Adriatic Sea, are in line with basal level for As, Ba, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, V, Zn identified by Fattorini et al. (2008). The trend of concentrations of trace elements in soft tissue of *Mytilus galloprovincialis* belonging to T0 is (Zn, Fe) > (As, Mn, Cu, Ni, Pb) > (Cd, Cr, Ag) > (Hg, Sb). The trend is confirmed also for average trace elements concentration among all sites: (Fe, Zn) > (As, Mn, Cu) > (Pb, Ag, Ni, Cd, Cr, Hg) > Sb. The only notable change is the Ag enrichment from 0.12 mg/kg in T0 tissues to a mean concentration of 0.80 mg/kg in tissues from experimental sites. Although Cd, Hg, Sb were not included, the same abundance trend was found by Giusti and Zang (2002) in the Venice Lagoon: Fe > Zn > (Cu, Mn, As) > Ag > Pb > (Ni, Cr). Absolute concentrations of Zn, Cu, Pb and Cd found in this study (Table 2) fall in the lowest part of the ranges summarized by Benali et al. (2017) for 10 locations in Mediterranean Sea.

Table 2. Results of the chemical analyses on mussel tissues (bioaccumulation). Data refers to T0 and to the 5 sites where mussels survived or remained after the exposure period.

	unit	T0	S2	S3	S5	S6	S7
Ag	mg/kg	0.12	0.99	0.98	1.21	0.62	0.82
Al	mg/kg	36	39	56	98	82	47
As	mg/kg	9.83	12.8	9.98	11.9	12.4	11.4
B	mg/kg	25.7	22.9	19.7	22.8	25.9	19.4

	Ba	mg/kg	1.95	0.35	0.49	0.54	0.52	0.36
1	Be	mg/kg	0.003	0.003	0.003	0.005	0.006	0.004
2	Bi	mg/kg	0.057	0.009	0.009	0.007	0.011	0.008
3	Ca	mg/kg	2004	1322	1299	1726	1679	1157
4	Cd	mg/kg	0.70	0.71	0.78	0.76	0.77	0.50
5	Ce	mg/kg	0.118	0.051	0.092	0.124	0.166	0.075
6	Co	mg/kg	0.54	0.323	0.3	0.303	0.511	0.315
7	Cr	mg/kg	0.57	0.6	0.53	0.67	0.53	0.64
8	Cs	mg/kg	0.022	0.014	0.016	0.022	0.019	0.015
9	Cu	mg/kg	4.65	2.71	2.63	2.81	2.66	1.46
10	Fe	mg/kg	67.5	68.1	77.4	116.0	103.4	91.7
11	Ga	mg/kg	0.018	0.007	0.011	0.019	0.013	0.009
12	Hg	mg/kg	0.082	0.35	0.32	0.41	0.27	0.16
13	K	mg/kg	11084	10988	9729	11129	11244	9535
14	La	mg/kg	0.138	0.035	0.057	0.074	0.109	0.06
15	Li	mg/kg	0.88	0.46	0.41	0.51	0.58	0.34
16	Mg	mg/kg	4198	3851	3467	3676	4422	2506
17	Mn	mg/kg	4.84	5.19	7.50	8.16	8.03	5.03
18	Mo	mg/kg	0.645	0.353	0.368	0.317	0.394	0.274
19	Na	mg/kg	22885	20201	15986	19714	24751	13438
20	Nb	mg/kg	0.002	0.001	0.002	0.003	0.003	0.002
21	Ni	mg/kg	3.61	0.72	0.69	0.73	0.92	0.64
22	Pb	mg/kg	1.41	0.87	0.52	0.57	0.94	0.64
23	Rb	mg/kg	5.29	4.08	4.32	4.27	4.3	3.78
24	Sb	mg/kg	0.018	0.038	0.031	0.03	0.048	0.039
25	Sc	mg/kg	0.04	0.04	0.03	0.05	0.07	0.04
26	Se	mg/kg	2.27	2.10	2.10	2.01	1.83	1.52
27	Sn	mg/kg	0.12	0.12	0.12	0.10	0.09	0.08
28	Sr	mg/kg	31.2	17.7	16.5	20.6	22.8	14.3
29	Th	mg/kg	0.019	0.011	0.014	0.025	0.031	0.016
30	Ti	mg/kg	0.49	0.34	0.62	1.07	0.88	0.50
31	Tl	mg/kg	0.003	0.001	0.002	0.002	0.001	0.001
32	U	mg/kg	0.127	0.049	0.040	0.054	0.065	0.032
33	V	mg/kg	1.15	0.44	0.49	0.55	0.80	0.47
34	Y	mg/kg	0.080	0.046	0.071	0.085	0.094	0.04
35	Zn	mg/kg	107.1	116	62.4	95.6	134	86.9
36	Zr	mg/kg	0.064	0.013	0.025	0.025	0.021	0.018

The bioaccumulation factor is graphically showed in Figure 3. Enrichments are notable for Ag, Hg and Sb in all the sites, with higher bioaccumulation at S5 located in the centre of the lagoon in a pond close to the outlet of a canal from the southern part of the lagoon, the most contaminated one. Although not high as those measured in mussels from other sites such as Northern England (Giusti et al., 1999), these three elements could be the most critical since other elements known for toxicity to mussels (e.g. Cd, Pb, Ni) (Benali et al. 2017; Yigit et al. 2017) are similar or depleted compared to T0 (Fig. 3).

The enrichment in mercury was relatively high, as expected and already verified both in living clams (Trombini et al., 2003) and mussels (Fabbri et al., 2006; Donnini et al., 2007). Moreover, the Hg cycling at the sediment-water interface (Covelli et al., 2011), could be a reason for the mercury enrichment observed in all sediment samples and may also reflect the high Hg concentrations observed in mussels exposed at S5 (Fig. 3).

Silver has recently attracted much interest since its increasing application as bactericidal and usage in numerous consumer products lead to contamination of aquatic ecosystems, often in form of Ag nanoparticles (Yuan et al., 2018; Outa et al., 2020). Silver speciation in environment is dependent by local environmental conditions and usually forms stable complexes with Lewis bases such as amines, halides, and thiolates and with dissolved organic matter altering its toxicity (Mousavi et al., 2015). In Pialassa Baiona, Greggio et al. (2020) showed dissolved concentrations ranging from 0.02 to 10 µg/L, with higher concentrations in basins with drainage water input. Concerning bioaccumulation, recent works reported the effect of Ag nanoparticles on soft tissues of mussels. *Mytilus galloprovincialis* accumulated Ag in digestive gland tubule cells and gills during the entire year (Duroudier et al., 2019) and intralysosomal metal accumulation and lysosomal membrane destabilisation were observed (Jimeno-Romero et al., 2017). Compare to this study (max 1.21 mg/kg dry wt), Giusti and Zang (2002) in Venice lagoon found silver concentrations up to 6.2 mg/kg dry wt that are aligned with outcomes in other species of the genus *Mytilus* as reported by Martin et al. (1988).

Antimony is pervasive in synthetic polymers (including textiles, plastics and rubbers) (Filella et al., 2019) and it is widely applied in alloy industries as flame retardant, as pigments for colour or colour protection (Filella et al., 2002; James and Turner 2020). Although rock weathering and soil runoff supply Sb to the environment, anthropogenic source is crucial. James and Turner (2020) demonstrated that Sb could also be mobilised via digestion and bioturbation by deposit-feeders in sediment contaminated by plastic or directly adsorbed from microplastics taken up by mussels (Van Cauwenberghe et al., 2015). Antimony concentrations in unpolluted waters are less than 1 µg/L (Filella et al., 2002). Greggio et al. (2020) reported Sb water concentrations in western ponds of the Pialassa Baiona ranging from 0.05 to 0.5 µg/L. Limited is the knowledge of Antimony concentration in mussels. In this study Sb in mussel tissues ranged from 0.01 to 0.05 mg/kg (Table 2) with a 2-3 as enrichment factor (Fig. 3). De Gregori et al. (2007) found tissue concentration ranging from 0.007 to 0.06 mg/kg in unpolluted area, while 0.23 to 1.030 mg/kg in harbour dock protected by antimony-lead alloy cover. Garcia (2015) measured Sb concentration ranging from 0.003 to 0.023 mg/kg in the tissues of *Corbicula fluminea* collected in a river affected by a decommissioned Antimony smelter.

It is worth noting the depletion relative to T0 of elements like Cu, Pb and Ni, (Fig. 3 and Table 2). This might suggest that these elements are not strongly mobilized in the lagoon environment, although slight differences can be recognized between S7 and the other sites. This is in contrast with results by Pignotti et al. (2018) that using sequential extraction, defined Cu and Pb as the most

mobile elements in sediments (~70 % and ~80 %, of the total, respectively) in the neighbouring Pialassa Piomboni. In agreement with Pignotti et al. (2018) is the Ni behaviour here characterised by low mobility and predominantly bound to the residual fraction of the sediment, suggesting a lithogenic origin.

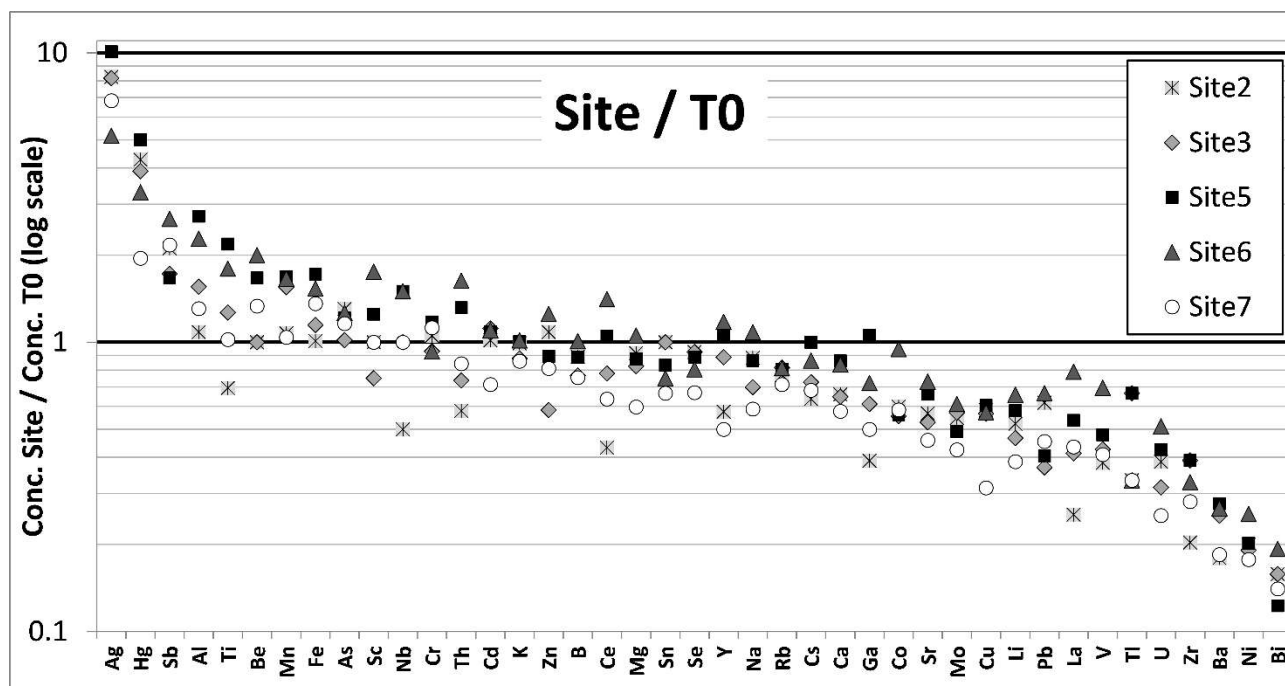


Figure 3. Bioaccumulation factor of metals in mussel tissues. The analysis has been performed on the tissue of mussels deployed for 30 days into different sites of the Pialassa Baiona lagoon compared to mussels analysed before the *in situ* deployment (T0). Elements are ranked according to average enrichment.

3.4 Biological effects on mussels

The mussel 30-day exposure within the Pialassa Baiona lagoon resulted in significant alterations of biological endpoints measured at different levels of the hierarchical ladder (from cell to organism) (Fig. 4). Among the investigated sites, adverse conditions for the mussel survival were observed in S1, with about 80 % mortality *versus* 10 % recorded elsewhere (Fig. 4A). Different from S2 – S7, survival (and biomarker) data were measured in S1 after just three days of exposure, in line with previous observations on mussels transplanted in the same area (Franzellitti et al., 2010). The short time spent *in situ* within S1 was not such to allow mussels to bioaccumulate metals at levels considered environmentally realistic, for which ≥ 28 days are usually required (Viarengo et al., 2007a); thus, data of metal bioaccumulation are missing for this site. Sediment concentrations of Hg, Cr, Zn, Cu and Ba, known for their toxicity on marine mussels (De Los Ríos et al., 2013) were

negatively and significantly correlated with mussel survival (Fig. 5). Relationships were also detected between survival and average salinity and temperature, which showed minima and maxima in S1, respectively (Table S3). Both parameters are known to be responsible for the mussel physiological fitness and to influence their response to toxicants synergistically (Fabbri and Dinelli, 2014; DeCourten and Brander, 2017). It is thus plausible that physico-chemical conditions of both natural and anthropogenic nature simultaneously influenced the survival of mussels across the lagoon.

Data of *in situ* survival follow trends similar to those from the SOS test, which showed significant reduction of survival functions in mussels from S1 (Fig. 4B). SOS is measured as the survival of mussels in dry conditions, which is an event regularly experienced by intertidal organisms due to tidal fluctuations. The SOS data display significant and negative correlation with a suite of metals measured in both sediments and mussel tissues, including Ba, Bi, Cu, Sr and Zn, among others (Fig. 5). These findings are in line with previous evidence obtained in *M. galloprovincialis*, which showed significant SOS decrease after 3 to 7 days of exposure to trace levels of Cu and Zn-based antifouling agents (Viarengo et al., 1995; Marcheselli et al., 2011). Further elements showing marked enrichment in S1 sediments, as Cd (0.68 mg/kg) and Hg (25.7 µg/kg), were significantly correlated with SOS (Fig. 4). SOS and survival data were found to be significantly and positively correlated to each other and showed positive and negative correlation with electrical conductivity and temperature, respectively (Table S3). This indicates that higher temperatures and salinity measured at S1 throughout the exposure period may have cumulatively increased the impact of metals on the mussel vital functions. Although it is acknowledged that SOS sensitivity is lower than other sub-lethal and general stress biomarkers (Viarengo et al., 2007a), data from this study highlight its suitability to detect causal relationships between metal contamination/bioavailability and adverse effects on mussels at the “organism” level.

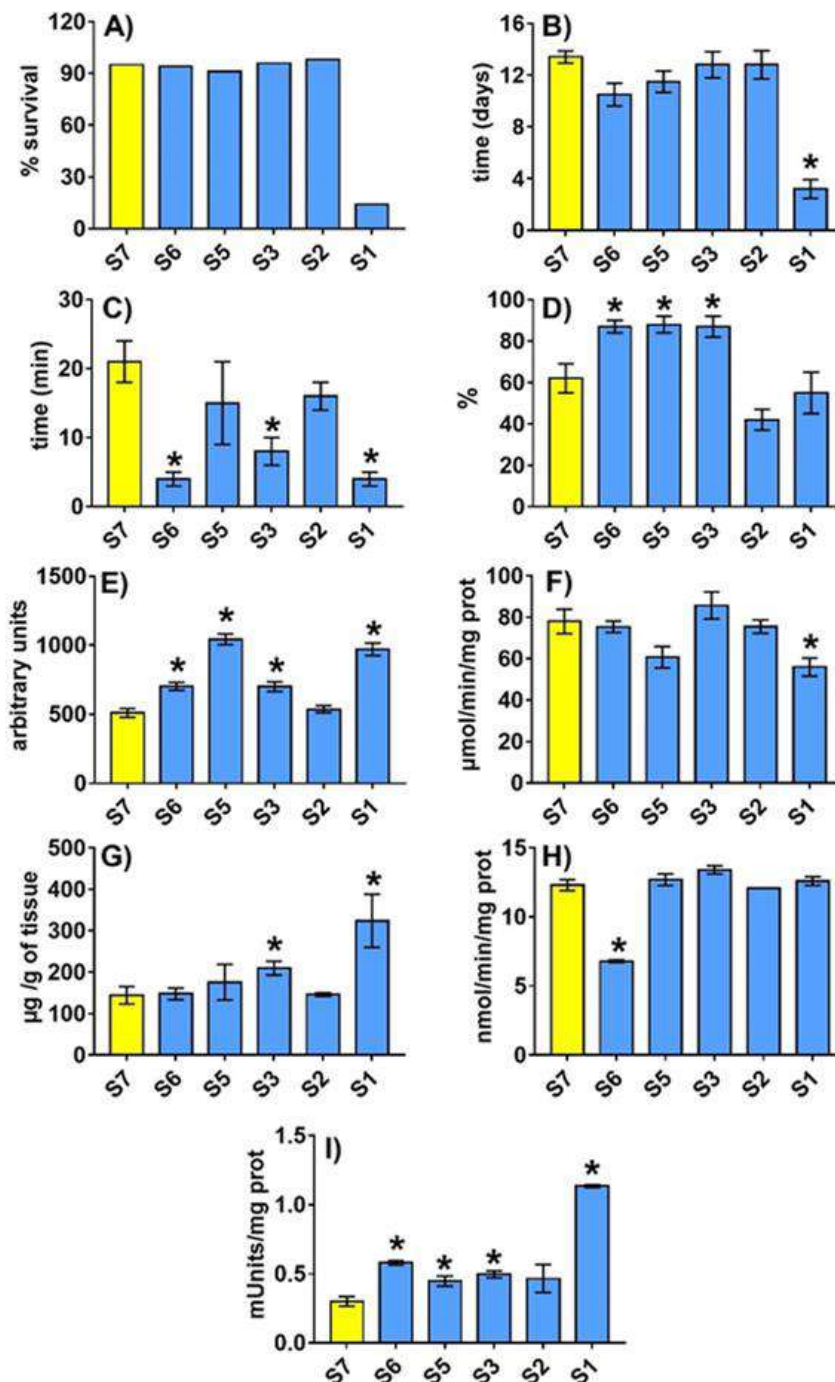


Figure 4. Biomarker modulation in mussels transplanted into the Pialassa Baiona lagoon. Data from all sites (mean \pm SEM) have been measured in mussels collected after 30 days of exposure, except for S1, referred to mussels collected after 3 days of exposure due to high mortality. A, survival (SURV); B, Stress on stress (SOS); C, lysosomal membrane stability (LMS); D, lysosome to cytoplasm volume ratio (LYS/CYT); E, lipofuscin (LIF); F, catalase activity (CAT); G, metallothionein content (MT); H, acetylcholinesterase activity (AChE); I, peroxisomal acyl-CoA oxidase (AOX). Asterisks indicate statistically significant differences compared to the control site S7 (One-Way ANOVA, Duncan's post-hoc test).

