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RESEARCH ARTICLE

Biochemical biomarkers and overall health status of mussels *Mytilus galloprovincialis* exposed to nickel and chromium

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This study examined the effects of nickel and chromium exposure on two biochemical biomarkers, glutathione peroxidase (GPX) and acetylcholinesterase (ACHE), in parallel with an indicator of overall health, Scope for Growth (SFG), in the mussel *Mytilus galloprovincialis* to evaluate their ecological relevance and to assess their potential use as biomarkers of Ni and Cr pollution. SFG integrates major physiological responses into an index that represents the energy status of the animals and is considered an ecologically relevant biomarker. Following exposure to a range of Ni and Cr concentrations effects of Ni and Cr were evident on GPX and SFG but not on ACHE. Accumulation in the mussels' tissues increased according to exposure concentrations. Adverse effects on the energy status and induction of GPX were found at the higher exposure concentrations while possible hormetic effects on the energy status and moderate GPX suppression were associated with lower exposure concentrations. Nevertheless GPX was negatively correlated with SFG suggesting that GPX responses reflect the overall health of the organisms and thus show potential as an early warning signal of population level effects of Ni and Cr. On the other hand, our results indicate that ACHE is not a suitable biomarker for Ni and Cr pollution.

Keywords: biomarkers; glutathione peroxidase; acetylcholinesterase; energy budget; mussels; metals

1. Introduction

Biomarkers represent molecular, biochemical, physiological or behavioural changes in organisms following exposure to environmental stressors that are increasingly used for the assessment of pollution impacts in aquatic ecosystems [1,2]. It is generally believed that suborganism responses occur prior to alterations at the population and community levels so biomarkers can be predictive and anticipatory [3]. Biochemical biomarkers range from general to specific, reflecting general cellular stress or exposure to specific classes of contaminants [4,5] but hardly provide evidence of consequences on the organisms and populations. In order to reveal potential ecological consequence, biochemical biomarkers should be related to changes in growth, reproductive output, or metabolic function that could lead to alterations in the health status of the organisms [6]. Hence linking biochemical biomarkers with biomarkers of the overall health of individual organisms is important for the evaluation of their utility in predicting population-level effects [7].

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Ni and Cr are common heavy metal contaminants of known toxicity and they are both considered carcinogenic [8,9]. These metals represent major contaminants produced by mining and smelting operations of lateritic nickel ores [10], an important industry in Greece. In coastal areas impacted by nickel ore processes, high Ni and Cr concentrations are found in water, sediments and biota [10–12]. Numerous studies describe adverse effects of Ni and Cr in aquatic organisms [8,9]. However, information on the effects of these metals on biomarkers in mussels of the genus *Mytilus* that are common sentinel organisms in pollution biomonitoring programmes [13] is lacking. The present study examined the effects of Ni and Cr on two biochemical biomarkers glutathione peroxidase (GPX) and acetylcholinestrease (ACHE), in parallel with a physiological condition index, Scope for Growth (SFG), in the Mediterranean mussel *Mytilus galloprovincialis* in order to evaluate their usefulness as predictive biomarkers of population level effects of Ni and Cr pollution.

GPX is an antioxidant enzyme, which contributes to the oxidative defence by catalysing the reduction of hydrogen and lipid peroxides [14,15]. Variations in antioxidant enzyme activities are considered as biomarkers of reactive oxygen species (ROS) production that may lead to oxidative stress caused by a wide range of environmental stressors including heavy metals [4]. Toxicity and carcinogenicity of Ni and Cr is known to occur due to production of ROS [16]. Ni and Cr undergo redox cycling resulting in production of ROS by reactions with hydrogen peroxide and organic hydroperoxides [16,17] and previous literature data on vertebrate species demonstrate that both metals affect GPX [18,19]. ACHE is an enzyme involved in nerve impulse transmission and its inhibition is widely used as a biomarker of neurotoxicity. Neutoroxic compounds such as organophospate and carbamate pesticides specifically inhibit ACHE [5,20] but more recently ACHE inhibition by several other chemicals including heavy metals has been demonstrated [21–25]. ACHE is thus currently suggested as a potential biomarker for detecting general stress in aquatic organisms [13]. Previous studies have shown inhibitory effects of Ni and Cr on ACHE activities in aquatic species [24,26,27] and in vitro ACHE inhibition by Cr in mussels [24]. To our knowledge in vivo effects of Ni and Cr on GPX and ACHE activities in mussels of the genus *Mytilus* have not been studied.

SFG is an established biomarker of the overall health of an organism that provides a general response to environmental stressors and is considered an ecologically relevant biomarker [28]. SFG integrates estimates of major physiological responses (feeding, food absorption, respiration and excretion) converted to energy equivalents into an index that represents the energy available for growth and reproduction [28].

The aim of this study was to assess the responsiveness of GPX and ACHE activities, in parallel with SFG, to Ni and Cr exposure in *M. galloprovincialis* and to evaluate the linkage of GPX and ACHE responses to effects on the overall health status of the organisms in order to assess their usefulness as early warning signals of population level effects of Ni and Cr.

2. Materials and methods

2.1. *Experimental exposure of mussels*

Mussels *Mytilus galloprovincialis* of similar shell length (5.5 ± 1.0 cm) were purchased from a local farm at Megara (Saronikos Gulf, Greece) in November 1997 and in April 1998 in order to avoid spawning and the winter period when energy reserves are low. In the area of Megara, heavy metal levels in mussels are similar to those found in non-polluted Mediterranean coastal areas [29,30]. The mussels were placed in 4 l tanks (5 mussels/l) and were acclimated in aerated artificial seawater (38‰, 18 ± 1 °C) for seven days prior to heavy metal exposure. Artificial seawater was prepared using artificial marine salt (Sera premium sea salt, Germany).

