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PAPER

A feeding inhibition based prediction of the toxic effect of dissolved metal mixtures upon *Echinogammarus marinus* (Crustacea: Amphipoda) at field relevant concentrations across a latitudinal gradient

M. Ramiro Pastorinho,*[a] Trevor C. Telfer,[b] Amadeu M. V. M. Soares[a] and António J. A. Nogueira[a]

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Risk assessment of metals in the environment is performed mainly with toxicity evaluations on single metals, which is largely inadequate since these substances occur in mixtures. The development of models predicting combined toxic effects on the basis of the concentration–response relationships of individual compounds has emerged as an answer. In the present study, metal effects on post-exposure anorexia (the concept of FdC_{50}—concentration causing 50% of feeding inhibition—is implemented) in *Echinogammarus marinus*, a widely distributed gammarid amphipod, were assessed and compared with modelled ones obtained through the application of the concentration addition (CA) model, which represents a reasonable worst-case scenario for the risk assessment of metal mixtures. Data were validated using *in situ* experiments performed along a latitudinal gradient (Iceland, Scotland and Portugal) aiming at establishing a geographic profile of autochthonous population susceptibilities to metals. For all of the metals studied concentrations in the water column at exposure sites were in good agreement with feeding inhibition levels. Models gave low to relatively high percentage agreement between predictions and experimental data. Boreal populations demonstrated higher susceptibility to single metals, but not to mixture exposures. Meridional populations denoted lower susceptibilities with higher FdC_{50}.

Introduction

Metals in the aquatic environment, either from anthropogenic or natural sources, rarely occur isolated. Instead they occur in complex mixtures,[1] which may exert effects upon aquatic organisms at concentrations well below their individual sublethal values.[2,3] However, chemical risk assessments for aquatic environments largely rely on toxicological data derived for single chemicals, since apportioning toxic effects of individual toxicants within a complex mixture is both difficult and intricate. Developing models for mixture toxicity based on concentration–response relationships of individual compounds is a satisfactory alternative to testing all possible combinations of a given set of chemicals.[4] Such toxicity models are based on two concepts: concentration addition (CA) and independent action (IA).[5,6] These vary in that a CA-based model assumes a shared, common target site and similar mechanisms of action for each chemical, whilst an IA-based model assumes different target sites and dissimilar mechanisms of action for all components in the mixture.[1] For a more detailed discussion see Faust *et al.*[7] and the literature cited therein.

Amphipods are being increasingly used for laboratory and field studies to evaluate metal contamination, with feeding inhibition being a common endpoint.[8,9] Sublethal effects on food acquisition

[a]*Centre for Marine Environmental Studies (CESAM), Department of Biology, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal. E-mail: rpastorinho@ua.pt; Fax: +351 234372587; Tel: +351 234370350/768*
[b]*Environmental Group, Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, UK*

Environmental impact

A CA (concentration addition) based predictive model for metal exposure is developed for autochthonous populations of the marine amphipod *Echinogammarus marinus* encompassing its known entire geographic distribution in European shores. Validated by means of *in situ* and *ex situ* (single metals and mixtures) bioassays performed at each location (Iceland, Scotland and Portugal), besides presenting a powerful tool in Environmental Risk Assessment, the model sheds light upon the differential metal susceptibility of the species populations. A direct correlation between local contamination levels and effects (measured by post-exposure anorexia) is reported. Moreover, indications of diminished or null influence of temperature on the toxicity of single metals were obtained.

influence production rates (*i.e.* growth and reproduction) and other life traits in several species, including amphipods.[10–13] Post-exposure feeding depression is a reliable and sensitive method of quantifying this endpoint.[10] Post-exposure anorexia, in particular, is caused by metal exposure[10,12] and when used in field studies (*in situ* toxicity tests), supported by laboratory exposures, allows linkage of physiological responses at the individual animal level to their populations or communities.[12,14] However, the use of unrealistic high metal concentrations in toxicity evaluations has frequently prevented such a linkage, undermining any laboratory–field extrapolation through lack of ecological relevance.[15] The need to provide regulators with sound data, from which guidelines can be developed, has also emphasized the necessity for environmentally realistic toxicity tests, notably with regard to chemical concentrations.[16,17] In the present work, we assessed the toxicity of environmental realistic concentrations of four individual metals (zinc, cadmium, copper and nickel) and their mixtures upon the marine gammarid amphipod *Echinogammarus marinus* (Leach 1815) at different geographical latitudes using feeding inhibition as endpoint. The observed toxicity was then compared to modelled predictions based on the CA concept, on the assumption of similar modes of action for the tested metals (all divalent cations), and validated by *in situ* deployments of the gammarid. These evaluations were performed over the entire latitudinal distribution of *E. marinus*, encompassing Iceland, Scotland and Portugal.

