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Sardine and anchovy as bioindicators of metal content in Greek coastal waters

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Abstract

Metal and element concentrations (Li, Na, Mg, P, Ca, V, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Mo, Pd, Cd, Cs, Ba, Hg, Tl, Pb, U) were monitored in the tissues of sardine (*Sardina pilchardus*) and anchovy (*Engraulis encrasicolus*) from six Greek coastal areas subjected to different natural and anthropogenic forcings in order to be assessed as bioindicator species of marine metal pollution. Sardine and anchovy provided a thorough view of the metal pollution load of each site in accordance with local pollution sources. The wide range of pressures applied in Elefsina Gulf and Thermaikos Gulf are depicted in the large number and the kind of elements reaching maximum concentrations among the sites (e.g. Pb, Cu, Hg, As in Elefsina Gulf, and Cd, Ni, P in Thermaikos Gulf) while in the rest of the sites (Amvrakikos Gulf, Strymonian Sea, Thracian Sea, Artemisium Straits), few elements were found at maximum levels. Statistically significant site-specific differences in metal content were detected. The differentiation in metal content of the fish tissues among the sites could be attributed to anthropogenic pressures, different background levels and environmental conditions. Sardine and anchovy can be evaluated as appropriate, reliable and useful bioindicator species of marine metal pollution.

Keywords: metal pollution; sardine; anchovy; bioindicators; site-specific variations.

Introduction

Marine ecosystems have been highly impacted by anthropogenic stressors (Romero *et al*., 2007; Cacador *et al*., 2012; Pavlidou *et al*., 2019; Mehana *et al*., 2020; Salvaggio *et al*., 2020) not only from the ecological but also from the socioeconomic point of view since they are crucial for the well-being of many countries (Lozano-Bilbao *et al*., 2019; Parra-Luna *et al*., 2020). Increasing anthropogenic pressure is promoted by economies built on encouraging the constant consumption of goods and services (Salvaggio *et al*., 2020) but the uncontrolled dumping of pollutants has detrimental effects on human health, society, and future generations (Parra-Luna *et al*., 2020). In the Mediterranean Sea, the degradation of the environment has been reported to have accelerated during the last decades (Basilone *et al*., 2018). Metal pollution assessment is essential before measures to conserve, protect and restore aquatic ecosystems can be taken. Biomonitoring is an effective means to that end (Zhou *et al*., 2008). Bioindicators are species selected to monitor the status or quality of aquatic ecosystems, that are able to offer a quantitative evaluation of pollutants (Whitfield & Elliott, 2002; Maulvault *et al*., 2015), allow identification of the pollutants' origin (natural or anthropogenic) (Zhou *et al*., 2008; Cacador *et al*., 2012), facilitate spatial and temporal comparisons of pollution levels, and, mainly, reveal the impact of pollutants on the ecosystems (Rainbow, 1995; Sarkar *et al*., 2008; Li *et al*., 2010).

The efficiency of a bioindicator species is based on its ability to accumulate pollutants in concentrations much higher than in water but mainly on their ability to be time integrating; they can reveal pollutants that are no longer present in water or are occasionally present (Rainbow, 1995; Saha *et al*., 2006; Zhou *et al*., 2008; Cunningham *et al*., 2019). Furthermore, bioindicators can indicate the impact of pollution on organisms and the ecosystem while this is not feasible when chemical analysis of water or sediment is conducted (Rainbow, 1995; Whitfield & Elliott, 2002; Zhou *et al*., 2008; Tsangaris *et al*., 2010; Cacador *et al*., 2012; Copat *et al*., 2012a). In order to obtain a complete evaluation of ecological status, the combination of biomonitoring and conventional chemical analysis is appropriate (Harris, 1995; Li *et al*., 2010).

Apart from the detection and quantification of pollutants and their impact on the ecosystem (Whitfield & Elliott, 2002), biomonitoring can also contribute to precursory actions, assessments of the measures taken (Zhou *et al*., 2008), and public awareness of the changes in ecosystems due to anthropogenic stressors (Whitfield & Elliott, 2002; Simboura *et al*., 2016; Parra-Luna *et al*., 2020). Rideout & Kosatsky (2017) argued that a collective consideration of planetary health, human health, animal welfare, and social and community factors is essential for fish consumption guidelines. The collective consideration of all these factors is also necessary when legislative measures for the protection of the marine environment are designed. At national and international levels, monitoring networks have been developed for the surveillance and control of pollution, the assessment of its ecological impact, and the conservation or restoration of aquatic ecosystems (Romero *et al*., 2007; Cacador *et al*., 2012). From a regulatory point of view, biomonitoring was introduced into the E.U. by the Water Framework Directive 2000/60/EC (WFD) (Romero *et al*., 2007; Simboura *et al*., 2016). The Marine Strategy Framework Directive 2008/56/EC (MSFD) also uses ecological indicators as tools that indicate the pressure the marine environment is being subjected to (Simboura *et al*., 2016). These directives have been established in order to accomplish good ecological status and good environmental status (Romero *et al*., 2007; Cacador *et al*., 2012; Paraskevopoulou *et al*., 2014).

Various species, such as algae, macrophyte, phytoplankton, zooplankton, parasites, bacteria, bivalve mollusks, fish, invertebrates, echinoderms, isopods, amphibians, and birds, have been suggested as bioindicators (Whitfield & Elliott, 2002; Romero *et al*., 2007; Zhou *et al*., 2008; Maulvault *et al*., 2015; Cunningham *et al*., 2019; O'Callaghan *et al*., 2019; Mehana *et al*., 2020; Parra-Luna *et al*., 2020). Fish have been reported as good bioindicators for aquatic metal pollution assessment (Harris, 1995; Whitfield & Elliott, 2002; Djedjibegovic *et al*., 2012; Metian *et al*., 2013; Cunningham *et al*., 2019; Salvaggio *et al*., 2020) since they are sensitive to changes due to anthropogenic pressures (Harris, 1995; Whitfield & Elliott, 2002; Li *et al*., 2010). Apart from accumulating large amounts of metals (Bat *et al*., 2014; Cunningham *et al*., 2019; Salvaggio *et al*., 2020), a major advantage is that there is wider public sensitization and understanding of information concerning fish (Harris, 1995; Whitfield & Elliott, 2002). Analyses of fish can contribute to identifying the dangers pollutants pose to organisms and through the food chain to humans (Cacador *et al*., 2012), and that is the most important reason they are considered good bioindicators (Zhou *et al*., 2008; Li *et al*., 2010).

To the authors' knowledge, there are a few studies that have used sardine or anchovy as bioindicator species for marine metal pollution in Egypt, Turkey, and Italy (Ahdy *et al*., 2007; Copat *et al*., 2012a, b; Bat *et al*., 2014). Thus, this study aims at evaluating the potential role of sardine (*Sardina pilchardus*) and anchovy (*Engraulis encrasicolus*) as bioindicators of marine metal content. To this end, the metals and elements Li, Na, Mg, P, Ca, V, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Mo, Pd, Cd, Cs, Ba, Hg, Tl, Pb, and U were monitored in the tissues of sardine and anchovy from six Greek coastal areas exhibiting natural variability and subjected to a gradient of anthropogenic stressors. Data regarding the pressures imposed, the pollution sources, and the overall status assessment data for each site were considered.