LMS is the most sensitive biomarker of general stress in bivalves and previous studies highlighted strict relationships between the xenobiotic-driven decrease of this parameter in *Mytilus*

spp. and alterations to higher functions, such as growth and/or reproduction (Moore et al., 2006; Martinez-Gomez et al., 2008). Data reported in Figure 4C indicate a significant LMS reduction in digestive glands from mussels collected at S1 (3-day exposure), S3 and S6 (30-day exposure) compared to control mussels from S7. Interestingly, none of the measured metals, physico-chemical factors and/or biological endpoints was correlated with trends of LMS in exposed mussels (Fig. 5, Table S3). Although positive relationships between (dissolved) metal exposure and LMS have been reported in mussels under laboratory conditions (Viarengo et al., 1997), no correlation has been highlighted following 28 days of *in situ* exposure into a nearby lagoon (i.e. Pialassa Piomboni, Ravenna, Italy; Capolupo et al., 2017).

The mussel exposure to contaminated environment is frequently associated to an augmented permeability of the lysosomal membrane and an overall lysosomal swelling. In the present study, mussels deployed into S3, S5 and S6 showed a significantly higher LYS/CYT compared to the control site S7 (Fig. 4D), following trends significantly correlated to tissue levels of Ba, Mn and Zr (Fig. 5). The S3 and S5 were located in proximity of streams ensuring the flowing of industrial wastewater from southern and eastern settlements to the Adriatic Sea, while S6 was located along a channel that receive water from Lamone river after having crossed the natural areas located to the west (Fig. 1); thus, these data outline the need for a better understanding of the spatial/temporal relationships between different source of lagoon water (industrial and continental), metal release into the environment and lysosomal dysfunction in the exposed biota.

Lysosomal biomarker of general stress, as LMS and LYS/CYT, describe the overall health status of tested organisms and may thus be modulated by a wide range of natural/anthropogenic stressors; therefore, considering the diverse array of human activities directly and indirectly linked to the Pialassa Baiona lagoon, it cannot be ruled out that further organic pollutants may have played a role in observed lysosomal responses.

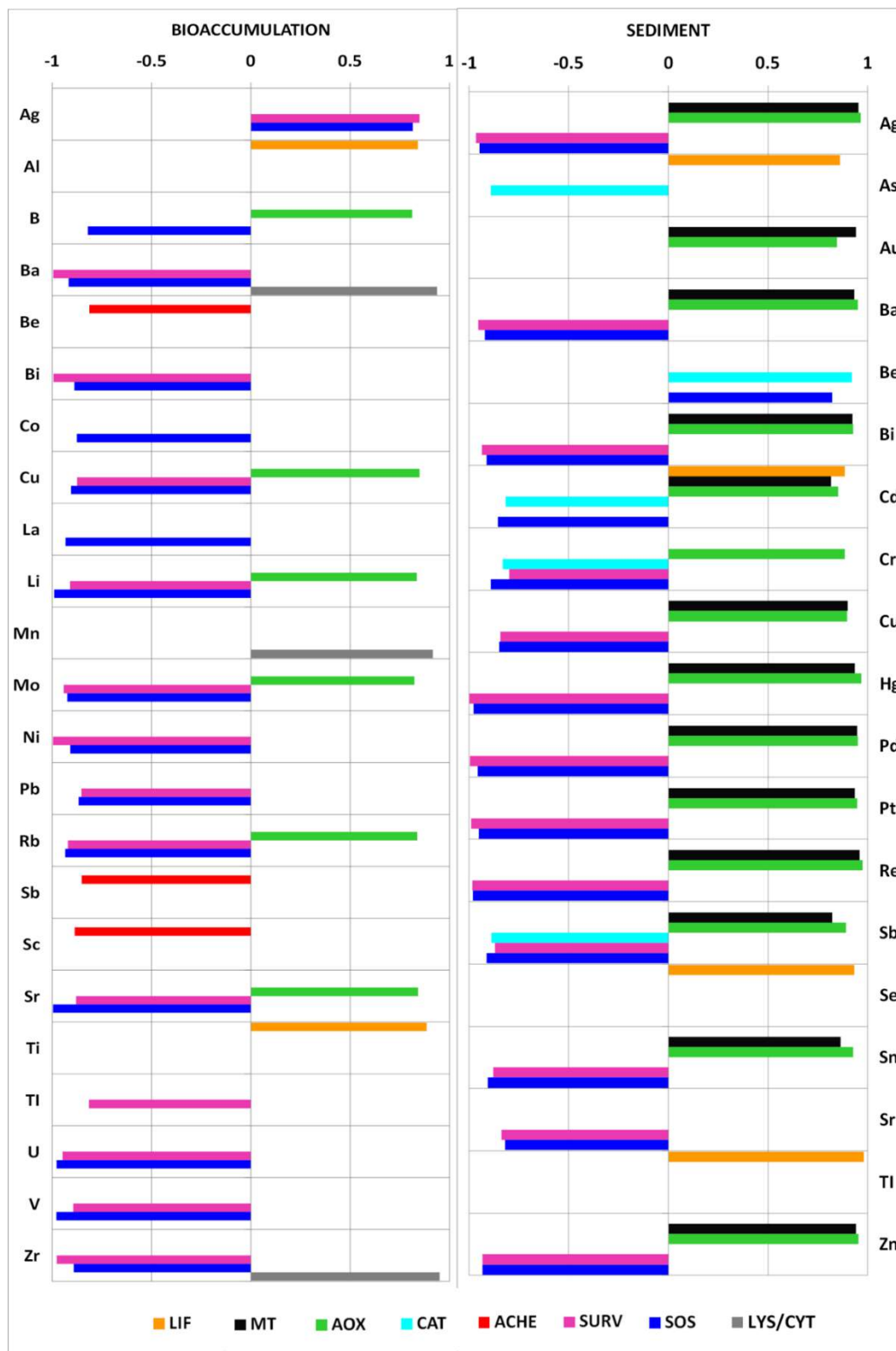


Figure 5. Correlation between biological data and metal concentrations. Pearson's coefficients calculated between biological data and levels of metals measured in mussel tissues (bioaccumulation) and sediment. Only coefficients showing a significant correlation ($p < 0.05$) are reported in the figure.

Mussels from all sites, except S2, showed a significant accumulation of LIF compared to those deployed into the reference site (S7) (Fig. 4E). LIF represent the ultimate products of the lipid peroxidation of biological membranes; they are composed by oxidatively modified proteins, lipid degradation by-products, carbohydrates and metals, and appear as granules within lysosomes of cells composing the digestive tubules epithelium (Moore et al., 2008). Previous biomonitoring studies highlighted clear relationships between LIF accumulation in mussels, the metal uptake and the metal spatial/temporal distribution into enclosed marine systems as bays and lagoons (Moschino et al., 2011; Capolupo et al., 2017). Accordingly, we identified significant and positive correlations between LIF, Al and Ti tissue content in mussels, as well as with As, Cd, Se and TI measured in sediments (Fig. 5). In addition, a significant and negative correlation has been observed between LIF and the activity of the antioxidant enzyme CAT (Table S3). The latter showed a significant decrease *versus* control (S7) in mussels exposed at S1 (Fig. 4F), suggesting that beside ROS formation, the exposure to relatively high metal concentrations might also down-regulate the overall mussel antioxidant defences. CAT also showed negative correlation with elements previously found to elicit pro-oxidant effects in mussels, as As, Cd, Cr, Sb (Franzellitti et al., 2012; Benali et al., 2017; Coppola et al., 2018), and with temperature trends within the lagoon. This indicates that both chemical and physical factors might have cumulatively influenced the mussel ROS scavenging activity over the exposure within the lagoon.

MT have been used as biomarker of exposure in this study owing to their high heavy-metal-binding capacity (Lavradas et al., 2016). Compared to control site S7, a significant MT synthesis induction has been recorded in the digestive gland of mussels from S3 and S1 (Fig. 4H), both located in the southern part of the lagoon in proximity of a large industrial complex (Fig.1). MT trends showed a positive and significant correlation with the sediment concentration of metals known as MT up-regulator, including Cd, Cu, Zn, Pd, Pt, Sn and Hg (Viarengo et al., 2001; Rocha et al., 2016; Zimmermann and Sures, 2018), and other less studied such as Ag, Au, Ba, Bi, Re, and Sb (Fig. 5). Positive relationships were detected MT trends and fluctuations of temperature and conductivity (Table S3), indicating that physico-chemical factors may interactively modulate MT in mussels from contaminated areas.

The activity of the enzyme AChE, which mediates the termination of the nervous impulses driven by the neurotransmitter acetylcholine, was significantly inhibited in mussels from the northern site S6 (Fig. 4H). AChE is typically inhibited by pesticides, although in mussels it may also be inhibited by high levels of metals (Lionetto et al., 2003). Apart from a negative relationship with

Be, AChE trends did not show significant relationships with metals measured in sediments and mussel tissues (Fig. 5), nor with other biological endpoints measured in deployed mussels (Table S3). Compared to the S7, sediments collected in S6 showed a 20 times higher enrichment of Hg (Fig. 2), which is known as a potent AChE inhibitor in mussels, notably when co-exposed to fluctuations of other physico-chemical parameters, such as temperature (Coppola et al., 2017).

The possible impact of organic xenobiotics on the physiology of transplanted mussels has been screened through the analysis of the Acyl-Co A oxidase (AOX) activity. *In situ* deployed mussels showed a significant AOX increase following exposure at all sites except S2 (Fig. 4I). These data corroborate previous evidence of a diffuse PAH contamination (notably pyrene, fluoranthene and anthracene) in sediment and mussels collected from the same area (Vassura et al., 2005; Fabbri et al., 2006). Vassura et al. (2005) also found clear relationships between patterns of PAHs and Hg in the lagoon sediments. Accordingly, many metals herein measured in sediments, including among others Ag, Ba; Hg, Cu, Cr, Cd and Zn, as well as mussel tissues concentrations of B, Cu, Li, Mo and Sr showed positive correlation with AOX (Fig. 5), suggesting a possible metal-induced peroxisome proliferation and a similar spatial distribution of metals and POPs within the lagoon.

The output produced by biomarkers data integration performed through the MES is reported in Figure 6. The LMS was chosen as guide parameter for the data integration. A good health status (HSI = A) was assigned by the Expert System to mussels *in situ* exposed at the reference site S7 and at S2. This output confirms the good environmental quality of the lagoon north-eastern area, as previously identified based on chemical and biological evidence (Franzellitti et al., 2010), and is in line with the lack of biomarker alterations observed in mussels from S2. Although this site is located in the southern part of the lagoon, i.e. in proximity of industrial settlements, it is directly exposed to the marine water entering the lagoon indicating the relevance of high water circulation and oxygenation to the fitness of the resilient biota. A moderate stress level (HSI = C) was assigned to mussels exposed at S5, while mussels from S1, S3 and S6 were classified as heavily stressed (HSI = D) (Fig. 6). In line with the metal contamination/bioaccumulation and coherently with MT levels measured in mussels, an excess of metal contamination was identified in S1 and S3. Moreover, the MES associated the health status alterations of mussels exposed at S1, S3, S5 and S6 to the presence of persistent organic compounds (POPs), in line with measured AOX trends. Although organics were not included in the analytical design of this study, this output is consistent with previous evidence of PCB and PAH contamination across the lagoon (Vassura et al., 2005; Fabbri et al., 2006; Guerra,

2012) and may confirm the co-occurrence of multiple stressors in different areas of the Pialassa Baiona lagoon

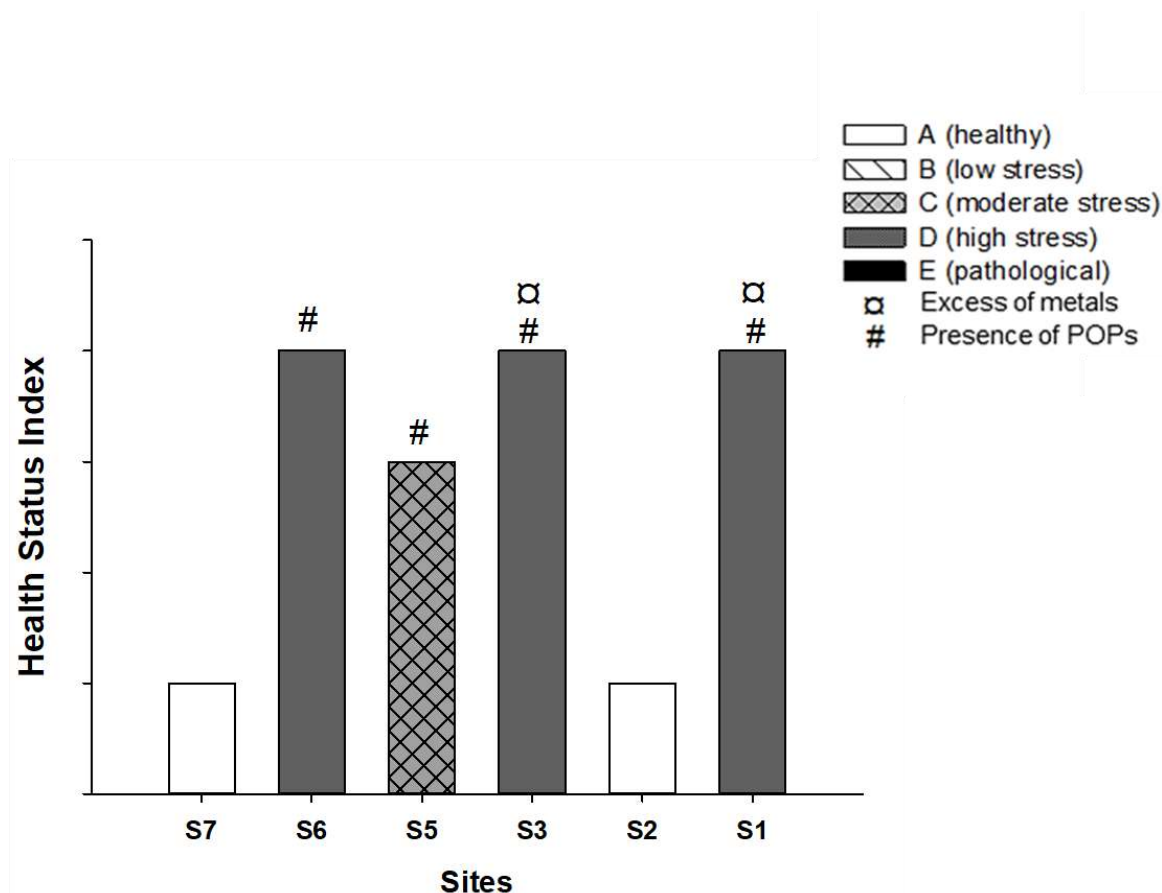


Figure 6. Biomarker data integration. A unique Health status index (HSI) has been generated using the Mussel Expert System (MES, developed by Dagnino et al., 2007) based on biomarker alterations observed at each site. The LMS was chosen as guide parameter for the HSI assessment.

4 Conclusions

The integrated approach implemented in this study provided accurate and reliable clues on the distribution of metals in the Pialassa Baiona lagoon, their bioavailability for exposed mussels and their effects on key biological functions, posing also attention to seldom considered elements such as Ag and Sb.

The physico-chemical data revealed clear differences among S1 and other investigated sites. The water in Magni Channel (S1) showed higher column temperature, reduced EC and a lower pH value, for the entire monitoring period generating local conditions aligned with the worst forecasted climate change scenarios.

The geochemical analysis of total elements indicated that sediments in Pialassa Baiona are a mixture of carbonate and silicates with variable amount of organic matter. The total concentrations of the investigated elements are within the range of regional background. Exception is made for Zn and Cu, and Pb respectively double (in S1) and slightly above (S3 and S5) the local natural background values.

The aqua regia digestion identified a south-north decreasing contamination trend, with highest enrichments of Hg, Pt, Au, Ag, Mo, Re, Cd, Pd, Zn; Sn, and Cu (in enrichment order, Fig. 2) being at least two folds enriched than S7 (internal control). Moreover, the most affected sites are in the south-western areas, which consistently receives wastewater effluents from a near petrochemical settlement (south) and drainage water from the inland (west).

Bioaccumulation analysis performed in transplanted mussels proved suitable to evaluate the bioavailability of metals in relation to their distribution across the lagoon. Integrating physical, chemical and biological measures allowed for defining causal relationships between observed mortality rates, lysosomal dysfunctions, oxidative stress induction, biomarker of exposure modulations and the metal distribution within the lagoon. Besides this, the different channels examined within the lagoon proved to be an excellent study system for evaluating climate change effects (i.e. modified temperature, pH, and salinity) combined with pollution. In addition, the use of the MES proved suitable to identify the impact induced by specific substances on the overall mussel fitness and to estimate the toxicological pathways occurring in highly pressure lagoons.

Overall, data from this study assign a general scarce environmental quality to the Pialassa Baiona lagoon; considering the heterogeneity of this area in terms of geological, physico-chemical and anthropogenic factors, regular biomonitoring surveys should be performed to control the onset of conditions which might exacerbate the impact of metals and other pollutants, notably on a seasonal timeframe. In this respect, the integrated strategy undertaken in this investigation represents a promising approach for the biomonitoring of this area and, more generally, for reliably assessing the environmental quality of highly pressured lagoons.