Materials and methods

Unless otherwise specified, the methods and procedures described are common to all three laboratories/field locations.

Experimental animals

Test gammarids were collected from local populations in Iceland (South of Sandgerði, Reikjanes Peninsula 64°02′N, 22°42′W), Scotland (Loch Fyne, 56°10′N, 5°05′W), and Portugal (Mondego estuary, 40°07′N, 8°49′W). These were transported to local laboratories (Sandgerði Marine Centre, Institute of Aquaculture University of Stirling and Department of Biology, University of Aveiro, respectively) in 50 L plastic buckets filled with local water and brown macro-algae as a substrate. The gammarids were allowed to acclimate and depurate[18] for one month (Portugal and Scotland) or three weeks (Iceland) in plastic containers (40 × 20 cm) filled with 4 L of continuously aerated artificial saltwater or natural seawater (see below). Water was changed twice a week and dry *Fucus vesiculosus* (obtained from local clean sites, oven dried at 50 °C for 48 h) supplied *ad libitum* as food. To provide shelter and simulate the gammarid's habitat, small black polyethylene sheet rectangles were placed in the tank.[19]

Artificial seawater (SERA PREMIUM® in de-ionized water) was used in Scotland and Portugal and natural seawater (taken from a 50 m deep bore hole, and free from any contaminants (Svavarsson, unpublished data)) used in Iceland. Salinity was set at 30‰ using deionized water. Acclimation and test temperatures were maintained in illuminated CT rooms at 10 ± 1 °C in Iceland, 15 ± 1 °C in Scotland and 20 ± 1 °C in Portugal, mirroring the typical average water temperatures of each region at the time of collection.[20–22] Photoperiod regime was 12 h light/12 h dark at all locations.

Laboratory toxicity tests

Stock solutions of four metals (zinc, cadmium, copper and nickel) were prepared from salts ($ZnSO_4 \cdot 7H_2O$, $CdCl_2 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$, $NiCl_2 \cdot 6H_2O$, Sigma-Aldrich) in ultra-pure Milli-Q water. Five nominal concentrations per metal (Zn: 19, 38, 75, 150, 300 μg L^{-1}; Cd: 0.2, 0.38, 0.6, 1.0, 1.75 μg L^{-1}; Cu: 2.25, 4.5, 9, 18, 36 μg L^{-1}; Ni: 5, 10, 20, 35, 50 μg L^{-1}) were obtained by adding appropriated aliquots of the stock solution to saltwater (see the "Experimental animals" section). In order to guarantee adequate ecological framing, the choice of concentrations was based on values obtained from a survey in the metal polluted Ria de Aveiro[23] as a benchmark. These values are surrogates for the maximum possible levels in the water column (the worst-case scenario). Individual metals and quaternary mixtures were tested (96 h static exposure) using five replicates per treatment (ten organisms per chamber). All materials (including the plastic test chambers) were acid washed and pre-soaked in the appropriate test medium for 24 h to saturate all adsorption sites.[24] No food was provided to the organisms during experiments. Immediately on completion of the 96 h exposure time, individuals were transferred to new chambers containing clean seawater and food. Feeding rate was measured over the subsequent 24 h (see the "Feeding assays" section).

In situ exposures

Environmental chambers were constructed from clear polyvinyl chloride cylindrical piping as described by McWilliam and Baird.[12] Twenty adult organisms (from the same laboratory culture as used in the laboratory toxicity assays) of approximate size (within ±2 mm) were introduced in each chamber and deployed *in situ* for a period of 96 h. The chambers were then taken back to the laboratory (<2 h drive in all cases) submerged in local water, where the gammarids were carefully retrieved from the chambers and immediately allocated to the post-exposure 24 h feeding assays (see the "Feeding assays" section). The *in situ* chambers were deployed in three countries (Iceland, Scotland, and Portugal) representing three different eco-regions associated with different latitudes (Fig. 1).

