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BIOLOGICAL INVASIONS, POLLUTION, AND CLIMATE WARMING IN *ARTEMIA*: ECOLOGICAL AND EVOLUTIONARY RESPONSES

Tese no âmbito do doutoramento em Biociência, área de especialização em Ecologia, orientada pelo Professor Doutor João Carlos Marques, pela Doutora Marta Isabel Sánchez, e pela Doutora Mónica Martinez-Haro, e apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Biological invasions, pollution, and climate warming in *Artemia*: ecological and evolutionary responses

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THESIS OUTLINE

The thesis is structured into eight sections: a general introduction section which sets the framework for the topics covered in the chapters of the thesis; four original research chapters, each corresponding to a paper published or submitted for publication; a general discussion section with a contextual analysis of the topics covered and main findings; a general conclusion section; and future research section.

The manuscripts resulting from the present research are the following:

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CONTENTS

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SUMMARY

The present Thesis aims to increase our understanding of the interactions between biological invasions and pollution in hypersaline coastal ecosystems and the mechanisms involved in thermal adaptation using the genera *Artemia* as model system. Hypersaline ecosystems are widely distributed habitats that provide invaluable economic (e.g., salt and *Artemia* cysts production) and non-economic services (e.g., conservation of avifauna). From a research point of view, hypersaline ecosystems represent excellent natural laboratories to address a variety of ecological, physiological, and evolutionary questions due to their simple web structure and ecological gradients (e.g., salinity). Moreover, these environments are under increasing threat due to anthropogenic pressure providing suitable contexts to study the individual and combined response to global change.

In **Chapter I**, I studied bioaccumulation of trace elements (As, Cd, Cu, Co, Cr, Mn, Ni, Pb and Zn) in native and invasive *Artemia* from hypersaline ecosystems in Southern Spain exposed to different sources of pollution. Overall, the invasive *Artemia franciscana* showed the highest potential to bioaccumulate trace elements, particularly As, compared to the native *Artemia parthenogenetica*. Given the importance of *Artemia* as food resource for many species of birds, the high bioaccumulation potential showed by *A. franciscana* represents an additional indirect impact that had not been previously recognised for the species. I also considered the effect of salinity on the bioaccumulation of trace elements by the different *Artemia* populations. I found that, generally, bioaccumulation of trace elements was the lowest at the highest salinity. For Odiel data, I also measured the bioaccumulation potential of trace elements from water and sediments by key primary consumers – *A. parthenogenetica*, *Chironomus salinarius* and *Ochthebius notabilis* – exposed to the same conditions of salinity and pollution. Overall, *A. parthenogenetica* and *C. salinarius* bioaccumulated the same elements from water and sediment, respectively. Moreover, *O. notabilis* showed the highest potential to bioaccumulate As and Cu, two of the elements of most concern in Odiel. This represents the most complete study on the bioaccumulation of trace elements in hypersaline ecosystems up to date.

In **Chapter II and III**, I studied the role of pollution in the invasion of *A. franciscana*, providing some evidence to the "pollution resistance hypothesis" (Rodrigues et al., 2012) from an experimental perspective. First (**Chapter II**), I investigated the individual effect of **acute toxicity** of Hg and Zn on the survival of native and invasive *Artemia* populations from the Iberian Peninsula, collected from saltpans with different levels of Hg- and Zn- contamination. I found that native *A. parthenogenetica* from Cabo de Gata are extremely resistant to Hg pollution, which may be

explained by the long-term history of Hg- contamination in that site (since ancient times). These results suggest that high levels of Hg may have played a key role in the persistence of this native *Artemia* population. However, no support was found to the "pollution resistance hypothesis" for the native population from Ria de Aveiro (Hg contamination of this complex derived from around 40 years of discharges from a chloralkali plant), which showed similar tolerance to Hg as the *A. franciscana* population from the same complex. Other factors may be limiting the invasion in this case. Regarding Zn, the pattern of acute sensitivity did not differ between *Artemia* species, suggesting that this element do not play an important role in the invasion process at that level. Second (**Chapter III),** I studied the effect of **chronic toxicity** to Zn on the life history and oxidative stress response of native and invasive *Artemia* species from saltpans with different levels of Zn contamination. Although no differences in tolerance to acute Zn exposure were detected between the populations tested (**Chapter II)**, the results of long-term exposure strongly suggested that the native *Artemia* from Odiel is locally adapted (at both reproductive and physiological levels) to Zn contamination and that *A. franciscana* is highly sensitive.

In **Chapter IV**, I investigated the role of genetic adaptation, epigenetic effects, developmental plasticity, and local microbiota on thermal adaptation. In the early 1980s, *A. franciscana* from San Francisco Bay, USA, was introduced in Vinh Chau, Vietnam, which represents a +10°C climate change. I used a resurrection ecology approach and compared the response of "ancestral" (from San Francisco) and "derived" populations (from Vinh Chau). I found that the "derived" population shows increased phenotypic tolerance to warming. Yet, strikingly, these changes were not genetic and were not caused by epigenetic marks set by adult parents exposed to heat stress. Further, I did not find any developmental plasticity in response to heat stress, nor any protective effect of heat-tolerant local microbiota. Thus, I concluded that the evolution of *Artemia* extreme thermal tolerance is only due to transgenerational (great) grandparental effects, possibly epigenetic marks set by parents who were exposed to high temperatures as juveniles.

RESUMO

A presente Tese visa o estudo das respostas ecológicas e evolutivas de *Artemia*, um género-chave nos ecossistemas hipersalinos, a três principais determinantes das alterações globais: invasões biológicas, poluição e alterações climáticas. Os ecossistemas hipersalinos são habitats amplamente distribuídos que proporcionam serviços com valor económico (por exemplo, produção de sal e cistos de *Artemia*), e não-económico (por exemplo, conservação da avifauna), inestimável. Do ponto de vista da investigação, os ecossistemas hipersalinos representam excelentes laboratórios naturais para abordar uma variedade de questões ecológicas, fisiológicas e evolutivas, devido à sua rede estrutural e gradientes ecológicos (por exemplo, salinidade) simples. Além disso, estes ambientes estão sob ameaça crescente devido à pressão antropogénica, o que proporciona contextos adequados ao estudo das respostas individuais e combinadas às mudanças globais.

No **Capítulo I**, estudei a bioacumulação de elementos-traço (As, Cd, Cu, Co, Cr, Mn, Ni, Pb e Zn) em *Artemia* nativa e invasora de ecossistemas hipersalinos do sul de Espanha expostos a diferentes fontes de poluição. De modo geral, a invasora *Artemia franciscana* mostrou o maior potencial para bioacumular os elementos-traço, e particularmente As, em comparação com a nativa *Artemia parthenogenetica*. Dada a importância de *Artemia* como recurso alimentar para muitas espécies de aves, o alto potencial bioacumulador de *A. Franciscana* representa um impacto indireto adicional que não tinha sido previamente reconhecido para esta espécie. Também considerei o efeito da salinidade na bioacumulação de elementos-traço pelas diferentes populações de *Artemia*. Descobri que, geralmente, a bioacumulação de elementos-traço era mais baixa na salinidade mais alta. Nos dados de Odiel, medi também o potencial de bioacumulação de elementos-traço a partir da água e dos sedimentos por consumidores primários principais – *A. parthenogenetica*, *Chironomus salinarius* e *Ochthebius notabilis* – expostos às mesmas condições de salinidade e poluição. De modo geral, *A. parthenogenetica* e *C. salinarius* bioacumularam os mesmos elementos da água e dos sedimentos, respetivamente. Além disso, *O. notabilis* mostrou o maior potencial para bioacumular As e Cu, dois dos elementos mais preocupantes em Odiel. Isto representa até à data o estudo mais completo sobre a bioacumulação de elementos-traço em ecossistemas hipersalinos.

Nos **Capítulos II e III**, estudei o papel da poluição na invasão de *A. franciscana*, fornecendo algumas evidências da "hipótese de resistência à poluição" (Rodrigues et al., 2012). Primeiro (**Capítulo II**), investiguei o efeito individual da toxicidade aguda de Hg e Zn na sobrevivência das populações de *Artemia* nativas e invasoras da Península Ibérica, recolhidas a partir de salinas com diferentes níveis de contaminação por Hg e Zn. Descobri que a nativa *A. parthenogenetica* de Cabo de Gata é extremamente resistente à contaminação por Hg, o que pode

ser explicado pela contaminação por Hg histórica naquele local. Estes resultados sugerem que altos níveis de Hg podem estar a desempenhar um papel fundamental na persistência desta população nativa de *Artemia*. No entanto, não foi encontrado qualquer apoio à "hipótese de resistência à poluição" para a população nativa da Ria de Aveiro (a contaminação por Hg deste complexo deriva das descargas de uma central de cloro-alcalinos), que apresentava tolerância ao Hg semelhante à população de *A. franciscana* do mesmo complexo. Neste caso, outros fatores podem estar a limitar a invasão. No que diz respeito ao Zn, o padrão de sensibilidade não diferiu entre as espécies de *Artemia*, sugerindo que este elemento não desempenha um papel importante no processo de invasão. Depois (**Capítulo III**), estudei o efeito da exposição a longo prazo ao Zn na história de vida e na resposta ao stress oxidativo das espécies de *Artemia* nativas e invasoras de salinas com diferentes níveis de contaminação por Zn. Contrariamente ao observado para a exposição aguda ao Zn (Capítulo II), os resultados da exposição a longo prazo sugeriram fortemente que a *Artemia* nativa de Odiel está adaptada localmente (em níveis reprodutivos e fisiológicos) à contaminação por Zn e que *A. franciscana* é altamente sensível.

No **Capítulo IV**, investiguei o papel da adaptação genética, dos efeitos epigenéticos, da plasticidade do desenvolvimento e do microbioma local na adaptação térmica. No início da década de 1980, *A. franciscana* da Baía de São Francisco, EUA, foi introduzida em Vinh Chau, Vietname, o que representa uma mudança climática de +10°C. Usei uma abordagem de ecologia da ressurreição e comparei a resposta das populações “ancestrais” (de São Francisco) e "derivadas" (de Vinh Chau). Descobri que a população "derivada" mostra uma maior tolerância fenotípica ao aquecimento. No entanto, surpreendentemente, estas mudanças não foram genéticas e não foram consequência de marcas epigenéticas definidas por pais adultos expostos ao stress térmico. Além disso, não encontrei qualquer plasticidade de desenvolvimento em resposta ao stress térmico, nem qualquer efeito protetor da microbiota local tolerante ao calor. Assim, concluí que a evolução da tolerância térmica extrema da *Artemia* se deve apenas aos efeitos transgeracionais dos (bi) avós, possivelmente marcas epigenéticas definidas por progenitores que foram expostos a altas temperaturas enquanto juvenis.

GENERAL INTRODUCTION

1. Biological invasions and interaction with pollution

Aquatic ecosystems comprise the largest part of the biosphere and are critical components of the global environment. They provide many essential ecosystem services to human well-being such as resource harvesting, coastal protection, erosion control, water purification, maintenance of fisheries, carbon sequestration, tourism and recreation (Grizzetti et al, 2016). Yet currently these systems are strongly affected by a variety of human impacts (Barbier et al., 2011).

Among the major threats to aquatic biodiversity and ecosystem function are biological invasions (Gallardo et al., 2016) and environmental pollution (Bernhardt et al., 2017). They are considered main drivers of global change with particular impact in aquatic ecosystems. Impacts of invasive species include alteration of nutrient cycling, changes of the community structure and dynamics (Mack et al., 2000; Ehrenfeld, 2003; Levine et al., 2003, Gallardo et al., 2016) as well as the displacement of native species (Viard and Comtet, 2015). 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. Environmental pollution can play an important role in ecosystems invasibility with different studies showing that invasive species have a superior tolerance to pollution when compared to their native counterparts (e.g., Piola and Johnston, 2009; Crooks et al., 2010). Though, most of these studies have considered environments where invasive species have succeeded (Sołtysiak and Brej, 2014; Guarnieri et al., 2017). Furthermore, they generally consider scenarios of recent environmental pollution or emerging pollutants (e.g., Varó et al., 2015). However, it has been proposed that local adaptation of native species to long-term environmental pollution may limit the establishment of invasive species as proposed by Rodrigues et al. (2012). For example, in areas where pollution dates from prehistoric or ancient times (e.g., from mining activities) native communities would have had the time to adapt to the presence of those pollutants by evolutionary acquisition of chemical tolerance (e.g., Barata et al., 2002; Lopes et al., 2006; Ruggeri et al., 2019), and therefore, would, possibly, be more resistant to the establishment of newly arriving invasive species (Faria et al., 2010; Sánchez et al., 2016a). Yet, little research has been done to study the potential role of pollution in limiting invasions.

2. Biological invasions as model systems to study global change

Biological invasions can also provide excellent model systems to study the evolutionary response of organisms to global change (Moran and Alexander, 2014). For decades, exotic species have been intentionally or unintentionally introduced by humans into ecosystems where environmental conditions – biotic (e.g., competitors, predators, or pathogens) and abiotic (e.g.,

temperature, water chemistry, salinity, etc) – are very different from their native habitats. Biological invasions can thus serve as 'natural' experiments to study evolution over contemporary timescales in novel environments affected by different drivers of global change, such as climate change, pollution and habitat fragmentation (Sax et al., 2007; Moran and Alexander, 2014; Colautti and Lau, 2015). However, few studies have considered evolution in the context of biological invasions.

Particularly interesting for the study of adaptation to environmental stress, are those species producing dormant stages (e.g., cysts, seeds, spores). Dormant stages carry crucial information of recent evolutionary changes and ecological histories of populations (Hairston et al., 1996; Brendonck and De Meester, 2003). They form propagule banks that can be stored either in situ (Hairston et al., 1996) or under laboratory conditions where they may remain viable for decades. Using a resurrection ecology approach (Weider et al., 2018), it is possible to revive long-dormant organisms via hatching of dormant stages to study evolutionary trajectories of historical populations in response to environmental stress. It allows to compare the response of ancestral and contemporary populations collected from the same site and raised under similar conditions (Davis et al., 2005), and make inferences whether species will be able to adapt quickly enough to keep pace with the projected environmental changes (Clegg et al., 2000). In particular, many species translocations are associated to abrupt temperature changes, so they are of especial interest to study adaptation to climate warming. In the 20th century, average annual temperature has increased 0.6 °C globally (Jones et al., 1999), and the worst-case scenarios predict a global increase of up to 6°C by 2100 (IPCC, 2013). Moreover, the frequency of temperature extremes is likely to increase both globally and in mountain areas (IPCC, 2014). In aquatic ecosystems increase in water temperature will alter lake mixing regimes (e.g., longer periods of summer stratification), reduce lake levels (because of excess evaporation), and shorten water residence time, among others, which will alter fundamental ecological processes and the geographic distribution of aquatic species. To cope with climate warming, species can either: (i) migrate to new locations where temperatures are suitable for their survival; (ii) adapt to the increased temperatures of its habitat through phenotypic changes (West-Eberhard, 2003; Parmesan, 2006; Visser, 2008) which may be genetic (Gienapp et al., 2008; Franks and Hoffmann, 2012), or non-genetic (e.g., epigenetic changes, i.e., changes in the gene expression that can be expressed within a single generation or transgenerationally inherited; Lind and Spagopoulou, 2018), or some combination of both (Holt, 1990; Visser, 2008); or (iii) go extinct. Thus, understanding whether species will be able to adapt quickly enough to keep pace to the projected temperature changes (Clegg et al., 2000) is of upmost importance. Biological invasions together with resurrection ecology experiments can offer useful contexts to test such questions and disentangle the contribution of different processes to global warming adaptation.

3. Hypersaline ecosystems and *Artemia* as model systems

Hypersaline ecosystems are widely distributed habitats, being found in all continents, including the Antarctica (Gajardo and Beardmore, 2012). They have unique aesthetic, cultural, economic (e.g., salt and *Artemia* cysts production), recreational, ecological, and conservational (e.g., conservation of avifauna) values (Williams, 1993, 1998) due to their distinctive geochemical properties, extremophile biodiversity and the ecosystem services they provide. Hypersaline systems are relatively simplified environments compared with much more complex freshwater and marine ecosystems, making them good model systems to address a variety of ecological, physiological, and evolutionary questions. Moreover, these environments are exposed to many anthropogenic pressures (Amat et al., 2005, 2007; Williams 2002; Paul and Mormile, 2017) providing suitable contexts to study the individual and combined response to global change.

For example, due to the lack of laws and awareness programs to protect these systems hypersaline environments are exposed to high levels of pollution (e.g., trace elements from mining activities). Given the simplicity of hypersaline food webs it is relatively easy to study the response of organisms to pollution and bioaccumulation processes through the food web. It is also possible to study the effect of salinity on bioaccumulation processes, due to the broad salinity gradient present in some hypersaline ecosystems such as saltpans. Salinity plays an important role in controlling bioavailability of trace elements as it may increase adsorption/precipitation and consequently immobilizing them in sediments thus, decreasing bioavailability (Nieto et al., 2007; Zhang et al., 2014). Transfer and circulation of trace elements through the sediment, water and organisms may also pose consequences for top consumers if biomagnification occurs. In hypersaline environments waterbirds, humans, or both, that direct or indirectly consume saltpans resources (salt, algae for medical use, fish, brine shrimps cysts for aquaculture, etc) may be at risk due to this environmental problem (e.g., Schroeder et al., 1988). This is why understanding the bioavailability/toxicity of trace elements in hypersaline ecosystems under different environmental conditions is of paramount importance (Violante et al., 2010).

The genus *Artemia*, the most conspicuous inhabitants in hypersaline environments, has already been recognized as a good model organism in Toxicology (see review by Libralato et al., 2016) - to determine the effect of contaminants on different species, as well as on finding reliable biomarkers for environmental risk assessment. The key features that make this genus a good model organism to use in ecotoxicology have been reviewed by Nunes et al. (2006) and include: short life cycle, high adaptability to adverse environmental conditions, high fecundity, bisexual/parthenogenetic reproduction strategy (with free-swimming nauplii (ovoviviparity), or

diapausing or resting eggs/cysts (oviparity) production), small body size, and adaptability to varied nutrient resources. *Artemia* are non-selective, continuous filter-feeders (Provasoli and Shiraishi, 1959; Dobbeleir et al., 1980), with the potential to bioaccumulate large quantities of certain contaminants (Petrucci et al., 1995; Varó et al., 2000). As an essential element in hypersaline food webs (Lenz and Browne, 1991), as primary consumer and main prey of a wide range of waterbirds (Sánchez et al., 2005; Varo et al., 2011), *Artemia* may play a key role in the flux of contaminants through the food web (Rodríguez-Estival et al., 2019). On the other hand, the species *Artemia franciscana* is today one of the main problems threatening biodiversity in hypersaline ecosystems, so it can allow the study of the interaction between invasions and pollution.

The genus *Artemia* comprises six known bisexually reproducing species, *A. franciscana* and *Artemia persimilis*, restricted to the New World; and *Artemia salina*, *Artemia sinica*, *Artemia tibetiana* and *Artemia urmiana* and also several obligate parthenogenetic strains identified as *Artemia parthenogenetica* restricted to the Old World (Triantaphyllidis et al., 1998; Gajardo et al., 2002; Baxevanis et al., 2006; Muñoz et al., 2010). Since the 1950's, commercial *A. franciscana* cysts (mainly from the San Francisco Bay and the Great Salt Lake, USA; Ruebhart et al., 2008) have been intentionally introduced in several parts of the world, for various economic reasons (e.g., to produce more cysts to use in the aquaculture industry; Dhont and Sorgeloos, 2002; Mura et al., 2006). Subsequent dispersion of cysts by waterbirds movements (transported in faeces and feathers) has extended the range of this invasive species along migratory flyways (Green et al., 2005; Sánchez et al., 2012). The establishment of *A. franciscana* in those new environments led to the gradual disappearance of native *Artemia* species in many parts of the globe (e.g., Amat et al., 2007; Horváth et al., 2018).

In the Iberian Peninsula, few populations of native *Artemia* still persist and most are in highly polluted areas. For example, in Portugal, one of the last refuges of native *A. parthenogenetica* is the saltpans complex of Ria de Aveiro which is considered a highly mercury polluted system (Pereira et al., 1998). The same was true for Odiel saltpans in Spain, one of the last refuges for *A. parthenogenetica* in one of the most polluted estuarine systems in Europe. Thus, these two places are interesting to test the potential role of pollution in preventing or delaying invasions for which information is extremely limited and fragmented.

Artemia also offers an exceptional model system to conduct resurrection ecology studies given its short generation time, cyst bank and collections, well-documented phylogeography, and ecology (Lenormand et al., 2018). It has been introduced from temperate to tropical regions, so it represents an ideal organism to test questions related to climate change.

4. Objectives

The general objective of this thesis was to increase our understanding of the interactions between biological invasions and pollution in hypersaline coastal ecosystems and the mechanisms involved in thermal adaptation using the genus *Artemia* as model system.

The specific objectives of the thesis were the following: Determine the bioaccumulation of trace elements in native and invasive *Artemia* under different degrees of pollution in relation to salinity (**Chapter I**). Provide insights into the "pollution resistance hypothesis" (Rodrigues et al., 2012) by which high levels of pollution may be slowing down or even avoiding the invasion by *A. franciscana* (**Chapters II and III**). Provide insights on the adaptation rate to temperature using the introduction of *A. franciscana* to the tropics as model system (**Chapter IV**).

5. Study sites

In this Thesis, I included some of the most important saltpans in the Iberian Peninsula, including the last refuges for native *Artemia* (*A. parthenogenetica*) in Spain and Portugal, and systems invaded by the exotic *A. franciscana* in both countries. Saltpans were selected on the basis of the presence of native and invasive *Artemia* and the different degrees of trace elements pollution. These study sites served to address objectives 1 and 2 (**Chapters I-III**). We also included *A. franciscana* cysts sampled from Vinh Chau saltpans in Vietnam and San Francisco in order to address objective 3 **(Chapter IV)**.

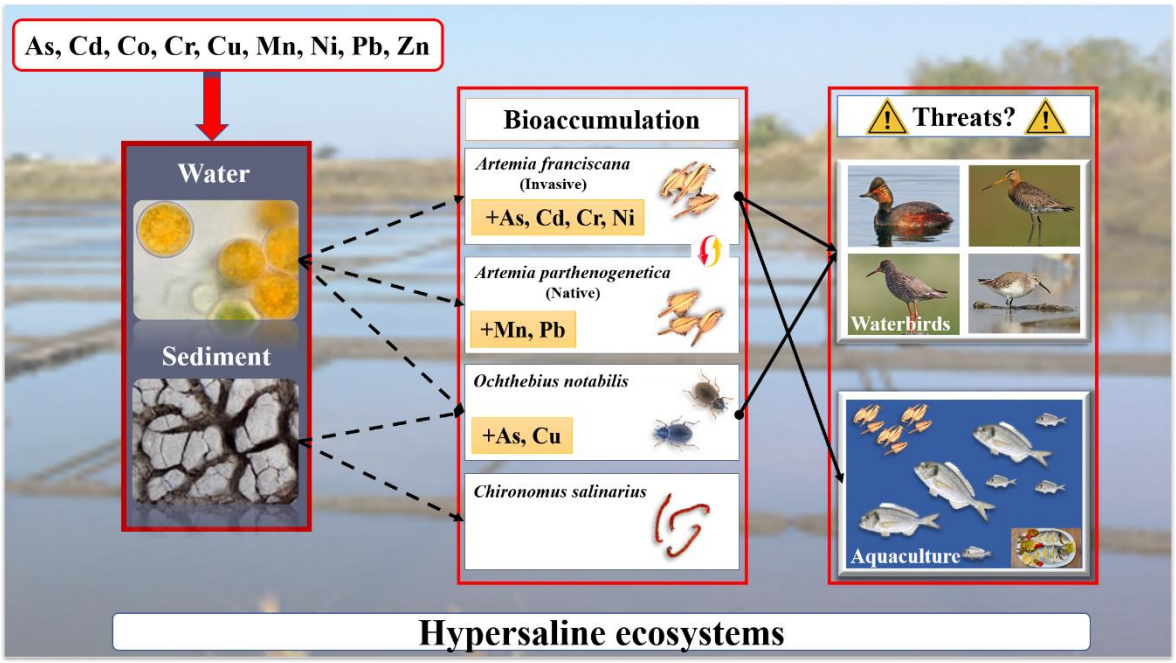
- ✓ The Cabo de Gata saltpans is one of the last refugia for native *A. parthenogenetica* in Spain. It is located at the southern end of the Cabo de Gata-Níjar Natural Park and southwest of Cartagena-Cabo de Gata metallogenic belt. This area has been exploited for mining since ancient times (>2000 years ago) until 1990 (Ruano et al., 2000). Trace elements extracted mostly included Hg, Pb, Zn, Ag, Au and Cu (Viladevall et al., 1999; Hernandez, 2005).
- ✓ The Odiel saltpans are located at the estuary of Odiel and Tinto rivers, one of the most polluted systems in Europe (Nelson and Lamothe, 1993; Ruiz, 2001). The Odiel and Tinto rivers flow through the Iberian Pyrite Belt, one of the largest polymetallic sulphide deposits in the world, where mining activity dates back to prehistoric times (Nocete et al., 2005). The Odiel and Tinto rivers transport enormous quantities of dissolved trace elements to the estuary, including As, Cu and Zn (Nelson and Lamothe, 1993; Ruiz, 2001; Sarmiento et al., 2009). Moreover, a big Industrial chemical complex significantly contributes to pollutants load to the estuary. Until recently, Odiel was one of the last refugia for native *A.*

parthenogenetica but during the course of this thesis, in 2016, it was replaced by the exotic *A. franciscana*.

- ✓ The La Tapa saltpans (hereafter referred to as the Cádiz saltpans), located at the Gulf of Cádiz, were invaded by *A. franciscana* > 40 years ago. This saltpan is comparatively less contaminated than other saltpans included in this Thesis (e.g., Odiel, Cabo de Gata and Aveiro saltpans) but some source of exposition include the trace elements discharged by the Odiel and Tinto rivers into the Gulf of Cádiz (Palanques et al., 1995; Hanebuth et al., 2018), and industrial and urban wastewater from Cádiz (Ligero et al., 2002; Carrasco et al., 2003).
- ✓ 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 located in Estarreja near Aveiro (Pereira et al., 1998). The saltpans complex of Ria de Aveiro harbours, in distinct locations, both native and invasive *Artemia*. The invasion of this saltpans has, probably, started in 1991 (Green et al., 2005). The Troncalhada saltpan, where native *A. parthenogenetica* still persists, (Pinto et al., 2013, 2014a,b), due to its location, is the most contaminated as it is one of the first saltpans to receive the contaminated effluents from the Ria (Rodrigues et al., 2012). The Tanoeira saltpan, already invaded by *A. franciscana* (Pinto et al., 2013, 2014a,b), is located much farther away from the main channels of the Ria, thus being less contaminated as it receives lower levels of contaminates compared to Troncalhada.
- ✓ The Rio Maior saltpans, the other saltpan 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).

CHAPTER I

Trace element bioaccumulation in hypersaline ecosystems and implications of a global invasion.



Pais-Costa, A.J., Sánchez, M.I., Taggart, M., Green, A.J., Hortas, F., Vinagre, P.A., Mata, M., Marques, J.C., Martinez-Haro, M. Trace element bioaccumulation in hypersaline ecosystems and implications of a global invasion. Science of the Total Environment 800, 149349. https://doi.org/10.1016/j.scitotenv.2021.149349.

I. Abstract

Hypersaline ecosystems are under increasing threat due to anthropogenic pressures such as environmental pollution and biological invasions. Here we address the ecotoxicological implications of the *Artemia franciscana* (Crustacea) invasion in saltpans of southern Spain. This North American species is causing the extinction of native *Artemia* populations in many parts of the globe. The bioaccumulation of trace elements (As, Cd, Cu, Co, Cr, Mn, Ni, Pb and Zn) in native populations (*Artemia parthenogenetica*) from Cabo de Gata and Odiel saltpans and invasive *Artemia* from Cádiz saltpans was studied at different salinities. Furthermore, in Odiel, the most polluted study site, we also analysed the bioaccumulation of trace elements by *Chironomus salinarius* larvae (Diptera) and *Ochthebius notabilis* adults (Coleoptera). High levels of trace elements were detected in the studied saltpans, many of them exceeding the recommended threshold guidelines for aquatic life. Bioaccumulation of trace elements by *Artemia* was lowest at the highest salinity. The invasive *A. franciscana* showed higher potential to bioaccumulate trace elements than its native counterpart (in particular for As, Cd, Cr and Ni). In Odiel, *O. notabilis* stood out as showing the highest potential to bioaccumulate As and Cu. Results showed that the shift from a native to an alien *Artemia* species with a higher bioaccumulation capacity may increase the transfer of trace elements in hypersaline food webs, especially for waterbirds that depend on *Artemia* as food. Thus, our study identifies an indirect impact of the *Artemia franciscana* invasion that had not previously been recognised.

Keywords:

Artemia franciscana; *Artemia parthenogenetica*; *Chironomus salinarius* larvae; *Ochthebius notabilis;* Bioaccumulation factors; Metals.

I.1. Introduction

Hypersaline ecosystems are widely distributed and include hypersaline lakes and lagoons, saltern ponds, and coastal and inland saltpans. They have distinctive geochemical properties and extremophile biodiversity providing invaluable economic (e.g., salt and *Artemia* cyst production) and non-economic services (e.g., conservation of avifauna) (Deocampo and Jones, 2014; Wurtsbaugh et al., 2017). Many hypersaline ecosystems are protected under national and international laws (e.g., the RAMSAR convention) as they are important complementary or alternative habitats for large numbers of migratory birds on stopovers, wintering, or breeding there (Masero, 2003). However, like many aquatic ecosystems, hypersaline systems are under increasing threat due to anthropogenic pressure. This includes threats from environmental pollution (e.g., by trace elements) and biological invasions (e.g., by *Artemia franciscana*) (Paul and Mormile, 2017; Horváth et al., 2018; Céspedes et al., 2019).

Trace elements (including those that are toxic/non-essential) obviously occur naturally in the environment and are ubiquitous, but their concentrations can often be amplified due to anthropogenic activities (e.g., industrial discharges, mining activities, agricultural practices, etc) (Gheorghe et al., 2017; Paul and Mormile, 2017). In aquatic ecosystems, trace elements are often ultimately deposited in sediments and immobilized through adsorption and co-precipitation (Du Laing et al., 2009; Lin et al., 2013). Furthermore, salinity, Eh (redox potential) and pH play important roles in controlling the geochemistry and thus bioavailability of all elements. Variations in these factors may increase adsorption/precipitation, and thus facilitate their deposition into sediments, consequently decreasing bioavailability (Nieto et al., 2007; Zhang et al., 2014). Hence, understanding the bioavailability/toxicity of elements (particularly toxicants) in the environment under differing environmental conditions is of paramount importance (Violante et al., 2010). Aquatic organisms may then accumulate trace elements, whether these are essential or not, through direct absorption from the abiotic environment (i.e., from water and suspended sediments) and/or from the biotic environment (i.e., food/prey) (Wang and Fisher, 1999).

In hypersaline ecosystems, crustaceans of the genus *Artemia* (Branchiopoda, Anostraca) are among its most conspicuous inhabitants (Van Stappen, 2002; Amat et al., 2005). Due to its geochemical properties, hypersaline ecosystems maintain simple food webs (Gajardo et al., 2006), with *Artemia* being the dominant primary consumers. *Artemia* are non-selective filter feeders and play a vital role in ecosystem functioning and nutrient cycling (Jellison and Melack, 1993, Sánchez et al., 2016a), being the main prey for a wide range of aquatic birds (Sánchez et al., 2005; Varo et al., 2011). Additionally, *Artemia* have the potential to bioaccumulate large quantities of certain

contaminants (Petrucci et al., 1995; Varó et al., 2000), and may potentially transfer them to their predators (Rodríguez-Estival et al., 2019). *Artemia* is also the most widely used live food item in the aquaculture industry (Dhont and Sorgeloos, 2002; Mura et al., 2006) due to its high nutritional content (Wache and Laufer, 1997). Consequently, the need of *Artemia* cysts increased leading several countries to import cysts and farm *Artemia* to boost cyst production (Sorgeloos et al., 1986). The main supplier of cysts was the United States, supplying the needs of different countries with *A. franciscana* cysts produced mainly in San Francisco Bay saltpans and Great Salt Lake. The intentional introduction of this exotic species beyond their native range (Dhont and Sorgeloos, 2002; Mura et al., 2006) and the subsequent dispersion of these by waterbirds carrying cysts in their faeces and feathers, extending its range along migratory flyways (Green et al., 2005), is leading to the extinction of native *Artemia* populations in many parts of the globe (Amat et al., 2007; Horváth et al., 2018).

Hypersaline ecosystems in southern Spain vary in the levels of pollution (by trace elements) and invasion status, providing model systems in which to study trace element bioaccumulation and biological invasion. Bioaccumulation is the enrichment of a contaminant in an organism relative to its environment, and occurs as the result of faster uptake and storage vs metabolism and excretion (Markich et al., 2001).

The aim of the present work was to deepen our understanding regarding ecotoxicological implications of biological invasion in hypersaline systems. We sampled sediment, water and *Artemia* (both native *A. parthenogenetica* and invasive *A. franciscana*) at different points along salinity gradients in saltpans in southern Spain. We also conducted a more detailed study in a highly polluted system (the Odiel saltpans), collecting two other abundant macroinvertebrates that play important roles in the food web and are consumed by waterbirds feeding in these saltpans (Sánchez et al., 2005, 2006). *Chironomus salinarius* larvae (Diptera, Chironomidae) are deposit-feeders, mainly feeding on detritus and microphytobenthos (Gamito, 1994). *Ochthebius notabilis* adults (Coleoptera, Hydraenidae) are benthic detritivores and herbivores (Abellán et al., 2009).

We tested the following hypotheses: **(1)** pollution by trace elements in sediment and water will vary among study sites due to different pollution inputs; **(2)** trace element assimilation and bioaccumulation will differ among *Artemia* populations (as each is subject to different pollution scenarios) and also due to physiological and ecological differences between native and invasive species; **(3)** increased salinity will decrease assimilation and bioaccumulation of trace elements in macroinvertebrates (due to element solubility, mobility and bioavailability shifts); **(4)** trace element concentrations in different macroinvertebrate species (*A. parthenogenetica* adults, *C. salinarius* larvae and *O. notabilis* adults) from the same site at the same salinity, will vary according to their

feeding habits. We focused on nine elements: arsenic (As), cadmium (Cd), cobalt (Co), copper (Cu), chromium (Cr), manganese (Mn), nickel (Ni), lead (Pb), and zinc (Zn). These elements were chosen mainly because this is a typical suite of metals that one might expect to be toxic at low (As/Pb/Cd) or higher levels (Cu/Zn/Co/Cr/Ni). Mn is the only one not quite in that 'category', but Mn and Fe oxides can often exert some 'control' over trace elements in wetlands (i.e., acting as sorption sites that could result in lower levels in solution).