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Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring

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Abstract:

Coastal lagoons are complex environments threatened by natural and anthropogenic stressors. Here, we tested the effectiveness of combining physical, geochemical and chemical measurements with biomarker data obtained in field-exposed marine mussels (*Mytilus galloprovincialis*) as a biomonitoring strategy for a highly pressured lagoon (Pialassa Baiona, Ravenna, Italy). Data showed a spatial trend of sediment contamination by Hg, Pt, Au, Ag, Mo, Re, Cd, Pd and Zn. Local conditions of high water temperature/low conductivity were detected among selected sites. After a 30-day in situ exposure, Ag and Hg were the most bioaccumulated elements (10 and 5 folds, respectively) in mussels followed by Sb, Al, Ti and Fe. Decreased survival, lysosomal dysfunctions, increased metallothionein content and peroxisome proliferation were observed in mussels in relation to metal spatial distribution and physico-chemical fluctuations. Overall, this study provides a further confirmation of the role of biomonitoring to reliably assess the environmental quality of highly pressured lagoons.

Keywords: Coastal lagoon; Sediment geochemistry; Metal bioaccumulation; Biomarkers; Biomonitoring;

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1 Introduction

Being at the boundary between terrestrial and marine ecosystems coastal lagoons are, by definition, unstable and complex systems morphologically dependent and shaped by natural continental evolution (rivers sediment transport and discharge), sea level variations, coastal dynamics, storminess and local geologic processes (e.g. subsidence) (Bird, 1994; Newton et al., 2014). In natural conditions, coastal lagoons receive fresh and nutrient-rich inputs from the land-side, that combined with shallow, warm and relatively stable water, sustain a complex food chain (from algae, to macro benthos, fish and swamps birds), providing a multitude of life-supporting (Aliaume et al., 2007; Dominik et al., 2014; Petry et al., 2016).

The naturally complicated equilibrium of these environments has often been impacted by strong anthropic development in the last century, especially in those lagoons close to major cities and harbours. With the beginning of demographic boom and of the correlated industrial development, lagoons have been converted into commercial ports or industrial areas and in the worst case as processing water disposal sites (Guerra, 2012; Guerra et al., 2014).

Water and sediment geochemistry of lagoon environments have been widely explored in order to define water status (Mollema et al., 2013; Greggio et al., 2020), sediment provenance and local background concentrations (Migani et al., 2015; Borghesi et al., 2016; Greggio et al., 2018a) or levels of occurring pollutants, notably heavy metals (Nazneen et al., 2019; Saidi et al., 2019), organic compounds (León et al., 2017; Mattes et al., 2018) and emerging contaminants (Pignotti et al., 2017; Pignotti and Dinelli, 2018).

The geochemical characterization of sediments and water is important, but alone often does not allow for an accurate and reliable evaluation of the environmental status of lagoon ecosystems (Carvalho et al., 2014; Dahms, 2014; Moreno-González et al., 2015). Physico-chemical properties and geological background of lagoons can influence the response of exposed organisms to pollutants, inducing potential interactive effects due to changes in bioavailability and toxicity (Migani et al., 2015; Piggott et al., 2015; Borghesi et al., 2016). Chemical assessments fail in detecting biotransformation products, eventually more toxic than the parental compounds (Buryškova et al., 2006; Braga et al., 2018), and in estimating synergistic/antagonistic effects induced on the fitness of the exposed biota (Lari et al., 2017).

For these considerations, multidisciplinary applications integrating geological/chemical parameters, pollutants bioaccumulation and biological effects may represent a promising approach to assess the environmental status of highly pressured coastal systems.

In the framework of biomonitoring programs of marine areas, particular attention has recently been addressed to the assessment of biomarkers of environmental stress (Turja et al., 2014; Capolupo et al., 2017; Mansour et al., 2020). Biomarkers are defined as indices of either exposure or effect of pollutants induced at biochemical, cellular and physiological level on exposed organisms (Viarengo et al., 2007a). They have successfully been used in association to bioaccumulation analyses to characterize “sub-organism” toxicological dynamics occurring in highly contaminated environments and provide crucial and early-warning information for planning urgent intervention or following the effects of remediation plans (Donnini et al., 2007; Capolupo et al., 2017). The simultaneous analysis of a wide set of biomarkers offers accurate information on the sub-lethal alterations induced by multiple stressors and may allow to correlate observed effects with specific classes of pollutants (Shaw et al., 2011; Turja et al., 2014). Moreover, biomarkers provide a unique contribution to determine the synergistic effect of pollutant mixtures, even when single contaminants occur at low concentrations. Biomarker-based assessments are thus currently promoted under the current EU legislation for monitoring surveys of marine and transitional environments (2000/60/EC; 2008/56/EC; Piló et al., 2017).

In the investigated area previous works mainly focused on organic pollutants and mercury in sediments (Trombini et al 2003; Guerra, 2012; Guerra et al., 2014). The bioaccumulation of PHAs in mussels (Fabbri et al., 2006) as well as mercury and methylmercury in clams (Trombini et al., 2003) are the only studies involving living organisms in the area. Inorganic pollutants are poor investigated in the area and when considered they are limited to the ordinary Cd, Cu, Hg, Ni, Pb and Zn in sediments (Matteucci et al., 2005; Guerra et al., 2014) without integration with biomarkers. Only a recent study by Capolupo et al. (2017) included, among others, metals bioaccumulation on mussels in an adjacent coastal lagoon. Moreover, a recent hydrogeological study reported water physico-chemical alterations and the abundance of seldom analysed metals (Ag and Sb among others) in surface drainage water entering in the investigated lagoon (Greggio et al., 2020).

For these reasons, the aim of this work was to evaluate the effectiveness of integrating water physico-chemical data, sediment geochemistry, metal bioaccumulation and biomarker measurements as a biomonitoring strategy for highly contaminated coastal lagoons. The study was conducted in a shallow and brackish basin located along the North-Western coast of the Adriatic Sea (Pialassa Baiona, Ravenna, Italy). In this area, previous works highlight the presence of organic pollutants in sediments (Fabbri et al., 2006; Guerra, 2012; Guerra et al., 2014; Trombini et al 2003; Vassura et al. 2005) and their potential for being bioaccumulated by mussels (Fabbri et al., 2006).

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and clams (Vassura et al., 2005). Inorganic pollutants were less investigated and the current information is limited to few elements, as Cd, Cu, Hg, Ni, Pb and Zn (Matteucci et al., 2005; Trombini et al., 2003; Guerra et al., 2014). A recent hydrogeological study reported water physico-chemical alterations and the abundance of seldom analysed metals (Ag and Sb among others) in surface drainage water flowing into the investigated lagoon (Greggio et al., 2020). Therefore, a wide spectrum of elements was measured in sediments collected from different sites within the lagoon and Biological parameters were assessed in adult specimens of the Mediterranean mussel *Mytilus galloprovincialis* transplanted into different sites in situ within at the same location the lagoon. Finally, a battery of biological endpoints was measured in mussels, including, and encompassed lysosomal endpoints responses, oxidative stress parameters and biomarkers of exposure to specific classes of pollutants.

2 Materials and methods

2.1 Study area and sampling sites

The Pialassa Baiona (Fig. 1) is a brackish lagoon located northward the town of Ravenna (Italy), between 44°27'55.5"N and 44°31'31.3"N and 12°14'9.7"E and 12°16'10"E that cover an area of about 10 km². The lagoon is part of a barrier-lagoon system that characterized the historical evolution of the Adriatic Sea coastline in the area (Amorosi et al., 1999). It is formed by small, shallow ponds (0.5–1 m depth) and deeper artificial channels (2–3 m depth) that permit the incessant water exchange with the Adriatic Sea driven by tides. It is connected to the Adriatic Sea only through the Candiano Channel that also is the Ravenna operative industrial harbour. The Pialassa Baiona is mainly adopted by local citizens as recreational fishing and hunting site and few clam farming activities exist in the northernmost portion.

The lagoon receives freshwaters from the west through three main channels (Fossatone, Cerba and Cupa-Magni channels) which drain agricultural lands and collect effluents from industrial and municipal treatment plants, located in the southernmost part (Fig. 1). The drainage water entering the lagoon are often rich in nutrients and dissolved metals (Mollema et al., 2013; Greggio et al., 2018b). The highest concentration of nutrients, as well as the highest bacteriological content, are observed in the southern part of the lagoon, in proximity of the via Cupa channel (Soprani et al., 1994). In addition, during the 60s and 70s, high quantities of chemical compounds were discharged

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into the lagoon through the via Cupa and Magni channels, carrying wastewater from the industrial area close to the southern border (Fabbri et al., 1998).

Previous investigations showed that high levels of metals, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and other pollutants occurred in surface, subsurface and suspended sediments with N-S increasing gradient (Trombini et al., 2003; Matteucci et al., 2005; Guerra, 2012; Guerra et al., 2014). On these bases, seven sites were selected within the Pialassa Baiona lagoon in order to cover its overall geochemical complexity and spatial trend of anthropogenic pressures (Fig. 1). All sites (S1 to S7) were investigated for sediment geochemistry, while metal bioaccumulation and biomarker responses were measured on mussels transplanted into six sites (S1, S2, S3, S5, S6 and S7), since *in situ* transplantation into site S4 was not possible for technical reasons. Mussels were exposed into the lagoon for 30 days, which is acknowledged as a suitable time for sessile organisms to bioaccumulate contaminants at environmentally representative levels and to develop physiological responses to “in site” pollution (Viarengo et al., 2007b). Since previous investigations highlighted a lesser incidence of metals and organic compounds in the north-western part of the lagoon (Trombini et al., 2003; Fabbri et al., 2006; Donnini et al., 2007; Franzellitti et al., 2010), Site 7 (S7), located at about 0.5 km from the north-western border, was defined as internal control site for both geochemical and biological assessments.

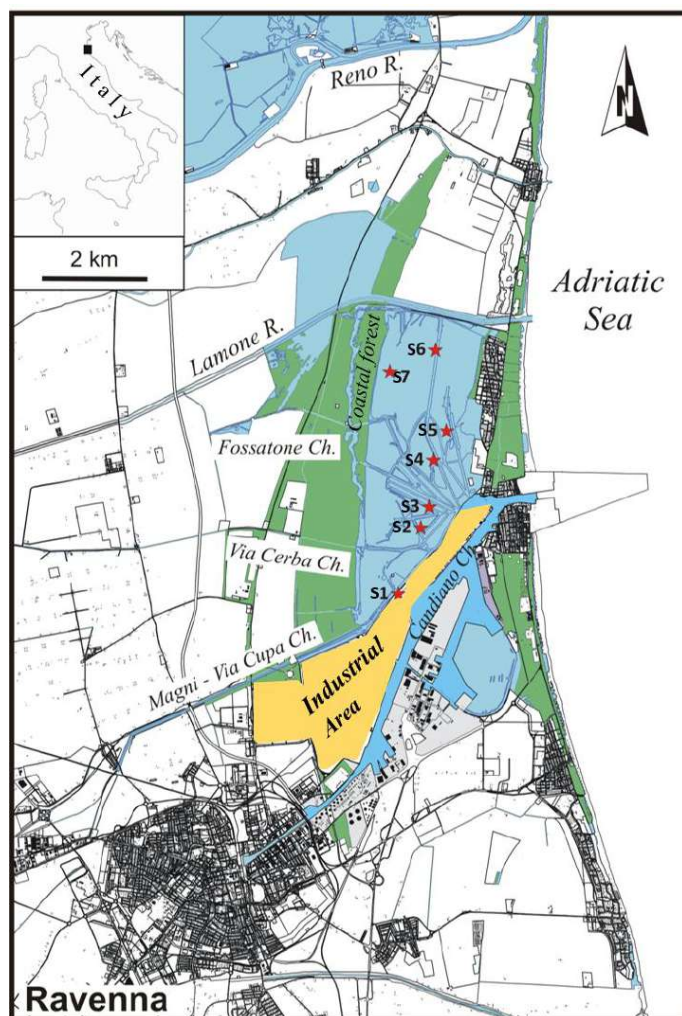


Figure 1. Location map and sampling points (S1 to S7) within the Pialassa Baiona lagoon (Ravenna, Italy). The abbreviations "R." and "Ch." refer to the main rivers and/or channels which supply freshwater to the lagoon. The yellow shape represents the industrial area settled at the south-eastern boundary of the Pialassa Baiona lagoon.

2.2 Field activities and measurements

In order to have a better characterization of the environmental conditions influencing the organisms' response, measurements for physico-chemical parameters were conducted at each site. During mussel field exposure (from June 1st to June 30th) six vertical profiles (10 cm resolution) of electrical conductivity, temperature and pH, using a CTD probe were conducted.

2.3 Sediment geochemistry

The bottom sediments were sampled at each site with a manual corer using a Plexiglas tube. After the extrusion, the sections 0 - 5 cm was retained for analysis, placed in polyethylene bottles, were stored at -25 °C. For geochemical analysis, sediment aliquots were dried at 60 °C and homogenized by grinding in agate mortar. On the bottom sediments total elements concentration by X-ray fluorescence spectrometry (XRF) and pseudo-total (mobile/available) concentrations by aqua regia digestion (AR) have been performed.

The total metal content of 29 chemical elements (SiO₂, TiO₂, Al₂O₃, Fe₂O₃, MnO, MgO, CaO, Na₂O, K₂O, P₂O₅, S, As, Ba, Ce, Co, Cr, Cu, La, Nb, Ni, Pb, Rb, Sc, Sr, Th, V, Y, Zn, Zr) has been measured by XRF, using a Philips PW 1480 spectrometer equipped with a Rh tube on pressed powder pellets following matrix correction methods suggested by Franzini et al. (1972; 1975) Leoni and Saitta (1976) and Leoni et al. (1982). Thirty international reference materials were used for the instrument calibration, while accuracy was evaluated using 4 international reference samples, namely BR, BCR-1, TB and AGV-1 (Govindaraju, 1984). The average accuracy was higher than 5 % for trace-element determinations. In order to quantify the pseudo total concentration, 15 g of sediment were digested with a modified aqua regia solution of equal parts concentrated HCl, HNO₃ and deionized H₂O for one hour in a heating block of hot water bath at the Bureau Veritas Laboratories in Vancouver (Canada). Fifty-three chemical elements (Ag, Al, As, Au, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Ge, Hf, Hg, In, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pd, Pt, Rb, Re, S, Sb, Sc, Se, Sn, Sr, Ta, Te, Th, Ti, Tl, U, V, W, Y, Zn, Zr) were analysed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

The volatile content (including humidity, organic matter, water incorporated in the lattice of clay minerals, carbon dioxide in the carbonate minerals) was evaluated by thermal analysis using a Setaram TAG24 double furnace apparatus, with simultaneous registration of thermogravimetric (TG), derivative thermogravimetric (DTG), differential thermal analysis (DTA). A CO₂ atmosphere was used to increase the temperature of carbonate decomposition and the carbonate content was estimated by quantifying the weight loss at temperature > 700 °C.

2.4 Mussel collection and handling

Adult specimens of the Mediterranean mussel (*M. galloprovincialis*) with a shell-length of 5 ± 1 cm were collected by professional fishermen of the "Cooperativa CONISUB" (Marina di Ravenna,

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Italy) along the north-western Adriatic Sea coast in high quality marine areas (Zone A) and immediately transferred to the laboratory in seawater tanks with continuous aeration. Prior to the *in situ* deployment, all animals were acclimatized for 6 days in aquaria containing 60 L of aerated natural seawater (at a ratio of 1 mussel/L) at 16 °C and natural photoperiod, and fed daily on an algal slurry (Coral Diet Filtrator, Xaqua, Italy) after water renewal. Seventy organisms were dissected prior to the *in situ* transplantations (time 0, T0) for bioaccumulation and biological endpoint analyses. Digestive glands and gills were immediately collected, frozen in liquid nitrogen and stored at -80 °C until analysed. At the same time six groups of seventy organisms each were put in fishnet bags, fixed to piles and driven into the sediment at sites S1, S2, S3, S5, S6 and S7 (Fig. 1) at about 50 cm from the bottom to ensure continuous submersion during the whole exposure period. Deployed mussels were regularly controlled at three days intervals and dead mussels were eventually recorded for survival assessment. Since previous investigations identified the Magni channel as a heavily polluted site (Fabbri et al., 2000; 2003; Matteucci et al., 2005), three additional fishnet bags, with seventy organisms each, were transplanted into S1 to execute biomarkers with shorter timestep.

2.5 Metal bioaccumulation analysis

Metal bioaccumulation was evaluated on mussels prior to the *in situ* deployment (Time 0, T0) and after 30 days of field exposure into the lagoon. Metal bioaccumulation from each site was used to calculate the bioaccumulation factor as enrichment/depletion compared to T0. Samples (0.2 g each) were digested in a microwave system with ultrapure HNO₃. The resulting fluid was diluted by adding deionized water to a final volume of 30 mL, at the Department of Environmental Science of the University of Siena. Analytical blanks and certified reference material were included in the analysis, specifically the mussel tissue ERM-CE278 (*Mytilus edulis*) from the Institute for Reference Materials and Measurements, Geel, Belgium.

The samples were analysed by inductively coupled plasma quadrupole mass spectrometry (ICP-QMS) using an Agilent 7500ce instrument at the laboratories of the Federal Institute for Geosciences and Natural Resources in Berlin. Atomic fluorescence spectrometry was used for the determination of mercury (Instrument PSA 10.035 Millennium Merlin 1631). The Table S1 reports the results for the investigated elements in the certified reference material. Additional details can be found in Capolupo et al. (2017).

