TITLE. Toxicity of seabird guano to sea urchin embryos and interaction with Cu and Pb

AUTHOR NAMES Diego Rial[1]*, Juan Santos-Echeandía[2], Xosé Antón Álvarez-Salgado[2], Antoni Jordi[3], Antonio Tovar-Sánchez[3,4]. Juan Bellas[5].

AUTHORS ADDRESSES

[1] CIIMAR Ecotoxicology, stress ecology and environmental health, Rua dos Bragas 289, 4050-123 Porto, Portugal

[2] CSIC Instituto de Investigaciones Marinas, Eduardo Cabello 6, 36208 Vigo, Spain

[3] CSIC Instituto Mediterráneo de Estudios Avanzados –University of the Balearic Islands, Miguel Marqués 21, 07190 Esporles, Balearic Islands, Spain

[4] CSIC Instituto de Ciencias Marinas de Andalucía, Campus Universitario Río San Pedro, 11510 Puerto Real, Cádiz, Spain

[5] Instituto Español de Oceanografía (IEO), Centro Oceanográfico de Vigo, Cabo Estai - Canido, 36200 Vigo, Spain

* Corresponding author e-mail: diegorialconde@gmail.com

CIIMAR Ecotoxicology, stress ecology and environmental health, Rua dos Bragas 289, 4050-123 Porto, Portugal

Abstract

Guano is an important source of marine-derived nutrients to seabird nesting areas. Seabirds usually present high levels of metals and other contaminants because the bioaccumulation processes and biotic depositions can increase the concentration of pollutants in the receiving environments. The objectives of this study were to investigate: the toxicity of seabird guano and the joint toxicity of guano, Cu and Pb by using the sea urchin embryo-larval bioassay. In a first experiment, aqueous extracts of guano were prepared at two loading rates (0.462 and 1.952 g L^{-1}) and toxicity to sea-urchin embryos was tested. Toxicity was low and not dependent of the load of guano used (EC_{50} 0.42±0.03 g L^{-1}). Trace metal concentrations were also low either in guano or in aqueous extracts of guano and the toxicity of extracts were apparently related to dissolved organic matter. In a second experiment, the toxicity of Cu-Pb mixtures in artificial seawater and in extracts of guano (at two loadings: 0.015 and 0.073 g L^{-1}), was tested. According to individual fittings, Cu added to extracts of guano showed less toxicity than when dissolved in artificial seawater. The response surfaces obtained for mixtures of Cu and Pb in artificial seawater, and in 0.015 g L^{-1} and 0.073 g L^{-1} of guano, were better described by Independent Action model adapted to describe antagonism, than by the other proposed models. This implied accepting that EC_{50} for Cu and Pb increased with the load of guano and with a greater interaction for Cu than for Pb.

Keywords. Antagonism; joint toxicity; response surface; sea urchin embryo; seabird guano; trace metals.

1. Introduction

Coastal areas and cliffs represent suitable areas for seabird nesting activities. Yellow-legged gull (*Larus michahellis*) is one of the main communities that forms these coastal colonies and tend to deposit large amounts of organic type material (faeces, feathers, corpses, foodstuff, etc.). Among these materials, faeces or guano represent around 85% of the dry weight (Wait et al., 2005). The guano is mainly rich in nutrients (N and P) so its inputs alter the surrounding environment. Several works have shown an increase in the nutrient content of soils (Bukacinski et al., 1994; Otero et al., 2015) but also in the adjacent coastal waters. This enrichment is associated to direct deposition (Kolb et al., 2010) or to rain and run-off events, sea spray or waves that wash guano from bird colonies into the sea. This may cause local nutrient enrichment (Bosman et al., 1986; Kolb et al., 2010) which may enhance marine phytoplankton production (Zelickman and Golovkin, 1972) and intertidal macroalgal growth (Bosman et al., 1986). It has been shown that the guano derived materials can reach several meters seawards (Kolb et al., 2010) .

In addition to the high nutrient contents, guano is also rich in organic matter, metals and other toxicants (Otero and Fernandez-Sanjurjo, 2000; Liu et al., 2006; Signa et al., 2013). The nitrogen content of seabird guano ranges from 8 to 21% of the fresh weight and consist mainly of uric acid (ca. 80%), proteins (ca. 10%), ammonia (ca. 7%) and nitrate (ca. 0.5%) (Szpak et al., 2012) . Concerning phosphorus, it represents from 0.1 to 10% of the fresh weight and consists of about 50 to 70% of phosphate (Smith and Johnson, 1995; Otero et al., 2015). Due to bioaccumulation, seabirds usually present high levels of metals and other contaminants, and their concentrations can increase in the biotic depositions to toxic levels with negative implications for the ecosystems (Sun et al., 2000; Michelutti et al., 2010; Signa et al., 2013).

Due to the high content of organic material present in seabird guano, it may also represent an important input of dissolved organic matter to these coastal environments. The organic complexation of trace metals drives their biogeochemical cycles in coastal waters representing up to 99% of trace metal dissolved species (van den Berg et al., 1987; Saito and Moffett, 2001; Santos-Echeandia et al., 2008). This binding reduces metal lability and toxicity for marine organisms. Thus, it may be expected that this extra input of organic matter could be a natural defence for coastal littoral species against punctual inputs of trace metals as it has been demonstrated with other sources of organic matter (i.e. sediments or sewage) (Sanchez-Marin et al., 2010).

Due to their environmental relevance, metals have received particular attention in toxicity studies with aquatic organisms, but focus has been mostly placed on individual toxicity. However, aquatic organisms are usually exposed simultaneously to a wide range of toxicants in the environment rather than to individual substances. Thus, the inclusion of the combined effects of toxicants resulting from multiple exposures in water quality regulations has been advocated (Bellas, 2008). Although, the experimental evaluation of all possible combinations of toxicants is not feasible, several models have been developed for the prediction of combination effects on the basis of the concentration-response relationships of individual mixture components. Mixture toxicity studies conducted with aquatic organisms have reported either antagonistic, additive or synergistic interactions, depending on the metals studied and on the test species (Meyer et al., 2015).