Iceland

Three sites which reflected different local hydrodynamic conditions (a strong coastal drift circulating clock-wise around the entire island)[25] were used. Sandgerði Harbour (64°02′N, 22°42′W) was used as a central point (first location) and the two other sites, chosen due to their sheltered conditions (where sedimentary deposition occurs), are at approximately 5 km to the North (Hafurbjarnastaður) and South (Hvalsnes). Despite these shores being considered pristine environments[26] there is indication of slightly elevated metal background values probably due to volcanic activity as a diffuse source of metals[27] and point sources from the sparse human settlements.[28]

Scotland

Strachur (Loch Fyne, 56°10′N 5°05′W), Ardentinny (Loch Long, 56°02′N 4°54′W) and Hound Point (Firth of Forth, 56°00′N 3°21′W) were used as field deployment sites. Loch Fyne is the longest, deepest fiordic sea loch of Scotland's Western coast.

Fig. 1 Generic depiction of *E. marinus* collection/*in situ* experiments deployment sites.

Despite being part of the Firth of Clyde watershed, which is customarily deemed as one of the country's most contaminated,[29] and possessing intensive fish farming, the sampling area shows signs of very low contamination, as the production of Class A oysters (the highest standard, the bivalves can go straight to the market) testifies. Loch Long is a remote system, equally a part of the Clyde watershed. It is the second most brackish sea loch in Scotland, and, despite the existence of a ship refuelling depot (MOD) and an oil terminal operated by one of the largest chemical companies in the world (INEOS), is regarded as mostly undisturbed.[30] The Firth of Forth (on the East coast) has a history of industrial pollution (chemical, oil refinery, pulp mill, and sewage).[31] It possesses a long record of metal pollution[32] that, nevertheless, has strongly abated in recent times.[21]

Portugal

Ria de Aveiro (40°38′N, 8°44′W), a coastal lagoon in the NW coast of the Iberian Peninsula, is 45 km long and 10 km wide.[33] Of the four main channels (S. Jacinto, Espinheiro, Mira and Ílhavo), the most hydrodynamically important are the S. Jacinto and the Espinheiro channels, as they are connected directly to the lagoon mouth and have the strongest currents, reaching values of about 2 m s^{-1} during spring tides and high rainfall. The remaining channels, which are mostly very narrow, are dominated by mud flats and salt marshes, characterized by a very asymmetrical topography, which contributes to a strong damping of the currents and an increase of the phase delay of the tidal wave.[33,34] Due to their unique characteristics, each channel may be regarded as an independent estuary connected to a common inlet. The area encircling this system is inhabited by 700 000 people, and besides intensive agriculture, industries such as chemical, metallurgic, ceramics, tannery and pulp milling are present, draining their effluents (collection and pre-treatment being performed only in recent years) into the lagoon.[35] Four sites inside the Ria were selected: (1) Areão situated in the most "isolated" channel, with tidally driven hydrodynamics and overall low level of metals;[23] (2) S. Jacinto situated in one of the most hydrodynamic channels, nearly oceanic conditions, with low metal levels; (3) Bico da Murtosa, the most contaminated of the sites, situated in the vicinity of the most polluted area of the lagoon—Laranjo Bay—with historical metal contamination;[36] and (4) Ovar Marina situated in the far reaches of the system, with low hydrodynamism, representing an intermediate case of contamination.[23]

Feeding assays

Pre-dried (50 °C until stable weight is attained) and weighed discs of *F. vesiculosus* were offered to test gammarids in clean saltwater immediately after the laboratory or field exposures and amphipods were allowed to feed for 24 h. Remaining algae were collected from the chambers, dried (same conditions) and reweighed. Feeding rate (in mg per individual) was obtained from the amount of food consumed (initial *F. vesiculosus* mass minus final mass) divided by the number of individuals feeding.

Metal analysis

Metals were analysed in acidified samples of field and laboratory test water by atomic absorption spectrophotometry (Unicam 939QZ Atomic Absorption Spectroscope with coupled Unicam GF90 Graphite Furnace (GF-AAS) and deuterium arc lamp or Zeeman© background correction). Calibration was obtained using MERCK CertiPUR standards and internal quality control was performed using the certified reference material SLEW-3 (National Research Council Canada). Certified values are 0.201 ± 0.037, 0.048 ± 0.004, 1.55 ± 0.12 and 1.23 ± 0.07 μg L^{-1} for zinc, cadmium, copper, and nickel, respectively, whilst measured values were 0.210 ± 0.005, 0.049 ± 0.003, 0.153 ± 0.016 and 1.24 ± 0.017 μg L^{-1}, respectively. Ammonium hydrogen orthophosphate ($NH_4H_2PO_4$) was used as matrix modifier for Zn analysis. Detection limits were 0.009 μg L^{-1}, 0.02 μg L^{-1}, 0.07 μg L^{-1}, and 0.2 μg L^{-1} for zinc, cadmium, copper, and nickel, respectively.

