

# Surviving an Invasion: Characterization of One of the Last Refugia for *Artemia* Diploid Parthenogenetic Strains

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**Abstract** The eradication of native populations of *Artemia* by the invasive *A. franciscana* constitutes one of the most conspicuous examples of biodiversity loss in hypersaline aquatic environments. Detailed information on the biological mechanisms that are supporting the invasion process, as well as on the importance of environment variables, is of paramount importance if adequate measures aiming at preventing

the eradication of native strains are to be successfully implemented. Although the role of environmental stress in benefiting invasions has recently been documented, there seems to be little information on the characterization of environments where invasion is delayed or has failed altogether. Given that both the biotic and abiotic parameters of salt ponds within the Aveiro's salinas complex (Portugal) presently occupied by *A. franciscana* have already been thoroughly characterised (Vieira and Bio, *Journal of Sea Research* 65:293–303, 2011), we will compare the same variables to those measured in an artisanal salina from the same complex, where native *Artemia* still occurs. Since there is no indication of salt ponds where both the native and invasive species co-occur, we hypothesise that explicit differences in environmental factors (e.g. salinity, temperature, pH, alkalinity, dissolved oxygen availability or nutrient concentrations) would help justify the observed distribution pattern of both *Artemia* species.

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## Introduction

One of the main examples of biodiversity loss in hypersaline aquatic environments is the eradication process, started three decades ago, of the Western Mediterranean native populations of the brine shrimp *Artemia* (Crustacea, Branchiopoda, Anostraca) by the invasive *Artemia franciscana* from San Francisco Bay (SFB) and the Great Salt Lake (GSL), United States (Amat et al. 2005, 2007).

Over the last few years, commercial *A. franciscana* cysts, originating from two major suppliers in the United States, have been increasingly used in hatcheries worldwide. Alternatively, several inoculations of *A. franciscana* in solar saltworks have taken place to improve salt production and/or produce cysts and biomass for use in the aquaculture

industry (Mura et al. 2006). The presence of *A. franciscana* in the Western Mediterranean region was first observed in the early 1980s in the salinas of southern Portugal (Hontoria et al. 1987) due to indiscriminate inoculations for aquaculture purposes (Narciso 1989). At that time, *A. franciscana* was absent in the northern Portuguese regions, and repeated observations failed to identify this species in the saltworks of Aveiro (Vieira and Amat 1985). Subsequent dispersion by waterfowl, towards the North and East, mediated by the existing Atlantic and Mediterranean flyways, has extended the species' range along the Atlantic coast and towards the Mediterranean, continuously expanding towards the Spanish, French and Italian saltworks (Amat et al. 2007).

The rate and success of the dissemination process of non-native *Artemia* species throughout Portuguese salinas was remarkable (Amat et al. 2007). Strains belonging to the autochthonous populations of *Artemia* seem to have been eradicated from all Portuguese salinas with exception of the inland rock salt salinas of Rio Maior (39°21'49.90"N; 8°56'38.93"W; Amat et al. 2007) and some salinas belonging to the Aveiro's salinas complex, which occupies a large part of the Ria de Aveiro coastal lagoon on the Atlantic Portuguese coast (40°39'43.78"N; 8°43'11.35"W) (Amat et al. 2007; Pinto et al. 2012). However, these Aveiro salinas are threatened since the exotic *A. franciscana* can already be found in the "Northern group" in the same salinas complex (e.g. in the Tanoeiras salina), where it replaced the native *Artemia* populations.

Apart from their substantial economic impacts, invasive species may alter the evolutionary trajectory of native species through competition, displacement, hybridization and even extinction (Mura et al. 2006). Because biological invasions are threatening global biodiversity worldwide by altering the structure and functioning of ecosystems (Traveset et al. 2006), a better understanding of the factors that positively or negatively affect the invasion process is urgently needed.

Establishment and subsequent range expansion of invasive species in a novel environment are undoubtedly related to the biological attributes of the invader and to biotic interactions with the host community (Mura et al. 2006). Studies on the phenomenon of biological invasions generally focus on the biological characteristics and demographic strategies of species that are successfully established in non-native environments. However, despite much investigation, it has been proven difficult to identify traits that predict the success of these species. This may largely be due to the fact that different traits favour invasiveness in different habitats (Hufbauer 2008). The role of environmental stress in potentiating invasions has also been addressed. For example, the spread of two species of algae along the Western Mediterranean coasts (*Caulerpa taxifolia* and *C. racemosa*) seems to have been favoured not only by their high intrinsic fitness but also by the decline of the native angiosperm *Posidonia*

*oceanica*, induced by both anthropogenic and natural pressures (Occhipinti-Ambrogi and Savini 2003).