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225 2.6 *Biological endpoints*
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227 2.6.1 Survival percentage and Stress on Stress
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229 The survival (SURV) was calculated as the percentage of mussels found alive over the exposure
230 period with respect to the initial number of individuals (70 at each site).
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232 For Stress on Stress (SOS) assessment, immediately after collection, 15 mussels per site were
233 subjected to anoxia by air exposure at 15 °C in a humidified chamber. Death symptoms were open
234 valves and absence of muscular activity. The survival was assessed daily, and results expressed as
235 the time at which 100% mortality was achieved (Viarengo et al., 2007a).
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237 2.6.2 Cytochemical parameters
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239 Lysosomal membrane stability (LMS), lysosome to cytoplasm volume ratio (LYS/CYT) and
240 lipofuscin content (LIF) were assessed in the digestive gland from 20 mussels per each site (N = 20).
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242 After dissection, glands were mounted on specific aluminium supports for cryostat microtomy
243 (chucks), frozen in N-hexane at -70 °C, and stored at -80 °C. All assessments were performed
244 according to the UNEP/RAMOGÉ manual (1999) on 10 µm-thick gland sections obtained using a
245 cryostat (MICROM HM 505 N) at -30 °C. The LMS was measured as the time necessary to produce
246 the maximum staining reaction between the lysosomal hydrolase N-acetyl-β-hexosaminidase and a
247 specific substrate (Capolupo et al., 2016). Briefly, sections were incubated at 37 °C in a shaking water
248 bath with a destabilization buffer (0.1 M Na-citrate, pH 4.5) for different times (0, 3, 5, 10, 15, 20,
249 30 and 40 min), exposed to the substrate naphtol AS-BI N-acetyl-β-D-glucosaminide (Sigma, N4006)
250 for 20 min and finally stained with the diazonium dye Fast Violet B (Sigma, F1631) (1 mg/ml in 0.1
251 M phosphate buffer, pH 7.4). For each site, slides not exposed to the destabilization buffer (0 min)
252 were screened for the LYS/CYT assessment, which was determined by evaluating the cytoplasmic
253 and lysosomal areas according to Capolupo et al. (2017). The LIF determination was performed
254 according to Martin-Diaz et al. (2009). Gland sections were fixed in calcium formol and immersed
255 for 5 min in an aqueous solution of 1 % ferric chloride and 1 % potassium ferricyanide in a 3:1 ratio.
256
257 For all lysosomal parameters, tissue sections were assessed under a light microscope
258 (Axioskop 40, Carl Zeiss, Milan, Italy) equipped with a 40X objective and a digital camera (AxioCam
259 MRC, Carl Zeiss, Milan, Italy) as reported by Capolupo et al. (2016). Results were expressed as
260 labilization period for LMS, percentage of lysosomal volume inside digestive cells for LYS/CYT and
261 arbitrary units for LIF.

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2.6.3 Enzymatic biomarkers

The acetylcholinesterase activity (AChE) activity was determined in mussel gills as previously reported (Valbonesi et al., 2003). Gills from 5 mussels per site (N = 5) were homogenized in a 0.1 M phosphate buffer, pH 7.4, and centrifuged at 9000 ×g at 4 °C for 30 min. Supernatant aliquots were incubated at 25 °C with 0.5 mM acetylthiocholine iodide (ASCh) and 0.33 mM DTNB (Ellman et al., 1961), and read at 405 nm for 10 min. Results are reported as nmoles/min/mg protein. The sample protein concentration was measured according to Lowry et al. (1951).

For catalase (CAT) assessment, about 50 mg of digestive gland was dissected from 5 mussels per site (N = 5), homogenized in 50 mM potassium-phosphate buffer (pH 7.0, 0.5 mM Na₂EDTA) and centrifuged at 15,000 g at 4 °C. The CAT activity was determined by measuring the time-dependent decrease of absorbance at 240 nm in the presence of 55 mM H₂O₂. Data were expressed as μmol/min/mg of total protein.

The Peroxisomal acyl-CoA oxidase (AOX) activity was determined on five pools of 2 digestive glands per site (N = 5) according to Orbea et al. (2006). Tissues were homogenized in four volumes of TVBE buffer (1 mM sodium bicarbonate, 1 mM EDTA, 0.1 % ethanol and 0.01 % Triton X-100), pH 7.6 and centrifuged at 500 × g for 15 min at 4 °C. Supernatants were diluted 1:10 in TVBE buffer and spectrophotometrically assayed for AOX activity at a wavelength of 502 nm. Data were expressed as milliunits/mg of AOX protein (equivalent to nmol H₂O₂/min x mg protein).

2.6.4 Metallothionein content (MT)

The metallothionein (MT) content was analysed on five pools of 5 mussel digestive glands (about 1.5 g of tissue) per site according to Viarengo et al. (1997). Final absorbance was measured at 410 nm, using reduced glutathione as reference standard. Data were expressed as μg of MT/mg tissue.

2.6.5 Mussel Expert System

Data from biological responses were integrated using the Mussel Expert System (MES) developed by Dagnino et al. (2007) in a unique and synoptic five-level Health Status Index (HSI) ranging from A (healthy) to E (pathological status). The system classification is based on (i) the number of biomarkers altered in the exposed organisms (significant changes greater than 20 % were utilized to evaluate alterations in the health status of the organisms); (ii) the magnitude of the alteration; (iii) the role of the autophagic process and (iv) the level of biological organization affected

(i.e., cell, tissue and organism level). For each biomarker/site, the system calculates an Alteration Factor (AF), which is the ratio between biomarker levels from treatments and control, and an Alteration Level (AL), which defines the magnitude of observed alterations based on the AFs magnitude and the biomarker toxicological profile (i.e. increasing, decreasing, bell-shaped). ALs and AFs are then processed by the system applying a set of rules in the “if...then” form in order to calculate the overall HSI associated to the exposure to each site. The simultaneous alterations of general stress parameters (i.e. lysosomal responses) and biomarkers of exposure to specific classes of compounds (e.g. MT for metals; AOX for POPs) is further computed and eventually recognized by the system as an early warning signal regarding the presence or excess of those pollutants in the monitored sites or tested condition.

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2.7 Data analysis

All data were tested for normal distribution using the Shapiro-Wilk test, while the Levene's test was employed to test the equal variance. Significant differences between S1 to S6 compared to the control site S7 were estimated by a One-Way ANOVA (SigmaStat, SPSS) followed by the Duncan's post-hoc test. The evaluation of potential linear relationships between biological endpoints and physico-chemical parameters (including pH, temperature, electrical conductivity, metal concentrations in sediments and mussel tissues) were conducted through the Pearson's correlation analysis. For all performed tests, significance was established at $p < 0.05$. All analyses were performed using the software SigmaPlot 13 (Systat Software Inc. San Jose, CA, USA).

3 Results and discussion

3.1 Physico-chemical data

The vertical profiles of temperature, pH and electrical conductivity (EC) measured during mussels field exposure as well as a graphical representation of the temperature, pH and EC vertical profiles for June 14th are presented respectively in Table S2 and in Figure S1 of supplementary material. The site S1, located in the Magni Channel at the southern side of the lagoon (Fig. 1), is different from the other sites for all monitored parameters. It shows higher water column temperature, reduced EC and a lower pH value, for the entire monitoring period (Table S2). Specifically, S1 shows the lowest EC among the sites with a marked vertical gradient (Fig. S1A) and

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it is also the site with the highest temperature, up to 32.3 °C. Moreover, it records the highest average profile temperature during the surveying period, at least 2 °C constantly higher compared to all the other sites (Fig. S1B). Site S1 receives freshwater from artificial drainage channel network surrounding the town of Ravenna (Greggio et al., 2018b). Previous investigations also reported water temperature fluctuation wider (10-24 °C) in the southernmost channel with respect to the lagoon average (16-18 °C), which was subjected to variations of about 10 °C within the 24 hours owing to discharges of cooling water from the nearby industrial site (Franzellitti et al., 2010). A thermal vertical gradient is also present in S5 probably associated to local limited water circulation favouring water warming up (Fig. S1A). The pH is the lowest in S1, ranging from 7.2 to 7.8, and with a marked vertical profile. In all the other sites the pH is instead similar and fairly constant around 8. Only at S5 the observed pH values are higher (8.0-8.3) for the entire surveying period (Table S2).

3.2 Sediment data

The results of the geochemical analyses are presented in Table 1. According to the geochemical composition of the major elements (expressed as %), and referencing to the work of Dinelli et al. (2007) which compared geochemical proxies with grain size analysing borehole samples in the same geographic area, the Pialassa Baiona bed sediment samples are classified as fine grained-sediments (with a recalculated SiO₂/Al₂O₃ ratio < 3) enriched in carbonates. The sediments are also rich in organic matter (OM in Table 1, ranging from 1.1 to 3.4) as already reported for adjacent coastal lagoons (Migani et al., 2015). The bulk geochemical composition is quite homogeneous among the different sites, with S1 being slightly enriched in carbonates (Table 1).

The Pialassa Baiona bed sediments composition is a mixture of carbonate and silicates with variable amount of organic matter. The geochemical results, in terms of total sediment composition, are comparable with those reported by Donnini et al. (2007) although the number of sites was different. As far as total concentrations are concerned, these are within the range of regional background, as proposed by Migani et al. (2015), taking into consideration Holocene data from boreholes in the area not affected by anthropogenic inputs (Amorosi et al., 2002; Curzi et al., 2006; Dinelli et al., 2012, Greggio et al., 2018a). Except for sites S6 and S7, Zn and Cu concentrations exceed the maximum value observed in the background sediments, respectively of 124 and 41 mg/kg (Table 1). In particular, for S1 site Zn and Cu total concentrations are double respect to background, respectively 316 and 87 mg/kg, in line with concentration detected in the neighbouring Pialassa Piomboni by Pignotti et al. (2018). Pb concentrations in sites S3 and S5 are 30 mg/kg being

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slightly higher respect to background level established at 29 mg/kg by Migani et al. (2015), but certainly lower than Pb concentration in the Pialassa Piomboni by Pignotti et al. (2018) where a maximum concentration of 257 mg/kg was measured.

Table 1. Results of the chemical analyses on sediments. The table includes either pseudo total Aqua Regia (AR) and total XRF data. Table includes also the ranges observed in boreholes in the area, considered representative of the natural background (Amorosi et al., 2002; Curzi et al., 2006). Bold elements exceed natural background. AR DL indicates Detection Limit for Aqua Regia extraction.

		S1		S2		S3		S4		S5		S6		S7		Natural background	
		AR	DL	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF		
Ag	µg/kg	2		991	-	269	-	268	-	173	-	275	-	179	-	62	-
Al	%	0.01		1.39	6.31	1.41	7.02	1.23	6.98	1.10	6.97	1.35	7.09	1.39	7.07	1.41	6.55
As	mg/kg	0.1		7.8	-	5.9	-	6.1	-	5.9	-	7.8	-	6.8	-	6.7	-
Au	µg/kg	0.2		16.2	-	5.4	-	11.1	-	2.6	-	5.1	-	3.4	-	1	-
B	mg/kg	1		25	-	28	-	26	-	24	-	38	-	33	-	22	-
Ba	mg/kg	0.5		61.9	263	43.7	218	41.8	260	42.6	322	40.1	258	38.7	294	36.5	258
Be	mg/kg	0.1		0.4	-	0.7	-	0.7	-	0.8	-	0.5	-	0.6	-	0.6	-
Bi	mg/kg	0.02		0.6	-	0.38	-	0.35	-	0.26	-	0.38	-	0.31	-	0.28	-
Cd	%	0.01		11.27	11.37	7.45	7.51	7.87	7.96	7.34	7.87	6.10	6.65	7.24	7.43	8.61	9.52
Ca	mg/kg	0.01		0.68	-	0.29	-	0.33	-	0.21	-	0.5	-	0.35	-	0.11	-
Ce	mg/kg	0.1		19.4	42	17	55	17.6	71	16.8	52	17.8	64	18.3	42	20.1	58
Co	mg/kg	0.1		10.2	12	10.9	11	10.7	12	9.9	11	11.7	14	11.5	12	10.6	11
Cr	mg/kg	0.5		70.6	131	57.5	103	51.4	132	47.7	131	59.9	138	59.3	130	47	100
Cs	mg/kg	0.02		1.36	-	1.15	-	1.08	-	1.16	-	1.11	-	1.39	-	1.57	-
Cu	mg/kg	0.01		84.1	87	49.1	52	49.5	53	36.7	44	51.1	52	37.5	38	22.4	24
Fe	%	0.01		2.17	3.42	2.46	3.92	2.23	3.86	2.00	3.69	2.54	3.83	2.30	3.64	2.36	3.72
Ga	mg/kg	0.1		4.4	-	4.2	-	4	-	3.9	-	4	-	4.2	-	4.4	-
Ge	mg/kg	0.1		<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-
Hf	mg/kg	0.02		0.03	-	0.03	-	0.04	-	0.03	-	0.03	-	0.03	-	0.05	-
Hg	mg/kg	0.005		25.7	-	1.42	-	1.48	-	1.89	-	2.38	-	2.26	-	0.1	-
In	mg/kg	0.02		0.03	-	0.03	-	0.02	-	0.02	-	0.03	-	0.02	-	0.03	-
K	%	0.01		0.33	1.77	0.36	1.98	0.31	2.06	0.28	2.04	0.39	1.97	0.36	2.04	0.35	2.03
La	mg/kg	0.5		8.3	19	7.7	26	7.3	22	6.9	18	8	20	8.5	34	9.5	20
Li	mg/kg	0.1		26.1	-	30.6	-	27.8	-	24.9	-	28.7	-	28	-	28.1	-
Mg	%	0.01		1.37	2.41	1.34	2.38	1.30	2.38	1.22	2.44	1.42	2.54	1.55	2.55	1.58	2.35
Mn	mg/kg	1		531	893	527	865	549	896	513	949	511	801	550	897	644	1396
Mo	mg/kg	0.01		2.67	-	3.33	-	2.61	-	2.28	-	3.34	-	1.57	-	0.54	-
Na	%	0.001		0.85	0.68	0.98	0.69	0.90	0.75	0.65	0.99	1.18	0.67	0.85	0.78	0.42	0.85
Nb	mg/kg	0.02		0.37	15	0.28	13	0.25	13	0.29	14	0.34	16	0.37	10	0.29	13
Ni	mg/kg	0.1		50.9	73	56.5	73	50.7	88	46.3	72	55.3	82	51.5	74	46.4	57
P	%	0.001		0.11	0.10	0.06	0.06	0.06	0.07	0.06	0.06	0.08	0.07	0.07	0.06	0.06	0.06
Pb	mg/kg	0.01		27.92	28	22.71	26	22.14	30	18.16	19	26.22	30	20.71	22	11.99	18
Pd	µg/kg	10		46	-	10	-	12	-	11	-	10	-	<10	-	<10	-
Pt	µg/kg	2		37	-	6	-	4	-	4	-	4	-	<2	-	<2	-
Rb	mg/kg	0.1		24.9	113	22.2	128	20.8	128	22.4	113	22.3	130	25.1	114	29.8	89
Re	µg/kg	1		7	-	1	-	2	-	<1	-	2	-	2	-	<1	-
S	%	0.02		0.6	-	0.55	-	0.6	-	0.54	-	0.72	-	0.71	-	0.34	-
Sb	mg/kg	0.02		0.51	-	0.29	-	0.25	-	0.19	-	0.37	-	0.29	-	0.19	-
Sc	mg/kg	0.1		3.4	11	3.6	11	3.4	6	3.2	8	3.7	10	3.7	8	4.1	13
Se	mg/kg	0.1		0.9	-	0.7	-	0.7	-	0.5	-	1.1	-	0.7	-	0.5	-
Si	%	-		-	17.72	-	19.43	-	19.90	-	20.61	-	19.31	-	20.95	-	20.03
Sn	mg/kg	0.1		3.2	-	1.8	-	1.5	-	1.3	-	1.9	-	1.5	-	0.8	-
Sr	mg/kg	0.5		353	368	274.4	300	224.8	252	218	265	259.1	281	281.6	291	290.5	310
Th	mg/kg	0.1		3.5	4	4.1	5	3.6	17	3.6	25	4	11	4.2	10	4.7	7
Ti	%	0.001		0.009	0.308	0.005	0.328	0.006	0.347	0.008	0.349	0.006	0.324	0.010	0.334	0.015	0.336
Tl	mg/kg	0.02		0.26	-	0.17	-	0.21	-	0.19	-	0.27	-	0.2	-	0.14	-
U	mg/kg	0.1		1.3	-	1.5	-	1.2	-	1.1	-	1.3	-	1.2	-	1.1	-
V	mg/kg	2		32	85	33	85	30	99	26	94	35	113	33	94	31	76
Y	mg/kg	0.01		10.02	18	9.39	23	9.28	24	8.86	20	9.7	24	9.63	19	10.58	19
Zn	mg/kg	0.1		306.6	316	129.8	152	133.1	154	101.2	126	146.9	174	109.3	124	58.2	67
Zr	mg/kg	0.1		1.1	108	1.2	91	1	105	1	124	1.1	86	1.2	92	1.9	106
H ₂ O ⁻	%	-		-	2.7	-	4.0	-	2.8	-	2.4	-	3.7	-	2.7	-	2.1
OM	%	-		-	2.8	-	1.8	-	2.0	-	1.7	-	3.4	-	2.2	-	1.1
H ₂ O ⁺	%	-		-	6.0	-	6.5	-	5.4	-	4.4	-	6.5	-	5.8	-	2.3
CO ₂	%	-		-	11.6	-	8.9	-	9.1	-	9.3	-	8.6	-	7.4	-	12.3

LOI	%	-	-	23.1	-	21.2	-	19.3	-	17.8	-	22.3	-	18.1	-	17.8	4.8-29.3
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As regards the Aqua Regia extraction, data showed the presence of elements seldom analysed in sediments of the area. The most striking features are the differences observed between S1 and all the other sites for a number of elements, in particular Ag, As, Au, Bi, Cd, Cr, Cu, Hg, Pb, Pd, Pt, Re, Zn (Table 1). According to Donnini et al. (2007), S7 should be considered as unpolluted site and if its results are taken as reference, a strong enrichment is clear for several elements (Fig. 2). Mercury, which is a well-known pollutant in the area (Miserocchi et al., 1993; Fabbri et al., 1998; Fabbri et al., 2001a, b; Trombini et al., 2003; Matteucci et al., 2005; Guerra et al., 2007; Covelli et al., 2011; Guerra, 2012; Dominik et al., 2014; Borghesi et al., 2016), is the element with the largest enrichment (up to 250 times compared to S7) and reaches relatively high concentrations in the Magni Channel site (S1, up to 25 mg/kg). Mercury dynamics in the lagoon is complex and although the maxima in concentrations are not at the surface, the element is heavily mobilized at the water/sediment interface in the southern part of the lagoon (Covelli et al., 2011). Borghesi et al. (2016) highlighted similar behaviour among Hg and Ag, Au, Cd, Cu and Zn for six different coastal lagoons in the same Region due to metal affinity for the fine fraction of the sediment.