It is generally accepted that embryos and larvae are the most sensitive developmental stages in the life cycle of marine invertebrates. Because of their high sensitivity, rapid response and ecological relevance, the embryo-larval bioassays with marine invertebrates, in particular with bivalves and sea urchins, have been used for decades in the toxicity evaluation of marine pollutants (Kobayashi, 1995; His et al., 2000). The sea urchin embryo test has also been used to investigate the toxicity of complex matrices as sediment or petroleum using different methods (porewater, elutriate, water accommodated fraction, chemical extraction…) (Losso et al., 2009; Beiras et al., 2012; Rial et al., 2013). We report here toxicity tests with the edible sea-urchin *Paracentrotus lividus* (Lamarck, 1816), a large regular sea-urchin widely distributed throughout the Mediterranean Sea and European Atlantic coast with important ecological roles in the functioning, dynamics and structure of benthic assemblages (Hayward and Ryland, 1990; Boudouresque and Verlaque, 2007). Also, several studies have shown the importance of sea-urchin pluteus larvae in the composition and biomass of zooplankton communities, playing a significant role in the pelagic food web (Luis et al., 2005). In some European countries *P. lividus* is also exploited for its highly valued gonads (Boudouresque and Verlaque, 2007).

The objectives of this study were to investigate: 1) the toxicity of seabird guano for sea-urchin embryos; and 2) the joint toxicity of two metals, Cu and Pb, in presence of seabird guano as a source of organic matter.

2. Material and methods

Around 60 plastic collectors were placed at the Islote dels Conills (Parque Nacional Marítimo-Terrestre del Archipiélago de Cabrera, Balearic Islands) in order to get the guano samples. A representative colony of yellow-legged gull (*Larus michahellis*), the most representative seabird in the archipelago, inhabits this small island. Guano samples were collected during 2013-2014 making a unique and homogenized sample. Samples were frozen at -20ºC just after collection until experiments were conducted.

Once in the lab, samples were freeze-dried, ground and preserved in plastic vials waiting for its chemical characterization and experiments.

2.1. Chemical characterization of the original guano samples

Metal determinations were carried out in the guano. Prior to analysis samples were microwave-digested (Milestone 1200 Mega, Milestone Inc., USA) in Teflon bombs using a mixture of HNO_3 and HF according to EPA guideline 3052 (USEPA, 1996).

According to the content level of metal in the samples, analyses were conducted using Electrothermal Atomic Absorption Spectrometry (Varian 220, Varian Inc., USA) equipped with Zeeman background correction for the determination of Cd, Co, Cr, Cu, Ni and Pb. On the other hand, Al, Fe and Zn were measured with Flame-Atomic Absorption Spectrophotometry (Varian SpectrAA 220FS). The accuracy of the analytical procedure was checked using the reference material PACS-2 (marine sediment reference material) and was in good agreement with the certified values. For all analysed metals, the recoveries were in the range of 94% to 103%.

Particulate organic carbon and nitrogen content of the freeze-dried guano sample was determined by high temperature (900ºC) catalytic oxidation in an Elemental Analyser Perkin Elmer 2400 (PerkinElmer Inc., USA).

2.2. Aqueous extracts of guano

Different proportions of guano:water were applied in order to discriminate if the load of guano determined the amount of dissolved substances in the aqueous extract and their toxicity (subsection 2.2.1).

The evaluation of the joint toxicity of Cu, Pb and guano required an experimental design adequate to describe the interactions of metals and the effect of the organic matter released from guano on their toxicity (subsection 2.2.2).

2.2.1. Loading rate test

Loading rate is defined as the ratio of guano to water used in the preparation (OECD, 2000). Aqueous extracts of guano were prepared at two loadings by adding 0.231 and 0.976 g of guano to 500 mL of artificial seawater (ASW) (0.462 and 1.952 g L^{-1} respectively). Subsequently, extracts were exposed to an ultrasonic bath for 30 minutes, to rotatory mixing for 30 min and finally to overnight decantation. The aqueous extract was filtered through a 0.45 μm polyethersulfone filter (Pall corp., USA) in order to remove particulate material and was diluted with ASW (0, 20, 40, 60, 80, 100, 200, 400, 600, 800 and 1000 mL L^{-1}). Diluted samples were used concurrently for chemical analyses and for toxicity tests.

2.2.2. Mixtures of Cu and Pb at different concentrations of guano

Aqueous extracts of freeze-dried guano were performed in triplicate by adding 0.181 g to 500 mL of ASW using the procedure mentioned above. The aqueous extracts were siphoned, filtered through a 0.45 μm polyethersulfone filter and diluted with ASW to final concentrations of 0 mL L^{-1}, 40 mL L^{-1} (0.015 g/L) and 200 mL L^{-1} (0.073 g L^{-1}) in 2 L clean acid plastic bottles.

20 mL of each diluted extracts (0, 40 and 200 mL L^{-1}) and 100 μL of a stock solution of Cu and/or Pb were added to 25 mL plastic vials. The resultant nominal concentrations were 0, 8, 12, 16, 20, 24, 30, 38, 50 and 100 μg L^{-1} for Cu and 12.5, 25, 50, 100, 130, 175, 250, 500 and 1000 μg L^{-1} for Pb. Mixtures corresponding to 4x4 combinations of Cu (12, 16, 24 and 38 μg L^{-1}) and Pb (25, 50, 130 and 250 μg L^{-1}) were also performed. Solutions were kept for 1 h in orbital shaking at 150 rpm to allow equilibration of the complexation reaction.

2.2.3. Chemical characterization of the elutriates and metal solutions

Samples for the analysis of inorganic nutrients were collected in acid-cleaned 50-mL polyethylene bottles; they were frozen at -20ºC until determination using standard segmented flow analysis with colorimetric detection in an Alliance Futura analyser (AMS S.p.A, Italy). The precisions were ±0.02 μM for nitrite and phosphate, ±0.1 μM for nitrate and ±0.05 μM for ammonium and silicate.

For the analysis of dissolved organic carbon (DOC), samples were taken in acid-cleaned 20-mL amber glass flasks with Teflon caps. After acidification with H_3PO_4 to pH <2, the flasks were capped and stored in the dark at 4 ºC until analysis. DOC was measured by high temperature (680 ºC) catalytic oxidation with a Shimadzu TOC-V organic carbon analyser (Shimadzu corp., Japan). The precision of the method was ± 1 μM.

Dissolved trace metal analyses were conducted for elutriates and selected solutions of Cu and Pb by means of stripping voltammetry using a Metrohm797 VA computrace equipped with a hanging mercury drop electrode as the working electrode, Ag/AgCl as the reference electrode, and a Pt wire as the counter-electrode. Prior to determination, samples were UV-digested for 1 h using an UV-Digestor equipped with a high-pressure mercury lamp of 200 W (Achterberg and van den Berg, 1994). The simultaneous determination of Cd, Cu and Pb in the dissolved phase was carried out using the method of standard additions by differential pulse anodic stripping voltammetry (DPASV) (Gardiner and Stiff, 1975) while the simultaneous determination of Co and Ni was performed by Adsorptive Cathodic Stripping Voltammetry (ACSV) (Santos-Echeandia, 2011). The solution was deaerated by purging (5 min) with nitrogen. Voltammetric parameters for the DPASV method were: deposition 300–900 s at −1.1 V whilst stirring; 10 s quiescence at −1.1 V; potential scan using the differential pulse modulation: pulse amplitude of 50 mV, a pulse duration of 40 ms, a pulse frequency of 5 s^{-1} and a scan rate of 20 mV s^{-1}, from −1.1 to 0 V. In the case of the ACSV method, voltammetric parameter were: deposition 30–120 s at −0.35 V whilst stirring; 10 s quiescence at −0.05 V; potential scan using the differential pulse modulation: pulse amplitude of 50 mV, a pulse duration of 40 ms, a pulse frequency of 5 s^{-1} and a scan rate of 20 mV s^{-1}, from −0.05 V to −1.2 V.