I.2. Material and Methods

I.2.1. Study sites

We studied three Spanish saltpan complexes (see **Figure 1**):

i) The Cabo de Gata saltpans (36°47′N, 2°14′W), where one of the last native *A. parthenogenetica* populations still persists in Spain, are located at the southern end of the Cabo de Gata-Níjar Natural Park and southwest of Cartagena. Cabo de Gata sits within a volcanic belt, an area naturally rich in trace elements exploited for mining from ancient times (>2000 years ago) until 1990 (Ruano et al., 2000). Trace elements extracted mostly included Pb, Zn, Ag, Au and Cu (Viladevall et al., 1999; Hernandez, 2005).

ii) The Odiel saltpans (37°15′29″N, 6°58′25″W) are located at the estuary of the Odiel and Tinto rivers that flow through the Iberian Pyrite Belt, one of the largest polymetallic sulphide deposits in the world, where mining activity dates back to prehistoric times (Nocete et al., 2005). Pollution derives from mine drainage and industrial discharges from the port of Huelva, resulting in high levels of trace elements, such as As, Cd, Cu, Pb and Zn (Nelson and Lamothe, 1993; Ruiz, 2001; Sarmiento et al., 2009). Until recently, Odiel was one of the last refugia for native *Artemia* but they have recently been replaced by *A. franciscana* (between 2014 and 2016, after the present study).

iii) La Tapa saltpans (hereafter referred to as the Cádiz saltpans; 36°35.799′N, 6°12.597′W), located in the Bay of Cádiz, were invaded by *A. franciscana* >30 years ago. Trace elements discharged by the Odiel and Tinto rivers produce a plume of contaminants into the Gulf of Cádiz, from where it enters Cádiz Bay (Palanques et al., 1995; Hanebuth et al., 2018). In addition, industrial and urban wastewater contributes to pollution by trace elements in this area (Ligero et al., 2002; Carrasco et al., 2003).

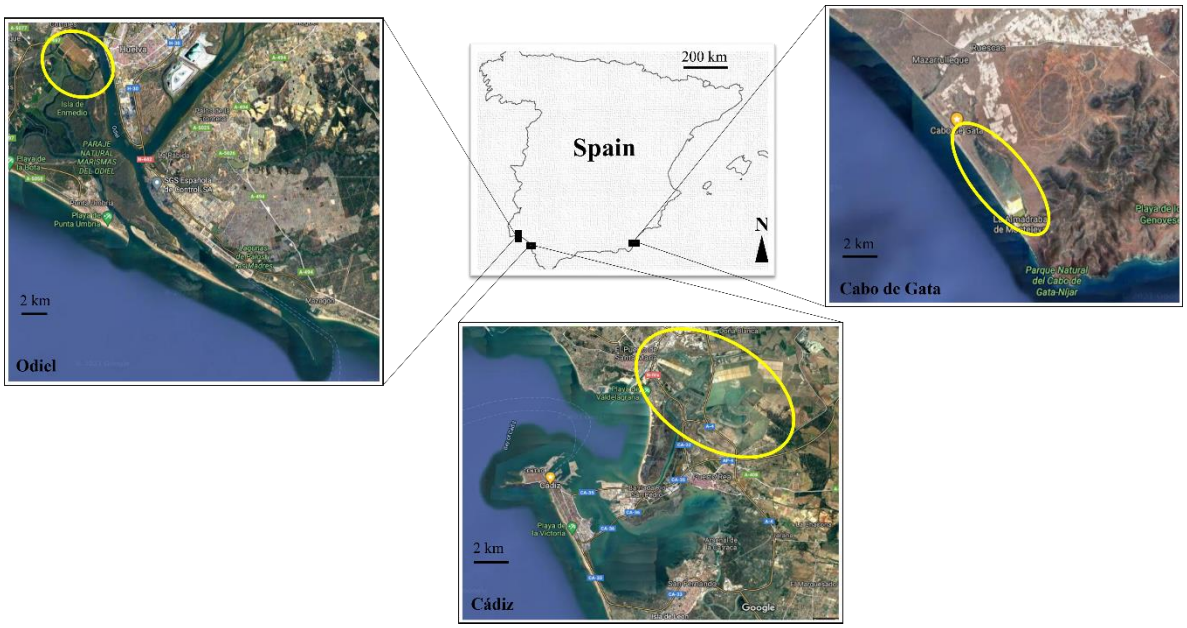


Figure 1: Location of the three study sites (Cabo de Gata, Odiel and Cádiz). [source: Google Maps 2021].

I.2.2. Sample collection

In April 2014, samples of sediment, water and *Artemia* (adults) were collected from different ponds along salinity gradients ('low' (S=135-145), 'medium' (S=155-165), and 'high' (S=180-190)) at each of the three study sites (**Table 1**). Although ionic composition of salts also has a strong effect on biota such as *Artemia* (Frisch et al., 2021), we did not consider it since all ponds were fed with sea water. *Artemia* adults were sampled with a sweep net (1 mm mesh size) and transported alive to the laboratory. The surface sediments (0-5 cm depth, i.e., those in most direct contact with our study organisms), and water samples were collected in pre-cleaned polyethylene containers. Furthermore, in Odiel only, we took three sediment-cores (using a 19.6 cm^2 corer to a depth of 3 cm) from the low salinity ponds where *A. parthenogenetica* were sampled to collect *C. salinarius* larvae, as well as samples of adult *O. notabilis* taken with a sweep net (1 mm mesh size). These two taxa were only collected at low salinity because that is where they are abundant (Sánchez et al., 2006). Once collected, macroinvertebrates were transported alive to the laboratory where *C. salinarius* larvae were extracted from the cores using a 0.5 mm sieve, hence retaining only the later instars. All macroinvertebrates were kept in filtered pond water for 24-h to eliminate gut contents (Brooke et al., 1996). Samples were then drained and frozen in clean plastic zip-lock bags until trace element analysis. Each sample of *Artemia* was composed of a pool of at least 50 individuals to ensure enough material for analysis.

Table 1. Description of the samples collected. *Artemia parthenogenetica* (Ap); *A. franciscana* (Af); *Chironomus salinarius* (Cs); *Ochthebius notabilis* (Och).

Study site	Salinity (S)	Biotic samples*	Abiotic samples*
Cabo de Gata (30/4/2014)	Low (S=140)	Ap	Sediment, Water
	Medium (S=155)	Ap	Sediment, Water
	High (S=180)	Ap	Sediment, Water
Odiel (22/4/2014)	Low (S=135)	Ap, Cs, Och	Sediment, Water
	Medium (S=160)	Ap	Sediment, Water
	High (S=190)	Ap	Sediment, Water
Cádiz (23/4/2014)	Low (S=145)	Af	Sediment, Water
	Medium (S=165)	Af	Sediment, Water
	High (S=187)	Af	Sediment, Water

* The number of replicates collected were 4 for each of *A. parthenogenetica* from Cabo de Gata, *A. franciscana* from Cádiz, *A. parthenogenetica* at 190 salinity, *C. salinarius*, *O. notabilis* and abiotic samples. For *A. parthenogenetica* from Odiel at 135 and 160, the number of samples collected were 6 and 8, respectively. At the time of the study, *A. franciscana* was not recorded in Odiel.

I.2.3. Samples processing for trace element analysis

Solid samples (sediments and macroinvertebrates) were dried to constant weight and then digested using an Anton Paar Multiwave Pro microwave digestion system with a 24 Teflon vessel carousel. Dry samples (0.2 g – 0.5 g) were weighed accurately into vessels, and ultra-trace element grade concentrated HNO_3 (2 mL) was added to pre-digest samples overnight at room temperature (~21 ºC). The next day, ultra-trace element grade hydrogen peroxide H_2O_2 (0.5 mL) was added, and the vessels were sealed and heated using a heating program with a maximum temperature of 160 °C. Finally, digests were diluted to 14 mL total volume with H_2O (Milli-Q grade) and stored at 4 °C until analysis. Water samples were digested as above (using HNO_3 and H_2O_2), but in open pure quartz digest tubes using a heated block digestor (Martín-Vélez et al., 2021).

Samples were analysed for a suite of nine elements – As, Cd, Co, Cu, Cr, Mn, Ni, Pb, and Zn – using inductively coupled plasma optical emission spectrometry (ICP-OES; Varian 720ES with SPS3 autosampler). Blanks and certified reference material (lobster hepatopancreas CRM: NRC-CNRC TORT-2, National Research Council, Canada) and spike solutions were processed and analysed at the same time for quality assurance/control purposes. Limits of detection (LODs; calculated from blanks) and recovery values (%; calculated from CRM and spikes) are shown in Supplementary Material (SM) (**Table S1)**. In solid samples (sediments and macroinvertebrates), trace elements concentrations were determined on a dry weight basis (Martín-Vélez et al., 2021).

I.2.4. Assessment of potential ecological risks

To assess risk, trace element levels found in the sediment and water of the three study sites were compared with international and local guidelines for the protection of aquatic life. The guidelines used for sediment were the Effect Range Low (ERL) and Effect Range Medium (ERM), levels proposed by Long et al. (1995), and the Lowest Effect Level (LEL) and Severe Effect Level (SEL), proposed by USEPA (2001). Values below the ERL and LEL indicated that adverse biological effects would be rare, whereas element concentrations above the ERM and SEL indicated that a probable biological effect would frequently occur. For water, the guidelines used were the Criteria of Continuous Concentration (CCC) and the Criteria of Maximum Concentration (CMC), established to prevent chronic and acute toxicity to aquatic organisms, respectively (USEPA, 2002), alongside values derived for regional government use, namely the Quality of Water for Limited waters (QoWL**)** values (Boja, 1997).

I.2.5. Bioaccumulation Factors

Biota versus sediment/water accumulation factors (BSAF and BAF, respectively) were calculated as below:

$$BSAF = C_O / C_S \quad (1)$$

$$BAF = C_O / C_W \quad (2)$$

where C_O is the concentration of an element in an organism, C_S is the concentration in the sediment, and C_W is the concentration in water. BAF was calculated for *A. parthenogenetica*, *A. franciscana* and *O. notabilis*. BSAF was calculated for *O. notabilis* and *C. salinarius*.

Dallinger (1993) proposed three groups of organisms based on BSAF values: macroconcentrator (BSAF > 2), microconcentrator (1 > BSAF < 2) and deconcentrator (BSAF < 1). USEPA (2011) presented a classification based on BAF values, which were: very highly bioaccumulative (BAF > 5000), highly bioaccumulative (1000 > BAF < 5000), moderately bioaccumulative (100 > BAF < 1000), and little bioaccumulative (BAF < 100).

I.2.6. Statistical analysis

For statistical purposes, for element concentrations < LOD, which were only found in water samples, a value of half of the respective LOD was assigned. For significant effects ($p < 0.05$), marginal mean pairwise tests were conducted for multiple comparisons. Final models were chosen

after testing for normal distribution of the residuals. Statistical analysis was performed with SPSS version 24 and Primer version 6.

I.2.6.1. Trace elements in sediments, water, and biota

First, trace elements concentration in sediment, water and *Artemia* were compared among study sites using one-way ANOVAs and Tukey post hoc test (for Cd, Ni, Pb and Zn in sediment; Ni in water; and As, Cr and Zn in *Artemia*) for multiple comparisons. When data fail to meet the assumption of homoscedasticity a Welch's ANOVA and Games-Howell post hoc test for multiple comparisons were chosen to perform the analysis. Next, with the samples collected from the three study sites (Cabo de Gata, Odiel and Cádiz), Principal Component Analysis (PCA) was performed to assess the spatial patterns associated with elements concentration in sediment, water or *Artemia* at different levels of salinity ('low', 'medium', and 'high'). The data used in the ANOVA and PCA analysis was log-transformed. Then, General Linear Models (GLMs), with normal distributions and identity link function, were used to test the effect of 'study site' on the concentrations of elements found in the different *Artemia* populations. For this, the element concentration in *Artemia* was the dependent variable; with 'study site' (Cabo de Gata, Odiel, Cádiz) and 'salinity level' ('low', 'medium', and 'high') as fixed factors; 'replicate' (i.e., number of samples of sediment, water or biota analysed for trace element content) as a random factor; and 'water element concentration' as covariate. A second set of GLMs were used to explore the differences in elements concentration in the three species sampled from Odiel at the same salinity. In this case, the element concentration in species was the dependent variable; 'species' (*C. salinarius*, *O. notabilis* and *A. parthenogenetica*) was a fixed factor; and 'replicate' was a random factor.

I.2.6.2. Bioaccumulation potential in biota

GLMs, with normal distribution and identity link function, were used to test the effect of study site on the bioaccumulation of trace elements in the different *Artemia* populations. For this, the bioaccumulation factor (BAF) value was used as the dependent variable; with 'study site' (Cabo de Gata, Odiel, Cádiz) and 'salinity level' ('low', 'medium', and 'high') as fixed factors; and 'replicate' as random factor. Data for BAF for Co, Cu, Mn and Pb were log-transformed to better fit the model. A second set of GLMs were used to explore the effect of 'species' (using data from Odiel) on the bioaccumulation of trace elements. In this case, the bioaccumulation factor (BSAF or BAF) value was used as the dependent variable; 'species' (*C. salinarius* and *O. notabilis* for BSAF; and *A. parthenogenetica* and *O. notabilis* for BAF) as fixed factor; and 'replicate' as random factor. BSAF values for As and Pb were log-transformed to better fit the model.

I.3. Results

I.3.1. Trace elements in sediment, water and *Artemia*

Sediment samples had significantly higher concentrations (µg g^{-1}, d.w.) of Pb in Cabo de Gata; As and Cu in Odiel; and Cr and Ni in Cádiz **(Table 2 and Table S2)**. Some trace elements exceeded sediment threshold guidelines, namely As, Pb and Zn (> ERL and LEL) in Cabo de Gata; As (> ERM and SEL), Cu, Zn (> ERL and LEL) in Odiel; and Ni (> ERL and LEL), Cr, Cu, and Pb (> LEL) in Cádiz (**Table 2**). In water, samples had significantly higher concentrations (µg L^{-1}) of As and Cu in Odiel (**Table 2 and Table S2**). Elements exceeding water threshold guidelines were Pb (> CCC and QoWL), Cu (> CCC) and Zn (> QoWL) in Cabo de Gata; As (> CMC and QoWL), Cu (> CMC), Pb (> CCC) and Zn (> QoWL) in Odiel; and As (> CCC and QoWL), Cu, Ni (> CCC), and Pb (> CCC and QoWL) in Cádiz (**Table 2**). *Artemia* samples showed significantly higher concentrations (µg g^{-1} d.w.) of Pb in Cabo de Gata; Cu and Zn in Odiel; and Cr and Ni in Cádiz **(Table 2 and Table S2)**.

Table 2. Element concentrations in sediment (mean ± SE; µg g^{-1}, d.w.), water (mean ± SE; µg L^{-1}) and *Artemia* (mean ± SE; µg g^{-1}, d.w.) samples collected from Cabo de Gata, Odiel and Cádiz. Sediment quality guideline (µg g^{-1}, d.w.), water quality guideline (µg L^{-1}) and reference values for *Artemia* (range; µg g^{-1}, d.w.). Values higher than those of the guidelines are in grey and marked with different numbers (1 if levels are above ERL; 2 if levels are above ERM; 3 if levels are above LEL; 4 if levels are above SEL; 5 if levels are above CCC; 6 if levels are above CMC; and 7 if levels are above QoWL).Different letters indicate significant differences ($p<0.05$) among study sites. (Continues)

	Type of sample	Study site			Sediment guidelines[a,b]				Water guidelines[c,d]			Reference values[**]
		Cabo de Gata	Odiel	Cádiz	ERL[1]	ERM[2]	LEL[3]	SEL[4]	CCC[5]	CMC[6]	QoWL[7]	Range
As	Sediment	11.9±1.5[1,3]B	89.6±12.0[1,2,3,4]A	4.3±0.3C	8.2	70	6	33	36	69	25	0.9 - 5.1[f,g]
	Water	**< LOD**	151.5±9.4[5,6,7]A	37.6±9.0[5,7]B								
	Artemia	16.6±0.9B	41.2±1.5A	37.6±2.5A								
Cd	Sediment	0.3±0.0A	0.4±0.0A	0.3±0.0A	1.2	9.6	0.6	10	7.9	33	2.5	0.04 - 0.54[e,f,g,h]
	Water	**< LOD**	**< LOD**	**< LOD**								
	Artemia	0.2±0.0A	0.1±0.0B	0.4±0.1A,B								
Co	Sediment	3.0±0.3B	8.1±0.8A	7.2±0.3A	-	-	-	-	-	-	-	2.3 - 2.9[g]
	Water	**< LOD**	**< LOD**	**< LOD**								
	Artemia	1.0±0.1B	1.5±0.1A	1.6±0.2A,B								
Cr	Sediment	12.6±1.0C	26.6±3.1[3]B	38.0±1.8[3]A	81	370	26	110	-	-	10	12.5 - 12.6[g]
	Water	9.0±4.7A	2.5±1.0A	**< LOD**								
	Artemia	1.7±0.2C	2.6±0.3B	5.1±0.7A								
Cu	Sediment	12.5±1.5C	84.9±3.8[1,3]A	18.9±1.2[3]B	34	270	16	110	3.1	4.8	20	0.09 - 27.2[e,f,g,h]
	Water	4.2±0.5[5]B	16.0±2.9[5,6]A	3.8±0.7[5]B								
	Artemia	14.5±1.5B	36.9±3.7A	19.1±2.8B								
Mn	Sediment	196.5±31.1B	298.9±34.6A,B	365.5±20.5A	-	-	460	1100	-	-	-	-
	Water	11.6±2.1B	421.7±72.4A	525.8±153.4A								
	Artemia	57.9±8.8B	106.2±9.8A	162.1±38.5A,B								

[*]< LOD: Levels below the analytical method.
[a]ERL (Effects Range Low) and ERM (Effects Range Median) (Long et al., 1995).
[b]LEL (Lowest Effect Level) and SEL (Severe Effect Level) (USEPA, 2001).
[c]CCC (Criteria of Continuous Concentration) and CMC (Criteria of Maximum Concentration) (USEPA, 2002).
[d]QoWL (Quality of Water: Limited waters) (Reference value based on local laws; BOJA, 1997).
[**]Reference values based on concentration range found in *Artemia* spp. from different unpolluted areas around the globe, using: [e]Olney et al. (1980); [f]Petrucci et al. (1995); [g]Leonova et al. (2007); [h]Aloui et al. (2012).

Table 2. Continued.

	Type of sample	Study site			Sediment guidelines[a,b]				Water guidelines[c,d]			Reference values[**]
		Cabo de Gata	Odiel	Cádiz	ERL[1]	ERM[2]	LEL[3]	SEL[4]	CCC[1]	CMC[2]	QoWL[3]	Range
Ni	Sediment	7.2±0.8**C**	15.2±1.1**B**	21.7±0.9[1,3]**A**	20.9	51.6	16	75	8.2	74	25	12.9 - 16.8[g]
	Water	8.2±1.8**A**	7.3±0.9**A**	9.9±1.4[5]**A**								
	Artemia	2.9±0.2**B**	2.2±0.1**C**	5.3±0.8**A**								
Pb	Sediment	133.7±17.1[1,3]**A**	29.0±3.8**B**	39.1±9.7[3]**B**	46.7	218	31	250	5.6	140	10	0.25 - 6.2[e,f,g,h]
	Water	15.4±2.7[5,7]**A**	10.0±1.0[5]**A**	10.8±1.3[5,7]**A**								
	Artemia	31.4±6.2**A**	4.0±0.5**B**	5.4±1.4**B**								
Zn	Sediment	166.7±32.2[1,3]**A**	211.9±27.9[1,3]**A**	59.4±4.9**B**	150	410	120	820	81	90	25	2.8 – 144[e,f,g,h]
	Water	31.7±3.3[7]**A**	59.4±10.4[7]**A**	14.3±3.7**B**								
	Artemia	138.1±18.0**B**	228.0±20.8**A**	110.9±14.0**B**								

[*]<LOD: Levels below the analytical method.
[a]ERL (Effects Range Low) and ERM (Effects Range Median) (Long et al., 1995).
[b]LEL (Lowest Effect Level) and SEL (Severe Effect Level) (USEPA, 2001).
[c]CCC (Criteria of Continuous Concentration) and CMC (Criteria of Maximum Concentration) (USEPA, 2002).
[d]QoWL (Quality of Water: Limited waters) (Reference value based on local laws; BOJA, 1997).
[**]Reference values based on concentration range found in *Artemia* spp. from different unpolluted areas around the globe, using: [e]Olney et al. (1980); [f]Petrucci et al. (1995); [g]Leonova et al. (2007); [h]Aloui et al. (2012).

In the PCA biplot for sediments (**Figure 2A; Table S3** of the SM), the first two principal components (PC1; PC2) accounted for 74.4 % of the total variance (PC1 explains 45.7 % and PC2 explains 28.7 % of the total variance). The variable As (with a coefficient of -0.75) had the strongest role in explaining the variation in PC1, whereas in PC2 the variable Pb explained most of the variation (with a coefficient of 0.78). In PC1, a general separation among salinities within sites is visible, but no clear pattern can be distinguished.

In the PCA biplot for water (**Figure 2B; Table S3** of the SM), the first two principal components accounted for 71.8 % of the total variance (PC1 explains 55.8 % and PC2 explains 16.0 % of the total variance). The variable Mn (with a coefficient of -0.90) had the strongest role in explaining the variation in PC1, whereas Zn explained most of the variation in PC2 (with a coefficient of 0.74). A general separation among salinities is visible within sites, with higher salinities being associated with a lower PC1.

In the PCA biplot for *Artemia* (**Figure 2C; Table S3** of the SM**),** the first two principal components accounted for 80.0 % of the total variance (PC1 explains 49.0 % and PC2 explains 31.0 % of the total variance). The variable Cd (with a coefficient of -0.60) had the strongest role in explaining the variation in PC1, whereas Pb explained most of the variation in PC2 (with a coefficient of -0.83). A general separation among salinities is visible, with the highest salinity having a consistently higher PC1 value than the lowest salinity.

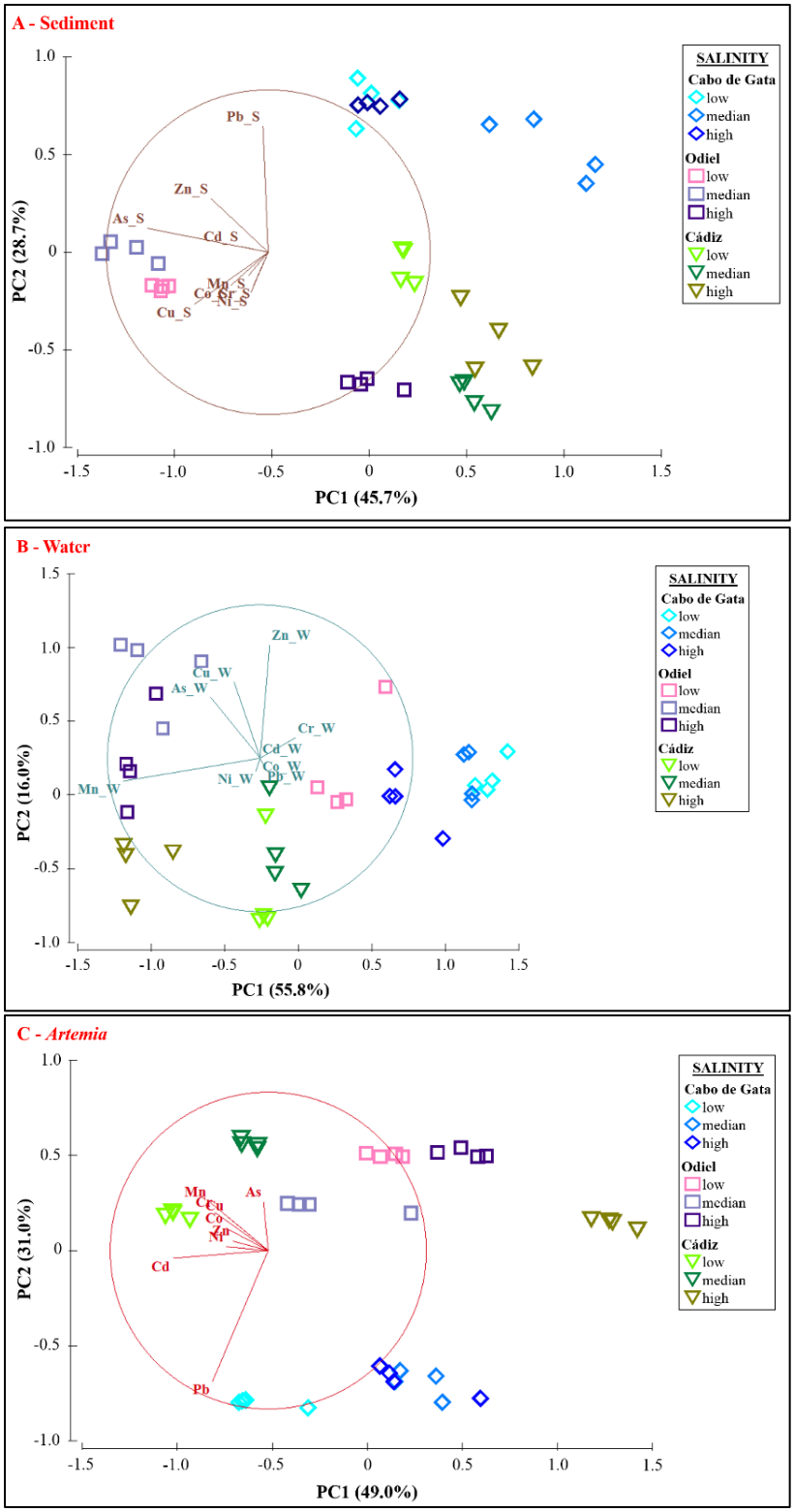


Figure 2. Principal Component Analysis (PCA) biplot of sediment (A), water (B) and (C) *Artemia* trace element concentrations, measured at three different sites (Cabo de Gata, Odiel and Cádiz) at different salinity levels (low, median, and high).

The GLM showed a significant main effect of 'study site' on the concentration of the nine trace elements analysed in *Artemia* (**Table S4a** of the SM). Marginal mean pairwise tests showed that *A. franciscana* from Cádiz had significantly higher concentrations of Cd, Cr, and Ni; *A. parthenogenetica* from Odiel had significantly higher concentrations of Cu and Zn; while *A. parthenogenetica* from Cabo de Gata had significantly higher concentrations of Pb and the lowest concentration of As, Co and Mn (**Figure 3**). A significant main effect of 'salinity' was observed for eight elements. Overall, pairwise results showed that the lowest concentration of elements measured in *Artemia* were found at 'high' salinity (**Table S4b** of the SM). 'Water element concentration' had a positive significant effect on concentrations in *Artemia* for Zn, and a negative effect for Mn (**Table S4** of the SM).

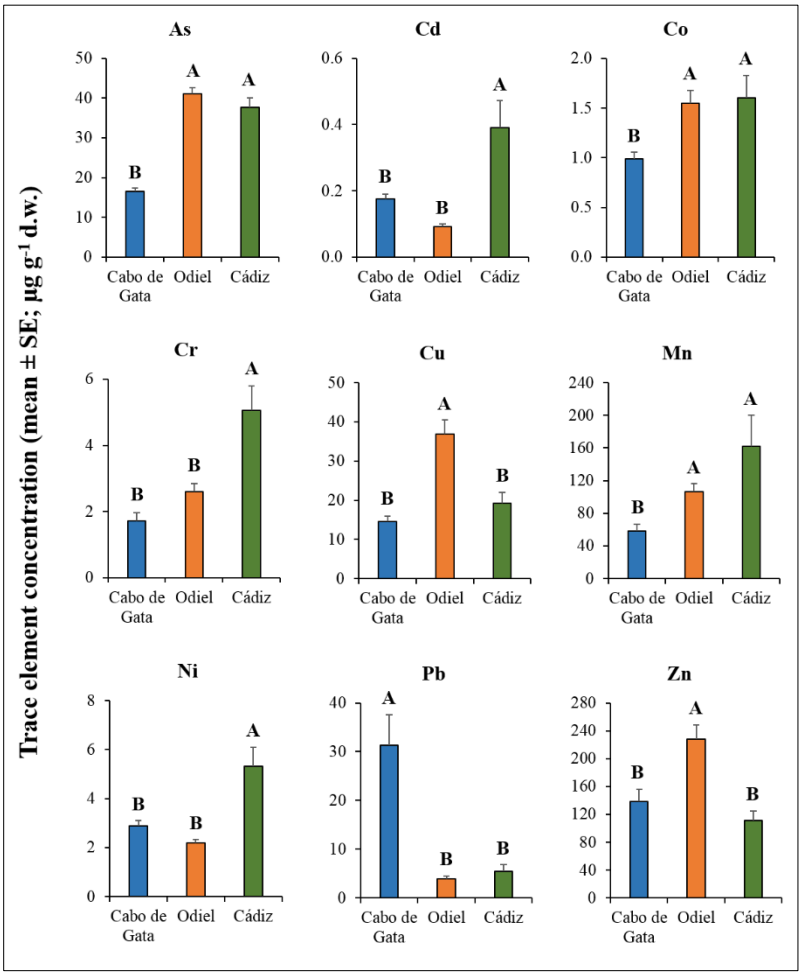


Figure 3. Trace element concentrations (mean ± SE; µg g^{-1}, d.w.) in *A. parthenogenetica* from Cabo de Gata and Odiel and *A. franciscana* from Cádiz. Different letters indicate significant differences ($p < 0.05$) among populations.

I.3.2. Bioaccumulation of trace elements by *Artemia*

BAF levels were the highest for Mn and Pb in Cabo de Gata; Cu in Odiel; and As, Cd, Co, Cr, Ni and Zn in Cádiz. Overall, results suggested that among the three studied populations, *A. franciscana* from Cádiz had higher potential, and *A. parthenogenetica* from Odiel had lower potential, to bioaccumulate trace elements from water (**Table S5** of the SM). GLM analysis found a significant main effect of 'study site' for the bioaccumulation of eight elements in *Artemia* (**Table S6a** of the SM). Marginal mean pairwise tests showed that *A. franciscana* from Cádiz bioaccumulated significantly more As, Cd, Cr and Ni compared to native populations; while *A. parthenogenetica* from Cabo de Gata bioaccumulated significantly more Mn, Pb and less Co than the other study populations (**Figure 4**). Furthermore, bioaccumulation in *Artemia* was, generally, the lowest at 'high' salinity (**Table S6b** of the SM).

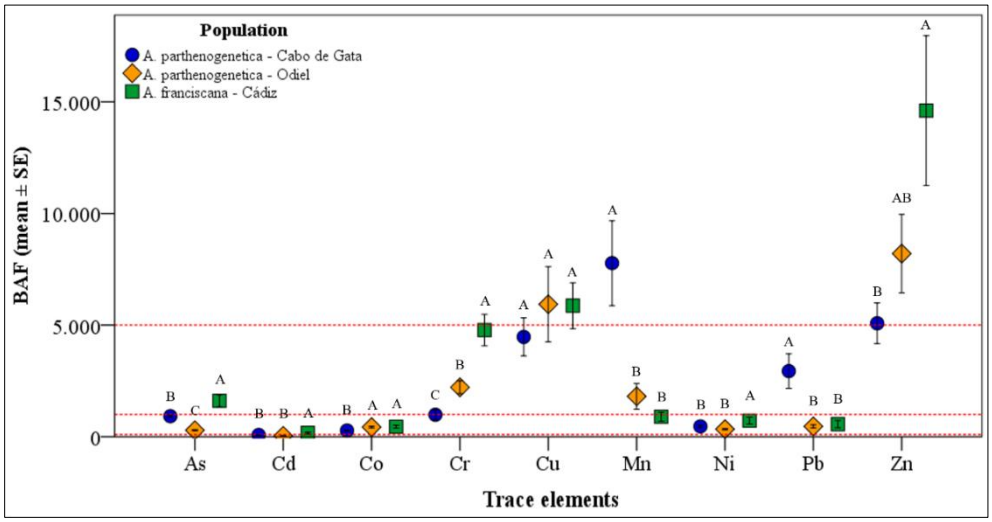


Figure 4. Bioaccumulation factor (BAF) calculated as the ratio of the trace element concentrations in *Artemia* and the element concentrations in water, from three study sites (Cabo de Gata, Odiel and Cádiz). Different letters indicate significant differences ($p < 0.05$) among populations. Reference values are represented in red lines BAF (USEPA, 2011): BAF > 5000 (very highly bioaccumulative), 1000 > BAF < 5000 (highly bioaccumulative), 100 > BAF < 1000 (moderately bioaccumulative), and BAF < 100 (little bioaccumulative).

I.3.3. Trace elements concentrations in different species from Odiel

Concentrations (µg g^{-1}, d.w.) of Cr, Mn and Pb were higher in *C. salinarius*; Co, Ni and Zn were higher in *A. parthenogenetica*; and As, Cd and Cu were higher in *O. notabilis* (**Table S7** of the SM). GLMs showed a significant main effect of 'species' in terms of element concentration for six elements (**Table S8** of the SM). Marginal mean pairwise tests showed that *A. parthenogenetica* had

significantly higher concentrations of Zn; *C. salinarius* had significantly higher concentrations of Cr and Pb; and *O. notabilis* had significantly higher concentrations of As and Cu (**Figure 5**). Both *A. parthenogenetica* and *C. salinarius* showed significantly higher concentrations of Ni than *O. notabilis*.

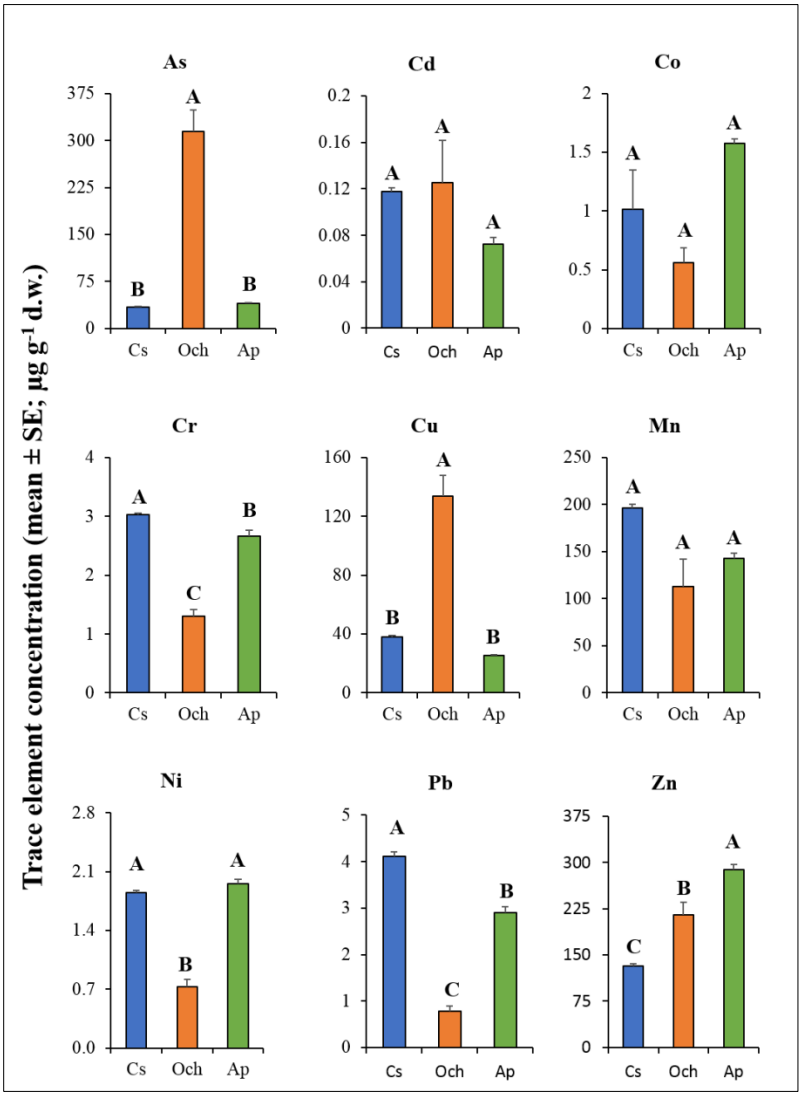


Figure 5. Trace element concentrations (mean ± SE; µg g^{-1}) in *Chironomus salinarius* (Cs), *Ochthebius notabilis* (Och) and *Artemia parthenogenetica* (Ap) from Odiel. Different letters indicate significant differences ($p < 0.05$) among species.