Materials and Methods

Study areas description and environmental pressures

Sampling locations are presented in Figure 1. The ecological status varies among the studied sites due to the different natural and anthropogenic forcings applied in each case. The pressure classification of the studied sites and the overall status assessment data (integrative status) reported are based on the results of Pavlidou *et al*. (2015) and Simboura *et al*. (2016). Both of these studies used analytical biological and environmental data derived from the Greek WFD coastal waters monitoring network and applied the pressure index as modified by Pavlidou *et al*. (2015) to assess the multiple stresses imposed on each site, including eutrophication, sewage discharge, organic enrichment-agriculture, industrial discharge–chemical pollution, physical and hydro-morphological alteration, dredging and sediment disposal–spoil wastes, aquaculture, and harbor–marina–port, featuring the main anthropogenic stressors reported in the Water Information System for Europe (WISE-SoE) for coastal and marine waters (EEA, 2015, Simboura *et al*., 2016).

The wetland complex of Thermaikos Gulf constitutes one of the most important and diverse ecosystems in Greece and is protected by the Ramsar Convention (1971) (Albanis *et al*., 1996). Thermaikos Gulf is a semi-enclosed area impacted by various anthropogenic activities: treated or partly treated sewage, industrial and agriculture discharges, and harbor and aquaculture activities (Christophoridis *et al*., 2009; Pavlidou *et al*., 2015; Simboura *et al*., 2016). The city of Thessaloniki, with a population of over one million, a developed industrial zone, and one of the biggest commercial harbors in Greece, is located in the inner part of the gulf.

The Strymonian Sea is a semi-enclosed water body adjacent to areas of agricultural and touristic activities and is considered among the most important nursery and fishing grounds in the North Aegean Sea for pelagic species like sardine and anchovy. It is the final recipient of the Strymon River discharges (agricultural wastes, sewage, effluents from Greek and Bulgarian industries situated along its banks) and the Richios River discharges

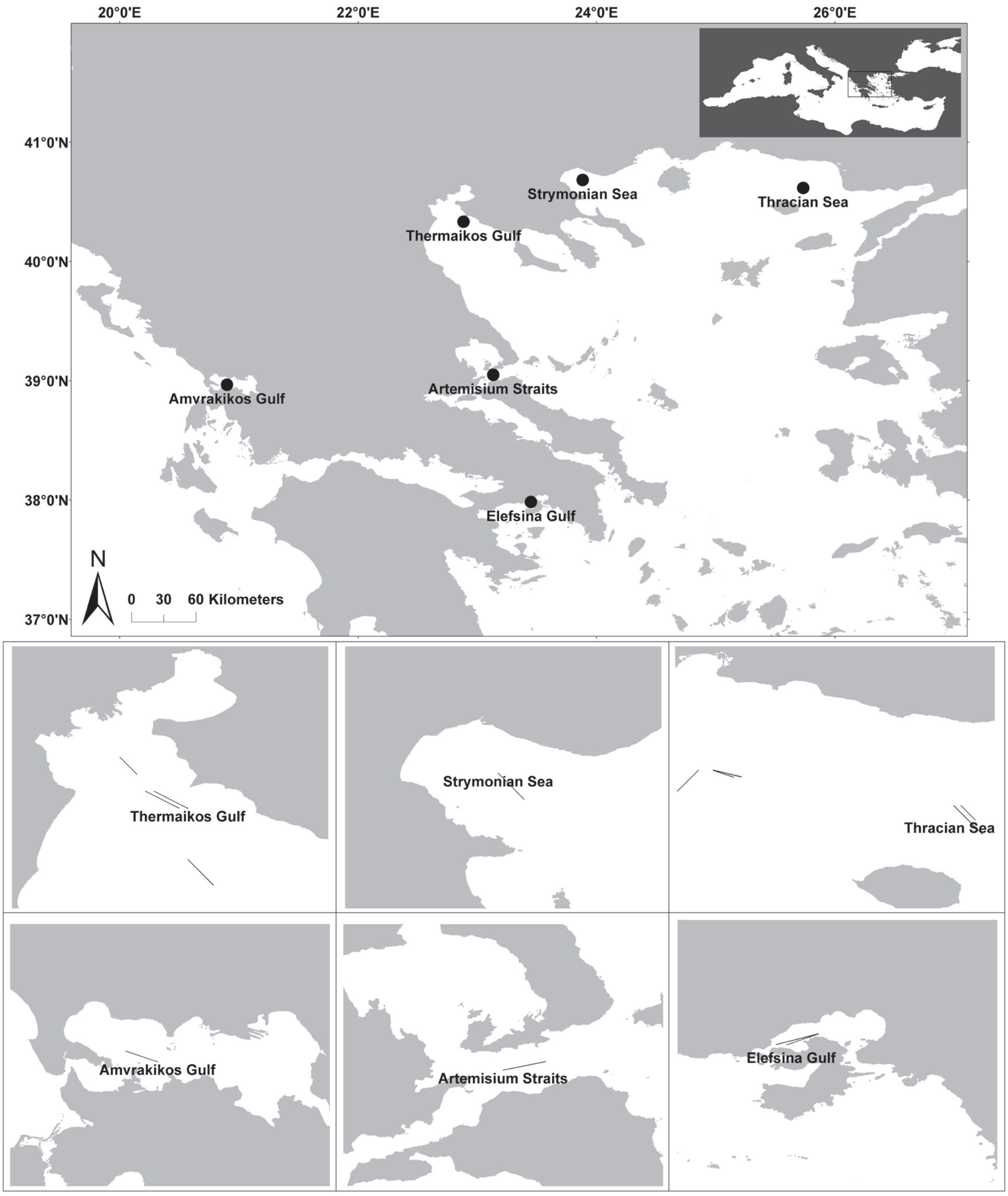

Fig. 1: Location of the six sampling sites in Greece, Eastern Mediterranean Sea.

(agricultural wastes) (Stamatis *et al*., 2002; Sylaios *et al*., 2006).

In the Thracian Sea, sampling took place in two spots: near the Evros River estuary and the coasts of Alexandroupolis and near the coasts of Porto Lagos and the Thasos Channel. Evros River is one of the most heavily polluted and, at the same time, most ecologically valuable transboundary rivers in the eastern Mediterranean. The Evros River Delta wetlands are protected by the Ramsar Convention (Boubonari *et al*., 2008; Skoulikidis, 2009; Pitta *et al*., 2014). Along the coasts of Porto Lagos and the Thasos Channel there are pressures due to eutrophication, agricultural discharges, dredging, and aquaculture (Simboura *et al*., 2016).

Elefsina Gulf is a heavily impacted shallow embayment formed in the north of Saronic Gulf and hosts the industrial zone of Athens, the population of which exceeds 4 million people. Major anthropogenic activities include shipyards, oil refineries, food industries, iron steelworks, cement factories, cable manufacturing, waste recycling plants, landfills, and military installations. One very important pollution source is the wastewater treatment plant (WWTP) on Psittalia Island, the second- largest treatment plant in Europe (Pavlidou *et al*., 2019), which discharges ~800,000 km^3 d^{-1} of treated waste at ~65 m depth. Other anthropogenic pressures include marinas, tourist facilities, fish farms, the treated or untreated effluents of smaller towns, and the illegal dumping of waste in wells. Most of these activities enter the gulf directly while others contaminate the soil or the groundwater, affecting the marine environment via runoff and submarine groundwater discharges (Paraskevopoulou *et al*., 2014; Pavlidou *et al*., 2015; Simboura *et al*., 2016; Karageorgis *et al*., 2020).