The mussels were exposed to a range of Ni (3 μg/l, 300 μg/l and 3000 μg/l) and Cr (0.5 μg/l, 5 μg/l, 50 μg/l) concentrations for 28 days. The lowest concentrations were chosen as being representative of the dissolved Ni and Cr seawater levels reported in contaminated areas; (0.52–0.79 μg Cr/l) [31], (0.116–3.5 μg Ni/l) [32]. The dosed metal concentrations although high were considered environmentally relevant, as in polluted environments such as areas of nickel mining and smelting, spills of ores and effluents may lead to high localised concentrations of Ni and Cr depending on currents and tidal actions. For example values up to 600 μg/l of Ni and 5000 μg/l of CrVI have been found in seawater leachates of nickel ores [10] while values of up to 27200 μg/l of Ni have been reported in wastewater from Ni mining, smelting and refinery operations [9]. All concentrations were sublethal according to earlier studies in mussels [33,34]. Ni and Cr were added in seawater as stock solutions of $NiCl_2$ and Na_2CrO_4. Cr (VI) was employed which is considered more toxic and biologically available than Cr (III) [8]. Artificial seawater with no added metal served as control. The seawater was renewed every second day and the mussels were fed daily with *Dunaniella tertiolecta* (60×10^6 cells/mussel). After 28 days of heavy metal exposure GPX and ACHE activities, SFG, as well as heavy metal -Ni and -Cr concentrations in the mussels were measured.

2.2. *Heavy metal analysis*

Whole mussel tissues were dissected, freeze-dried and digested with concentrated HNO_3 in Teflon digestion vials under pressure at 120° for 12 h [35]. Three to five individual organisms were analysed per treatment. Ni and Cr analysis was performed by atomic absorption spectrophotometry (AAS) on a Varian Spectra AA 20plus spectrophotometer (Varian, Victoria, Australia). Heavy metal concentrations were expressed as μg/g dry weight tissue. Analytical quality control was achieved using certified reference material (DORM-2) provided by the National Research Council of Canada.

2.3. *Glutathione peroxidase (GPX) activity*

GPX was measured as described by Livingstone et al. [36] in the digestive gland, which is the major detoxication tissue in mussels, and where the highest GPX activities are found [37]. Digestive glands from 4–6 individuals (four replicates per treatment) were pooled and homogenised using a Potter-Elvehjem homogeniser (Heidolph Electro GmbH, Kelheim, Germany) in 1:4 (w:v) 20 mM Tris-HCl buffer, pH 7.6, containing 1 mM EDTA, 1 mM dithiothietrol, 0.5 M sucrose and 0.15 M KCl. Homogenates were centrifuged at 500 *g* for 15 min and 10,000 *g* for 30 min. All preparation procedures were carried out at 4 °C. Se-dependent GPX activity was assayed in the 10,000 *g* supernatant by the rate of NADPH oxidation monitored at 340 nm by the coupled reaction with glutathione reductase using H_2O_2 as a substrate ($GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$, NADPH extinction coefficient $= 6.2\,mM^{-1}/cm$). GPX activity was expressed as nmoles NADPH consumed per min per mg protein. Total protein content in the homogenate supernatants was measured by the Bradford method [38] using a Bovine Serum Albumin (BSA) standard curve.

2.4. *Acetylcholinesterase (ACHE) activity*

ACHE activity was measured in the adductor muscle as ACHE has an essential role in nerve transmission processes at neuromuscular junctions and this tissue has been previously used for ACHE measurements in mussels [39]. Samples were prepared as described by Bocquené et al. [39]. Tissues from 4–6 individuals (four replicates per treatment) were pooled and homogenised using a Potter-Elvehjem homogeniser in 1:5 (w:v) 0.1 M Tris-HCl buffer, pH 7. Homogenates

were centrifuged at 15,000 g for 20 min. All preparation procedures were carried out at 4 °C. ACHE activity was assayed at 412 nm by the colorimetric method of Ellman [40]. ACHE activity was expressed as nmoles of acetylthiocholine hydrolysed per min per mg protein. Total protein content in the homogenate supernatants was measured by the Bradford method [38].

2.5. *Scope for Growth (SFG)*

The procedure described by Widdows and Salked [41] with the modification of Cotou et al. [42] was used to measure Scope for Growth. Clearance rate, respiration rate and food absorption efficiency were measured for each mussel individually (three to six mussels per treatment) under laboratory standardised conditions (artificial seawater of 38‰, 18 ± 1 °C). For clearance rate and absorption efficiency measurements, mussels were placed in individual chambers in a closed circulation system at a water flow rate of 160–180 ml/min, where *Dunaniella tertiolecta* was added at a concentration of 6500–7000 cells/ml. Algal cell concentrations were measured from the outfall of all chambers including the controls (two chambers without mussels) four times at 45 min intervals, using a Z1 Coulter Counter (Coulter Electronics, Luton, UK). Clearance rate (Cl) was calculated as follows:

$$\mathrm{Cl} = \mathrm{Fl\ X\ } (\mathrm{C_0} - \mathrm{C_1})/\mathrm{C_0},$$

where Fl = flow rate, C_0 = inflow concentration (control chamber), C_1 = outflow concentration (experimental chamber).

Faeces were collected from each chamber after mussels remained in the system for 12 h. Absorption efficiency (AE) was measured by the ratio of Conover [43]:

$$\mathrm{AE} = (\mathrm{F} - \mathrm{E})/((1 - \mathrm{E}) \times \mathrm{F}),$$

where F = ash free dry mass/dry mass of food, E = ash free dry mass/dry mass of faeces.

Respiration rate was measured for each mussel in a transparent plastic respirometer (i.e. modified Quickfit chamber) containing 750 ml of air saturated synthetic seawater circulated with a magnetic stirrer. Each mussel was settled in the respirometer for 15 min and subsequently the decline in oxygen tension was monitored over a period of 30–45 min using a Strathkelvin Model 781 oxygen meter (Strathkelvin Instruments, Glasgow, UK).

Physiological rates were converted to energy equivalents and were used to calculate SFG, the energy available for growth and reproduction, according to the equation:

$$\mathrm{SFG} = \mathrm{A} - \mathrm{R} = (\mathrm{C} \times \text{absorption efficiency}) - \mathrm{R},$$

where A = energy absorbed from food, C = energy consumed from food, R = energy lost via respiration.