I.3.4. Trace element bioaccumulation in species from Odiel

GLM analysis on the bioaccumulation of trace elements from sediment by biota showed a significant main effect of 'species' for the BASF for six elements (**Table S9a** of the SM). Marginal

mean pairwise tests showed that *O. notabilis* bioaccumulated significantly more As, Cu and Zn, whereas *C. salinarius* bioaccumulated significantly more Cr, Ni and Pb (**Figure 6A; Table S10a** of the SM).

Regarding bioaccumulation of trace elements from water by biota, GLM analysis found a significant main effect of 'species' for the BAFs for six elements analysed (**Table S9b** of the SM). Marginal mean pairwise tests showed that *A. parthenogenetica* bioaccumulated significantly more Co, Cr, Ni and Pb, whereas *O. notabilis* bioaccumulated significantly more As and Cu (**Figure 6B; Table S10b** of the SM).

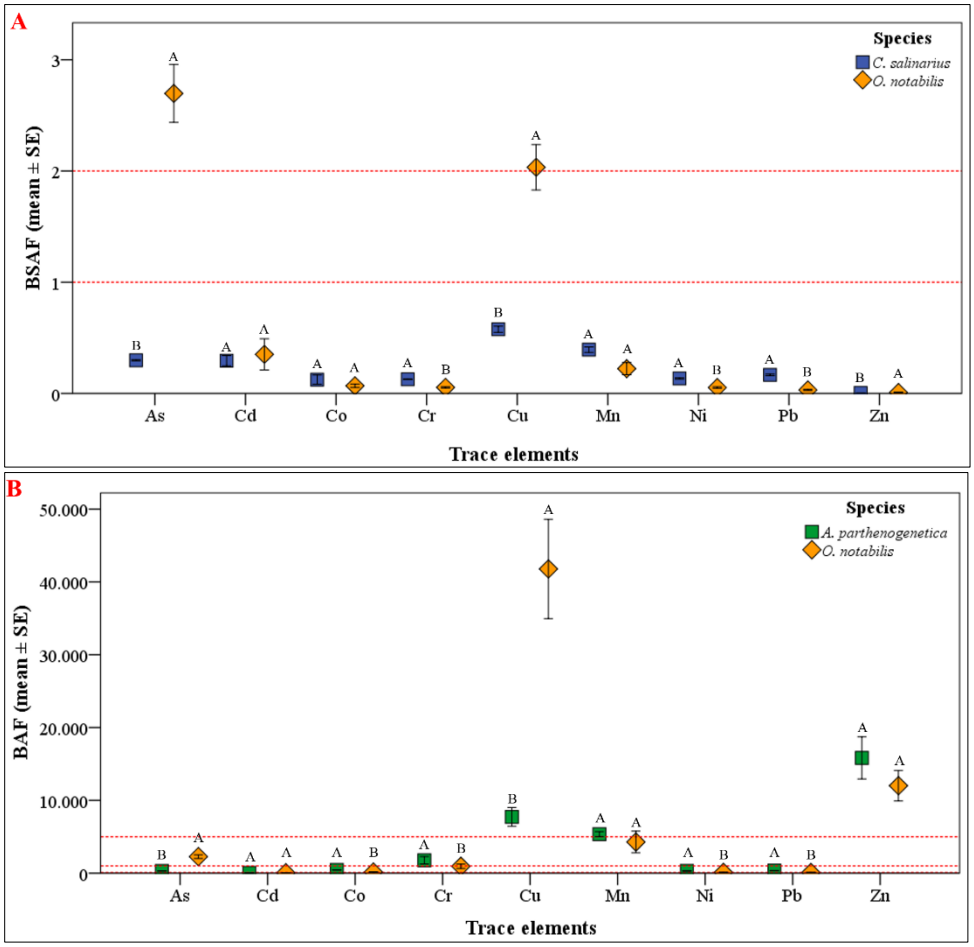


Figure 6. Bioaccumulation factors calculated as the ratio of trace element concentrations in biota (*A. parthenogenetica*, *C. salinarius* or *O. notabilis*) and the element concentrations in sediment (**A**; BSAF) or water (**B**; BAF) collected from Odiel. Different letters indicate significant differences ($p < 0.05$) among species. Reference values are represented in red lines BSAF (Dallinger, 1993): BSAF > 2 (macroconcentrator), 1 > BSAF < 2 (microconcentrator) and BSAF < 1 (deconcentrator); and BAF (USEPA, 2011): BAF > 5000 (very highly bioaccumulative), 1000 > BAF < 5000 (highly bioaccumulative), 100 > BAF < 1000 (moderately bioaccumulative), and BAF < 100 (little bioaccumulative).

I.4. Discussion

As far as we know, this is the most detailed study to date regarding trace element bioaccumulation in biota from hypersaline aquatic ecosystems (accounting for salinity) and its implications in a scenario of biological invasion.

I.4.1. Sediment and water quality

In general, we found high levels of trace elements in the study saltpans, occasionally exceeding threshold guidelines. Patterns varied among saltpans, depending on pollution sources at the respective sites. In Cabo de Gata, As and Zn in the sediments and Cu in the water showed concentrations slightly above those defined to cause adverse effects in aquatic communities, whereas the concentrations of Pb far exceeded those guidelines (Long et al., 1995; USEPA, 2001, 2002). These results may be related to the fact that this area is located in a volcanic belt naturally reach in trace elements, particularly Pb, Zn and Cu (Viladevall et al., 1999; Hernandez, 2005). Moreover, the area may be affected by the erosion of tailings from the Almeria mining district which present high concentrations of As, Cu, Pb and Zn (among other trace elements; Flores and Rubio, 2010). In the Odiel saltpans, the elements of most concern in the sediments were Cu, Zn and especially As, and As and Cu in water. These results were expected, given contamination from the Odiel and Tinto rivers. This contamination is well documented and derives from centuries of drainage of abandoned mines (along the Iberian Pyrite Belt) and also from industrial discharges (Nelson and Lamothe, 1993; Ruiz, 2001; Sarmiento et al., 2009). Finally, in the case of the Cádiz saltpans, high concentrations of Cr, Mn and Ni in sediments and Mn in water were detected, but most notable was the detection of As above regional guidelines. Trace elements discharged by the Odiel and Tinto rivers, which drain the Iberian Pyrite Belt (Leistel et al., 1997), produce a plume of contaminants into the Gulf of Cádiz (Elbaz-Poulichet et al., 2001; Laiz et al., 2020). However, the lower levels of trace elements detected in Cádiz saltpans, compared with Odiel saltpans, suggest a low impact of the polluted plume, as this will be diluted by the Atlantic Ocean before contaminating the Cádiz coast (Laiz et al., 2020). Furthermore, Cádiz saltpans may also be affected by pollution from the Guadalete River. For nearly 25 years (from the 1960s until 1988, when the recovery started) this river was subjected to intense pollution from urban, industrial, and agricultural discharges (Campana et al., 2005).

I.4.2. Trace elements in *Artemia* from different saltpans

Artemia, a key filter feeder in hypersaline ecosystems, is able to assimilate trace elements (essential and non-essential) from its aquatic environment even if they are present at extremely low concentrations (Petrucci et al., 1995; Varó et al., 2000). There are many laboratory studies investigating the effects of trace elements on *Artemia* (see Libralato et al., 2016 for a review); however, there is a paucity of information regarding exposure in natural environments.

Here, concentrations of trace elements found in *Artemia* varied by site, and the elements that showed the highest concentration in each *Artemia* population were in line with the highest concentrations found in water from the saltpan where they were sampled. Thus, *Artemia* from Cabo de Gata had the highest concentrations of Pb, *Artemia* from Odiel the highest As, Cu and Zn, and *Artemia* from Cádiz the highest Mn and Ni. Overall, the concentration of As in all populations, Pb in *A. parthenogenetica* from Cabo de Gata, and Cu and Zn in *A. parthenogenetica* from Odiel were significantly above the reference values found for *Artemia* from unpolluted saltpans (Olney et al., 1980; Petrucci et al., 1995; Aloui et al., 2012; Leonova et al., 2007; details in **Table 2**). Furthermore, similar As concentrations were recorded in native *A. parthenogenetica* from Odiel and invasive *A. franciscana* from Cádiz, even though As in water and sediment was lower in Cádiz than in Odiel (nearly four-times lower in water). This may relate to As speciation (not determined here), or physiological mechanisms developed by the different *Artemia* species to regulate or detoxify this element. In addition, high concentrations of As can be related to assimilation and biotransformation to organo-As species (methylated species, arseno-sugars, arseno-betaine, etc.) or through complexation with other elements (such as Se) (Caumette et al., 2012; Glabonjat et al., 2020). Hence, to fully understand the biogeochemical As cycle in these complex systems, more research is needed.

Regarding bioaccumulation in *Artemia*, the elements with BAFs > 1000 are of most concern, as they are more readily accumulated (USEPA, 2011). Here, bioaccumulation of Zn and Cu (for native and invasive *Artemia*), Mn (for native species), Cr (for native and invasive *Artemia* from Odiel and Cádiz, respectively), Pb (for native species from Cabo de Gata) and As (for invasive species) were noted. The elements Zn, Cu, Cr and Mn are considered essential and are actively regulated by living organisms (Rainbow, 2007). However, Cr may be toxic to *Artemia* depending on its speciation since Cr (VI) is notably highly toxic, while Cr III is essential (Devi et al., 2015). On the other hand, Pb and As are considered non-essential elements, and have no known biological functions (Rainbow, 2007). Thus, it is important to monitor the bioaccumulation of these elements in these populations in the future, as they may trigger deleterious effects in the aquatic life inhabiting Cabo de Gata and Odiel, respectively.

Overall, the invasive *A. franciscana* from Cádiz stood out as having a higher potential to bioaccumulate both essential (Cr) and non-essential elements (As, Cd and Ni) than its native counterpart. A possible explanation could be the feeding rate, given that this has been shown to be higher for *A. franciscana* than for native species (Sánchez et al., 2016a). The differences in bioaccumulation of elements between native and invasive *Artemia* might also be related to differences in growth rate and age. *Artemia franciscana* has shorter pre-reproductive and between-brood periods, and by the time of the native species first brood, the invasive one is already producing its second brood (Amat et al., 2007). This may explain the lower levels of trace elements in *A. franciscana* as adults will likely be younger on average compared to adult *A. parthenogenetica*, although this was not quantified during our study. Bioaccumulation is not only related to feeding rate or life-history traits, and detoxification mechanisms (among other factors) could also have an influence (Rainbow, 2002). Thus, the high BAF values in *A. franciscana* may be the result of an inefficient detoxification mechanism, or alternatively much higher tolerance, as reported in previous works on aquatic invertebrates (Yu and Wang, 2002; Ng and Wang, 2004; Sanchéz et al., 2016b). Future work could investigate metallothionein in this species, as this can play an important role in the homeostatic regulation of essential trace elements (e.g., Cu and Zn) and in the detoxification of toxicants (e.g., see Amiard et al., 2006 for a review). Finally, despite the high levels of contamination found in Odiel, the native *A. parthenogenetica* inhabiting these saltpans generally had lower BAFs than other *Artemia* populations. Mining activities in the Odiel catchment date from prehistoric times (Nocete et al., 2005), so native *A. parthenogenetica* from this area are likely selected to tolerate high trace element concentrations. As such, they may have developed appropriate strategies for efficient metabolism of these elements (homeostasis).

Until recently, Odiel was one of the last refugia for native *Artemia* in southern Spain, but they have now been replaced by the invasive *A. franciscana* (between 2014 and 2016, authors personal observation). Since we found that *A. franciscana* appears to have a higher potential to bioaccumulate trace elements, especially As, the shift in *Artemia* species during an invasion to one with a higher bioaccumulation capacity may increase the bio-transfer of trace elements in hypersaline food webs. The more recent invasion of Odiel by *A. franciscana* opens up a future opportunity to study the potential effects that the replacement of native species by invasive ones has on this ecosystem. This can be particularly important in relation to waterbirds, for which *Artemia* is the main prey item (Sánchez et al., 2006). In fact, migratory grebes (*Podiceps nigricollis*) have already been shown to have high blood levels of As, Hg and Zn in Odiel, with food chain transfer through the consumption of *A. parthenogenetica* as the most likely route of exposure (Rodríguez-Estival et al., 2019). Other studies have suggested that high Se and Hg levels in grebes from Great

Salt Lake (Utah, USA) were transferred by their *A. franciscana* prey (Conover and Vest, 2009; Wright et al., 2020). In addition, because *A. franciscana* is used worldwide in aquaculture, the potential high capacity to bioaccumulate trace elements detected here should be of interest and concern because of its potential transfer to fish larvae (e.g., Wang and Wong, 2003; Soto-Jiménez et al., 2011).

I.4.3. Effect of salinity in the bioaccumulation of trace elements

Trace elements bioavailability and toxicity are known to be affected by various factors including salinity (Gheorghe et al., 2017), which has a strong influence on the precipitation/binding of trace elements within sediments (Nieto et al., 2007). Considering the bioaccumulation factors data here (as indicative of bioavailability), we found generally that, at the highest salinity sampled, bioaccumulation of trace elements by *Artemia* was the lowest. This is in accordance with the study of Nieto et al. (2007) which found that bioavailability of elements often decreases with increasing salinity due to trace elements complexation, although this earlier work was performed at lower salinities (10-35). Future work should also take into consideration other important factors known to affect trace element bioavailability, notably (i) sediment particle size, (ii) the dynamics of adsorption and mobilization of metals between water and sediment (Du Laing et al., 2009; Lin et al., 2013), (iii) pH/Eh, and (iv) the ionic composition of salts which can vary greatly inland (Frisch et al., 2021), as element solubility will vary markedly with respect to these variables (Nieto et al., 2007; Zhang et al., 2014).

I.4.4. Trace elements in macroinvertebrates from Odiel and Ecotoxicological implications

In aquatic environments, trace elements ultimately deposit into sediments through precipitation/adsorption, etc. (Du Laing et al., 2009; Lin et al., 2013). Therefore, we may expect benthic *C. salinarius* and *Ochthebius* to have higher trace element loads than *Artemia.* However, from the nine trace elements measured, only the concentration of Pb and Cr in *C. salinarius* and As and Cu in *O. notabilis* were higher than in *A. parthenogenetica*. The concentration of As found in *O. notabilis* was surprisingly high (243±50 μg g^{-1}, d.w.), seven to nine-fold higher than that measured in *C. salinarius* (26±5 μg g^{-1}, d.w.) and *A. parthenogenetica* (33±2 μg g^{-1}, d.w.).

Another important factor is the bioavailability of the element transferred from the abiotic environment (sediment or water) to the organism, which is ultimately translated in values calculated using bioaccumulation factors (BSAF or BAF, respectively). Studies on freshwater Diptera and Chironomidae suggest they are good accumulators of trace elements (e.g., Pastorino et al., 2020) due to their feeding and living habits (Gamito, 1994; Abellán et al., 2009) that put them into direct

contact with contaminated substrates (Santoro et al., 2009). Here, bioaccumulation of trace elements, from both sediment and water, varied with invertebrate species and element analysed. Results for BSAF showed that *C. salinarius* larvae is a deconcentrator of all the trace elements analysed (all at BSAF < 1). On the other hand, *O. notabilis* adults have the highest potential to bioaccumulate As and Cu, being macroconcentrators of those elements (BSAF > 2). We could not find any previous literature on As levels for *Ochthebius* or the Hydraenidae family. Regarding bioaccumulation of trace elements in *C. salinarius*, this showed higher BSAF values compared to those previously reported in freshwater *Chironomus* sp. (e.g., Desrosiers et al., 2008; Bervoets et al., 2016). However, all concentrations were below the critical body burdens described by Bervoets et al. (2016) in *Chironomus* sp. from freshwater systems, which may explain the low bioaccumulation of trace elements by this genus. On the other hand, *A. parthenogenetica* showed the highest potential to bioaccumulate Co, Cr, Ni and Pb, and *O. notabilis* the highest potential to bioaccumulate As and Cu from water. The elements of most concern, with BAFs > 1000 (as they are more readily accumulated; USEPA, 2011), were Cu, Zn, Cr and Mn (plus As in the case of *O. notabilis*). Again, bioaccumulation of essential elements, such as Cu, Cr and Zn, are often needed for normal metabolism in organisms and are highly regulated (Rainbow, 2007). Overall, feeding behaviour does not seem to be a major factor influencing bioaccumulation of trace elements among species, nor does habitat choice (water column or sediment) since the most striking differences were related to the bioaccumulation of As and Cu in *O. notabilis* from both sediment and water. Brown (1977) reported that aquatic invertebrates mostly uptake, and may bioaccumulate, trace elements through their diet. However, in aquatic insects (as beetles and chironomids) it has been demonstrated that some elements, such as As, can be bound either directly or indirectly – i.e., in association with iron oxide deposits – on the surface of the chitinous exoskeleton (see Hare, 1992 for a review). The load of elements bound on the Fe oxide incrustations will depend on the extent of time passed since the previous molt (see Hare, 1992 for a review). Furthermore, it has also been suggested that surface area-to-volume ratio play an important role on the concentration of trace elements in these organisms, with the ratio decreasing with increased body size. Thus, organisms with smaller sizes will have higher proportions of trace elements bound externally (see Hare, 1992 for a review). However, we do not know the timing of the last molt of *Ochthebius* or *Chironomus*, and we did not measure the size of the organisms. Still, this may help explain the high loads of trace elements found in *O. notabilis* adults because they may live longer, have no further moults (unlike insect larvae) and are the smallest species of the three. For this species, the physiological mechanisms of trace element bioaccumulation are unknown, as well as the existence (or not) of efficient detoxification mechanisms, which needs to be further investigated.

Overall, the high levels of trace elements, particularly As, assimilated and bioaccumulated by the invasive *A. franciscana* and *O. notabilis* suggest that these two species may be important sources of trace elements to waterbirds. This is of particular interest because all three study sites are Ramsar sites supporting more than 20,000 waterbirds. Among them are threatened species such as the black-tailed godwit (*Limosa limosa*), the curlew sandpiper (*Calidris ferruginea*), and the common redshank (*Tringa totanus*) (BirdLife International, 2015), for which these invertebrates may be an important source of contamination to them (Sánchez et al., 2005). Furthermore, birds may act as pollutant biovectors transporting contaminants transferred to them from their prey through feces and feathers, releasing them into new ecosystems, influencing ecological processes and contamination levels even in remote areas (e.g., Blais et al., 2007; Martín-Vélez et al., 2021).

I.5. Conclusion

The assimilation of specific trace elements in brine shrimp *Artemia* is related to pollution in the surrounding abiotic environment. However, we found that bioaccumulation depends on the species of *Artemia* and the trace element, and the invasive *A. franciscana* appears to have a higher potential to bioaccumulate trace elements than the native *A. parthenogenetica*. Bioaccumulation in *Artemia* was lowest at extreme salinities. In Odiel saltpans, bioaccumulation also depends on the species (*A. parthenogenetica*, *C. salinarius* and *O. notabilis*) and the trace element involved. The high potential bioaccumulation of As by *A. franciscana*, and especially by *O. notabilis*, is of particular concern due to its high toxicity. During a biological invasion, the shift to an *Artemia* species with a higher bioaccumulation capacity may increase the transfer of trace elements in hypersaline food webs, with potential impacts on waterbirds. Hypersaline aquatic systems are important conservation areas for waterbirds, and the ingestion of macroinvertebrates by waterbirds feeding in these Spanish saltpans, protected as Ramsar sites, may be an important source of toxic trace elements, such as As. The higher potential to bioaccumulate trace elements represents an additional indirect impact of the *A. franciscana* invasion that had not been previously recognised for this invasive species.

I. Supplementary Material

Table S1. Limits of detection (LOD) and recovery rates of spike solutions and the certified reference materials.

Element	LOD (ppb)			Recovery rates (%)	
	Water (µg/L)	Biota (µg/kg)	Sediment (µg/kg)	Spike solution	CRM TORT-2
As	36	226	471	99	96
Cd	5	29	60	97	94
Co	7	45	94	97	84
Cr	2	13	28	99	81
Cu	5	32	67	104	83
Mn	12	74	155	100	85
Mo	10	66	137	102	94
Ni	11	71	149	99	80
Pb	17	104	218	99	111
Se	44	276	576	98	107
Zn	9	57	119	101	98

Table S2. Results of the (a) one-way / Welch's ANOVA with element concentration in samples (sediment, water and *Artemia*) populations as the test field; and 'study site' / location (Cabo de Gata, Odiel, Cádiz) as group; and (b) post-hoc results (Tukey / Games-Howell) on multiple comparisons among 'study site'. *n.c.: not computed as all values were lower than LOD.; **n.s.d.: no statistical differences.

a)

Element	Sediment		Water		*Artemia*	
	F	p-value	F	p-value	F	p-value
As	69.36	**<0.001**	614.61	**<0.001**	92.77	**<0.001**
Cd	1.69	0.198	n.c.		16.26	**<0.001**
Co	21.81	**<0.001**	n.c.		6.23	**0.007**
Cr	60.05	**<0.001**	3.77	**0.043**	10.56	**<0.001**
Cu	238.71	**<0.001**	8.21	**0.002**	23.40	**<0.001**
Mn	9.83	**0.001**	65.80	**<0.001**	6.33	**0.007**
Ni	43.78	**<0.001**	1.64	0.207	11.48	**<0.001**
Pb	18.15	**<0.001**	1.58	0.228	43.08	**<0.001**
Zn	12.42	**<0.001**	8.46	**0.002**	8.39	**0.001**

b)

Element	Comparison	Sediment		Water		*Artemia*	
		S.E.	p-value	S.E.	p-value	S.E.	p-value
As	Cabo de Gata vs Odiel	0.12	**<0.001**	0.03	**<0.001**	0.04	**<0.001**
	Cabo de Gata vs Cádiz	0.07	**<0.001**	0.09	0.101	0.03	**<0.001**
	Odiel vs Cádiz	0.11	**<0.001**	0.09	**<0.001**	0.03	0.151
Cd	Cabo de Gata vs Odiel	n.s.d.		n.s.d.		0.05	**<0.001**
	Cabo de Gata vs Cádiz					0.21	0.979
	Odiel vs Cádiz					0.21	0.308
Co	Cabo de Gata vs Odiel	0.08	**<0.001**	n.s.d.		0.05	**0.005**
	Cabo de Gata vs Cádiz	0.06	**<0.001**			0.08	0.197
	Odiel vs Cádiz	0.05	0.964			0.09	0.976
Cr	Cabo de Gata vs Odiel	0.07	**0.002**	n.s.d.		0.09	**0.045**
	Cabo de Gata vs Cádiz	0.04	**<0.001**			0.10	**<0.001**
	Odiel vs Cádiz	0.07	**0.009**			0.09	**0.005**
Cu	Cabo de Gata vs Odiel	0.06	**<0.001**	0.12	**0.004**	0.06	**<0.001**
	Cabo de Gata vs Cádiz	0.07	**0.016**	0.08	0.674	0.10	0.813
	Odiel vs Cádiz	0.03	**<0.001**	0.12	**0.001**	0.10	**0.010**
Mn	Cabo de Gata vs Odiel	0.09	0.090	0.17	**<0.001**	0.08	**0.005**
	Cabo de Gata vs Cádiz	0.08	**0.002**	0.15	**<0.001**	0.15	0.156
	Odiel vs Cádiz	0.06	0.071	0.20	0.678	0.15	0.982
Ni	Cabo de Gata vs Odiel	0.05	**<0.001**	n.s.d.		0.04	**0.019**
	Cabo de Gata vs Cádiz	0.05	**<0.001**			0.08	**0.040**
	Odiel vs Cádiz	0.05	**0.001**			0.08	**0.002**
Pb	Cabo de Gata vs Odiel	0.12	**<0.001**	n.s.d.		0.10	**<0.001**
	Cabo de Gata vs Cádiz	0.13	**<0.001**			0.13	**<0.001**
	Odiel vs Cádiz	0.12	0.580			0.12	0.950
Zn	Cabo de Gata vs Odiel	0.10	0.305	0.13	0.575	0.08	**0.013**
	Cabo de Gata vs Cádiz	0.11	**0.001**	0.13	**0.006**	0.09	**0.245**
	Odiel vs Cádiz	0.10	**<0.001**	0.15	**0.002**	0.08	**<0.001**

Table S3. Summary of the eigenvectors obtained for the factors emerged after Principal Component Analysis (PCA) for sediment, water and *Artemia*.

Variable	Sediment		Water		*Artemia*	
	PC1	PC2	PC1	PC2	PC1	PC2
As	-0.75	0.15	-0.32	0.40	-0.03	0.31
Cd	-0.16	0.03	0.00	0.00	-0.60	-0.05
Co	-0.23	-0.21	0.00	0.00	-0.27	0.14
Cr	-0.09	-0.21	0.23	0.14	-0.34	0.24
Cu	-0.46	-0.32	-0.17	0.50	-0.26	0.22
Mn	-0.12	-0.14	-0.90	-0.15	-0.37	0.31
Ni	-0.11	-0.25	-0.03	-0.09	-0.27	0.02
Pb	-0.03	0.78	0.03	-0.07	-0.35	-0.83
Zn	-0.35	0.33	0.06	0.74	-0.22	0.06
Eigenvalues	0.50	0.31	0.83	0.24	0.46	0.29
%Variation explained	45.70	28.70	55.80	16.00	49.00	31.00
Cumulative %Variation explained	45.70	74.40	55.80	71.80	49.00	80.00

Table S4. Results of the (a) GLM analysis with element concentration in *Artemia* as the dependent variable; 'study site' / location (Cabo de Gata, Odiel, Cádiz) and 'salinity' (low, median, and high) as fixed factors; 'replicate' as random factor; and 'sediment element concentration' and 'water element concentration' as covariates; and (b) pairwise comparison among salinity levels. Estimates for "Cádiz", sample "8" and salinity "high" are not included as they were aliased, but they are effectively zero. Significant values are in bold. (Continues)

a) (Continues)

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
As	Intercept		33.56	7.50	98.26	1	<**0.001**	0.75
	Study site	Cabo de Gata	-21.99	2.80	30.89	2	<**0.001**	0.67
		Odiel	9.76	6.66				
	Salinity	low	3.30	3.37	0.79	2	0.461	0.05
		median	0.60	3.31				
	Replicate	1	4.33	7.52	0.50	7	0.829	0.10
		2	6.29	7.42				
		3	4.08	7.41				
		4	3.54	7.24				
		5	2.95	7.74				
		6	6.79	8.19				
		7	-5.44	9.11				
	Water element concentration		-47.76	49.80	0.92	1	0.345	0.03
Cd	Intercept		0.16	0.16	40.02	1	<**0.001**	0.53
	Study site	Cabo de Gata	-0.21	0.06	15.47	2	<**0.001**	0.50
		Odiel	-0.30	0.06				
	Salinity	low	0.17	0.05	10.18	2	<**0.001**	0.40
		median	0.23	0.05				
	Replicate	1	0.09	0.15	0.16	7	0.991	0.04
		2	0.11	0.15				
		3	0.09	0.15				
		4	0.09	0.15				
		5	0.09	0.16				
		6	0.04	0.17				
		7	-0.01	0.19				
Co	Intercept		0.77	0.50	168.14	1	<**0.001**	0.82
	Study site	Cabo de Gata	-0.61	0.18	6.87	2	<**0.001**	0.31
		Odiel	-0.10	0.18				
	Salinity	low	0.89	0.17	18.09	2	<**0.001**	0.54
		median	0.89	0.17				
	Replicate	1	0.23	0.48	0.36	7	0.920	0.01
		2	0.31	0.48				
		3	0.19	0.48				
		4	0.23	0.48				
		5	0.24	0.51				
		6	0.45	0.54				
		7	-0.30	0.62				

Table S4. Continued.

a) Continued.

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
Cr	Intercept		2.82	1.47	94.79	1	<**0.001**	0.74
	Study site	Cabo de Gata	-3.28	0.56	19.67	2	<**0.001**	0.57
		Odiel	-2.48	0.52				
	Salinity	low	2.63	0.50	15.48	2	<**0.001**	0.51
		median	2.16	0.50				
	Replicate	1	0.66	1.42	0.14	7	0.994	0.03
		2	0.66	1.41				
		3	0.49	1.41				
		4	0.74	1.41				
		5	0.48	1.51				
		6	0.64	1.60				
		7	-0.45	1.81				
	Water element concentration		-6.11	23.25	0.07	1	0.795	0.00
Cu	Intercept		-0.06	11.23	24.19	1	<**0.001**	0.40
	Study site	Cabo de Gata	-4.81	3.90	5.87	2	**0.007**	0.28
		Odiel	11.27	4.76				
	Salinity	low	10.98	4.12	13.18	2	<**0.001**	0.47
		median	19.10	3.75				
	Replicate	1	9.43	10.69	0.69	7	0.677	0.14
		2	8.10	10.57				
		3	5.63	10.58				
		4	6.71	10.56				
		5	12.20	11.55				
		6	7.72	11.87				
		7	-12.01	13.78				
	Water element concentration		456.89	243.20	3.53	1	0.070	0.11
Mn	Intercept		152.15	67.03	61.55	1	<**0.001**	0.65
	Study site	Cabo de Gata	-155.36	28.32	15.06	2	<**0.001**	0.50
		Odiel	-57.00	22.78				
	Salinity	low	57.97	28.26	6.60	2	**0.004**	0.31
		median	-23.68	23.62				
	Replicate	1	56.14	60.99	0.19	7	0.985	0.04
		2	53.76	61.04				
		3	54.23	60.99				
		4	39.35	60.79				
		5	42.13	64.90				
		6	36.69	68.97				
		7	39.79	79.46				
	Water element concentration		-99.60	33.47	8.85	1	**0.006**	0.23

Table S4. (Continued)

a) Continued.

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
Ni	Intercept		4.36	1.62	56.40	1	**<0.001**	0.65
	Study site	Cabo de Gata	-2.54	0.56	18.25	2	**<0.001**	0.55
		Odiel	-3.28	0.57				
	Salinity	low	2.07	0.55	7.22	2	**0.003**	0.33
		median	1.26	0.53				
	Replicate	1	0.33	1.50	0.08	7	0.999	0.02
		2	0.56	1.52				
		3	0.25	1.50				
		4	0.41	1.50				
		5	0.35	1.60				
		6	0.31	1.71				
		7	-0.39	1.92				
	Water element concentration		-52.59	49.45	1.13	1	0.296	0.04
Pb	Intercept		7.10	13.24	12.75	1	**0.001**	0.29
	Study site	Cabo de Gata	26.92	4.59	23.21	2	**<0.001**	0.61
		Odiel	-1.79	4.32				
	Salinity	low	14.28	4.69	7.17	2	**0.003**	0.32
		median	1.00	4.72				
	Replicate	1	-3.71	11.70	0.89	7	0.999	0.02
		2	-3.88	11.66				
		3	-4.13	11.67				
		4	-6.26	11.66				
		5	-4.18	12.43				
		6	-5.66	13.10				
		7	-0.89	14.95				
	Water element concentration		-211.86	339.12	0.39	1	0.537	0.01
Zn	Intercept		5.24	51.76	50.30	1	**<0.001**	0.59
	Study site	Cabo de Gata	14.07	18.99	8.52	2	**0.001**	0.36
		Odiel	80.35	21.25				
	Salinity	low	132.58	17.31	29.58	2	**<0.001**	0.66
		median	84.60	20.74				
	Replicate	1	26.06	50.01	0.55	7	0.793	0.11
		2	30.68	49.74				
		3	23.14	49.89				
		4	10.06	49.22				
		5	25.46	52.62				
		6	42.42	55.19				
		7	-48.45	63.16				
	Water element concentration		754.75	314.21	5.77	1	**0.023**	0.16

Table S4. Continued.

b)

Element	Comparison	SE	p-value
As	low vs median	2.434	0.275
	low vs high	3.375	0.336
	median vs high	3.309	0.858
Cd	low vs median	0.051	0.262
	low > high	0.053	**0.003**
	median > high	0.053	**0.000**
Co	low vs median	0.164	0.984
	low > high	0.169	**0.000**
	median > high	0.169	**0.000**
Cr	low vs median	0.485	0.334
	low > high	0.500	**0.000**
	median > high	0.501	**0.000**
Cu	low vs median	4.262	0.066
	low > high	4.123	**0.012**
	median > high	3.747	**0.000**
Mn	low > median	22.490	**0.001**
	low > high	28.262	**0.049**
	median vs high	23.617	0.324
Ni	low vs median	0.524	0.133
	low > high	0.548	**0.001**
	median > high	0.530	**0.024**
Pb	low > median	3.997	**0.002**
	low > high	4.693	**0.005**
	median vs high	4.716	0.834
Zn	low > median	20.206	**0.024**
	low > high	17.314	**0.000**
	median > high	20.743	**0.000**

Table S5. Bioaccumulation factors (BAF; mean ± SE) in *Artemia parthenogenetica* from Cabo de Gata and from Odiel and *Artemia franciscana* from Cádiz. Hazard level BAF values and classification (USEPA, 2011). Values higher than reference ones are in different colours.

