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Combining chemometric tools for assessing hazard sources and factors acting simultaneously in contaminated areas. Case study: "Mar Piccolo" Taranto (South Italy)

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Abstract

Almost all marine coastal ecosystems possess complex structural and dynamic characteristics, which are influenced by anthropogenic causes and natural processes as well. Revealing the impact of sources and factors controlling the spatial distributions of contaminants within highly polluted areas is a fundamental propaedeutic step of their quality evaluation. Combination of different pattern recognition techniques, applied to one of the most polluted Mediterranean coastal basin, resulted in a more reliable hazard assessment. PCA/CA and factorial ANOVA were exploited as complementary techniques for apprehending the impact of multi-sources and multi-factors acting simultaneously and leading to similarities or differences in the spatial contamination pattern, thus defining areas of most concern.

Keywords. Marine sediments, hazard assessment, chemometrics, PCA, factorial ANOVA, contamination pattern

1. Introduction

Coastal areas are the most impacted natural environments, being subjected to various forms of contamination. Thousands of pollutants can affect the quality of both water column and bottom sediments, triggering severe changes in marine ecosystem and posing high risks to its structure and function (Kennish et al. 2014). The continuous loading of multiple disturbances needs to be monitored and evaluated by means of accurate and complex hazard assessment procedures calling for multidisciplinary competences and knowledges. Moreover, advanced tools are needed in order

to extract the most relevant informations from the extensive amount of bio-geo-chemical data usually acquired in the characterization phase aimed at identifying the pollution pattern in the study area.

In recent years, the use of chemometrics has grown significantly, becoming a discipline well recognized for environmental evaluation of complex matrixes (Simeonov et al. 2002; 2003; Kowalkowski et al. 2006; Shrestha et al. 2007; Venugopal et al. 2009; Zhou et al. 2007; Varol et al. 2011; Dabioch et al. 2013; Mali et al. 2016; Mali et al. 2017a, Mali et al. 2017b). Pattern recognition techniques, either supervised or unsupervised, and generally all modern multivariate analyses, have been widely exploited for generating continuous spatial pollution maps at global/local scales and for identifying anthropogenic multi-sources (Einax et al., 1997; Gredilla et al. 2013; Hopke K.P., 2015). The main reason for this large use lies in the possibility to extract hidden informations from large data sets and the easy-way of graphically resolving relationships between multiple samples and variables. Nevertheless, identifying pollution sources is often not enough to describe the possible recovery trajectories of contaminants and, thus, to define the best remediation actions, especially in high-polluted areas where complicated interaction processes occur and a plethora of factors control the spatial pollution pattern (Clements et al. 2010).

Sea sediments are known to be very complex matrixes since they act as sink for all contaminants discharged in water column (Long. et al. 1995; 2006; Caeiro et al. 2005; Burton G.A. 2013, Chapman et al. 2013; Mali et al. 2015). The distribution of contaminants within sediments depends not only on anthropogenic and natural sources but also upon the textural characteristics, organic matter content, mineralogical composition and other factors. The evaluation of effects exerted by these factors is surely necessary, but getting insights into simultaneous interaction phenomena could enhance the robustness of the hazard estimation.

In this paper we present the application of two different statistical techniques to assess the hazard degree in a highly contaminated area. The chosen statistical techniques are Principal Component Analysis/Cluster Analysis (PCA/CA) and factorial Analysis of Variance (ANOVA). The combination of these complementary techniques is considered useful for emphasizing interaction effects between factors controlling pollution spectrum. Indeed, PCA/CA was applied in order to obtain information about the existing site-clusters with similar pollution character and to identify the most important discriminant contaminants (variables) within the same cluster. On the other side, ANOVA was used in order to assess the impact of some main factors, such as organic matter

63 and textural sediment, when they act simultaneously. The combination of the two techniques were
64 thus suggested as useful approach for a meaningful hazard assessment procedure.

65 As case study area we selected one of the most industrialized and contaminated zone in the
66 Mediterranean: "Mar Piccolo", a marine basin located in the Ionian Sea of Southern Italy near city
67 of Taranto, a densely populated town subjected to a high environmental risk. Due to the
68 extraordinary industrialization activity affecting the quality of soil and groundwater, Taranto and
69 the whole catchment area were declared contaminated Site of National Interest (SIN). Continuous
70 monitoring campaigns have been conducted in the area and the present work is based on the
71 characterization campaign carried out by ISPRA, Italian Agency of Environment Protection of
72 Coastal Areas and committed by the Environmental Ministry for the SIN Taranto area in 2010
73 (ISPRA. 2010).

74 **2. Design and methods**

75 **2.1 Study area and sampling programs**

76 Taranto's Mar Piccolo is a semi-enclosed basin with a surface area of approximately 21 km^2 and a
77 maximum depth of 13 m. It is divided into two inlets and it is connected to Mar Grande and to
78 Taranto Gulf (Ionian Sea) through two channels, one artificial named Navigable Channel and the
79 other one natural called Porta Napoli's Channel. In this study, the relative large channel
80 connecting the two inlets is called Punta Penna Channel, like the bridge name over the channel.
81 (Figure 1). A peculiar characteristic of Mar Piccolo is the presence of several submarine
82 freshwater springs called "citri" (Caroppo and Cardellicchio, 1995) which contribute to freshwater
83 inputs together with small karstic rivers. The two most significant "citri" are located within the
84 first inlet and are called citro-Galeso and citro-Citrello. The area is characterized by a low water
85 exchange and hypoxic conditions as consequence of oxygen consumption by microbial
86 degradation due to large inputs of organic matter (from wastewater discharges, mussel farming
87 and agricultural soil drainage) which also contribute to an excess of nutrients in the system
88 (Umgiesser et al. 2007). Previous studies refer also high sulphide concentrations in the bottom
89 sediments as a result of anaerobic degradation of organic matter (Cardellicchio et al. 2006).

