Effect of acute exposure of Hg and Zn on survival of native and invasive *Artemia* from wild populations exposed to different degrees of environmental contamination

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Highlights

1. Native *Artemia* from Cabo de Gata (Spain) was extremely resistant to Hg
2. Native and invasive *Artemia* from Aveiro showed similar Hg tolerance
3. Hg may play a role limiting/delaying invasion in some native populations
4. All studied *Artemia* populations showed similar tolerance to Zn

Abstract

In recent decades, brine shrimps of the genus *Artemia* has suffered a major biodiversity loss in the Mediterranean region due to the introduction of the highly invasive *A. franciscana*. Pollution has been proposed as an important factor limiting this global invasion. Contrary to the general acceptation that pollution tends to favour invasive species, it has been postulated that local adaptation of native *Artemia* to pollution may prevent or delay colonization by the exotic species. To provide insight into this “pollution resistance hypothesis”, we investigated the individual effect of acute toxicity of mercury (Hg) and zinc (Zn) on the survival of six different native and invasive *Artemia* populations from the Iberian Peninsula collected from areas with different levels of Hg- and Zn-pollution. The Hg and Zn 24h-LC50 values for *Artemia* nauplii of the different populations varied between 20 and 70 mg Hg L^{-1}, and between 350 and 450 mg Zn L^{-1}, respectively. Native *Artemia* from Cabo de Gata (SW Spain) showed significantly higher survival at high Hg concentrations than other populations, which may be explained by the longer history of Hg-pollution in that area from mining activities, compared to the other sites. In contrast, differences between populations in response to high Zn levels were weak, and inconsistent with the environmental differences in Zn concentrations. Discussion of the results of this work was done in relation to the “pollution resistance hypothesis” and conclude that Hg pollution may limit the invasion by *A. franciscana* in some study sites for an uncertain length of time.

Key words: metal pollution; biological invasion; pollution resistance hypothesis; *Artemia franciscana*; *Artemia parthenogenetica*

1. Introduction

Biological invasions are a major threat to biodiversity and ecosystem functioning worldwide (Simberloff et al., 2013). Therefore, it is crucial to understand the factors affecting the invasibility of ecosystems (Ruiz et al., 2001) and the attributes allowing native populations to survive invasions. Most studies up to now show that environmental contamination enhance invasions (e.g., Piola and Johnston, 2009; Crooks et al., 2010). However, most of these studies consider scenarios of recent environmental pollution or emerging pollutants (e.g., Varó et al., 2015), and environments where invasive species have succeeded (Soltysiak and Brej, 2014; Guarnieri et al., 2017); little is known about how local adaptation of native species to pollution may limit the establishment of invasive species. In areas with historic pollution (e.g., with prehistoric or ancient mining) native communities have had time to adapt to the presence of pollutants by evolutionary acquisition of chemical tolerance (e.g., Barata et al., 2002; Lopes et al., 2006; Ruggeri et al., 2019), and therefore may be more resistant to the establishment of newly arriving invasive species (Sánchez et al., 2016; Pais-Costa et al., 2019).

The brine shrimp *Artemia* (Branchiopoda, Anostraca), a key taxon in hypersaline ecosystems, is an interesting model system to study interactions between contaminants and invasions. This genus is suffering a major biodiversity decline worldwide (e.g., Amat et al., 2007; Horváth et al., 2018) due to the introduction, since the 1950s, of the North American *Artemia franciscana* for aquaculture purposes (Amat et al., 2005; Muñoz et al., 2014). In Europe, *A. franciscana* was first detected in Portugal in the 1980s (Hontoria et al., 1987) and a decade later in France (Thiery and Robert, 1992). Since then, it has progressively invaded most hypersaline ecosystems of the Mediterranean basin, including those of Spain and Italy (Amat et al., 2005, 2007; Horváth et al., 2018), North Africa (Morocco, Tunisia) (Amat et al., 2005, 2007; Naceur et al., 2010), and has reached the

Middle East (Iran, Egypt, Arab Emirates) (Hajirostamloo and Pourrabbi, 2011; Sheir et al., 2018; Saji et al., 2019). It is also present in Australia, Brazil, India, China and Kenya (Ruebhart et al., 2008; Camara, 2001; Zheng et al., 2004; Krishnakumar and Munuswamy, 2014; Ogello et al., 2014). The establishment of the exotic species in the Mediterranean region has led to a rapid local extinction of native *A. salina* and *A. parthenogenetica*; currently these species are listed as Endangered and Vulnerable respectively, in the Iberian Peninsula (IUCN red list; García-de-Lomas et al., 2017).

In SW Europe, few populations of native *Artemia* persist and most of them are in highly polluted areas. In Portugal, one of the last refuges of native *A. parthenogenetica* is the saltpans complex of Ria de Aveiro (Portugal), highly polluted by mercury (Hg). However, in the same saltpans complex there are *A. franciscana* populations and the reasons for the resistance of the native species to the invasion are unknown. Rodrigues et al. (2012) studied the physicochemical and biological parameters that may explain the distribution of these native and invasive species but found that their environments were rather similar. They then hypothesized (but did not demonstrate) that the presence of pollutants (as Hg) may play a decisive role in the prevention of the invasion. Pinto et al. (2013, 2014) subsequently studied the effects of water temperature, salinity, photoperiod and food supply on the survival and reproduction of these native populations and concluded that their persistence remained an unexplained phenomenon, pointing out again to the potential role of a chemical barrier related to the pollution. This “pollution resistance hypothesis” has been partially supported for contaminants other than Hg, for some populations from the southern Iberian Peninsula (arsenic (As): Sánchez et al., 2016; zinc (Zn): Pais-Costa et al., 2019). However, information is extremely limited and fragmented, and more data are critical to understand the role of pollution in preventing or delaying the colonization of the last native *Artemia* populations by the exotic *A. franciscana* (Pinto et al., 2014).