Data analysis

The absolute-rate theory[37] describes the rates of elementary chemical reactions by assuming a special type of equilibrium (quasi-equilibrium) with an equilibrium constant existing between reactants and activated complexes. This can be used to describe the inhibition (I) of a biological process (like feeding) as a function of toxicant concentration, with observed values ranging from the control values to zero,

$$I = I_0 \times \frac{EC_{50}{}^k}{EC_{50}{}^k + [C]^k} \quad (1)$$

where I is the measured value of the biological process, I_0 is the maximum value measured for the biological process (*i.e.* the average response in the control), EC_{50} is the half saturation constant (*i.e.* concentration that causes an inhibition of 50% in the biological process), C is the concentration of the metal, and k is the decay index. Rearranging the equation, we get

$$\frac{I}{I_0} = \frac{{EC_{50}}^k}{{EC_{50}}^k + [C]^k} \quad (2)$$

and

$$\frac{I_0 - I}{I} = \frac{[C]^k}{{EC_{50}}^k} \quad (3)$$

By applying logarithms to both sides of the equation a linear equation is obtained (*i.e.* $Y = mX + b$):

$$\log\left(\frac{I_0 - I}{I}\right) = k \log([C]) - k \log(EC_{50}) \quad (4)$$

Thus, if we use feeding (F) as the biological process in eqn (3) we get

$$\frac{1 - F}{F} = \frac{[C]^k}{{EC_{50}}^k}, \text{ or } \frac{1 - F}{F} = TU^k \quad (5)$$

where $TU = [C]/EC_{50}$ refers to the toxic units of an individual chemical as defined by Sprague.[38] Solving eqn (5) in relation to feeding (F) we get

$$F = \frac{1}{1 - TU^k} \quad (6)$$

Considering a mixture of n chemicals, where each chemical contributes to the overall toxicity proportionally to the concentration (expressed as TU) of each chemical, the expected feeding for the mixture (F_{mix}) can be calculated as

$$F_{mix} = \frac{1}{1 + \left(\sum_{i=1}^{n} TU_i\right)^{\kappa'}} \quad (7)$$

where

$$\kappa' = \sqrt[z]{\prod_{i=1}^{n} K_i^{TU_i}}, \text{ and } z = \sum_{i=1}^{n} TU_i \quad (8)$$

i.e. κ' is the weighted geometric mean of the K_i obtained for each chemical in the mixture.

E. marinus feeding under exposure to toxicants does not follow this general pattern (*i.e.* feeding rate decaying to zero with increasing exposure of the toxicant), instead feeding decays to a minimum value (F_{min}) for increasing concentrations of the toxicant (Fig. 2).

Since some of the original concepts have been adapted (EC_{50} and TU) and do not have the same meaning, to avoid misinterpretations it is suggested that this particular EC_{50} is referred as FdC_{50}, and the TU are referred as fTU:

$$fTU = \frac{[C]}{FdC_{50}} \quad (9)$$

Thus, for this species, eqn (6) must be rewritten as

$$F = (1 - F_{min}) \times \frac{1}{1 + fTU^k} \quad (10)$$

and consequently eqn (7) becomes

$$F_{mix} = (1 - F_{min}) \times \frac{1}{1 + \left(\sum_{i=1}^{n} fTU_i\right)^{\kappa'}} \quad (11)$$

Single metal feeding inhibition parameters were calculated fitting the experimental data to eqn (10) with SigmaPlot 10 (SPSS, Inc.). The model parameters from single metal exposures were integrated in eqn (11) to calculate the expected feeding inhibition for the quaternary mixtures and field exposures.

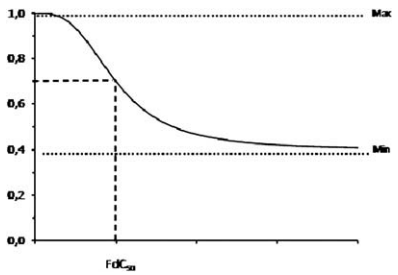

Fig. 2 Theoretical sigmoid function (with an offset) describing *E. marinus* feeding decay, and the 50% feeding decay (FdC_{50}) derivation.

Results

Chemical analysis

Measured concentrations of the test solutions for single and mixtures of metals were within 10% of nominal concentrations for laboratory experiments. Responses to all metals are therefore based on nominal concentrations.