Several studies have been carried out on growth, survival, reproductive and life span characteristics of *Artemia* populations from different parts of the world, cultured under standardized laboratory conditions. Of the many conditions that affect *Artemia*'s reproductive success, temperature and salinity have been shown to exert a pronounced impact (e.g. Browne et al. 1984; Vanhaecke et al. 1984; Wear and Haslett 1986; Browne et al. 1988; Barata et al. 1996b; Browne and Wanigasekera 2000). However studies specifically aimed at characterizing the environments where invasions are delayed, or fail altogether, are uncommon.

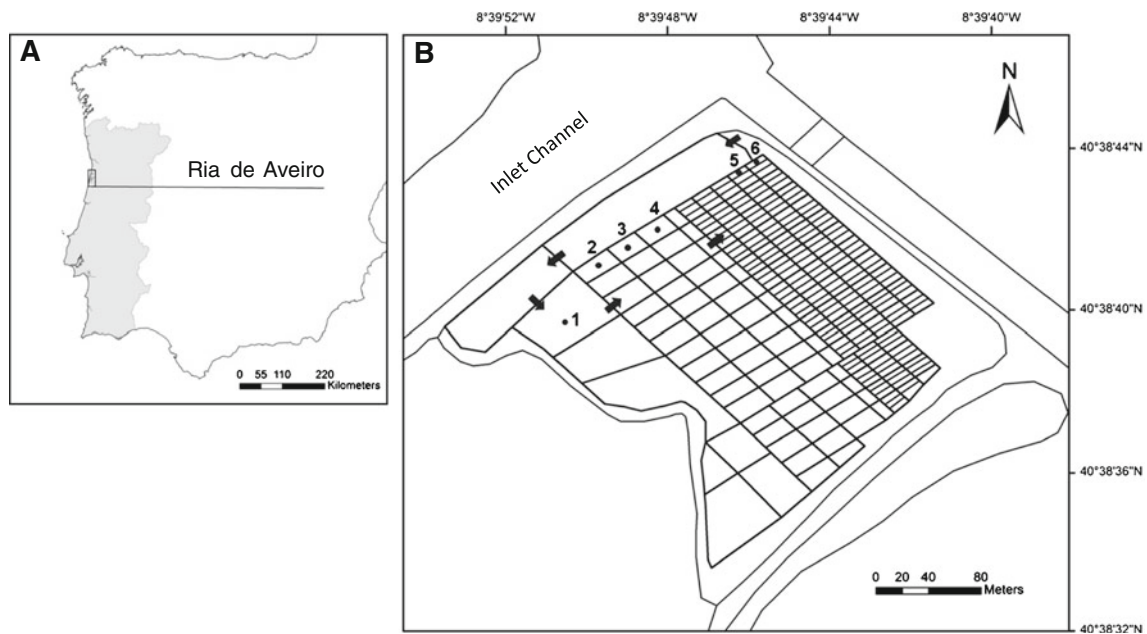
Given that both the biotic and abiotic parameters of salt ponds within the Aveiro's salinas complex, presently occupied by *A. franciscana*, have already been thoroughly characterised (Vieira and Bio 2011), we will compare the same variables to those measured in an artisanal salina where native *Artemia* still occurs (the Troncalhada in Aveiro, Portugal). Since there is no indication of salt ponds where both the native and invasive species co-occur, we hypothesise that explicit differences in environmental factors (e.g. salinity, temperature, pH, alkalinity, biochemical oxygen demand, dissolved oxygen availability or nutrient concentrations) would justify the observed distribution pattern of both *Artemia* species. For instance, an inadequate food supply might be hampering colonisation from the invasive *A. franciscana*, while allowing the native species to endure. This salina, where native *Artemia* still persists, albeit surrounded by the invasive *A. franciscana*, seems to be an invaluable opportunity to expand our understanding on the role played by the environment in the spread of non-native *Artemia*.