The Artemisium Straits lie between Evia Island and continental Greece. There are no major industries on the coasts; only small towns and resorts with minor environmental impact (e.g. sewage discharges). Fishing activities are limited and there are no rivers draining into the straits. To the north lies Pagassitikos Gulf, where the port and the industrial city of Volos, with a population exceeding 100,000 residents, are located. Pagassitikos Gulf is connected to the Artemisium Straits through the Trikeri Channel. The integrative status of the Trikeri Channel (in the north of the sampling area) has been assessed as good, as has the integrative status of the Oraioi Channel (in the west of the sampling area, in the straits) (Simboura *et al*., 2016). The integrative status of the sampling area has not been assessed.

Amvrakikos Gulf is considered one of the most important regions of the NATURA 2000 Network. Characterized by a complex system of lagoons, extensive delta formations, salt meadows, marshlands, and swamps, it is a shallow semi-enclosed gulf connected with the Ionian Sea through a narrow channel. It is protected by the Ramsar, Barcelona, Berne, and Bonne Conventions and has been declared a national park (2008) (Tsangaris *et al*., 2010; Vasileiadou *et al*., 2016). The rivers Vovos, Krikeliotis, Arachthos, and Louros drain into the gulf, with the two latter discharging contaminants carried from agricultural land (Tsangaris *et al*., 2010; Karaouzas *et al*., 2021; Tzempelikou *et al*., 2021). Agriculture and aquaculture activities are the main pressures imposed on the gulf, while less pressure is applied due to hydromorphological stressors, sewage, and industrial discharges according to the respective pressure indices estimated by Simboura *et al*. (2016).

Sample collection and analysis

A total of 180 samples of sardine (*S. pilchardus*) and anchovy (*E. encrasicolus*) were collected from the above-mentioned sites (30 individuals per site, 15 for each species) during September and the beginning of October 2013. The time and date of sampling at each site were the same for sardine and anchovy. The body weight of the sardines ranged from 5.6 g to 19.1 g and the body weight of the anchovies ranged from 2.9 g to 11.7 g. Total length ranged from 91 mm to 141 mm for the sardines and from 82 mm to 125 mm for the anchovies. Fish were beheaded, eviscerated, and freeze-dried to a constant weight. For each species, three composites, consisting of five homogenized individuals each, were created for each site. All edible parts (skin, flesh and bone) were included in the homogenization. For each composite, metal determinations conducted in triplicate.

Metal and element concentrations were determined based on a modified version of the USEPA method 3052 (1996). An Inductively Coupled Plasma – Mass Spectrometer (ICP–MS NexION300, PerkinElmer, Shelton, CT, U.S.) was used. The protocol is described in detail in Sofoulaki *et al*. (2018; 2019).

For metal determination in seawater (Co, Ni, Cu, Zn, Cd and Pb), data from the Greek WFD coastal waters monitoring network were used (Simboura *et al*., 2016). A total of 124 seawater samples were collected during the years 2012–2015 from the six sites studied. The samplings covered a grid of two to five stations in each site. The protocols of metal determinations are presented in detail by Tzempelikou *et al*. (2021).

Criteria for evaluating sardine and anchovy as bioindicators of marine metal pollution

Various assessment criteria have been established for the selection of appropriate bioindicator species. The ones examined in this study are:

1. Ability to produce site-specific measurements (Rainbow, 1995; Sarkar *et al*., 2008; Zhou *et al*., 2008; Li *et al*., 2010; Metian *et al*., 2013; O'Callaghan *et al*., 2019) that enable the comparison of the sites regarding their metal contamination.
2. Sensitivity to environmental variations so that the impact of the pressures applied can be depicted (Romero *et al*., 2007; Zhou *et al*., 2008; Li *et al*., 2010; Mehana *et al*., 2020).
3. Correlation between the metal pollution load of the organism and environmental data (Boubonari *et al*., 2008); in this case, metal concentrations in seawater.
4. Ability to accumulate pollutants without dying, resulting in concentrations higher than those in seawater that can facilitate measurements (USEPA, 2000; Sarkar *et al*., 2008; Zhou *et al*., 2008; Cacador *et al*., 2012; Cunningham *et al*., 2019; O'Callaghan *et al*., 2019; Mehana *et al*., 2020; Salvaggio *et al*., 2020).
5. Abundance and wide geographical distribution so that sampling can be repeatable and comparison of the sites can be facilitated (Rainbow, 1995; USEPA, 2000; Sarkar *et al*., 2008; Zhou *et al*., 2008; Li *et al*., 2010; Cunningham *et al*., 2019; Parra-Luna *et al*., 2020).
6. Common consumption in the area under investigation (USEPA, 2000; Cunningham *et al*., 2019).
7. Important position in the food chain (Zhou *et al*., 2008; Salvaggio *et al*., 2020).
8. Easy recognition and classification and known ecological characteristics (Rainbow, 1995; USEPA, 2000; Li *et al*., 2010; Cunningham *et al*., 2019; Parra-Luna *et al*., 2020).
9. Sufficient lifespan to integrate the pollution preceding the sampling (Rainbow, 1995; Sarkar *et al*., 2008; Zhou *et al*., 2008).

To examine whether these criteria are met, statistical analysis was conducted (regarding the 1st, 2nd and 3rd criterion) and certain traits of the species are discussed. In order to further investigate the 2nd criterion and assess the metal pollution at each site, metal concentrations of both species were also compared to data regarding the pressures imposed (both anthropogenic and natural) at each site, the pollution sources reported and the overall status assessment data.

Statistical analysis

Values of metals below the limit of detection (LOD) were set equal to 0.5*LOD if more than 50% of the metal concentration of the samples exceeded the LOD. If this assumption was not met, they were excluded from the analysis (USEPA, 1991). For the statistical analysis, only the mean value of each measurement was considered for each composite sample. Statistical analysis (nMDS ordination plots, analysis of similarities (ANOSIM), Kruskal-Wallis, Spearman correlation matrix) was performed to investigate the above-mentioned criteria and to examine the differences in metal pollution among sites. Specifically, non-metric multidimensional scaling (nMDS) ordination was used to obtain a general view of the elemental distribution among sites considering both species. The nMDS ordination was based on Euclidean distance of log(x+1) transformed data. The ANOSIM of the Euclidean distance matrice was used to test the significance of differences in overall metal concentrations between sites considering both species. Pairwise comparisons were tested for each combination of sites. An nMDS ordination plot was created using proximate composition and size data found in Sofoulaki *et al*. (2018) to explore whether the differentiation in metal content among the sites could be attributed to or explained by proximate composition and size differentiation. These analyses were performed using PRIMER v6 software (Plymouth Marine Laboratory, Natural Environmental Research council, UK) (Clarke & Warwick, 1994). The non-parametric Kruskal-Wallis test followed by multiple comparisons of mean ranks for all groups (Dunn's test) was used to explore the differences among sites in each metal and element concentration for both fish tissues and seawater more deeply. In order to explore possible correlations between metal concentrations in seawater and in fish species, the Spearman correlation was used. The STATISTICA v.8.0 (StatSoft Inc.) software was used for the univariate analysis.