The aim of the present study was to provide insights into the pollution resistance hypothesis (Rodrigues et al., 2012; Pinto et al., 2013, 2014; Sánchez et al., 2016) by which high levels of pollution may be slowing down or even avoiding the invasion by *A. franciscana*. For that, native and invasive populations of *Artemia* were exposed to acute concentrations of Hg and Zn. Among the studied populations, native and invasive *Artemia* populations collected from the same locations as in Rodrigues et al. (2012) were used to evaluate if potential differences in environmental factors could explain the distribution pattern of both *Artemia* species. This work hypothesis is that native *Artemia* from highly Hg- and Zn-polluted areas would be locally adapted more resistant to the invasion than populations from less polluted areas.

2. Material and Methods

2.1 Study sites

The selected *Artemia* populations were sampled in six different saltpans, located in the Iberian Peninsula: Ria de Aveiro (Troncalhada and Tanoeira saltpans) and Rio Maior in Portugal; and Odiel, Cádiz bay and Cabo de Gata in Spain (Figure 1).

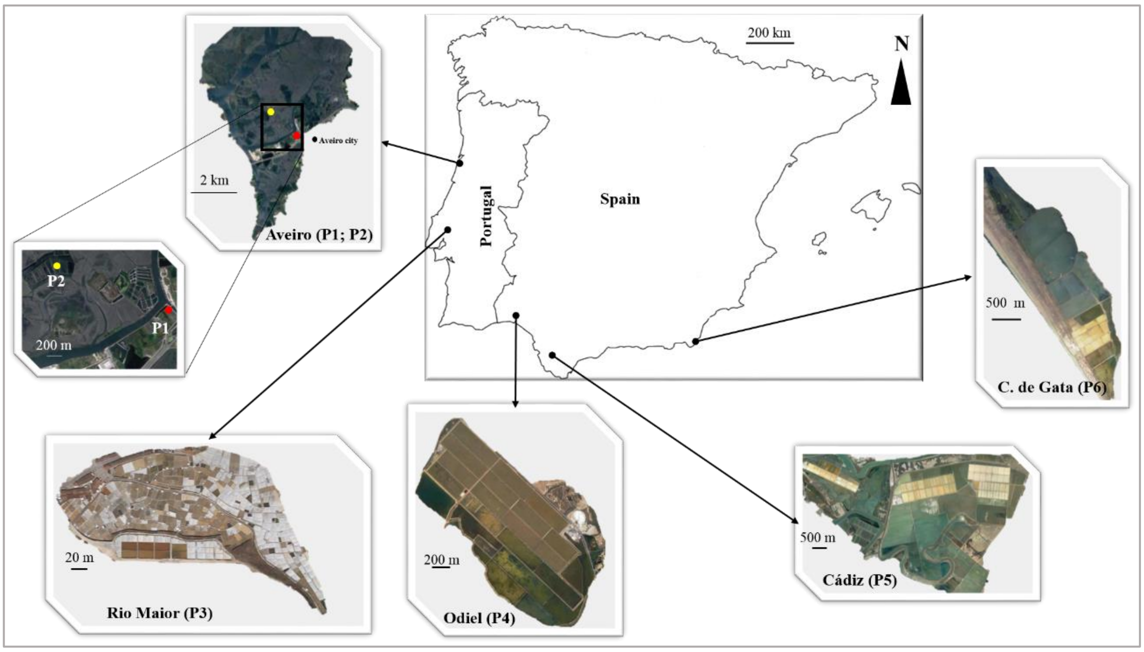

Figure 1: Location of the six populations (P1-P6) of *Artemia* selected for this study. Cysts of *A. parthenogenetica* were collected from Aveiro (Troncalhada saltpan), Rio Maior, Odiel and Cabo de Gata saltpans; and *A. franciscana* from Aveiro (Tanoeira saltpan) and Cádiz saltpans. [source: Google Maps 2020].

Ria de Aveiro is recognized as one of the most Hg-contaminated aquatic systems in Europe (Pereira et al., 1998). The Hg contamination of this lagoon derived from discharges of a chloralkali plant (active from 1950s to 1994) located in Estarreja near Aveiro (Pereira et al., 1998). Troncalhada saltpan (N, Portugal; 40°38′41.52″N; 8°39′45.81″W), where native *A. parthenogenetica* still persists, (Pinto et al., 2013, 2014), due to its location (Figure 1), is one of the first areas to receive the contaminated effluents from the Ria (Rodrigues et al., 2012). On the other hand, Tanoeira saltpan (N, Portugal, 40°39′0.70″N, 8°40′46.95″W), already invaded by *A. franciscana* (Pinto et al., 2013, 2014), is located much farther away from the main channels of the Ria, thus receiving lower levels of contamination compared to Troncalhada. Ria de Aveiro also presents high concentrations of Zn (Martins et al., 2015; Cachada et al., 2019), which may be related to the Pb-Zn-(Cu-Ag) hydrothermal veins deposits in Portugal, known and exploited since the 19th century (Guimarães dos Santos, 1948).

The Rio Maior saltpans (NW, Portugal, 39°21′49.90″N, 8°56′ 38.93″W), the other area where native *A. parthenogenetica* persist in Portugal, are considered a low polluted system due to its inner/inland location and the fact that the brine supply comes from a long and deep streak of rock salt located in Serra de Aires e Candeeiros Natural park (Calado and Brandão, 2009). However, Rio Maior presents high concentrations of Zn naturally present in the soil and subsoil of the area (Duarte, 1979) or perhaps due to nearby coal mining (Suárez-Ruiz et al., 2006).

The Odiel and Tinto estuary (SW, Spain, 37°15′29″N, 6°58′25″W), where native *A. parthenogenetica* cysts were collected, is considered one of the most polluted estuarine systems in the world, due to high concentrations of As, cadmium (Cd), copper (Cu), lead (Pb), antimonium (Sb) and Zn (Nelson and Lamothe, 1993; Ruiz, 2001). The pollution in this area derives from drainage from abandoned mines and from industrial discharges (Nelson and Lamothe, 1993; Ruiz, 2001). Although, this system presents very high concentration of Zn, it has low concentrations of Hg (Rosado et al., 2015; Elbaz-Poulichet et al., 2001; Bermejo et al., 2003).

