Trace metals in mussels, in contact with ships laid-up within the gulf of Elefsis, Greece

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Abstract The use of organotin compounds as biocides in antifouling paints has been banned since 2008 by the International Convention on the Control of Harmful Antifouling Systems on Ships of the International Maritime Organization. As a result, copper-based anti-fouling paints have been extensively used. Antifouling residues represent a significant, heterogeneous source of metallic contamination in the marine environment, where boating activities occur. However, it is often difficult to discriminate between metal inputs from ship and other sources, such as urban run-off. Laid-up ships, therefore, provide an opportunity for studying metal inputs from the specific source in the absence of other major sources.

Results from a field project within the Elefsis Gulf, Greece, demonstrate a statistically significant difference (one way ANOVA, $p<0.05$) characterizing As, Co, Cu, Mn and Zn levels in mussels (*M. galloprovincialis*) attached to ships laid-up at several anchorage sites, in comparison to those from other sites. In mussels attached on a ship laid-up nearby a small ship maintenance facility significant concentrations of Cd, Mn, Pb and Zn ($p<0.05$) were determined. In the case of a sunk ship relatively high concentrations of Co, Cr, Fe, Mn, Ni, Pb, V and Zn ($p<0.05$) in mussels were measured, probably attributed to the faster corrosion of the metallic surfaces of the ship as well as to fuels and oils leakage.

Keywords: trace metals; seawater; mussels; antifouling

1. Introduction

Antifouling materials were used since ancient times constituting of natural products such as wax, tar and asphalt (Callow 1990). During the second half of the 20th century the main types of antifouling paints used are systematically based on the dispersion of toxicants in different types of polymeric binders, differing according to their mechanisms of releasing toxicants in seawater (Almeida *et al*. 2007).

Tributyltin (TBT) compounds are organotin compounds being the most effective antifouling agents discovered so far. They have been applied for 40 years as biocides in antifouling paints, commonly known as the bottom paint applied to the hulls of ocean going vessels. In the 1970s, TBT-based antifouling paints were first recognized as an important source of pollution in Arcachon Bay, France, when oyster production nearly collapsed because of elevated concentrations of TBTs (Alzieu 2000).

The International Maritime Organization (IMO) has agreed on the implementation of regulations phasing out the use of TBT antifouling paints on all vessels by 2008 (IMO 2001). As a consequence, most contemporary marine antifouling paints contain a Cu(I)-based biocidal pigment (e.g. cuprous oxide or, less commonly, cuprous thiocyanate). Also, zinc oxide is sometimes applied as the principal, albeit relatively weak, biocidal pigment, but is generally used more in combination with Cu, increasing toxicity (Turner 2010; Cao *et al*. 2011). Hence, antifouling paints containing heavy metals are generally considered as an important pollution source.

Since antifouling is affected by the slow, controlled leaching of biocides from painted surfaces, elevated environmental concentrations of these chemicals including metals are more significant in semi-enclosed marine systems, such as harbors, marinas and estuaries, where the transport, berthing or docking of vessels is significant. These features characterize the gulf of Elefsis, a protected semi-closed embayment, being in contact with Saronic Gulf through two narrow channels to the east and west. Its total area is calculated to cover approximately 67 km^2, with a maximum depth of 33 m, consisting approximately of 1.2 km^3 of water. It is the most significant anchorage of temporarily laid-up and confiscated ships in Greece, with their number fluctuating, but usually exceeding 40. Sunk ship within the Elefsis gulf are calculated at approximately 30. The period 1982-84 the highest number of anchored ships was recorded in Greece, with the highest reported within the Elefsis gulf (435).

This study, hence, attempts to demonstrate a potential link between the presence of vessels and the concentrations of metals (As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, V, Zn) determined in mussels (*Mytilus galloprovincialis*) attached and multiplied, under the sea surface, on ship hulls and in the seawater of anchorage sites. Under this perspective,

laid-up vessels were studied in relation to a vessel anchored nearby the area, where restricted ship maintenance activities take place, as well as to a vessel half-sunk for approximately 15 years within the gulf. For comparison purposes, mussel samples were also obtained by a mussel culture of the study area and by a distant sampling site within the gulf, considered as reference site.

2. Materials and methods

2.1. Sampling

Sampling of seawater and mussels was performed by scuba diving in May 2016. Seawater temperature, pH and salinity were measured *in situ* with a YSI 63 pH/conductivity meter (YSI, Brannan Lane, Ohio, USA).