The accuracy of the analytical procedure was assessed by the analysis of two different certified reference materials (CASS-4 and SLEW-3), obtaining good agreement with the certified concentrations. Recoveries for all the elements were between 96-102%.

2.3. Sea urchin embryo test

The sea urchin embryo test was performed in accordance with the method of Saco-Álvarez et al.(2010). Gametes of *Paracentrotus lividus* were obtained by dissection of two adult sea urchins and their maturity (ovum sphericity and sperm mobility) checked with a microscope. The ova were transferred to a 100 mL graduated cylinder containing ASW (5-10 ova μL^{-1}). Then a few drops of sperm (30-100 μL), taken from the male gonad, were added, and the mixture was shaken gently to facilitate fertilisation. The fertilisation rate was determined in a Sedgewick-Rafter counting chamber in quadruplicate (n=100), as the proportion of eggs with a fertilisation membrane (control fertilisation success was always > 97%). Within 30 minutes, the fertilised eggs were transferred to vials with 4 mL of ASW containing the experimental solutions. Each vial received 40 eggs per mL and each treatment was performed in quadruplicate.

Eggs were incubated in the dark at 20 ºC for 48 hours, until larvae reached the four-arm pluteus stage. After the incubation period larvae were fixed and preserved by adding a few drops of 40% formalin. In each vial the maximum length of 35 individuals was measured using an inverted microscope and Leica QWIN image analysis software, version 3.4.0 (Leica Microsystems, Germany). The inhibition of growth in length was quantified as:

$$R_i = 1 - \frac{\Delta L_i}{\Delta L_0} \tag{1}$$

where ΔL_0 and ΔL_i are the mean length increases in control and in the i$^{\mathrm{th}}$ dose, respectively.

2.4. Mathematical models

The cumulative function of the Weibull distribution was used as a dose-response model (denoted by ${}^{m}W$):

$$R = K\left\{1-\exp\left[-\ln 2\left(\frac{C}{m}\right)^{a}\right]\right\}\text{; or briefly: } R = {}^{m}W\left(C; K, m, a\right) \tag{2}$$

where R is the response (with K as maximum value), C is the concentration, m is the dose corresponding to the semi-maximum response and a is a shape parameter related to the maximum slope of the response.

Equation 2 can be re-parameterized to estimate the slope at the median abscissa of Weibull density function:

$$R = K\left\{1-\exp\left[-\ln 2\left(\frac{C}{Ka\ln 2 / 2v_{med}}\right)^{a}\right]\right\} \tag{3}$$

A four-parameter model can also be used for those data sets in which a response higher than 0 is observed for the control (R_c):

$$R = R_c + K\left\{1 - \exp\left[\ln 2\left(\frac{C}{m}\right)^a\right]\right\} \tag{4}$$

To directly obtain the confidence intervals of the EC_{50}, equation 4 was re-parameterized to make explicit the corresponding dose:

$$R = R_c + K\left\{1 - \exp\left[\ln\left(1 - \frac{0.5 - R_c}{K}\right)\left(\frac{C}{EC_{50}}\right)^a\right]\right\} \tag{5}$$

2.4.1. CA hypothesis

The inverse of equation 2 is required to apply the Concentration Addition (CA) model:

$$C_i = f_i^{-1}(R) \,\text{or}\, C_i = m_i\left[\frac{\ln\left(1 - \frac{R}{K_i}\right)}{-\ln 2}\right]^{1/a_i} \tag{6}$$

where C_i is the concentration of the effector *i* that produces the response *R* and $f^{-1}(R)$ is the inverse function of the Weibull model. The CA model has to be solved by iteration to find the parameters of the single equations and the values of the predicted response (*R*) which minimizes the residual sum of squares and satisfy the following condition (Berenbaum, 1985):

$$\sum_{i=1}^{n}\frac{c_i}{f_i^{-1}(R)}=1;\ \text{or more commonly}\ \sum_{i=1}^{n}\frac{c_i}{C_i}=1 \tag{7}$$

where, c_i is the concentration of chemical i in the mixture and C_i is given by expression (6).

A unique maximum response (K) is assumed in the CA model (Jonker et al., 2005). The equation for three toxic agents is as follows:

$$\frac{c_1}{f_1^{-1}(R)}+\frac{c_2}{f_2^{-1}(R)}+\frac{c_3}{f_3^{-1}(R)}= \tag{8}$$

$$\frac{c_1}{m_1\left[\dfrac{\ln\left(1-\dfrac{R}{K}\right)}{-\ln 2}\right]^{1/a_1}}+\frac{c_2}{m_2\left[\dfrac{\ln\left(1-\dfrac{R}{K}\right)}{-\ln 2}\right]^{1/a_2}}+\frac{c_3}{m_3\left[\dfrac{\ln\left(1-\dfrac{R}{K}\right)}{-\ln 2}\right]^{1/a_3}}=1$$

A simplified version of the CA model (hereinafter called CA_M), which makes possible to obtain directly the function parameters by non linear regression, reported by Murado and Prieto (2013), was adapted here to three effectors:

$$R=W\left[\left(C_1+u_2C_2+u_3C_3\right);K,m,a\right] \tag{9}$$

where C_1, C_2 and C_3 are the concentrations of the effector 1, 2 and 3, respectively, and u_2 and u_3 are the factors that show the relative toxic potency of effectors 2 and 3 regarding to effector 1. The same maximum effect (K) and shape parameter (a) is assumed for all effectors.