## Material and Methods

### Field Sampling and Analysis

Sampling took place in the Troncalhada salina (Fig. 1b), which belongs to the so-called southern group of Aveiro's salinas complex (Fig. 1a). Covering an area of 42.000 m<sup>2</sup>, this salina is fed by water from a big channel (Canal das Pirâmides) that connects the lagoon to the city of Aveiro.

The salina has three types of compartments: supply, evaporation and crystallizer ponds (Fig. 1b). Similarly to salinas of some other European countries (e.g. France, Spain), in the Aveiro's salinas complex, the crystallizer section is subdivided into crystallizer ponds and an extra type of compartment, the condenser ponds, created to obtain purer sodium chloride through repeated water exchanges between these and the crystallizers. Salt (NaCl) harvesting takes place in the crystallizers and only exceptionally (i.e. during very hot summers) in the condensers (Rodrigues et al. 2011).



**Fig. 1** **a** Location of Ria de Aveiro (*square*) on the Portuguese Atlantic coast; **b** Scheme of the Troncalhada salina and the location of the sampling sites: 1) Supply pond (SP), 2) 1st Evaporation pond (EV1), 3)

2nd Evaporation pond (EV2), 4) 3rd Evaporation pond (EV3), 5) Condenser pond (CON), 6) Crystallizer pond (CRY). Water flow is indicated by *arrows*

Water samples (2 l) were collected monthly, from April 2009 to March 2010, at six different sampling sites (Fig. 1b) – in a supply pond (SP), three evaporation ponds (EV1, EV2, EV3), a condenser pond (CON) contiguous to EV1 and a crystallizer pond (CRY). Water temperature (°C), pH (Sorensen scale), dissolved oxygen (DO, mg/l), salinity (ppt) and alkalinity (ppt) were measured *in situ* using a HI 9142 probe (Hanna Instruments) to determine the first two parameters, a HI 98129 (Combo pH and EC) probe (Hanna Instruments) for the dissolved oxygen and a refractometer (model Zuvi, serie 300) to determine the salinity (ppt) and a HI 755 Ckecker®HC Handheld Colorimeter (Hanna Instruments) to determine the alkalinity (ppt). Given the small water depth in most ponds during the salt production season, water samples were obtained at the surface throughout the year, to allow data comparisons between seasons. Immediately after the water sampling, 1 l of water was filtered through a 4.5 cm of diameter glass filter (Whatman GF/C), for pigment assay. Chlorophyll *a* (mg/l) was determined in the laboratory according to the INAG protocol of phytoplankton analysis and sampling (INAG 2009) and estimated according to the Lorensen equation (Lorensen 1967). The Pigment Diversity Index (PDI) was also determined according to Margalef (Margalef 1960). Nitrate (NO<sub>3</sub>, μgat/l), nitrite (NO<sub>2</sub>, μgat/l), orthophosphate (PO<sub>4</sub>, μgat/l) and silicate (SiO<sub>4</sub>, μgat/l) were determined according to the Standard Methods of seawater analyses protocols (Strickland and Parsons 1972); their concentrations were expressed as microgram-atoms (μgat), where 1 μgat/l = 1 μg/l = 1 × 10<sup>-6</sup>g/l divided by the

atomic weight of nitrate, nitrite, orthophosphate and silicate, depending on the compound. The biochemical oxygen demand (BOD<sub>5</sub>, mg/l) was determined by the Winkler method (Strickland and Parsons 1972).

#### Data Analysis

Physicochemical and biological (pigments) data were summarized and plotted. Data were analysed in time, comparing the salt production (May to September) and non production (October to April) seasons, and in space, comparing conditions in the different salina sections. The variability and distribution of values of each variable was presented using Tukey's boxplots (Tukey 1977) with outliers (i.e. points that differ more than 1.5 times the interquartile range from the respective quartiles) presented as separate circles. Mean values obtained from different seasons (salt production and non-production) were compared using a Wilcoxon rank sum test (Hollander and Wolfe 1973) since most variables failed the Shapiro-Wilk normality test (Royston 1982).

Multivariate analyses were used to compare conditions between samples from different seasons (salt production, non-production) and sections (ponds). A correlation-based Principal Component Analysis (PCA) of the physicochemical variables, chlorophyll *a* and PDI, was carried out for each salina pond. The distinction between seasons was tested through Analysis of Similarity (ANOSIM) following a 2D Non-Metric Multidimensional Scaling (NMDS) based on standardized and normalized Euclidian distances.