Samples were obtained from five (5) stations within the Elefsis gulf (Figure 1). Sampling station No. 1 was located at a ship half-sunk for ~15 years; samples were collected both in contact (site 1A) and at a distance of 30 m (site 1B) from the ship. Sampling site No. 2 corresponds to a group of three (3) ships laid-up within the gulf for different time periods; the first ship has been laid-up for 12 years (site 2A) and the second for 8 months (site 2B). Sampling station No. 3 was located at a ship laid-up for ~6 years nearby the area where restricted ship maintenance activities take place (site 3), No. 4 at the area of a mussel culture (site 4), whereas No. 5 corresponds to the reference site (site 5) located at the centre of the western part of the gulf, at a distance from the ships anchored within the eastern part of the gulf.

Seawater samples were placed in FEP-Teflon containers previously thoroughly cleaned with HNO_3 10% and Milli-Q water 18.2 MΩ cm (Millipore, Bedford, MA, USA). Samples were transported (refrigerated) immediately to the laboratory, where they were filtered through 0.4 μm polycarbonate filters rinsed with HCl (10%) and Milli-Q water 18.2 MΩ cm (Millipore).

Mussel samples were immediately transferred to the laboratory in plastic containers within a portable refrigerator. The sizes of collected samples were 3-5 and 5-6 cm. Soft tissues were dissected, pooled (10-15 specimens per site and size), lyophilized, homogenized and stored inside a desiccator until analysis.

2.2. Analysis

Total dissolved metals in seawater were determined using the solvent extraction technique described by Danielsson *et*

al. (1982). Manipulations were performed inside a clean room in order to minimize the risk of metal contamination.

For metal determinations in mussel specimens, samples were digested using a mixture of HNO_3 (supra pure 65%) (Merck, Darmstadt, Germany) and H_2O_2 (supra pure 30%) (Merck), according to the procedures described by Copat *et al* (2013) and Santos *et al* (2013), slightly modified.

Metal determination in both the preconcentrated seawater and digested mussels samples was carried out through inductively coupled plasma mass spectrometry (ICP-MS) by a Thermo Scientific ICAP Qc (Waltham, MA USA). Measurements were carried out in a single collision cell mode, with kinetic energy discrimination (KED) using pure He.

The limits of detection (LOD) of metals in seawater (in ng L^{-1}) were 0.04 for Co, 0.20 for Cd, Cr, Ni and Pb, 0.40 for Cu and Mn, 0.30 for V, 0.80 for As, 2.0 for Zn and 6.0 for Fe and those in biota (in ng g^{-1}) 0.25 for Co, 1.2 for Cd, Cr, Ni and Pb, 2.5 for Cu and Mn, 1.9 for V, 5.0 for As, 12 for Zn and 38 for Fe (USEPA, 1997).

2.3. Quality assurance

Quality control measures included the use of laboratory reagent blanks, sample triplicates and certified reference materials (CRM), which were analysed for the evaluation of measurement accuracies. For every batch of six (for seawater) and ten (for biota) samples, a reagent blank and a CRM sample were run.

For seawater analysis the CRM "NASS-6, Seawater reference material for trace metals" (National Research Council Canada) was analysed, whereas for biota analysis the "ERM® - CE278k, Mass fractions of elements in mussel tissue (Joint Research Centre, EC, Geel, Belgium). In both cases, except Co and V not included in ERM® - CE278k, the obtained recoveries were calculated in the range ±20%.

2.4. Statistical analysis

One-way ANOVA and principal component analysis (PCA) were carried out using the software package IBM SPSS Statistics 24. A value of $p < 0.05$ (95% confidence level) was considered to indicate a significant difference in the statistical analysis of the data.
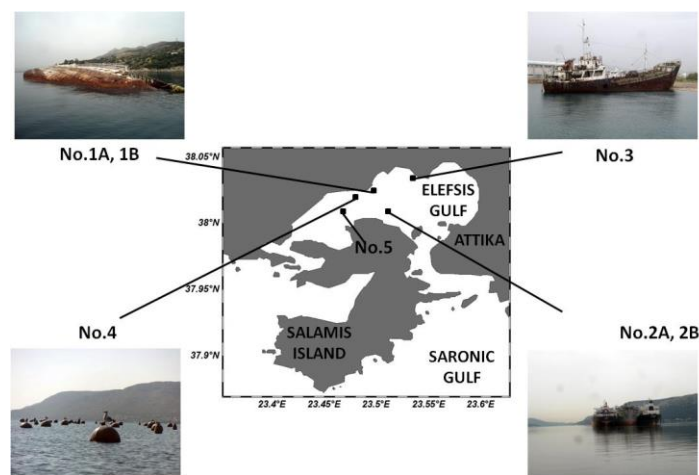
Figure 1. Sampling stations