Results

Metal and other element (Li, Na, Mg, P, Ca, V, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Mo, Pd, Cd, Cs, Ba, Hg, Tl, Pb, U) concentrations in sardine and anchovy in each of the six sites are presented in Figure 2. The metal concentrations (Co, Ni, Cu, Zn, Cd and Pb) in seawater are presented in Figure 3. Despite the differences detected among the sites, the general pattern of the distribution of concentrations in the fish tissues is as follows: Na, P, Ca and Mg are found in higher concentrations, followed by Fe and Zn, followed by As and Sr, Mn and Rb then, in descending order, the concentrations of Se and Cu, Ni, Li, V and Ba, Pb, Hg, Co and Mo and finally U, Ga, Pd, Cs and Tl.

Site-specific variations of the metal pollution

Potential differences in elemental distribution between sites were explored by non-metric multidimensional scaling (nMDS) ordinations considering both species (Figure 4). Metal concentrations in each site are grouped separately, clearly differentiating the sites. Metal concentrations found in fish show no differentiation only in the Strymonian and Thracian seas. These results are further supported by the analysis of similarity (ANOSIM) (Table 1).

To obtain a more detailed view, potential significant differences in concentrations between sites were further explored for each metal and element separately. The non-parametric Kruskal-Wallis test revealed site-specific variations in almost all metal and element concentrations in fish tissues among sites (Table 2) ($p<0.01$). It should be noted, though, that site differentiation in seawater is not clear; few statistically significant differences arise among sites (Table 2) ($p<0.05$, $p<0.01$).

In Thermaikos Gulf, Cd and Ni in both fish samples and seawater; Sr, U, Li, V, Ba in both fish samples; and Ca, Mn, P, Co, Ga and Tl in sardine were found at maximum levels among sites. A very small number of metals

Table 1. ANOSIM results of the differences between sites in overall metal and elemental concentrations considering both fish species. Pairwise comparisons were made for each possible combination of sites.

	Sardine & anchovy	
Global R	0.651	
p	***	
Groups	Statistic R	p
THE, STR	0.691	***
THE, THR	0.698	***
THE, ELE	0.957	***
THE, ART	0.567	***
THE, AMV	0.750	***
STR, THR	0.011	ns
STR, ELE	0.991	***
STR, ART	0.417	***
STR, AMV	0.698	***
THR, ELE	0.989	***
THR, ART	0.420	***
THR, AMV	0.722	***
ELE, ART	0.976	***
ELE, AMV	0.694	***
ART, AMV	0.157	*

*: $p < 0.05$; ***: $p < 0.005$; Elefsina Gulf (ELE); Amvrakikos Gulf (AMV); Artemisium Straits (ART); Thermaikos Gulf (THE); Strymonian Sea (STR); Thracian Sea (THR).

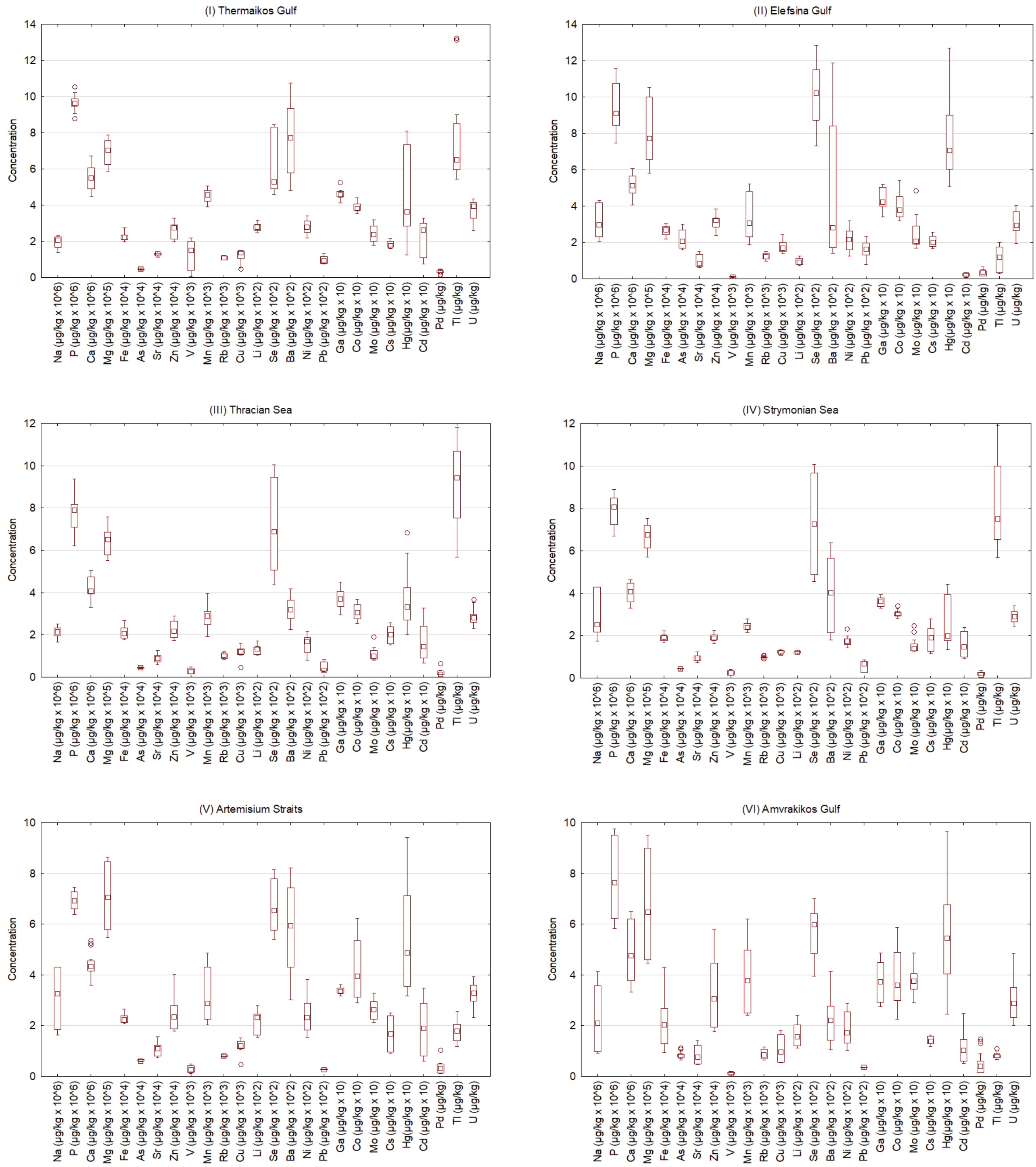

Fig. 2: Box-Whiskers plots of metal and elemental concentrations (wet weight) in sardine and anchovy from each of the 6 sampling sites (n=30 individuals per site).

(Se, Na, Cu, As) were found at minimum levels in sardine, anchovy, or seawater (Table 2) ($p<0.05$, $p<0.01$).