The Puerto de Santa María saltpans (S, Spain, 36°35.799′N, 6°12.597′W), where *A. franciscana* cysts were collected, are in Cádiz Bay (Spain), within the Gulf of Cádiz. The amount of highly toxic heavy metals discharged by the Odiel and Tinto Rivers produce a plume of contaminants in the Gulf of Cádiz (Palanques et al., 1995; Hanebuth et al., 2108) reaching the Strait of Gibraltar (Elbaz-Poulichet et al., 2001, Periáñez, 2009; Pérez-López et al., 2011). Thus, this area has high levels of some contaminants such as As (Suñer et al., 1999) but moderate concentrations of Zn and low concentrations of Hg (Hanebuth et al., 2018; Morillo et al., 2007; Carrasco et al., 2003).

Cabo de Gata, where one of the last native *A. parthenogenetica* still persist in Spain, is located at southern of Cabo de Gata-Níjar Natural Park and southwest of Cartagena-Cabo de Gata metallogenic belt, an historical mining area exploited in the 19th century for Hg extraction (Viladevall et al., 1999). The area presents high levels of Hg (Navarro et al., 2006, 2009; Bori et al., 2016; Ramos-Miras et al., 2019) and moderate concentrations of Zn (Bori et al., 2016; Navarro et al., 2006, 2009; Flores and Rubio, 2010).

Based on the study site description the level of resistance to metals for the different *Artemia* populations should follow the descending order of Cabo de Gata > Troncalhada (Aveiro) > Tanoeira (Aveiro) > Odiel > Cádiz > Rio Maior for Hg exposure; and Odiel > Troncalhada (Aveiro) > Tanoeira (Aveiro) > Cádiz > Cabo de Gata > Rio Maior for Zn exposure.

2.2 Cyst sampling

Cysts from six *Artemia* populations were collected in 2014 from the shores of evaporation ponds of low-medium salinity (90–150 g L^{-1}). The selected six populations were sampled in the above sites located in the Iberian Peninsula (Figure 1). The Junta de Andalucía and Câmara Municipal de Aveiro provided permission to sample. Cysts were transported to the laboratory and sieved through 500, 300, and 100 mm sieves (cyst size is normally ~250 mm). Retained cysts were cleaned by differential flotation in freshwater and saturated brine (after Sorgeloos et al., 1977; Amat, 1985). Cysts were then dried at 45 ºC for 24 h and stored at 5 ºC until use in experiments.

2.3 Hatching of cysts

Before the toxicity tests, cysts were hatched in artificial seawater prepared with 35 g L^{-1} of sea salt (Tropic Marin - Wartenberg, Germany), under a photoperiod of continuous illumination and aeration, at 28 ± 1°C. After hatching, the nauplii were immediately separated from their shells and transferred to clean media with continuous air supply 25±1°C, where they were kept for subsequent acute toxicity experiments. For every population studied, the toxicity tests were performed with nauplii at an age when at least 90% of the population was of instar II (the most sensitive stage of the *Artemia* life cycle; Leis et al., 2014), as checked by observation under a stereomicroscope.

2.4 Acute toxicity test

The endpoint relative mortality nauplii (24-h median lethal concentration [LC50]) was used to quantify the toxicity independently for Hg and Zn in the six *Artemia* populations. A stock solution of mercury chloride (HgCl2 (99.9% purity) from Sigma-Aldrich (Germany)) and of zinc sulphate heptahydrate (ZnSO4*7H2O (Merck Millipore)) (40 g Hg L^{-1} and 100 g Zn L^{-1}, respectively) was prepared in milliQ water for the LC50 experiment. Experimental solutions were prepared from this stock by diluting with artificial seawater to obtain the desired concentrations. Preliminary range-finding tests were conducted to determine the concentration ranges to be used in definitive tests (ASTM, 2014). Based on that preliminary tests, the ranges used for the definitive tests for Hg were 0-40 mg Hg L^{-1} for *A. parthenogenetica* from Odiel and *A. franciscana* from Cádiz, 0-50 mg Hg L^{-1} for *A. parthenogenetica* from Rio Maior, 0-80 mg Hg L^{-1} for *A. parthenogenetica* and *A. franciscana* from Aveiro, and 0-200 mg Hg L^{-1} for *A. parthenogenetica* from Cabo de Gata; and for Zn 0-1150 mg Zn L^{-1} for all populations except, for *A. parthenogenetica* from Odiel for which 0-1100 mg Zn L^{-1} was used. Details of the eight nominal concentrations use for each population are given in Table S1 of the supplementary material. Nauplii were divided into the control and the different treatments. Three replicates per concentration were tested in groups of 15 animals per well of 24-well microplates (volume of 1 ml per well). Plates were covered during the assays to prevent evaporation and accidental contaminations. Bioassays were conducted for 24 h in a temperature-controlled room (25 ± 1°C), in dark conditions. After 24 h of incubation, nauplii mortality was checked under a stereomicroscope. Lack of movement for 10 seconds was the criterion for animal death determination. The percentage mortality in the controls did not exceed 10%.