Murado and Prieto (2013) have also proposed a modification of the CA model to describe synergism or antagonism. In that model it is assumed that a toxic agent can increase or decrease the effective dose of other effector. That model was adapted here to three effectors and to a particular condition: an unidirectional interaction of the toxic agent 3 (guano as source of organic matter) over the effective concentrations of agents 1 (Cu) and 2 (Pb) (hereinafter called CA_SA):

$$R = W\left[\left(C_1\pi_{C1} + u_2C_2\pi_{C2} + u_3C_3\right); K, m, a\right] \qquad (10)$$

$$\pi_{C1} = \left(1 + b_{c1}C_3\right)/\left(1 + c_{c1}C_3\right);\ \pi_{C2} = \left(1 + b_{c2}C_3\right)/\left(1 + c_{c2}C_3\right)$$

2.4.2. IA hypothesis

The independent action (IA) model for three agents is formulated as follows (Bliss, 1939):

$$R(C_{\text{mix}}) = 1 - \left[1 - R(C_1)\right]\left[1 - R(C_2)\right]\left[1 - R(C_3)\right] = \qquad (11)$$

$$= 1 - \left[1 - W(C_1; K_1, m_1, a_1)\right]\left[1 - W(C_2; K_2, m_2, a_2)\right]\left[1 - W(C_3; K_3, m_3, a_3)\right]$$

where $R(C_{\text{mix}})$ is the response corresponding to the mixture, and $R(C_1)$, $R(C_2)$and $R(C_3)$ are the response to the agents 1, 2 and 3.

Murado and Prieto (2013) have also proposed a modification of the IA model to describe synergy or antagonism, in which it is assumed that the concentration of each toxic agent may alter the parameters value of the toxicity model of the other agents considered. This model is adapted here to three effectors and an unidirectional interaction of an agent over the other two effectors (IA_SA):

$$R = 1 - \left[1 - W\left(C_1; K_1\pi_{K1}, m_1\pi_{m1}, a_1\pi_{a1}\right)\right]\left[1 - W\left(C_2; K_2\pi_{K2}, m_2\pi_{m2}, a_2\pi_{a2}\right)\right] \left[1 - W\left(C_3; K_3, m_3, a_3\right)\right] \qquad (12)$$

$$\pi_{\theta 1} = \left(1 + b_{\theta 1}C_3\right)\big/\left(1 + c_{\theta 1}C_3\right);\ \pi_{\theta 2} = \left(1 + b_{\theta 2}C_3\right)\big/\left(1 + c_{\theta 2}C_3\right);\ \theta = K, m, a$$

2.5. Statistical analyses

Fitting procedures and parametric estimations from the experimental results were performed by minimisation of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro '*Solver*' of the *Microsoft Excel* spreadsheet. Confidence intervals from the parametric estimations (Student's *t* test) were determined with the freely available '*SolverAid*' macro and the consistence of mathematical models assessed by a Fisher's *F* test.

The Akaike´s information criterion (AIC) was used for comparing models. AIC statistics summarize goodness-of-fit as residual sum of squares (*RSS*) against the number of parameters (*p*) for the same data set (*n*) with the aim of avoiding over-fitting. AIC can be defined as (Motulsky and Christopoulos, 2003):

$$AIC = n\ln\left(\frac{RSS}{n}\right) + 2\left(p+1\right) + \left[\frac{2\left(p+1\right)\left(p+2\right)}{n-p-2}\right] \qquad (13)$$

The model with the lowest AIC is the one with the highest likelihood of being correct.

3. Results

3.1. Chemical characterization of the original guano samples

The reason for analysing the chemical composition of guano was to check for the potential toxicity of some of its elements. Trace metal concentrations in guano were: 12.7±0.20 mg g^{-1} for Al, 0.55±0.05 µg g^{-1} for Cd, 3.62±0.09 µg g^{-1} for Co, 19.1±1.51 µg g^{-1} for Cr, 21.6±0.6 µg g^{-1} for Cu, 5.21±0.39 µg g^{-1} for Fe, 6.29±0.62 µg g^{-1} for Ni, 8.30±0.01 µg g^{-1} for Pb and 154±10 µg g^{-1} for Zn.

Regarding carbon and nitrogen composition of guano, 26.4±0.2% (w/w) was C and 7.26±0.12% was N.

3.2. Composition of aqueous extracts of guano

In order to check the solubility of trace metals, these elements were analysed in the elutriates. Results are shown in Table 1 together with nutrients and dissolved organic carbon concentrations.

3.3. Loading rate test

The inhibition of sea urchin larval growth exhibited a similar pattern for the extracts of guano obtained at different loadings (Figure 1, left) and the parameters EC_{50}, EC_{10} and a of the Weibull model showed overlapping confidence intervals (Table 2). An F-test was used to assess whether a single model (null hypothesis) or two separate curves for each load described better the data (alternative hypothesis) and it was accepted the null hypothesis (p=0.093).

The dissolved organic carbon (Figure 1, right) may contribute to the observed toxicity for the extract of guano (Figure 1, left). How6ever, the parameter m (g L^{-1}) for the inhibitory response (0.37±0.03) (Figure 1, left) was less than that of DOC (0.59±0.07) (Figure 1, right), and the relative slope (v_m/K) was markedly greater for toxicity (1.3) than for DOC (0.8). So other factors may have contributed to the toxicity of guano.

The drop in pH value for undiluted extracts of low (8.02) or high load (7.52), with respect to ASW (8.11), does not serve to explain an increase in toxicity due to this parameter (Saco-Álvarez et al., 2010). The NH_3 concentrations calculated for dilutions of the extracts in the treatment of low (1.0-39.4 μg L^{-1}) or high load (0.3-31.7 μg L^{-1}) did not explain the observed toxicity either (Saco-Álvarez et al., 2010).

The concentrations of metals measured in undiluted extracts of low and high loads were not relevant from a toxicological point of view except for Cu (Table 1). For Cu, 0.2 toxic units were calculated in the undiluted extracts of both low and high loads.

3.4. Mixtures of Cu and Pb at different concentrations of guano

A good agreement was found between nominal and measured concentrations (in brackets) for Cu 12 (11.8±0.2) μg L^{-1}, 24 (23.8±0.1) μg L^{-1} and 50 (49.7±0.1) μg L^{-1} and Pb 25 (24.4±0.4) μg L^{-1}, 130 (129.6±0.3) μg L^{-1} and 500 (499.2±0.1) μg L^{-1} in ASW.

Cu toxicity in ASW (EC_{50} = 33.09 μg L^{-1} or 0.52 μM) was greater than that of Pb (355.87 μg L^{-1} or 1.72 μM) (Table 3). The slope of the curve (v_m) was also significantly higher for Cu (0.0192 L μg^{-1} or 1.22 μM^{-1}) than for Pb (0.0010 L μg^{-1} or 0.21 μM^{-1}) (Table 3).

The extracts of guano presented moderate toxicity in the absence of added Cu or Pb, showing inhibitory responses (R) of 0.18 and 0.30 for the low and high concentration respectively (Figure 2). Metal concentrations measured in the extracts of guano did not serve to explain the observed toxicity (Table 1), since toxic units could only be calculated for Cu and the values were quite low (0.1 and 0.2 for low and high concentration respectively).