Energy lost via excretion was not included in the above equation because it accounted for less than 5% of the total energy loss. Calculation of C, A and R was as follows:

$$\mathrm{C(J/g/h)} = \text{clearance rate (L/g/h)} \times \text{algae cells concentration (mg ash-free dry weight/l)} \times 23.5\ \mathrm{J/mg\ ash\ free\ dry\ weight},$$

where 23.5 J/mg ash free dry weight is the energy content of phytoplankton cells [44].

$$\mathrm{A(J/g/h)} = \mathrm{C} \times \text{ absorption efficiency}$$

$$\mathrm{R(J/g/h)} = \text{respiration rate } (\mu\mathrm{molO_2/g/h}) \times 0.456\ \mathrm{J/\mu mol\ O_2},$$

where 0.456 J/ml O_2 is the energetic equivalent of respiratory oxygen consumption [45].

2.6. *Statistical analysis*

Data are presented as mean ± standard error of the mean ($n = 3-6$ for metal analysis and SFG and $n = 4$ for ACHE and GPX). Kolmogorov-Smirnoff test and Levene's test were applied to test normal distribution and homogeneity of variance respectively. Data were log-transformed where necessary. Significant differences between means were tested by one-way ANOVA followed by the Dunnett test to detect differences between heavy metal treated and control mussels. To examine relationships between GPX, ACHE and SFG linear regression was applied. Statistical analysis was performed using the SPSS statistical package. Significance level was set at $p < 0.05$.

3. Results

3.1. *Ni and Cr bioaccumulation*

Ni and Cr concentrations in whole tissues of mussels exposed for 28 days to a range of Ni and Cr concentrations in seawater are shown in Table 1. Ni concentrations in the mussel tissues increased accordingly to Ni exposure concentrations and were significantly different from concentrations of control mussels at 300 and 3000 μg Ni/l. Cr tissue concentrations increased with increasing Cr exposure concentrations and were significantly different from controls in mussels exposed to 50 μg Cr/l.

3.2. *Effects of nickel exposure*

SFG of Ni exposed mussels significantly decreased to 41 and 21% of controls at Ni tissue concentrations of 59.9 and 219 μg Ni/g dw, respectively (Figure 1a). On the contrary, a significant increase in SFG to 173% of controls was found in Ni exposed mussels at the lowest Ni tissue concentration of 9.5 μg Ni/g dw (Figure 1a). These changes in SFG of Ni exposed mussels were primarily due to corresponding changes in the energy absorbed from food (Table 2). As shown in Table 2 the energy absorbed from food varied with respect to Ni tissue concentrations while changes in the energy consumed by respiration were not significant. GPX activity of Ni exposed mussels significantly increased to 180% of control levels at the highest Ni tissue concentration of 219 μg Ni/g dw (Figure 1b). At lower Ni tissue concentrations of Ni exposed mussels, GPX activities decreased (69–76% of controls) although differences compared to controls were

Table 1. Ni and Cr concentrations (μg/g dw) in whole tissues of mussels exposed to a range of Ni and Cr concentrations in seawater for 28 days.

Ni added in seawater (μg/l)	Ni in mussel tissues (μg/g dw)
0	8.8 ± 1.0
3	9.5 ± 1.5
300	59.9 ± 3.6*
3000	219.2 ± 6.6*
Cr added in seawater (μg/l)	**Cr in mussel tissues (μg/g dw)**
0	1.1 ± 0.2
0.5	1.4 ± 0.2
5	2.5 ± 0.4
50	15.4 ± 6.1*

Mean ± standard error, $n = 3$–5.
*Significantly different from control, $p < 0.05$.

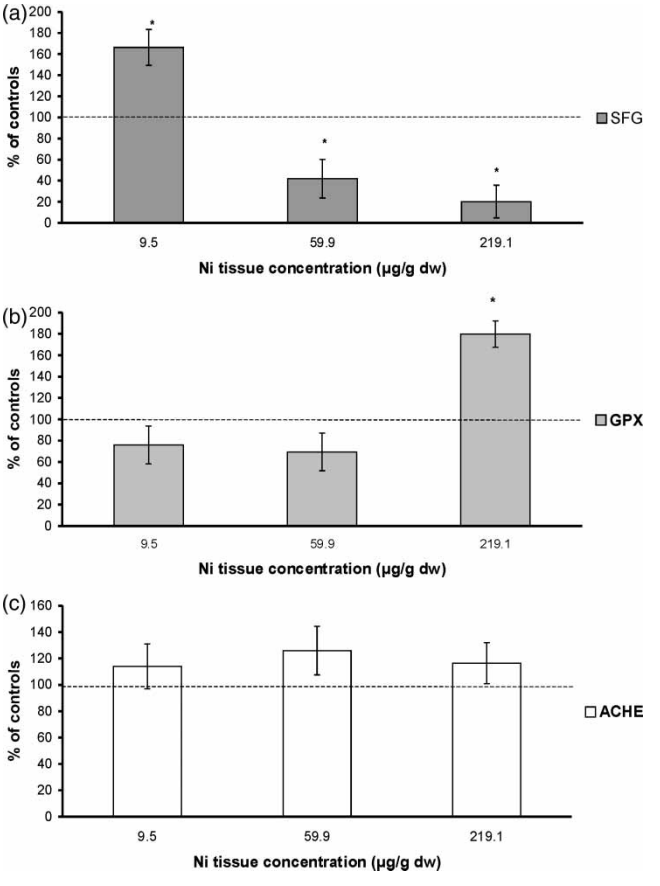

Figure 1. Effects of Ni concentrations in the tissues (µg Ni/g dw) on Scope for Growth (SFG) (a), glutathione peroxidase activities (GPX) (b), and acetylcholinesterase activities (ACHE) (c), of mussels exposed to Ni for 28 days. Results are expressed as percentage of the control values. Control SFG = 19.7 ± 2.3 J/h/g; Control GPX = 10.3 ± 2.4 nmoles/min/mg protein; Control ACHE = 6.4 ± 0.6 nmoles/min/mg protein. Mean ± standard error, $n = 6$ (a), $n = 4$ (b), (c). *Significant difference from control, $p < 0.05$.

Table 2. Components of the energy budget, A (J/h/g): energy absorbed from food and R (J/h/g): energy consumed by respiration, used for the calculation of SFG (J/h/g) of mussels exposed to a range of Ni and Cr concentrations in seawater for 28 days.