Notable enrichments are then evident in Figure 2 for valuable metals like Pt, Au, Ag, Re and Pd, which showed substantial increase (up to 10-fold) compared to S7 and to median values reported by Cicchella et al. (2015), that represent a useful reference for metal concentrations in mineral matrix obtained with the same analytical technique and at the same laboratory. In particular, Platinum and Palladium are of recent technogenic use and their anthropogenic fluxes greatly exceed the natural ones (Ruchter and Surer, 2015; McGillicuddy et al., 2017). Platinum and Palladium are largely used in automobile catalytic converters (Bossi and Gediga, 2017) and their dispersion increases particularly around urban areas in roadside soils (Ravindra et al., 2004; Ruchter and Surer, 2015; Leopold et al., 2018; Zuzolo et al., 2018), but also in marine areas close to urban settings (Cobelo-García et al., 2011; Abdou et al., 2016).

The Silver concentration reaches a peak at S1 with 0.99 mg/kg, at least four times more than the other sites in the Pialassa Baiona and ten times more than other coastal lagoons in the same Region as resulted by Borghesi et al. (2016). The Ag enrichment is controlled by many factors such as pH, OM, as well as the source material. At typical environmental pH values, Ag is adsorbed onto Fe hydroxides, in preference to Cu and Zn (Lottermoser et al., 1999). In the near Venice lagoon, Giusti and Zang (2002) measured Ag sediment concentrations 5-6 fold higher than this study,

justifying the abundance with both famous glassmaking firms in Murano island and the large volume of untreated sewage sludges discharged into the lagoon from the nearby Marghera industrial area.

Moderate enrichments (four times compared to S7) are shown by Mo, Cd, Zn, Sn and Cu that can be more directly linked to industrial effluents as reported by Giusti and Zang (2002) in Venice lagoon and Borghesi et al. (2016) for the same Region. Enrichments in all these elements also characterize the other sites, although at an order of magnitude lower than S1 (around 2 times).

Many of the metals detected in sediments show a decreasing concentration with increasing distance from the industrial area located in the southern part of the lagoon. This suggests that industrial discharge effluents play a major role in the lagoon contamination compared to effluents from urban and agricultural activities. In facts, the distribution of some elements, including Zn, Cr, Cu, Mo, Ag, and Hg, follow trends similar to those reported for other organic pollutants of industrial origin in the same area (Fabbri et al. 2006; Vassura et al. 2005). Vassura et al.(2005), for instance, detected a clear South-North increasing pattern of prioritized PAHs, with sediments from the Magni channel area (the present S1) showing concentrations of pyrene, fluoranthene, anthracene and phenanthrene up to 10,000 times higher compared to the northern sites.

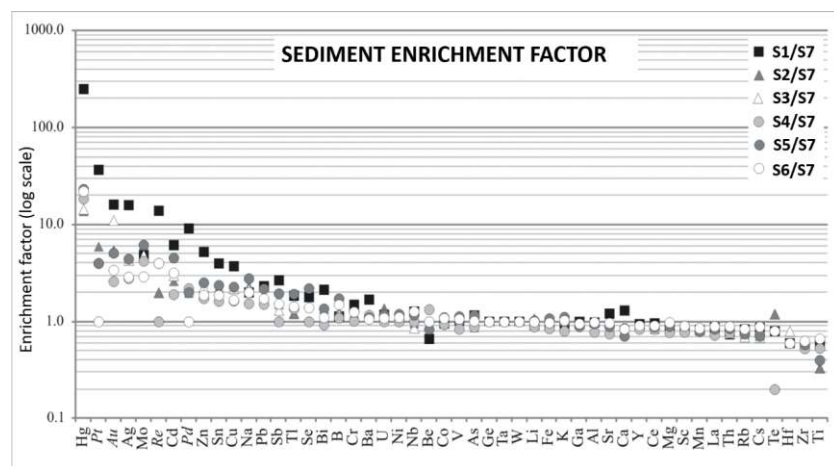


Figure 2. Metal enrichment in sediments from selected sites within the Pialassa Baiona lagoon. Data are normalized on Site 7 (S7), which was selected as internal reference based on previous literature (Trombini et al., 2003; Fabbri et al., 2006; Donnini et al., 2007; Franzellitti et al., 2010). Elements in italics (*Pt, Au, Re, Pd*) were not investigated in the mussel tissues.

3.3 Metal bioaccumulation

Results of the chemical analyses on mussel tissues are presented in Table 2. Data were obtained for all sites except S1, which was characterized by a high mortality rate (more details in section 3.4). T0 data, originating from mussels growing in the Adriatic Sea, are in line with basal level for As, Ba, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Se, V, Zn identified by Fattorini et al. (2008). The trend of concentrations of trace elements in soft tissue of *Mytilus galloprovincialis* belonging to T0 is (Zn, Fe) > (As, Mn, Cu, Ni, Pb) > (Cd, Cr, Ag) > (Hg, Sb). The trend is confirmed also for average trace elements concentration among all sites: (Fe, Zn) > (As, Mn, Cu) > (Pb, Ag, Ni, Cd, Cr, Hg) > Sb. The only notable change is the Ag enrichment from 0.12 mg/kg in T0 tissues to a mean concentration of 0.80 mg/kg in tissues from experimental sites. Although Cd, Hg, Sb were not included, the same abundance trend was found by Giusti and Zang (2002) in the Venice Lagoon: Fe > Zn > (Cu, Mn, As) > Ag > Pb > (Ni, Cr). Absolute concentrations of Zn, Cu, Pb and Cd found in this study (Table 2) fall in the lowest part of the ranges summarized by Benali et al. (2017) for 10 locations in Mediterranean Sea.

Table 2. Results of the chemical analyses on mussel tissues (bioaccumulation). Data refers to T0 and to the 5 sites were mussels survived or remained after the exposure period.

	unit	T0	S2	S3	S5	S6	S7
Ag	mg/kg	0.12	0.99	0.98	1.21	0.62	0.82
Al	mg/kg	36	39	56	98	82	47
As	mg/kg	9.83	12.8	9.98	11.9	12.4	11.4
B	mg/kg	25.7	22.9	19.7	22.8	25.9	19.4
Ba	mg/kg	1.95	0.35	0.49	0.54	0.52	0.36
Be	mg/kg	0.003	0.003	0.003	0.005	0.006	0.004
Bi	mg/kg	0.057	0.009	0.009	0.007	0.011	0.008
Ca	mg/kg	2004	1322	1299	1726	1679	1157
Cd	mg/kg	0.70	0.71	0.78	0.76	0.77	0.50
Ce	mg/kg	0.118	0.051	0.092	0.124	0.166	0.075
Co	mg/kg	0.54	0.323	0.3	0.303	0.511	0.315
Cr	mg/kg	0.57	0.6	0.53	0.67	0.53	0.64
Cs	mg/kg	0.022	0.014	0.016	0.022	0.019	0.015
Cu	mg/kg	4.65	2.71	2.63	2.81	2.66	1.46
Fe	mg/kg	67.5	68.1	77.4	116.0	103.4	91.7
Ga	mg/kg	0.018	0.007	0.011	0.019	0.013	0.009
Hg	mg/kg	0.082	0.35	0.32	0.41	0.27	0.16
K	mg/kg	11084	10988	9729	11129	11244	9535
La	mg/kg	0.138	0.035	0.057	0.074	0.109	0.06
Li	mg/kg	0.88	0.46	0.41	0.51	0.58	0.34
Mg	mg/kg	4198	3851	3467	3676	4422	2506
Mn	mg/kg	4.84	5.19	7.50	8.16	8.03	5.03
Mo	mg/kg	0.645	0.353	0.368	0.317	0.394	0.274
Na	mg/kg	22885	20201	15986	19714	24751	13438
Nb	mg/kg	0.002	0.001	0.002	0.003	0.003	0.002
Ni	mg/kg	3.61	0.72	0.69	0.73	0.92	0.64
Pb	mg/kg	1.41	0.87	0.52	0.57	0.94	0.64
Rb	mg/kg	5.29	4.08	4.32	4.27	4.3	3.78
Sb	mg/kg	0.018	0.038	0.031	0.03	0.048	0.039
Sc	mg/kg	0.04	0.04	0.03	0.05	0.07	0.04
Se	mg/kg	2.27	2.10	2.10	2.01	1.83	1.52
Sn	mg/kg	0.12	0.12	0.12	0.10	0.09	0.08

Sr	mg/kg	31.2	17.7	16.5	20.6	22.8	14.3
Th	mg/kg	0.019	0.011	0.014	0.025	0.031	0.016
Ti	mg/kg	0.49	0.34	0.62	1.07	0.88	0.50
Tl	mg/kg	0.003	0.001	0.002	0.002	0.001	0.001
U	mg/kg	0.127	0.049	0.040	0.054	0.065	0.032
V	mg/kg	1.15	0.44	0.49	0.55	0.80	0.47
Y	mg/kg	0.080	0.046	0.071	0.085	0.094	0.04
Zn	mg/kg	107.1	116	62.4	95.6	134	86.9
Zr	mg/kg	0.064	0.013	0.025	0.025	0.021	0.018

The bioaccumulation factor is graphically showed in Figure 3. Enrichments are notable for Ag, Hg and Sb in all the sites, with higher bioaccumulation at S5 located in the centre of the lagoon in a pond close to the outlet of a canal from the southern part of the lagoon, the most contaminated one. Although not high as those measured in mussels from other sites such as Northern England (Giusti et al., 1999), these three elements could be the most critical since other elements known for toxicity to mussels (e.g. Cd, Pb, Ni) (Benali et al. 2017; Yigit et al. 2017) are similar or depleted compared to T0 (Fig. 3).

The enrichment in mercury was relatively high, as expected and already verified both in living clams (Trombini et al., 2003) and mussels (Fabbri et al., 2006; Donnini et al., 2007). Moreover, the Hg cycling at the sediment-water interface (Covelli et al., 2011), could be a reason for the mercury enrichment observed in all sediment samples and may also reflect the high Hg concentrations observed in mussels exposed at S5 (Fig. 3).

Silver has recently attracted much interest since its increasing application as bactericidal and usage in numerous consumer products lead to contamination of aquatic ecosystems, often in form of Ag nanoparticles (Yuan et al., 2018; Outa et al., 2020). Silver speciation in environment is dependent by local environmental conditions and usually forms stable complexes with Lewis bases such as amines, halides, and thiolates and with dissolved organic matter altering its toxicity (Mousavi et al., 2015). In Pialassa Baiona, Greggio et al. (2020) showed dissolved concentrations ranging from 0.02 to 10 µg/L, with higher concentrations in basins with drainage water input. Concerning bioaccumulation, recent works reported the effect of Ag nanoparticles on soft tissues of mussels. *Mytilus galloprovincialis* accumulated Ag in digestive gland tubule cells and gills during the entire year (Duroudier et al., 2019) and intralysosomal metal accumulation and lysosomal membrane destabilisation were observed (Jimeno-Romero et al., 2017). Compare to this study (max 1.21 mg/kg dry wt), Giusti and Zang (2002) in Venice lagoon found silver concentrations up to 6.2 mg/kg dry wt that are aligned with outcomes in other species of the genus *Mytilus* as reported by Martin et al. (1988).

Field Code Changed

Antimony is pervasive in synthetic polymers (including textiles, plastics and rubbers) (Filella et al., 2019) and it is widely applied in alloy industries as flame retardant, as pigments for colour or colour protection (Filella et al., 2002; James and Turner 2020). Although rock weathering and soil runoff supply Sb to the environment, anthropogenic source is crucial. James and Turner (2020) demonstrated that Sb could also be mobilised via digestion and bioturbation by deposit-feeders in sediment contaminated by plastic or directly adsorbed from microplastics taken up by mussels (Van Cauwenberghe et al., 2015). Antimony concentrations in unpolluted waters are less than 1 µg/L (Filella et al., 2002). Greggio et al. (2020) reported Sb water concentrations in western ponds of the Pialassa Baiona ranging from 0.05 to 0.5 µg/L. Limited is the knowledge of Antimony concentration in mussels. In this study Sb in mussel tissues ranged from 0.01 to 0.05 mg/kg (Table 2) with a 2-3 as enrichment factor (Fig. 3). De Gregori et al. (2007) found tissue concentration ranging from 0.007 to 0.06 mg/kg in unpolluted area, while 0.23 to 1.030 mg/kg in harbour dock protected by antimony–lead alloy cover. Garcia (2015) measured Sb concentration ranging from 0.003 to 0.023 mg/kg in the tissues of *Corbicula fluminea* collected in a river affected by a decommissioned Antimony smelter.

It is worth noting the depletion relative to T0 of elements like Cu, Pb and Ni, (Fig. 3 and Table 2). This might suggest that these elements are not strongly mobilized in the lagoon environment, although slight differences can be recognized between S7 and the other sites. This is in contrast with results by Pignotti et al. (2018) that using sequential extraction, defined Cu and Pb as the most mobile elements in sediments (~70 % and ~80 %, of the total, respectively) in the neighbouring Pialassa Piomboni. In agreement with Pignotti et al. (2018) is the Ni behaviour here characterised by low mobility and predominantly bound to the residual fraction of the sediment, suggesting a lithogenic origin.

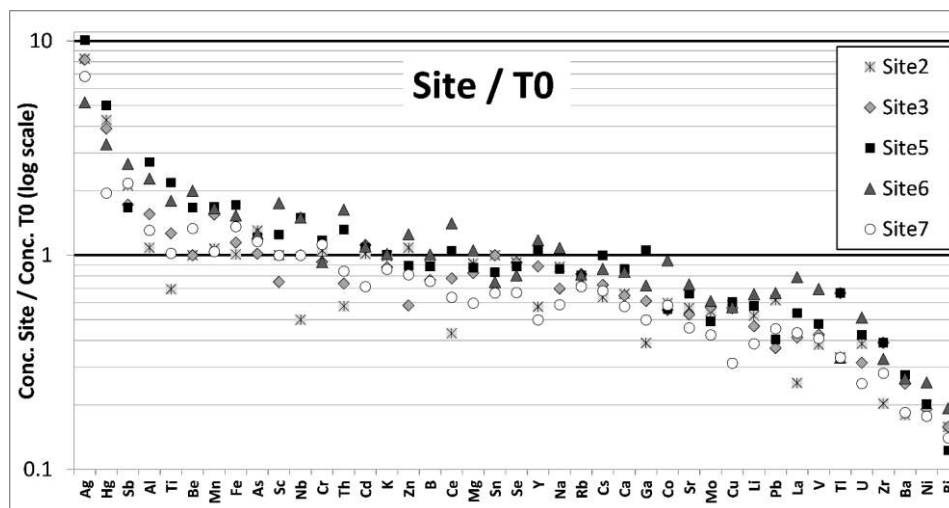


Figure 3. Bioaccumulation factor of metals in mussel tissues. The analysis has been performed on the tissue of mussels deployed for 30 days into different sites of the Pialassa Baiona lagoon compared to mussels analysed before the *in situ* deployment (T0). Elements are ranked according to average enrichment.

3.4 Biological effects on mussels

The mussel 30-day exposure within the Pialassa Baiona lagoon resulted in significant alterations of biological endpoints measured at different levels of the hierarchical ladder (from cell to organism) (Fig. 4). Among the investigated sites, adverse conditions for the mussel survival were observed in S1, with about 80 % mortality *versus* 10 % recorded elsewhere (Fig. 4A). Different from S2 – S7, survival (and biomarker) data were measured in S1 after just three days of exposure, in line with previous observations on mussels transplanted in the same area (Franzellitti et al., 2010). The short time spent *in situ* within S1 was not such to allow mussels to bioaccumulate metals at levels considered environmentally realistic, for which ≥ 28 days are usually required (Viarengo et al., 2007a); thus, data of metal bioaccumulation are missing for this site. Sediment concentrations of Hg, Cr, Zn, Cu and Ba, known for their toxicity on marine mussels (De Los Ríos et al., 2013) were negatively and significantly correlated with mussel survival (Fig. 5). Relationships were also detected between survival and average salinity and temperature, which showed minima and maxima in S1, respectively (Table S3). Both parameters are known to be responsible for the mussel physiological fitness and to influence their response to toxicants synergistically (Fabbri and Dinelli, 2014;

DeCourten and Brander, 2017). It is thus plausible that physico-chemical conditions of both natural and anthropogenic nature simultaneously influenced the survival of mussels across the lagoon.

Data of *in situ* survival follow trends similar to those from the SOS test, which showed significant reduction of survival functions in mussels from S1 (Fig. 4B). SOS is measured as the survival of mussels in dry conditions, which is an event regularly experienced by intertidal organisms due to tidal fluctuations. The SOS data display significant and negative correlation with a suite of metals measured in both sediments and mussel tissues, including Ba, Bi, Cu, Sr and Zn, among others (Fig. 5). These findings are in line with previous evidence obtained in *M. galloprovincialis*, which showed significant SOS decrease after 3 to 7 days of exposure to trace levels of Cu and Zn-based antifouling agents (Viarengo et al., 1995; Marcheselli et al., 2011). Further elements showing marked enrichment in S1 sediments, as Cd (0.68 mg/kg) and Hg (25.7 µg/kg), were significantly correlated with SOS (Fig. 4). SOS and survival data were found to be significantly and positively correlated to each other and showed positive and negative correlation with electrical conductivity and temperature, respectively (Table S3). This indicates that higher temperatures and salinity measured at S1 throughout the exposure period may have cumulatively increased the impact of metals on the mussel vital functions. Although it is acknowledged that SOS sensitivity is lower than other sub-lethal and general stress biomarkers (Viarengo et al., 2007a), data from this study highlight its suitability to detect causal relationships between metal contamination/bioavailability and adverse effects on mussels at the “organism” level.