All statistical tests considered the significance level  $\alpha=0.05$ . Multivariate analyses were performed with CANOCO 4.5 (Ter Braak and Smilauer 1998) with the exception of NMDS and ANOSIM that were carried out in Primer 5 (Clarke and Warwick 2001). All other statistical analyses were done in R (R Dev. Core Team 2009).

## Results

Results obtained from the Troncalhada salina show marked temporal and spatial variability. The salt production (i.e. May to September) and non-production (October to April) seasons constitute two distinct environments in terms of physicochemical and biological variability (Fig. 2, Tables 1 and 2).

With the exception of pH and Pigment Diversity Index (PDI), temporal (or seasonal) differences were significant ( $p < 0.05$ ) for all of the determined parameters (Tables 1 and 2). The variation of the studied physicochemical parameters was more accentuated during the period in which the salina was active, i.e. producing salt (Table 2; Fig. 2). Next to the seasonal differences, there were also spatial variations of the determined parameters, reflecting the distinct conditions that exist in the different salina ponds (Tables 1 and 2, Figs. 2 and 3). Considering the several parameters individually, it seems clear that temperature and salinity clearly reached higher values in all salina sections during the salt production period. These parameters showed an increasing trend from the supply to the crystallizer ponds, with particularly high salinities in the condenser and crystallizer ponds.

The pH values were relatively stable during the non-production seasons, although more variable in the condenser and crystallizer ponds. During salt production there are some lower pH values in the supply pond and lower and more variable pH values in the crystallizer, which are associated with very variable alkalinity. Alkalinity was, in general, higher during the production season, with significantly different values between seasons in the condenser pond, where the highest mean values were achieved. Though statistically non-significant, alkalinity inside the crystallizer is much higher during salt production (150 ppt; Table 2) than in the non-production season (79 ppt; Table 1).

Dissolved oxygen (DO) values were lower during the salt production, with poorly oxygenated water in the different salina sections. During this season, the condenser exhibited the highest DO values and the crystallizer the lowest values, including some situations of anoxia. The BOD<sub>5</sub> values were, in general, higher during the non-production period, with quite significant seasonal differences between the condenser and crystallizer ponds.

Nitrate (NO<sub>3</sub>) and nitrite (NO<sub>2</sub>) concentrations were generally higher during the non-production season. Seasonal