Ni added in seawater (µg/l)	A (J/h/g)	R (J/h/g)	SFG (J/h/g)
0	32.8 ± 2.3	13.1 ± 0.5	19.7 ± 2.3
3	43.1 ± 3.7*	9.0 ± 1.1	34.0 ± 4.1*
300	18.9 ± 2.7*	10.4 ± 1.3	8.6 ± 1.5*
3000	20.0 ± 2.5*	15.8 ± 3.1	4.1 ± 3.2*
Cr added in seawater (µg/l)	A (J/h/g)	R (J/h/g)	SFG (J/h/g)
0	38.7 ± 5.7	8.6 ± 0.6	30.1 ± 5.4
0.5	52.5 ± 4.9*	8.0 ± 0.6	44.5 ± 4.6
5	57.9 ± 6.6*	9.3 ± 1.6	48.6 ± 5.4*
50	39.4 ± 4.5	4.4 ± 2.1*	25.1 ± 4.7

Mean ± standard error, $n = 3$–6.
*Significantly different from control, $p < 0.05$.

not statistically significant (Figure 1b). There were no significant differences in ACHE activities between Ni exposed and control mussels (Figure 1c).

3.3. *Effects of chromium exposure*

SFG of Cr exposed mussels at the highest Cr tissue concentration of 15.4 μg Cr/g dw only slightly decreased compared to controls (83% of controls) (Figure 2a). On the contrary, at lower Cr tissue concentrations, SFG values of Cr exposed mussels increased to 148 and 162% of controls respectively, this increase being significant at 2.5 μg Cr/g (Figure 2a). These increased SFG values were primarily due to increased energy absorption from food while the slight decrease in SFG at the highest Cr concentration was due to a decrease in the energy consumed by respiration (Table 2). GPX activities of Cr exposed mussels showed a decreasing tendency (63–69% of controls) at the lower Cr tissue concentrations of 1.4 and 2.5 μg Cr/g dw however differences from controls were not significant (Figure 2b). Cr exposure had no effect on ACHE of mussels as similar ACHE activities were found in Cr exposed and control mussels (Figure 2c).

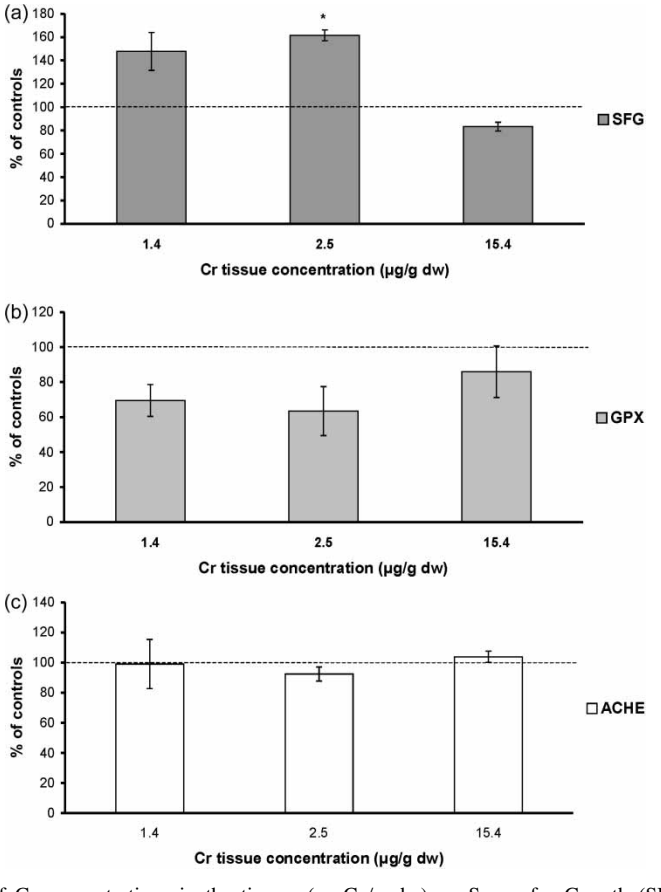

Figure 2. Effects of Cr concentrations in the tissues (μg Cr/g dw) on Scope for Growth (SFG) (a), glutathione peroxidase (GPX) activities (b), and acetylcholinesterase (ACHE) activities (c), of mussels exposed to Cr for 28 days. Results are expressed as percentage of the control values. Control SFG = 30.1 ± 5.4 J/h/g; Control GPX = 8.4 ± 1.4 nmoles/min/mg protein; Control ACHE = 8.5 ± 0.6 nmoles/min/mg protein. Mean ± standard error, $n = 3$ (a), $n = 4$ (b), (c). *Significant difference from control, $p < 0.05$.

3.4. *Relationship between biochemical biomarkers and scope for growth*

Using the combined data of both Ni and Cr exposure experiments (means of treatment groups were used, $n = 8$) a significant linear relationship was found between GPX activities and SFG ($R^2 = 0.539$, $p < 0.05$) whereas there was no significant relationship between ACHE activities and SFG ($R^2 = 0.050$, $p > 0.05$).