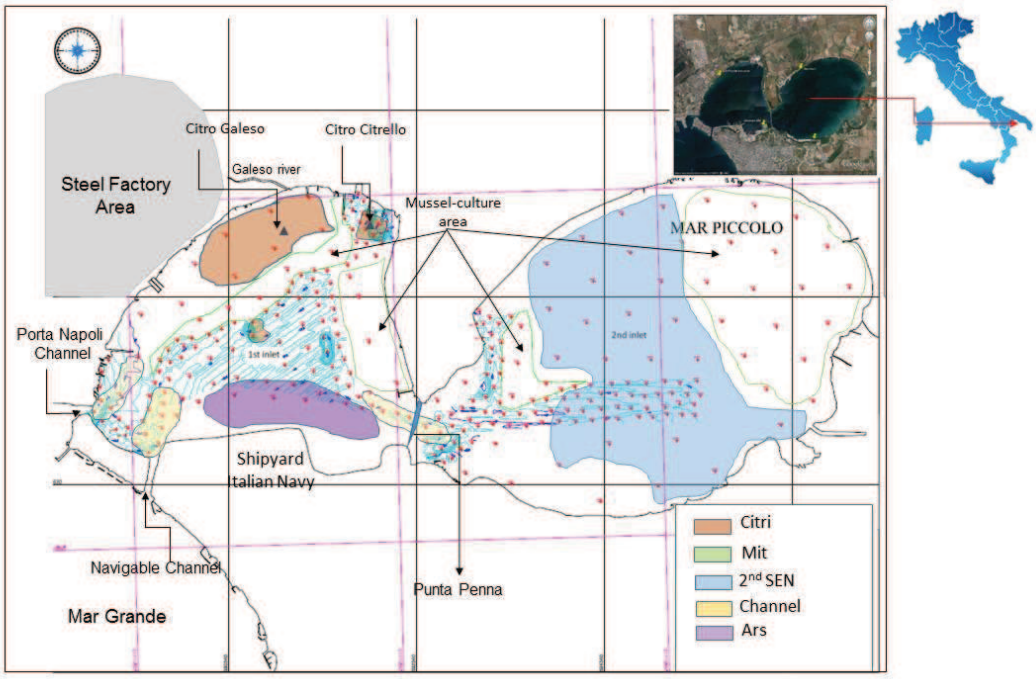


Figure 1. Sampling map within two inlets of Mar Piccolo. The figure reports the location of the main anthropogenic sources and freshwater springs within the two inlets.

Many anthropogenic activities load on the first inlet, affecting adversely the quality of the basin. The most relevant ones are: intensive mussel cultures and fishery facilities, an abandoned shipyard (built at the beginning of the twentieth century) and a very large Navy port (Buccolieri et al. 2006). Furthermore, urban discharges and agricultural freshwater are transported within Mar Piccolo basin through a number of pipes and karstic rivers. Finally, nearby the first inlet one of the most important European steel factory is still active. Although nowadays some pollution sources are no longer active, the quality of the area is strongly compromised (Bellucci et al. 2016).

A sampling grid matrix on regular basis was defined according national guidelines (ICRAM–APAT (2007)) including 266 samples collected within the two inlets at different depth levels as shown in Figure 1.

2.2 Chemical analysis

For the purpose of the present study the following specific variables were considered: *i)* 11 metals and metalloids (As, Cd, Cu, Cr, Hg, Ni, Pb, V, Zn, Al and Fe); *ii)* Sum of Polychlorinated Biphenyls (PCB), sum of Polycyclic Aromatic Hydrocarbons (PAHs) and sum of Total HydroCarbons (THC), the latter split into the following two sub-groups: sum of Light molecular

Total HydroCarbons LTHC (C<12) and sum of High molecular Total HydroCarbons, HTHC (C>12); *iii*) main physico-chemical parameters, i.e. grain size (in terms of % of coarse, % of sand, % of mud, % of clay), water content, Total Organic Carbon (TOC), Total Nitrogen (N_{tot}) and Total Phosphorous (P_{tot}). The TOC/N_{tot} ratio was also included in the database being a useful indicator of the organic matter provenance (Mayer 1993; Meyers 2003; Gao et al. 2012). For the 266 sampling points, sediments were collected at five different depths (0.10 cm; 10-30 cm; 30-50 cm; 100-120 cm; 180-200 cm; 280-300 cm), for a total of 1021 samples. The analytical procedures for each contaminant's class are reported in Table S1.

2.3 Statistical analysis

Two software were utilized for statistical analysis: Soft Independent Modeling of Class Analogy (SIMCA) 10.2 for PCA/CA analyses and STATISTICA 10.0 for two-way factorial ANOVA and for normality-test of the raw and log-transformed data. The SIMCA software was used due to its very intuitive graphical interphase. Two-way ANOVA was preferred with respect to the three ANOVA in order to reduce the risk of getting false positive effects with increasing the independent factors (McKillup, 2012). The Type II ANOVA model was chosen because the levels assigned to main independent factors were randomly selected. In addition the model was considered suitable for the purpose of the manuscript (*vide infra*).

3. Statistical treatment and results

3.1 Preliminary data elaboration

All multivariate analyses are sensitive to outliers and to non-normality of data sets (Reimann and Filzmoser, 2000; Reimann et al. 2005; Micò et al. 2006; Filzmoser et al. 2009; Gałuszka et al. 2015). Usually environmental data are not normally distributed, but their logarithmically transformed values can follow a normal distribution (Leardi et al. 2000; Rostron & Ramsey 2012). Prior to performing multivariate analyses, grain size and/or geochemical normalization is also recommended (Covelli and Fontolan, 1997; Ho et al. 2012). In the present study, we normalized metal concentrations using Al as proxy (Horowitz, 1997; Mali et al. 2015), before checking the normality features of all variables. The results of normality tests are presented in Appendix A, while the basic statistical parameters for the whole dataset (1021x24) are reported in Table S2.

In order to enhance the performance of the statistical analyses, additional variables were defined, grouped as "spatial" and "qualitative" variables. The "spatial" variables include "Depth" and

"Basin-type". "Depth" is referred to the depth of core-sampling and three classes were thus defined: "surficial" (S) including samples collected at 0-10cm; 20-30cm and 30-50 cm, "intermediate" (I) including samples collected at 100-120 cm, and "deep" (P) including samples collected at 180-200 and 280-300 cm. "Basin-type" distinguishes sample zones influenced by different neighboring industrial activities and human pressure loading. Six "Basin-type" classes were defined (Figure 1): 1) "*channel*" indicates those samples located at the entrance of the three existing channels; 2) "*citro*" indicates samples located around citro-Galeso and citro-Citrello freshwater fluxes and in the zone where the Galeso river flows; 3) "Ars" refers to samples located nearby Navy area; 4) "Mit" belongs to all core-samples located in the mussel-culture activity; 5) "2nd Sen" refers to samples sited within the second inlet; 6) "In" indicates all remaining samples not included in previous groups.