2.5 Statistical analysis

Relative mortality of nauplii was used to quantify the toxicity to Hg and Zn in the six study populations. The test validation criterion was a percentage mortality in controls of less than 10%. The median acute lethal concentration (24h-LC50) and its 95% confidence limits were calculated and compared between the different *Artemia* populations, using Trimmed Spearman-Karber (TSK) analysis for lethal tests (Hamilton et al, 1977). Higher LC50 (lethal concentration causing the death of 50% of the group of test animals) values are less toxic because greater concentrations are required to produce 50% mortality in exposed animals. Statistical differences among LC50 values were based on non-overlapping confidence limits (CL) (APHA, 1995). Generalized Linear Models (GLMz) with binomial distribution and logit link function were used to test the effect of populations and replicates as fixed factors, and concentrations as covariates, and the population x concentration interaction on mortality (dependent variable, with a fixed number of 15 individuals per replicate). A backward stepwise procedure was used to select the final models, excluding predictor variables (except replicate) when they had non-significant effects, except for predictors implicated in a significant interaction. For significant effects, marginal mean pairwise tests were conducted for multiple comparisons. Results were considered significant when $p < 0.05$. Analyses were performed in SPSS (IBM SPSS Statistics for Windows, Version 23).

3. Results

3.1 Acute test – Hg

The values of 24h-LC50 for nauplii from the different *Artemia* population tested were between 18 and 70 mg Hg L^{-1}. The sensitivity to Hg varied in the following direction: *A. franciscana*-Cádiz = *A. parthenogenetica*-Odiel > *A. parthenogenetica*-Rio Maior = *A. franciscana*-Aveiro = *A. parthenogenetica*-Aveiro > *A. parthenogenetica*-Cabo de Gata (Figure 2). The LC50 values for Hg indicate that the invasive *A. franciscana* from Cádiz (18.44 mg Hg L^{-1}) together with the native *A. parthenogenetica* from Odiel (19.90 mg Hg L^{-1}) were the most sensitive populations, whereas native *A. parthenogenetica* from Cabo de Gata (70.54 mg Hg L^{-1}) was the most tolerant one.

Based on non-overlapping 95% Confidence Limits (CL), Hg acute exposure showed a significant effect on the percentage of mortality in different *Artemia* populations. The 24h-LC50 was significantly higher for *A. parthenogenetica* from Cabo de Gata, a Hg-polluted site, almost four-fold higher compared to the *Artemia* populations from other sites – Aveiro: Hg-polluted; Rio Maior, Odiel and Cádiz: comparatively much less Hg-polluted. On the other hand, *A. franciscana* from Cádiz and *A. parthenogenetica* from Odiel presented a significantly higher percentage of mortality compared to the *A. parthenogenetica* from Rio Maior (Figure 2).

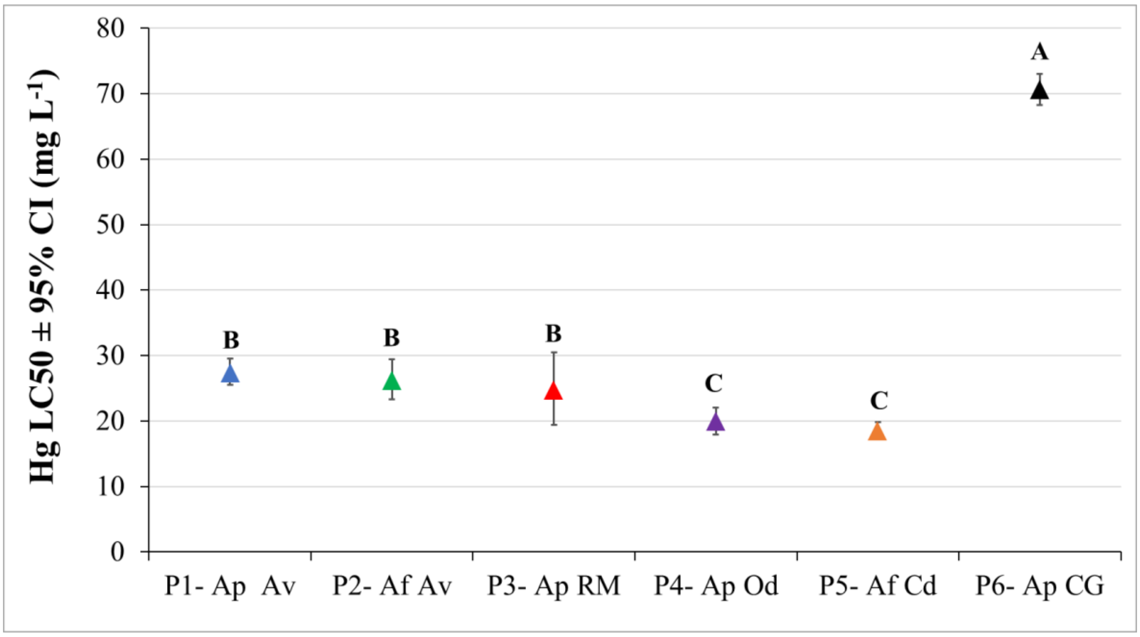

Figure 2. Values of Hg concentrations that were lethal for 50% of individuals over 24-h (LC50) recorded for the six *Artemia* populations (P1-P6) tested, with 95% confidence intervals. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd). Different letters indicate significant differences among *Artemia* populations.

GLMz analysis showed that there were no differences among replicates but that the interaction between Hg concentration and population was highly significant (Table 1; Figure 3), i.e. that the relationship between Hg concentration and the probability of *Artemia* survival varied among populations.

Table 1. Generalized linear model (GLMz) on nauplii mortality of the six *Artemia* populations (P1-P6) under different mercury (Hg) concentrations within 24h, using a Binomial error distribution and logit link. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd). Estimates for "*Af*-CG" and replicate "3" are not included as they were aliased, but they are effectively zero.