4. Discussion

Laboratory studies are useful to assess the responsiveness of biomarkers to different types of contaminants and to investigate relationships between biomarkers at different levels of biological organisation in order to provide information on their relevance and applicability for the assessment of environmental pollution. The present study revealed effects of Ni and Cr on GPX and SFG of mussels following laboratory exposure to a range of Ni and Cr concentrations while no effects on ACHE were noted. The observed effects did not show a clear dose response but rather followed a biphasic pattern while Ni and Cr accumulation in the tissues of mussels increased according to exposure concentrations in agreement with previous studies in *Mytilus* sp. [34,46]. At the higher exposure concentrations, Ni and Cr body burdens were markedly elevated (59.9, 219 μg Ni/g dw and 15.4 μg Cr/g dw) and higher or comparable to those found in mussels at contaminated areas [8,9]. These elevated Ni body burdens negatively affected the overall health status of mussels as indicated by the decrease in SFG to 41 and 21% of control levels. SFG represents the energy budget of the animals and is considered a good indicator of the overall health status [28]. Adverse effects of Cr on health status were not prominent; however the lowest SFG value (83% of controls) coincided with the highest Cr body burden. These results are in agreement with previous studies that have shown inverse relationships between SFG and heavy metal body burdens in mussels [47,48]. On the contrary, unexpectedly positive effects on the energy status were detected in metal exposed mussels with lower Ni and Cr body burdens (9.5 μg Ni/g dw, 1.4 and 2.5 μg Cr/g dw). SFG values of these mussels increased by 48–66% above control levels suggesting a hormetic response. Hormesis is described as stimulation of organism performance occurring at low levels of exposure to agents that are harmful or toxic at high levels of exposure. Hormetic responses are induced by a variety of stressors and include a wide range of endpoints, with growth responses being the most common [49]. Positive effects on shell growth of mussels during toxic exposure have been previously reported for low Cd [50] and low hydrocarbon concentrations [51] and they have been also assigned to hormesis. SFG is an indirect estimate of growth and its increases both in Ni and Cr exposed animals of this study were mainly due to increased clearance rates that resulted in increased energy absorption from food (Table 2). A similar increase was recorded in the filtration rate of clams *Anadara granosa* during the first 24 hours of Cd exposure whereas after prolonged Cd exposure that rate was decreased [52]. Previous studies have reported similar pattern of responses with stimulation at low levels of exposure and adverse effects occurring at higher levels of exposure and have drawn attention to hormesis implications in toxicity testing [53,54]. Unexpected responses occasionally observed in field investigations of contaminant impacts may also be related to hormetic responses [55,56]. Our results imply that consideration has to be taken into account in case of unexpectedly high SFG values at areas of low Ni and Cr contamination as they might be related to hormetic type responses. Hormesis is regarded as an overcompensation response to a disruption in homeostasis [57] and its importance in ecotoxicology is underlined by Chapman [58]. Stebbing [49] suggested that hormesis might not be only symptomatic of the effects of sub inhibitory levels of toxicants, but it could be a useful index of such effects.

GPX in metal exposed mussels showed a similar to SFG biphasic pattern of response, with increased GPX activities at the highest Ni body burden and moderately decreased activities at

lower Ni and Cr body burdens. The increased GPX activities indicate enzyme induction possibly as a protection mechanism against ROS that can be produced by Ni [16,17]. Generally, enzymes of the antioxidant defence system are induced by contaminant-mediated production of ROS as a protection mechanism against oxidative stress or inhibited when deficiency of the system occurs [14]. No data are currently available on the effects of Ni and Cr on GPX in mussels but both metals have been reported to affect GPX in vertebrate species as a response to formation of ROS [18,19]. Our results indicate that GPX responses of mussels to Ni and Cr vary depending on exposure dose. Literature data on mussels exposed to various contaminants in field or laboratory conditions report variations [15,59–62] as well as no changes of GPX activities [63]. Thus, these different contaminant-mediated effects could be explained by a different dose or time response of GPX to different contaminants. A depletion of certain antioxidant enzymes as a first response to contaminant exposure followed by a later induction has been reported in bivalve species in field [15,64] and laboratory experiments [65]. GPX reduces hydrogen peroxide and organic peroxides using the glutathione pool in order to prevent oxidative damage [14,15]. Géret et al. [65] suggested that the decrease of GPX activity of clams *Ruditapes dessucatus* in the first days of Cd exposure could be attributed to a depletion of cellular glutathione, which is used as a cofactor in GPX reactions, due to formation of Cd complexes [15]. After some days of Cd exposure, metallothioneins that capture free Cd ions in the cells were induced and the antioxidant system was functional. Ni also binds to glutathione and metallothioneins, thus the effects of different Ni doses on GPX in the present study could be similarly explained. Cr binds to glutathione but not to metallothionein, which may explain the lack of GPX induction in Cr exposed mussels. Nevertheless, GPX is a component of a complex antioxidant defence system and its response is accompanied by responses of other enzymes and scavenger molecules that could have been responsible for the protection against ROS. For example, a reciprocal compensation between decreasing GPX and increasing catalase activity, that are both hydrogen peroxide metabolising enzymes, has been reported [66].

The ability of biochemical markers to provide early warning signals of ecosystem-level damage can be evaluated by their correlation with ecologically relevant biomarkers such as SFG [7,48]. In the present study, despite the different responses of SGF and GPX of mussels to different Ni and Cr doses, a significant linear regression relationship was found between the two biomarkers suggesting that GPX responses may reflect the overall health of the organism. The concurrent marked reduction in SFG with the increase in GPX activity at the highest Ni body burdens suggests that the decline in the overall health of the organisms may be due, to some extent, to ROS production. Furthermore, it associates GPX induction with a real harmful effect on the organism thus fulfilling the requirements of a biochemical biomarker to have a significant ecological consequence. On the other hand the unexpected hormetic-like responses of SFG in parallel with consistent, even though moderate decreases in GPX activity is a subject for further research. This may be due to energy conservation or energy allocation towards other defence mechanisms or components of the antioxidant defence when SFG was increased. However, even if the significance of this finding needs to be clarified, our results suggest that GPX may be useful as an early warning signal of population level effects.

Conversely, the present study showed no effect of Ni or Cr exposure on ACHE activity of mussels and consequently no relationship between ACHE and overall health status of the organisms. Based on studies that have shown inhibitory effects of Ni and Cr on ACHE in aquatic species [24,26,27] ACHE inhibition was expected in this study. Literature data on mussels report in vitro ACHE inhibition by Cr [24] but no data are available on ACHE inhibition by Ni. Previous laboratory studies have shown ACHE inhibition by heavy metals such as Cu, Cd, Fe and Zn in mussels in vitro [21] and by Zn and Cu in clams in vivo [22]. However, the concentrations used in the above experiments were much higher than those occurring in the marine environment. Payne et al. [25] suggested that inhibition of ACHE by heavy metals is likely to occur at very high concentrations, rarely found in the environment. Field studies in Mediterranean coastal waters

have related decreases in ACHE activities of mussels with heavy metal pollution nevertheless in most cases additive effects of several contaminants possibly occurred [67,68]. However, the concentrations used in the above experiments were much higher than those occurring in the marine environment. It has to be noted that ACHE activities of mussels in the above-mentioned studies were measured in total soft tissues [21,67,68] and haemolymph [24] whereas in this study, ACHE activity was measured in the adductor muscle considering the importance of nerve impulse transmission in muscle tissues and that previous studies have shown ACHE activity variations in this tissue of field collected mussels that could relate to pollution levels [39,60]. In the present study, the lack of ACHE inhibition in mussels with elevated Ni and Cr tissue concentrations, higher or comparable to those found in mussels at contaminated areas [8,9], suggests that ACHE in *M. galloprovincialis* may not be suitable as a biomarker of Ni and Cr effects.