In the Strymonian Sea, in contrast, none of the metals in anchovy and seawater were found at maximum levels and only 2 out of 26 metals (Cs and Tl) in sardine while the majority of metals for anchovy, 5 out of 26 metals for sardine and 3 out of 6 for seawater (Hg, As, Mn, Zn, Cu, Ni, Co, Pd, Se, P, Ga, Sr, Ca, Fe) were found at minimum levels among sites (Table 2) ($p<0.05$, $p<0.01$).

Likewise, in the Thracian Sea, only 4 metals were found at maximum levels among sites in anchovy, sardine or seawater while a large number of metals were found at minimum levels. Namely, Tl concentration in both species, Cs concentration in sardine, and Cd and Pb concentration in seawater were at maximum levels while Cu, Zn Mo, Ni, Pb, Se, Mg, Sr, U, Ba, Na, As, Mn and Co concentrations in sardine, anchovy or seawater were at minimum levels among the sites (Table 2) ($p<0.05$, $p<0.01$).

The most elements, both toxic (Pb, Cu, Hg, As, Rb, Sr) and essential (Se, Mg, P, Fe, Zn), were found at maximum levels in Elefsina Gulf. Specifically, 16 out of 26 metals and elements were found at maximum levels in at

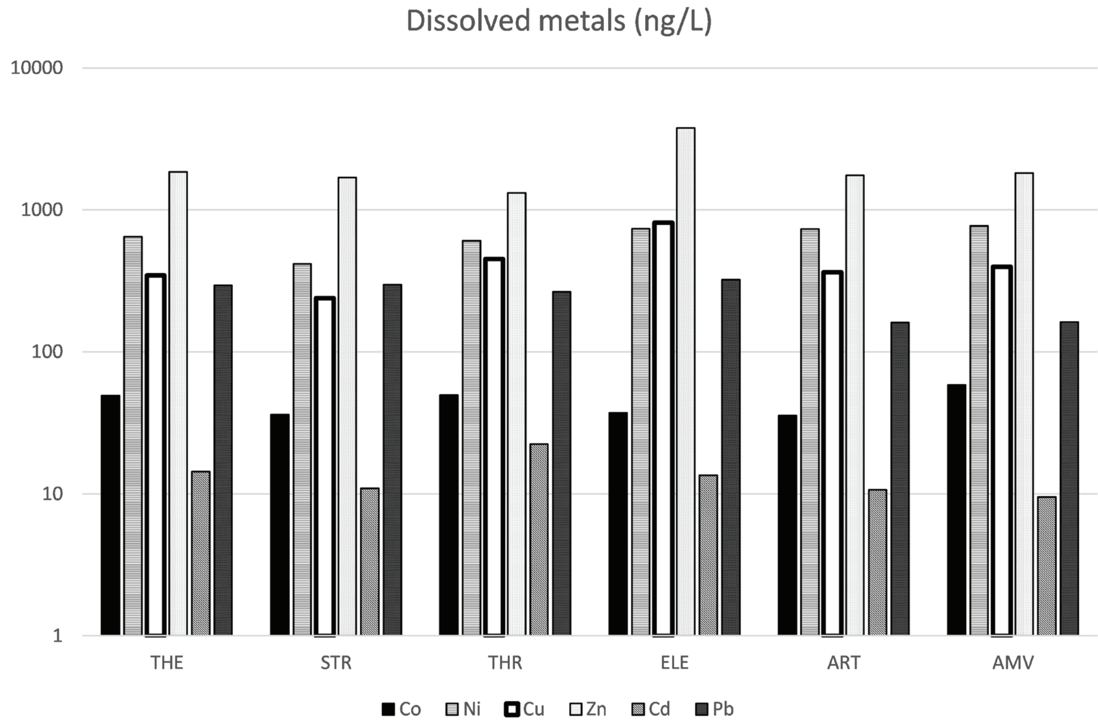

Fig. 3: Metal concentrations in seawater (ng/L) from the different sampling sites. Thermaikos Gulf: THE; Strymonian Sea: STR; Thracian Sea: THR; Elefsina Gulf: ELE; Artemisium Straits: ART; Amvrakikos Gulf: AMV.

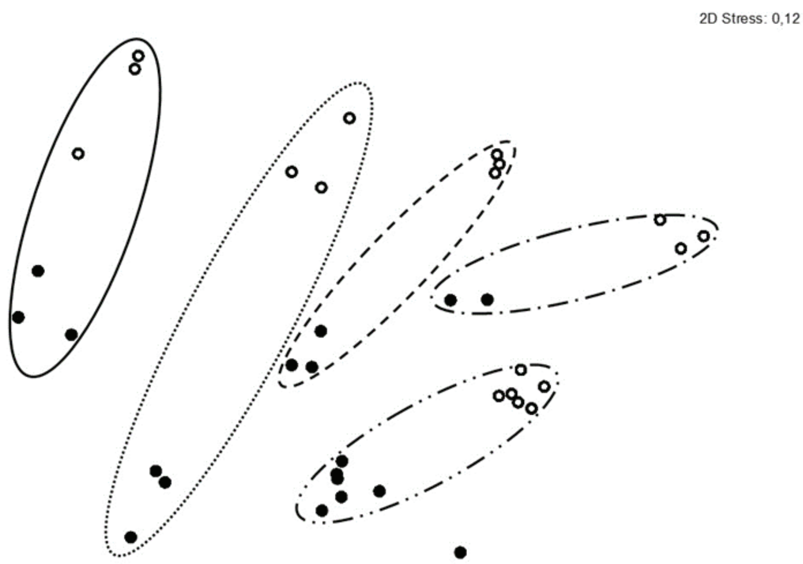

Fig. 4: nMDS ordination plots of the elemental distribution among the different sites in sardine and anchovy. In plot, the metal concentrations of sardine are symbolized with (●) and of anchovy with (○). Metal concentrations which are grouped together are marked with: (—) for Elefsina Gulf; (…) for Amvrakikos Gulf; (– –) for Artemisium Straits; (— • —) for Thermaikos Gulf; (— •• —) for Strymonian Sea & Thracian Sea.

least one of the two fish species or seawater. Only a small number of metals were found at minimum levels for anchovy, sardine or seawater (Cd, Li, V, Ba, Mn, Co) (Table 2) ($p<0.05$, $p<0.01$).

In the Artemisium Straits, a small number of metals were found at maximum levels: Ni in anchovy and seawater; Sr, Cd, Na, and Co in anchovy, and Cs and Pd in sardine. Minimum levels were observed in Pb, Cu, Rb, Cs, P, Ga, Zn and Mn in sardine, anchovy, or seawater (Table 2) ($p<0.05$, $p<0.01$).

In Amvrakikos Gulf, a small number of metals were found at minimum (Ba and Tl) or maximum levels (Mo, Ca, Mn, Fe, Zn, Co) in anchovy whereas in sardine the majority of metals were found at minimum levels (Cu, Se, Mg, Rb, Cs, Na, Fe, Zn, U, Ba, Ca, Mn, P, Ga, Sr, Tl) and only 2 metals at maximum levels (Pd and Mo). In seawater, maximum levels were observed in 2 metals (Co and Ni) and 3 metals (Cu, Cd, Pb) at minimum levels (Table 2) ($p<0.05$, $p<0.01$).