It is well known that contaminant (especially trace elements) concentrations within sediments are strongly affected by textural characteristics, organic matter content, mineralogical composition and depositional environment. The generally accepted opinion is that the smaller the size of sediment fraction, the larger the amount of trace elements and pollutants bound to this fraction. In particular, trace elements are mainly present in the clay/mud particles with grain size smaller than 0.063 mm diameter. For the importance of the granulometric distribution, an additional variable named "Grain size class" was included and three classes were defined: the finest fraction including samples with fraction $\boldsymbol{\theta < 63\ \mu m}$ more than 55%; the coarse/sand fraction including samples with $\boldsymbol{\theta > 63\ \mu m}$ more than 55%; and the **mix** fraction accounting for the remaining cases.

Organic matter content (in terms of TOC) and nutrients (in terms of P_{tot} and N_{tot}) play an important role in the marine chemistry (van Nugteren, 2009). The distribution of TOC within sediments in Mar Piccolo spans an unusually large range (from 0.13 up to 8.10 $\%_w$) even more evident within different depths. For this reason, we included TOC concentration as an additional "qualitative" variable, defining four different range-concentrations: "low", "medium", "high" and "very high" for TOC concentration ranging from 0-1 $\%_w$, 1-3 $\%_w$, 3-5 $\%_w$ and > 5 $\%_w$, respectively.

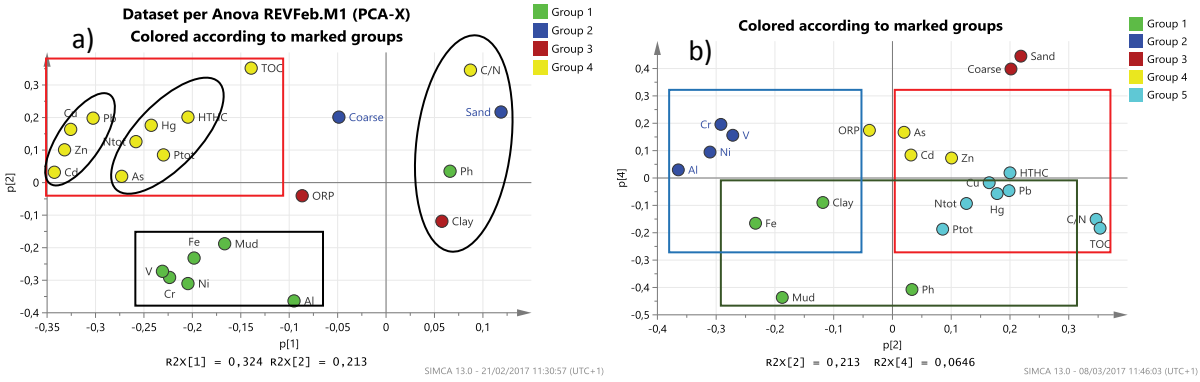
3.2 Spatial Distribution of Contaminants through PCA/CA analyses

A model with four principal components (PCs) accounting for a cumulative variance explained of 70% was considered. Despite additional elaborations (such as the exclusion of "spike" concentrations and variables with negligible influence on PCs), the cumulative variance explained

by the first four PCs, remained almost unchanged. We presume that this circumstance is related to the occurrence of too many simultaneous and multi-source effects, each of them marginally affecting contamination pattern. Therefore, in order to evaluate how the unexplained variance could affect the results obtained, we decided to perform cluster analysis (CA) considering both the whole dataset and data coming from the selected PCs. The clusters obtained by both approaches were then compared (*vide infra*).

Inspection of loadings PCs projections evidenced different variable groups (Figure 2). In PC1/PC2 space (Figure 2a), elevated negative loadings on PC1 are marked for four metals grouped in a cluster: Cd, Zn, Cu and Pb. A second cluster, displaying less negative PC1 loadings and constituted by two toxic elements, As and Hg, is also clearly visible. This cluster resulted highly correlated with nutrients (N_{tot}, P_{tot}) and with HTHC. Both clusters fall in the same PC1/PC2 plane and show a positive correlation with TOC, even if the latter displays higher positive loadings on PC2. The obtained results suggest different distribution fate (partially affected by organic matter) and similar anthropogenic sources for contaminants grouped in the two clusters.

Indeed, all metals in both clusters are sulphophile elements, that is those elements which show a high affinity to sulfur, forming insoluble sulfides. However, differently from Cu, Zn, Cd and Pb, As and Hg are also subjected to easy methylation (facilitated by high levels of TOC and nutrients) and therefore are more mobile in the sediment phases with respect to Pb, Cu, Zn and Cd. Similar conclusions were drawn by Chakraborty et al. (2015) and (Liu et al. 2013). In addition, it was demonstrated that the strong affinity of As and Hg for iron and manganese (hydr)oxides controls their sequestration and their bioavailability in sediments and aquifers, in strong relation to microbial activity and physico-chemical conditions (Burnol et al. 2011).