Element	Cabo de Gata	Odiel	Cádiz	BAF	Hazard level
As	920 ± 48	291 ± 20	1607 ± 272	>5000	very highly bioaccumulative
Cd	78 ± 7	41 ± 3	173 ± 37		
Co	277 ± 19	432 ± 37	448 ± 63		
Cr	985 ± 218	2215 ± 292	4780 ± 707	1000-5000	highly bioaccumulative
Cu	4471 ± 853	5935 ± 1689	5871 ± 1028		
Mn	7778 ± 1901	1811 ± 580	894 ± 204	100-1000	moderately bioaccumulative
Ni	464 ± 58	340 ± 26	721 ± 156		
Pb	2941 ± 775	465 ± 69	562 ± 162	<100	little bioaccumulative
Zn	5082 ± 907	8204 ± 1761	14606 ± 3356		

Table S6. Results of the (a) GLM analysis with value of the bioaccumulation factor BAF as the dependent variable; 'study site' / location (Cabo de Gata, Odiel, Cádiz) and 'salinity' (low, median, and high) as fixed factor; and 'replicate' as random factor; and (b) pairwise comparison among salinity levels. Estimates for "Cádiz", replicate "8" and salinity "high" are not included as they are aliased, but they are effectively zero. Significant values are in bold. (Continues)

a) (Continues)

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
As	Intercept		1421.68	547.24	69.33	1	<**0.001**	0.65
	Study site	Cabo de Gata	-686.99	193.91	24.08	2	<**0.001**	0.61
		Odiel	-1322.64	190.58				
	Salinity	low	658.64	187.02	6.34	2	**0.005**	0.29
		median	271.45	187.02				
	Replicate	1	-205.79	523.70	0.30	7	0.947	0.06
		2	-98.72	523.70				
		3	-228.51	523.70				
		4	35.43	520.43				
		5	-226.75	588.62				
		6	-243.60	588.62				
		7	-74.71	671.74				
Cd	Intercept		65.20	68.78	32.62	1	<**0.001**	0.47
	Study site	Cabo de Gata	-94.91	24.37	15.30	2	<**0.001**	0.50
		Odiel	-128.46	23.95				
	Salinity	low	78.55	23.51	10.51	2	<**0.001**	0.40
		median	104.51	23.51				
	Replicate	1	42.99	65.83	0.23	7	0.976	0.05
		2	51.02	65.83				
		3	44.52	65.83				
		4	47.83	65.41				
		5	16.24	73.99				
		6	18.67	73.99				
		7	-4.74	84.43				
Co	Intercept		2.34	0.16	5977.74	1	<**0.001**	0.99
	Study site	Cabo de Gata	-0.15	0.06	4.99	2	**0.013**	0.24
		Odiel	0.00	0.06				
	Salinity	low	0.32	0.05	20.77	2	<**0.001**	0.57
		median	0.30	0.05				
	Replicate	1	0.02	0.15	0.27	7	0.960	0.06
		2	0.07	0.15				
		3	0.03	0.15				
		4	0.03	0.15				
		5	0.05	0.17				
		6	0.09	0.17				
		7	-0.09	0.20				

Table S6. Continued.

a) Continued.

Element	Model	Level of effect	Estimates	SE	F	df	p - value	η^2
Cr	Intercept		2790.84	1678.22	49.44	1	<**0.001**	0.58
	Study site	Cabo de Gata	-3794.76	594.67	21.17	2	<**0.001**	0.58
		Odiel	-2536.52	584.45				
	Salinity	low	1880.55	573.54	7.94	2	**0.002**	0.34
		median	2107.38	573.54				
	Replicate	1	837.70	1606.03	0.19	7	0.986	0.04
		2	562.68	1606.03				
		3	585.61	1606.03				
		4	652.22	1595.99				
		5	701.85	1805.12				
		6	-217.04	1805.12				
		7	-424.18	2060.00				
Cu	Intercept		3.07	0.26	1688.65	1	<**0.001**	0.99
	Study site	Cabo de Gata	-0.13	0.09	1.66	2	0.207	0.10
		Odiel	-0.16	0.09				
	Salinity	low	0.63	0.09	23.99	2	<**0.001**	0.61
		median	0.34	0.09				
	Replicate	1	0.41	0.25	3.54	7	**0.007**	0.44
		2	0.30	0.25				
		3	0.29	0.25				
		4	0.17	0.25				
		5	0.80	0.28				
		6	0.01	0.28				
		7	-0.25	0.32				
Mn	Intercept		1.52	0.53	608.36	1	<**0.001**	0.94
	Study site	Cabo de Gata	1.22	0.19	24.44	2	<**0.001**	0.61
		Odiel	0.22	0.18				
	Salinity	low	1.44	0.18	33.65	2	<**0.001**	0.69
		median	0.51	0.18				
	Replicate	1	0.32	0.50	0.55	7	0.791	0.11
		2	0.29	0.50				
		3	0.33	0.50				
		4	0.41	0.50				
		5	0.15	0.56				
		6	0.15	0.56				
		7	-0.51	0.64				

Table S6. Continued.

a) Continued.

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
Ni	Intercept		557.38	339.49	50.49	1	**<0.001**	0.58
	Study site	Cabo de Gata	-257.33	120.30	5.05	2	**0.013**	0.25
		Odiel	-368.00	118.23				
	Salinity	low	412.07	116.02	6.44	2	**0.005**	0.29
		median	172.02	116.02				
	Replicate	1	8.72	324.89	0.23	7	0.974	0.05
		2	-108.07	324.89				
		3	-24.80	324.89				
		4	1.40	322.86				
		5	-60.14	365.16				
		6	-218.00	365.16				
		7	-68.34	416.72				
Pb	Intercept		2.35	0.33	1781.79	1	**<0.001**	0.95
	Study site	Cabo de Gata	0.73	0.12	28.96	2	**<0.001**	0.65
		Odiel	-0.06	0.11				
	Salinity	low	0.66	0.11	17.68	2	**<0.001**	0.53
		median	0.46	0.11				
	Replicate	1	-0.25	0.31	0.30	7	0.948	0.06
		2	-0.10	0.31				
		3	-0.10	0.31				
		4	-0.16	0.31				
		5	-0.12	0.35				
		6	-0.05	0.35				
		7	-0.09	0.40				
Zn	Intercept		6328.86	8254.13	12.88	1	**0.001**	0.35
	Study site	Cabo de Gata	-9524.80	2924.81	5.31	2	**0.010**	0.26
		Odiel	-5061.91	2874.52				
	Salinity	low	7034.35	2820.88	3.69	2	**0.037**	0.19
		median	1242.40	2820.88				
	Replicate	1	8881.16	7899.06	1.73	7	0.137	0.28
		2	5863.38	7899.06				
		3	7786.10	7899.06				
		4	-456.34	7849.68				
		5	7830.87	8878.27				
		6	-547.42	8878.27				
		7	-667.58	10131.84				

Table S6. Continued.

b)

Element	Comparison	SE	p-value
As	low > median	179.529	**0.039**
	low > high	187.023	**0.001**
	median vs high	187.023	0.157
Cd	low vs median	22.565	0.259
	low > high	23.507	**0.002**
	median > high	23.507	**<0.001**
Co	low vs median	0.053	0.668
	low > high	0.055	**<0.001**
	median > high	0.055	**<0.001**
Cr	low vs median	550.557	0.683
	low > high	573.539	**0.003**
	median > high	573.539	**0.001**
Cu	low > median	0.087	**0.002**
	low > high	0.090	**<0.001**
	median > high	0.090	**0.001**
Mn	low > median	0.172	**<0.001**
	low > high	0.179	**<0.001**
	median > high	0.179	**0.008**
Ni	low > median	111.373	**0.039**
	low > high	116.022	**0.001**
	median vs high	116.022	0.148
Pb	low vs median	0.108	0.073
	low > high	0.112	**<0.001**
	median > high	0.112	**<0.002**
Zn	low > median	2707.848	**0.040**
	low > high	2820.881	**0.018**
	median vs high	2820.881	0.663

Table S7. Trace elements concentration (mean ± SE) in sediment (µg g^{-1}, dry weight), water (µg L^{-1}) and biota (*Chironomus salinarius*, *Ochthebius notabilis* and *Artemia parthenogenetica*; µg g^{-1}, dry weight) samples collected from Odiel at the same salinity (low).

Sample type	Element								
	As	Cd	Co	Cr	Cu	Mn	Ni	Pb	Zn
Sediment	115.70±1.10	0.43±0.03	8.14±0.07	23.84±0.26	65.80±0.72	503.02±10.77	13.79±0.21	24.61±0.24	185.30±1.83
Water	136.97±3.96	**<LOD**	**<LOD**	4.98±1.72	3.84±0.56	28.17±1.69	7.78±1.14	**<LOD**	22.41±3.64
C. salinarius	34.63±0.45	0.12±0.00	1.01±0.34	3.03±0.02	37.77±0.99	196.05±4.07	1.85±0.02	4.12±0.10	132.65±3.53
O. notabilis	314.40±33.65	0.13±0.04	0.56±0.13	1.30±0.11	133.64±14.35	112.76±29.59	0.72±0.09	0.78±0.12	214.49±20.73
A. parthenogenetica	40.30±0.69	0.07±0.01	1.57±0.04	2.67±0.10	25.27±0.64	142.70±5.45	1.95±0.06	2.90±0.13	288.43±8.35

*<LOD: Levels below the analytical method

Table S8. Results of the GLM with trace elements concentration in biota as the dependent variable; 'species' (*Chironomus salinarius*, *Ochthebius notabilis* and *Artemia parthenogenetica*) as fixed factors; and 'replicate' as random factor. Estimates for "*O. notabilis*", replicate "6" are not included as they were aliased, but they are effectively zero. Significant values are in bold. (Continues)

Element	Model	Level of effect	Estimates	SE	F	df	p - value	η^2
As	Intercept		313.84	47.54	136.51	1	<**0.001**	0.93
	Species	*A. parthenogenetica*	-273.39	27.44	67.73	2	<**0.001**	0.96
		C. salinarius	-279.76	27.44				
	Replicate	1	20.94	47.54	0.61	5	0.700	0.34
		2	-13.10	47.54				
		3	-23.96	47.54				
		4	18.35	47.54				
		5	-3.09	54.89				
Cd	Intercept		0.12	0.05	61.08	1	<**0.001**	0.87
	Species	*A. parthenogenetica*	-0.05	0.03		2	0.219	0.40
		C. salinarius	-0.01	0.03				
	Replicate	1	0.04	0.05		5	0.439	0.48
		2	-0.03	0.05				
		3	-0.01	0.05				
		4	0.00	0.05				
		5	-0.01	0.05				
Co	Intercept		0.61	0.57	89.11	1	<**0.001**	0.89
	Species	*A. parthenogenetica*	1.04	0.33	4.93	2	0.054	0.62
		C. salinarius	0.45	0.33				
	Replicate	1	0.05	0.57	0.26	5	0.921	0.18
		2	0.05	0.57				
		3	-0.01	0.57				
		4	-0.29	0.57				
		5	-0.24	0.66				
Cr	Intercept		1.08	0.15	883.34	1	<**0.001**	0.99
	Species	*A. parthenogenetica*	1.48	0.08	246.69	2	<**0.001**	0.99
		C. salinarius	1.73	0.08				
	Replicate	1	0.34	0.15	4.93	5	**0.039**	0.80
		2	0.06	0.15				
		3	0.34	0.15				
		4	0.16	0.15				
		5	-0.23	0.17				

Table S8. Continued.

Element	Model	Level of effect	Estimates	SE	F	df	p - value	η^2
Cu	Intercept		136.73	20.65	199.19	1	<**0.001**	0.95
	Species	*A. parthenogenetica*	-109.36	11.93	50.00	2	<**0.001**	0.94
		C. salinarius	-95.87	11.93				
	Replicate	1	1.68	20.65	0.56	5	0.733	0.32
		2	-10.58	20.65				
		3	-11.12	20.65				
		4	7.67	20.65				
		5	-0.24	23.85				
Mn	Intercept		109.11	49.25	256.82	1	<**0.001**	0.96
	Species	*A. parthenogenetica*	32.96	28.44	4.35	2	0.068	0.59
		C. salinarius	83.29	28.44				
	Replicate	1	-0.89	49.25	0.23	5	0.934	0.16
		2	8.46	49.25				
		3	-12.76	49.25				
		4	19.82	49.25				
		5	-10.85	56.87				
Ni	Intercept		0.59	0.15	972.64	1	<**0.001**	0.99
	Species	*A. parthenogenetica*	1.30	0.09	135.74	2	<**0.001**	0.98
		C. salinarius	1.12	0.09				
	Replicate	1	0.19	0.15	1.52	5	0.310	0.56
		2	0.06	0.15				
		3	0.08	0.15				
		4	0.19	0.15				
		5	-0.15	0.17				
Pb	Intercept		1.23	0.29	782.74	1	<**0.001**	0.99
	Species	*A. parthenogenetica*	2.11	0.17	205.54	2	<**0.001**	0.99
		C. salinarius	3.34	0.17				
	Replicate	1	-0.35	0.29	1.59	5	0.294	0.57
		2	-0.53	0.29				
		3	-0.46	0.29				
		4	-0.46	0.29				
		5	-0.88	0.33				
Zn	Intercept		252.79	27.91	581.75	1	<**0.001**	0.99
	Species	*A. parthenogenetica*	61.65	16.12	39.89	2	<**0.001**	0.93
		C. salinarius	-81.84	16.12				
	Replicate	1	-24.50	27.91	1.65	5	0.279	0.58
		2	-50.29	27.91				
		3	-54.79	27.91				
		4	-23.63	27.91				
		5	-2.84	32.23				

Table S9. Bioaccumulation factors (a) BSAF and (b) BAF (mean ± SE) in *Artemia parthenogenetica*, *Chironomus salinarius* and *Ochthebius notabilis* and from Odiel. Values higher than reference ones are in different colours.

a)

Element	*C. salinarius*	*O. Notabilis*	Reference values	Classification
As	0.30 ± 0.01	2.70 ± 0.26	>2	macroconcentrator
Cd	0.29 ± 0.05	0.35 ± 0.14		
Co	0.12 ± 0.04	0.07 ± 0.02		
Cr	0.13 ± 0.00	0.05 ± 0.00	1-2	microconcentrator
Cu	0.58 ± 0.03	2.03 ± 0.20		
Mn	0.39 ± 0.03	0.22 ± 0.06		
Ni	0.13 ± 0.01	0.05 ± 0.01	<1	deconcentrator
Pb	0.17 ± 0.01	0.03 ± 0.01		
Zn	0.00 ± 0.00	0.01 ± 0.00		

b)

Element	*O. Notabilis*	*A. parthenogenetica*	Reference values	Classification
As	2274 ± 249	302 ± 12	>5000	very highly bioaccumulative
Cd	56 ± 16	32 ± 3		
Co	157 ± 35	440 ± 10		
Cr	946 ± 303	1771 ± 513	1000-5000	highly bioaccumulative
Cu	41785 ± 6822	7727 ± 1292		
Mn	4283 ± 1488	5360 ± 345	100-1000	moderately bioaccumulative
Ni	109 ± 27	310 ± 39		
Pb	94 ± 15	351 ± 15	<100	little bioaccumulative
Zn	12015 ± 2097	15829 ± 2894		

Table S10. Results of the GLM analysis with value of the bioaccumulation factor (a) BSAF or (b) BAF as the dependent variable; 'species' (*C. salinarius* and *O. notabilis* for BSAF; and *C. salinarius* and *A. parthenogenetica* for BAF) as fixed factor; and 'replicate' as random factor. Estimates for "*O. notabilis*", replicate "4" (BSAF) or replicate "6" (BAF) are not included as they are aliased, but they are effectively zero. Significant values are in bold. (Continues)

a) (Continues)

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
As	Intercept		0.46	0.05	6.36	1	0.086	0.68
	Species	*C. salinarius*	-0.95	0.05	426.32	1	**<0.001**	0.99
	Replicate	1	-0.01	0.07				
		2	-0.07	0.07	0.75	3	0.590	0.43
		3	-0.07	0.07				
Cd	Intercept		0.41	0.11	11.95	1	0.041	0.80
	Species	*C. salinarius*	-0.06	0.09	0.37	1	0.585	0.11
	Replicate	1	0.18	0.13				
		2	-0.23	0.13	3.97	3	0.144	0.80
		3	-0.19	0.13				
Co	Intercept		0.03	0.06	45.98	1	**0.007**	0.94
	Species	*C. salinarius*	0.06	0.06	0.99	1	0.394	0.25
	Replicate	1	0.06	0.08				
		2	0.06	0.08	0.26	3	0.852	0.21
		3	0.05	0.08				
Cr	Intercept		0.05	0.00	644.64	1	**<0.001**	1.00
	Species	*C. salinarius*	0.07	0.00	360.30	1	**<0.001**	0.99
	Replicate	1	0.01	0.01				
		2	0.00	0.01	3.53	3	0.164	0.78
		3	0.01	0.01				
Cu	Intercept		2.20	0.24	171.68	1	**0.001**	0.98
	Species	*C. salinarius*	-1.46	0.21	47.20	1	**0.006**	0.94
	Replicate	1	0.02	0.30				
		2	-0.35	0.30	0.88	3	0.539	0.47
		3	-0.32	0.30				
Mn	Intercept		0.28	0.08	135.10	1	**0.001**	0.98
	Species	*C. salinarius*	0.17	0.07	6.40	1	0.085	0.68
	Replicate	1	-0.04	0.10				
		2	-0.08	0.10	0.62	3	0.649	0.38
		3	-0.12	0.10				
Ni	Intercept		0.06	0.01	286.57	1	**<0.001**	0.99
	Species	*C. salinarius*	0.08	0.01	145.32	1	**0.001**	0.98
	Replicate	1	0.01	0.01				
		2	-0.02	0.01				
		3	-0.02	0.01	2.68	3	0.219	0.73
		2	0.00	0.00				
		3	0.00	0.00				

Table S10. Continued.

a) Continued.

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
Pb	Intercept		-1.44	0.09	845.46	1	<**0.001**	1.00
	Species	*C. salinarius*	0.74	0.08	89.41	1	**0.003**	0.97
	Replicate	1	-0.03	0.11				
		2	-0.10	0.11	1.02	3	0.495	0.50
		3	-0.18	0.11				
Zn	Intercept		0.01	0.00	414.71	1	<**0.001**	0.99
	Species	*C. salinarius*	0.00	0.00	65.47	1	**0.004**	0.96
	Replicate	1	0.00	0.00				
		2	0.00	0.00	1.74	3	0.330	0.64
		3	0.00	0.00				

b) (Continues)

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
As	Intercept		2298.66	427.84	158.24	1	<**0.001**	0.96
	Species	*A. parthenogenetica*	-1975.38	247.01	63.95	1	**0.004**	0.96
	Replicate	1	-9.43	445.31				
		2	-230.09	445.31				
		3	-186.44	445.31	0.63	5	0.698	0.70
		4	325.34	445.31				
		5	-25.35	494.03				
Cd	Intercept		54.49	23.71	0.00	1	**0.001**	0.84
	Species	*A. parthenogenetica*	-22.06	13.69	0.21	1	0.205	0.46
	Replicate	1	26.35	24.67				
		2	-16.25	24.67				
		3	-8.90	24.67	1.14	5	0.489	0.66
		4	3.16	24.67				
		5	-6.49	27.37				
Co	Intercept		170.74	71.03	371.75	1	<**0.001**	0.98
	Species	*A. parthenogenetica*	289.36	41.01	49.79	1	**0.006**	0.94
	Replicate	1	-24.96	73.93				
		2	-9.50	73.93				
		3	-44.84	73.93	0.47	5	0.785	0.44
		4	24.77	73.93				
		5	-66.31	82.02				

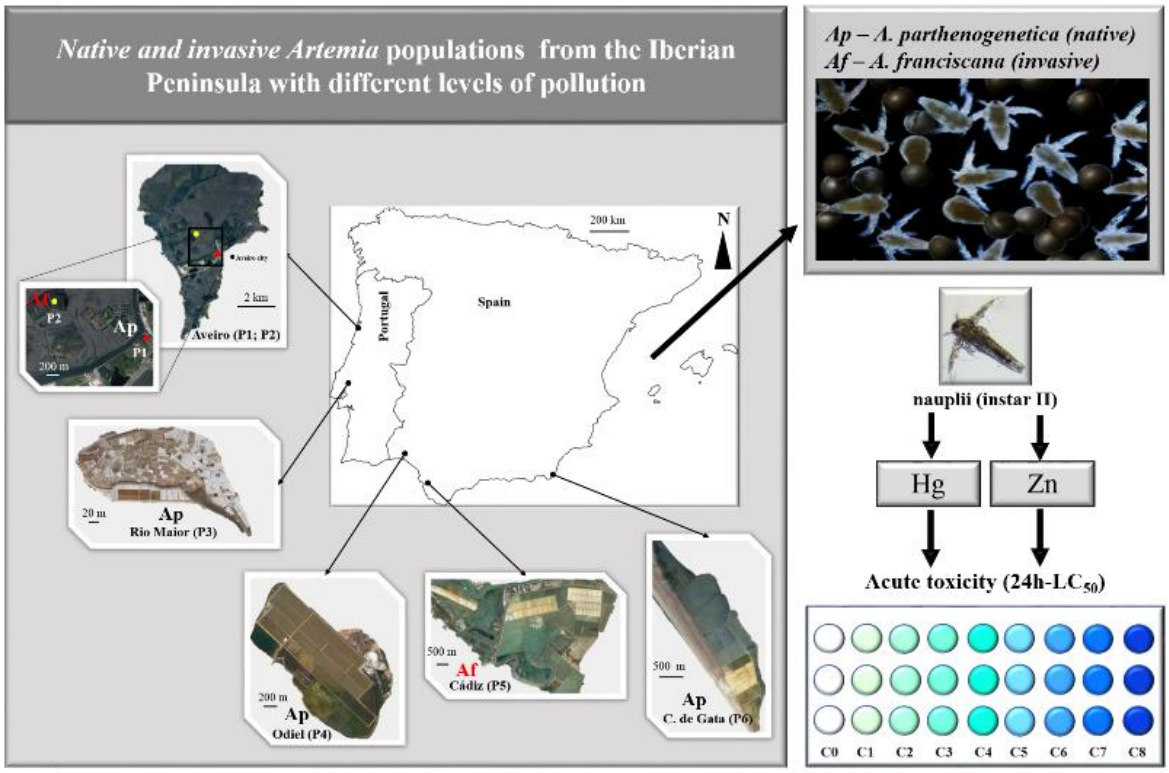
Table S10. Continued.

b) Continued.

Element	Model	Level of effect	Estimates	SE	F	df	p-value	η^2
Cr	Intercept		-944.39	599.97	8.29	1	**0.032**	0.61
	Species	*A. parthenogenetica*	1116.98	346.39	10.40	1	**0.048**	0.78
	Replicate	1	2470.52	624.47	6.89	5	0.071	0.92
		2	2110.47	624.47				
		3	2460.83	624.47				
		4	517.74	624.47				
		5	2031.12	692.78				
Cu	Intercept		37707.23	9612.67	60.03	1	**0.001**	0.91
	Species	*A. parthenogenetica*	-33817.95	5549.88	0.01	1	**0.009**	0.93
	Replicate	1	12533.80	10005.19	0.42	5	0.422	0.70
		2	5762.54	10005.19				
		3	5107.80	10005.19				
		4	-7094.69	10005.19				
		5	6717.41	11099.76				
Mn	Intercept		5279.02	2523.41	53.04	1	**<0.001**	0.88
	Species	*A. parthenogenetica*	938.29	1456.89	0.42	1	0.565	0.12
	Replicate	1	-2755.92	2626.44	0.82	5	0.606	0.58
		2	-766.93	2626.44				
		3	-1633.23	2626.44				
		4	1170.06	2626.44				
		5	-1159.45	2913.78				
Ni	Intercept		139.55	74.37	46.12	1	**0.001**	0.89
	Species	*A. parthenogenetica*	195.67	42.94	20.77	1	**0.020**	0.87
	Replicate	1	21.42	77.41	2.35	5	0.256	0.80
		2	-154.79	77.41				
		3	-11.55	77.41				
		4	22.53	77.41				
		5	-25.96	85.88				
Pb	Intercept		148.73	42.54	334.08	1	**<0.001**	0.98
	Species	*A. parthenogenetica*	256.14	24.56	108.75	1	**0.002**	0.97
	Replicate	1	-51.93	44.28	0.98	5	0.542	0.62
		2	-59.68	44.28				
		3	-59.67	44.28				
		4	-46.91	44.28				
		5	-106.58	49.12				
Zn	Intercept		3217.57	2668.60	30.98	1	**0.002**	0.86
	Species	*A. parthenogenetica*	4082.02	1540.72	7.02	1	0.077	0.70
	Replicate	1	11182.16	2777.57	12.21	5	**0.033**	0.95
		2	11877.61	2777.57				
		3	11090.64	2777.57				
		4	1040.50	2777.57				
		5	15985.68	3081.44				

CHAPTER II

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.



Pais-Costa, A.J., Sánchez, M.I., Vieira, N., Green, A.J., Marques, J.C., Martínez-Haro, M., 2020. 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. Ecological Indicators, 118, 106739.
https://doi.org/10.1016/j.ecolind.2020.106739.

II. Abstract

In recent decades, brine shrimps of the genus *Artemia* have suffered a major biodiversity loss in the Mediterranean region due to the introduction of the highly invasive *Artemia 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 the 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 saltpans 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 site, 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.

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

II.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., 2016a; 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 (Thiéry 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 *Artemia salina* and *Artemia 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 saltpans. In Portugal, one of the last refuges of native *A. parthenogenetica* is in the saltpan complex of Ria de Aveiro (Portugal), highly polluted by mercury (Hg). However, in that same

saltpan 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, 2014a) 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., 2016a; 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., 2014a).

The aim of the present study was to provide insights into the pollution resistance hypothesis (Rodrigues et al., 2012) 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* species collected from the same locations as in Rodrigues et al. (2012) were used to evaluate if potential differences in environmental factors could explain their distribution pattern. This work hypothesis is that native *Artemia* from highly Hg- and Zn-polluted sites would be locally adapted therefore more resistant to the invasion than populations from less polluted sites.

II.2. Material and Methods

II.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**).

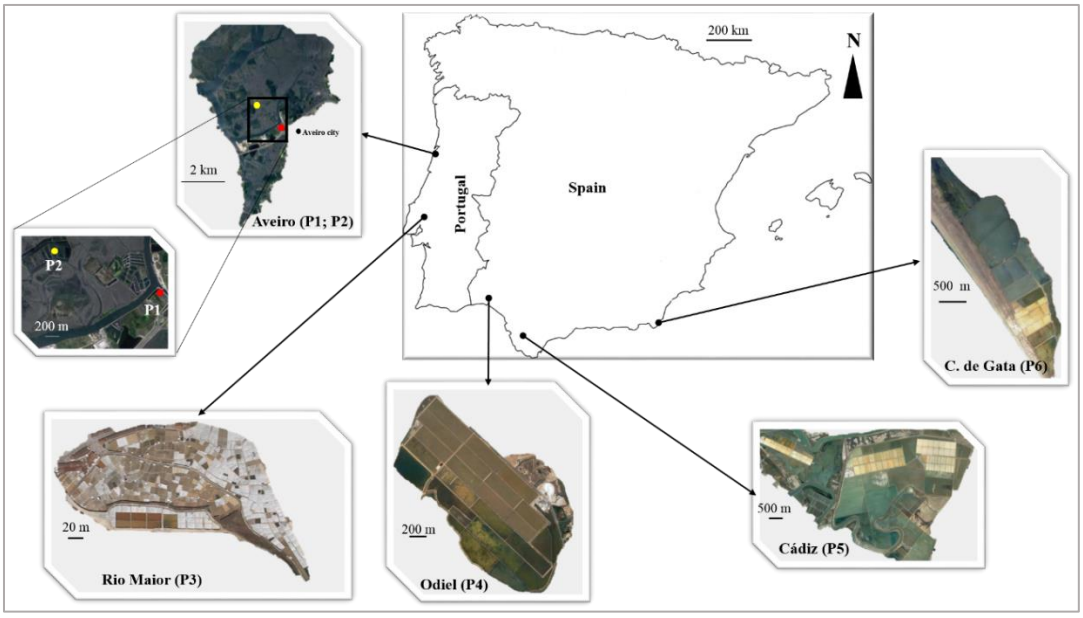


Figure 1: Location of the six populations (P1-P6) of *Artemia* selected for this study. Cysts of *A. parthenogenetica* were collected from Aveiro (Troncalhada saltpan), Rio Maior, Odiel and Cabo de Gata saltpans; and *A. franciscana* from Aveiro (Tanoeira saltpan) and Cádiz saltpans. [source: Google 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, 2014a,b), due to its location (**Figure 1**), is one of the first saltpans 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, 2014a,b), 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 lead-zinc-(copper-silver) (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 saltpan 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 the 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), Cu, 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 trace elements 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 trace elements 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.

II.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). The selected six populations were sampled in the above sites located in the Iberian Peninsula (**Figure 1**). 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.

II.2.3. Hatching of cysts

Before the toxicity tests, cysts were hatched in artificial seawater 35 g L^{-1}, prepared with 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 at room temperature (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.

II.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 ($HgCl_2$ 99.9 % purity; from Sigma-Aldrich (Germany)) and of zinc sulphate heptahydrate ($ZnSO_4*7H_2O$ (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 by diluting the stock in artificial seawater to obtain the desired concentrations. Preliminary range-finding tests were conducted to determine the concentration ranges to be used in the definitive tests (ASTM, 2014). Based on those 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 contamination. 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 %.

II.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 (24 h 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 (GLM) with binomial distribution and logit link function were used to test the effect of 'populations' and 'replicate' as fixed factors, and 'concentration' as covariate, 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).

II.3. Results

II.3.1. Acute test – Hg

The values of the LC50 (24 h) 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 the 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 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**).

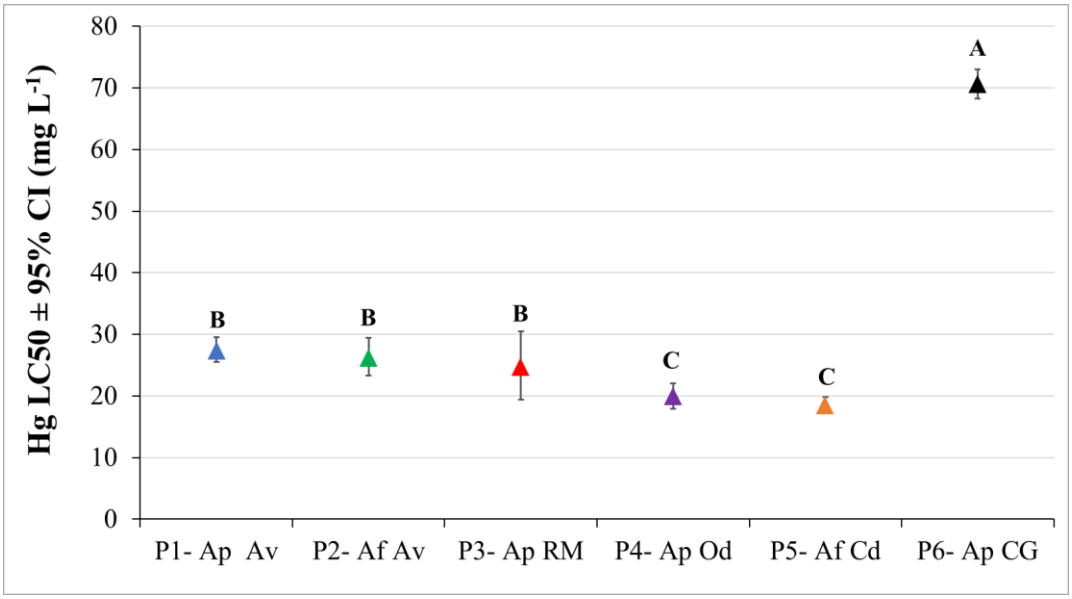


Figure 2. Values of Hg concentrations that were lethal for 50 % of individuals over 24 h (LC50) recorded for the six *Artemia* populations (P1–P6) tested, with 95 % confidence intervals. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive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

GLM analysis showed no differences among the study populations but 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 (GLM) on nauplii mortality of the six *Artemia* populations (P1–P6) under different mercury (Hg) concentrations within 24 h, 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 (Av) and Cádiz (Cd). Estimates for "Af CG" and replicate "3" are not included as they were aliased, but they are effectively zero. Significant values are in bold.

Effect	Level of effect	Estimates	SE	df	Wald Chi-Square	p-value
Intercept		-2.406	0.283	1	546.725	**<0.001**
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	**<0.001**
Population * Concentration	P1-Ap Av*Concentration	0.046	0.014	5	158.187	**<0.001**
	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	<0.001	0.134	2	0.719	0.698
	2	0.098	0.134			

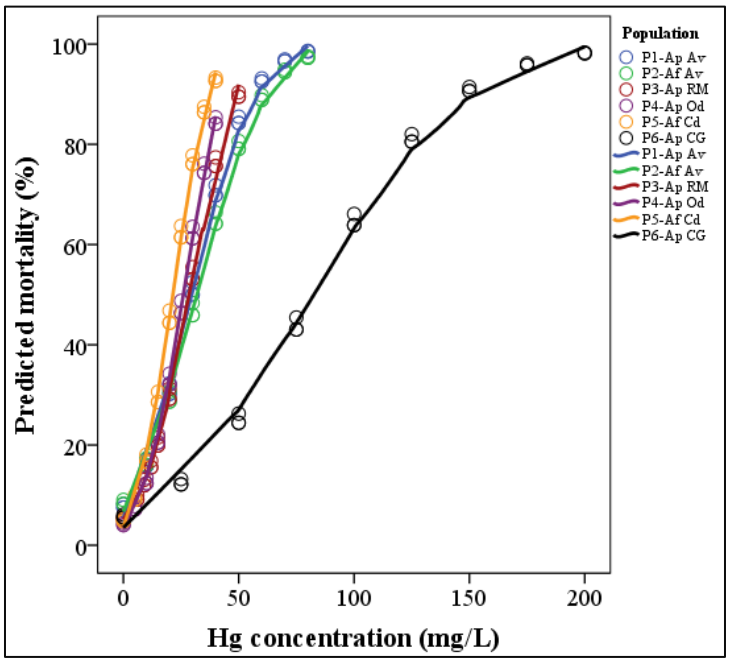


Figure 3. Relationship between the predicted mortality at different mercury (Hg) concentrations for the six studied populations (P1-P6). Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro and Cádiz (Cd).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

II.3.2. Acute test – Zn

The values of LC50 (24 h) for nauplii from the different *Artemia* populations ranged from 354 to 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*–Cádiz ≥ *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 ones (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**).

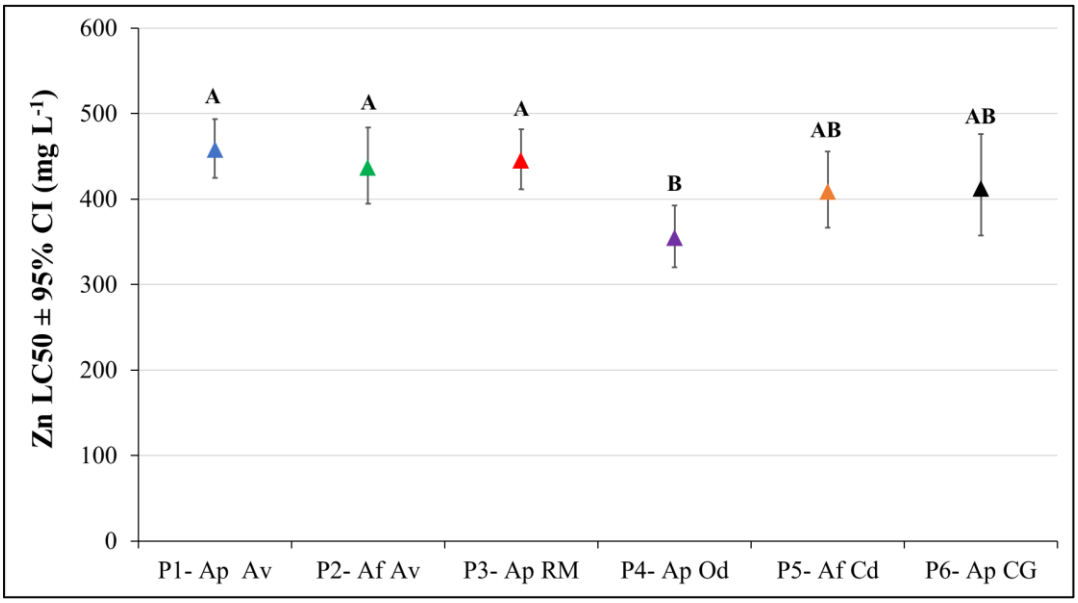


Figure 4. Values of Zn concentrations that were lethal for 50 % of individuals over 24 h (LC50) for six *Artemia* populations (P1-P6), with 95 % confidence intervals. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af)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

GLM analysis showed no differences among 'replicate' 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. Significant values are in bold.