The nMDS ordination plot showed that, unless there

Table 2. Kruskal-Wallis results of the differences among sites in metal and elemental concentrations in sardine, anchovy and seawater. Non-significant different elements are omitted. The statistically higher concentrations found among the sites are marked in bold.

Species	Anchovy									Sardine								
	Variable	H	p	Sites						Variable	H	p	Sites					
				THE	STR	THR	ELE	ART	AMV				THE	STR	THR	ELE	ART	AMV
Metals and elements	Hg	11.0	*	db	f	edf	**a**	b	b	Hg	13.5	*	d	f	d	**a**	b	b
	Pb	14.0	*	b	dcf	f	**a**	f	c	Pb	15.7	**	b	c	c	**a**	f	e
	Cu	12.4	*	c	f	c	**a**	c	b	Cu	10.6	*	f	b	f	**a**	f	f
	As	16.4	**	d	f	e	**a**	c	b	As	14.6	*	f	f	f	**a**	c	b
	Se	14.9	*	f	f	f	**a**	c	b	Se	15.8	**	d	b	b	**a**	d	f
	Mg	13.7	*	d	ed	fe	**a**	c	ba	Mg	10.1	*	ba	cbd	dbc	**a**	dbc	f
	Rb	15.4	**	b	e	cb	**a**	f	dc	Rb	13.8	*	b	b	b	**a**	e	f
	Cs	15.8	**	b	d	c	**a**	f	ed	Cs	12.5	*	e	**a**	**a**	**a**	**a**	f
	P	14.6	*	bac	f	d	**a**	f	c	Na	11.8	*	e	c	cda	**a**	de	f
	Ga	14.4	*	b	f	b	**a**	f	b	Fe	15.7	**	b	d	d	**a**	b	f
	Sr	9.7	*	**a**	f	f	**a**	**a**	**a**	Zn	13.4	*	b	f	f	**a**	f	f
	Cd	13.4	*	**a**	dc	c	f	**a**	ed	Pd	11.8	*	da	f	e	**a**	**a**	**a**
	U	12.6	*	**a**	ef	f	cb	cb	ba	Cd	13.6	*	**a**	ba	ca	f	dc	e
	Li	15.5	**	**a**	d	d	f	b	c	U	8.9	*	**a**	dae	ba	ef	ba	f
	V	15.6	**	**a**	d	bc	f	bc	e	Li	14.9	*	**a**	c	c	f	b	c
	Ba	13.5	*	**a**	db	f	ba	cb	f	V	8.6	*	**a**	dc	b	f	bc	ef
	Mo	15.4	**	d	e	f	b	b	**a**	Ba	15.0	*	**a**	c	c	f	b	f
	Ca	13.6	*	c	f	ed	ba	dc	**a**	Ca	12.4	*	**a**	cd	df	b	df	f
	Mn	12.5	*	ba	f	e	cab	dc	**a**	Mn	8.7	*	**a**	f	f	f	f	f
	Fe	11.1	*	c	f	c	ba	c	**a**	P	14.9	*	**a**	b	d	b	d	f
	Zn	14.3	*	dc	f	ec	b	c	**a**	Co	13.6	*	**a**	dce	f	ba	ce	ef
	Ni	13.4	*	ba	f	f	cb	**a**	dcb	Ni	10.1	*	**a**	dbf	f	b	cb	ef
	Na	14.2	*	f	cbad	f	b	**a**	d	Ga	13.8	*	**a**	cd	de	b	e	f
	Co	12.2	*	dc	f	ec	cb	**a**	ba	Sr	14.5	*	**a**	b	cbe	e	c	f
	Tl	15.7	**	c	ba	**a**	f	d	f	Tl	15.0	*	**a**	**a**	**a**	e	d	f
										Mo	15.6	**	b	e	f	d	c	**a**
SEAWATER	Co	18.0	***	cf	f	cf	f	f	**a**									
	Ni	19.9	***	ab	f	b	**a**	**a**	**a**									
	Cu	27.5	***	f	f	f	**a**	f	f									
	Zn	25.7	***	d	df	f	**a**	df	d									
	Cd	18.4	***	**a**	af	**a**	af	af	f									
	Pb	13.1	*	af	af	**a**	**a**	f	f									

Thermaikos Gulf (THE); Strymonian Sea (STR); Thracian Sea (THR); Elefsina Gulf (ELE); Artemisium Straits (ART); Amvrakikos Gulf (AMV); a = max; f = min; *: p < 0.05; **: p<0.01

was no significant differentiation of proximate composition and size, concentrations of various metals significantly differentiated among sites (Figure A1).

Correlation to seawater

The potential correlation between each metal concentration in seawater and fish samples was explored (Table 3) ($p<0.05$). Given the significant correlations found, both sardine and anchovy seem to be appropriate for monitoring Zn in the studied sites while sardine seems to be appropriate for also monitoring Pb and anchovy for monitoring Ni and Cu.

Discussion

Site-specific variations in metal and elemental content

The first criterion for assessing sardine and anchovy as bioindicators of marine metal pollution is whether these species can offer site-specific measurements (Rainbow, 1995; Sarkar *et al*., 2008; Zhou *et al*., 2008; Li *et al*., 2010; Metian *et al*., 2013; O'Callaghan *et al*., 2019). The sites under investigation are subjected to different anthropogenic pressures, exhibit different geophysical features, and have different overall status (integrative status) based on site pressures (Pavlidou *et al*., 2015; Simboura *et al*., 2016). These differences have been depicted in the metal load found in sardine and anchovy from the studied sites. Statistical analysis demonstrated strong site-specific differentiation not only in overall metal concentrations but also in each metal and element, indicating different elemental distribution and metal pollution load among sites.

Species-dependent accumulation of metals has been reported (Saha *et al*., 2006; Maanan, 2008; Sarkar *et al*., 2008; Uysal *et al*., 2008; Djedjibegovic *et al*., 2012; Galitsopoulou *et al*., 2012; Kalantzi *et al*., 2013) and significant variations in metal content have been found between the studied species (Sofoulaki *et al*., 2018). However, the results of this study clearly showed that the differentiation in metal content among sites could not be attributed to or explained by proximate composition or size differentiation since there were significant differences in metal concentrations and such differences were not detected in proximate composition among sites. Thus, according to the first criterion set, sardine and anchovy can be considered appropriate bioindicator species of marine metal pollution.

Table 3. Spearman correlation matrix between metal concentrations in seawater – sardine and seawater – anchovy. All examined sites are included ($p<0.05$). The statistically significant correlations are underlined.

	Seawater – Sardine	Seawater – Anchovy
Co	0.04	0.36
Ni	-0.02	<u>0.67</u>
Cu	0.14	<u>0.69</u>
Zn	<u>0.78</u>	<u>0.49</u>
Cd	0.29	0.14
Pb	<u>0.44</u>	-0.04

Site-specific differentiation in metal concentrations has been reported by Galitsopoulou *et al*. (2012) for Cd and Pb in sardine and anchovy and by Hayase *et al*. (2009) for Sr and As in Japanese sardine *(Sardinops melanosticus)* and Pacific sardine *(Sardinops sagax)*. Nunes *et al*. (2015) found significant differences among sites using enzymatic biomarkers of sardine, and Guerranti *et al*. (2016) using anchovy as a bioindicator for Polycyclic Aromatic Hydrocarbons (PAHs). Growth, reproduction, and abundance of anchovy have also been associated to environmental conditions (Martin *et al*., 2008).