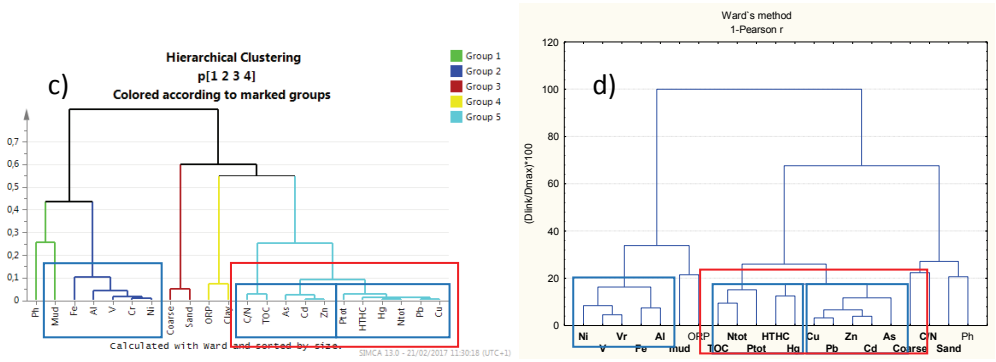


Figure 2. PCA loading plots for: a) PC1/PC2, b) PC2/PC4, c) partial hierarchical clustering calculated with Ward's method considering four PCs; d) hierarchical clustering calculated with Ward's method considering the whole dataset. *C/N stands for TOC/N_{tot}

As to the pollution sources, damping of contaminated material and untreated terrestrial discharges can be considered possible causes affecting Cd, Cu, Pb, Zn, As, Hg contents (the variables displaying positive loadings on PC2), as suggested by previous studies in the area (Cardellicchio et al. 2007; Petronio et al. 2012). Nevertheless, these metals resulted to be the main elements contained in dust emission from steel factories (EC Remus et al. 2013, Arruti et al. 2010, Dore et al.2014). For this reason, we can reasonably assume that metal-reached atmospheric input should be an important contamination source of these metals, being the study area located very near to a steel factory. As far as Pb and Hg are concerned, both metals could derive also from oil spilling and ship-fuel losses. The presence of "Navy" in the area can contribute to fuel losses from military ships during maneuvering (Mali et al. 2017b) and thus can explain the association of Pb and Hg with HTHC.

Continuing analyzing of PC2 negative loadings (Figure 2a), it is apparent another metal cluster constituted by V, Ni, Cr and Fe, strongly correlated with Al and mud fraction (4μm < mud < 63μm) and in contrast trend with respect to Cd, Zn, Cu, Pb, As, Hg and HTHC. Both circumstances suggest that the presence of V, Ni, Cr and Fe might be attributed to natural origin. Probably, the geochemical composition of the parent rocks which generated Mar Piccolo sediments, could be the main factor controlling the variability of V, Ni, Cr and Fe. In fact, statistically significant positive correlations (R) were found between these metals and the finest grain size sediments (Table S4), supporting our hypothesis of their enrichment predominantly due to sedimentary processes (Dung et al. 2013; Lofu et al. 2016; Mali et al. 2015; Mali et al. 2016).

Interesting data derived from PC2/PC4 space plot (Figure 2b), where some differences among anthropogenic enriched metals are emphasized. On the negative PC4 plan, Hg and Pb (and Cu in less extent) resulted associated with nutrients (N_{tot} and P_{tot}) and TOC. All these variables display positive correlation with Fe, fine sediments (mud and clay) and pH. On the contrary, As, Cd and Zn are displayed on the positive PC4 plan as well as lithogenic metals.

We propose that the observed dissimilarities between these two groups (Hg, Pb and Cu group with respect to the As, Cd and Zn one) might be caused by different affinity of these metal for different phases (ion-exchangeable, carbonate, organic, iron-oxy-hydroxide, sulphides etc.) (Calloy et al., 2002; Bucolieri et al. 2006). Hg, Pb and Cu are probable bound to the more mobile phases with respect to As, Cd and Zn. This hypothesis is supported by previous studies: Calace et al. (2008) found that Pb in Mar Piccolo is present mainly as ion-exchangeable species or bound to carbonate while Hg and Cu result predominantly associated to organic matter. Cardellicchio et al. 2006 found that, except for Hg and Pb, metals such as Zn, Cd and As in Mar Piccolo result to be present mainly as insoluble sulphides encouraged by high sulphide concentrations and negative redox (ORP) values.

In order to reveal the hidden sample clusters (scores) and to identify the discriminant contaminants (variables-loadings) for each cluster, we considered meaningful the analysis of biplot graphs colored according to "TOC levels", "Depth" and "Basin Type" (Figure 3 a,b,c,d).

Analysis of the PC1/PC2 biplot colored according to TOC levels (Figure 3a), reveals that almost all samples containing high and very high TOC concentration (yellow and green) resulted associated with anthropogenically influenced metals (As, Cd, Cu, Hg, Zn, Pb) and display positive relationship with TOC and (Corg/N). On the contrary, samples containing low and medium TOC concentrations (blue and red) resulted associated with lithogenic metals (V, Cr, Ni, Al and Fe), following positive correlation with the finest sediment grain size (mud and clay). In particular, the samples with low TOC concentration (blue) are strongly associated with clay. Exceptions were observed for some medium TOC samples (red arrow) that display the same trend of high and very high TOC levels. The circumstance suggests that probably in these samples anthropogenic contamination could take place and/or synergistic effects of TOC with other variables might control their contamination pattern.