Cu added to extracts of guano showed less toxicity than Cu dissolved in ASW. Figure 2 shows how guano inhibits sea-urchin growth at low concentrations of Cu and reduces Cu toxicity at higher concentrations. A reduction of Cu toxicity was observed in terms of both EC_{50} and slope (v_m) for the concentrated extract of guano and a lower value of v_m was also found for the diluted extract (Table 3 and Figure 2). For Pb, unlike Cu, significant differences were only found in terms of v_m (Table 3 and Figure 2). Lower EC_{50} values for Pb were found in guano treatments than in ASW, though these values did not differ significantly (Table 3). However, the comparison of the EC_{50} values is not straightforward, due to the intrinsic toxicity of guano in the absence of added metals and the impossibility of separating its inhibitory effects of the considered metal (Figure 2).

The model of Independent Action (IA) for two effectors (AIC = -286.82, adj. R^2 = 0.978) described better the joint toxicity of Cu and Pb in ASW than Concentration

Addition (CA) (AIC = -275.9, adj. R^2 = 0.972) or Concentration Addition Modified (CA_M) (AIC = -249.34, adj. R^2 = 0.948).

The joint inhibition of Cu, Pb and extract of guano was well described by the model IA_M_SA (Figure 3). The lowest value of the Akaike Information Criterion and the maximum value of adjusted R^2 were obtained for this model (Table 4). The parameters of the model were significant for Cu and Pb, but K for guano was not significant (Table 4). The model IA_M_SA implies accepting that m for Cu or Pb increases with the concentration of guano according to the expression (12), and the following values of b_{m1} = 11.580 (interaction parameter of guano on m of Cu) and b_{m2} = 5.974 (interaction parameter on m of Pb) were obtained (Table 4). The value of b_{m1} was approximately double that of b_{m2}, indicating a greater interaction of guano for Cu than for Pb. A similar description and identical values of b_{m1} and b_{m2} was obtained using model 3 as the core of model 12.

4. Discussion

Trace metal concentrations in guano were low. In fact, our values were lower than those determined by Otero (1998) in faeces collected from fishing ports of Galicia for the same species and which could be related to the low concentrations of heavy metals – except mercury– measured in sediments from archipelago of Cabrera (Tovar-Sánchez et al., 2011). Concentrations of Cd, Cr, Cu, Ni and Pb were lower than the Effects Range Low (ERL), indicative of concentrations below which adverse effects in marine sediments rarely occur, although Zn showed a slightly higher value (154 µg g^{-1}) than of this guideline (150 µg g^{-1}) (Long et al., 1995).

Guano showed low toxicity to sea urchin embryos and its toxicity seems to be related in part to the dissolved organic matter released from the guano. The two treatments assayed in the loading rate test were homogeneous in terms of growth inhibition (Figure 1, left) and the dissolution of organic carbon in seawater showed a pattern which is apparently independent from the load of guano used (Figure 1, right). The toxic contribution of NH_3 and metals in the extracts of guano was low, but it cannot be ruled out that other unmeasured toxicants may have contributed to the growth inhibition observed in the sea urchin test. *NOEC* and EC_{10} for unionized ammonia were 40 and 68.4 μg L^{-1} according to Saco-Álvarez et al. (2010) and the unionized ammonia measured in the extracts were 0.3-39.4 μg L^{-1} for the loading rate test and 3.8-13.3 μg L^{-1} for the mixture toxicity test; so unionized ammonia did not explain the observed toxicity. A slight drop in pH was observed in the loading rate test for undiluted extracts of guano (pH: 7.52-8.02) compared to control (8.11), which may be due to the acids contained in guano (mainly uric acid). However, these values are well above the *NOEC* (pH = 7) for the sea urchin test (Saco-Álvarez et al., 2010). The concentrations of DOC measured in the mixture toxicity test were 149 and 375 µM-C for low (0.015 g L^{-1}) and high load (0.073 g L^{-1}) of guano respectively. These values were comparatively greater than those obtained in the loading rate test (Table 1 and Figure 1). When the ratio DOC/load of guano was calculated, a higher value was shown for the mixture toxicity test (5163-10297 µmol-C g_{guano}^{-1}) than for the loading rate test (3167-5367 µmol-C g_{guano}^{-1}), which might be related to the use of a lyophilized powder of guano instead of dry guano in the former. The toxicity of guano in the mixture toxicity test (lyophilized) was also considerably higher than that of the loading rate test (dry), but it was not explained by the increase of solubilization. It was not possible to achieve a convincing interpretation for the difference in the toxicity values obtained in both tests, although two explanations might be given: a) an increase of colloidal concentration in the mixture toxicity test associated to a higher toxicity, or b) different ratio of Dissolved Organic Carbon/Dissolved Organic Matter for the two tests.

The EC_{50} of Cu (33.1 μg L^{-1}) was significantly higher than that of Pb (355.9 μg L^{-1}), and is indicative of different mechanisms of toxicity or affinity for specific receptors. These values are similar to those found by Lorenzo et al. (2006) (35.6 μg L^{-1}) and Sánchez-Marín et al. (2010) (22.2 μg L^{-1}) for Cu, and by Sánchez-Marín et al. (2010) (406.1 μg L^{-1}) for Pb. The difference in slope (v_m) between Cu (0.0192 L μg^{-1}) and Pb (0.0010 L μg^{-1}) is even higher than for EC_{50} values. It is well known that metals can react with enzymes, cell membranes and specific cellular components. Tellis et al. (2014a, b) have pointed out that both Cu and Pb impair the function of the ionic regulation (especially Ca homeostasis), interact with sulfhydryl groups of enzymes and generate free radicals in sea-urchin larvae. However, Radenac et al. (2001) showed that although Pb was accumulated more than Cu in *P. lividus* larvae, its toxicity was much lower than that of Cu, which suggested different mechanisms of toxicity for both metals.

The joint toxicity of Cu and Pb is determined by their specific mechanisms of toxicity. The observed response for binary mixtures of Cu and Pb in ASW was better described by IA (11) than by CA (8) or CA_M (9) models. Either of the reference models -CA or IA- allow to predict the joint toxicity of a mixture but with different assumptions: CA assumes that the components of the mixture present the same or similar mode of action while diverse or "dissimilar" modes of action are expected with the IA model (Kortenkamp and Altenburger, 2010). Thus, dissimilar modes of action are presumed for Cu and Pb according to our results. It should be noted that CA described significantly better the observed response than CA_M; this latter model is a simplification of the CA that assumes equal curve shape but different toxic potency between Cu and Pb, which could not be accepted in this case. Xu et al. (2011) also evaluated the joint toxicity of Cu and Pb for embryos of the sea-urchin *Strongylocentyotus intermedius* and reported a weak antagonistic effect for these mixtures on the basis of the CA model. Some authors have pointed out the need to predict the toxicity of mixtures of metals in a more accurate way (e.g. (Meyer et al., 2015), but it would be necessary to emphasize that the interpretation of the results is conditioned by the experimental design previously performed. The experimental design used here allowed to perform an adequate description of the results and choose unambiguously between the reference models proposed.