Results for the analysis of field water samples are presented in Table 1. Metal concentrations in Icelandic water samples were unexpectedly high, particularly for Hafurbjarnastaður. This site was near the northernmost tip of the Reykjanes Peninsula where rock outcrops form an area of shallow waters abating the currents. The circulation patterns of the area were therefore different from the other two stations where a strong northwards current was felt. The settlement of particulate materials due to current abatement at this location could partly explain the higher metal concentrations. In contrast, metal concentrations from the

Table 1 Concentration of metals from *in situ* collected water samples (μg L^{-1}) with the indication of EU EQS: quality standards adopted for transitional and salt waters by the European Union for "dangerous substances"

		Zn	Cd	Cu	Ni
Iceland	Hafurbjarnastaður	589	5.26	35.2	242.2
	Sandgerði Harbor	717	0.59	35.9	75.6
	Hvalsnes	22	0.56	22.5	30.4
Scotland	Hound point	8	0.02	6.5	4.5
	Strachur	5	0.02	6.2	3.7
	Ardentinny	6	0.03	5.4	3.5
Portugal	Ovar Marina	324	1.02	22.0	6.3
	B. Murtosa	246	2.30	34.1	9.4
	S. Jacinto	12	0.06	2.9	1.7
	Areão	11	0.02	1.5	2.8
EU EQS		40[a]	0.2	5[a]	20.0

[a] Values proposed by the UKTAG, 2008 for "specific pollutants" in brackish and salt waters. (UKTAG -Technical Advisory Group on the Water Framework Directive - Proposals for Environmental Quality Standards for Annex VIII substances, 2008)

Scottish sites were low. Though emission reduction strategies of the Scottish Environment Protection Agency (SEPA) were a likely contributor, records for the historically polluted Firth of Forth reported consistently low metal concentrations in waters, even during the mid-1990s when discharges were severe.[39] The turbid nature of the Firth is likely to be a significant factor in decreasing the concentration of dissolved metals in the water column through particle binding. Analysis results for water taken from the Portuguese stations presented a very diverse scenario, mirroring the complex hydrodynamic conditions and particle circulation patterns within the lagoon. Stations at Ovar Marina and B. Murtosa clearly showed considerably higher metal concentrations than S. Jacinto and Areão (Table 1).

Single metal toxicity

Results for the single metal toxicities are given in Table 2. In all single metal exposures no mortality occurred in either controls or treatments. The decay curves describing the proportional feeding were statistically significant with three exceptions (zinc experiments in Scotland and Portugal and copper experiments in Portugal). Parameter estimates for single metal exposures obtained from eqn 10 (Table 2) show that toxicity varied according to the metal and location. In all cases residuals of the regression model obtained were normally distributed (Kolmogorov–Smirnov test: $p > 0.05$). Consistent reproducibility of results between controls and individual metals was observed for individuals from all locations.

Iceland

High susceptibility to all metals was observed. Low values of FdC_{50} (73.43 μg L^{-1} for Zn, 0.21 μg L^{-1} for Cd, 1.78 μg L^{-1} for Cu and 10.8 μg L^{-1} for Ni) corroborate this finding, with cadmium and, to a higher extent, copper giving the strongest feeding inhibition (Table 2) at the ecologically relevant concentrations tested.

Scotland

Gammarids from Scotland showed high susceptibility to all metals (low FdC_{50}), except for zinc (Table 2) to which no significant sensitivity was shown. As for Iceland results, cadmium and copper gave the highest inhibitory effects. Calculated FdC_{50} values were 0.28 μg L^{-1} for Cd, 2.02 μg L^{-1} for Cu and 8.39 μg L^{-1} for Ni.

Portugal

The similarities in sensitivities shown by gammarids from Iceland and Scotland were not, with the exception of cadmium (FdC_{50} = 0.87 μg L^{-1}), present for gammarids tested in Portugal. No toxicity was detected to zinc and copper at the tested concentrations (Table 2), and susceptibility to nickel was much lower than to cadmium. The range of concentrations tested was low, leading to an estimation of FdC_{50} at 44.96 μg L^{-1}, almost the highest tested concentration (50 μg L^{-1}). Nevertheless, susceptibility to both cadmium and nickel was observed in comparison with zinc and copper.

Mixture and field toxicity

Mortality rates were never higher than 10% in laboratory exposures to mixtures and no mortality in controls occurred. In field exposures, mortality rates were never higher than 5% at any location. Comparisons of the predicted and observed quaternary mixture toxicities with those of the individual components and field data are given in Fig. 3. Both the IA and the CA models were tested, yielding virtually similar predictions. The CA model was pursued given the divalent nature of all the metals tested pointing towards an additive in detriment of an independent mode of action. Predicted mixture toxicity, as feeding response, was calculated using eqn (11), with parameter estimates derived from single metal exposures (Table 2).