Effect	Level of effect	Estimates	SE	df	Wald Chi-Square	Sig.
Intercept		-2.406	0.283	1	546.725	0.000
Population	P1-*Ap* Av	-0.764	0.416	5	5.033	0.412
	P2-*Af* Av	-0.582	0.406			
	P3-*Ap* RM	-0.423	0.408			
	P4-*Ap* Od	-0.107	0.395			
	P5-*Af* Cd	-0.499	0.376			
Concentration		0.075	0.007	1	573.128	0.000
	P1-*Ap* Av*Concentration	0.046	0.014	5	158.187	0.000

Population * Concentration	P2-*Af* Av*Concentration	0.063	0.015			
	P3-*Ap* RM*Concentration	0.041	0.008			
	P4-*Ap* Od*Concentration	0.009	0.011			
	P5-*Af* Cd*Concentration	0.026	0.012			
Replicate	1	<0.0001	0.134	2	0.719	0.698
	2	0.098	0.134			

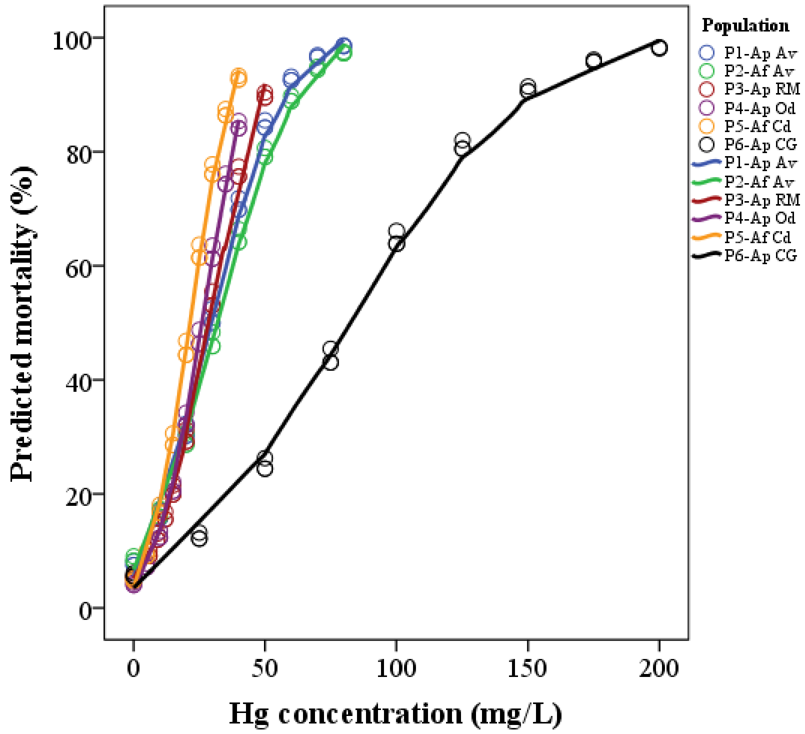

Figure 3. Relationship between the predicted mortality at different mercury (Hg) concentrations for the six studied populations (P1-P6). Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd). Lines show locally estimated scatterplot smoothing (LOESS) for each population.

3.2 Acute test – Zn

The values of 24h-LC50 for nauplii from the different *Artemia* populations ranged from 354 and 458 mg Zn L^{-1}. The sensitivity to Zn varied among the different populations tested: *A. parthenogenetica*-Odiel > *A. parthenogenetica*-Aveiro, *A. parthenogenetica*-Rio Maior, *A. franciscana*-Aveiro; and sensitivity of *A. parthenogenetica*-Cabo de Gata = *A. franciscana* Cd ≥ *A. parthenogenetica*-Aveiro = *A. parthenogenetica*-Rio Maior =

A. franciscana-Aveiro (Figure 4). The LC50 values for Zn indicate that *A. parthenogenetica* from Odiel (354.51 mg Zn L^{-1}) was the most sensitive population, and *A. parthenogenetica* from Aveiro, *A. franciscana* from Aveiro and *A. parthenogenetica* from Rio Maior were the most tolerant (458.06, 436.86, 445.33 mg Zn L^{-1}; Figure 4).

Based on non-overlapping 95% CL, Zn exposure showed a significant effect on the percentage of mortality in the different *Artemia* populations tested. After 24 h of Zn exposure, *A. parthenogenetica* from Odiel, a highly Zn-polluted site, showed significantly higher percentage of mortality compared with *A. parthenogenetica* and *A. franciscana* from Aveiro, and *A. parthenogenetica* from Rio Maior, less Zn-polluted sites (Figure 4).

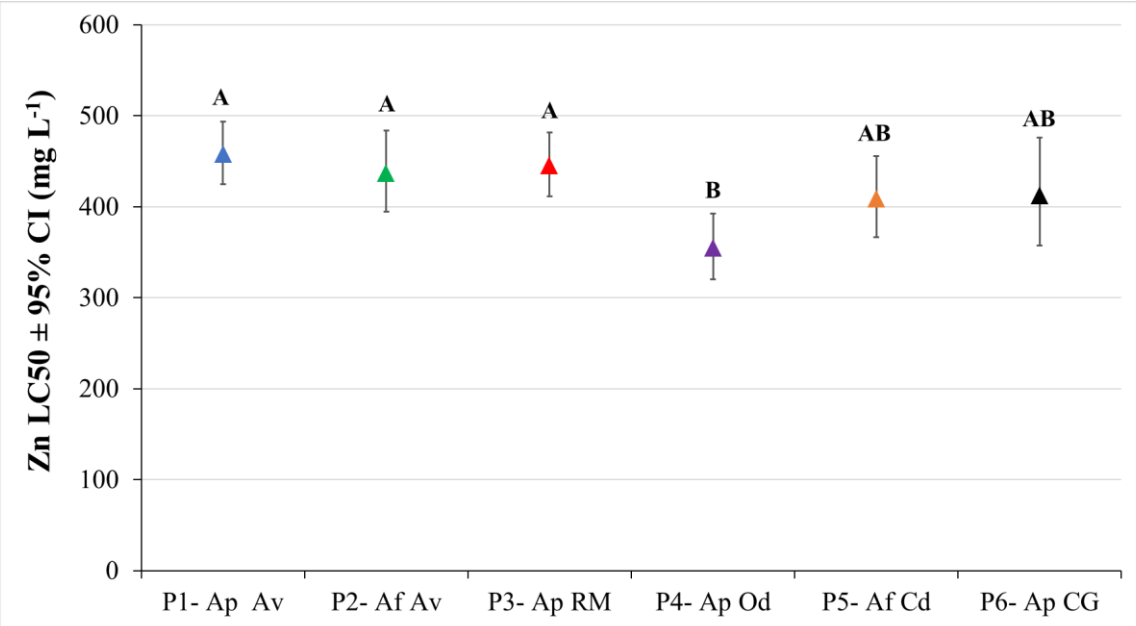

Figure 4. Values of Zn concentrations that were lethal for 50% of individuals over 24-h (LC50) for six *Artemia* populations (P1-P6), with 95% confidence intervals. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd). Different letters indicate significant differences among *Artemia* populations.