The model used to describe the joint toxicity of Cu, Pb and guano (12) shows the particularity of including the guano as a source of organic matter and as an additional toxicant. The inhibitory response of guano in absence of Cu and Pb at low (0.18) and high (0.30) concentration can be seen in Figure 2. Hence, a marked toxic contribution of guano in the treatments of guano and metal cannot be discarded at a response level of 0.5, which prevents a direct comparison of EC_{50} values for the three treatments shown in Figure 2. In this figure it can be seen clearly how guano inhibits sea-urchin larval growth at low concentrations of Cu and reduces Cu toxicity at higher concentrations. It is known that dissolved organic matter from different origins may cause dose-dependent inhibition effects in the sea-urchin embryo development (Sanchez-Marin et al., 2010; Nadella et al., 2013), which justifies its inclusion in the model of joint toxicity. This allowed us to infer the inhibitory effects of guano, metals and their interaction.

The interaction described by model (12) involves an increase in *m* (EC_{50}) values, for both Cu and Pb, with increasing concentrations of guano (Figure 3). The magnitude of the interaction is greater for Cu (b_{m1} = 11.580) than for Pb (b_{m2} = 5.974), which indicates a higher affinity of Cu than Pb for dissolved organic matter from guano. A decrease in Cu toxicity to *P. lividus* larvae in the presence of organic matter from different origins, including humic and fulvic acids, has been previously reported (Lorenzo et al., 2002, 2006; Sanchez-Marin et al., 2010). However, the interaction of dissolved organic matter with Pb is less clear: increased toxicity with humic and fulvic acids (Sanchez-Marin et al., 2010; Sánchez-Marín and Beiras, 2012) and different effects depending on the type of organic matter tested (Sanchez-Marin et al., 2010). Sánchez-Marín et al. (2010) also reported that the complexing capacity of dissolved organic matter from different origins was lower in all cases for Pb than for Cu, which is consistent with the values found here for b_{m1} and b_{m2}.

Dissolved organic matter from guano differs from other sources, such as humic or fulvic acids, since it presents a lower heterogeneity of groups that can act as ligands of metal cations. Birds excrete nitrogenous waste primarily as uric acid and some of this uric acid is degraded by bacteria; although the proportions may vary depending on the species and diet (Lindeboom, 1984; Fugler, 1985). Proteins are also excreted by seabirds in lower proportions (Szpak et al., 2012). The major form of excreted phosphorus in guano is phosphate but only a reduced proportion is soluble (Smith and Johnson, 1995; Otero et al., 2015). Therefore, major chemical compounds present in the extracts of guano are expected to be uric acid, proteins, ammonia and phosphate (Smith and Johnson, 1995), so it is likely that the drop in toxicity observed for Cu and Pb may be due to complexation or precipitation by these species. Hydrogen urate is the predominant form of uric acid at pH = 8 and it has complexing capacity on Cu or Pb, forming a non-electrolitic complex due to charge neutralization of the cation (Cu^{+2} or Pb^{+2}) (Wilcox et al., 1972; Tak et al., 1981; Moawad, 2002). The solubility product constants (K_{sp}) of the hydrogen urates were 5.8×10^{-5} for Cu (Moawad, 2002) and 1.2×10^{-14} for Pb (Tak et al., 1981). Hydrogen phosphate is the predominant phosphate species at pH = 8 and shows K_{sp} values at 25 ºC of 4.5×10^{-7} for Cu and 5.3×10^{-12} for Pb (Markich et al., 2001).. Therefore, according to these values, precipitation of Pb is possible by either hydrogen phosphate or hydrogen urate but is unlikely for Cu. Therefore, the reduction of the Cu toxicity is possibly due to the complexing capacity of other dissolved species in the extract of guano such as protein materials given the recognised capability of amino acids to bind with Cu and Pb (Sovago et al., 1993).

The guano is an important source of marine-derived nutrients in seabird breeding islands, but shows little relevance in other coastal areas regarding alternative sources of nutrients (Bedard et al., 1980; Bosman et al., 1986). Wootton (1991) found that guano had a positive influence on 4 of 18 taxonomic groups from intertidal communities on cliffs and led to a reduction of biomass in some groups which could have been caused by ammonia from guano. The results of this study do not point in that direction as the observed toxicity appeared to be related to the dissolved organic matter or other unmeasured toxic agents.

5. Conclusions

The toxicity of guano to sea urchin embryos has been tested at environmentally relevant concentrations. Low toxicity of guano was found which was apparently related to the dissolved organic matter and independent from the load of guano used. The compounds dissolved from guano diminished both Cu and Pb toxicity. The response surfaces obtained for mixtures of Cu and Pb in artificial seawater, low and high load of guano were better described by Independent Action model adapted to describe antagonism than by the other proposed models. The magnitude of the interaction of guano was greater for Cu than for Pb, which indicated a higher affinity of the former for the dissolved organic matter from guano than the latter.

Acknowledgements

This work was partly supported by the Spanish Ministry of Agriculture, Food and Environment Grant PN384/2011. Diego Rial was funded by a postdoctoral contract from the Xunta de Galicia (ED481B 2014/098-0 Plan I2C), Spain (Plan I2C, 2014). The work of A.J. was supported by a Ramón y Cajal grant from the Spanish Ministry of Economy and Competitiveness. The CSIC, under the program JAE (Junta para la Ampliación de Estudios) co-funded by the Fondo Social Europeo (FSE), is greatly acknowledge for the post-doctoral fellowships to J. Santos-Echeandía.