Iceland

Toxicity predictions for mixture exposures are clearly overestimated by the model. Therefore, toxicity predictions for the ranges of metal concentrations used in the mixtures and measured on all *in situ* water samples were near the minimum values. The percentage of observations falling between ±25% of the predicted value are: 93% for the control, 88% for single metals, 60% for mixtures, and 33% for field data (Fig. 3a).

Table 2 Parameter estimates for single metal exposures (eqn (9)) using non-linear regression (these estimates were used to predict mixture toxicity with eqn (10))

Country	Metal	EC_{50} (FdC_{50})[a]	k[a]	F_{min}[a]	n	r^2	Regression significance
Iceland	Zn	73.42 (151.58)	0.88 (0.92)	0.44 (0.44)	39	0.42	$p < 0.001$
	Cd	0.21 (0.22)	0.88 (1.19)	0.44 (0.28)	39	0.61	$p < 0.001$
	Cu	1.78 (1.70)	0.88 (1.58)	0.44 (0.25)	39	0.36	$p < 0.001$
	Ni	10.80 (23.55)	0.88 (1.19)	0.44 (0.47)	39	0.46	$p < 0.001$
Scotland	Zn	1335.68 (nd)	0.58 (3.39)	0.44 (nd)	26	0.14	$p = 0.21$[a]
	Cd	0.28 (2.05)	0.58 (1.95)	0.44 (1.18)	26	0.52	$p < 0.001$
	Cu	2.02 (5.92)	0.58 (1.43)	0.44 (0.56)	26	0.64	$p < 0.001$
	Ni	8.39 (59.32)	0.58 (1.76)	0.44 (1.11)	26	0.61	p <0.001
Portugal	Zn	nd (nd)[c]	nd (nd)[c]	nd (nd)[c]	41	0	$p = 1$[b]
	Cd	0.73 (5.38)	0.72 (1.76)	0.44 (1.33)	41	0.16	$p < 0.05$
	Cu	nd (nd)[c]	nd (nd)[c]	nd (nd)[c]	41	0	$p = 1$[b]
	Ni	76.25 (483.92)	0.72 (0.89)	0.44 (nd)	41	0.22	$p < 0.05$

[a] Parameter estimate with standard error of the estimate between brackets. [b] This metal was not considered for calculation of expected toxicity from mixtures. [c] Fit not possible since responses were fairly stable throughout the range of concentrations tested; nd: parameter not determined.

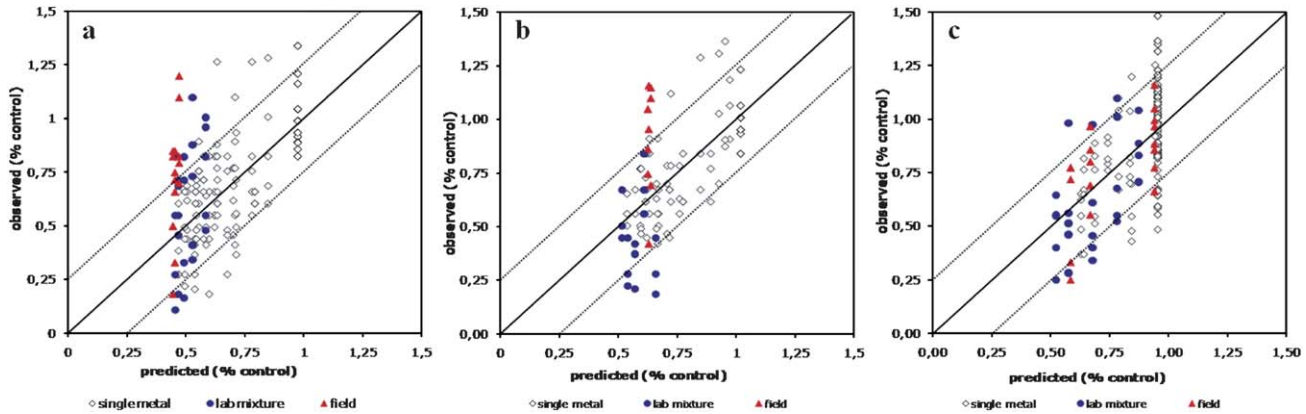

Fig. 3 Predicted and observed joint action relationships for feeding response of a quaternary mixture (Zn, Cd, Cu and Ni—dots), individual constituents (diamonds) and field collected data (triangles) upon the post-exposure feeding activity of *Echinogammarus marinus* Icelandic (a), Scottish (b) and Portuguese (c) populations. The identity line (solid line) and 25% deviation intervals (dotted lines) are also depicted. Each data point corresponds to a single observation and the corresponding prediction was obtained from eqn (11), using parameters from Table 2.