The ability of an organism to produce site-specific measurements is usually attributed to species of low mobility (Sarkar *et al*., 2008; Zhou *et al*., 2008; Li *et al*., 2010; Metian *et al*., 2013; O'Callaghan *et al*., 2019). However, when relatively wider geographical areas, instead of specific stations, are investigated, species that exhibit relatively higher mobility than sedentary species (e.g. mussels) seem to be more suitable (Whitfield & Elliott, 2002). This is consistent with the results of the present study since sardines and anchovies, which have relatively high mobility, provided a detailed view of the differences in marine metal pollution among sites.

Metal pollution at each site

In order to assess metal pollution and investigate whether the influence of local stressors is depicted in sardine and anchovy metal content, the results of the present study are discussed considering data about the pressures imposed on each site, the pollution sources and the overall status assessment data for each site (integrative status). Maximum levels were found for most of the metals in fish from Elefsina and Thermaikos Gulf due to the wide range of stressors while a small number of metals were found at minimum levels there. In all the other sites, few metals were found at maximum levels.

Even though the overall pressure imposed on the outer part of Thermaikos Gulf and the integrative status of the gulf has been classified as moderate (Pavlidou *et al*., 2015; Simboura *et al*., 2016), a wide range of metals and elements (13 out of the 26 studied) and mainly toxic ones (Cd, Ni, Sr, U, Li, V, Ba, Tl) were found at maximum concentrations among the sites in sardine, anchovy or seawater. These results indicate probable influence of the heavy pressure imposed on the inner part of Thermaikos Gulf and Thessaloniki Bay (Pavlidou *et al*., 2015; Simboura *et al*., 2016;. Industrial, sewage, and agricultural discharges, aquaculture, and harbor activities are the main stressors in Thermaikos Gulf (Christophoridis *et al*., 2009; Skoulikidis, 2009; Pavlidou *et al*., 2015; Simboura *et al*., 2016; Karaouzas *et al*., 2021; Tzempelikou *et al*., 2021). All the above-reported pressures seem to have resulted in the maximum levels observed among the sites of Cd (Castro-Gonzalez & Mendez-Armenta, 2008; Maanan, 2008; Djedjibegovic *et al*., 2012; Simboura *et al*., 2016; Afandi *et al*., 2018; Karaouzas *et al*., 2021; Tzempelikou *et al*., 2021), Ni (Bradi, 2005; Maanan, 2008; Metian *et al*., 2013) and P. In line with the results of this study, similar concentrations of Cd and Pb in sardine and anchovy were reported in Thermaikos Gulf by Galitsopoulou *et al*. (2012). Above background concentrations has also been documented for Cd and Ni in saline water in Thermaikos Gulf (Tzempelikou *et al*., 2021).

Significant differences were found between the Strymonian and Thracian seas, although not as strongly pronounced as among other sites. Only two metals (Cs and Tl) were found at maximum levels among the sites in sardine or anchovy while many metals were found at minimum levels. This is consistent with the overall pressure imposed on the Strymonian Sea, which has been classified as slight (mainly due to agricultural activities) and its integrative status assessed as good (Simboura *et al,*. 2016). Furthermore, metal concentrations have been documented to decrease in the central areas of this gulf and increase near the shore probably due to the tidal water circulation (Stamatis *et al*., 2002). The main pressures imposed on the coastal areas of the Thracian Sea are agricultural discharges while industrial discharges should also be considered in the coasts of Alexandroupolis. The total pressure imposed on all the coastal areas near the sampling area has been classified as moderate (Simboura *et al*., 2016). It is noteworthy that although Evros River is a major source of heavy metals (Boubonari *et al*., 2008; Karaouzas *et al*., 2021; Tzempelikou *et al*., 2021), this pressure is not evident in the present study. This may be explained by the fact that the Thracian Sea is an open sea compared to the rest of the studied sites, which are semi-closed, and the metal pollution effect may be mitigated. This argument is consistent with the observations of Boubonari *et al*. (2008), who reported a decrease in the concentrations of Zn, Cu, Pb and Cd in *Ulva rigida* with increasing distance from Evros River. Malea *et al*. (2019) also suggested there was a potential gradient of trace element levels with increasing distance from the coast but concluded that there was no marked anthropogenic enrichment in Cd, Co, Cr, Cu, Ni, Pb along the Thracian coastal area. Galitsopoulou *et al*. (2012) found Cd concentrations in anchovy from this area in agreement with those reported in this study.

Many toxic metals were found at maximum concentrations among sites in fish from Elefsina Gulf, which is consistent with the heavy total pressure applied (Pavlidou *et al*., 2015; Simboura *et al*., 2016). Sampling took place at the industrial zone of the most intensely urbanized regions in Greece, next to one of the biggest harbors in the Mediterranean Sea (Port of Piraeus). The maximum levels of Pb, Cu, Hg, and As found in this study can be attributed to industrial/ chemical pollution and harbor activities, sewage, and agricultural discharges (Castro-Gonzalez & Mendez-Armenta, 2008; Maanan, 2008; Nadal *et al*., 2008; Christophoridis *et al*., 2009; Copat *et al*., 2013; Longo *et al*., 2013; Pavlidou *et al*., 2015; Bat *et al*., 2014; Paraskevopoulou *et al*., 2014 and references therein; Simboura *et al*., 2016; Karaouzas *et al*., 2021). Tzempelikou *et al*. (2021) reported concentrations of Cu and Pb above seawater levels from Elefsina Gulf while Karageorgis *et al*. (2020) also reported an enrichment of sediments, with Cu and Pb in the gulf associated with an anthropogenic origin. To the extent that comparisons can be made, the same range of Cu, Fe, Pb, and Zn concentrations or even higher have been reported in mussels, *Mytilus galloprovincialis,* and deep-water rose shrimps, *Parapenaeus longirostris,* from Elefsina Gulf (Vlahogianni *et al*., 2007; Kalogeropoulos *et al*., 2012).

Even though the wastewater treatment plant (WWTP) on Psittalia Island in Elefsina Gulf has been reported as a source of Cd (Paraskevopoulou *et al*., 2014), Cd was found at minimum levels in both species from Elefsina Gulf in the present study. In a 38-year comparative study (1977–2015) from Elefsina Gulf by Scoullos *et al*. (2015), a dramatic decrease in Cd and a considerable decrease in Cu, Fe, Mn, Pb, and Zn levels in seawater were reported because dumping of the waste of the phosphate fertilizers plant stopped in the mid-1990s when the plant closed down. This is consistent with the lower Cd levels found in this study compared to the Cd levels in *Mytilus galloprovincialis* and *Parapenaeus longirostris* from earlier studies in Elefsina Gulf (Vlahogianni *et al*., 2007; Kalogeropoulos *et al*., 2012). In another 20-year sedimentary record in Elefsina Gulf (1999–2018), decreasing trends in metal levels were also detected (Karageorgis *et al*., 2020). The decreasing trends were associated with deindustrialization, an improvement in production processes, environmental policies implementation, reduction of land-based metal discharges, use of unleaded gasoline for vehicles, and the shutting down of industries following the economic crisis. It should be noted, however, that in spite of the decreasing trend throughout the years, the levels of most metals in Elefsina Gulf in the present study are significantly higher compared to sites with less anthropogenic pressure.