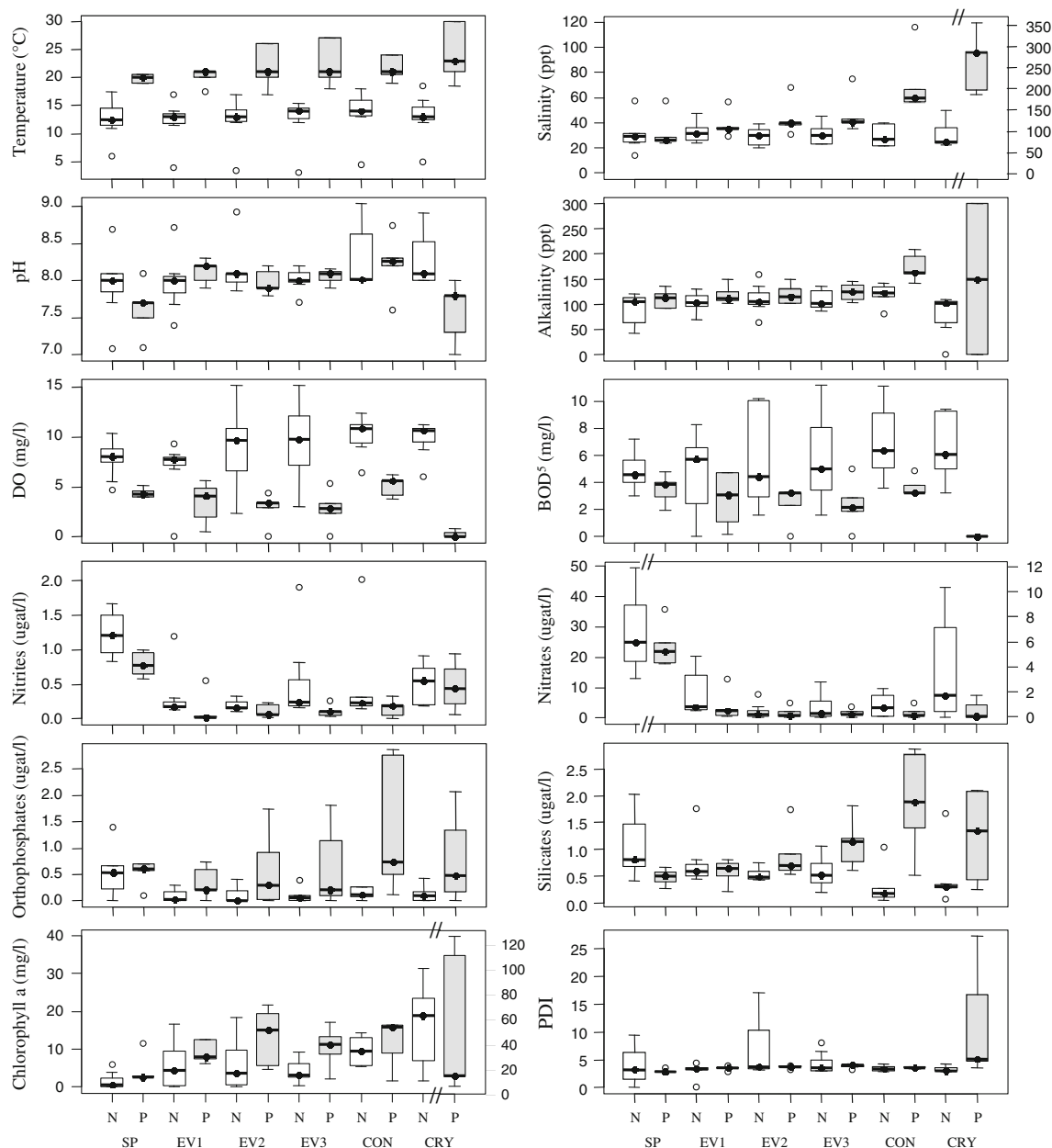
differences are significant for the supply pond (especially in terms of nitrates), which presented very high NO<sub>3</sub> and NO<sub>2</sub> values during the non-production season. On the contrary, the amount of orthophosphate (PO<sub>4</sub>) was largely higher during the salt production season. The differences in orthophosphate values between seasons was significant in the condenser pond, which showed the highest and most variable PO<sub>4</sub> values during the production season.

In the evaporators 2 and 3, the condenser and crystallizer, the silicate concentration (SiO<sub>4</sub>) was higher during salt production, whilst in the supply pond and evaporator 1 the values were higher during the non-production season. Seasonal differences were statistically significant for most of the ponds.

The Chlorophyll *a* values were, in general, higher during the salt production season, while the PDI values were relatively stable in both seasons. However, during the production period, the crystallizer pond exhibited higher and very variable values for both pigment parameters.

The principal components analysis (PCA) of the studied parameters measured during salt production (Fig. 3), showed that variability between samples was mainly characterized by a salinity/PDI and inverse DO/BOD<sub>5</sub> gradient (on the 1st axis), and by a NO<sub>2</sub>/NO<sub>3</sub> and inverse SiO<sub>4</sub>/PO<sub>4</sub>/pH gradient (2nd axis). In terms of spatial patterns, samples from the supply pond (SP) and samples from the crystallizer pond (CRY) are well separated from samples collected in the evaporators (EV1, EV2 e EV3) and in the condenser pond (CON). This distinction is also clear from the analysis of similarity (Table 3). There is an increasing discrimination between samples from the supply pond and those from increasingly distant successive ponds, with R-values ranging from 0.532 (SP-EV1) to 0.934 (SP-CRY). Samples from the crystallizer pond are well discriminated from those collected in any other pond, with  $R \geq 0.720$ . Comparisons between evaporator and condenser ponds were not significant, with very overlapping data ( $R \leq 0.252$ ).

The PCA applied to the results obtained for the non-production season shows a 1st axis dominated by a gradient of all nutrients and a 2nd axis dominated by DO/pH and inverse temperature gradient. During this season the samples from the different ponds are highly clustered (more similar). Only the samples that correspond to the supply pond appear segregated, characterized by higher nutrient concentrations. This is further confirmed by the analysis of similarity (Table 3). The R-values for the comparisons with the supply pond samples showed some discrimination between ponds, although data were quite overlapping ( $260 < R < 343$ ). All other comparisons showed practically complete overlap ( $R \leq 0.150$ ).