3. Results and discussion

Considering all the sampling sites examined in this work, the obtained concentrations of dissolved metals in seawater were in the range <100 ng L^{-1} for As, Cd, Co, Cr, <500 ng L^{-1} for Mn, Pb, V, <1000 ng L^{-1} for Cu, Fe, Ni whereas in the case of Zn some samples exceeded 1000 ng L^{-1} (Table 1). Dissolved metal levels are considered as typical of coastal marine environments. Despite the fact that the lowest dissolved metal concentrations were obtained for the reference station No.5, the corresponding differences with the other sampling sites examined were not significant.

The mean concentrations of the studied metals in the soft tissues of the mussel M. *galloprovincialis* are shown in Table 2. Considering the total of metals and sampling sites of this work, the obtained mean concentrations decreased in the following order: Fe>Zn>As>Pb>Mn>Cu>V>Ni>Co>Cr>Cd. This order is in accordance with that referring to metal concentrations in mussels from the coast of Apulia (Southern Italy) (Spada *et al.*, 2013), with the single exception of Pb, since higher Pb levels were determined in the present study.

In mussels attached on the hull surface of the sunk ship (sampling station 1A) relatively high mean concentrations were obtained for Fe, Ni, Cr, V, Mn, Zn, Pb and Co ($p<0.05$; Table 2). Regarding the hull of the temporarily anchored vessel (sampling station 2A) relatively high mean concentrations in mussels were obtained for Co, Zn and Pb ($p<0.05$; Table 2), whereas regarding station 2B for Cu, Co, As, Zn, Pb and Mn ($p<0.05$; Table 2). In the case of a ship anchored nearby an area where restricted ship maintenance activities take place relatively high concentrations in mussels were measured for Cd, Zn, Pb and Mn ($p<0.05$; Table 2). It is noteworthy that in mussels from the site of the ship wreck the highest metal concentrations were recorded (Table 2), whereas for some metals such as Fe, Ni, Cr and V the concentrations obtained for other sites, also characterized by the presence of ships, were lower. In the case of the ship-wreck a faster corrosion-oxidation of the extended metallic surfaces of the vessel occurs, with a continuous release of small quantities of petrol and oils simultaneously taking place,

significantly affecting the concentrations of some metals in the mussels attached. Consequently, the specific metals are probably not related to antifouling compounds. Regarding the anchored ships (stations 2A, 2B, 3), relatively elevated metal concentrations in attached mussels were measured for Cu and Zn, which are known to relate with antifouling compounds, as well as of Pb, Co and Mn. The relatively high concentrations of As are apparently linked to the presence of vessels, however further investigation would be required on this matter. The significant Cd concentration measured at station 3 is probably attributed to terrestrial sources, due to the presence of an important stream flowing at the specific area and to restricted local shipyard activities.

In order to evaluate data trends and gain an overview of the relationships among the metals examined, principal component analysis (PCA) was performed for the mussel samples analysed. In factor analysis the value of the Keiser-Meyer-Olkin (KMO) statistical criterion is equal to 0.771, sampling adequacy values (MSA) are all >0.63 and two principal components (PCs) with eigenvalues >1 are extracted. PC1 explained up to 61% of the total variance and PC2 explained up to 20%. According to the component plot obtained (Figure 2), Co, Cd, As, Cu seem to correlate, according to PC1 and Fe with V according to PC2.

4. Conclusions

Due to the low release rate of metals from the hulls of laid-up ships, the determination of these metals in mussels, which multiply and habit in contact with laid-up ships hulls, appears to be a sensitive method for the investigation of metal release. In the specific field study within the gulf of Elefsis, it was demonstrated that laid-up vessels contribute to Cu and Zn, as well as to Co, Mn and Pb. In the case of a sunk ship, relatively high concentrations of Fe, Ni, Cr and V were also determined.

Table 1. Mean concentrations of dissolved metals in seawater samples (N=3), in ng L^{-1} for Cd, Co, As, Cr and Pb and in µg L^{-1} for Cu, Ni, Fe, V, Zn and Mn.