GLMz analysis showed no differences among replicates and no significant interaction between Zn concentration and population. However, there was a significant main effect of population, and a positive significant effect of Zn concentration on mortality (Table 2; Figure 5), indicating that independently of Zn concentration there are significant differences on survival between some of the populations. In this sense, pairwise comparisons (Table 3) showed higher mortality of *A. franciscana* from Cádiz compared to *A. parthenogenetica* from Rio Maior and both of *A. parthenogenetica* and *A. franciscana* from Aveiro, and higher mortality of *A. parthenogenetica* from Odiel compared to *A. parthenogenetica* from Rio Maior.

Table 2. Generalized linear model (GLMz) on nauplii mortality of the six *Artemia* population (P1-P6) under different zinc (Zn) concentrations within 24h, using a Binomial error distribution and logit link. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd). Estimates for "P6-*Ap*_CG" and replicate "3" are not included as they were aliased, but they are effectively zero.

<table>
<tr><th>Effect</th><th>Level of effect</th><th>Estimates</th><th>SE</th><th>df</th><th>Wald Chi-Square</th><th>Sig.</th></tr>
<tr><td>Intercept</td><td></td><td>-2.655</td><td>0.190</td><td>1</td><td>527.618</td><td>0.000</td></tr>
<tr><td rowspan="5">Population</td><td>P1-Ap Av</td><td>-0.270</td><td>0.197</td><td rowspan="5">5</td><td rowspan="5">16.318</td><td rowspan="5">0.006</td></tr>
<tr><td>P2-Af Av</td><td>-0.309</td><td>0.197</td></tr>
<tr><td>P3-Ap RM</td><td>-0.367</td><td>0.197</td></tr>
<tr><td>P4-Ap Od</td><td>0.071</td><td>0.197</td></tr>
<tr><td>P5-Af Cd</td><td>0.271</td><td>0.197</td></tr>
<tr><td>Concentration</td><td></td><td>0.006</td><td>0.0002</td><td>1</td><td>713.316</td><td>0.000</td></tr>
<tr><td rowspan="2">Replicate</td><td>1</td><td>0.048</td><td>0.139</td><td rowspan="2">2</td><td rowspan="2">2.558</td><td rowspan="2">0.278</td></tr>
<tr><td>2</td><td>0.212</td><td>0.139</td></tr>
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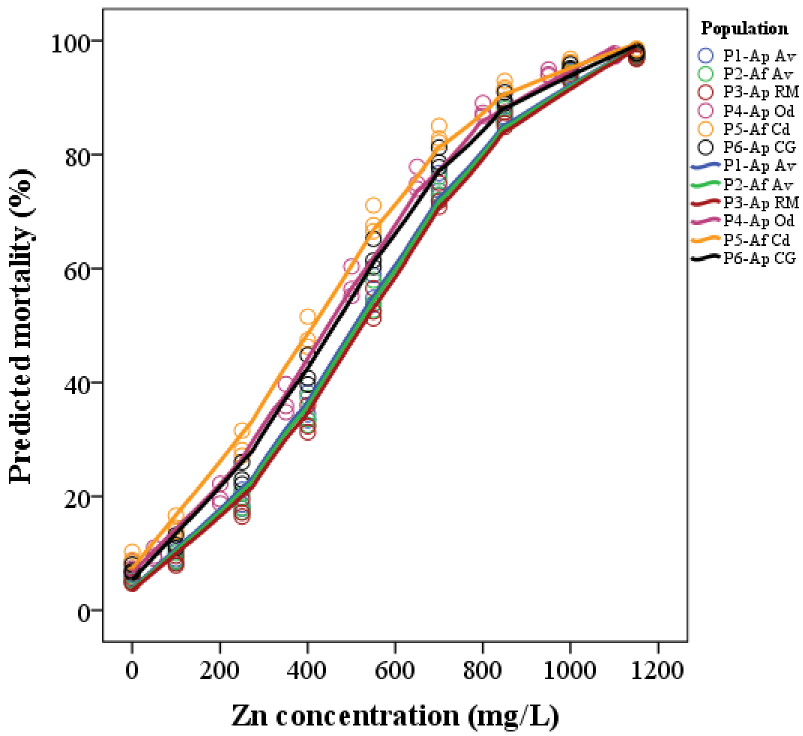

Figure 5. Predicted mortality at different zinc (Zn) concentrations for the six studied populations (P1-P6) of *Artemia*. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd). Lines show locally estimated scatterplot smoothing (LOESS) for each area.

Table 3. Pairwise comparisons of nauplii mortality among populations (P1-P6) after acute exposure to zinc (Zn). Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd).