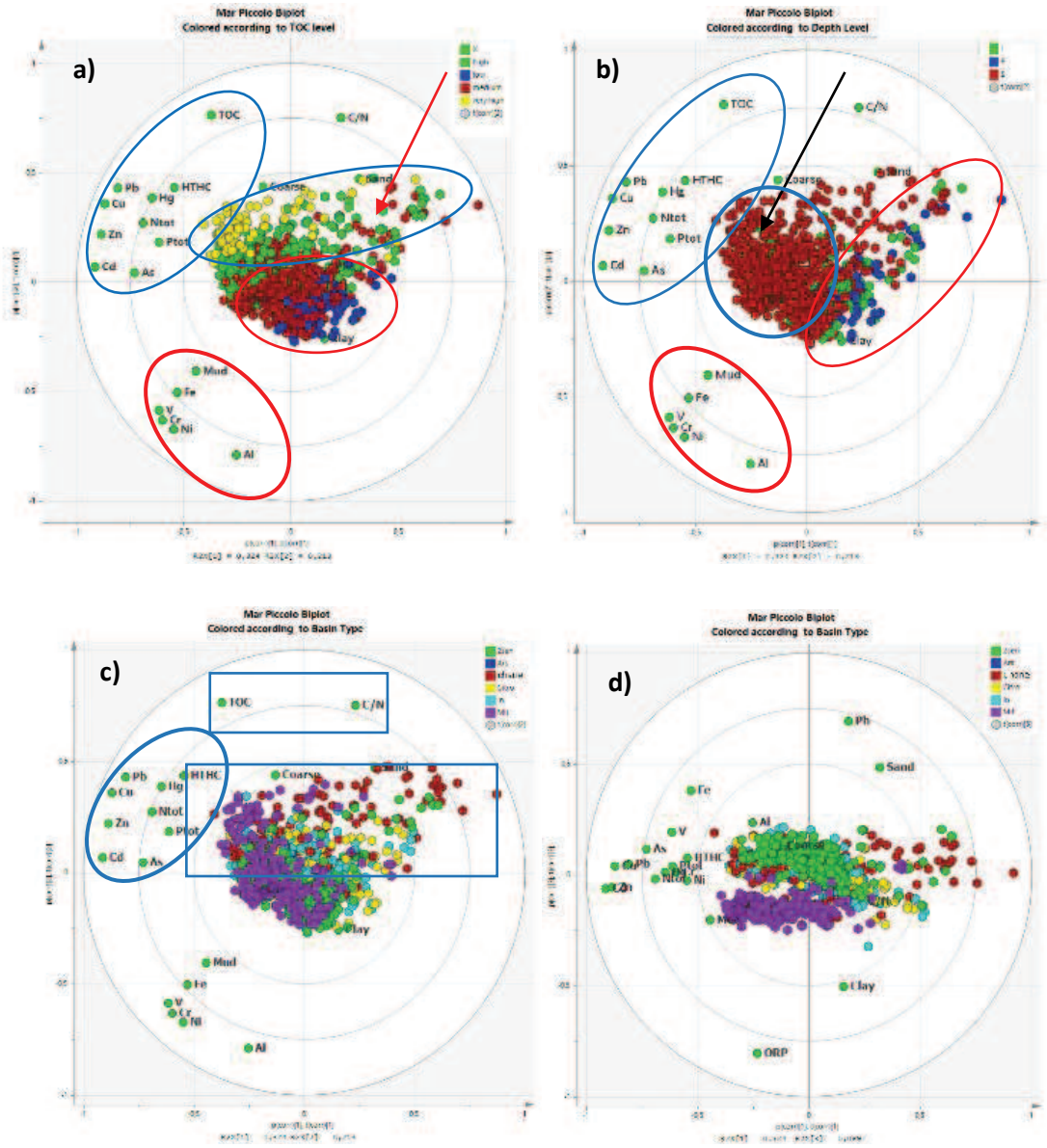


Figure 3. Biplot PC1/PC2, a) colored according "TOC level", b) colored according Depth, c) colored according "basin type", d) Biplot PC1/PC3 colored according to "basin type"

The biplot graph colored according to "depth" (Figure 3b) points out that almost all superficial (S) sediments are associated with anthropogenic-influenced metals Pb-Cu-Zn-Cd-As-Hg and HTHC, N_{tot}, P_{tot}, while the majority of intermedium (I) and deep (P) samples are associated with two grain size phases (sand and clay). Also in this case some exceptions are detected: few I samples (green) show the same trend of the superficial ones (indicated with black arrow, Figure 3b), suggesting an anthropogenic metal contamination also in the intermedium layer for these sites. These exceptional cases belong all to "channels" and "Citro" basin type areas (Figure S1a,b). For this reason, we decided to study the biplot PC1/PC2 projection colored according to "Basin type"

(Figure 3c). The graph confirms that almost all samples coming from Channels (red) and the predominance of samples from "Citro" (yellow) are affected by contamination, irrespective of the depth. We can reasonably assume that these samples are more subjected to dynamic conditions, *i.e*. high hydrodynamic circulation and bio-perturbation phenomena, that might promote an active vertical transport of metal contaminants versus the deepest layers (Pascali et al. 2015; Mali et al. 2017b). In addition, it is evident that the superficial layer of "channel" and "Citro" resulted more affected by terrestrial organic matter (high values of C_{org}/N ratio) (Figure S1c) than by metals suggesting that the provenance of organic matter in sediments might control the distribution of contaminants (Calace et al. 2008).

The biplot projection of PC1/PC3 (Figure 3d) shows evidence of another interesting factor: samples located in the mussel (Mit) culture activity (violet) resulted well separated from the rest of basin groups. Indeed, Mit-samples are characterized by low pH values and increasing ORP (less negative values), suggesting probable metal leaching processes from sediment to water column (Miao et al. 2006; Malviya & Chaudhary, 2006), although high concentrations of Cd, Hg and Zn persist in these sediments, as shown by the variable loading of PC1/PC3.

Finally, Cluster Analysis (CA) considering the whole dataset was carried out, using Ward′s method. By comparing the cluster dendrograms (Figure 2d) with cluster obtained by analyzing only 70 % of information of four PCs (Figure 2c), it is apparent that the clusters found in both CA and PCA are almost the same (the only difference is constituted by As that belongs to Cd, Cu, Pb, and Zn cluster in CA). This evidence corroborates our conclusions based on the selected PCA model.

3.3 ANOVA analysis

PCA elaborations discussed so far allowed understanding the influence of several factors on the contamination pattern. Nonetheless, when the hazard assessment is required, the simultaneous effect, synergistic or antagonist, of different loading factors needs to be clarified. We decided to combine PCA elaboration with the factorial ANOVA in order to determine the factors that most significantly control the chemical compositions of marine sediments and to gain insights into their possible synergistic effects. The ANOVA procedure is detailed in Appendix B. The independent factors selected for ANOVA discussed in the present paper are: organic matter content, expressed in terms of TOC range concentration (TOC level), grain size distribution and sampling depth, whose classes are described in the section 3.1. We performed the two-way ANOVA considering

two different pairs of independent factors (A, B, AxB), *i.e.* TOC Level *and* Depth, Gran Size *and* TOC level and Grain Size *and* Depth.