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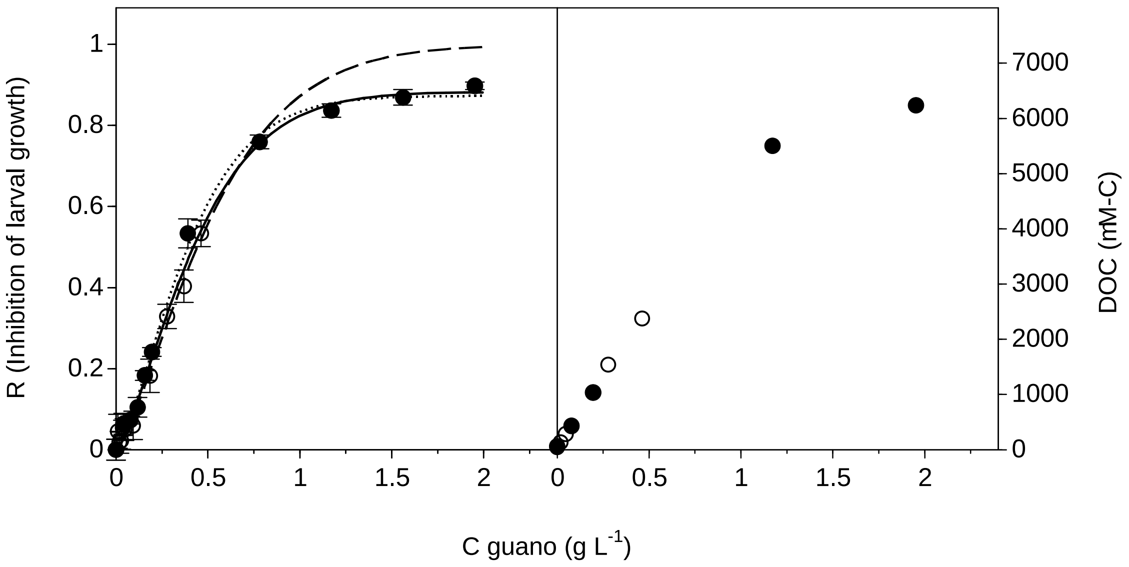
Figure captions

Figure 1. Inhibition of sea urchin larval growth (R) by aqueous extracts of guano obtained at different loads (left) and dissolved organic carbon measured on the extracts (right). The symbols represent the loads of 0.462 g L^{-1} (○) and 1.952 g L^{-1} (•). The lines represent the predictions of model 2 for the loads of 0.462 g L^{-1} (--), 1.952 g L^{-1} (···) and all the results obtained (—). C$_{guano}$, concentration of guano in g/L. Error bars are standard errors.

Figure 2. Inhibition of sea urchin larval growth (R) by Cu (left) and Pb (right) in Artificial Sea Water (ASW) (—○—) and aqueous extracts of guano at low (--◇--) and high concentration (···□···). The lines represent prediction of models 2 and 4 obtained by individual fitting and the symbols represent the observed values. Concentrations of Cu and Pb in μg L^{-1}. Error bars are standard errors.

Figure 3. Inhibition of sea urchin larval growth (R) by joint action of Cu and Pb in Artificial Sea Water (ASW) (up), aqueous extract of guano at low concentration (middle) and aqueous extract of guano at high concentration (down). The symbols represent the observed values and the response surface the prediction of model IA_SA (12). Concentrations of Cu and Pb in μg L^{-1}.

Figure 1


R (Inhibition of larval growth)
0
0.2
0.4
0.6
0.8
1
0
0.5
1
1.5
2
0
0.5
1
1.5
2
0
1000
2000
3000
4000
5000
6000
7000
DOC (mM-C)
C guano (g L^{-1})

756 **Figure 2**

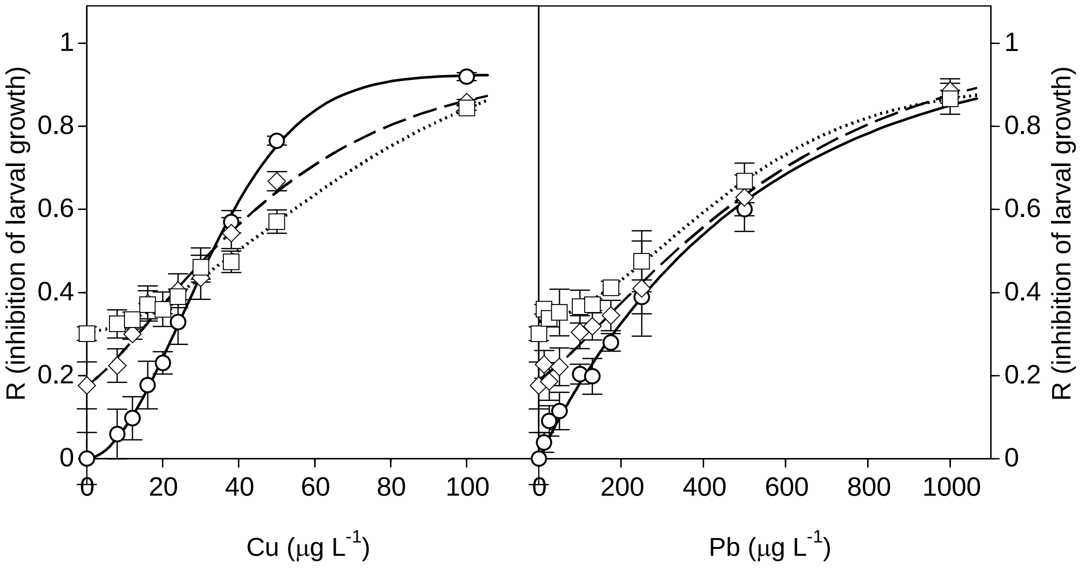


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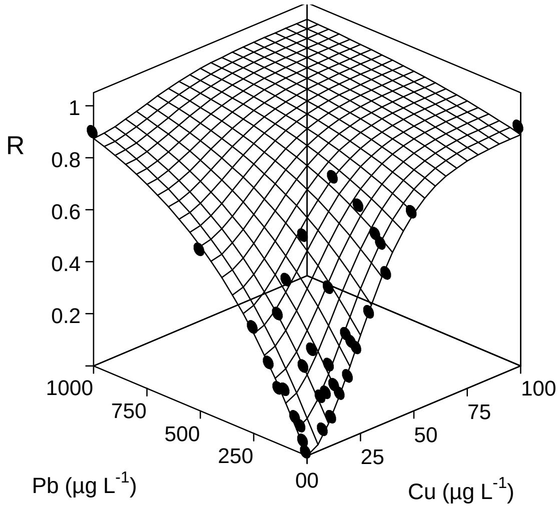
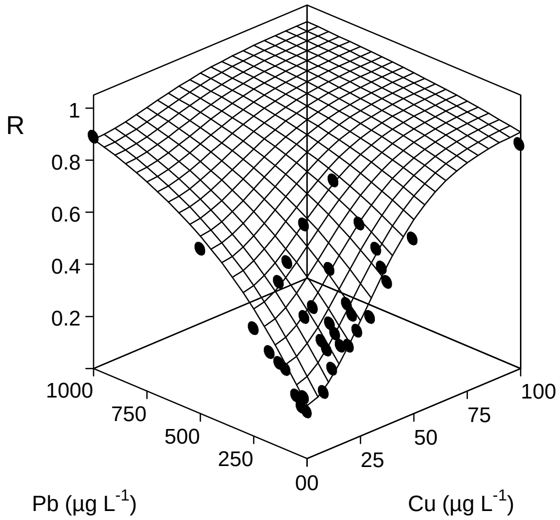
Figure 3