Effect	Level of effect	Estimates	SE	df	Wald Chi-Square	p-value
Intercept		-2.655	0.190	1	527.618	**<0.001**
Population	P1-Ap Av	-0.270	0.197	5	16.318	**0.006**
	P2-Af Av	-0.309	0.197			
	P3-Ap RM	-0.367	0.197			
	P4-Ap Od	0.071	0.197			
	P5-Af Cd	0.271	0.197			
Concentration		0.006	0.0002	1	713.316	**<0.001**
Replicate	1	0.048	0.139	2	2.558	0.278
	2	0.212	0.139			

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). Significant values are in bold.

Comparison	SE	p-value
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

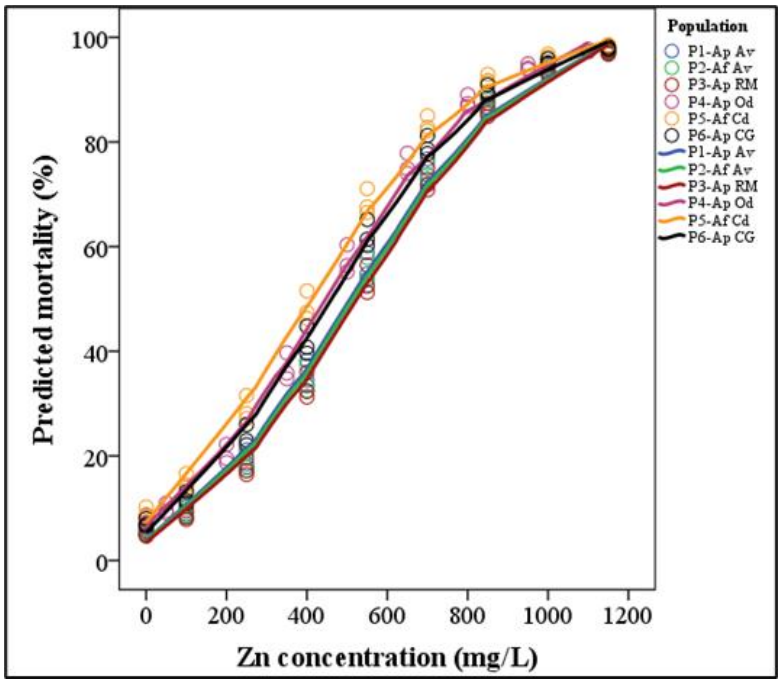


Figure 5. Predicted mortality at different zinc (Zn) concentrations for the six studied populations (P1-P6) of *Artemia*. Native *A. parthenogenetica* (Ap) from Aveiro (Av), Rio Maior (RM), Odiel (Od) and Cabo de Gata (CG), and invasive *A. franciscana* (Af) from Aveiro (Av) and Cádiz (Cd). Lines show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

II.4. Discussion

The present work hypothesized that native *Artemia* from highly Hg-polluted sites and highly Zn-polluted sites would be locally adapted and consequently be more resistant to the invasion than native populations from less polluted sites ('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 study sites – would present the highest resistance to Hg, and native *Artemia* from Odiel, – the most Zn-polluted study sites – would present the highest resistance to Zn.

II.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 trace elements 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, 2014a,b). Rodrigues et al. (2012) and Pinto et al. (2013, 2014a) 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 the 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). Thus, the persistence of this native *A. parthenogenetica* population could be related to other contaminants present in Ria de Aveiro as other trace elements (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 saltpan 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, 2014b) 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 did not identified factors involved in the persistence

and remained on the idea that a chemical barrier related to trace elements or pesticides may be preventing the invasion.

II.4.2. Effect of Zn on the survival of *Artemia*

The LC50 (24 h) 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 trace element. 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 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 trace element necessary for normal physiological and biochemical process of organisms, unlike Hg which has no biological function (Clarkson and Magos, 2006), and 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 GLM analysis 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; **Chapter III**) 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.

II.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., 2014b). Recent studies examining different abiotic factors highlight the necessity to study the potential role of contaminants (Rodrigues et al., 2012, Pinto et al., 2013, 2014a). 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 the *A. franciscana* population from the same complex, but different saltpans. 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 on these relict native populations to preserve the remaining *Artemia* biodiversity and limit the probability of *A. franciscana* introduction.

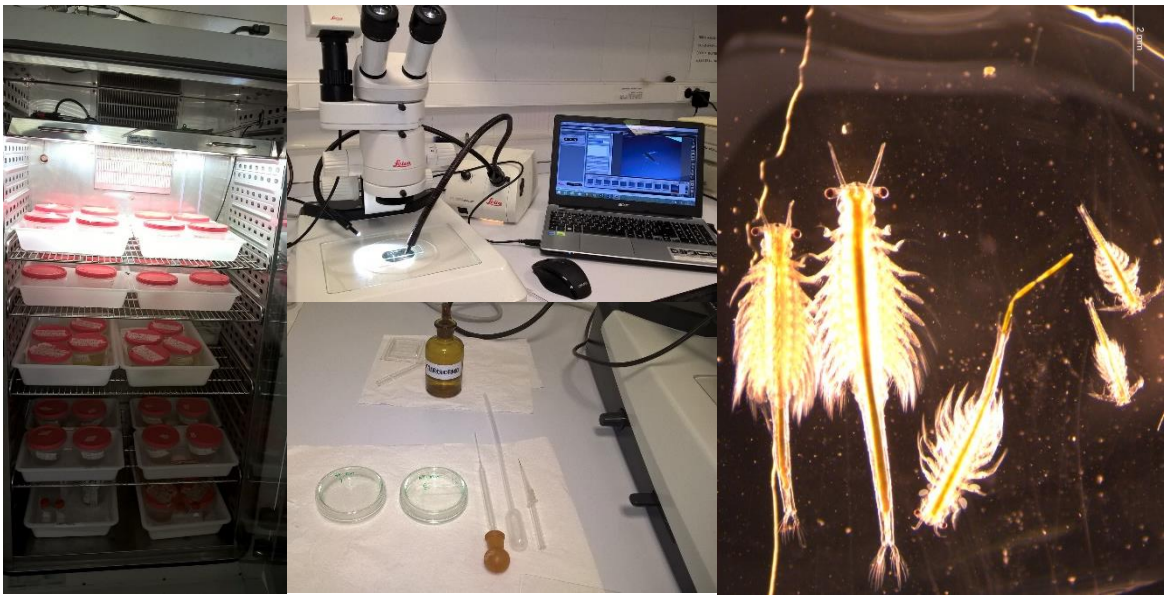
II. Supplementary Material

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).

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

CHAPTER III

Life history and physiological responses of native and invasive brine shrimps exposed to zinc.



Pais-Costa, A.J., Varó, I., Martinez-Haro, M., Vinagre, P.A., Green, A.J., Hortas, F., Marques, J.C., Sánchez, M.I., 2019. Life history and physiological responses of native and invasive brine shrimps exposed to zinc. Aquatic Toxicology 210, 148-157.
https://doi.org/10.1016/j.aquatox.2019.02.023.

III. Abstract

Although a substantial amount of research exists on pollution and biological invasions, there is a paucity of understanding of how both factors interact. Most studies show that pollution favours the establishment of invasive species, but pollution may also promote local adaptation of native species and prevent the establishment of new incomers. However, evidence for this is extremely limited because most studies focus on successful invasions and very few on cases where an invasion has been resisted. Here we provide evidence of local adaptation of native species to pollution combining life history and physiological data. We focused on the invasion of the North American brine shrimp *Artemia franciscana*, which is causing a dramatic biodiversity loss in hypersaline ecosystems worldwide, and one of the last native *Artemia* populations in SW Europe (*Artemia parthenogenetica* from the historically polluted Odiel estuary, SW Spain). Life table response experiments were carried out in the laboratory to compare the demographic responses of *A. parthenogenetica* and a nearby *A. franciscana* population to long-term Zn exposure (0.2 mg Zn L^{-1}). We also evaluated oxidative stress by measuring antioxidant defences (catalase, glutathione reductase and superoxide dismutase) and lipid peroxidation (thiobarbituric acid reactive substances). A high concentration of Zn induced strong mortality in *A. franciscana*, which also showed high levels of lipid peroxidation, suggesting relatively poor physiological resistance to pollution compared with *A. parthenogenetica*. The age at maturity was shorter in *A. parthenogenetica*, which may be an adaptation to the naturally high mortality rate observed in the Odiel population. Exposure to Zn accelerated age at first reproduction in *A. franciscana* but not in *A. parthenogenetica*. In contrast, Zn had a stimulatory effect on offspring production in *A. parthenogenetica*, which also showed higher reproductive parameters (number of broods, total offspring, and offspring per brood) than *A. franciscana*. Overall, the results of this study strongly suggest that native *Artemia* from Odiel estuary is locally adapted (at both, reproductive and physiological levels) to Zn contamination and that *A. franciscana* is highly sensitive. This is a good example of how pollution may play a role in the persistence of the last native *Artemia* populations in the Mediterranean.

Keywords: *Artemia franciscana*, *Artemia parthenogenetica*, Natural populations, Zinc, Biological invasions, Sublethal concentration, Oxidative stress.

III.1. Introduction

Biological invasions are threatening global biodiversity worldwide and altering the structure and functioning of ecosystems (Simberloff et al., 2013). Hence, a better understanding of the factors that make ecosystems susceptible to invasions is a major challenge in ecology (Elton, 1958; Crooks et al., 2010). This knowledge is also crucial to predict and prevent future invasions. While biotic factors, such as competition or parasitism, have been the focus of many studies (e.g., Georgiev et al., 2007; Olyarnik et al., 2009; Sánchez et al., 2013), less attention have been devoted to abiotic determinants (Crooks et al., 2010). On the other hand, most studies to date have focused on the mechanisms allowing an invasive species to colonize a new environment, and much less attention has been devoted to factors allowing native populations to survive invasions (Sánchez et al., 2016a). For example, pollution is considered a major cause of human-induced environmental stress (Freedman, 1995) and many studies show that it can increase the invasibility of ecosystems (Crooks et al., 2010). However, the potential role of pollution in limiting invasions has been much less studied, even though it may be a common phenomenon when native species are locally adapted to high levels of environmental pollution. An interesting model to study interactions between pollution and invasions is the brine shrimp *Artemia* (Crustacea, Branchiopoda). Despite the extensive literature on dose-effect relationships of toxicants on *Artemia* hatched from commercially available dry cysts, studies addressing realistic ecological/environmental problems involving natural populations of *Artemia* are very scarce. The genus *Artemia* is suffering an important biodiversity loss at a global scale due to the introduction of the North American brine shrimp *Artemia franciscana* (Amat et al., 2007; Horváth et al., 2018), used worldwide for aquaculture (Lavens and Sorgeloos, 2000; Dhont and Sorgeloos, 2002). Currently few populations of native *Artemia* persist in SW Europe and most of them occupy highly polluted environments. Despite its endangered status at the Iberian Peninsula level (IUCN red list; García-de-Lomas et al., 2017) and the recognised importance of preserving highly threatened and diverse native *Artemia* populations in the Mediterranean (Muñoz et al., 2008), little effort has been devoted to study those populations to provide insight on factors promoting or limiting the invasion.

A common pollutant in hypersaline water bodies where *Artemia* occur is Zn (Usero et al., 2004). There are several studies evaluating the effect of Zn in *Artemia* using commercial cysts (Seebaugh and Wallace, 2004; Brix et al., 2006; Nováková et al., 2007), which come mainly from Great Salt Lake, or from introduced populations in China. Studies considering natural populations are extremely scarce (to our knowledge, only Sarabia et al., 2008). The aim of the present study was to explore the effect of chronic exposure to Zn to assess possible differences in adaptation to

pollution, in two *Artemia* populations of South Spain: the invasive *A. franciscana* from Cádiz bay, which arrived more than 30 years ago, and the native *Artemia parthenogenetica* from Odiel and Tinto estuary, one of the most polluted ecosystems in Europe (Grande et al., 1999; Vallés et al., 2017).

We measured life history traits (mortality, growth rate and reproductive performance) under realistic long-term Zn exposure conditions. Life history traits are important to determine the viability of populations (Sibly and Calow, 1989; Snell and Serra, 2000). We also measured antioxidant enzyme activities, and lipid peroxidation as indicators of damage produced by oxidative stress. Oxidative stress has become a topic of significant interest for environmental, toxicological, and ecological studies because sublethal concentrations of pollutants can cause cellular responses before detrimental effects are detected at higher levels of biological organization (Hook et al., 2014). Based on previous studies (Rodrigues et al., 2012 and Sánchez et al., 2016a), we predicted that *A. parthenogenetica* from Odiel should be less sensitive to chronic exposure to Zn than *A. franciscana* in terms of 1) mortality, 2) growth rate, 3) reproduction, and 4) oxidative stress.

III.2. Material and methods

III.2.1. Study sites

This study was conducted in the historically and highly contaminated Odiel and Tinto estuary (Huelva province Spain, 37°15′29 ″N, 6°58′25 ″W) where one of the last native *A. parthenogenetica* populations in southern Europe persists, and the less contaminated Puerto de Santa María (Cádiz bay, 36°35.799 ′N, 6°12.597 ′W) with occurrence of *A. franciscana*. The Odiel and Tinto rivers flow through the Iberian Pyrite Belt, one of the largest polymetallic sulphide deposits in the world, whose mining activity dates back to prehistoric times (Nocete et al., 2005). The Odiel and Tinto rivers transport enormous quantities of dissolved trace elements to the estuary, including 3500 t Zn yr^{-1} (Nieto et al., 2007) which represents 37 % of the global gross flux of dissolved Zn transported by rivers into the ocean (based on estimations published by GESAMP, 1987). It indicates the extreme degree of contamination that the estuary suffers (Nieto et al., 2007).

These two wetlands are separated by less than 100 km. Both are important feeding and roosting areas for birds using the East Atlantic Flyway (Sánchez et al., 2006) through which more than 15.5 million shorebirds migrate (Stroud et al., 2004). Dispersal of *A. franciscana* is strongly favoured by waterbirds which can transport viable cysts over long distances (Green et al., 2005; Sánchez et al., 2012). Previous studies suggest that the current distribution of this exotic crustacean

is consistent with an expansion via waterbirds (Green et al., 2005). Shorebirds such as redshank and godwits fly at 56–60 km h^{-1} (Welham, 1994) and cyst retention time can be up to 12 h (Sánchez et al., 2012), so the probability that birds flying from Cádiz Bay excrete *A. franciscana* cysts in Odiel marshes is very high.

III.2.2. Cysts sampling

Cysts of native *A. parthenogenetica* from Odiel saltpans and cysts of invasive *A. franciscana* from Cádiz saltpans were sampled in January 2015 in several evaporation ponds of low-medium salinity (90–150). Sampling was authorized by the Junta de Andalucía (Authorization DGGMN 1059). Cysts were transported to the laboratory and sieved through 500-, 300-, and 100-μm sieves (cyst size is usually ~250 μm). They were cleaned by differential flotation in freshwater and saturated brine (Sorgeloos et al., 1977; Amat, 1985). Cysts were then dried at 45 °C for 24 h and stored at 5 °C until use in experiments.

III.2.3. Hatching and culture conditions

Cysts were hatched in seawater (salinity of 35) at 28 °C under continuous illumination (1500–2000 lux, using fluorescent lights) and constant aeration by air bubbling for 24 h. Hatching success was high for both *Artemia* populations, with approximately 90 % of hatching. Newly hatched nauplii were separated from their empty floating shells and transferred to clean medium at 25 °C. Hatched nauplii (< 24 h old) were immediately used for the toxicity test. Remaining unhatched cysts were discarded.

The cultures were kept at constant conditions of temperature and photoperiod in a climatic chamber (25 °C, 16 h : 8 h photoperiod). Salinity was gradually increased (over 10 days) up to 100. During the first six days of culture, *Artemia* were fed with the microalgae *Tetraselmis suecica* (salinity of 45), from days 6–9 we used a mixture of the microalgae *Dunaliella salina* and *T. suecica* (salinity of 65) and from days 10–21 we used only *D. salina* (salinity of 100) (**Table S1**). Microalgae final density was 2.5×10^5 cells ml^{-1} for the first three days, and 5×10^5 cells ml^{-1} afterwards. Water was replaced three times per week to maintain the exposure conditions and to minimize infection by fungi and bacteria.

III.2.4. Life table response experiments under long-term exposure to Zn

Brine shrimp from the two study populations were exposed to 0 (control) and 0.2 mg Zn L^{-1} (treatment) for 21 days. Concentrations of 0.1 mg Zn L^{-1} were registered in Odiel saltpans (**Table S2**). Concentration was kept constant throughout the experiment, and we proceeded in the same

way (medium renewal and feeding) for each treatment and species. Stock solution of 40 mg Zn L^{-1} was prepared using $ZnSO_4*7H_2O$ (Merck Millipore) and nanopure water. The test concentration of 0.2 mg Zn L^{-1} was obtained by diluting the stock solution with seawater. Zinc concentrations in the test solutions were analysed by means of atomic absorption spectroscopy (AAnalyst800, PerkinElmer). The minimum detection limit was 3.6 μg Zn L^{-1}. The Zn concentration in the controls was below the limit of detection. Real and nominal concentrations of the Zn solutions differed by less than 10%.

Each combination of treatment and species consisted of four replicates with 140 nauplii (i.e., 560 individuals per species and treatment) placed in plastic containers filled with 140 ml of experimental solution (see **Table S1** for a summary of experimental conditions). Data on survival and growth were recorded three times per week for 21 days. *Artemia* size was measured (body length, mm) randomly selecting 20 individuals from each combination of species and treatments. Specimens were anaesthetized with a few drops of distilled water saturated with chloroform and measured from the top of the head to the furca using a stereomicroscope. Given the stress caused during measurements, these individuals were then removed and discarded from the experiment.

After 21 days of exposure, a total of 105 females showing signs of vitellogenesis (30 *A. parthenogenetica* control, 30 *A. parthenogenetica* treatment, 25 *A. franciscana* control and 20 *A. franciscana* treatment) were monitored individually for reproductive parameters, together with randomly selected males in the case of the bisexual *A. franciscana*. Males recognised and attached to females very rapidly (less than 1 h) due to the small container where the two individuals were placed. The other individuals were gently washed in distilled water and, after removing excess water, stored at -80 °C until biochemical analysis. Reproductive parameters were monitored as follows:

Individualized females were placed in 50 ml polypropylene flasks filled with 20 ml of experimental solution (0 and 0.2 mg Zn L^{-1}) (in the case of the bisexual *A. franciscana* a male was placed together with each female). Individuals were kept under the same conditions of temperature, salinity, photoperiod and feeding regime as indicated above (protocol from day 10 onwards, **Table S1**). Survival and reproduction were examined every 2–3 days. Dead specimens were removed and replaced during the first three days after their individualization. The reproductive parameters examined were age at first reproduction (pre-reproductive period), number of broods, total number of offspring, and % of viable nauplii (alive/dead). Due to different times of maturation and mortality throughout the experiment, *A. parthenogenetica* was monitored for 47 days and *A. franciscana* for 43 days (time from hatching).

III.2.5. Physiological response: oxidative stress analysis

The following biomarkers of oxidative status were quantified: Catalase (CAT; EC 1.11.1.6), Glutathione reductase (GR; EC 1.8.1.7) and Superoxide dismutase (SOD; EC 1.15.1.1). The SOD catalyses the conversion of the toxic O_2^- radical to H_2O_2 and O_2 (Xie et al., 2015). H_2O_2 can be subsequently converted by CAT into H_2O and O_2 (Xie et al., 2015). The GR activity catalyses the reduction of the oxidized form of glutathione (glutathione disulfide (GSSG) to glutathione (GSH)), which is a critical molecule in combating oxidative stress in cells (Farhat et al., 2018). We also measured thiobarbituric acid reactive substances (TBARS) as an indicator of lipid peroxidation caused by oxidative stress (Akbulut et al., 2014). Lipid peroxidation occurs when antioxidant pathways are overwhelmed or inactivated by excess of reactive oxygen species (ROS), causing oxidative degradation of lipids (Akbulut et al., 2014).

A total of 600 individuals (of the different species and treatments) were analysed for oxidative stress after 21 days of experimental exposure to treatments. To assure enough volume to perform the biochemical analysis, we made pools of 20 individuals. In this way we obtained eight samples of *A. parthenogenetica* control, eight of *A. parthenogenetica* treatment, seven of *A. franciscana* control and seven of *A. franciscana* treatment. Individuals were homogenized with an electrical homogenator (Miccra D-1 Art Moderne Labor Technik) in cool buffer (Tris-HCl 100 mM, EDTA 0.1 mM, Triton X-100 0.1 %, pH = 7.8) using 200 µL of buffer per sample. Samples were centrifuged at 14,000 g at 4 °C for 30 min, and supernatant was stored at -80 °C until enzymatic determination. Total protein content in the supernatant fluid was determined following Bradford's procedure (Kruger, 1994). All the biomarkers were determined colorimetrically at the Ecophysiology Laboratory of the EBD-CSIC using a Victor 3 multiplate reader (PerkinElmer, Massachusetts, USA). Enzyme activity was expressed as µmol per minute per mg protein (CAT and SOD) or nmol per minute per mg protein (GR). Lipid peroxidation level was expressed as nmol of TBARS per ml.

III.2.6. Statistical analysis

Cox regression models were used to compare 'cumulative survival' between *Artemia* species (*A. parthenogenetica* vs *A. franciscana*) and 'treatments' (control vs Zn). The interaction 'species x treatment' was also included in the models. For this analysis we excluded individuals that were removed for measurements, so it was based on 380 individuals per species and treatment. Repeated measures ANOVA were performed to test the effect of *Artemia* species and treatments on growth rate. A GLM was also used to analyse the effect of the former variables on *Artemia* 'final size', using a normal error distribution and an identity link. A similar GLM was conducted on the length

of the 'pre-reproductive period' (age at first reproduction). For the fecundity traits ('number of broods', 'total offspring', 'offspring per brood' and '% of viable nauplii') we included 'time of monitoring' (in days) as a covariate to control for variation in the length of the monitoring period (time until death or until the end of the experiments for individuals that survived the entire period). Two-way interactions were also included in the models. Residuals were normally distributed.

GLM analyses were performed on biomarkers of oxidative status ('CAT', 'SOD' and 'GR' activities, and 'TBARS'; sqr-transformed variables) with *Artemia* 'species' (*A. parthenogenetica* vs *A. franciscana*) and 'treatment' (control vs Zn) as predictor variables, using a normal error distribution and an identity link. Two-way interactions ('species x treatment') were also included in the models. All statistical analyses were conducted using Statistica software version 13.

III.3. Results

III.3.1. Survival

The effect of Zn on cumulative survival of *A. parthenogenetica* and *A. franciscana* is shown in **Figure 1**. According to the cox regression, survival was significantly different between *Artemia* species and between treatments (**Table 1**). Survival was naturally higher in *A. franciscana* compared with *A. parthenogenetica* (**Figure 1**, **Table 1**). However, exposure to Zn significantly reduced survival in *A. franciscana* but did not affect *A. parthenogenetica* (significant interaction 'species x treatment'; **Figure 1, Table 1**).

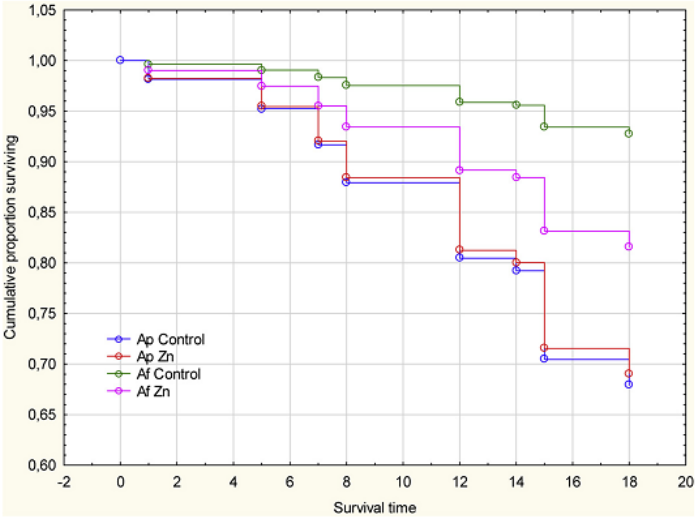


Figure 1. Cumulative survival of *Artemia franciscana* (Af) and *A. parthenogenetica* (Ap) after long-term exposure to Zn (0 and 0.2 mg Zn L^{-1}, i.e., control and treatment, respectively).

Table 1. *Artemia* mortality during long-term exposure to Zn. Results of Cox proportional hazard regression analysis on *Artemia* survival after Zn exposure, based on different *Artemia* species (*A. franciscana* and *A. parthenogenetica*) and Zn concentration (0.2 and 0 mg Zn L^{-1}). Coefficients for '*A. franciscana'* and 'Zn treatment' (0.2 mg Zn L^{-1}) are not included because they are aliased (i.e., they would be redundant) and are effectively zero. Significant values are in bold.

Effect	Level of effect	Estimate	SE	Chi-square	P-value
Species	*A. parthenogenetica*	0.559	0.0642	75.776	< **0.001**
Treatment	Control	-0.239	0.0642	13.842	< **0.001**
Species x Treatment		0.261	0.0642	16.564	< **0.001**

III.3.2. Growth rate and final size

The results of the repeated-measures ANOVA showed significant effects of *Artemia* species, treatment, and time on growth rate (**Table 2**). On average, *A. franciscana* grew faster than *A. parthenogenetica* but both species attained similar final size (**Figure 2**, GLM, $F_{1,76} = 0.912$, $p = 0.342$). Zinc exposure significantly reduced growth rate in both *Artemia* species (Table 2) but it did not affect final size (**Figure 2**, GLM, $F_{1,76} = 0.959$, $p = 0.331$). The interaction 'species x treatment' was not significant in either of the models (growth rate and final size, $p > 0.486$).

Table 2. Growth rate of native and invasive *Artemia* under long term exposure to Zn. Results of a repeated-measures ANOVA with *Artemia* growth rate as the dependent variable, *Artemia* species (*A. franciscana* and *A. parthenogenetica*) and treatments (0.2 and 0 mg Zn L^{-1}) as between subject fixed factors, and TIME (with 10 levels, from time 1 to time 10) as the within-subject variable. Partial eta-squared values (η^2) are presented as a measure of effect sizes. Significant values are in bold.

Effect	SS	df	MS	F	P-value	η^2
Intercept	5171.015	1	5171.015	5112.466	<**0.001**	0.985
Species	5.007	1	5.007	4.951	**0.029**	0.061
Treatment	11.014	1	11.014	10.889	**0.001**	0.125
Error	76.870	76	1.011			
Time	1749.954	9	194.439	246.540	<**0.001**	
Error	539.452	684	0.789			0.764

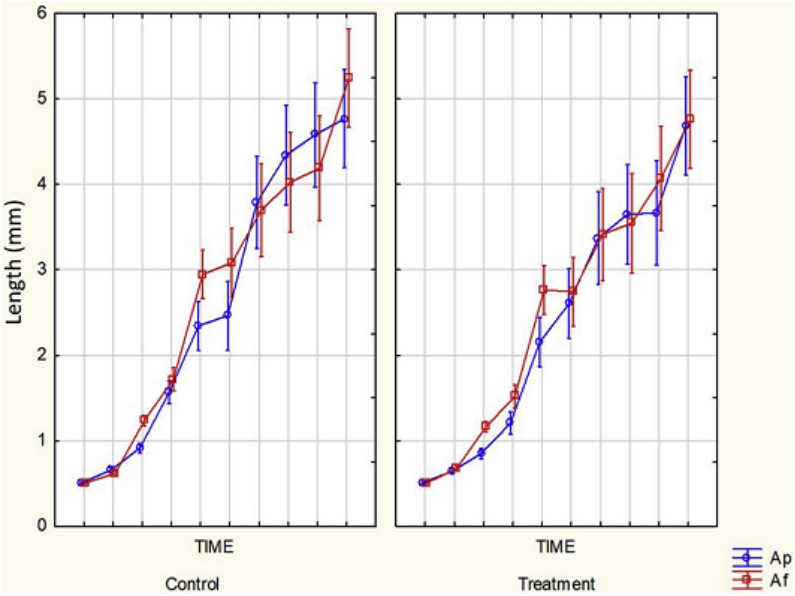


Figure 2. *Artemia franciscana* (Af) and *A. parthenogenetica* (Ap) growth rates after long-term exposure to Zn (0 and 0.2 mg Zn L^{-1}, i.e., control and treatment, respectively). Vertical bars denote 95 % confidence intervals.

III.3.3. Reproductive parameters

III.3.3.1. Pre-reproductive period

Pre-reproductive period (i.e., age at first reproduction) was significantly different between *Artemia* species and treatments (**Figure 3, Table 3**). It was shorter in *A. parthenogenetica* compared to *A. franciscana*, and in Zn exposed *Artemia* compared to the controls. The interaction 'species x treatment' was also statistically significant, with pre-reproductive period in *A. franciscana* being higher in controls (39.43 ± 0.25, mean days ± SE) compared to treatments (33.71 ± 1.35, mean days ± SE), but not in *A. parthenogenetica* (29.13 ± 0.55 and 28.23 ± 0.26, mean days ± SE, for controls and Zn exposure, respectively).

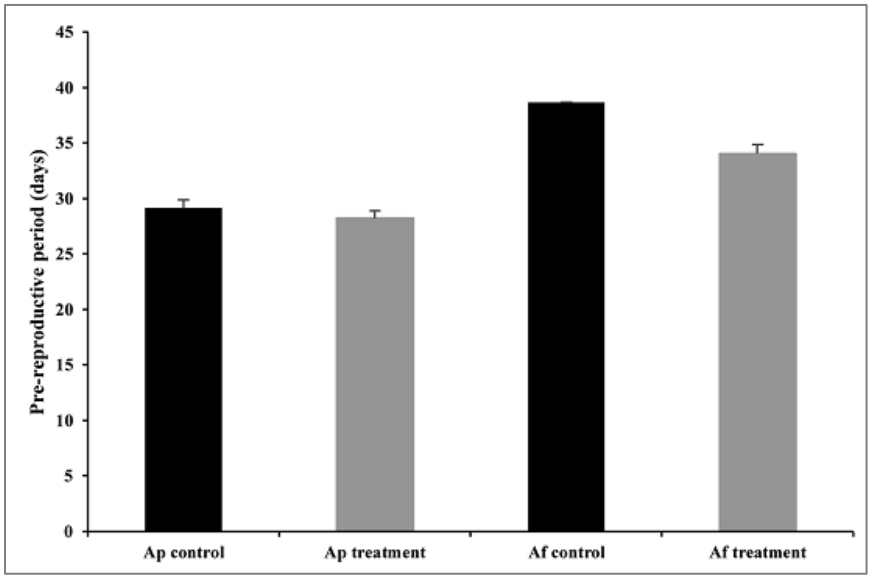


Figure 3. *Artemia franciscana* (Af) and *A. parthenogenetica* (Ap) pre-reproductive period (in days) (mean ± SE) after long-term exposure to Zn (0 and 0.2 mg Zn L^{-1}, i.e., control and treatment, respectively).

Table 3. Results of GLM analysis on *Artemia* pre-reproductive period after long-term Zn exposure (0 and 0.2 mg Zn L^{-1}, control and treatment, respectively). Coefficients for '*A. franciscana*' and 'Zn treatment' (0.2 mg Zn L^{-1}) are not included because they are aliased (i.e., they would be redundant) and are effectively zero. Significant values are in value.

Effect	Level of effect	Estimates	SE	df	F	P-value
Intercept		32.625	0.343	1	9045.785	< **0.001**
Species	*A. parthenogenetica*	-3.942	0.343	1	132.056	< **0.001**
Treatment	Control	1.656	0.343	1	23.296	< **0.001**
Species x Treatment		-1.206	0.343	1	12.354	< **0.001**

III.3.3.2. Number of broods

Number of broods was significantly different between species, with higher values in *A. parthenogenetica* compared with *A. franciscana*. However, they did not differ between treatments (mean ± SE: 2.93 ± 0.12 vs 3.07 ± 0.10, *A. parthenogenetica* control and exposed, respectively; and 1.00 ± 1.03 vs 1.24 ± 0.75, *A. franciscana* control and exposed, respectively). The interaction 'species x treatment' was not statistically significant (**Figure 4**; **Table 4**), and neither was the 'time of monitoring' (**Table 4**).

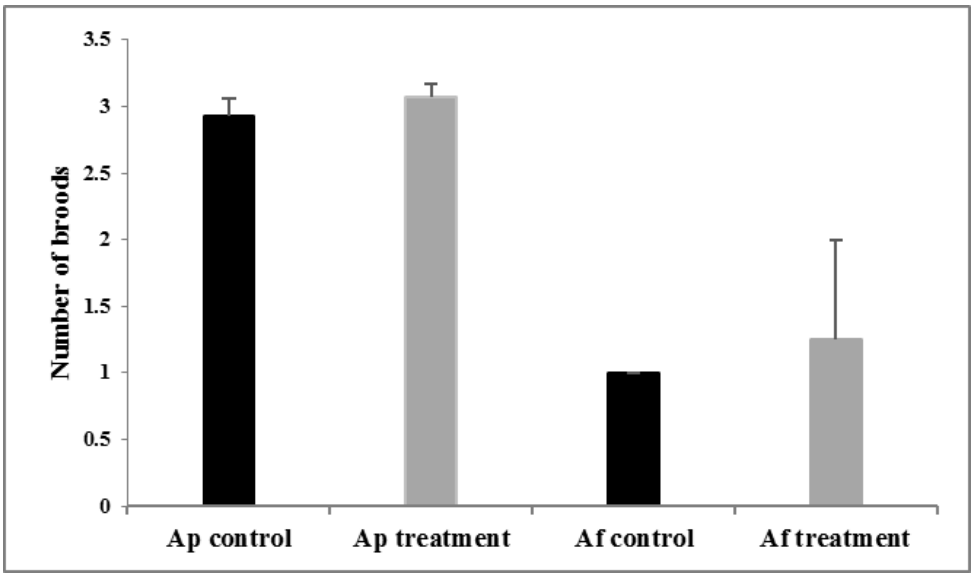


Figure 4. Total number of broods (mean ± SE) for females *A. franciscana* (Af) and *A. parthenogenetica* (Ap) after long-term exposure to Zn (0 and 0.2 mg Zn L^{-1}, i.e., control and treatment, respectively).

Table 4. Results of GLM analysis comparing number of broods between *Artemia* species and treatments. Coefficients for '*A. franciscana*' and 'Zn treatment' (0.2 mg Zn L^{-1}) are not included because they are aliased (i.e., they would be redundant) and are effectively zero. Non-significant interactions were excluded from the final model. Significant values are in bold.