Sampling sites	Cd	Cu	Co	As	Ni	Fe	Cr	V	Zn	Pb	Mn
1A (S[*])	52.2	0.575	39.7	59.1	0.331	0.347	44.4	0.081	0.621	62.2	0.067
1A (B[**])	55.6	0.552	36.6	58.6	0.308	0.711	20.4	0.088	1.15	109	0.026
1B (S)	80.8	0.761	44.5	62.1	0.632	0.356	19.0	0.124	0.668	156	0.081
1B (B)	70.8	0.846	42.3	59.9	0.396	0.389	19.1	0.115	1.21	77.3	0.159
2A (S)	37.9	0.803	41.2	61.0	0.422	0.650	28.8	0.088	0.708	102	0.075
2A (B)	57.6	0.570	46.9	58.8	0.420	0.233	22.8	0.103	0.646	120	0.395
2B (S)	40.3	0.573	37.4	59.3	0.275	0.238	26.1	0.113	0.602	60.8	0.079
3 (S)	51.4	0.636	42.3	60.5	0.377	0.386	20.0	0.119	0.831	116	0.167
4 (S)	49.3	0.111	38.0	58.7	0.387	0.277	19.7	0.134	0.601	73.1	0.072
4 (B)	74.3	0.500	36.2	58.2	0.291	0.385	23.1	0.110	0.638	73.7	0.088
5 (S)	41.2	0.500	34.9	48.8	0.230	0.167	16.5	0.074	0.699	63.2	0.059

[*]Surface [**]Bottom

Table 2. Metal mean concentrations in mussel samples (µg g^{-1} dry weight)

Sampling sites	1A[d]	1B[e]	2A[f]	2B[g]	3[a]	4[b]	5[c]
As	21.0	16.4	19.6	23.7[bc]	19.1	13.6[g]	13.2[g]
Cd	0.492	0.382	0.502	0.472	0.606[bc]	0.329[a]	0.272[a]
Co	1.60[bce]	0.701[dg]	1.35[c]	1.55[abce]	0.935[g]	0.801[dg]	0.493[dfg]
Cr	1.67[abcefg]	0.477[d]	0.566[d]	0.688[d]	0.691[d]	0.399[d]	0.363[d]
Cu	7.36	5.45	7.83	10.0[c]	8.41	6.47	4.90[g]
Fe	3350[abcefg]	596[bcdf]	106[de]	239[d]	244[d]	130[de]	35.0[de]
Mn	21.1[abcefg]	6.70[d]	10.6[d]	11.3[cd]	13.3[cd]	7.92[d]	4.33[adg]
Ni	2.79[abcefg]	0.932[d]	1.16[d]	1.51[d]	1.37[d]	0.817[d]	0.540[dg]
Pb	7.60[bcefg]	2.52[ad]	4.11[acd]	3.73[acd]	6.56[bcefg]	3.51[ad]	1.73[adfg]
V	12.2[abcefg]	3.52[abcefg]	1.46[de]	1.32[de]	1.43[de]	1.01[de]	0.703[de]
Zn	132[bc]	82.0[a]	113[abc]	101[abc]	176[bcefg]	36.0[adfg]	25.2[adfg]

*letters (a, b, c, d, e, f, g) indicate that the mean difference is significant at the 0.05 level (Tukey Post Hoc test)

**Sampling site No.1A (N=3); 1B (N=3); 2A (N=6); 2B (N=10); 3 (N=5); 4(N=4); 5 (N=4)
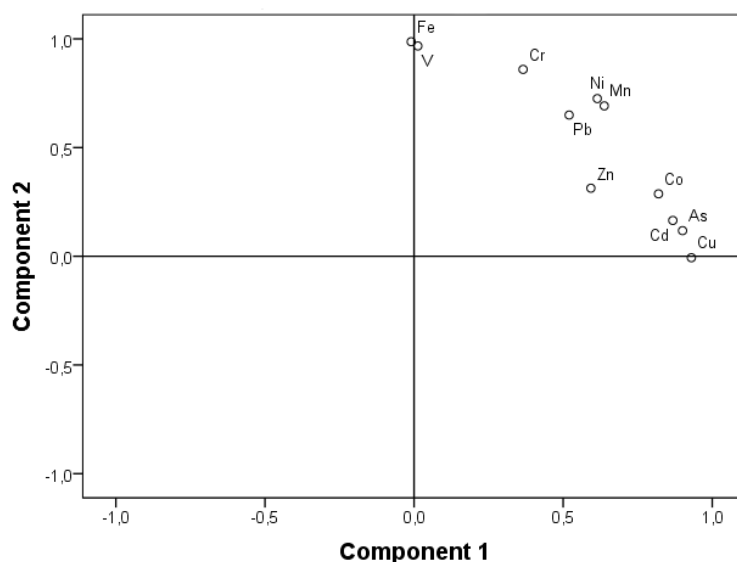
Figure 2. Principal component analysis plot of metals in mussels from all sampling sites.

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