Scotland

The mixture toxicity predictions based on laboratory data show that 67% of the observed values are located within ±25% of the predicted values. However, predicted values for field exposures were overestimated. The percentage of observations falling between ±25% of the predicted values were 100% for control, 88% for single metals and 45% for the field (Fig. 3b).

Portugal

The Portuguese dataset produced the most consistent predictive scenario of the three locations. Predictions for laboratory based mixture toxicity showed 68% of the observed values to be within ±25% of the predicted value, whereas for field data 75% of observed values were within ±25% of the predicted value for the same interval. In addition, the percentage of observations falling between ±25% of the predicted value was 88% for the control, and 80% for the single metals. The high density of data for single metals at the maximum predictive value is a consequence of the high number of control observations (Fig. 3c).

Discussion

Single metal exposures and mixture exposure show a consistent degree of variability between replicates which is independent of the metal concentration. A good agreement (over 80%) was generally found between predictions and observations (observed values tended to be located within an interval of ±25% of predictions). However, predictions associated with field exposures in Scotland overestimated toxicity probably due to interaction with other environmental parameters.

Temperature can influence the sensitivity of organisms to toxicants. Almost every biological rate is affected by temperature (*e.g.* biochemical reaction rates, metabolic rates) having its activity increased exponentially,[40] and inevitably so are the metabolic pathways involved in sequestration and secretion of toxic substances. Nevertheless, available data are insufficient to attribute a uniform role to temperature in toxicity mechanisms in nature,[40,41] and to pronounce temperate species as more sensitive to contaminants than boreal ones as some authors defend.[42] For this reason in our experimental design, we opted to adjust test temperatures according to local annual averages.

Given the latitudinal breadth of the present study, temperature was one of the variables that could strongly contribute to influence results. For the concentrations tested, a transition in toxicity was observed with change in latitude: Icelandic organisms were highly sensitive to all metals, Scottish organisms were equally sensitive to the metals except to zinc, and Portuguese individuals were insensitive to zinc and copper and the calculated FdC_{50} value for the other two metals was much higher (fourfold). Portuguese gammarids, while more sensitive to cadmium and nickel, were not as sensitive to these metals as at the other locations. Data seem to disprove the general rule of higher temperature = higher toxicity[43] and it can be concluded that, for *E. marinus*, temperature is not a major influence in single metal toxicity. When considering mixture exposures the results are different with Icelandic gammarids being less sensitive than the gammarids from the other locations, despite high standard deviations. This lowered sensibility could be a result of metal interaction during uptake. Despite being sensitive to all metals tested, the Icelandic gammarids were particularly sensitive to copper. Daka and Hawkins[44] demonstrated an (intermittent) antagonistic effect between Cu and Zn for the gastropod *Littorina saxatilis*. It is possible that during mixture exposure uptake of Cu was diminished by competition with Zn, thereby reducing overall toxic effects. However, the high standard deviations in toxic effect for *E. marinus* illustrated a highly variable antagonistic interaction between these metals.

Metal interactions have been at the centre of the debate around the validity of use of the CA model for metals. Poynton *et al.*[45] used gene expression profiles to show that Cu, Cd and Zn had distinct modes of action to *Daphnia magna* when previously the contrary was believed to be true. Despite this the CA-based model has been considered as a good estimator for worst-case scenario of metal toxicity due to overestimated predictions,[46] something that happened in the present study.

Aside from occasional work using specific species it has not been possible to accurately predict general interactions among

metals, let alone interactions between metals and other contaminants (the case of field exposures), without targeted site-specific testing.[47] Less than additive and more than additive responses for metal–metal interactions are as likely as strictly additive responses;[47] multiple metal effects are not always additive[48] and metal uptake is influenced by the specific metals and their ratios in a mixture. The existence of different strategies towards metals, between metals, and between individuals[49] modulates these realities, creating a complex web of interaction. Therefore, predictions returned by the application of the CA-based model for mixtures present only a satisfactory level of accuracy, with 60 and 70% of the predictions falling between ±25% of the predicted value for the three locations.