Effect	Level of effect	Estimates	SE	df	F	P-value
Intercept		2.060	0.061	1	1135.435	< **0.001**
Species	*A. parthenogenetica*	0.940	0.061	1	236.086	< **0.001**
Treatment	Control	-0.084	0.058	1	2.094	0.151

III.3.3.3. Offspring

Total offspring was significantly different between *Artemia* species and treatments. *Artemia parthenogenetica* produced more offspring than *A. franciscana*, and offspring were more abundant in treatments than in controls (**Figure 5**). The interaction 'species x treatment' was also statistically significant (**Table 5a**) denoting a stronger increase in *A. parthenogenetica* compared to *A. franciscana*. We included in the model 'time of monitoring', which was also significant.

Similar results were observed for offspring per brood, with *A. parthenogenetica* producing more nauplii than *A. franciscana* and under exposure to Zn (**Figure 5**, **Table 5b**). The significant interaction 'species x treatment' reflected a stronger effect of exposure in *A. parthenogenetica* (33.02 ± 1.72 vs 48.29 ± 2.63, mean ± SE, nº offspring for control and treatment, respectively)

compared to *A. franciscana* (13.40 ± 2.14 vs 16.69 ± 2.64, mean ± SE, nº offspring for control and treatment, respectively; **Figure 5**). 'Time of monitoring' was not significant.

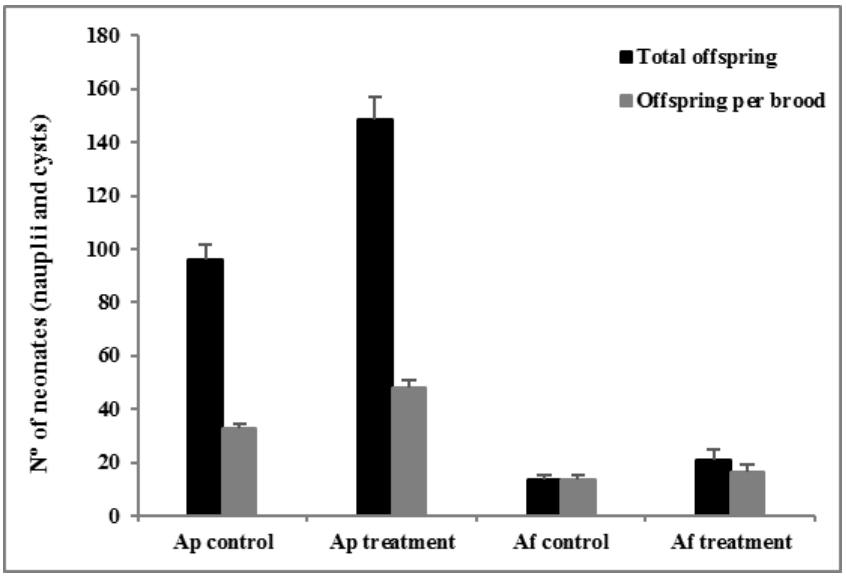


Figure 5. Fecundity in terms of total offspring and offspring per brood, per female (mean ± SE), obtained from *A. franciscana* (Af) and *A. parthenogenetica* (Ap) after long-term exposure to Zn (0 and 0.2 mg Zn L^{-1}, i.e., control and treatment, respectively).

Table 5. Results of GLM analysis comparing (a) total offspring and (b) offspring per brood between different *Artemia* 'species' (*Artemia franciscana* and *A. parthenogenetica*) and 'treatments'. Coefficients for '*A. franciscana*' and 'Zn treatment' (0.2 mg Zn L^{-1}) are not included because they are aliased (i.e., they would be redundant) and are effectively zero. Non-significant variables and/or interactions were excluded from the final models. Significant values are in bold.

a)

Effect	Level of effect	Estimates	SE	df	F	P-value
Intercept		-97.139	69.299	1	1.965	0.165
Species	*A. parthenogenetica*	44.464	5.039	1	77.867	**<0.001**
Treatment	Control	-14.708	3.717	1	15.655	**<0.001**
Species x Treatment		-11.921	3.718	1	10.283	**0.002**
Time of monitoring		3.789	1.571	1	5.814	**0.018**

b)

Effect	Level of effect	Estimates	SE	df	F	P-value
Intercept		-11.104	23.694	1	0.220	0.641
Species	*A. parthenogenetica*	10.902	1.723	1	40.047	**<0.001**
Treatment	Control	-4.259	1.271	1	11.230	**0.001**
Species x Treatment		-3.466	1.271	1	7.433	**0.008**

III.3.3.4. Percentage of non-viable nauplii

GLM analysis showed a significant effect of *Artemia* species on the % of non-viable nauplii (dead at birth during ovoviviparous reproduction), with lower proportion in *A. parthenogenetica* than in *A. franciscana* (**Table S3**, **Figure S1**). Exposure to Zn did not affect viability of nauplii. The interaction 'species x treatment' was not significant.

III.3.4. Oxidative stress

Results of GLM on biomarkers of oxidative stress (sqr-transformed) showed no significant differences ($p > 0.05$) between treatments and species in CAT and SOD. However, in the case of GR, results for species were marginally significant ($F_{1, 26} = 3.331$, $p = 0.070$), with levels for *A. franciscana* being higher than for *A. parthenogenetica*. The interaction 'species x treatment' was non-significant for enzymatic activities ($p > 0.643$). This was probably due to the low sample size. However, in the case of TBARS, the interaction 'species x treatment' was statistically significant (**Table 6**, **Figure 6**), reflecting an increase of lipid peroxidation in *A. franciscana* when exposed to Zn but the opposite effect in *A. parthenogenetica*.

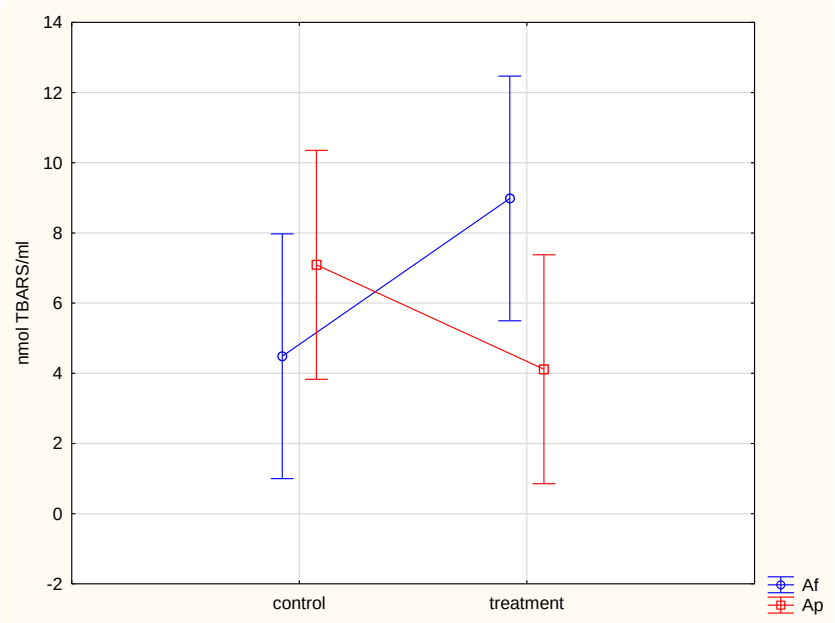


Figure 6. Comparison of TBARS levels (mean ± SE) of *A. parthenogenetica* (Ap) and *A. franciscana* (Af) after long-term exposure to Zn (0 and 0.2 mg Zn L^{-1}, i.e., control and treatment, respectively).

Table 6. Results of GLM analysis on TBARS levels with different *Artemia* 'species' (*Artemia franciscana* and *A. parthenogenetica*) and 'treatments' (Zn and control). Coefficients for '*A. parthenogenetica*' and 'treatment' (0.2 mg Zn L^{-1}) are not included because they are aliased (i.e., they would be redundant) and are effectively zero. Non-significant variables and/or interactions were excluded from the final model. Significant values are in bold.

Effect	Level of effect	Estimates	SE	df	F	P-value
Intercept		6.170	0.821	1	56.446	< **0.001**
Species	*A. franciscana*	0.565	0.821	1	0.474	0.497
Treatment	control	-0.381	0.821	1	0.215	0.647
Species x Treatment		-1.868	0.821	1	5.176	**0.031**

III.4. Discussion

Pollution and invasive species are among the most prevalent and pervasive anthropogenic stressors in aquatic ecosystems worldwide, with major ecological and economic impacts (Vitousek et al., 1996; Ginebreda et al., 2014; Laws, 2017). However, despite the substantial amount of research that exists on these factors, there is a paucity of understanding of how pollution might modulate invasion success. Several studies show that polluted ecosystems are more prone to invasions (Piola and Johnston, 2009; Crooks et al., 2010). Characteristics such as phenotypic plasticity and adaptation to stressful conditions can also facilitate invasion success (Blackburn et al., 2009; Oliveira et al., 2015). On the other hand, pollution may also promote local adaptation of native species and prevent the establishment of new incomers (Rodrigues et al., 2012; but see Boets et al., 2012). However, evidence for this is extremely limited because most studies focus on successful invasions and very few on cases where invasion has been resisted (Sánchez et al., 2016a). In the present study we found evidence that native species can be more adapted to pollution than invasive species, by combining life history and physiological data. We specifically evaluated the effect of pollution by Zn (under real ecological concentrations) in the invasive crustacean *A. franciscana* and one of the few remaining native *Artemia* populations in the Mediterranean.

III.4.1. Survival

Our results show higher survival in *A. franciscana* compared to *A. parthenogenetica* irrespective of the treatment. However, exposure to Zn (0.2 mg Zn L^{-1}) strongly reduced survival in *A. franciscana* but did not affect *A. parthenogenetica*. This suggests local adaptation of the native population to high levels of Zn, supporting the hypothesis of Rodrigues et al. (2012) that pollution may have a role limiting the invasion. Low sensitivity to trace element pollution has been previously shown in crustaceans from polluted areas and can be acquired through genetic processes

(Ward and Robinson, 2005) or acclimation (Muyssen et al., 2002). However, it is not a general rule. For example, Boets et al. (2012) hypothesized that gammarid populations originating from historically trace element-polluted sites would be less sensitive to cadmium than populations of the same species originating from non-polluted sites, and that alien gammarids would be more resistant than native ones. They found no significant differences in Cd sensitivity between populations from trace element polluted systems and non-polluted sites, and alien gammarids were not more tolerant to Cd than indigenous species, concluding that they would probably not have a competitive advantage in Cd contaminated environments, compared to indigenous species.

Our results on *A. parthenogenetica* survival also contrasts with those of Sarabia et al. (2008) who reported significant mortality of this species when experimentally exposed to 0.08 mg L^{-1} Zn. However, they used a population from a relatively unpolluted system of Eastern Spain (Varó unpublished data) which would not be adapted to Zn.

III.4.2. Growth rate

At low levels, Zn is essential for the normal growth and reproduction of plants and animals, including humans (Goyer and Clarkson, 2001). In addition, it plays a vital role in many biological processes, including the stabilization of DNA, gene expression, functionality of many enzymes (Frassinetti et al., 2006; DeForest et al., 2007; Caplat et al., 2012) and immune function (Frassinetti et al., 2006). However, at the concentrations used in our study it significantly reduced growth rate in both *Artemia* species, with no differences in the strength of the effect among species (no significant interaction between species and treatment). Similar results (showing lower growth rate under Zn exposure) have been reported for other crustaceans, e.g., *Daphnia lumholtzi* (Dao et al., 2017), *Daphnia longispina* (Martins, 2017) and *Corophium volutator* (Conradi and Depledge, 1999). This effect can be explained by the reduction in the amount of metabolic energy available for growth, since Zn affects feeding, respiration, and excretion ratios, as well as energy absorbed and moulting frequency in crustaceans (Conradi and Depledge, 1999). Lower growth rate can have important implications for survival because of size/stage-dependent sensitivity to pollution observed in many organisms (See Sánchez et al., 2016a for *Artemia* and Iwasaki et al., 2015 for fishes, with opposing patterns). However, contrary to the general prediction linking size with stress (i.e., chronically stressed environments would select for smaller organisms, Cattaneo et al., 1998), both species attained similar final size, suggesting that size did not trade off against other traits.

III.4.3. Reproductive parameters

There is strong evidence that environmental stress can affect the evolution of life-history patterns which in turn are determined by trade-offs between traits (Roff et al., 2006). An increase in one life history trait that enhances fitness should be counteracted by a shift in another trait that decreases fitness (Stearns, 1977, 1992; Charlesworth, 1980; Smith et al., 1985; Roff, 1992). For example, age at first reproduction can vary among different species and populations exposed to different environmental conditions (Roff, 1984; Stearns, 1992), and is an important determinant of future growth and survival. Our results showed firstly a short pre-reproductive period and high fecundity (number of broods, total offspring and % of non-viable nauplii) in *A. parthenogenetica*, which also exhibited a high mortality. This is in accordance with the life history theory, which predicts that high adult mortality should lead to early maturity and fecundity (Stearns, 1992). Early maturation and high fecundity may be the optimal strategy for *A. parthenogenetica* to maximise fitness in the highly polluted Odiel to compensate for the high mortality. High mortality can be also the cause of an early maturation (as a cost of reproduction at smaller size caused by lower growth rate). Younger/smaller individuals would have fewer resources to allocate to growth, which would also result in a higher mortality. Several studies have shown that a mortality cost of reproduction is a common phenomenon (Roff, 1992) and may be a plausible explanation for our observations.

Our results showing higher fecundity of *A. parthenogenetica* compared to *A. franciscana* contrast with several studies showing that *A. franciscana* has the greatest fecundity of all species of the genus (Browne et al., 1984; Lenz and Browne, 1991; Browne and Wanigasekera, 2000; Amat et al., 2007; Pinto et al., 2013, 2014a; Varó et al., 2015). However, as shown in other studies (e.g., Sarabia et al., 2002; Varó et al., 1998) life history traits are very population- and environment-dependent.

The second point of our results is that *Artemia* species responded differently to Zn exposure; for example, while it did not affect age at first reproduction in the case of the native *Artemia* it accelerated reproduction in the exotic one. Early maturation has an adaptive significance as a key mechanism ensuring an increased rate of population reproduction under conditions of high mortality (see for example Moiseenko, 2010). Several studies show that animals exposed to high levels of pollution mature at younger age and produce an increased number of small eggs, compensating for the toxic effect of pollutants and maintaining their population size (see for example Collier et al., 1998). On the other hand, Zn increased offspring in both *Artemia* species, with a stronger effect in *A. parthenogenetica* (three times more than in *A. franciscana*). These effects on reproduction have the potential to affect population densities and consequently the probability of a successful invasion. The stimulatory effect on reproductive parameters may be related to the phenomenon of hormesis

(Stebbing, 1982). Low dose stimulation has been also reported in a broad range of organisms (from bacteria to vertebrates) and biological traits (e.g., growth, survival, reproduction) (Calabrese and Baldwin, 2003) and in particular for crustaceans exposed to Zn. Zinc has been shown to have stimulatory effects on the reproduction and protein levels of *Daphnia magna* (Canli, 2005), and Zn deficiency causes an important reduction in the reproduction of this species (Caffrey and Keating, 1997).

Sarabia et al. (2008) reported different results when studying the chronic effect of Zn (0.08 mg Zn L^{-1}) in *A. parthenogenetica* populations from Eastern Spain. They found detrimental effects on *A. parthenogenetica* fecundity and concluded that Zn toxicity may represent a limitation for their study population, preventing it from colonizing Zn-polluted environments. However, as stated previously, Sarabia et al. (2008) studied a relatively unpolluted *A. parthenogenetica* population, which is a fundamental difference compared to our system.

Our results show that a similar concentration of Zn can induce toxicity or hormesis depending on the endpoint or on the development stage. Nascarella et al. (2003) demonstrated that queen blowfly, *Phormia regina*, presented a hormetic response in an early developmental stage (pupation), while adults displayed a stage-specific toxicity. This was possibly owed to gene expression of some binding trace element proteins or metallothioneins implicated in trace element detoxification mechanisms that occur in a particular moment of the life cycle (Akoi and Suzuki, 1984). This may also be the case in *Artemia*. Trace element detoxification systems may activate only after a period of exposure, explaining the increased mortality during the 21 days of the survival experiment and hormesis after this period, during the reproductive phase.

III.4.4. Oxidative stress

In our study, no significant changes were noted in the levels of enzymatic activity (CAT, SOD, GR) between *Artemia* species or treatments. These enzymes are considered to play a key antioxidant role in invertebrates (Simmons and Jamall, 1988; Livingstone et al., 1992) and we suspect that absence of significant differences is owed to the low sample size, because individuals needed to be pooled to attain a suitable volume for analysis. However, TBARS content (as a biomarker of lipid peroxidation) strongly increased in *A. franciscana* under exposure to Zn, while the opposite effect was observed in *A. parthenogenetica*. These results suggest that general antioxidant response to Zn exposure was enough to detoxify excess of ROS in *A. parthenogenetica* but not in *A. franciscana*. Failure of antioxidant defences to detoxify excess ROS production can lead to significant oxidative damage with apoptosis and death (Halliwell and Gutteridge, 1999). Results of TBARS can explain the high mortality experienced by *A. franciscana* when exposed to

Zn and the lack of effect in the case of *A. parthenogenetica*. These results are consistent with those of life history and represent an additional support to the pollution resistance hypothesis (McMahon, 2002; Rodrigues et al., 2012). To our knowledge, only Faria et al. (2010) have previously provided physiological data to support this hypothesis. These authors showed that the invasive Asiatic clam *Corbicula fluminea* had higher levels of lipid peroxidation than native bivalves when exposed to mercury, and the exotic zebra mussel *Dreissena polymorpha* presented higher levels of DNA fragmentation caused by oxidative stress compared with native bivalves. As suggested by McMahon (2002), having strong physiological defensive mechanisms is of limited adaptive interest to invasive species whose ecological success is based on life history traits that guarantee a rapid population growth. Therefore, the important physiological stress due to the inefficiency to neutralize ROS in highly polluted environments may be a critical limitation for *A. franciscana*, preventing the colonization of highly polluted environments where native species are locally adapted. The global antioxidant response of *A. parthenogenetica* was sufficient to prevent lipid peroxidation in exposed individuals, which performed better in polluted conditions than in controls.

III.5. Conclusions

In this study we show clear differences in the response of native and invasive *Artemia* to Zn. Our results suggest that Zn affects resource allocation between metabolism maintenance (important for survival), reproduction, growth, and defence against oxidative stress, with important implications for *Artemia* population growth. They suggest that *A. parthenogenetica* from the contaminated Odiel is better adapted to Zn than the invasive *A. franciscana*. This may affect the probability of invasion by the exotic species due to the high physiological stress, the concomitant mortality and low fecundity. Physiological resistance is key to the development of viable populations of newly arriving species to stressed ecosystems (Faria et al., 2010). On the other hand, populations with low growth rates would be particularly sensitive to the effect of environmental stress (Belovsky et al., 1999). However, *A. franciscana* has a high capacity for local adaptation (Lenormand et al., 2018), so it remains possible that it will acquire greater resistance to trace element pollution in the future. Our results also suggest that *A. franciscana* has the potential to change maturation patterns under exposure to Zn.

To draw firmer inferences in terms of invasion probability, future studies should consider a mixture of pollutants as this is the norm in natural conditions. For example, Sánchez et al. (2016a) showed that *A. parthenogenetica* experienced stronger mortality than *A. franciscana* when

chronically exposed to arsenic, and that both species accelerated growth rate, which is the opposite result to what we found here for Zn.

III. Supplementary material

Table S1. Experimental conditions for the long-term exposure to Zinc.

Time (days)	Temperature (ºC)	Salinity (g L^{-1})	Individuals (nº)	Replicates (nº)	Volume (ml)	Microalgae (spp.)	Algae concentration (cell ml^{-1})
1-3	25	45	140	4	140	*T. suecica*	$2,5*10^5$
4-6	25	45	140	4	140	*T. suecica*	$5*10^5$
6-9	25	65	140	4	280	*T. suecica* + *D. salina*	$5*10^5$
10-16	25	100	140	4	280	*D. salina*	$5*10^5$
17-21	25	100	140	4	560	*D. salina*	$5*10^5$

Table S2. Real concentrations of Zinc measured in Odiel saltpans water.

Zn (mg L^{-1})	Study site	Date	Sampling point	Salinity	Replicate	Type of sample
0.1099	Odiel	22/04/2014	Canal Bomba 2	160	1	water
0.1158	Odiel	22/04/2014	Canal Bomba 2	160	2	water
0.1121	Odiel	22/04/2014	Canal Bomba 2	160	3	water
0.974	Odiel	22/04/2014	Canal Bomba 2	160	4	water

Table S3. Percentage (%) of non-viable nauplii (dead at birth during ovoviviparous reproduction) during long-term exposure to Zn. Results of GLM analysis on 'non-viable nauplii' in the offspring after Zn exposure, for different *Artemia* 'species' (*A. franciscana* and *A. parthenogenetica*) and 'treatments' (0 and 0.2 mg L^{-1}). 'Time of monitoring' was included as a covariable. Coefficients for '*A. franciscana*' and 'Zn treatment' are not included because they are aliased and are effectively zero.

	Level of Effect	Estimates	SE	df	F	p-value
Intercept		24.035	2.086	1	132.794	< 0.001
Species	*A. parthenogenetica*	-4.438	2.086	1	4.528	0.036
Treatment	Control	1.303	1.978	1	0.434	0.512

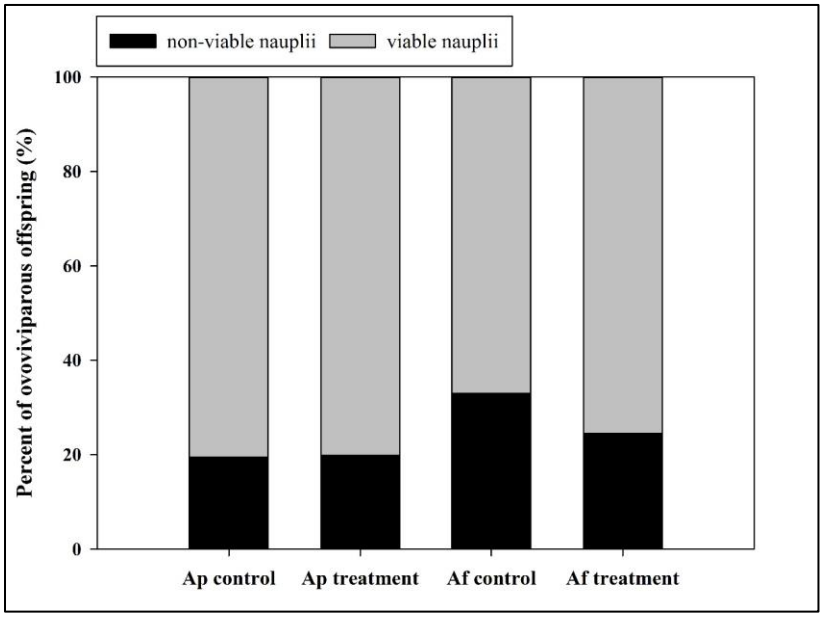
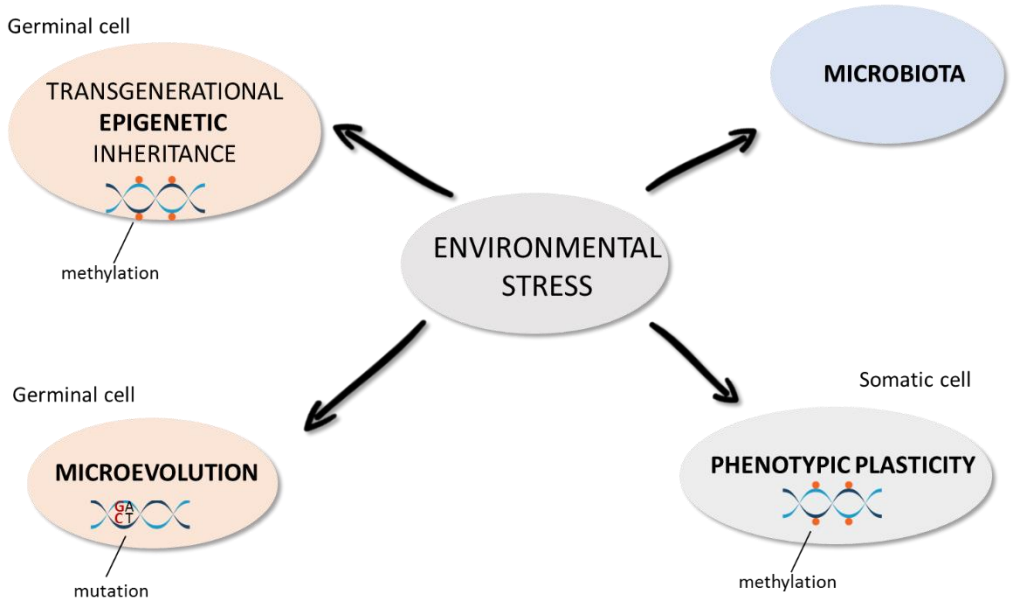


Figure S1. Qualitative differences in ovoviviparous offspring related to percentages of viable and non-viable nauplii, obtained from *Artemia franciscana* (Af) and *Artemia parthenogenetica* (Ap) after long-term exposure to Zn (0 and 0.2 mg L^{-1}, i.e., control and treatment, respectively).

CHAPTER IV

Phenotypic but no genetic adaptation in zooplankton 24 years after an abrupt +10°C climate change.



Pais-Costa, A.J., Lievens, E.J.P., Redón, S., Sánchez, M.I., Jabbour-Zahab, R., Joncour, P., Hoa, N.V., Stappen, G.V., Lenormand, T. Dissecting phenotypic adaptation to +10°C in *Artemia* introduced to the tropics: (epi)genetics, plasticity, and microbiota. Accepted in Evolution Letters.

IV. Abstract

The climate is currently warming fast, threatening biodiversity all over the globe. Adaptation is often rapid when the environment changes quickly, but for climate warming very little evidence is available. Here, we investigate the pattern of adaptation to an extreme +10 °C climate change in the wild, following the introduction of brine shrimp *Artemia franciscana* from San Francisco Bay, USA, to Vinh Chau saltpan in Vietnam. We use a resurrection ecology approach, hatching diapause eggs from the 'ancestral' population and the 'derived' population after 13 and 24 years (resp. ~54 and ~100 generations). In a series of coordinated experiments, we determined whether the introduced *Artemia* show increased tolerance to higher temperatures, and the extent to which genetic adaptation, developmental plasticity, transgenerational effects, and local microbiome differences contributed to this tolerance. We find that introduced brine shrimp do show increased phenotypic tolerance to warming. Yet strikingly, these changes appear to i) not have an additive genetic component, ii) to be caused by mitochondrial genetic variation, and iv) to be caused by epigenetic marks set by adult parents exposed to warming. Further, we do not find any developmental plasticity in response to warming, nor any protective effect of heat-tolerant local microbiota. The evolved thermal tolerance might therefore be entirely due to transgenerational (great)grandparental effects, possibly epigenetic marks set by parents who were exposed to high temperatures as juveniles. This study is an outstanding example of "missing heritability", where a large adaptive phenotypic change, not due to phenotypic plasticity, is not accompanied by additive genetic effects.

Keywords: additive genetic effect, microbiota, transgenerational epigenetic effects, plasticity, climate change, missing heritability, thermal tolerance, resurrection ecology.

IV.1. Introduction

Understanding how biodiversity responds to global warming, and anticipating whether species will be able to adapt quickly enough to keep pace with the projected changes, have become major scientific challenges (Hoffmann and Sgrò, 2011). While rapid genetic adaptation to novel human-made environmental changes—pollution, pesticides, antibiotics— has been extensively documented (Hendry et al., 2017), much less has been observed for climate warming (Hoffmann and Sgrò, 2011; Merilä and Hendry, 2014; Franks and Hoffmann, 2012; Stoks et al., 2014; Gienapp et al., 2008). This discrepancy might be due to the (yet) modest climate change or to the fact that many pre-existing mechanisms are already in place in most species to cope with the current range of climatic variation.

Theoretically, several mechanisms may cause a phenotypic response to climate warming (Gienapp et al., 2008; Franks and Hoffmann, 2012). First, organisms may genetically evolve to better tolerate high temperatures, and this process may extend their tolerance outside their current thermal niche. They may also phenotypically adjust to these changes using pre-existing plastic responses, within (Lande, 2015; Chevin and Hoffmann, 2017) or across generations (maternal effects, transgenerational epigenetic effects (Auge et al., 2017; Lind and Spagopoulou, 2018)). Finally, they may also benefit from symbionts / microbiota adapted to these new conditions (Vannier et al., 2015; Nougué et al., 2015; Frankel-Bricker et al., 2020), without adapting to these conditions themselves. These sources of variation are mutually non-exclusive and can interact in ways that are difficult to disentangle (for instance maternal effects may be mediated by transmitted symbionts, epigenetic marks, or maternal plastic responses (Schlichting and Wund, 2014; Palumbi et al., 2014; Vannier et al., 2015)).

We investigated whether species could adapt in the wild beyond their climatic niche, with the aim of disentangling these different effects. We used a resurrection ecology approach to assess the thermal adaptive potential of natural populations of the brine shrimp *Artemia franciscana* over 24 years (ca. 100 generations) following an abrupt climatic shift (Lenormand et al., 2018). In the early 1980s, *A. franciscana* from San Francisco Bay, USA (hereafter SFB) were introduced into Vinh Chau salterns, Vietnam (hereafter VCH), where mean (air) temperatures are +10 °C higher (Clegg et al., 2000; Frankenberg et al., 2000). This far exceeds the worst IPCC climate warming scenario for the 21st century (RCP8.5 Model predicts +6 °C (IPCC 2013)), yet the brine shrimp have thrived (Van Hoa 2014), and show phenotypic adaptation to high temperatures (Clegg et al. 2000; Kappas et al. 2004). Indeed, VCH *Artemia* are now commonly used to inoculate other (sub)tropical salterns. We used a series of coordinated experiments to determine the extent to which the introduced

Artemia's phenotypic adaptation to higher temperatures resulted from genetic changes, pre-existing plastic responses, transgenerational effects or the effect of locally adapted microbiota.

IV.2. Material and Methods

Experiments were performed with three populations of *A. franciscana*: one from San Francisco Bay (SFB), USA, collected in 1984 (SFB_{84}); a second from Vinh Chau (VCH) saltpan, Vietnam, collected in 1997 (VCH_{97}); and a third, also from VCH, collected in 2008 (VCH_{08}).

IV.2.1. Decapsulation and hatching of the cysts:

The parental generation of experimental individuals (see below) was hatched from dormant cysts. Cyst decapsulation and hatching protocols were modified from Bengtson et al. (1991). Cysts were rehydrated in deionized water (2 h to 3 h). After rehydration, cysts were decapsulated by a 10 min exposure to a sodium hypochlorite solution, then rinsed with running water (10 min) and deionized water (5 min). Decapsulated cysts were incubated for 48 h at 28 °C (± 1 °C), with constant light and aeration, in a medium with a salinity of 5 (see below). After emergence, first-instar nauplii were moved to 23 °C (±1 °C) and natural light conditions. Salinity was gradually increased to 80–90 over 8–9 days. This procedure was performed independently for the three different *Artemia* populations.

IV.2.2. Baseline experimental conditions

Throughout the preparation and execution of the experiments, *Artemia* were kept in an 80–90 g L^{-1} saline medium prepared by diluting field-collected concentrated brine (280 g L^{-1}, Camargue Pêche, France) from Aigues-Mortes saltpan with deionized water. Organisms were fed a solution of *Tetraselmis chuii* algae (Fitoplankton marino, Spain), prepared by dissolving 1 g of lyophilized algae in 1 L of deionized water (ca. $6.8x10^9$ *T. chuii* cells L^{-1}). Stock individuals were fed ad libitum. Food was added daily (1 ml of algae / group of juveniles / day; and 1ml of algae / couple / day) before exposure and 3 times a week (2 ml of algae / group of juveniles / 2 days) during exposure to the temperature treatments. Unless specifically mentioned, individuals were kept at 23 °C (±1 °C), under natural light conditions. Juvenile survival tests were all performed in the dark in incubators and thermostatic chambers. Mortality was checked twice (5 and 10 days after the beginning of the treatment, i.e., midway through and at the end of the thermal treatment).

IV.2.3. SFB and VCH temperature regimes

The same temperature regimes were applied in each experiment. Two temperature cycles were used: i) cycle of temperatures based on the air temperatures from SFB (T_{SFB}): 16 ºC (2 h); 22 ºC (8 h); 27 ºC (4 h); 22 ºC (8 h); 16 ºC (2 h); and ii) cycle of temperatures based on the air temperatures from VCH saltpan (T_{VCH}): 26 ºC (2 h); 32ºC (8 h); 37 ºC (4 h) for experiment 2 and 35 ºC (4h) for the remaining experiments; 32 ºC (8 h); 26 ºC (2 h).

IV.2.4. Experiment 1: microevolution/adaptation

This experiment was performed to measure the additive genetic effect of thermal adaptation, removing maternal lineage effects. For this experiment, we collected virgin females from a laboratory population of *A. franciscana* from SFB, hatched from cysts collected in 2003 (SFB_{03}). The SFB_{03} population was maintained in the laboratory for over 2 years, so it was well acclimated to the standard laboratory temperature conditions (23 °C ± 1°C). For SFB_{84}, VCH_{97}, and VCH_{08}, we hatched individuals from field cysts. Before individuals reached sexual maturity, their sex was assigned based on sexual dimorphism. After maturity, males from the three study populations of *Artemia* were mass crossed (animals divided into 4 replicates) with the virgin stock females (SFB_{03}) to produce an F1 generation. Starting 24 h after the first nauplii were seen, we collected daily batches of nauplii from the mass crosses. This ensured that the organisms used in each replicate were born within the same period. New-born nauplii from each cross were maintained in 50 ml Falcon tubes (max. 30 nauplii per tube) filled with 30 ml of brine solution for a period of 7 days. After 7 days, all meta-nauplii from the same cross were mixed and then separated into replicate groups of 10 individuals. Each group was placed in a 50 ml Falcon tube filled with 30 ml of brine solution and exposed to T_{SFB} or T_{VCH} for 10 days (7^{th} to 17^{th} day). 30–32 groups per population were exposed to each cycle of temperatures (1830 individuals in total).

IV.2.5. Experiment 2: Parental acclimation

This experiment was designed to investigate the possibility that thermal exposure in the parents could influence juvenile performance at high temperature. Individuals were hatched from SFB_{84}, VCH_{97}, and VCH_{08} field cysts. Before they reached sexual maturity, their sex was assigned based on sexual dimorphism. After maturity, single pairs of males and females from each population were isolated in 50 ml Falcon tubes filled with 30 ml of brine solution to produce an F1 generation. We collected the first brood of nauplii produced by each parental couple. Each brood of nauplii was isolated from their parents after confirming, under a stereomicroscope, that the female

ovisac was empty. In this way, we ensured that the organisms used in each replicate were born within the same period. Immediately after the first clutch (CL_1) was born, the parents were separated, and one of three treatments was applied: i) mother exposed to high temperature; ii) father exposed to high temperature; and iii) control (none exposed to high temperature). The 'high temperature' treatment consisted of 8 h at 35 °C (±1 °C) in the dark. Afterwards, the couples were put back together to produce a second clutch (CL_2), which we collected in the same way. New-born nauplii were kept in 50 ml Falcon tubes (max. 30 nauplii per tube) filled with 30 ml of brine solution for a period of 7 days. After 7 days, meta-nauplii from each family were separated into groups of 10 individuals and placed in 50 ml Falcon tubes filled with 30 ml of brine solution. A minimum of 5 replicate groups were tested per couple. Each replicate was exposed to T_{SFB} or T_{VCH} for 10 days (7^{th} to 17^{th} day). Overall, 39.3 groups (SD 9.4) were used per treatment, population, and temperature regime combination (survival of 7080 individuals assayed in total).