In conclusion, our results indicate that SFG and GPX were affected by Ni and Cr levels of the present study although a clear dose response was not evident; adverse effects on the energy status and induction of GPX were found at the higher exposure levels while possible hormetic effects on the energy status and moderate GPX suppression were associated with lower exposure levels. Nevertheless GPX responses were linked to changes in the overall health of the organisms suggesting GPX in *M. galloprovincialis* shows potential to signal population level effects of Ni and Cr. Our findings imply potential complications in the use of these biomarkers for the assessment of Ni and Cr pollution in marine environments as the type of response appeared to be related to exposure levels. A better understanding of such complex responses may help interpret some conflicting results in biomarker application for the assessment of environmental pollution. On the other hand, ACHE was not affected by Ni and Cr exposure and is not considered a suitable biomarker for Ni and Cr pollution.

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References

[1] M.H. Depledge and M.C. Fossi, *The role of biomarkers in environmental assessment* (2). *Invertebrates*, Ecotoxicology 3 (1994), pp. 161–172.
[2] S.M. Adams, J.P. Giesy, L.A. Tremblay, and C.T. Eason, *The use of biomarkers in ecological risk assessment: Recommendations from the Christchurch conference on Biomarkers in Ecotoxicology*, Biomarkers 6 (2001), pp. 1–6.
[3] C.H. Walker, S.P. Hopkin, R.M. Sibly, and D.B. Peakal, *Principles of Ecotoxicology*, Boca Raton, FL, CRC Press, Taylor & Francis Group, 2006, 315 pp.
[4] D.R. Livingstone, *Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms*, Mar. Pollut. Bull. 42 (2001), pp. 656–666.
[5] M.H. Fulton and P.B. Key, *Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects*, Environ. Toxicol. Chem. 20 (2001), pp. 37–45.
[6] M.H. Depledge, A. Aagard, and P. Györkös, *Assessment of trace metal toxicity using molecular, physiological and behavioral biomarkers*, Mar. Pollut. Bull. 31 (1995), pp. 19–27.
[7] J.I. Allen and M.N. Moore, *Environmental prognostics: Is the current use of biomarkers appropriate for environmental risk evaluation?*, Mar. Environ. Res. 58 (2004), pp. 227–232.
[8] R. Eisler, *Chromium hazards to fish, wildlife and invertebrates: a synoptic review*, US Fish and Wildlife Service Biological Report 85 (1.6), 1986.
[9] R. Eisler, *Nickel hazards to fish, wildlife, and invertebrates: A synoptic review*, US Geological Survey, Biological Resources Division, Biological Science Report USGS/BRD/BSR–1998-0001, Contaminant Hazard Reviews Report 34, 1988.
[10] T.M. Florence, J.L. Stauber, and M. Ahsanullah, *Toxicity of nickel ores to marine organisms*, Sci. Total Environ. 148 (1994), pp. 139–155.
[11] A. Nicolaidou and J.A. Nott, *Metals in sediments, seagrass and gastropods near a nickel smelter in Greece: Possible interactions*, Mar. Pollut. Bull. 36 (1998), pp. 360–365.