The results of two-way ANOVA (3x4) considering Depth as factor A and TOC level as factor B, are shown in Table 1, reporting the values of variance ratios (F), probabilities (p), and effect size (η^2). Usually, when the estimated probability (p) is lower than 0.01, the considered independent factor makes a significant difference in the contaminant concentration. In our case, almost all factors resulted statistically significant ($p < 0.01$). This is because a statistical test with a large amount of input data (n = 1021) is highly sensitive to very small differences. Therefore, we decided to calculate also the partial effects given by η^2 to form a plausible estimation of p value irrespective of sample number. Magnitudes of $\eta^2 < 0.01$, $0.01 \leq \eta^2 < 0.05$, $0.05 \leq \eta^2 < 0.10$, and $\eta^2 \geq 0.10$ represent no effect, a small effect, an intermediate effect, and a significant effect, respectively (Ohta et al. 2015). In the present study, we assume that each factor with $\eta^2 \geq 0.05$ has a *significant effect* on the contamination patterns of the sediments. On the other hand, the interaction effect (AxB) refers to the influence of a factor on the other one, helping thus in quantifying how and to what extent both A and B factors synergistically affect the concentration of contaminants. We assume synergistic interaction effects *insignificant*, *significant* and *dominant* for $\eta^2_{(axb)} < 0.01$, <0.01 $\eta^2_{(axb)} < 0.05$ and $\eta^2_{(axb)} > 0.05$, respectively. This estimation is conform to the main purpose of the ANOVA elaboration, aiming at understanding the causes of synergistic effects and not at explaining the 100% variability of the contaminant concentration (our dependent variables) (Shaw & Mitchell-Olds 1993; Ohta et al. 2015).

Type II	A=Depth B= TOC (3x4)								
	F			p			η2		
	A	B	AxB	A	B	AxB	A	B	AxB
Ph	4.00	24.2	1.50	0.02	0.00	0.17	0.01	0.07	0.01
W	25.1	28.3	2.90	0.00	0.00	0.01	0.04	0.07	0.01
ORP	0.30	28.4	1.40	0.74	0.00	0.22	0.00	0.08	0.01
C/N	12.0	197	5.43	0.00	0.00	0.00	0.01	**0.36**	0.02
Al	18064	79.1	2.41	0.00	0.00	0.03	0.03	**0.18**	0.01
As	182	2.48	12.0	0.00	0.06	0.00	**0.25**	0.01	**0.06**
Cd	126	45.0	10.2	0.00	0.00	0.00	**0.17**	**0.09**	**0.05**
Cr	26.1	33.5	3.30	0.00	0.00	0.00	**0.04**	**0.09**	0.02
Fe	29.6	18.7	5.64	0.00	0.00	0.00	**0.05**	**0.05**	0.03
Hg	73.9	59.4	4.40	0.00	0.00	0.00	**0.11**	**0.13**	0.02
Ni	15.0	47.3	5.30	0.00	0.00	0.00	0.02	**0.12**	0.03
Pb	138	123	5.20	0.00	0.00	0.00	**0.16**	**0.22**	0.02
Cu	146	101	14.0	0.00	0.00	0.00	**0.17**	**0.18**	**0.05**
V	41.1	22.6	1.89	0.00	0.00	0.08	**0.07**	**0.06**	0.01
Zn	133	59.5	7.94	0.00	0.00	0.00	**0.18**	**0.12**	0.03
Ptot	18.1	52.2	3.73	0.00	0.00	0.00	0.03	**0.13**	0.02
Ntot	18.7	156	4.16	0.00	0.00	0.00	**0.02**	**0.30**	0.02

TOC	3.45	1951	0.69	0.03	0.00	0.66	0.00	**0.85**	0.00
HTHC	27.9	73.3	4.86	0.00	0.00	0.00	0.04	**0.17**	**0.02**

Table 1. The variance ratios (F), probabilities (*p*), and partial effect size (η^2) due to each main factor (factor A: Depth), (factor B: TOC level), and the interaction effect (factor A×B) of two-way ANOVA 3x4 - Type II)

The two-way model reveals that sampling depth (Factor A) strongly influences the concentration of all metals, while TOC level (Factor B) has a dominant influence on the contamination distribution of almost all metals except for As (η^2 = 0.01, p>0.01). Furthermore, the interaction effects of both factors (η^2_{AxB}) resulted important for all metals but the weight of interaction effect is different for two metal-groups. For As, Cd and Cu the interaction effect is *dominant* (η^2>0.05), while it resulted *significant* (η^2>0.01) for the other metals (Cr, Fe, Ni, Pb, Zn, Hg), nutrients and HTHC. In order to facilitate the interpretation of data and to get insights into the interaction effects, the results of two way ANOVA are graphically reported in Figure 4.

Inspection into the graphs reveals that the concentrations trend of Cd, Zn, Cu, Pb, As and Hg within superficial layers (blue line) differs substantially from the trend determined in the intermedium and deep levels (green and red lines, respectively): in superficial samples the higher the TOC content the higher the metal concentration while in intermedium (I) and deep (P) layers, the higher the TOC content the lower the metal concentration (Figure 4). The circumstance suggests a different source of TOC within different layers. TOC of superficial layers seems to derive from organo-metallic or organic contaminants (like HTHC), while TOC of the deeper layers seems to be not containing metal-binding substances, for instance unreactive humin or degraded organic matter.

The concentrations of nutrients (N_{tot} and P_{tot}) and HTHC within samples of superficial layers (S) follow the same trend displayed by Cd, Zn, Cu, Pb, As and Hg, *i.e.* they increase when TOC level increases. This trend is in line with Kenney study (Kenney et al., 2002), that reported an enhancement of primary productivity when nutrient supply increases, which in turn leads to an increase of TOC.