IV.2.6. Experiment 3: Juvenile acclimation

This experiment was conducted to study if very early exposure of the organisms to a thermal regime would increase their performance as juveniles under the same regime. Individuals were hatched from SFB_{84}, VCH_{97}, and VCH_{08} field cysts. Before individuals reached sexual maturity, their sex was assigned based on sexual dimorphism. After maturity, single pairs of males and females from the same population were placed in 50 ml Falcon tubes filled with 30 ml of brine solution to produce an F1 generation. We collected new-born nauplii from the parental couples. Each brood was isolated after confirming, under a stereomicroscope, that the female ovisac was empty. In this way, it was ensured that the organisms used in each replicate were born within the same period. New-born nauplii were counted and separated into 50 ml tubes containing 30 ml brine solution (maximum 30 nauplii per tube). Nauplii were then maintained under the same conditions of light, food, and temperature as the parents for a period of 5 days. After 5 days, meta-nauplii entered the experiment, which was divided into 2 phases (P_1 and P_2). At day 5, a first temperature regime was applied for two days (P_1). Meta-nauplii from each family were separated into 50 ml Falcon tubes (max. 30 nauplii per tube) filled with 30 ml of brine solution and assigned to either T_{SFB} or T_{VCH}. Broods were discarded whenever it was impossible to obtain two replicates (one per temperature regime) with a minimum of 10 individuals each. After this first phase (P_1), mortality was checked, and surviving meta-nauplii were separated into groups and placed into 50 ml Falcon tubes (no more than 14 individuals per falcon) filled with 30ml of brine solution. Broods were discarded whenever it was impossible to obtain two replicates (one per temperature regime) with a minimum of 5 individuals each. Meta-nauplii from each population and temperature regime were

again assigned to T_{SFB} or T_{VCH} for the second phase (P_2). Hence, different individuals were exposed to different temperature histories: $T_{SFB} \rightarrow T_{SFB}$, $T_{SFB} \rightarrow T_{VCH}$, $T_{VCH} \rightarrow T_{SFB}$, $T_{VCH} \rightarrow T_{VCH}$. Survival during this second phase was recorded for a period of 10 days (7^{th} to 17^{th} day). Overall, 48.8 (SD 12.0) groups were used per temperature history ($P_1 \rightarrow P_2$) and population combination (survival of 5315 individuals assayed in total).

IV.2.7. Experiment 4: Microbiota

This experiment was designed to investigate whether exposing organisms to microbiota adapted to different climates lent their hosts different performance in those climates. SFB_{84}, VCH_{97}, and VCH_{08} field cysts were rehydrated in sterile deionized water (2 h to 3 h). After rehydration, cysts were decapsulated by a 10 min exposure to a sodium hypochlorite solution, then rinsed with deionized water (10 min) and sterile deionized water (5 min). Decapsulated cysts were then incubated for 3 days at 28 °C (±1 °C) and under constant light, in sealed bottles containing 400 mL autoclaved brine solution (salinity of 5). After emergence, first-instar nauplii were placed at 23 °C (±1 °C) under constant light and fed with sterilized *T. chuii* solution. This procedure was performed independently for the three different populations. Salinity was gradually increased to 80–90 over 8–9 days. When salinity reached 40, nauplii from each population were separated into 3 groups and inoculated with i) microbiota from SFB ii) microbiota from VCH or iii) microbiota from containers in the laboratory. The microbiota inoculum was obtained by mixing crushed live adult individuals collected in 2017 in two sites in both Vinh Chau saltpan (salinity 70 and 90) and San Francisco Bay Estuary Field Station (salinity 70 and 130). These 2017 microbiota might differ from the original 1984 situation, but the thermal background did not significantly change between 1984 and 2008 and those microbial communities should reflect this climatic difference. Each inoculation bottle was filled with 400 ml of sterile deionized water and 100 ml of microbiota solution. Sterilized *T. chuii* was added ad libitum. When individuals reached sexual maturity, 12 males and 12 females from each population and treatment were separated into new sterile containers and mass crossed to produce a F1 generation and kept under the same conditions as the stock. New-born nauplii were checked daily. Each batch of nauplii was isolated within 24 h after the first nauplius was seen, to ensure that organisms used in the experiment were born within the same period. New-born nauplii were separated into sterile 50 ml tubes (max. 30 nauplii per tube) containing 26 ml of sterile brine solution, 2 ml of microbiota solution, and 2 ml of autoclaved algae solution. Nauplii were then maintained under natural light at 23 °C (±1 °C) for a period of 7 days. After 7 days, all meta-nauplii from the same treatment were mixed and separated into replicate groups of 10 individuals. Each group was placed in a sterile 50 ml tube containing 26 ml of sterile brine solution, 2 ml of

microbiota solution, and 2 ml of algae solution. To maintain the comparison with the other experiments, only the water was autoclaved to prepare the food solution for the rest of the experiment (i.e., not the lyophilized algae, which would have significantly altered the food source). Each replicate was exposed to T_{SFB} or T_{VCH} for 10 days (7^{th} to 17^{th} day). Overall, 87–103 groups (27–39 groups per microbiota treatment) per population were exposed to each temperature regime (5630 individuals in total). All feeding and transfers were performed under a laminar flow hood to prevent microbial contamination. During the experiment, the containers were closed to limit contamination, but not sealed to allow gas and oxygen exchange.

IV.2.8. Mitochondrial genome sequencing and analyses

In order to determine whether increased heat tolerance of the Vietnamese populations could be caused by mitochondrial genetic variation, we sequenced the full mitochondrial genome of 10 individuals (individuals 1–5 sampled in 1984, and individuals 6–10 sampled in 2008). We also sequenced pools of cysts sampled in Vinh Chau saltpan (25mg of cysts, ca 6500 cysts per pool) from eight years (1984, 1987, 1988, 1993, 1994, 1997, 1998, 2008). Three of these were replicated twice, with independent DNA extraction (1984, 1997, 2008). For each sample, mitochondrial DNA was extracted using an Abcam ab65321 Mitochondrial DNA isolation kit, following the manufacturer's instructions. NGS libraries were constructed using a Nextera DNA flex illumina kit (ref 20018704) and sequenced (PE 150) on an Illumina NovaSeq 6000 (MGX platform, Montpellier).

For each sample, paired reads were mapped onto an *A. franciscana* reference sequence (NC_001620.1) with *bowtie2*, trimming 10 bases in 5'. Read duplicates were removed with *Picard MarkDuplicates*. Reads with a mapping quality over 20 and in proper pairs were kept with *samtools view*. The program *pysamstats* was used to get the raw percentage of each base and the total coverage at each position of the reference sequence. These steps were done twice, on the original reference genome and on a version that was cut in the middle and had the two parts reordered. This was done to avoid border effects and obtain a good mapping for the reference extremities of this circular genome. A dedicated *R* script was written to concatenate the *pysamstats* output files, keeping 50 % middle positions of the two reference versions, to obtain two tables with all samples: one with the percentages of alternative bases at each position and one with the coverages. SNP calling was done using a dedicated Mathematica 10.1 (*Wolfram*) script. Genome coverage was ~3000X on average for cyst pool samples (range 1000X–6000X) and was ~200X on average for individual samples (range 42X–336X). Three regions showed a drop in coverage on the reference

genome and were excluded from further analyses (region 1: 14045–14394; region 2: 14682–14835; region 3 15409–15806).

Forty variable positions were identified that distinguished the 10 sampled individuals. One of them was an ambiguous insertion of a variable number of T's at position 1247 and was removed. Among the 39 remaining SNPs, 7 were shared by at least two individuals and 32 were private to a single individual. The shared-SNPs defined 6 non-ambiguous haplotypes (hereafter 'mitotypes'), three being characterized by a combination of at least two shared-SNPs (individuals 7 and 10; individuals 5 and 6; individuals 2, 4, and 8) and three by the absence of shared-SNPs (individuals 1, 3, and 9). The frequency envelopes of the former were obtained using the frequency of their most frequent shared-SNP, while the frequency envelope for the latter was based on the frequency of their most frequent private-SNP (as in the absence of recombination, the sum of the frequency of private SNPs cannot exceed that of shared SNPs within a mitotype).

The frequency of each of the 39 SNPs was estimated from the cyst pool-seq data in eight separate years (**Figure S2**). Frequencies at all shared and private SNPs were very highly correlated between replicates (R^2 = 0.995 for years 1984, 1997, 2008), showing that the pool-seq data provided very precise information (**Figure S3**). Frequency data from consecutive years also showed very consistent frequency estimates (**Figure S2**). The cumulative frequency of the 6 mitotypes identified represented ~80 % of the population. Other SNPs were identified in the dataset but were not used as they could not be easily clustered or assigned to a mitotype due to the lack of important temporal frequency variation. Overall, the frequency pattern of the different mitotypes was remarkably stable, ruling out that the genetic composition of the mitochondrial population changed significantly over the study period. This therefore rules out that mitochondrial genetics explain the increased heat tolerance in the Vietnamese *Artemia* through time.

IV.2.9. Statistical analyses

We first analysed the overall temperature tolerance of the VCH populations compared to the ancestral SFB_{84}. To maximize our power to detect differences between populations, we pooled the 'control' data from the acclimation and microbiome experiments. Specifically, we used: the first clutches from the 'Parental acclimation' experiment, the second clutches from the 'Parental acclimation' experiment whose parents were not exposed to high temperature; the individuals from the 'Microbiome' experiment who were inoculated with the lab microbiome; and the organisms from the 'Juvenile acclimation' experiment who had undergone the same temperature regime in Phases 1 and 2. There are of course some small differences between these experiments (i.e. the 'Juvenile acclimation' organisms had undergone a slightly longer exposure to the temperature

regimes, the 'Microbiome' organisms were cultured differently), but the meta-analysis approach accounts for this additional variation. We used a multilevel meta-analysis model (R Core Team, 2014; Viechtbauer, 2010), and meta-analysed the two temperature regimes separately. Survival relative to the SFB_{84} population was taken as the response variable because it is the ancestral population. Effect sizes were obtained by fitting binomial models like those described below to the control data for each experiment and extracting the log odds ratio of each VCH population relative to SFB_{84} (more details in **Supplementary Table 2**). Standard errors extracted from the same models were used to weight the meta-analysis. The full meta-analysis model contained *VCH population* (VCH_{97} or VCH_{08}) as a fixed effect, and *Experiment* as a random effect controlling for non-independence within experiments. The significance of *VCH population* was then tested using likelihood ratio tests. Where relevant, post-hoc Tukey tests were performed to compare the two populations.

To analyse the individual experiments, we used generalized linear mixed models (R Core Team, 2014; Bates et al., 2015), with the number of surviving vs. dead *Artemia* in each replicate as the response variable (binomial response with logit link). The two temperature regimes were analysed separately (i.e., the following was repeated for T_{SFB} and T_{VCH}). First, we constructed a full model that included all the experimentally manipulated factors and their interactions. The 'Additive genetic effects' models included only *Population*. The 'Parental acclimation' models included *Population*, *Clutch* (a dummy variable, with the first clutch coded as '0' and the second clutch as '1'), and their interaction, and the interactions between these and the factor *Parental treatment*. By using the dummy variable and restricting *Parental treatment* to the interaction terms, we avoided generating spurious (and biologically impossible) estimates of the effect of *Parental treatment* on the first clutch. We also included a random *Family* term to group replicates collected from the same parental couple. In the 'Juvenile acclimation' experiment, we analysed the survival in Phase 2, which was conditional upon survival in Phase 1. The models included *Population*, *Temperature in Phase 1*, and their interaction, as well as a random *Family* term to group replicates collected from the same parental couple. For the 'Microbiome' experiment, the models included *Population*, *Microbiome*, and their interaction. Where necessary, the full models were corrected for overdispersion by including an observation-level random effect (Harrison, 2015). Finally, the significance of the predictors was tested using likelihood ratio tests. For the 'Parental acclimation' experiment, where we were only interested in the effects of *Population* and *Parental treatment* on the difference between the first and second clutch, we only tested the significance of the interaction terms.

IV.3. Results and Discussion

We compared the temperature tolerance of an ancestral population from SFB (cysts collected in 1984; hereafter SFB_{84}) with that of two populations from VCH (cysts collected in 1997 and 2008; hereafter VCH_{97} and VCH_{08}). We resurrected an F0 generation from each population and kept them at a standardized lab temperature, thus removing plastic maternal effects. We then measured juvenile survival in the F1 generation in common garden experiments under temperatures mimicking daily thermal conditions in SFB and VCH (hereafter T_{SFB} and T_{VCH}). This experiment was repeated several times as the 'control' treatment in the juvenile acclimation, parental acclimation, and microbiota experiments (**see methods**, and **Figure 1A**, solid points, for possible outcomes). Very consistently in these controls, VCH populations raised in the laboratory showed increased juvenile survival compared to the original SFB_{84} population, but only when exposed to a VCH climate (meta-analysis $\chi^2(1) = 9.6$, $p = 0.002$ at T_{VCH} and $\chi^2(1) = 0.8$, $p = 0.38$ at T_{SFB}; **Figure 1B** solid points, **Table S1a**). The VCH populations are thus phenotypically adapted to high temperatures, consistent with previous studies (Clegg et al., 2000; Frankenberg et al., 2000; Kappas et al., 2004), and this is not due to direct plastic maternal effects since all F0 females were raised in the same conditions. Furthermore, VCH_{08} juveniles had significantly higher survival at T_{VCH} than VCH_{97} juveniles (post-hoc $z = 3.1$, $p = 0.002$; **Figure 1B**), so phenotypic adaptation increased over time in VCH.

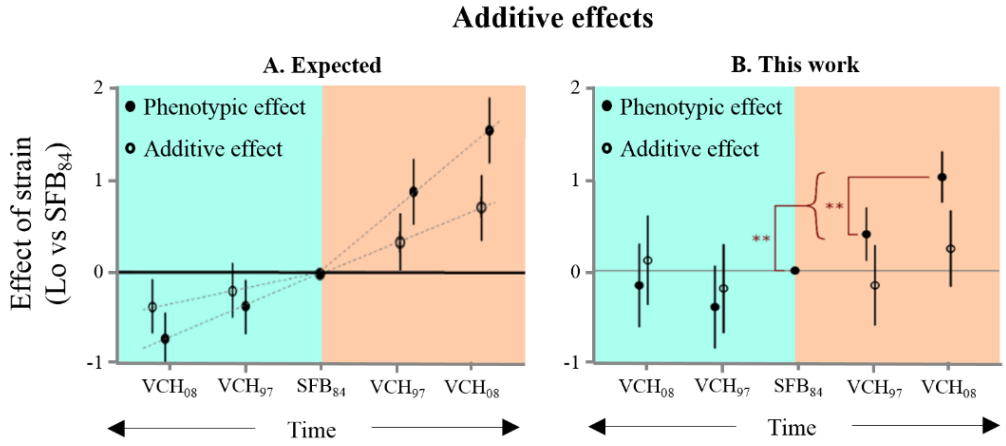


Figure 1. Survival of the VCH strains compared to the ancestral SFB_{84}, when mothers belonged to the own population (solid points) and to an SFB reference population ('crossed' populations, empty points). **A:** Illustration of a possible outcome for this experiment. **B:** results found in this work. The '0' point for SFB_{84} is included for reference. Blue and orange backgrounds represent assays run at T_{SFB} and T_{VCH}, respectively. The grey line corresponds to a lack of effect; bars are CIs. To maintain clarity, only significant differences relevant to the phenotypic adaptation to high temperature in VCH are shown. Abbreviations: LO, log odds ratio of survival.

A second, crucial, step was to determine whether this increased performance resulted from genetic changes. If so, VCH_{97} and VCH_{08} males should be able to transmit at least part of this increased performance to their progeny when crossed with reference SFB females from a stock cultured for over two years under standardized experimental conditions (**see methods**). This cross removed any maternal and (great)-grandmaternal effects that might have contributed to the observed phenotypic variation. Assuming that adaptation to warmer climate is a polygenic trait, we expect roughly half of the additive genetic effects to be transmitted through males (see **Figure 1A**, open points, for possible outcomes; the additive genetic effect is expected to be ~half of the phenotypic effect). We would therefore expect to see increased performance at T_{VCH} for the crossed VCH_{97} and VCH_{08} populations but not for the crossed SFB_{84} population in the same juvenile survival test. Despite the strong phenotypic change observed in the uncrossed F1s, survival was not significantly different in juveniles from crossed SFB_{84}, VCH_{97} and VCH_{08} populations in either temperature treatment (p = 0.44 for a population-level difference at T_{SFB}; p = 0.16 at T_{VCH}; **Table S2**, **Figure 1B** open points, **Table S1a**). This suggests that the increased performance of VCH *Artemia* at T_{VCH} did not result from additive genetic effects. Instead, it may have resulted from i) fully recessive genetic effects, ii) mitochondrial evolution, or iii) plastic grandmaternal (or earlier great-grandmaternal, etc.) effects, e.g., the transmission of epigenetic marks acquired in VCH. Although not formally excluded, the recessivity hypothesis is unlikely as thermal adaptation likely involves numerous quantitative traits with at least some additive effects, and as recessive beneficial alleles (here conferring thermal tolerance) are not expected to sweep quickly in a large population as would be required to explain the rapid phenotypic change observed here (**Figure S3**). We investigated the possibility of mitochondrial evolution by sequencing the mitochondrial genome of 10 individuals from SFB_{84} and VCH_{08}, as well as sequencing pooled cysts from VCH collected at eight dates between 1984 and 2008. SNP analyses show that mitotype frequencies were remarkably stable over that period, excluding a role for adaptation via the mitochondrial genome (**Figure S2**). Hence, it is most likely that the VCH populations have not adapted genetically to higher temperatures. This finding is surprising, but other studies on adaptation to climate warming have also reported an absence of genetic response (Merilä et al., 2014; Gienapp et al., 2008; Franks et al., 2014). Frankenberg et al. (2000) also showed that VCH *Artemia* populations (hatched from field cysts collected in 1994) had increased survival at high temperature (compared to SFB cysts collected in 1978), but this increased performance was not apparent in later laboratory generations. Such a finding could result from transgenerational effects. Grandmaternal effects are also supported by the study of Norouzitallab et al. (2014), who report transgenerational epigenetic effects on

thermal tolerance in laboratory *A. parthenogenetica*, which were transmitted up to the F3 generations.

To further investigate these transgenerational effects, we tested whether thermal exposure of adult parents to T_{VCH} could influence progeny performance at T_{SFB} vs T_{VCH} (see **Figure 2A** for possible outcomes). If so, we would have a mechanism for the grandparental effects (provided they could be maintained for one more generation). We compared juvenile survival in clutches produced before and after exposing their parents to high temperatures ("Parental acclimation" experiment). Hence, by design, this experiment ensures that no genetic change occurs, which greatly simplifies interpretation. We exposed the mother, the father, or neither parent. Comparing within the same family controlled for biases resulting from differential mortality of parents exposed to the different treatments; comparisons with families where neither parent was exposed controlled for a second clutch effect. Results showed no significant differences in survival between clutches from the different parental treatments at T_{SFB} or T_{VCH} for any *Artemia* population ($0.08 \leq p \leq 0.36$ for a population, parental treatment, or interaction effect at T_{SFB}; $0.15 \leq p \leq 0.41$ at T_{VCH}; **Table S2**, **Figure 2B**, **Table S1b**), indicating that thermal exposure in the parent does not influence the thermal tolerance of its progeny. This experiment rules out that epigenetic marks are set in adults in the time window preceding clutch production. However, it does not exclude epigenetic marks that are set during the juvenile development of the parents (or grandparents, etc.) (Norouzitallab et al., 2014; Donelson et al., 2018). The imprint may be set early during meiosis in the female germ line, which occurs during juvenile development (Lenormand et al., 2016). Indeed, the epigenetic effects referenced above were found after exposing juvenile *A. parthenogenetica* to a heat shock (Norouzitallab et al., 2014). Doing a similar experiment, but where the parents are exposed to the environmental stress as juveniles is much more difficult to interpret. Indeed, exposing juveniles to an environmental stress usually cause some mortality, and it is therefore difficult to discard that selection operates within the parent generation in the treatment compared to the control. If stress-tolerant genotypes become overrepresented in the treatment compared to the control, then, in the following generation, offspring from stressed parents may better tolerate stress. Hence distinguishing transgenerational effects from selection in the previous generation becomes difficult. This type of methodological issue is likely to be a major limitation to evaluate the extent of transgenerational effects in many situations.

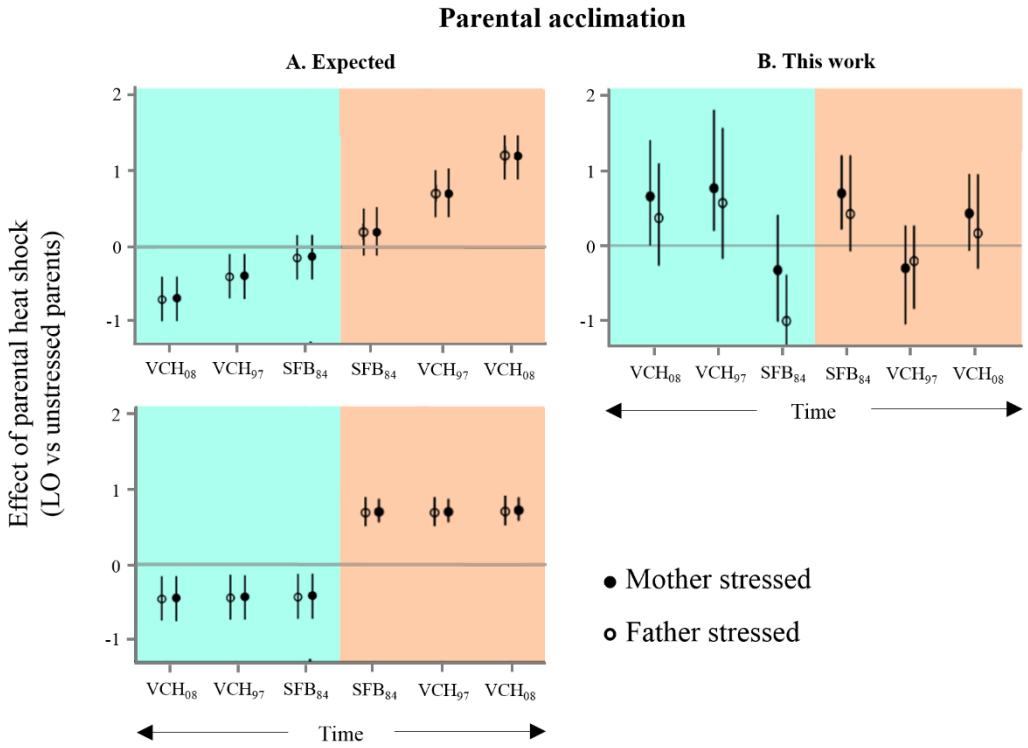


Figure 2. Difference in survival between the second and first clutches, when parents were exposed to high temperature between clutches 1 and 2. The effect of the second clutch itself (which may have differed in survival compared to the first) is controlled for using the second vs. first clutch effect observed for the unexposed control parents. **A:** Illustration of a possible outcomes for this experiment**.** Top: expected result if acclimation effects were present for both parents but did not evolve. Heat-stressed parents leave a beneficial (response deleterious) imprint to offspring exposed to heat (response to cold). Bottom: expected result if both parents acclimation effects evolved in the tropics. Heat-stressed parents leave a beneficial (response deleterious) imprint to offspring exposed to heat (response to cold). **B:** results found in this work. Blue and orange backgrounds represent assays run at T_{SFB} and T_{VCH}, respectively. The grey line corresponds to a lack of effect; bars are CIs. Abbreviations: LO, log odds ratio of survival.

Next, we investigated whether *Artemia* have a developmental plasticity response to the thermal environment. Such a plastic response would not be sufficient to explain the phenotypic effects that we observed since these experiments did not include an acclimation phase before measurement. However, if plastic adjustment to cope with high temperatures pre-existed in SFB, or evolved in VCH, this would help explain the lack of genetic change in VCH. This possibility is reinforced by previous studies in *Artemia*, which demonstrated plastic response to thermal stress (Clegg et al., 2000; Frankenberg et al., 2000). To investigate this possibility, we exposed 5-day-old juveniles to T_{SFB} or T_{VCH} for 2 days, and then tested whether pre-exposure increased performance in each environment ("Juvenile acclimation" experiment) during the same age window used for the other experiments (see **Figure 3A** for possible outcomes). Strikingly, we found that early exposure

to T_{VCH} did not increase juvenile survival at T_{VCH} in any of the *Artemia* populations ($p \geq 0.62$ for an effect of pre-exposure or its interaction with population; **Table S2**, **Figure 3B**, **Table S1c**). In contrast, pre-exposure to T_{VCH} significantly increased survival at T_{SFB} ($p < 0.0001$ for a pre-exposure effect; **Table S2**, **Figure 3B**) for all three *Artemia* populations ($p = 0.31$ for an interaction with population; **Table S2**), indicating that there is indeed a plastic response (e.g. activation of heat shock proteins (Clegg et al., 2000; Frankenberg et al., 2000)). However, this plasticity does not appear to confer an improved performance at T_{VCH}, so we can rule out that it plays a major role in the thermal adaptation at VCH.

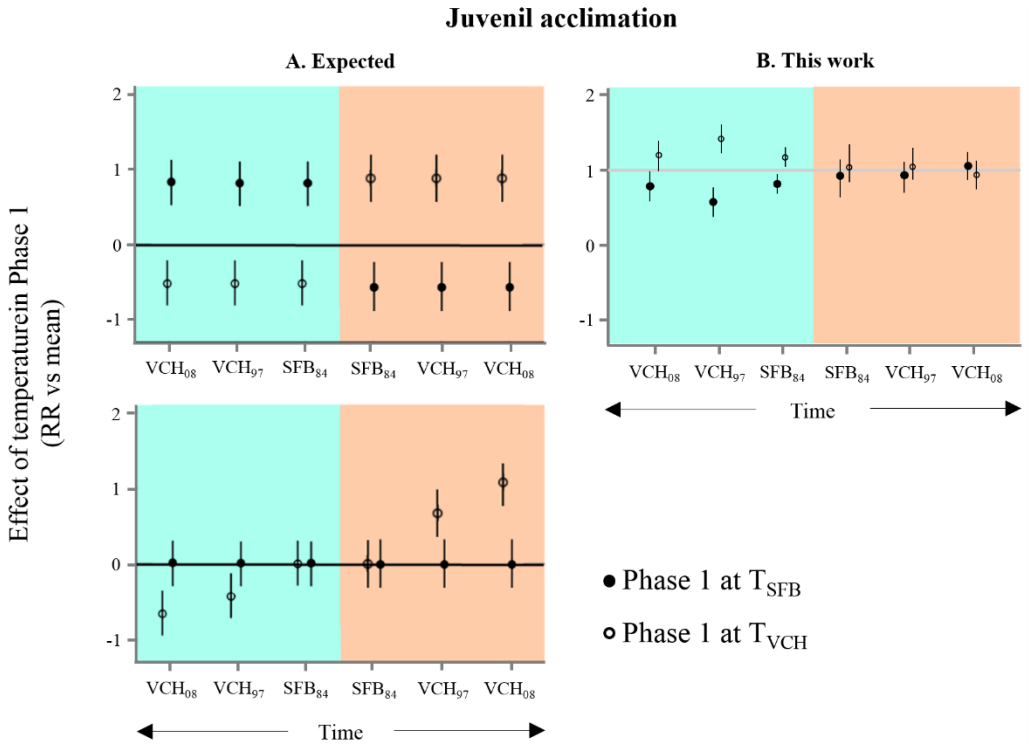


Figure 3. Survival in Phase 2, after exposure to T_{SFB} or T_{VCH} in Phase 1. Here, 'mean' is the mean survival in Phase 2 for each strain. **A:** Illustration of a possible outcomes for this experiment. Top: Expected result if adaptative early life acclimation was present but did not evolve in the tropics. Early life exposure to a given climate improves tolerance later at that climate but reduces tolerance to another climate. Bottom: Expected result if adaptative early live acclimation evolved in the tropics in response to heat. **B:** results found in this work. Blue and orange backgrounds represent assays run at TSFB and TVCH, respectively. The grey line corresponds to a lack of effect; bars are CIs. Abbreviations: RR, relative risk of survival.

Last, we investigated whether performance at T_{SFB} and T_{VCH} could be affected by the presence of microbiota adapted to those climates ("Microbiota" experiment). In corals, for example, the temperature niche is controlled by that of their symbionts (Littman et al., 1990). *Artemia* host many gut bacteria that are essential for the proper digestion of unicellular algae, their main food

source. Adaptation of this microbiota to high salinity has been shown to determine their host's salinity niche (Nougué et al., 2015). Hence, it is possible that *Artemia*'s thermal niche is controlled in part by the thermal niche of its microbiome. Such a finding would also help explain the lack of genetic change in VCH. To evaluate this possibility, we investigated the thermal tolerance of axenic *Artemia* from SFB_{84}, VCH_{97}, and VCH_{08} populations inoculated with microbes sampled from live *Artemia* in SFB, VCH, or our reference laboratory cultures. If microbes contribute to thermal tolerance, we expect VCH microbes to increase juvenile survival at T_{VCH}, but not T_{SFB}, whereas SFB microbes should increase survival at T_{SFB} but not T_{VCH} (see **Figure 4A** for possible outcomes). We did not find this pattern. Instead, we found that having microbes from VCH increased survival for all *Artemia* populations at both T_{SFB} and T_{VCH}, while having lab microbes decreased survival in all circumstances (p = 0.003 for an interaction between population and microbiome at T_{SFB}; p = 0.001 at T_{VCH}; **Table S2**, **Figure 4B**, **Table S1d**). Hosting VCH microbes appears to simply be better than hosting lab microbes. For the SFB microbes, we found that they conferred the same survival as VCH microbes in SFB_{84} but were equally poor as the lab microbes for both VCH populations. Hence, our results are consistent with the idea that i) microbes have a large impact on survival, ii) microbes from our three stocks are different and iii) their effect depends on the *Artemia* population. We did not find any indication that the microbes play a major role in thermal adaptation. Interestingly, we found that *Artemia* had no problems when exposed to microbiota from a tropical climate: they are available, and there is no need to specifically adapt to them (as SFB_{84} performed equally well with VCH microbes). All our findings are consistent with a loss of function in the laboratory microbes, and by a loss of ability of the Vietnamese *Artemia* to benefit as much from their ancestral SFB microbes.

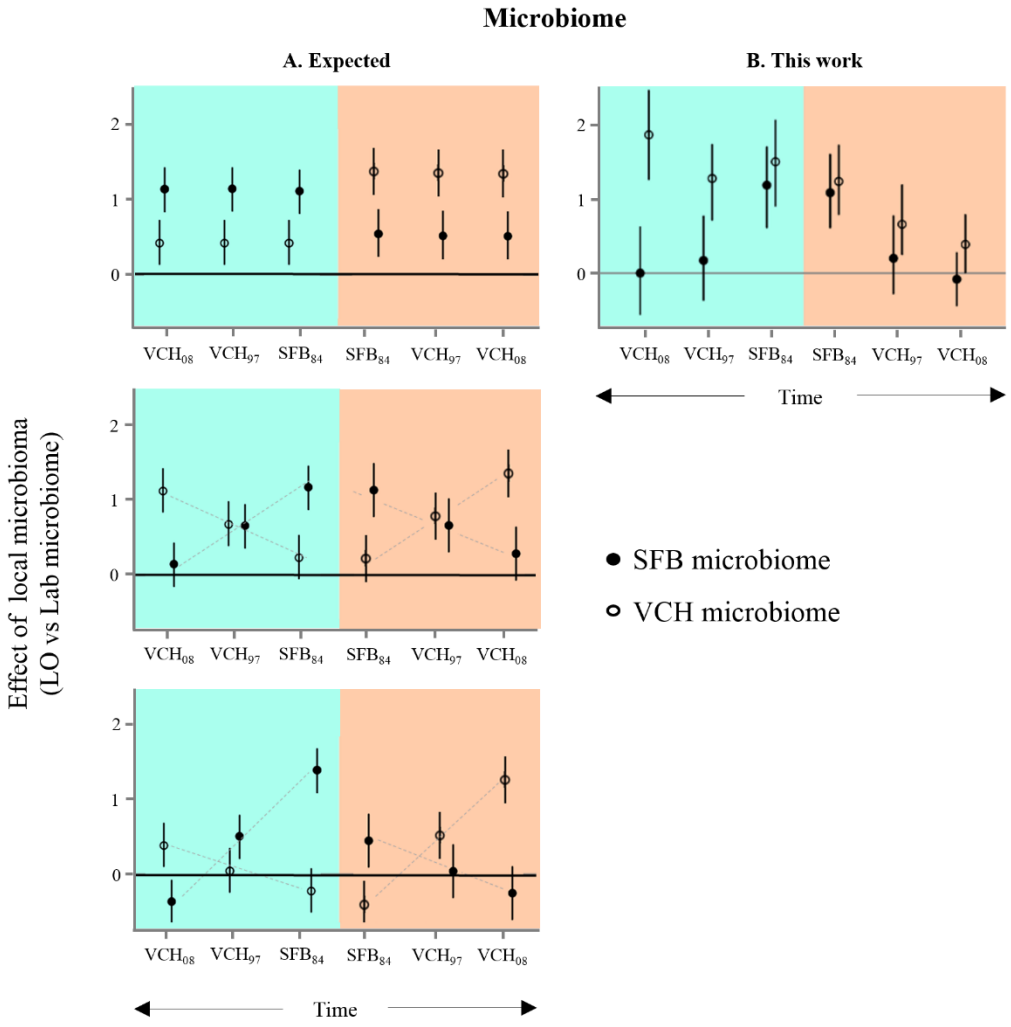


Figure 2. Survival after inoculation with a local microbiome, compared to survival with the reference lab microbiome. **A:** Illustration of a possible outcomes for this experiment**.** Top: Tropical (response temperate) microbes benefit everyone under tropical (response temperate) conditions. Natural microbiota are better than lab-impoverished microbiota. Middle: Microbiota improves fitness of their local host (SFB microbes benefit SFB *Artemia* and VCH microbes benefits VCH *Artemia*, but this effect has nothing to do with temperature. All microbiota are better than lab-impoverished microbiota. Bottom: Tropical (response temperate) microbiota confer better tolerance to tropical (response temperate) temperatures, bit hosts have to adapt to this microbiota to accrue this benefit. Without this adaptation, 'foreign' microbiota is worse than lab microbiota. **B:** results found in this work. Blue and orange backgrounds represent assays run at T_{SFB} and T_{VCH}, respectively. The grey line corresponds to a lack of effect; bars are CIs. Abbreviations: LO, log odds ratio of survival.