[12] J-M. Fernandez, S. Ouillon, C. Chevillon, P. Douillet, R. Fichez, and R. Le Gendre, *A combined modelling and geochemical study of the fate of terrigenous inputs from mixed natural and mining sources in a coral reef lagoon (New Caledonia)*, Mar. Pollut. Bull. 52 (2006), pp. 320–331.
[13] K.K. Lehtonen, D. Schiedek, A. Köhler, T. Lang, P.J. Vuorinen, L. Förlin, J. Barsiené, J. Pempkowiak, and J. Gercken, *The BEEP project in the Baltic Sea: Overview of results and outline for a regional biological effects monitoring strategy*, Mar. Pollut. Bull. 53 (2006), pp. 523–537.
[14] C. Cossu, A. Doyotte, M.C. Jacquin, M. Babut, A. Exinger, and P. Vasseur, *Glutathione reductase, selenium-dependent glutathione peroxidase, glutathione levels and lipid peroxidation in freshwater bivalves,* Unio tumidus*, as biomarkers of aquatic contamination in field studies*, Ecotoxicol. Environ. Saf. 38 (1997), pp. 122–131.
[15] F. Regoli and G. Principato, *Glutathione, glutathione-dependent and antioxidant enzymes in mussel,* Mytilus galloprovincialis, *exposed to metals under field and laboratory conditions: Implications for the use of biochemical biomarkers*, Aquat. Toxicol. 31 (1995), pp. 143–164.
[16] S.J. Stohs and D. Bagchi, *Oxidative mechanisms in the toxicity of metal ions*, Free Radical Biol. Med. 18 (1995), pp. 321–336.
[17] S. Lynn, F.H. Yew, K.S. Chen, and K.Y. Jan, *Reactive oxygen species are involved in nickel inhibition of DNA repair*, Environ. Mol. Mutag. 29 (1997), pp. 208–216.
[18] M. Misra, R. Rodriguez, and K.S. Kasprzak, *Nickel-induced lipid peroxidation in the rat: correlation with nickel effect on antioxidant defense systems*, Toxicology 64 (1990), pp. 1–17.
[19] S. Nobuyuki, U. Shunji, F. Yoshinori, and S. Masayasu, *Protective effect of deferoxamine on chromium(VI)-induced DNA single-strand breaks, cytotoxicity, and lipid peroxidation in primary cultures of rat hepatocytes*, Arch. Toxicol. 71 (1997), pp. 345–350.
[20] G. Bocquené and F. Galgani, *L'acétylcholinésterase chez les organismes marins, outil de surveillance des effets des pesticides organophosphorés et carbamates*, Océanis 17 (1991), pp. 439–448.
[21] S. Najimi, A. Bouhaimi, M. Daubèze, A. Zekhini, J. Pellerin, J.F. Narbonne, and A. Moukrim, *Use of Acetylcholinesterase in* Perna perna *and* Mytilus galloprovincialis *as a biomarker of pollution in Agadir Marine Bay (South of Morocco)*, Bull. Environ. Contam. Toxicol. 58 (1997), pp. 901–908.
[22] Hamza-Chaffai, M. Romèo, M. Gnassia-Barelli, and A. El Abed, *Effect of copper and lindane on some biomarkers measured in the clam Ruditapes decussatus,* Bull. Environ. Contam. Toxicol. 61 (1998), pp. 397–404.
[23] M. Elumalai, C. Antunes, and L. Guilhermino, *Single metals and their mixtures on selected enzymes of* Carcinus maenas, Water, Air, Soil Pollut. 141 (2002), pp. 273–280.
[24] L. Guilhermino, P. Barros, M.C. Silva Amadeu, and M.V.M. Soares, *Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned?*, Biomarkers 3 (1998), pp. 157–163.
[25] J.F. Payne, A. Mathieu, W. Melvin, and L.L. Fancey, *Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland*, Mar. Pollut. Bull. 32 (1996), pp. 225–231.
[26] L. Martinez-Tabche, L. Gomez-Olivan, M.C.R. Castillo, and A.M. Santiago, *Toxicity of nickel in artificial sediment on acetylcholinesterase activity and hemoglobin concentration of the aquatic flea* Moina macrocopa, J. Environ. Hydrol. 8 (2000), Paper 4.
[27] T.C. Diamantino, L. Guilhermino, E. Almeida, and A.M.V.M. Soares, *Toxicity of sodium molybdate and sodium dichromate to* Daphnia magna Straus *evaluated in acute, chronic and acetylcholinesterase inhibition tests*, Ecotoxicol. Environ. Saf. 45 (2000), pp 253–259.
[28] J. Widdows and P. Donkin, *Mussels and environmental contaminants: bioaccumulation and physiological aspects*, in *The Mussel* Mytilus, E. Gosling, ed., Elsevier Press, Amsterdam, 1992, pp. 383–424.
[29] NCMR, Pollution research and monitoring programme in Saronikos Gulf. MED-POL programme, Technical Report, Athens, November 1998.
[30] C. Tsangaris, E. Stroggyloudi, and E. Papathanassiou, *Measurements of biochemical markers of pollution in mussels* Mytilus galloprovincialis *from coastal areas of the Saronikos Gulf (Greece)*, Medit. Mar. Sci. 5 (2004), pp. 175–186.
[31] O.A. Dahab and H. Al-Madfa, *Chromium distribution in waters and sediments of the eastern side of the Qatari Peninsula*, Sci. Total Environ. 196 (1997), pp. 1–11.
[32] A.Turner, M. Nimmo, and KA. Thuresson, *Speciation and sorptive behaviour of nickel in an organic-rich estuary (Beaulieu, UK)*, Mar. Chem. 63 (1998), pp. 105–118.
[33] A.R. Friedrich and F.P. Filice, *Uptake and accumulation of the nickel ion by* Mytilus edulis, Bull. Environ. Contam. Toxicol. 16 (1976), pp. 750–755.
[34] H. Parlak, S. Katalay, and B. Büyükisik, *Accumulation and loss of chromium by mussels (*M. galloprovincialis), Bull. Environ. Contam. Toxicol. 62 (1999), pp. 286–292.
[35] UNEP, Determination of total Cd, Zn, Pd and Cu in selected marine organisms by atomic absorption spectrophotometry in Reference Methods for Marine Pollution Studies, No 11, Rev 1. UNEP 1984.
[36] D.R. Livingstone, F. Lips, P. Garcia Martinez, and R.K. Pipe, A*ntioxidant enzymes in the digestive gland of the common mussel* Mytilus edulis, Mar. Biol. 112 (1992), pp. 265–276.
[37] S.C. Gamble, P.S. Goldfarb, C. Porte, and D.R. Livingstone, *Glutathione peroxidase and other antioxidant enzyme function in marine invertebrates* (Mytilus edulis, Pecten maximus, Carcinus maenas *and* Asterias rubens), Mar. Environ. Res. 39 (1995), pp. 191–195.
[38] M. Bradforb, *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*, Anal. Biochem. 772 (1976), pp. 248–264.