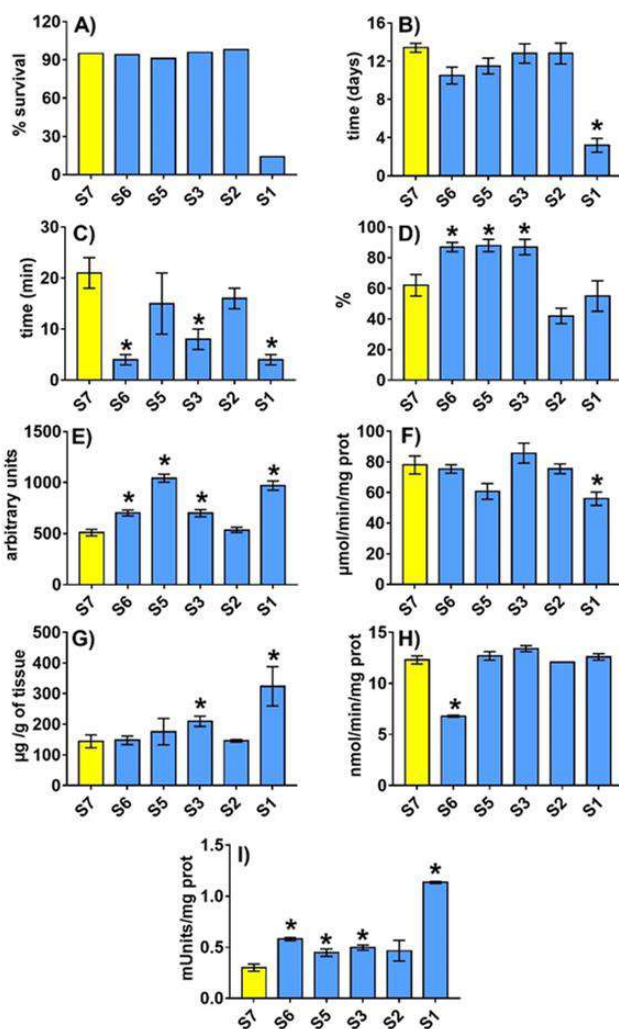


Figure 4. Biomarker modulation in mussels transplanted into the Pialassa Baiona lagoon. Data from all sites (mean \pm SEM) have been measured in mussels collected after 30 days of exposure, except for S1, referred to mussels collected after 3 days of exposure due to high mortality. A, survival (SURV); B, Stress on stress (SOS); C, lysosomal membrane stability (LMS); D, lysosome to cytoplasm volume ratio ([LYS/CYT](#)); E, lipofuscin (LIF); F, catalase activity (CAT); G, metallothionein content (MT); H, acetylcholinesterase activity (ACHE); I, peroxisomal acyl-CoA oxidase (AOX). Asterisks indicate statistically significant differences compared to the control site S7 (One-Way ANOVA, Duncan's post-hoc test).

LMS is the most sensitive biomarker of general stress in bivalves and previous studies highlighted strict relationships between the xenobiotic-driven decrease of this parameter in *Mytilus*

spp. and alterations to higher functions, such as growth and/or reproduction (Moore et al., 2006; Martinez-Gomez et al., 2008). Data reported in Figure 4C indicate a significant LMS reduction in digestive glands from mussels collected at S1 (3-day exposure), S3 and S6 (30-day exposure) compared to control mussels from S7. Interestingly, none of the measured metals, physico-chemical factors and/or biological endpoints was correlated with trends of LMS in exposed mussels (Fig. 5, Table S3). Although positive relationships between (dissolved) metal exposure and LMS have been reported in mussels under laboratory conditions (Viarengo et al., 1997), no correlation has been highlighted following 28 days of *in situ* exposure into a nearby lagoon (i.e. Pialassa Piomboni, Ravenna, Italy; Capolupo et al., 2017).

The mussel exposure to contaminated environment is frequently associated to an augmented permeability of the lysosomal membrane and an overall lysosomal swelling. In the present study, mussels deployed into S3, S5 and S6 showed a significantly higher LYS/CYT compared to the control site S7 (Fig. 4D), following trends significantly correlated to tissue levels of Ba, Mn and Zr (Fig. 5). The S3 and S5 were located in proximity of streams ensuring the flowing of industrial wastewater from southern and eastern settlements to the Adriatic Sea, while S6 was located along a channel that receive water from Lamone river after having crossed the natural areas located to the west (Fig. 1); thus, these data outline the need for a better understanding of the spatial/temporal relationships between different source of lagoon water (industrial and continental), metal release into the environment and lysosomal dysfunction in the exposed biota.

Lysosomal biomarker of general stress, as LMS and LYS/CYT, describe the overall health status of tested organisms and may thus be modulated by a wide range of natural/anthropogenic stressors; therefore, considering the diverse array of human activities directly and indirectly linked to the Pialassa Baiona lagoon, it cannot be ruled out that further organic pollutants may have played a role in observed lysosomal responses.

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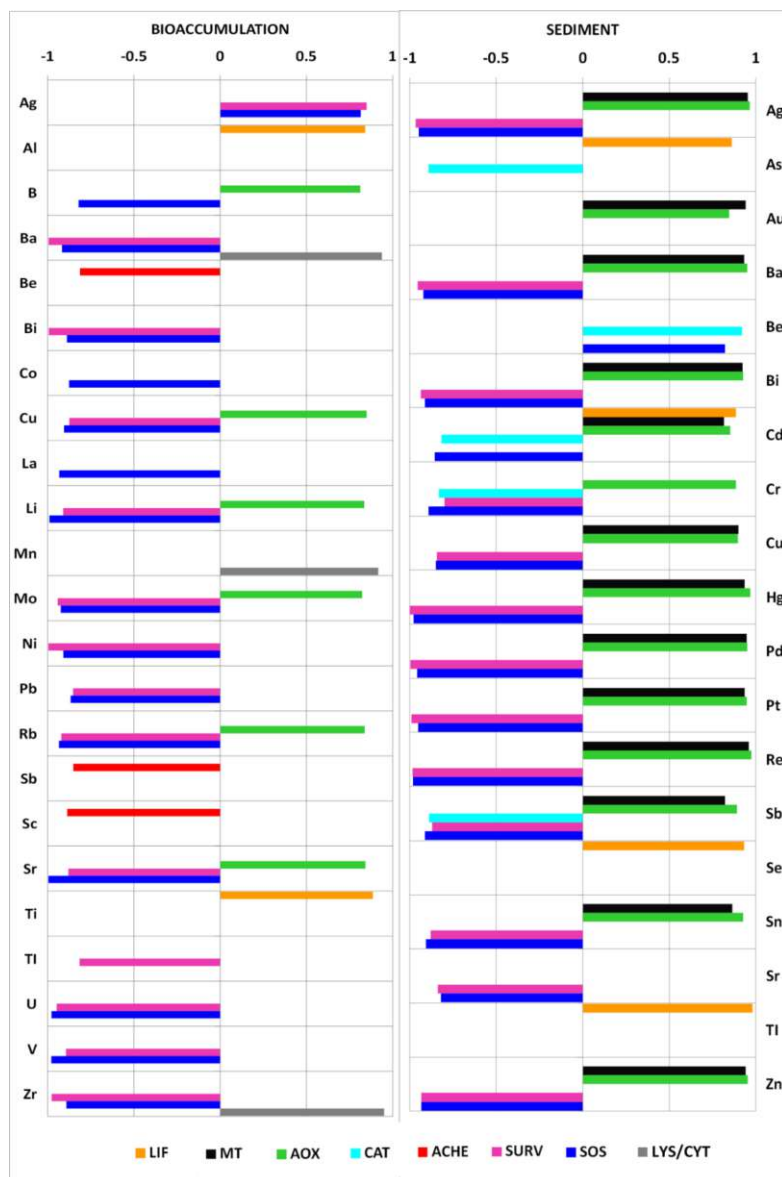


Figure 5. Correlation between biological data and metal concentrations. Pearson's coefficients calculated between biological data and levels of metals measured in mussel tissues (bioaccumulation) and sediment. Only coefficients showing a significant correlation ($p < 0.05$) are reported in the figure.

Mussels from all sites, except S2, showed a significant accumulation of LIF compared to those deployed into the reference site (S7) (Fig. 4E). LIF represent the ultimate products of the lipid peroxidation of biological membranes; they are composed by oxidatively modified proteins, lipid degradation by-products, carbohydrates and metals, and appear as granules within lysosomes of cells composing the digestive tubules epithelium (Moore et al., 2008). Previous biomonitoring studies highlighted clear relationships between LIF accumulation in mussels, the metal uptake and the metal spatial/temporal distribution into enclosed marine systems as bays and lagoons (Moschino et al., 2011; Capolupo et al., 2017). Accordingly, we identified significant and positive correlations between LIF, Al and Ti tissue content in mussels, as well as with As, Cd, Se and Ti measured in sediments (Fig. 5). In addition, a significant and negative correlation has been observed between LIF and the activity of the antioxidant enzyme CAT (Table S3). The latter showed a significant decrease *versus* control (S7) in mussels exposed at S1 (Fig. 4F), suggesting that beside ROS formation, the exposure to relatively high metal concentrations might also down-regulate the overall mussel antioxidant defences. CAT also showed negative correlation with elements previously found to elicit pro-oxidant effects in mussels, as As, Cd, Cr, Sb (Franzellitti et al., 2012; Benali et al., 2017; Coppola et al., 2018), and with temperature trends within the lagoon. This indicates that both chemical and physical factors might have cumulatively influenced the mussel ROS scavenging activity over the exposure within the lagoon.

MT have been used as biomarker of exposure in this study owing to their high heavy-metal-binding capacity (Lavradas et al., 2016). Compared to control site S7, a significant MT synthesis induction has been recorded in the digestive gland of mussels from S3 and S1 (Fig. 4H), both located in the southern part of the lagoon in proximity of a large industrial complex (Fig.1). MT trends showed a positive and significant correlation with the sediment concentration of metals known as MT up-regulator, including Cd, Cu, Zn, Pd, Pt, Sn and Hg (Viarengo et al., 2001; Rocha et al., 2016; Zimmermann and Sures, 2018), and other less studied such as Ag, Au, Ba, Bi, Re, and Sb (Fig. 5). Positive relationships were detected MT trends and fluctuations of temperature and conductivity (Table S3), indicating that physico-chemical factors may interactively modulate MT in mussels from contaminated areas.

The activity of the enzyme AChE, which mediates the termination of the nervous impulses driven by the neurotransmitter acetylcholine, was significantly inhibited in mussels from the northern site S6 (Fig. 4H). AChE is typically inhibited by pesticides, although in mussels it may also be inhibited by high levels of metals (Lionetto et al., 2003). Apart from a negative relationship with

Be, AChE trends did not show significant relationships with metals measured in sediments and mussel tissues (Fig. 5), nor with other biological endpoints measured in deployed mussels (Table S3). Compared to the S7, sediments collected in S6 showed a 20 times higher enrichment of Hg (Fig. 2), which is known as a potent AChE inhibitor in mussels, notably when co-exposed to fluctuations of other physico-chemical parameters, such as temperature (Coppola et al., 2017).

The possible impact of organic xenobiotics on the physiology of transplanted mussels has been screened through the analysis of the Acyl-Co A oxidase (AOX) activity. *In situ* deployed mussels showed a significant AOX increase following exposure at all sites except S2 (Fig. 4I). These data corroborate previous evidence of a diffuse PAH contamination (notably pyrene, fluoranthene and anthracene) in sediment and mussels collected from the same area (Vassura et al., 2005; Fabbri et al., 2006). Vassura et al. (2005) also found clear relationships between patterns of PAHs and Hg in the lagoon sediments. Accordingly, many metals herein measured in sediments, including among others Ag, Ba; Hg, Cu, Cr, Cd and Zn, as well as mussel tissues concentrations of B, Cu, Li, Mo and Sr showed positive correlation with AOX (Fig. 5), suggesting a possible metal-induced peroxisome proliferation and a similar spatial distribution of metals and POPs within the lagoon.

The output produced by biomarkers data integration performed through the MES is reported in Figure 6. The LMS was chosen as guide parameter for the data integration. A good health status (HSI = A) was assigned by the Expert System to mussels *in situ* exposed at the reference site S7 and at S2. This output confirms the good environmental quality of the lagoon north-eastern area, as previously identified based on chemical and biological evidence (Franzellitti et al., 2010), and is in line with the lack of biomarker alterations observed in mussels from S2. Although this site is located in the southern part of the lagoon, i.e. in proximity of industrial settlements, it is directly exposed to the marine water entering the lagoon indicating the relevance of high water circulation and oxygenation to the fitness of the resilient biota. A moderate stress level (HSI = C) was assigned to mussels exposed at S5, while mussels from S1, S3 and S6 were classified as heavily stressed (HSI = D) (Fig. 6). In line with the metal contamination/bioaccumulation and coherently with MT levels measured in mussels, the MES associated the health status alteration to a significant excess of metal contamination was identified in S1 and S3. Moreover, the MES associated the health status alteration of mussels exposed at S1, S3, S5 and S6 to the presence of organic xenobiotics/persistent organic compounds (POPs) has been identified in S1, S3, S5 and S6, in line with measured AOX trends. Although organics were not included in the analytical design of this study, this output is consistent with previous and evidence of PCB and PAH contamination across the

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lagoon reported in previous studies (Guerra, 2012; Vassura et al., 2005; Fabbri et al., 2006; Vassura et al., 2005) and may confirm the co-occurrence of multiple stressors in different areas of the Pialassa Baiona lagoon.

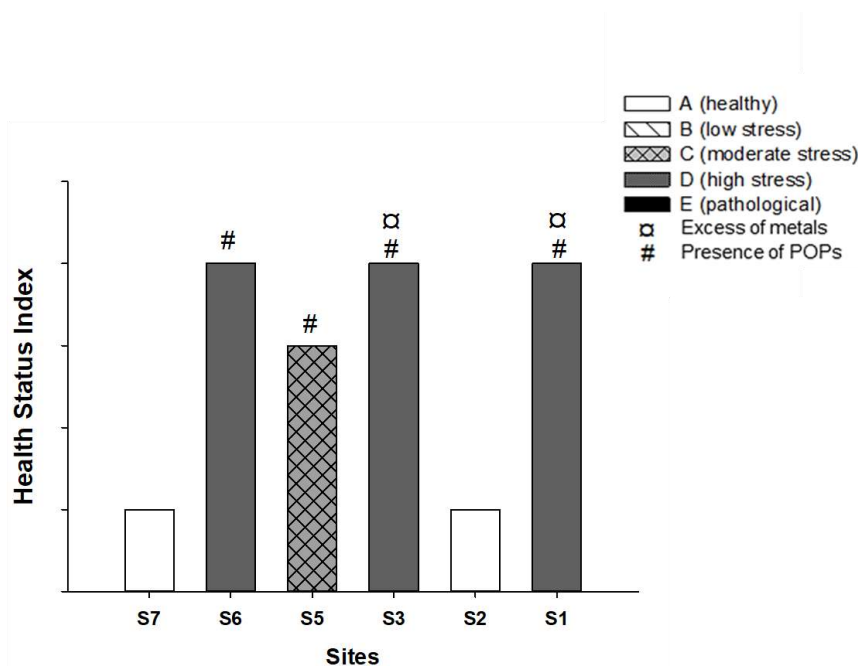


Figure 6. Biomarker data integration. A unique Health status index (HSI) has been generated using the Mussel Expert System (MES, developed by Dagnino et al., 2007) based on biomarker alterations observed at each site. The LMS was chosen as guide parameter for the HSI assessment.

4 Conclusions

The integrated approach implemented in this study provided accurate and reliable clues on the distribution of metals in the Pialassa Baiona lagoon, their bioavailability for exposed mussels and their effects on key biological functions, posing also attention to seldom considered elements such as Ag and Sb.

The physico-chemical data revealed clear differences among S1 and other investigated sites. The water in Magni Channel (S1) showed higher column temperature, reduced EC and a lower pH

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value, for the entire monitoring period generating local conditions aligned with the worst forecasted climate change scenarios.

The geochemical analysis of total elements indicated that sediments in Pialassa Baiona are a mixture of carbonate and silicates with variable amount of organic matter. The total concentrations of the investigated elements are within the range of regional background. Exception is made for Zn and Cu, and Pb respectively double (in S1) and slightly above (S3 and S5) the local natural background values.

The aqua regia digestion identified a south-north decreasing contamination trend, with highest enrichments of Hg, Pt, Au, Ag, Mo, Re, Cd, Pd, Zn; Sn, and Cu (in enrichment order, Fig. 2) being at least two folds enriched than S7 (internal control). Moreover, the most affected sites are in the south-western areas, which consistently receives wastewater effluents from a near petrochemical settlement (south) and drainage water from the inland (west).

Bioaccumulation analysis performed in transplanted mussels proved suitable to evaluate the bioavailability of metals in relation to their distribution across the lagoon. Integrating physical, chemical and biological measures allowed for defining causal relationships between observed mortality rates, lysosomal dysfunctions, oxidative stress induction, biomarker of exposure modulations and the metal distribution within the lagoon. Besides this, the different channels examined within the lagoon proved to be an excellent study system for evaluating climate change effects (i.e. modified temperature, pH, and salinity) combined with pollution. In addition, the use of the MES proved suitable to identify the impact induced by specific substances on the overall mussel fitness and to estimate the toxicological pathways occurring in highly pressure lagoons.

Overall, data from this study assign a general scarce environmental quality to the Pialassa Baiona lagoon; considering the heterogeneity of this area in terms of geological, physico-chemical and anthropogenic factors, regular biomonitoring surveys should be performed to control the onset of conditions which might exacerbate the impact of metals and other pollutants, notably on a seasonal timeframe. In this respect, the integrated strategy undertaken in this investigation represents a promising approach for the biomonitoring of this area and, more generally, for reliably assessing the environmental quality of highly pressured lagoons.