Comparison	SE	Sig.
P1-*Ap* Av vs. P2-*Af* Av	0.049	0.844
P1-*Ap* Av vs. P3-*Ap* RM	0.049	0.623
P1-*Ap* Av vs. P4-*Ap* Od	0.047	0.081
P1-*Ap* Av << P5-*Af* Cd	**0.046**	**0.006**
P1-*Ap* Av vs. P6-*Ap* CG	0.047	0.168
P2-*Af* Av vs. P3-*Ap* RM	0.049	0.768
P2-*Af* Av vs. P4-*Ap* Od	0.047	0.052
P2-*Af* Av << P5-*Af* Cd	0.046	**0.003**
P2-*Af* Av vs. P6- *Ap* CG	0.048	0.115
P3-*Ap* RM << P4-*Ap* Od	**0.047**	**0.025**
P3-*Ap* RM << P5- *Af* Cd	**0.046**	**0.001**
P3-*Ap* RM vs. P6-*Ap* CG	0.048	0.061
P4-*Ap* Od vs. P5- *Af* Cd	0.044	0.306
P4-*Ap* Od vs. P6-*Ap* CG	0.046	0.718

P5-*Af* Cd vs. P6-*Ap* CG	0.045	0.168

4. Discussion

The present work hypothesized that native *Artemia* from highly Hg-polluted areas and highly Zn-polluted areas would be locally adapted and consequently be more resistant to the invasion than native populations from less polluted areas (pollution resistance hypothesis) (Rodrigues et al, 2012). Based on this hypothesis it was expected that native *Artemia* from Cabo de Gata and Troncalhada (Aveiro) – two of the most Hg-polluted areas – would present the highest resistance to Hg, and native *Artemia* from Odiel, – the most Zn-polluted area – would present the highest resistance to Zn.

4.1 Effect of Hg on the survival of *Artemia*

The results from the acute toxicity tests showed that, of the six brine shrimp populations, native *A. parthenogenetica* from Cabo the Gata was the most tolerant species to Hg, whereas the native *A. parthenogenetica* from Odiel and the invasive *A. franciscana* from Cádiz were the most sensitive populations. Our results suggest that *A. parthenogenetica* from Cabo de Gata is locally adapted to withstand high levels of Hg pollution, which may explain the persistence of this relict native population in south Spain, where most A. *parthenogenetica* and *A. salina* populations have been replaced by the exotic species. Cabo de Gata is in the Cartagena-Cabo de Gata volcanic belt and contains high levels of metals including Hg (Navarro et al., 2006, 2009). This area has been exploited for mining since ancient times, more than 2,000 years ago (Ruano et al., 2000). In particular, Hg was extracted from the Valle del Azogue Hg mines from 1873 to 1890. Gold exploitation in the Cartagena-Cabo de Gata volcanic belt (Ruano et al., 2000) has also contributed for

Hg contamination in the area, as Hg is commonly used for gold extraction (Esdaile and Chalker, 2018). Waste produced by mine activity poses a threat to the surrounding areas even after the mines are closed (Dudka and Adriano, 1997). Mercury-rich mine tailings are prone to erosion (Henriques and Fernandes, 1991) and may be dispersed by atmospheric emissions, mechanical dispersion, or leachates from waste deposits (Navarro et al., 2006, 2009). On the other hand, despite Odiel and Cádiz are considered contaminated systems (especially Odiel, with high levels of As), both have very low concentrations of Hg (Elbaz-Poulichet et al., 2001; Bermejo et al., 2003). This could explain the high sensitivity to Hg of these *Artemia* populations. The LC50 values of *A. parthenogenetica* from Odiel were close to those reported by Leis et al. (2014) for *A. parthenogenetica* collected from a non-contaminated site in Italy (19.9 mg Hg L^{-1} and 17.9 mg Hg L^{-1}, respectively).

Artemia populations from Aveiro (*A. parthenogenetica* from Troncalhada saltpan and *A. franciscana* from Tanoeira saltpan) and Rio Maior (*A. parthenogenetica)* showed intermediate resistance to Hg acute exposure compared to the *Artemia* populations mentioned above. Unlike Cabo de Gata, Ria the Aveiro is considered a recent highly Hg-contaminated system, caused by 44 years (1950s until 1994) of discharges from a chloralkali plant (Pereira et al., 1998). In the case of Rio Maior, it is considered non-polluted system (Calado and Brandão, 2009). These two systems are the last refugia of native *Artemia* in Portugal. Ria de Aveiro saltpan complex currently harbours both native and invasive *Artemia* species (Pinto et al., 2013, 2014). Rodrigues et al. (2012) and Pinto et al. (2013, 2014) tried to explain the persistence of native *Artemia* in Troncalhada based on differences related to environmental factors between both saltpans and to the physiological response for each species under a variety of environmental conditions. They concluded that native strain´s survival remained an unexplained phenomenon, pointing out to the potential role of other unstudied local factors, as a chemical barrier related to the pollution, as the main driver, mainly based on the different location of these saltpans within Aveiro complex. The results of the present work do not support this hypothesis for Hg, as native and invasive *Artemia* from Rio Aveiro showed similar sensitivity to this pollutant. However, this similar sensitivity detected in the present study could be related to the fact that the invasive strain from Ria de Aveiro is the only population of *A. franciscana* in the Mediterranean more closely related genetically to the population from Great Salt Lake (Utah, USA) (Muñoz et al., 2014), a system which also has a recent history of Hg contamination (Naftz et al., 2008). The persistence of this native *A. parthenogenetica* population could be related to other contaminants present in Ria de Aveiro as other metals (Martins et al., 2015; Cachada et al., 2019), persistent organic pollutants (Ribeiro et al., 2016; Rocha and Palma, 2019) and/or sewage contaminants (Rada et al., 2016; Rocha et al., 2016).

Both *A. parthenogenetica* and *A. franciscana* from Aveiro showed sensitivity to Hg comparable with *A. parthenogenetica* from Rio Maior (24.7 mg Hg L^{-1}). This is surprising since Rio Maior has no known relevant chemical contamination (Calado and Brandão, 2009). The LC50 values of *A. parthenogenetica* from Rio Maior, are significantly higher than those observed by Leis et al. (2014) for *A. parthenogenetica* collected from a non-contaminated area in Italy (24.7 mg Hg L^{-1} and 17.9 mg Hg L^{-1}, respectively), suggesting that the population from Rio Maior may be naturally more resistant to Hg. Pinto et al. (2013, 2014) suggested that *A. parthenogenetica* from Rio Maior is a very well adapted population to its specific biotope characteristics, which, together with its inland localisation (far from the main bird migration routes and fish farming), may favours the resistance to the invasion. However, they didn’t identified factors involved in the persistence and remained on the idea that a chemical barrier related to heavy metals or pesticides may be preventing the invasion.