Artificial Sea Water



Low concentration of guano



High concentration of guano

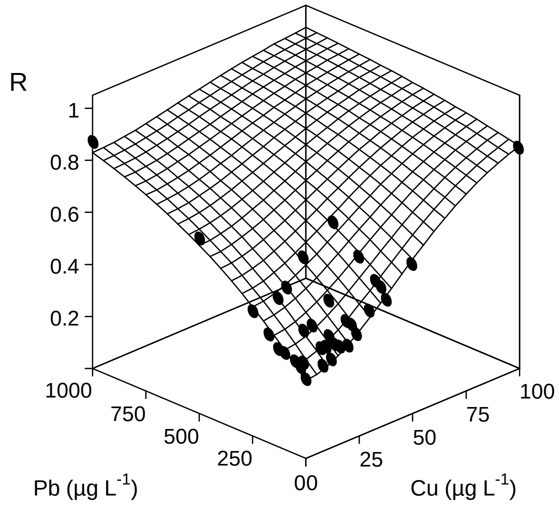


Table captions

Table 1. Concentrations of dissolved organic carbon, nutrients and metals measured in the aqueous extracts obtained in the two tests performed.

Table 2. Summary of the parameters obtained by fitting model 2 to the toxicity results of the loading rate test. ns, not significant.

Table 3. Pb and Cu toxicity in: Artificial Sea Water (ASW), low concentration of extract of guano (Low guano) and high concentration of extract of guano (High guano). Summary of the parameters obtained by individual fitting of models 2, 3, 4 and 5 to Cu or Pb inhibition observed in the treatments indicated above.

Table 4. Parameters and goodness of fit of Concentration Addition (CA), Concentration Addition Simplified (CA_M), Concentration Addition Simplified adapted to describe Synergism or Antagonism (CA_SA), Independent Action (IA) and Independent Action Synergism or Antagonism (IA_SA). adj. R^2, adjusted R^2; AIC, Akaike Information Criterion; ns, not significant.

Table 1.

Experiment	Treatment	C_{guano} (g L^{-1})	DOC (µM-C)	NO_3^- (mM)	NO_2^- (mM)	NH_4^+ (mM)	PO_4^{3-} (mM)	SiO_2 (mM)	Cd (nM)	Cu (nM)	Pb (nM)	Ni (nM)	Co (nM)
Loading rate	Control	0	55	1.15	0.05	1.77	0.18	1.14	0.5	7.7	1.1	15.82	0.0655
	Low loading rate	0.01848	137	1.26	0.10	5.69	0.81	1.26					
		0.0462	285	1.20	0.18	11.40	1.99	1.05					
		0.2772	1543	0.84	0.82	48.66	10.60	1.64					
		0.462	2376	0.68	1.33	66.34	17.26	2.10	1.3	99.1	1.3	29.42	1.51
	High loading rate	0.07808	433	0.78	0.21	30.84	6.07	0.58					
		0.1952	1036	0.68	0.30	60.33	15.34	0.92					
		1.1712	5504		1.57	173.24	85.54	5.86					
		1.952	6236		2.58	168.70	102.60	10.75	4.0	104.9	2.9	31.66	2.32
Mixtures	Control	0	15	10.69	0.08	1.82	0.14	3.65	0.5	5.9	1.3	9.28	0.038
of Cu and Pb	Diluted aqueous extract of guano	0.015	149	2.72	0.16	8.46	1.73	4.04	0.6	23.9	1.8	10.16	0.115
	Concentrated aqueous extract of guano	0.073	375	0.29	0.58	31.24	8.39	4.33	0.8	79.6	5.4	11.59	0.295

Table 2.

Parameters	Low loading rate (0.462 g L^{-1})	High loading rate (1.952 g L^{-1})	Joint curve
K	1 ns	0.87±0.03	0.88±0.04
m (g L^{-1})		0.33±0.03	0.37±0.03
EC_{50} (g L^{-1})	0.44±0.03	0.39±0.03	0.42±0.03
EC_{10} (g L^{-1})	0.11±0.02	0.09±0.02	0.10±0.01
a	1.33±0.23	1.37±0.18	1.35±0.15

Table 3

Metal	Parameter	Treatment		
		ASW	Low Guano	High Guano
Cu	EC_{50} (μg L^{-1})	33.09±0.73	32.86±3.54	39.69±3.16
	a	1.87±0.12	1.29±0.55	1.63±0.70
	v_m (L μg^{-1})	0.0192±0.0013	0.0086±0.0037	0.0063±0.0025
Pb	EC_{50} μg L^{-1})	355.87±181.76	331.75±40.15	285.03±34.63
	a	0.98±0.23	1.24±0.44	1.69±0.54
	v_m (L μg^{-1})	0.0010±0.0004	0.0008±0.0003	0.0008±0.0002

Table 4

		Concentration Addition				Independent Action	
		Null interaction CA	Simplified CA_M	Antagonism CA_SA		Null interaction IA	Antagonism IA_SA
Cu	K	0.907±0.107	1.000±0.227	1.000±0.191	K_1	0.875±0.102	0.890±0.065
	m_1 (µg L^{-1})	32.2±4.8	36.9±13.8	31.6±9.4	m_1 (µg/L)	32.3±4.3	28.4±2.2
	a_1	1.703±0.447	0.931±0.195	1.065±0.195	a_1	1.575±0.272	1.807±0.199
	c_1 (L g^{-1})			12.162±7.570	b_{m1} (L/g)		11.580±3.162
Pb					K_2	1.000±0.376	1.000±0.220
	m_2 (µg L^{-1})	315.2±77.4			m_2 (µg/L)	376.6±248.8	352.7±131.1
	a_2	1.198±0.315			a_2	0.958±0.270	1.051±0.193
	u_2		0.106±0.018	0.083±0.015	b_{m2} (L/g)		5.974±3.977
Guano					K_3	0.396 ns	1.000 ns
	m_3 (g L^{-1})	0.7 ns			m_3 (g/L)	0.4 ns	0.8±0.8
	a_3	0.182 ns			a_3	0.044 ns	0.280±0.089
	u_3 (µg g^{-1})		131.863±1.846	175.631±42.103			
	adj. R^2	0.933	0.843	0.880		0.904	0.959
	AIC	-605.7	-544.0	-518.0		-564.5	-653.3