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Tables of the paper:

Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring

Authors:

Greggio Nicolas*, Capolupo Marco, Filippo Donnini, Manfred Birke, Fabbri Elena, Dinelli Enrico

Table 1. Results of the chemical analyses on sediments. The table includes either pseudo total Aqua Regia (AR) and total XRF data. Table includes also the ranges observed in boreholes in the area, considered representative of the natural background (Amorosi et al., 2002; Curzi et al., 2006). Bold elements exceed natural background. AR DL indicates Detection Limit for Aqua Regia extraction.

		S1			S2			S3			S4			S5			S6			S7			Natural background
		AR DL	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	AR	XRF	
Ag	µg/kg	2	991	-	269	-	268	-	173	-	275	-	179	-	179	-	62	-	-	-	-	-	-
Al	%	0.01	1.39	6.31	1.41	7.02	1.23	6.98	1.10	6.97	1.35	7.09	1.39	7.07	1.41	6.55	4.4-9.7	-	-	-	-	-	-
As	mg/kg	0.1	7.8	-	5.9	-	6.1	-	5.9	-	7.8	-	6.8	-	6.7	-	-	-	-	-	-	-	-
Au	µg/kg	0.2	16.2	-	5.4	-	11.1	-	2.6	-	5.1	-	3.4	-	1	-	-	-	-	-	-	-	-
B	mg/kg	1	25	-	28	-	26	-	24	-	38	-	33	-	22	-	-	-	-	-	-	-	-
Ba	mg/kg	0.5	61.9	263	43.7	218	41.8	260	42.6	322	40.1	258	38.7	294	36.5	258	220-530	-	-	-	-	-	-
Be	mg/kg	0.1	0.4	-	0.7	-	0.7	-	0.8	-	0.5	-	0.6	-	0.6	-	-	-	-	-	-	-	-
Bi	mg/kg	0.02	0.6	-	0.38	-	0.35	-	0.26	-	0.38	-	0.31	-	0.28	-	-	-	-	-	-	-	-
Ca	%	0.01	11.27	11.37	7.45	7.51	7.87	7.96	7.34	7.87	6.10	6.65	7.24	7.43	8.61	9.52	0.3-17.2	-	-	-	-	-	-
Cd	mg/kg	0.01	0.68	-	0.29	-	0.33	-	0.21	-	0.5	-	0.35	-	0.11	-	-	-	-	-	-	-	-
Ce	mg/kg	0.1	19.4	42	17	55	17.6	71	16.8	52	17.8	64	18.3	42	20.1	58	14-83	-	-	-	-	-	-
Co	mg/kg	0.1	10.2	12	10.9	11	10.7	12	9.9	11	11.7	14	11.5	12	10.6	11	5-29	-	-	-	-	-	-
Cr	mg/kg	0.5	70.6	131	57.5	103	51.4	132	47.7	131	59.9	138	59.3	130	47	100	96-270	-	-	-	-	-	-
Cs	mg/kg	0.02	1.36	-	1.15	-	1.08	-	1.16	-	1.11	-	1.39	-	1.57	-	-	-	-	-	-	-	-
Cu	mg/kg	0.01	84.1	87	49.1	52	49.5	53	36.7	44	51.1	52	37.5	38	22.4	24	4-41	-	-	-	-	-	-
Fe	%	0.01	2.17	3.42	2.46	3.92	2.23	3.86	2.00	3.69	2.54	3.83	2.30	3.64	2.36	3.72	2.1-5.3	-	-	-	-	-	-
Ga	mg/kg	0.1	4.4	-	4.2	-	4	-	3.9	-	4	-	4.2	-	4.4	-	-	-	-	-	-	-	-
Ge	mg/kg	0.1	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-	<0.1	-	-	-	-	-	-	-	-
Hf	mg/kg	0.02	0.03	-	0.03	-	0.04	-	0.03	-	0.03	-	0.03	-	0.05	-	-	-	-	-	-	-	-
Hg	mg/kg	0.005	25.7	-	1.42	-	1.48	-	1.89	-	2.38	-	2.26	-	0.1	-	-	-	-	-	-	-	-
In	mg/kg	0.02	0.03	-	0.03	-	0.02	-	0.02	-	0.03	-	0.02	-	0.03	-	-	-	-	-	-	-	-
K	%	0.01	0.33	1.77	0.36	1.98	0.31	2.06	0.28	2.04	0.39	1.97	0.36	2.04	0.35	2.03	0.9-3.3	-	-	-	-	-	-
La	mg/kg	0.5	8.3	19	7.7	26	7.3	22	6.9	18	8	20	8.5	34	9.5	20	17-47	-	-	-	-	-	-
Li	mg/kg	0.1	26.1	-	30.6	-	27.8	-	24.9	-	28.7	-	28	-	28.1	-	-	-	-	-	-	-	-
Mg	%	0.01	1.37	2.41	1.34	2.38	1.30	2.38	1.22	2.44	1.42	2.54	1.55	2.55	1.58	2.35	2.2-4.2	-	-	-	-	-	-
Mn	mg/kg	1	531	893	527	865	549	896	513	949	511	801	550	897	644	1396	230-1470	-	-	-	-	-	-
Mo	mg/kg	0.01	2.67	-	3.33	-	2.61	-	2.28	-	3.34	-	1.57	-	0.54	-	-	-	-	-	-	-	-
Na	%	0.001	0.85	0.68	0.98	0.69	0.90	0.75	0.65	0.99	1.18	0.67	0.85	0.78	0.42	0.85	0.5-2.1	-	-	-	-	-	-
Nb	mg/kg	0.02	0.37	15	0.28	13	0.25	13	0.29	14	0.34	16	0.37	10	0.29	13	8-20	-	-	-	-	-	-
Ni	mg/kg	0.1	50.9	73	56.5	73	50.7	88	46.3	72	55.3	82	51.5	74	46.4	57	39-148	-	-	-	-	-	-
P	%	0.001	0.11	0.10	0.06	0.06	0.06	0.07	0.06	0.06	0.08	0.07	0.07	0.06	0.06	0.06	0.03-0.08	-	-	-	-	-	-
Pb	mg/kg	0.01	27.92	28	22.71	26	22.14	30	18.16	19	26.22	30	20.71	22	11.99	18	5-29	-	-	-	-	-	-
Pd	µg/kg	10	46	-	10	-	12	-	11	-	10	-	<10	-	<10	-	-	-	-	-	-	-	-
Pt	µg/kg	2	37	-	6	-	4	-	4	-	4	-	<2	-	<2	-	-	-	-	-	-	-	-
Rb	mg/kg	0.1	24.9	113	22.2	128	20.8	128	22.4	113	22.3	130	25.1	114	29.8	89	56-200	-	-	-	-	-	-
Re	µg/kg	1	7	-	1	-	2	-	<1	-	2	-	2	-	<1	-	-	-	-	-	-	-	-
S	%	0.02	0.6	-	0.55	-	0.6	-	0.54	-	0.72	-	0.71	-	0.34	-	-	-	-	-	-	-	-
Sb	mg/kg	0.02	0.51	-	0.29	-	0.25	-	0.19	-	0.37	-	0.29	-	0.19	-	-	-	-	-	-	-	-
Sc	mg/kg	0.1	3.4	11	3.6	11	3.4	6	3.2	8	3.7	10	3.7	8	4.1	13	12-21	-	-	-	-	-	-
Se	mg/kg	0.1	0.9	-	0.7	-	0.7	-	0.5	-	1.1	-	0.7	-	0.5	-	-	-	-	-	-	-	-
Si	%	-	-	17.72	-	19.43	-	19.90	-	20.61	-	19.31	-	20.95	-	20.03	14.7-29.3	-	-	-	-	-	-
Sn	mg/kg	0.1	3.2	-	1.8	-	1.5	-	1.3	-	1.9	-	1.5	-	0.8	-	-	-	-	-	-	-	-
Sr	mg/kg	0.5	353	368	274.4	300	224.8	252	218	265	259.1	281	281.6	291	290.5	310	113-442	-	-	-	-	-	-
Th	mg/kg	0.1	3.5	4	4.1	5	3.6	17	3.6	25	4	11	4.2	10	4.7	7	<3-19	-	-	-	-	-	-
Ti	%	0.001	0.009	0.308	0.005	0.328	0.006	0.347	0.008	0.349	0.006	0.324	0.010	0.334	0.015	0.336	0.02-0.58	-	-	-	-	-	-
Tl	mg/kg	0.02	0.26	-	0.17	-	0.21	-	0.19	-	0.27	-	0.2	-	0.14	-	-	-	-	-	-	-	-
U	mg/kg	0.1	1.3	-	1.5	-	1.2	-	1.1	-	1.3	-	1.2	-	1.1	-	-	-	-	-	-	-	-
V	mg/kg	2	32	85	33	85	30	99	26	94	35	113	33	94	31	76	54-169	-	-	-	-	-	-
Y	mg/kg	0.01	10.02	18	9.39	23	9.28	24	8.86	20	9.7	24	9.63	19	10.58	19	18-43	-	-	-	-	-	-
Zn	mg/kg	0.1	306.6	316	129.8	152	133.1	154	101.2	126	146.9	174	109.3	124	58.2	67	43-124	-	-	-	-	-	-
Zr	mg/kg	0.1	1.1	108	1.2	91	1	105	1	124	1.1	86	1.2	92	1.9	106	76-260	-	-	-	-	-	-
H₂O⁻	%	-	-	2.7	-	4.0	-	2.8	-	2.4	-	3.7	-	2.7	-	2.1	-	-	-	-	-	-	-
OM	%	-	-	2.8	-	1.8	-	2.0	-	1.7	-	3.4	-	2.2	-	1.1	-	-	-	-	-	-	-
H₂O⁺	%	-	-	6.0	-	6.5	-	5.4	-	4.4	-	6.5	-	5.8	-	2.3	-	-	-	-	-	-	-
CO₂	%	-	-	11.6	-	8.9	-	9.1	-	9.3	-	8.6	-	7.4	-	12.3	-	-	-	-	-	-	-
LOI	%	-	-	23.1	-	21.2	-	19.3	-	17.8	-	22.3	-	18.1	-	17.8	4.8-29.3	-	-	-	-	-	-

Table 2. Results of the chemical analyses on mussel tissues (bioaccumulation). Data refers to T0 and to the 5 sites where mussels survived or remained after the exposure period.

	unit	T0	S2	S3	S5	S6	S7
Ag	mg/kg	0.12	0.99	0.98	1.21	0.62	0.82
Al	mg/kg	36	39	56	98	82	47
As	mg/kg	9.83	12.8	9.98	11.9	12.4	11.4
B	mg/kg	25.7	22.9	19.7	22.8	25.9	19.4
Ba	mg/kg	1.95	0.35	0.49	0.54	0.52	0.36
Be	mg/kg	0.003	0.003	0.003	0.005	0.006	0.004
Bi	mg/kg	0.057	0.009	0.009	0.007	0.011	0.008
Ca	mg/kg	2004	1322	1299	1726	1679	1157
Cd	mg/kg	0.70	0.71	0.78	0.76	0.77	0.50
Ce	mg/kg	0.118	0.051	0.092	0.124	0.166	0.075
Co	mg/kg	0.54	0.323	0.3	0.303	0.511	0.315
Cr	mg/kg	0.57	0.6	0.53	0.67	0.53	0.64
Cs	mg/kg	0.022	0.014	0.016	0.022	0.019	0.015
Cu	mg/kg	4.65	2.71	2.63	2.81	2.66	1.46
Fe	mg/kg	67.5	68.1	77.4	116.0	103.4	91.7
Ga	mg/kg	0.018	0.007	0.011	0.019	0.013	0.009
Hg	mg/kg	0.082	0.35	0.32	0.41	0.27	0.16
K	mg/kg	11084	10988	9729	11129	11244	9535
La	mg/kg	0.138	0.035	0.057	0.074	0.109	0.06
Li	mg/kg	0.88	0.46	0.41	0.51	0.58	0.34
Mg	mg/kg	4198	3851	3467	3676	4422	2506
Mn	mg/kg	4.84	5.19	7.50	8.16	8.03	5.03
Mo	mg/kg	0.645	0.353	0.368	0.317	0.394	0.274
Na	mg/kg	22885	20201	15986	19714	24751	13438
Nb	mg/kg	0.002	0.001	0.002	0.003	0.003	0.002
Ni	mg/kg	3.61	0.72	0.69	0.73	0.92	0.64
Pb	mg/kg	1.41	0.87	0.52	0.57	0.94	0.64
Rb	mg/kg	5.29	4.08	4.32	4.27	4.3	3.78
Sb	mg/kg	0.018	0.038	0.031	0.03	0.048	0.039
Sc	mg/kg	0.04	0.04	0.03	0.05	0.07	0.04
Se	mg/kg	2.27	2.10	2.10	2.01	1.83	1.52
Sn	mg/kg	0.12	0.12	0.12	0.10	0.09	0.08
Sr	mg/kg	31.2	17.7	16.5	20.6	22.8	14.3
Th	mg/kg	0.019	0.011	0.014	0.025	0.031	0.016
Ti	mg/kg	0.49	0.34	0.62	1.07	0.88	0.50
Tl	mg/kg	0.003	0.001	0.002	0.002	0.001	0.001
U	mg/kg	0.127	0.049	0.040	0.054	0.065	0.032
V	mg/kg	1.15	0.44	0.49	0.55	0.80	0.47
Y	mg/kg	0.080	0.046	0.071	0.085	0.094	0.04
Zn	mg/kg	107.1	116	62.4	95.6	134	86.9
Zr	mg/kg	0.064	0.013	0.025	0.025	0.021	0.018

Figures of the paper:

Integration of physical, geochemical and biological analyses as a strategy for coastal lagoon biomonitoring

Authors:

Greggio Nicolas*, Capolupo Marco, Filippo Donnini, Manfred Birke, Fabbri Elena, Dinelli Enrico

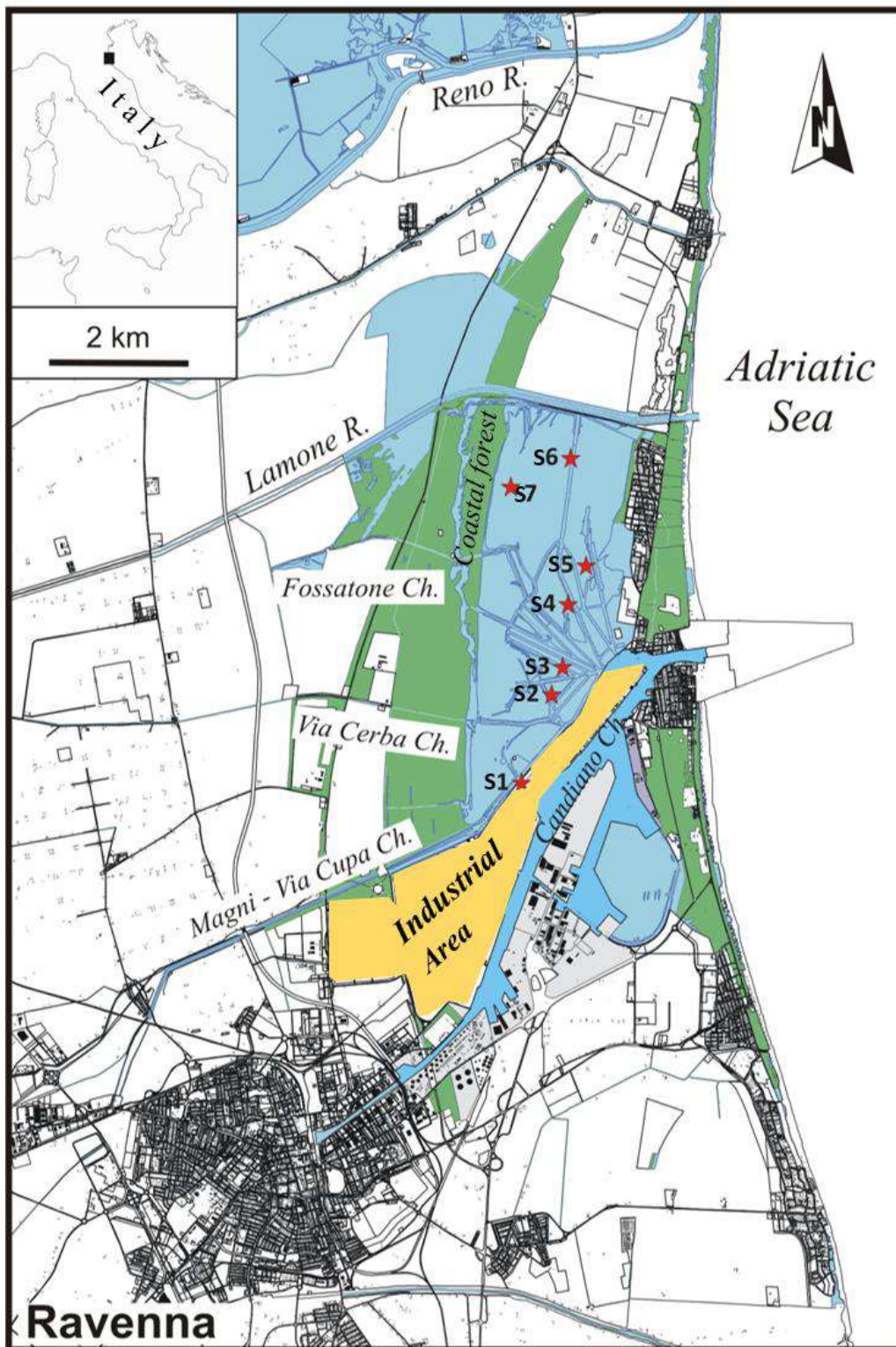


Figure 1. Location map and sampling points (S1 to S7) within the Pialassa Baiona lagoon (Ravenna, Italy). The abbreviations "R." and "Ch." refer to the main rivers and/or channels which supply freshwater to the lagoon. The yellow shape represents the industrial area settled at the south-eastern boundary of the Pialassa Baiona lagoon.