In the Artemisium Straits, few metals (Ni, Sr, Cd, Na, Co, Cs, Pd) were found at maximum levels in sardine, anchovy, or seawater. In all areas in the vicinity of the Artemisium Straits, only slight pressures are imposed from agricultural activities and aquaculture (Trikeri Channel and Oraioi Channel) (Simboura *et al*., 2016). This may explain the low number of metals at maximum levels, and also the maximum Cd levels, probably from Cd use in fertilizers (Castro-Gonzalez & Mendez-Armenta, 2008; Maanan, 2008; Djedjibegovic *et al*., 2012; Simboura *et al*., 2016; Karaouzas *et al*., 2021; Tzempelikou *et al*., 2021). Ni was found at maximum levels among sites in anchovy and seawater from the Artemisium Straits. In other studies (Karageorgis *et al*., 2002; Tsangaris *et al*., 2013), high Ni concentrations in sediments from Pagassitikos Gulf and the Trikeri Channel have been attributed to high background levels of Ni and natural enrichment from weathering. In addition, this enrichment may be attributed to the mining of Ni –Co for several decades on the mainland and in the center of Evia Island and the dumping of mineral waste into the sea (Tzempelikou *et* *al.*, 2021).

In Amvrakikos Gulf, both the low number of metals found at maximum levels (7 out of the 26 studied) and the fact that these were mainly nutrients (Mo, Mn, Ca, Fe, Zn, and Co in anchovy) are consistent with the relatively low pressure due to industrial activities (Pavlidou *et al.*, 2015; Simboura *et al.*, 2016). Similarly, high concentrations of Mn in sediments from Amvrakikos Gulf have been reported due to the interaction between seawater and suspended hydrate manganese oxide carried by the Arachthos River (Voutsinou-Taliadouri & Balopoulos, 1991). Similar conclusions about the natural origin of Mn and Fe found in seawater and sediments from the estuary of the Louros River were drawn by Scoullos *et al.* (1996). In the current study, Fe was also found at maximum levels in anchovy from Amvrakikos Gulf. Tzempelikou *et al.* (2021) reported that the water in several lagoons in Amvrakikos Gulf and the outflowing rivers Arachthos and Louros are affected by Co and Zn, which are also found at maximum levels in anchovy in this study. Regarding Zn, high levels were also reported by Karageorgis (2007) in sediments from Amvrakikos Gulf and were associated with the natural weathering of ultra-basic rocks (pre-industrial sediments were analyzed, indicating high background levels). High Zn concentrations were also found by Strogyloudi *et al.* (2014) in the sea urchin *Paracentrotus lividus* used as a bioindicator species in Amvrakikos Gulf. Karageorgis (2007) and Vasileiadou *et al.* (2016) highlighted that the metal enrichment in the gulf results from natural processes rather than anthropogenic stressors, which seems consistent with the results of the present study.

Considering all the sites studied, the metal load identified in sardine and anchovy agrees with the respective pressures applied, both natural and anthropogenic. Therefore, the second criterion set for their assessment as bioindicator species is also satisfied. Likewise, Copat *et al.* (2012b), who used sardine and anchovy in a biomonitoring study in Sicily to assess the pollution of Cd, Pb, Hg, and Cr, argued that the two species reflect the expected levels of pollution in the sampling areas regarding both natural and anthropogenic contributions.

Correlation between the metal content of fish and seawater

The third criterion set for assessing sardine and anchovy as potential bioindicators is the existence of correlations between the metal load of the organisms and the respective load in the environment (Boubonari *et al.*, 2008). The significant positive correlations between seawater and sardine, anchovy, or both of the species corroborate sardine and anchovy efficiency in monitoring Zn, Pb, Ni, and Cu (four out of the six metals examined in seawater). Hence, the third criterion of assessment is satisfied.

As can be observed by the few significant differences among seawater sites, site differentiation in seawater is not clear and not as strongly pronounced as in the metal concentrations in sardine and anchovy. Therefore, the combination of analysis of seawater and the two fish species provides a much more reliable measure of the differences in metal load among sites. In their exploration of the use of *P. leopardus* as a bioindicator species in New Caledonia, Metian *et al.* (2013) also stressed the importance of examining organisms to assess the contamination levels more efficiently.

Overall evaluation of sardine and anchovy as bioindicators of marine metal pollution

The relatively higher mobility of sardines and anchovies than sedentary species, their diet (based on plankton), short life span, and high fecundity make them particularly sensitive to environmental variations (Martin *et al.*, 2008; Bat *et al.*, 2014; Nikolioudakis *et al.*, 2014), a trait that enables them to effectively depict the impact of the pressures applied (Romero *et al.*, 2007; Zhou *et al.*, 2008; Li *et al.*, 2010; Bat *et al.*, 2014; Mehana *et al.*, 2020). They are species of particular importance for the pelagic ecosystems (Nunes *et al.*, 2015; Savoca *et al.*, 2020) due to their intermediate position in the food web (Ganias, 2014; Nikolioudakis *et al.*, 2014; Afandi *et al.*, 2018). They are the main food source for many larger marine species (Savoca *et al.*, 2020) and transfer energy from the plankton on which they feed to larger marine species (Ganias, 2014; Nikolioudakis *et al.*, 2014), thus influencing their health and abundance (Ganias, 2014). Therefore, the criteria set for their assessment as bioindicator species are satisfied. Furthermore, according to Rainbow (1995), it is advisable to use a suite of biomonitors to acquire a complete picture of the metal pollutants in marine habitats and allow identification of the sources. This is confirmed by results of the present study since the combination of sardine and anchovy seems to be quite effective. The importance of using a combination of several bioindicator species (Bonanno & Orlando-Bonaca, 2018) and integrating information provided by different sources at different scales (Doray *et al.*, 2018; Pavlidou *et al.*, 2019) for effective biomonitoring of marine ecosystems is also stressed in more recent studies.

Conclusions

Significant site-specific differences in metal pollution load were detected in the tissues of sardines and anchovies. They showed a positive correlation to metal concentrations measured in seawater and met all major assessment criteria. Differentiation in metal content among sites could not be attributed to or explained by species-specific differences, their proximate composition, or size differentiation. It seems to be strongly determined by the characteristics of each site, resulting from differentiation of the anthropogenic pressures applied, the different background levels, and the prevailing environmental conditions. Therefore, sardine and anchovy can be evaluated as appropriate, reliable, and useful bioindicator species of marine metal pollution.

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Supplementary Data

The following supplementary information is available online for the article:

Fig. A1: nMDS ordination plots of the proximate composition (weight, length, lipid content, protein content and ash) distribution among the different sites in both sardine and anchovy. (▲) for Thermaikos Gulf; (■) for Strymonian Sea; (○) for Thracian Sea; (●) for Elefsina Gulf; (Δ) for Artemisium Straits; (*) for Amvrakikos Gulf.