Since the organisms used in the present study were of the same species at all latitudes, any differences cannot be attributed to differing phylogenies. However, there has been growing attention to the influence that previous exposure scenarios have on the uptake of metals by organisms.[24,49,50] It has been shown that when environmental metal concentrations are sufficiently elevated, from either anthropogenic or natural sources, selection for metal-resistant populations can occur,[17] with resultant inheritable genetic adaptations.[51–53] Nevertheless, genetically determined metal resistance to one metal does not endow resistance to all metals nor to other stressors.[54] Alternatively, mechanisms that reduce metal uptake or accumulation and detoxification may also allow tolerance without genetic selection for metal-resistant populations[55] with concomitant loss of genetic variability. These mechanisms are energetically costly metabolic processes and, given organisms' energetic constraints, metal tolerance can quickly disappear once metal contamination is removed.[56] We believe this scenario fits our data: Icelandic organisms live in an energetically more demanding environment where any savings in maintenance costs (*e.g.* detoxification) would set free energy for growth. As natural selection favours individuals for energy efficiency and maximized growth,[57] it is unlikely that there would be selection for genetically metal-tolerant species. In addition, the main source of metal contamination in Iceland is volcanism[27] that exerts its influence in pulses, corresponding to an intermittent scenario of exposure. Conversely, the gammarid used in the experiments in Portugal were taken from an area in the Mondego estuary where metal-rich fertilizers and pesticides[58] are extensively used on rice crops.[59]

These circumstances are consistent with a continuous exposure to metals at sub-lethal levels (one of the necessary pre-requisites for selection mechanisms to act[60]) and together with a lower environment constriction for energy, create conditions for the emergence of a genetically selected metal-tolerant specimen profile.[61]

The context of the above becomes clear when considering that laboratory depuration is, from a physiological point of view, a period of non-exposure (the equivalent to the absence of field pulses) when no detoxification is required. On first metal exposure, those organisms in possession of genetic metal tolerance (*i.e.* Portuguese gammarids) can adapt to the toxicity more readily than those requiring to divert energy to detoxification strategies (*i.e.* Icelandic gammarids). Seen through this perspective, the higher consistency of prediction by the CA-based model to Portuguese field data (75% agreement to ±25%) than to Icelandic field data (33% agreement to ±25%) becomes clear. Scotland had an intermediate value (45%), reflecting an added sensitivity of the specimens to copper.

Table 3 Ranking of sites used in Iceland, Scotland and Portugal according to "contamination" (enhanced amounts of metal) and "effect" (effect upon organisms) scoring. Contamination and effect are used *lato sensu* and not as textbook definitions (Spearman Rank Correlation: $r_s = 0.81$, $p = 0.007$)

		Ranks	
		Contamination	Effect
Iceland	Hafurbjarnastaður	1	2
	Sandgerði Harbor	2	1
	Hvalsnes	2	3
Scotland	Hound point	1	1
	Strachur	2	2
	Ardentinny	3	3
Portugal	Ovar Marina	2	2
	B. Murtosa	1	1
	S. Jacinto	3	3
	Areão	4	4

The validity of this bioassay as a basis to apply the CA-based model can be demonstrated using the relationship between the presence of metal and post-exposure feeding performance in the field. Scores can be awarded to each metal individually, according to the recorded concentration and to the feeding performance (food uptake), and summed for each station. Thus, the lower the score the more "contaminated" will the site be, working similarly for effect (lowest score = highest feeding depression). This allows the establishment of two rankings: one for "contamination" (enhanced levels of metals) and another for "effect" (effect upon organisms). The ranking obtained for the study sites at each location is shown in Table 3, being highly significant ($r_s = 0.81$, $p = 0.007$). Here the ranking for "contamination" is almost exactly the same as for "effect", indicating that stations possessing overall higher metal concentrations in the water column are the ones exerting higher levels of biological effects (feeding inhibition) upon *E. marinus*. This ranking also allows comments upon metal bioavailability: unlike Scotland and Portugal, in Iceland the highest metal concentrations did not result in the highest verified effects, and similar pollution ranks resulted in opposite effects (highest and lowest), showing there was only partial bioavailability of the metals at each location, a reflex of the complex geochemistry of Icelandic waters, mostly driven by volcanism.

Conclusions

Amphipod feeding inhibition levels were in good agreement with concentrations of the metals studied in the natural waters of all exposure sites. Higher latitude populations possess higher susceptibility to single metal exposure. The same was not verified for mixture exposures. Southern populations denoted overall lower sensitivities (single and mixture exposures) reflected in higher values for concentrations causing feeding inhibition. Populations at median latitudes patented an intermediate record.

Selection for metal-tolerance fits the collected data regarding meridional populations. Models gave moderate to relatively high percentage agreement between predictions and experimental

data. Finally, indications of diminished influence of temperature on the toxicity of single metals were obtained.

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