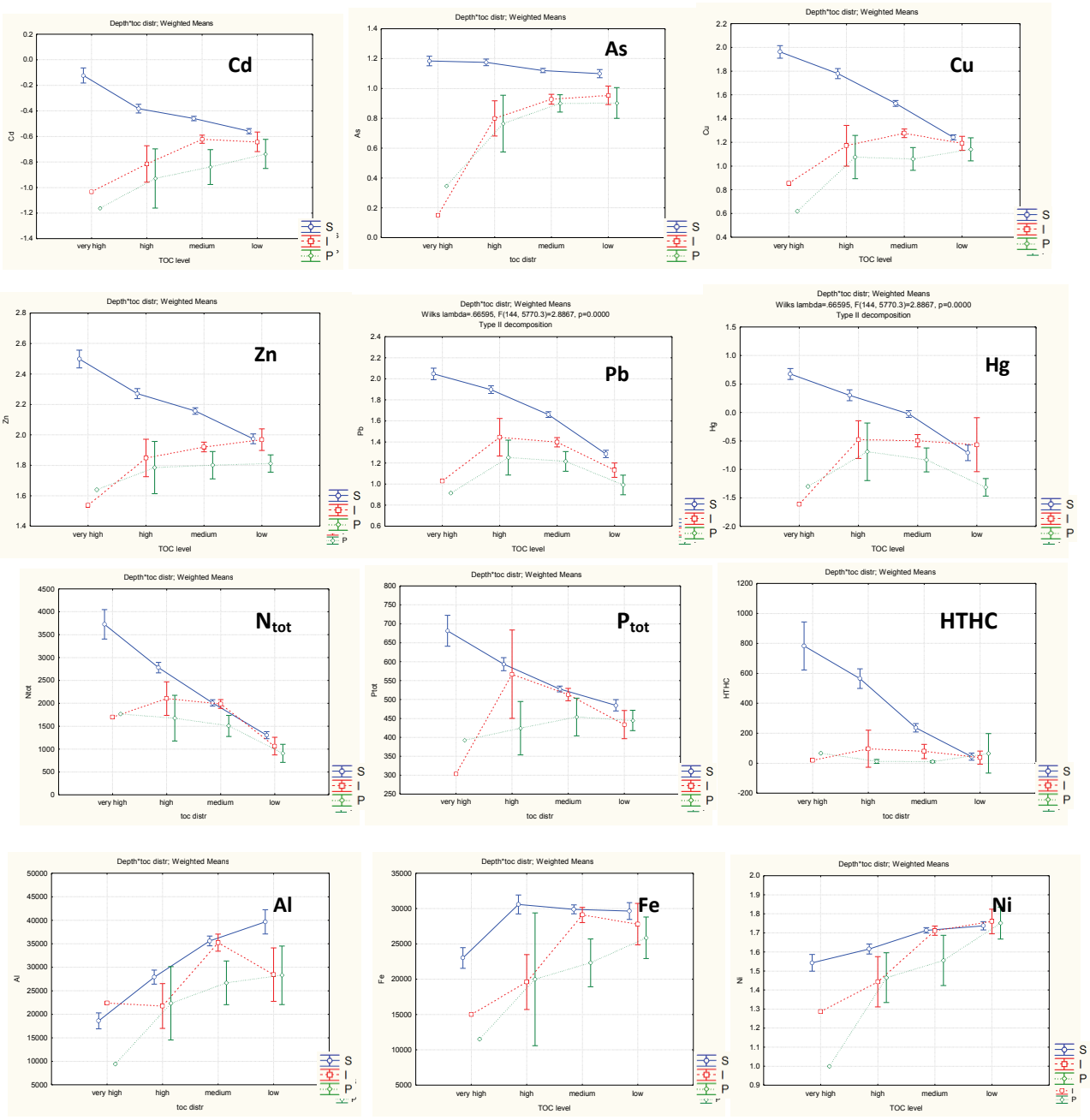


Figure 4. Mean concentrations (calculated as weighted means) of Cd, As, Cu, Zn, Pb, Hg, Ntot, Ptot, HTHC, Al, Fe and Ni according to two main effects, Depth (Factor A in ordinate, with three classes, superficial (S), intermedium (I) and deep (P)) and TOC concentration level (Factor B, in abscissa, with four classes, "low", "medium", "high" and "very high") (F, p, η^2 are reported in Table 1).

Nevertheless, the distribution trend of N_{tot} and P_{tot} *vs* TOC is different for the three depth layers. In the intermedium layer (red line), concentrations of N_{tot} and P_{tot} increase when TOC increases from "low" to "high" level, but they decay immediately when TOC passes from "high" to "very high"

level. On the other hand, for the P level (green line), the increase of TOC corresponds to a decrease of nutrients. This circumstance reinforces the hypothesis of different nature of organic matter in the different layers and suggests also that synergistic effects may occur in sediments of intermedium layer when the TOC level become high (more than 3%).

As to HTHC, their concentration in the intermedium and deep layers does not change with increasing of TOC, indicating almost a surficial sediment contamination by HTHC.

Analyzing two-way ANOVA graphs for lithogenic metals Cr, Ni, V, Fe and Al, it is apparent that an increase of TOC goes with a depletion of metal concentration irrespective to depth, except for the intermedium layer (red line), in which metal concentrations increase when TOC passes from "low" to "medium" level. Also in this case, the intermedium layer present an irregular pattern.

Concerning the role of the granulometry in the distribution of contaminants within different depth layers, two-way ANOVA considering as independent variables the Grain Size (Factor A) and Depth (Factor B) was carried out. Both factors influence the concentration of metals as indicated by high η^2 values for almost all metals (Table S3), while their interaction effects resulted to be minor significant with respect to the interaction of TOC *with* Depth. Inspection of graphs (Figure S2) reveals that anthropogenically influenced metals (As, Cd, Cu, Pb, Hg and Zn) display higher concentration in the surficial layers (blue lines) with respect to the deepest layers (red and green lines) irrespective to the grain size. Clear exceptions are again evident for samples in intermedium layer (red lines) (in Figure 5a Hg distribution is shown as representative of the anthropogenically-influenced metal cluster), that display an increase of metal concentrations passing from finest (<63μm) to mixed grain size. This anomalous trend is followed also by N_{tot} (Figure 5b) and all lithogenic metals such as Al (Figure 5c), that display increasing concentrations with increasing of grain size from "fine" to "mix". On the contrary, the amount of TOC (Figure 5d) unexpectedly decreases with the decrease of the grain size.