IV.4. Conclusion

In summary, we found no indication of genetic adaptation to increased temperature in a field situation which should *a priori* be very favourable for the evolution of thermal tolerance (Reznick and Ghalambor, 2001): a large and isolated sexual population without initial bottleneck, exposed to a large and abrupt environmental shift over 100 generations. However, we did find a phenotypic difference when testing individuals whose grandmothers were exposed to high temperatures, and this difference was larger for the VCH_{08} population than for VCH_{97}. We conclude that VCH *Artemia* have higher heat tolerance possibly due to transgenerational effects, and that these effects increased through time, e.g., by being better maintained through generations in more recent Vietnamese populations. Such effects are not entirely unexpected, as they are found more often in short-lived, dispersal-limited organisms, for juvenile traits, and in conditions where environmental variation is predictable over several generations (Yin et al., 2019). Further work is necessary to see whether these transgenerational effects are due to epigenetic marks, as in (Norouzitallab et al., 2014), or due to other mechanisms. Their presence could explain the apparent lack of genetic changes in VCH: transgenerational effects could keep the population phenotype close to a thermal optimum, thereby reducing directional selection and genetic changes. Such interference with genetic adaptation has been found in many studies reporting within-generation plasticity (Gienapp et al., 2008; Merilä and Hendry, 2014), but the transgenerational mechanism found here is much less documented. The resurrection ecology approach is among the most powerful methods to study adaptation to climate change (Orsini et al. 2013; Lenormand et al. 2018; Nogués-Bravo et al. 2018; Weider et al. 2018). However, here the possibility to perform crosses between the evolved populations with the ancestral one turned out to be crucial. Without such crosses we would likely have concluded that genetic adaptation had taken place (as in Geerts et al. 2015; Yousey et al. 2018). Compared to other studies, our adaptive transgenerational effects are large (Jeremias et al., 2018; Yin et al., 2019; Sánchez-Tójar et al., 2020), and contrast strikingly with the apparent absence of adaptive genetic and within-generation plastic effects, providing an example where adaptation most likely, involves traits whose heritability is entirely "missing" (Trerotola et al., 2015). Overall, this work represents one of the most complete studies jointly addressing the different factors associated with thermal adaptation in the wild, namely genetic effects, epigenetic effects, plasticity, and microbiota. It suggests that phenotypic adaptation to an extreme environmental change can be achieved by transgenerational effects and that these effects can be so large that no genetic adaptation takes place.

IV. Supplementary material

Table S1. Percentage of survival of the different *Artemia* strains exposed to two different temperature cycles (T_{SFB} and T_{VCH}) found for the a) phenotypic and additive effects, c) parental acclimation, d) juvenile acclimation and e) microbiome experiments.

a)

Strain	Phenotypic effect		Additive effect	
	T_{SFB}	T_{VCH}	T_{SFB}	T_{VCH}
SFB_{84}	51.0	15.4	36.0	17.0
VCH_{97}	41.9	16.9	32.2	14.8
VCH_{08}	43.9	21.6	38.3	20.7

b)

Strain	Parental Treatment	T_{SFB}		T_{VCH}	
		Clutch 1	*Clutch 2*	*Clutch 1*	*Clutch 2*
SFB_{84}	Control	66.2	64.8	6.9	5.0
	Female stress	76.0	69.2	7.3	11.8
	Male stress	67.1	58.0	10.0	9.5
VCH_{97}	Control	55.7	47.0	6.6	8.8
	Female stress	63.2	65.0	11.9	8.6
	Male stress	56.4	60.0	9.6	8.0
VCH_{08}	Control	64.3	49.2	25.1	22.2
	Female stress	71.8	68.8	23.0	26.6
	Male stress	69.6	58.0	17.8	20.0

c)

Strain	Juvenile treatment *Phase 2*	Juvenil treatment *Phase 1*	
		T_{SFB}	T_{VCH}
SFB_{84}	TSFB	56.4	76.1
VCH_{97}		29.1	51.7
VCH_{08}		38.5	50.5
SFB_{84}	TVCH	24.6	27.4
VCH_{97}		12.0	13.8
VCH_{08}		32.2	29.7

d)

Strain	Temperature cycle	Microbiota origin		
		Lab	SFB	VCH
SFB84	SFB	42.0	65.3	71.1
VCH97		37.9	42.8	64.3
VCH08		40.3	40.3	77.7
SFB84	VCH	12.0	28.7	31.9
VCH97		18.7	21.9	30.7
VCH08		24.3	23.5	32.1

Table S1. Models used to generate effect sizes and variances for the meta-analysis of phenotypic adaptation (solid points, **Figure 2A**), which compared the overall temperature tolerance of VCH and SFB populations. Effect sizes are the log odds ratio of survival compared to SFB_{84}. Where necessary, observation-level random effects were added to control for overdispersion.

Experiment	Fixed and random terms	T_{SFB}	T_{VCH}
		Effect size ± SE	Effect size ± SE
Parental acclimation	~ *VCH population* + (1|*Family*/*Clutch*[2])	VCH_{97}: -0.77 ± 0.27 VCH_{08}: -0.30 ± 0.27	VCH_{97}: 0.35 ± 0.21 VCH_{08}: 1.18 ± 0.19
Juvenile acclimation[1]	~ *VCH population* + (1|*Family*/*Observation*)	VCH_{97}: 0.38 ± 0.68 VCH_{08}: -0.25 ± 0.81	VCH_{97}: 0.19 ± 0.46 VCH_{08}: 0.85 ± 0.48
Microbiome	~ *VCH population* + (1|*Observation*)	VCH_{97}: -0.18 ± 0.28 VCH_{08}: -0.06 ± 0.28	VCH_{97}: 0.52 ± 0.23 VCH_{08}: 0.86 ± 0.22

[1]To estimate overall survival in this experiment, we fit models for survival in Phase 1 and Phase 2, then transformed, multiplied, and back-transformed the predicted survival rates. Standard errors were obtained by resampling.
[2]These models no longer have *Clutch* as a fixed term, so we control for possible variation in survival between the first and second clutches by including it here as a (non-dummy) random effect.

Table S2. Significance of the tested effects for the individual experiments. Temp., temperature; treatm., treatment.

Experiment	Fixed-effect term	T_{SFB}		T_{VCH}	
		Test statistic	p-value	Test statistic	p-value
Additive genetic effects	*Population*	$\chi^2_{(2)} = 1.7$	0.44	$\chi^2_{(2)} = 3.6$	0.16
Parental acclimation	*Clutch : Population*	$\chi^2_{(2)} = 2.1$	0.36	$\chi^2_{(2)} = 1.8$	0.41
	Clutch : Parental treatm.	$\chi^2_{(2)} = 3.3$	0.19	$\chi^2_{(2)} = 3.8$	0.15
	Clutch : Population : Parental treatm.	$\chi^2_{(4)} = 8.3$	0.08	$\chi^2_{(4)} = 5.6$	0.23
Juvenile acclimation	*Population*	$\chi^2_{(2)} = 28.8$	< 0.0001	$\chi^2_{(2)} = 44.4$	< 0.0001
	Temp. in Phase 1	$\chi^2_{(1)} = 30.8$	< 0.0001	$\chi^2_{(1)} = 0.0$	0.91
	Population : Temp. in Phase 1	$\chi^2_{(2)} = 2.3$	0.31	$\chi^2_{(2)} = 1.0$	0.62
Microbiome	*Population*	-	-	-	-
	Microbiome	-	-	-	-
	Population : Microbiome	$\chi^2_{(4)} = 15.7$	0.003	$\chi^2_{(4)} = 18.4$	0.001

Figure S1. Illustration of possible outcomes for the different experiments, with simple scenarios described next to the figures**.**

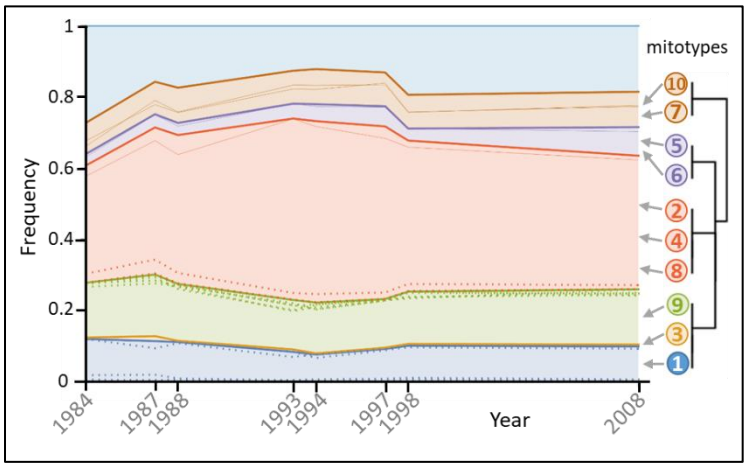


Figure S2. Mitotype frequency variation through time. Sampled years are shown on the *x*-axis, the *y*-axis expresses cumulative frequency. The relationship between the different mitotypes (based on shared-SNP, methods) is shown by the dendrogram on the right. Mitotypes are shown with different colours; numbers identify the individual sequenced (1–5 from 1984 and 6–10 from 2008). The mitotypes' frequency envelope is that of their most frequent shared-SNP. Individuals 1, 3, and 9 do not have shared-SNPs, and are therefore grouped on this dendrogram. Their frequency envelope is that of their most frequent private-SNP. Thin lines represent other shared-SNP frequencies within mitotypes. Dotted lines represent private-SNPs within groups (only those reaching a frequency >1 % are shown).

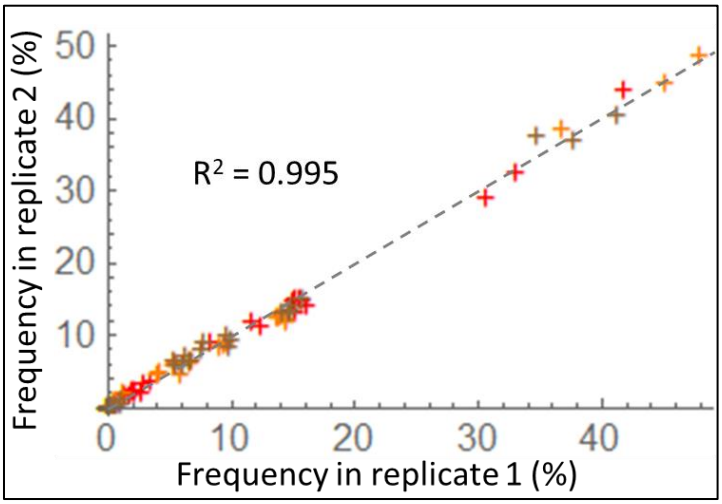


Figure S3. SNP frequency data quality. SNP frequency was independently estimated twice for years 1984 (red), 1997 (orange), and 2008 (brown). The figure reports the correlation between these replicated values for all SNPs used in **Figure S2** (all private and shared SNPs among sequenced individuals). $R^2 = 0.995$ over all replicated measures. Dashed line is the 1:1 line.

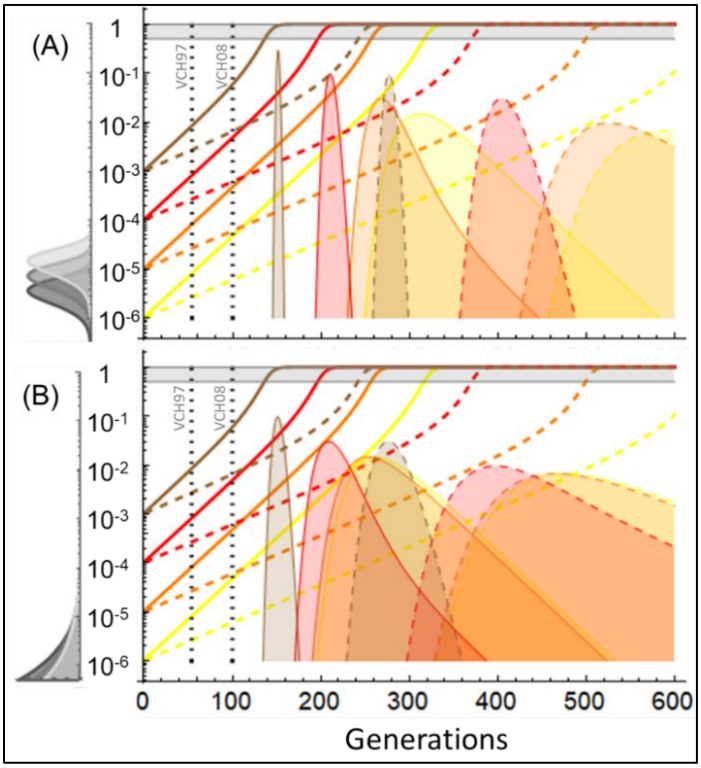


Figure S4. Frequency of a strongly beneficial recessive allele (s = 0.3) through time in a population of $N = 10^7$ (panel A) or $N = 10^6$ (panel B) individuals. Plain and dashed lines: exact deterministic frequency change for this beneficial allele given a dominance coefficient of h = 0.13 and h = 0.06, respectively. For these curves, the x-axis represents time in generations, and the y-axis is the frequency in log-scale. Different colours illustrate different initial frequencies, from 10^{-6} (yellow) to 10^{-3} (brown). For comparison, a newly arising mutant would start at 10^{-7} or 10^{-6} in panel A and B, respectively. The horizontal grey bar represents the [0.5,1] frequency range for the beneficial allele, within which it starts having a detectable effect on the mean fitness of the population (~10% increase in mean fitness). The vertical dotted lines correspond to our two dates of measurement (1997, at generation ~54, and 2008, at generation ~100). Coloured surface areas show the corresponding probability density of half-sweep times (i.e., the number of generations to reach a frequency of ½) from a stochastic model with $N_e = N$ (from Eq. 8 in (Martin and Lambert et al., 2015), same line and colour code for dominance and initial frequency as for the deterministic curves). Here, the x-axis shows this half-sweep time and the y-axis the corresponding probability density. Distributions in grey to the left of the y-axis show the frequency distribution of the heat tolerance allele in its population of origin (SFB), at mutation ($u = 10^{-7}$) selection balance, assuming that it reduces fitness at cold temperatures by a modest amount (hs = 0.02 dark grey, 0.01 grey or 0.005 light grey) compared to its strong advantage at high temperature. This Wright's distribution is computed from Eq. 9.3.4 in (Crow and Kimura, 1970) with $N_e = N$. y-axis: frequency, x-axis: probability density (rescaled for readability on panel A by the inverse of the density at mean frequency). Overall, the figure shows that even in the most favourable conditions, adaptation caused by a recessive beneficial allele cannot be detected within the short 54 or 100 generations of our study. These are the most favourable conditions, as we consider (1) incompletely recessive beneficial alleles, with h = 0.13 corresponding to the point estimate based on observed survival in our experiment 1 for VCH_{08} which is certainly a maximum given that the point estimate in VCH_{97} would be h = 0 at most); (2) a very strong beneficial effect s = 0.3, comparable to the highest published field estimates of fitness effects in situations of intense selection pressures (e.g. insecticide resistance (Lenormand et al., 1999)); (3) plausible population sizes of *Artemia*

populations, as census sizes in the field far exceed 10^6 in Vinh Chau saltpan; (4) a modest deleterious fitness effect of these beneficial alleles at cold temperature and standard mutation rate (10^{-7}; a ten-fold larger mutation rate would not alter the conclusion, so that these mutations are unlikely to segregate at a higher frequency than 10^{-3} in the population of introduction). In addition, with such intense selection, beneficial alleles spread extremely fast when they reach a frequency above 0.2 (they then reach a frequency of 0.8 in ~20 generations), so that in this scenario, with a strongly beneficial allele we would be likely to observe a fitness change only between 1984 and 1997 or 1997 and 2008, but not both.

GENERAL DISCUSSION

This Thesis aimed to increase the understanding of the ecotoxicological implications of the interactions between biological invasions and pollution in hypersaline coastal ecosystems and the mechanisms involved in thermal adaptation. For that *Artemia*, a key species of hypersaline ecosystem, was selected as model organism. The work developed under this Thesis is not only relevant for fundamental research reasons, but also from a conservation point of view, as native *Artemia* populations in the Mediterranean region are highly threatened by the invasive *A. franciscana*. Moreover, from an applied perspective, this Thesis provides a powerful model for the field of resurrection ecology to study adaptation of natural populations to climate change. It is increasingly important to have model organisms where to study different problems and questions related with adaptation to global environmental change, both in laboratory experiments and under the full complexity of the field. *Artemia* cysts (long lived dormant stages) offer the opportunity to study adaptation over long periods of time, providing natural archives of recent ecological and evolutionary histories of populations.

Pollution, biological invasions and climate warming are among the most important drivers of global change and understanding how organisms respond to them is of paramount importance to predict the persistence and stability of natural populations. Due to its physiological, biological and ecological characteristic, *Artemia* offers a timely opportunity to address the objectives of this thesis.

Ecotoxicological implications of the *A. franciscana* invasion

Hypersaline ecosystems are widely distributed habitats that provide valuable direct economic benefits (e.g., *Artemia* cysts production, salt production, nature tourism) and indirect non-economic services (e.g., waterbirds conservation, cultural and aesthetic values). However, they are under increasing threat due to anthropogenic pressure, such as environmental pollution, and biological invasions. In addition to the consequences that biological invasions can have on native biodiversity, the replacement of species by invasive ones can also have ecotoxicological implications. It can be particularly important for trace element transfer in the case of keystone species, with an important role in food web and ecosystem functioning. This question is addressed in **Chapter I**, where I studied the ecotoxicological implications of the invasion by *A. franciscana* in hypersaline ecosystems of Southern Spain, with different degrees of trace element pollution and invasion status, through the study of the bioaccumulation of nine trace elements (As, Cd, Cu, Co, Cr, Mn, Ni, Pb and Zn). The most studied impact of the introduction of the North American brine shrimp *A. franciscana* is the extinction of native *Artemia* populations from all over the world (Amat et al., 2007; Horváth et al., 2018). However, this is the first time that the ecotoxicological implications of this invasion is addressed. Overall, results suggested that the invasive *A. franciscana* has a greater

ability to bioaccumulate trace elements than the native *A. parthenogenetica* which may result from different factors such as differences in life history traits. Furthermore, these can be related to feeding rate, which is higher in *A. franciscana* than *A. parthenogenetica* (Sánchez et al., 2016b), detoxification mechanisms, which may be more efficient in native *A. parthenogenetica* from Odiel (Sánchez et al., 2016a) since this population has been exposed to high levels of contamination since prehistoric times, and the presence of parasites that increase antioxidant potential (Sánchez et al., 2016c).

The high capacity for bioaccumulation of trace elements shown by the invasive *A. franciscana* together with their presence all year round (*A. parthenogenetica* disappears during the winter) may increase the trophic transfer of trace elements through the hypersaline food web. Of particular concern are the possible implications for waterbirds, for which *Artemia* is the main prey (Sánchez et al., 2006). In fact, previous studies have already associated high blood levels of trace elements in waterbirds with the consumption of *Artemia* (Rodríguez-Estival et al., 2019; Conover and Vest, 2009; Wright et al., 2020).

Is pollution limiting/delaying the invasion of *A. franciscana*?

Most studies up to now show that environmental contamination may increase the invasibility of ecosystems (e.g., Piola and Johnston, 2009; Crooks et al., 2010). However, the potential role of pollution in limiting invasions has been much less studied, even though it could seem a common phenomenon when native species from areas with historic pollution (e.g., with prehistoric or ancient mining) are locally adapted (e.g., Barata et al., 2002; Lopes et al., 2006; Ruggeri et al., 2019).

In hypersaline ecosystems the deliberately introduction of *A. franciscana* cysts (USA), for commercial purposes (for aquacultural purposes and to aid in salt production; Dhont and Sorgeloos, 2002; Mura et al., 2006) and subsequent dispersion of cysts from this species, mainly, via waterbirds movements (cysts transported in faeces and feathers; Green et al., 2005; Sánchez et al., 2012) has critically hampered the biodiversity of the genus all over the globe (e.g., Amat et al., 2007; Horváth et al., 2018). Interestingly, in the Iberian Peninsula, few populations of native *Artemia* still persist and most are in highly polluted areas. However, the reasons for the resistance of these native species to the invasion are unknown. Rodrigues et al. (2012) hypothesised that the presence of pollutants (namely Hg) may play a decisive role in the prevention of the invasion. Later, Pinto et al. (2013, 2014a) also highlighted the potential role of a chemical barrier related to pollution as a limiting factor of the invasion. This "pollution resistance hypothesis" was first tested by Sánchez et al. (2016) using contaminants other than Hg and populations of the south of Spain, and their results partially supported the hypothesis. Nonetheless, more data is crucially needed to

provide valuable insight into the role of pollution in preventing or delaying the colonization of the last refuges of native *Artemia* populations by *A. franciscana*. That is why in this Thesis I have studied the response of different *Artemia* populations (native and invasive) to those elements more abundant in the last refuges of native *Artemia* species in Portugal and Spain. First through an acute toxicity perspective **(Chapter II),** and then, through a chronic toxicity perspective (under realistic environmental concentrations, **Chapter III**). Acute toxicity tests have low cost and test duration. They imply a short–time exposure of organisms to high concentrations of toxicants and the endpoint measured is the percent of mortality. Acute tests are generally used to access the concentration that is lethal to 50% of the test organisms exposed (calculated and expressed as LC50 value). On the other hand, chronic tests are more expensive and time consuming. These tests are extended through either a life cycle or a critical developmental phase of the test organism. Chronic toxicity, the most common in the environment, involves lower concentration of toxicants, resulting in cumulative toxicity and sublethal responses. These tests have greater resolution than acute tests, thus results may be contrasting between them. Organisms exposed to high concentrations of a toxic, will die, whereas, when exposed to lower concentrations of that same toxic, they may survive, yet some life traits (e.g., reproductive performance, growth) may be impaired when compared with the control organisms exposed to an uncontaminated medium (USEPA, 1994).

In **Chapter II**, I investigated the individual effect of acute toxicity of mercury (Hg) and zinc (Zn) on the survival of natural native and invasive *Artemia* populations from the Iberian Peninsula collected from saltpans with different levels of Hg- and Zn-pollution. The endpoint relative mortality of *Artemia* nauplii (24 h median lethal concentration [LC50]) was used to quantify the toxicity independently for Hg and Zn in six *Artemia* populations: native *A. parthenogenetica* populations from Aveiro, Odiel, Rio Maior, and Cabo de Gata; and invasive *A. franciscana* populations from Aveiro and Cádiz. Here I expected that native *Artemia* from highly Hg-polluted sites and highly Zn-polluted sites would be locally adapted and consequently be more resistant (lower mortality) to the invasion. Thus, native *Artemia* from Cabo de Gata and Troncalhada (Aveiro), two of the most Hg-polluted study sites in this Chapter, would present the highest resistance to Hg. Similarly, native *Artemia* from Odiel, the most Zn-polluted study site in this Chapter, would present the highest resistance to Zn.

The results found in **Chapter II** partially support the chapter hypothesis, which was based on the "pollution resistance hypothesis" of Rodrigues et al. (2012). The native *Artemia* from Cabo de Gata, a historically Hg-polluted system, presented the highest resistance (highest LC50 value) to Hg acute exposure from all the populations analysed. However, contrary to what I expected, contamination by Hg did not explain the persistence of native *Artemia* in the saltpan complex of Ria

de Aveiro, since both native and invasive species from this complex showed similar LC50 values, that translate in similar sensitivity to Hg. Nonetheless, as hypothesised, the resistance of these populations from Aveiro was higher than that found for the populations from Odiel and Cádiz, both of which with very low levels of Hg contamination (e.g., Bermejo et al., 2003; Carrasco et al., 2003). The similar sensitivity to Hg of both native and invasive species from Aveiro could be explained by the fact that the population of *A. franciscana* from these saltpans is the one in the Mediterranean more closely genetically related to the population from Great Salt Lake (Utah, USA) (Muñoz et al., 2014), a system that also has a recent history of Hg contamination (Naftz et al., 2008). The persistence of native *A. parthenogenetica* populations in Ria de Aveiro could thus be related to other contaminants present in this ecosystem, such as other trace elements (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).

Regarding Zn, the differences in survival among populations were weak and not related to the concentrations of the element registered in water and sediment from the environment. All six populations studied showed high tolerance to Zn acute exposure, with no statistical differences among the LC50 values of the different populations being found, which might be explained by the fact that Zn is an essential trace element necessary for normal physiological and biochemical process of organisms (Frassinetti et al., 2006; Valko et al., 2005) and actively regulated (Rainbow, 2007). In fact, acute Zn toxicity is rare, and has only been reported at very high concentrations (Frassinetti et al., 2006; Valko et al., 2005). However, as mentioned previously, the absence of differences found in the response to short-term lethal concentrations (acute toxicity) of Zn among the different populations does not rule out sublethal effects that may occur when organisms are exposed to long-term sub-lethal concentrations (chronic toxicity) of that same toxicant. In order to explore this in more depth, I carried out the **Chapter III**, through which the demographic responses of native *A. parthenogenetica* from Odiel and *A. franciscana* from Cadiz, exposed to realistic long-term Zn exposure (0.2 mg L^{-1} – based on environmental concentrations reported in **Chapter I)**, were compared to evaluate possible differences in adaptation to pollution. It included differences in **(i)** life history traits, namely mortality, growth rate and reproductive performance, **(ii)** antioxidant enzyme activities as biomarkers of oxidative status, and **(iii)** lipid peroxidation as an indicator of damage produced by oxidative stress.

Clear differences in the response of the different *Artemia* species to Zn were found, supporting the hypothesis of Rodrigues et al. (2012). For instance, I found that long-term Zn exposure strongly impaired the cumulative survival, measured over 21 days (which contrast with the 24 h period used to measure mortality in **Chapter II),** of the invasive *A. franciscana* but caused

no effect in the cumulative survival of *A. parthenogenetica*, over the same period of time. Furthermore, Zn exposure had positive effects on the reproductive performance of the native *A. parthenogenetica* (showing higher number of broods, total offspring, and offspring per brood than *A. franciscana*). The results on survival and reproductive parameters contrast with those of Sarabia et al. (2008) who reported that Zn exposure (0.08 mg Zn L^{-1}) significantly reduced survival and had detrimental effects on the fecundity of *A. parthenogenetica*. However, they used a population from a relatively unpolluted system of Eastern Spain (Varó unpublished data) which would not be adapted to Zn, a fundamental difference compared to our system.

Another important finding from **Chapter III** was the difference in the levels of lipid peroxidation between both species. The experimental concentration of Zn strongly increased lipid peroxidation in *A. franciscana* while the opposite effect was observed in *A. parthenogenetica*. This suggests that the general antioxidant response to Zn exposure was enough to detoxify excess of reactive oxygen species (ROS) in *A. parthenogenetica* but not in *A. franciscana*. This might explain the high mortality experienced by the invasive species from Cádiz as well as the lack of effect in *A. parthenogenetica* after long-term Zn exposure. These results are consistent with those of the life history traits and represent an additional support to the pollution resistance hypothesis. Physiological resistance is key to the development of viable populations of newly arriving species to stressed ecosystems (Faria et al., 2010). Thus, inefficiency to neutralize ROS in highly polluted environments may be a critical limitation for invasive species, preventing/delaying the colonization of highly polluted environments where native species are locally adapted (see McMahon, 2002). This finding may explain the high bioaccumulation of Zn in *A. franciscana* found in **Chapter I** and highlights the importance of testing both acute and chronic toxicity of pollutants as they are the most common impact in ecosystems (e.g., accidental catastrophic spills and continuous discharges). Here I found that even if no effect on mortality is registered under acute exposure, sublethal effects under long-term exposure can have an important impact on populations.

Using *A. franciscana* to understand the mechanisms of thermal adaptation

In this thesis I was also interested by other factors related with global change, such as global warming. One of the main questions in the climate change debate is if species will be able to adapt fast enough to cope with global increase of temperature. However, very few studies are available that provide sufficient long-term data to evaluate species thermal adaptation. Moreover, the few available studies, which mostly concern few generations, report phenotypic adjustments (plasticity), but very little genetic responses. The use of a resurrection ecology approach (the revival of dormant stages allowing examination of phenotypes over time spans longer than the human lifespan), has

emerged as a powerful method to study the course of evolution in nature. As climate is currently warming at a rapid pace, this approach is becoming particularly useful for understanding how biodiversity responds to global warming and anticipating whether species will be able to adapt quickly enough to keep pace with the projected changes (Lenormand et al., 2018).

The successful introduction of *A. franciscana* from temperate regions into tropical regions alongside with *Artemia* intrinsic characteristics, such as short generation time, high fecundity, and specially the production of dormant cysts (encapsulated embryos able to resist extreme environmental conditions and remain viable during decades), make them an ideal organism to study adaptation in the field (Lenormand et al., 2018). For example, in the early 1980s, *A. franciscana* cysts from San Francisco Bay (USA) were introduced to Vinh Chau (Vietnam) saltpans, where mean air temperatures are nearly 10 °C higher compared with the their native habitat. This population was introduced in an area lacking other *Artemia* and therefore represents a case study where gene flow can be excluded as a significant factor of genetic change. The exact date of introduction was 1984 (De Graaf, 1985) and successful cyst production was recorded in 1986 (Rothuis, 1986), remaining large since then (Van Hoa, 2014). This suggests that drift can also be excluded as a significant factor of genetic change.

In order to investigate whether species can adapt in the wild beyond their climatic niche, in **Chapter IV** I studied the effects that could contribute to the thermal adaptive potential of *A. franciscana* introduced in Vietnam. A resurrection ecology approach was used to determine whether the introduced *A. franciscana* showed increased tolerance to higher temperatures and the extent to which this phenotypic adaptation resulted from genetic changes, pre-existing plastic responses, transgenerational effects, or the effect of locally adapted microbiota.

Temperature tolerance was compared among three *Artemia* populations: an ancestral population from San Francisco Bay (cysts collected in 1984) and two evolved populations from Vinh Chau (cysts collected in 1997 and 2008), to investigate if the Vinh Chau populations were indeed phenotypically adapted to high temperatures. The results of this first experiment showed, when testing individuals whose grandmothers were exposed to high temperatures, that the Vinh Chau populations (evolved) had higher survival compared to the San Francisco population and this difference was larger for the youngest population (*A. franciscana* from Vietnam collected in 2008). These results showed that there is indeed a phenotypic adaptation of the Vinh Chau populations and were consistent with other studies (Clegg et al., 2000; Frankenberg et al., 2000). However, the results suggest that that this increased phenotypic tolerance to warming through the years, was not accompanied by genetic changes (neither nuclear nor mitochondrial), increased plasticity, the acquisition of heat tolerant symbionts or epigenetic marks (after stressing the adult parents). Still, as

reported by Norouzitallab et al. (2014), some epigenetic marks could be imprinted during the juvenile development of the parents or grandparents. Thus, the most plausible conclusion is that the phenotypic adaptation seen in the Vinh Chau populations can only accrue from transgenerational epigenetic effects (not tested in this thesis due to time constrains). Such effects were not entirely unexpected, as they have been found often in short-lived, dispersal-limited organisms, for juvenile traits, and in conditions where environmental variation is predictable over several generations (Yin et al., 2019).

This is an outstanding example of the power of epigenetic effects over genetic responses in phenotypic adaptation to warming. This is the first time that no real evidence of genetic change has been found after so many generations facing such a large environmental change. This case study therefore represents an outlier in our current knowledge, prompting us to generally reconsider the relative importance of the different possible mechanisms by which phenotypic change can occur.

GENERAL CONCLUSION

The aim of this thesis was to increase the understanding of interactions between biological invasions and pollution in hypersaline coastal ecosystems and the mechanisms involved in species thermal adaptation. Through **Chapter I**, I found that the assimilation of specific trace elements by *Artemia* is related to pollution in the surrounding abiotic environment, particularly from water. I showed that the bioaccumulation potential of trace elements was higher in the invasive *A. franciscana* than in native *A. parthenogenetica*. This finding is of great relevance because the displacement of native species by *A. franciscana* may increase the transfer of trace elements in the hypersaline food webs. The higher potential to bioaccumulate trace elements represents an additional indirect impact of the *A. franciscana* invasion that had not been previously recognised for this invasive species. Pollution has been proposed as an important factor preventing or delaying colonization by the invasive species *A. franciscana*, for an uncertain period of time, due to local adaptation of the native *Artemia* to historical pollution (pollution resistance hypothesis). Here, through **Chapter II,** I found some support to this hypothesis, with native *Artemia* from a site with the longest history of Hg-pollution showing the highest resistance to acute exposure to that trace element. More support was found through **Chapter III,** after testing the effect of long-term exposure to Zn. Native *Artemia* from a Zn-contaminated site showed higher survival, and better reproductive and physiological response than the invasive *A. franciscana* from a low Zn-contaminated site. However, *A. franciscana* has a high capacity for local adaptation (Lenormand et al., 2018), so it remains possible that it will acquire greater resistance to trace element pollution in the future. Management efforts should focus on these relict native populations to preserve the remaining *Artemia* biodiversity and limit the probability of *A. franciscana* introduction. All together, these results represent the most detailed ecotoxicological study in hypersaline ecosystems up now, including the most complete evidence that pollution can limit/delay the invasion of *A. franciscana* in highly polluted environments and the first study showing the ecotoxicological implications of an invasion. Finally, through **Chapter IV**, I found that the introduced *Artemia* do show increased phenotypic tolerance to warming. Yet strikingly, these changes do not appear to i) have an additive genetic component, ii) be caused by mitochondrial genetic variation, and iv) be caused by epigenetic marks set by adult parents exposed to warming. I did not find any developmental plasticity in response to warming, nor any protective effect of heat-tolerant local microbiota. Overall, this work represents one of the most complete studies jointly addressing the different factors associated with thermal adaptation in the wild. It suggests that the evolution of *Artemia's* extreme thermal tolerance is only due to transgenerational (great)grandparental effects,

possibly epigenetic marks set by parents who were exposed to high temperatures as juveniles. This finding challenge standard models of genetic and plastic adaptive responses, and our conception of how species may cope with climate warming.

FUTURE RESEARCH

1. The results from **Chapter I** represent the most detailed study on hypersaline ecosystems and the ecotoxicological implications of *A. franciscana* invasion. Still more work needs to be done in this field. For example, given the recent invasion of Odiel saltworks, future work should address the potential effects that the replacement of native *A. parthenogenetica* by *A. franciscana* may have on the Odiel food webs. Moreover, in the future the expression of metallothionein in native and invasive *Artemia* should be investigated, as this can play an important role in the homeostatic regulation of essential trace elements and in the detoxification of toxicants. Finally, in this work I have only considered the effect of salinity on the bioavailability of trace elements. However, other important factors are known to affect trace element bioavailability, not least (i) sediment particle size, (ii) the dynamics of adsorption and mobilization of metals between water and sediment, (iii) pH/Eh, and (iv) the ionic composition of salts which can vary greatly inland. Thus, future work should also consider the effect of those factors as element solubility will vary markedly with respect to them.

2. Future work should consider the effects of different pollutants or a mixture of different pollutants, in native and invasive *Artemia* populations, to provide a more realistic ecological context. Given the results found in **Chapter II** and **III**, future work should focus on chronic effects as they are the most common type of contaminant impact found in the environment.

3. Further work is necessary to see whether the transgenerational effects found in **Chapter IV** are due to epigenetic marks, as in (Norouzitallab et al., 2014), or due to other mechanisms.

4. Future research should use *Artemia* as a model organism and a resurrection ecology approach to study how populations change response to pollution or other effects of climate change (e.g., decrease of pH, increase of CO_2).

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