[39] G. Bocquené, F. Galgani, T. Burgeot, L. Le Dean, and P. Truquet, *Acetylcholinesterase levels in marine organisms along French coasts*, Mar. Pollut. Bull. 26 (1993), pp. 101–106.
[40] G.L. Ellman, K.D. Courtney, V. Andres, Jr. and R.M Featherstone, *A new and rapid colorimeric determination of acetylcholinesterase activity,* Biochem. Pharmacol. 7 (1961), pp. 88–95.
[41] J. Widdows and P. Salked, *Practical procedures for measurement of Scope for Growth,* in *UNEP/FAO/IOC: Selected techniques for monitoring biological effects of pollutants in marine organisms*. MAP Technical Report Series No. 71, Athens, UNEP, 1993.
[42] E. Cotou, E. Papathanassiou, and C. Tsangaris, *Assessing the quality of marine coastal environments: comparison of scope for growth and Microtox ® bioassay results of pollution gradient areas in eastern Mediterranean (Greece)*, Environ. Pollut. 119 (2002), pp. 141–149.
[43] R.J. Conover, *Assimilation of organic matter by zooplankton*, Limnol. Oceanogr. 11 (1966), pp. 338–354.
[44] J. Widdows, P. Fieth, and C.M. Worrall, *Relationships between seston, available food and feeding activity in the common mussel* Mytilus edulis, Mar. Biol. 50 (1979), pp. 195–207.
[45] E. Gnaiger, *Heat dissipation and energetic efficiency in animal anoxibiosis: Economy contra power*, J. Exp. Zool. 228 (1983), pp. 471–490.
[46] G.E. Zaroogian and M. Johnson, *Chromium uptake and loss in the bivalves* Crassostrea virginica *and* Mytilus edulis, Arch. Environ. Contam. Toxicol. 13 (1984), pp. 411–418.
[47] J. Widdows, D.K. Phelps, and W. Galloway, *Measurement of physiological condition of mussels transplanted along a pollution gradient in Narragansett bay*, Mar. Environ. Res. 4 (1981), pp. 181–194.
[48] B.M. Sanders, L.S. Martin, W.G. Nelson, D.K. Phelps, and W. Welch, *Relationships between accumulation of a 60 kDa stress protein and scope-for-growth in* Mytilus edulis *exposed to a range of copper concentrations*, Mar. Environ. Res. 31 (1991), pp. 81–97.
[49] A.R.D. Stebbing, *Hormesis – stimulation of colony growth in* Companularia flexuosa *(hydrozoa) by copper, cadmium and other toxicants*, Aquat. Toxicol. 1 (1981), pp. 227–238.
[50] T. Strömgren, *Effects of heavy metals (Zn, Hg, Cu, Cd, Pb, Ni) on the length growth of* Mytilus edulis, Mar. Biol. 72 (1982), pp. 69–72.
[51] T. Strömgren, M.V. Nielsen, and K. Ueland, *Short term effect of microencapsulated hydrocarbons on shell growth of* Mytilus edulis, Mar. Biol. 91 (1986), pp. 33–39.
[52] B. Patel and K. Anthony, *Uptake of cadmium in tropical marine lamellibranchs, and effects on physiological behavior*, Mar. Biol. 108 (1991), pp. 457–470.
[53] A.D. Correira and F. Costa, *Sub-lethal effects of copper-spiked sediments on the marine amphipod Gammarus locusta: Evidence of hormesis?*, Ecotoxicol. Environ. Restor. 4 (1991), pp. 32–38.
[54] M.B. Duchemin, M. Auffret, N. Wessel, M. Fortier, Y. Morin, J. Pellerin, and M. Fournier, *Multiple experimental approaches of immunotoxic effects of mercury chloride in the blue mussel,* Mytilus edulis*, through in vivo, in tubo and in vitro exposures*, Environ. Pollut. 153 (2008), pp 416–423.
[55] M. Martinez-Madrid, P. Rodriguez, and J.I. Perez-Iglesias, *Sediment toxicity bioassays for assessment of contaminated sites in the Nervion River (Northern Spain). 1. Three-brood sediment chronic bioassay of* Daphnia magna straus, Ecotoxicology 8 (1999), pp. 97–109.
[56] E. Guerlet, K. Ledy, and L. Giambérini, *Is the freshwater gammarid,* Dikerogammarus villosus, *a suitable sentinel species for the implementation of histochemical biomarkers?*, Chemosphere 72 (2008), pp. 697–702.
[57] E.J. Calabrese, *Evidence that hormesis represents an "overcompensation" response to a disruption in homeostasis*, Ecotoxicol. Environ. Saf. 42 (1999), pp. 135–137.
[58] P.M. Chapman, *Ecological risk assessment (ERA) and hormesis*, Sci. Total Environ. 288 (2002), pp. 131–140.
[59] M. Solé, C. Porte, and J. Albaiges, *Mixed function oxygenase system components and antioxidant enzymes in different marine bivalves: its relation with contaminant body burdens*, Aquat. Toxicol. 30 (1994), pp. 271–283.
[60] C. Tsangaris, E. Cotou, and E. Papathaassiou, *Multiple biomarker assessment for marine pollution: A case study to distinguish the type of pollutants in Amvrakikos Gulf (Greece)*, Mar. Environ. Res. 54 (2002), p. 845.
[61] I. Lima, S.M. Moreira, J. Rendon-Vón Osten, A.M.V.M. Soares, and L. Guilhermino, *Biochemical responses of the marine mussel* Mytilus galloprovincialis *to petrochemical environmental contamination along the North-western coast of Portugal*, Chemosphere 66 (2007), pp. 1230–1242.
[62] A. Orbea and M.P. Cajaraville, *Peroxisome proliferation and antioxidant enzymes in transplanted mussels of four Basque Estuaries with different levels of polycyclic aromatic hydrocarbon and polychlorinated biphenyl pollution*, Environ. Toxicol. Chem. 25 (2006), pp. 1616–1626.
[63] A. Orbea, M. Ortiz-Zarragoitia, and M. Cajaraville, *Interactive effects of benzo(a)pyrene and cadmium and effects of di(2-ethylhexyl) phthalate on antioxidant and peroxisomal enzymes and peroxisomal volume density in the digestive gland of mussel Mytilus galloprovincialis Lmk*, Biomarkers 7 (2002), pp. 33–48.
[64] F. Regoli, G. Frenzilli, R. Bocchetti, F. Annarumma, V. Scarcelli, D. Fattorini, and M. Nigro, *Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels,* Mytilus galloprovincialis, *during a field translocation experiment,* Aquatic Toxicol. 68 (2004), pp. 167–178.
[65] F. Géret, A. Serafim, L. Barreira, and M.J. Bebianno, *Effect of cadmium on antioxidant enzyme activities and lipid peroxidation in the gills of the clam* Ruditapes decussates, Biomarkers 7 (2002), pp. 342–356.
[66] P. Łaszczyca, M. Augustyniak, A. Babczyñska, K. Bednarska, A. Kafel, M. Pawel, G. Wilczek, and I. Witas, *Profiles of enzymatic activity in earthworms from zinc, lead and cadmium polluted areas near Olkusz (Poland)*, Environ. Int. 30 (2004), pp. 901–910.

[67] M.G. Lionetto, R. Caricato, M.E. Giordano, M.F. Pascariello, L. Marinosci, and T. Schettino, *Integrated use of biomarkers (acetylcholinesterase and antioxidant enzymes activities) in* Mytilus galloprovincialis *and* Mullus barbatus *in an Italian coastal marine area*, Mar. Pollut. Bull. 46 (2003), pp. 324–330.
[68] M. Dellali, M. Gnassia-Barelli, M. Romeo, and P. Aissa, *The use of acetylcholinesterase activity in* Ruditapes decussatus *and* Mytilus galloprovincialis *in the biomonitoring of Bizerta lagoon*, Comp. Biochem. Physiol. 130C (2001), pp. 227–235.