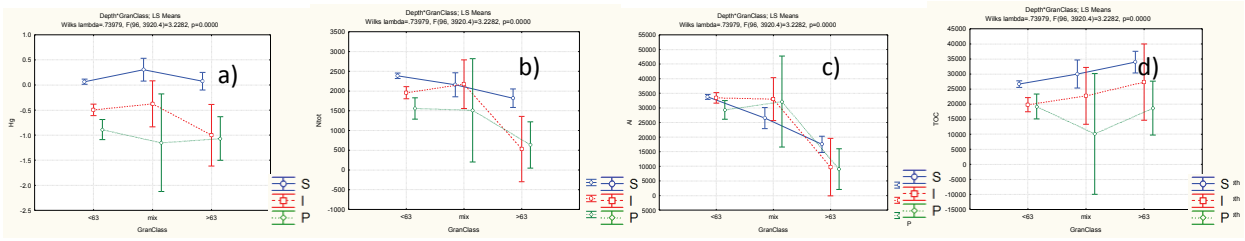


Figure 5. Mean concentrations of Hg (a), N_{tot} (b), Al (c) and TOC (d) in different Grain Size classes (Factor A, represented in ordinate with three classes θ<63μm, mix, θ>63μm) and different

depth (Factor B, reported in abscise with three classes, surficial (S), intermedium (I) and deep (P)), obtained from the two-way ANOVA (3x3). (F,p, η^2 are reported in Table S3).

It is clear that the intermedium layer represents a discontinuity point between the surficial and deepest levels and we can put forward the following hypothesis:

i. sediments with "mixed grain size" in the intermedium layer (I) can be interested by contamination of both metals and nutrients coming probably by channels that causes the anomalous contamination pattern of this layer (see PCA biplot graphs 3);

ii. synergistic effects of organic matter and grain size can occur, especially considering salt water medium. In fact, Koltyar et al. (Koltyar et al. 1996) found that concentration of salinity above 0.5 % in sediments with relevant organic matter acts as a coagulant of fine particles, promoting a fast aggregating process. In the salt water of Mar Piccolo, increase of fine particles coupled with certain levels of organic matter (from 3-5%), could drive to aggregation process of finest sediment, that turn in higher concentration of metals in these samples.

iii. in the deepest layer (P), fluxes of un-reactive and nitrogen-poor organic matter in sediments with >63μm, might be present. The depletion of *all* metal contents (including lithogenic ones) with increase of quartz-rich phases (sand) suggests that a dilution effect might occur (Hedges et al. 1984; 1995). However, quartz dilution effects can only partially control the metal distribution in our case, as revealed by the negative, but not statistically significant R values found for the sand fraction *vs* lithogenic metals correlation (R<–0.50, Table S4) (Bern R.C. 2009).

In order to verify the advanced hypothesis of coupling effects of TOC with Grain Size, we completed the statistical analysis performing two way ANOVA (TOC *and* Grain Size). The values of Wilks lambda=0.83, F (90, 5602.1)=2.0568, η^2=0.33 confirmed significant main effects of both TOC and Grain Size (Table S5), revealing that TOC concentration exerts more dominant effects with respect to the Grain Size. Inspection of graphs for each contaminant, reported in Appendix C, confirms the odd metal pattern in samples with "mixed" grain size and "medium" TOC concentration. Consequently, we can reasonable suppose that the unusually high metal concentrations found in the samples with "mixed" grain size and "medium to high" TOC level are probable due to the synergistic effects of grain size, organic matter and salinity as previously

403 reported (Koltyar et al. 1996). Further studies are still necessary to better understand the effect of
404 salinity as independent factor.

405 **Conclusion**

406 This study demonstrates that chemometric approach is an advantage for assessing and modeling
407 contamination patters of highly contaminated areas on a large scale, and thus could contribute to
408 effective monitoring their quality. The spatial distribution of contaminants within Mar Piccolo,
409 analyzed using two complementary chemometric techniques, PCA/CA and factorial ANOVA,
410 reflected the complex contamination pattern, arisen from the superposition of natural and
411 anthropogenic processes and by multiple factors acting simultaneously on a local scale.

412 PCA/CA technique gave the following informations:

413 i. Contaminants can be divided in two clusters. The first one contains all sulphofilic metals (As,
414 Cd, Cu, Hg, Pb and Zn), N_{tot}, P_{tot} and organic pollutants (HTHC). All of them resulted highly
415 associated with TOC. Within the group, dissimilar fates are also distinguishable: Cd, Cu, Pb
416 and Zn resulted differently influenced by absorption/desorption mechanisms onto iron-
417 sulphides or iron-oxyhydroxides with respect to As and Hg that, on the contrary, resulted
418 strongly influenced by alkylation phenomena promoted by TOC. Furthermore, slight
419 differences were noticed for Cd, Zn and Hg, which resulted highly concentrated in the organic
420 matter-reached sediments of the mussel culture area despite the low pH conditions here
421 determined. Relationship with textural sediment phases seems to influence the
422 mobilization/immobilization mechanisms of these elements. The second cluster includes
423 lithogenic elements (Cr, V, Fe, Al and Ni) originated by parent rocks of Mar Piccolo
424 sediments, thus not anthropogenically enriched.

425 ii. The areas of most concern resulted to be: those close to "Navy" characterized by high
426 concentration of toxic metals, those exposed to natural waterways and channels when a
427 vertical transport of contaminants versus the deepest layers occur due to high hydrodynamic
428 condition and due to high organic matter contents, and, finally, the areas hosting the mussel
429 activity plants characterized by high organic matter and low pH values, thus subjected to
430 leaching phenomena.

431 iii. The depth interested by high contamination either by metals and by organic compounds seems
432 to be the surficial one (up to 50 cm), while the distribution of contaminants within the deepest

layers resulted influenced by different processes and factors acting simultaneously (absorption/desorption phenomena, quartz or organic matter dilution processes etc.).

On the other hand, ANOVA analyses pointed out the main factors controlling the contaminant pattern that can be summarized as following:

- The nature and origin of organic matter within different depth layers
- The rates of dilution by terrigenous sediments, being the principal diluents quartz and/or organic matter coming with material flow through channels;
- The grain size fractions and aggregation phenomena promoted in salt water;
- The original composition/mineralogy of the sediment parent rocks.

The combination of both techniques revealed that the intermedium layer represents a discontinuity point in terms of contamination trend between the surficial and the deepest levels related to synergistic effects of independent factors such as TOC and Grain Size. Nevertheless, TOC concentration resulted to be dominant conditioning factor with respect to Grain Size.

The evaluation of future scenarios and associated environmental risks in the study area need investigation of conjunction effects of salinity and pH on contamination pattern.

The analyses show the importance of a spatially representative overview given by PCA-CA/ANOVA to infer the historical anthropogenic source loading on the area and new factors affecting areas of most concern, making more complex the dynamic of the whole ecosystems.

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