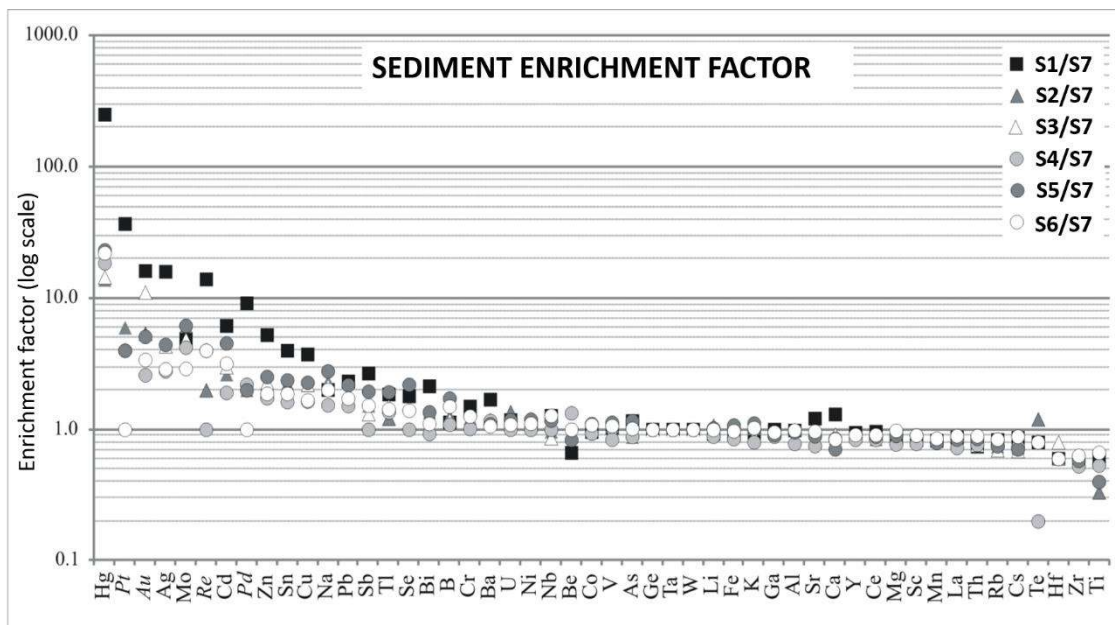


Figure 2. Metal enrichment in sediments from selected sites within the Pialassa Baiona lagoon. Data are normalized on Site 7 (S7), which was selected as internal reference based on previous literature (Trombini et al., 2003; Fabbri et al., 2006; Donnini et al., 2007; Franzellitti et al., 2010). Elements in italics (*Pt*, *Au*, *Re*, *Pd*) were not investigated in the mussel tissues.

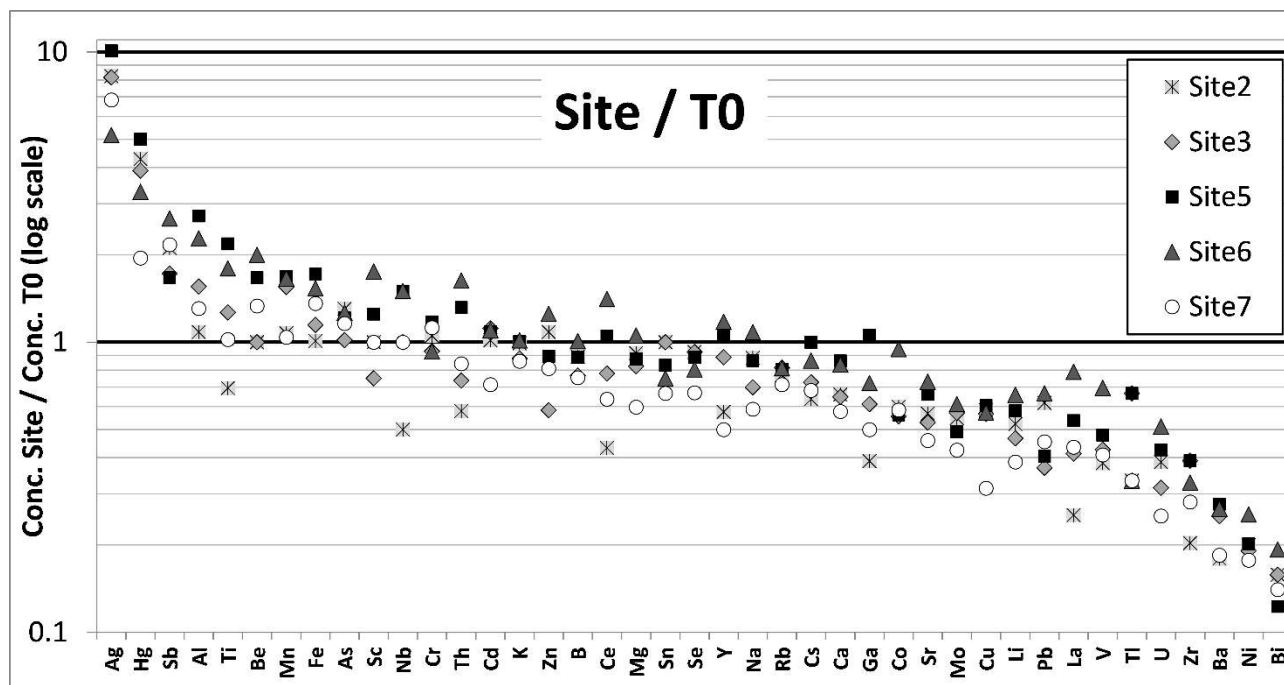


Figure 3. Bioaccumulation factor of metals in mussel tissues. The analysis has been performed on the tissue of mussels deployed for 30 days into different sites of the Pialassa Baiona lagoon compared to mussels analysed before the *in situ* deployment (T0). Elements are ranked according to average enrichment.

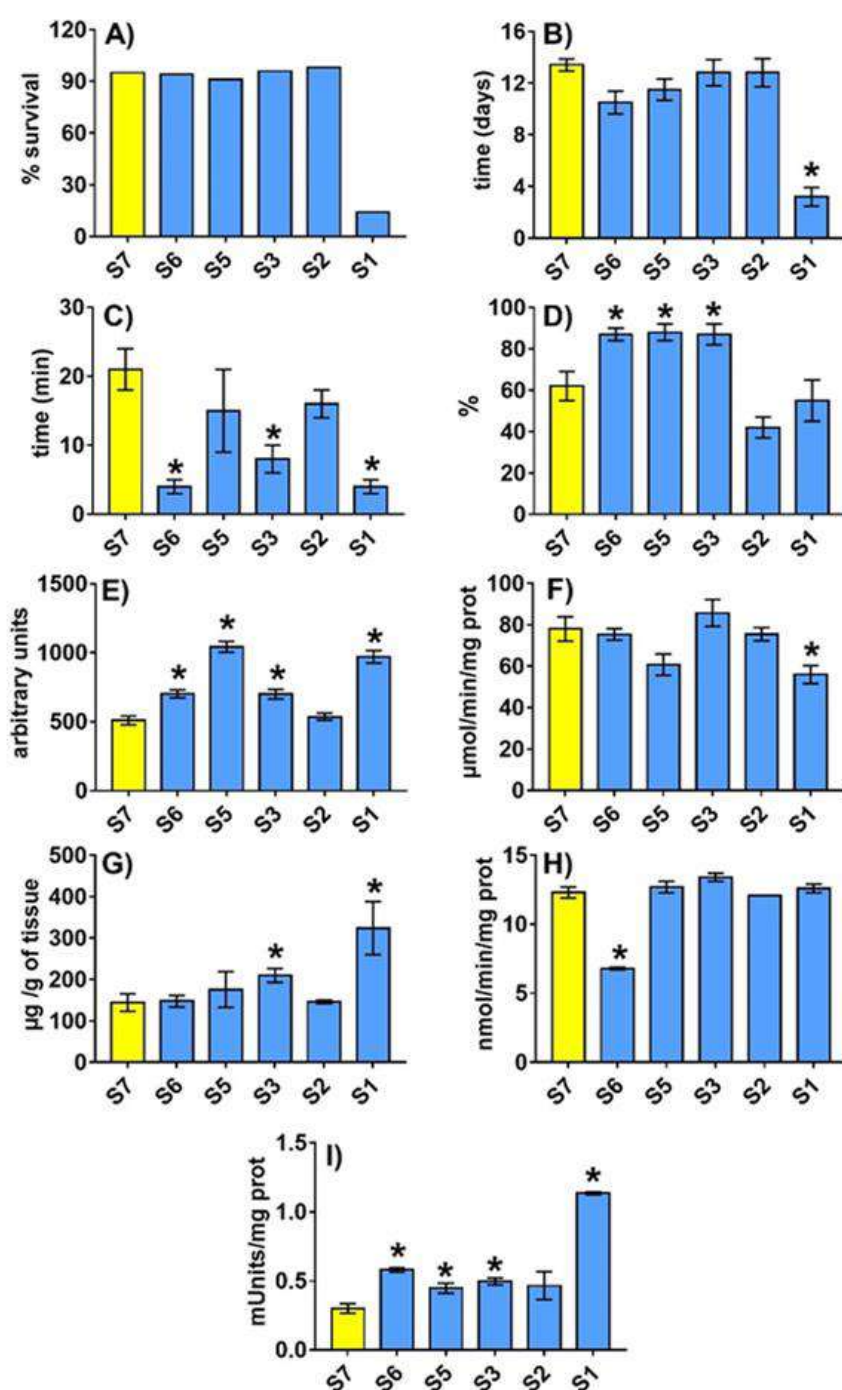


Figure 4. Biomarker modulation in mussels transplanted into the Pialassa Baiona lagoon. Data from all sites (mean \pm SEM) have been measured in mussels collected after 30 days of exposure, except for S1, referred to mussels collected after 3 days of exposure due to high mortality. A, survival (SURV); B, Stress on stress (SOS); C, lysosomal membrane stability (LMS); D, lysosome to cytoplasm volume ratio; E, lipofuscin (LIF), F, catalase activity (CAT); G, metallothionein content (MT); H, acetylcholinesterase activity (ACHE); I, peroxisomal acyl-CoA oxidase (AOX). Asterisks indicate statistically significant differences compared to the control site S7 (One-Way ANOVA, Duncan's post-hoc test).

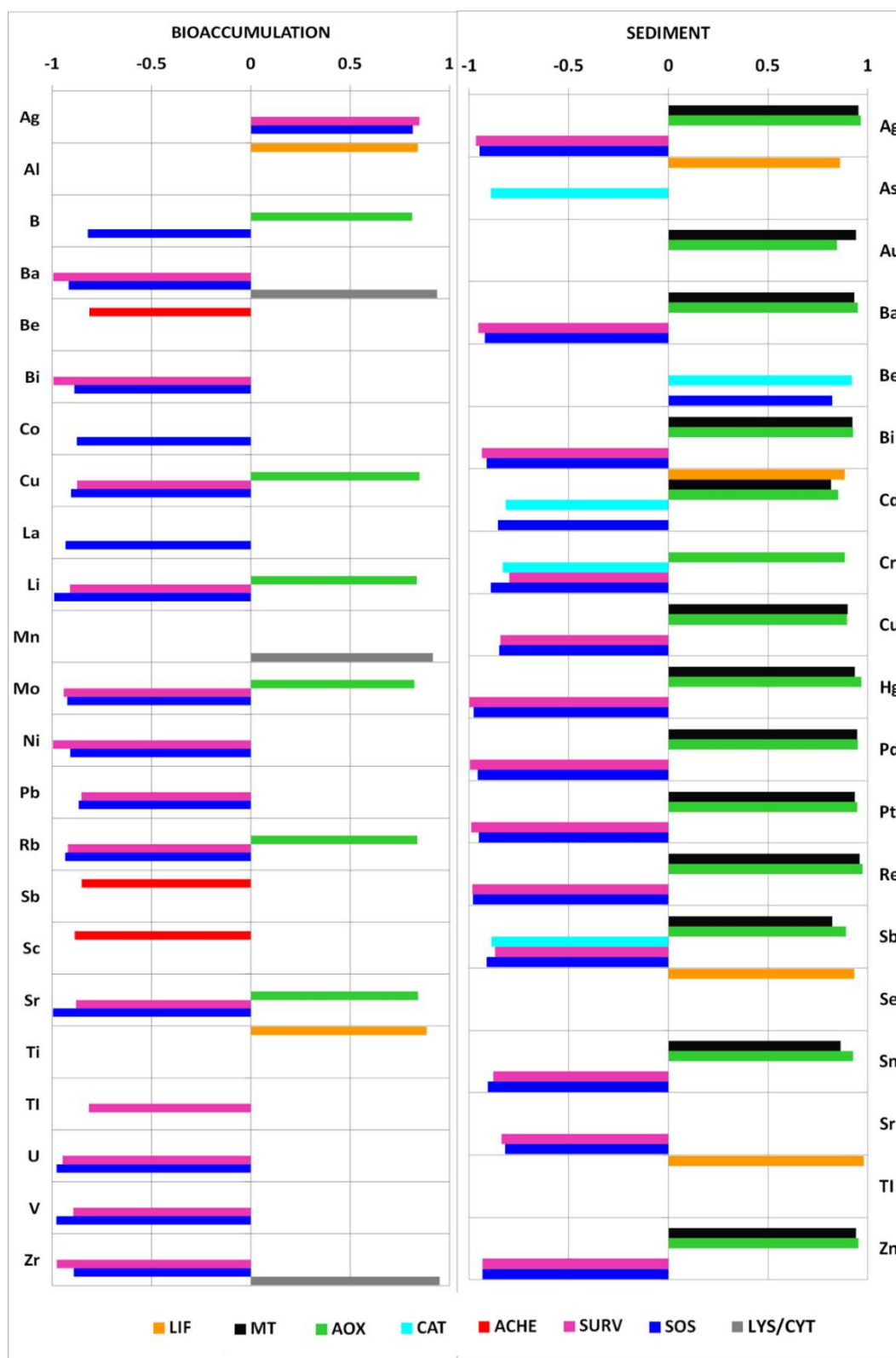


Figure 5. Correlation between biological data and metal concentrations. Pearson's coefficients calculated between biological data and levels of metals measured in mussel tissues (bioaccumulation) and sediment. Only coefficients showing a significant correlation ($p < 0.05$) are reported in the figure.

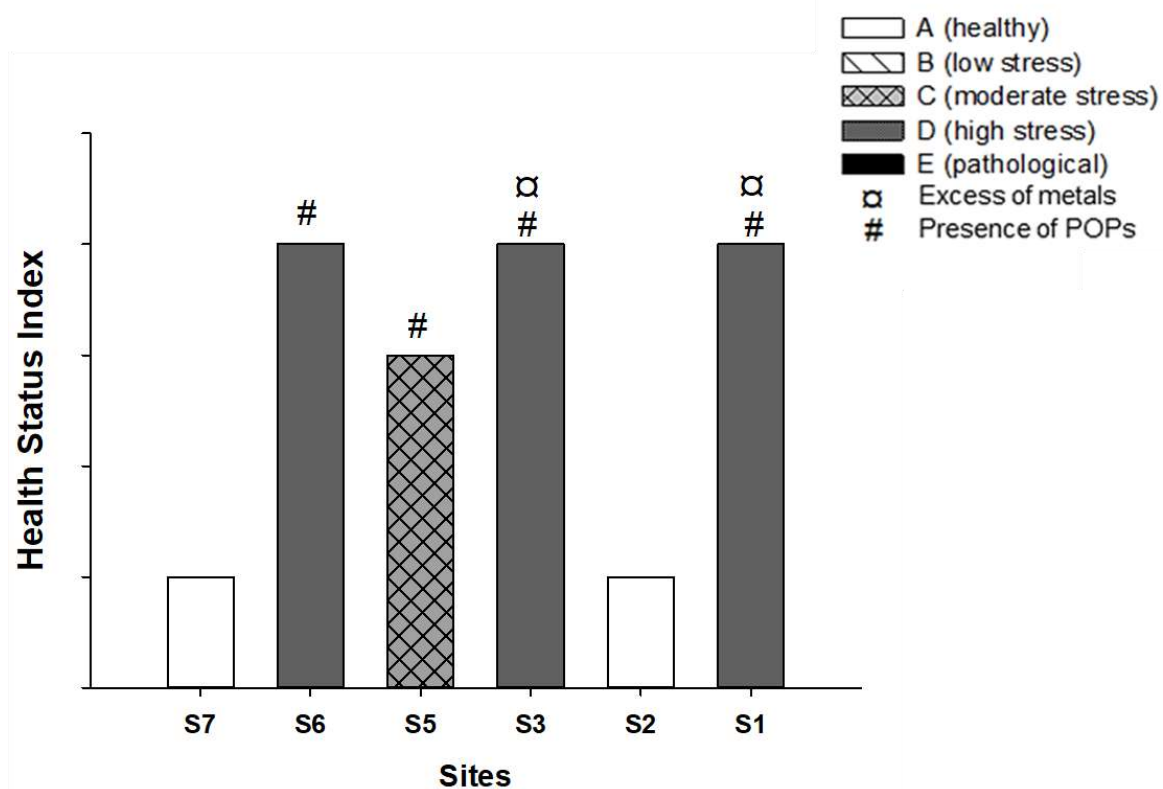


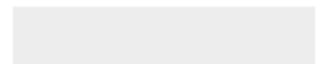
Figure 6. Biomarker data integration. A unique Health status index (HSI) has been generated using the Mussel Expert System (MES, developed by Dagnino et al., 2007) based on biomarker alterations observed at each site. The LMS was chosen as guide parameter for the HSI assessment.



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Supplementary Data

Revised Supplementary Material Greggio et al.
2020.docx



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Nicolas Greggio: Conceptualization, Data Curation, Formal analysis, Investigation, Visualization, Writing - Original Draft, Writing - Review & Editing. **Marco Capolupo:** Data Curation, Formal analysis, Investigation, Visualization, Writing - Original Draft, Writing - Review & Editing. **Filippo Donnini:** Conceptualization, Formal analysis, Investigation. **Manfred Birke:** Formal analysis Writing - Review & Editing. **Elena Fabbri:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - Review & Editing. **Enrico Dinelli:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - Review & Editing.