4.2 Effect of Zn on the survival of *Artemia*

The 24-h LC50 results for nauplii showed that *A. parthenogenetica* from Odiel population appears to be the most sensitive to Zn among the six populations tested. However, although the LC50 value was lower than those for the Portuguese populations, it showed no differences with the Spanish populations. This results contrast, in part, with the fact that, according to the literature, the Odiel estuary presents the highest Zn concentrations among the study sites analysed, thus it was expected that this population would present the highest tolerance to this metal. Zn concentrations in the Odiel estuary are very high, with means around 2,000-2,800 mg Zn Kg^{-1} in sediments (Rosado et al., 2015), much higher than Zn concentrations for the other study sites, where Zn concentrations ranged from 100-400 mg Zn Kg^{-1} (i.e., Aveiro: 400 mg Zn Kg^{-1}, Cachada et al., 2019; Martins et al., 2015; Cádiz: 100-200 mg Zn Kg^{-1}, Hanebuth et al., 2018; Cabo de Gata: 240-430 mg Zn Kg^{-1}, Navarro et al., 2009; Flores and Rubio, 2010). Furthermore, Zn concentrations reported in Odiel estuary (e.g., Rosado et al., 2015) are just below the concentrations of 3000 mg Zn Kg^{-1}, suggested by the Spanish Center for Studies and Experimentation of Public Works (CEDEX, 1994) as corresponding to action level 2 (limit or intervention level) for dredged materials, from which sediments must be isolated into containers or into a contained area.

The absence of a clear separation of the *Artemia* populations regarding Zn sensitivity suggests, therefore, that Zn contaminated systems would not potentially limit the *A. franciscana* invasion. Overall, in this work, the 24h- LC50 values ranged between 354-

458 mg Zn L^{-1} and similar values were reported by Jiménez et al. (2006; ~300 mg Zn L^{-1}) and Damasceno et al. (2017; 401 mg Zn L^{-1}) for commercial *A. franciscana*. On the other hand, the LC50 values are half of those found by Kokkali et al. (2011; 1,000 mg Zn L^{-1}) for *A. salina*. This high tolerance shown by *Artemia* to Zn acute exposure might be explained because Zn is an essential metal necessary for normal physiological and biochemical process of organisms, unlike Hg which has no biological function (Clarkson and Magos, 2006), its deficiency results in severe health consequences, being acute Zn toxicity rare, and only reported at very high concentrations (Frassinetti et al., 2006; Valko et al., 2005).

The GLMz showed significant differences on mortality between some of the populations, which do not seem to be explained by those Zn concentrations used, suggesting intrinsic differences on mortality among populations, or the influence of other factors. Our results contrast with a recent study by Pais-Costa et al. (2019) who provided evidence of local adaptation of native species to Zn pollution based on life history and physiological data under realistic chronic Zn exposure conditions (0.2 mg Zn L^{-1}). These findings highlight the importance of testing both chronic and acute exposure to the same contaminant and to different contaminants for more conclusive results.

5. Conclusion

Artemia is suffering a dramatic biodiversity loss at global scale due to the invasion of *A. franciscana*, so the conservation and characterization of last refuge of native *Artemia* have been pointed out as a priority (Pinto et al., 2014). Recent studies examining different abiotic factors highlight the necessity to study the potential role of contaminants (Rodrigues et al., 2012, Pinto et al., 2013, 2014). The results of the present study showed that *A. parthenogenetica* from Cabo de Gata are extremely resistant to Hg pollution, and it may explain its resistance to the invasion by the exotic *A. franciscana*. However, no support was found to the "pollution resistance hypothesis" for the native population from Ria de Aveiro, which showed similar tolerance to Hg than *A. franciscana* population from the same area. Regarding Zn, differences between populations in response to high levels were weak, and inconsistent with the environmental differences in Zn concentrations. However, previous studies have shown that chronic exposure to Zn may limit the invasion of *A. franciscana* due to physiological resistance (Pais-Costa et al., 2019). Future studies should test i) the effects of other contaminants in native and invasive *Artemia* populations, ii) the effects of a mixture of different pollutants to provide a more realistic ecological context, and iii) expose populations to chronic effects, which are the most common type of contaminant impact found in the environment. Management efforts should focus in these relict native populations to preserve the remaining *Artemia* biodiversity and limit the probability of *A. franciscana* introduction.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Material

Table S1: Mercury (Hg) and Zinc (Zn) concentrations (mg L^{-1}) used in LC50 tests for *Ap* (*A. parthenogenetica*) *Af* (*A. franciscana*), from Av (Aveiro, P1 and P2), RM (Rio Maior, P3) Od (Odiel, P4), Cd (Cádiz, P5) and CG (Cabo de Gata, P6). Different letters indicate significant differences among *Artemia* populations.

P1- *Ap* Av		**P2 *Af* Av**		**P3- *Ap* RM**		**P4- *Ap* Od**		**P5- *Af* Cd**		**P6- *Ap* CG**	
Hg	**Zn**	**Hg**	**Zn**	**Hg**	**Zn**	**Hg**	**Zn**	**Hg**	**Zn**	**Hg**	**Zn**
0	0	0	0	0	0	0	0	0	0	0	0
10	100	10	100	6	100	5	50	5	100	25	100
20	250	20	250	9	250	10	200	10	250	50	250
30	400	30	400	12	400	15	350	15	400	75	400
40	550	40	550	15	550	20	500	20	550	100	550
50	700	50	700	20	700	25	650	25	700	125	700
60	850	60	850	30	850	30	800	30	850	150	850
70	1000	70	1000	40	1000	35	950	35	1000	175	1000
80	1150	80	1150	50	1150	40	1100	40	1150	200	1150