**Fig. 2** Physicochemical and biological (Chl.a: chlorophyll *a*, PDI: Pigment Diversity Index) data for each salina section (SP: supply pond, EV1: 1st evaporation pond, EV2: 2nd evaporation pond, EV3: 3rd evaporation pond, CON: condenser pond, CRY: crystallizer pond),

during the non-production (N, white boxes) and the salt production seasons (P, grey boxes). Notice the two different y-axes for salinity, nitrates and chlorophyll, applied to better visualize lower values

There is a clear seasonal (salt production versus non-production periods) pattern in all salina sections (Fig. 4), which dominates the 1st axis of the PCA. This axis is characterized by variable gradients that vary along the sequence of salina sections: DO, NO<sub>3</sub> and SiO<sub>4</sub> versus alkalinity and temperature in SP; BOD<sub>5</sub>, DO and NO<sub>3</sub> versus PDI in EV1; PO<sub>4</sub>, Chl. *a*. and temperature versus DO and BOD<sub>5</sub> in EV2; PO<sub>4</sub>, SiO<sub>4</sub> and temperature versus DO and BOD<sub>5</sub> in EV3; SiO<sub>4</sub> versus BOD<sub>5</sub> and DO in CON. In the CRY, the first

axis is characterized by temperature and salinity versus DO and BOD<sub>5</sub> gradients.

According to the ANOSIM tests (Table 4), all ponds, except EV1, show significant seasonal distinction. There is a spatial trend for the differences between samples from the salt production and non-production seasons, with differences increasing from the evaporator ponds to the condenser and crystallizer ponds. Only these last two ponds in the system show well discriminated samples ( $R > 0.500$ ).

**Table 1** Means and standard deviations (SD) of physicochemical (Temp.: temperature in °C; Sal.: salinity in ppt; DO and BOD<sub>5</sub> in mg/l; pH at Sorensen scale; Alk.: alkalinity in ppt; NO<sub>2</sub>, NO<sub>3</sub>, PO<sub>4</sub> and SiO<sub>4</sub> in µgat/l) and biological (Chl.*a*: chlorophyll *a* in mg/l; PDI: Pigment Diversity Index) data observed during the non-productive season in the different salina ponds (SP: supply pond, EV1: 1st

evaporation pond, EV2: 2nd evaporation pond, EV3: 3rd evaporation pond, CON: condenser pond, CRY: crystallizer pond). The significance of the difference between mean values from samples collected during the non-productive season (this Table) and the salt production season (Table 2), according to Wilcoxon rank sum tests, is indicated in superscripts (ns: non significant; \*: α=0.05; \*\*: α=0.01; \*\*\*: α=0.001)

Parameter	Salina section						
	All Mean (SD)	SP Mean (SD)	EV1 Mean (SD)	EV2 Mean (SD)	EV3 Mean (SD)	CON Mean (SD)	CRY Mean (SD)
Temp.	12.67 (3.91)***	12.57 (3.70)**	12.07 (3.98)**	12.36 (4.24)**	12.43 (4.31)**	13.57 (4.36)**	13.00 (4.17)**
Sal.	30.63 (9.14)***	30.43 (13.43) <sup>ns</sup>	32.62 (8.20) <sup>ns</sup>	29.16 (7.75) <sup>ns</sup>	30.84 (8.87) <sup>ns</sup>	29.57 (8.37)**	31.01 (10.18)**
DO	8.86 (3.10)***	8.08 (1.56)**	6.77 (3.08)*	8.88 (4.32)*	9.49 (4.27)*	10.15 (1.98)**	9.79 (1.86)**
BOD <sub>5</sub>	5.86 (2.98)***	4.88 (1.40) <sup>ns</sup>	4.57 (3.03) <sup>ns</sup>	6.04 (3.94) <sup>ns</sup>	5.85 (3.66) <sup>ns</sup>	7.08 (2.87)*	6.75 (2.60)**
pH	8.12 (0.39) <sup>ns</sup>	7.95 (0.48) <sup>ns</sup>	7.98 (0.40) <sup>ns</sup>	8.15 (0.35) <sup>ns</sup>	8.01 (0.17) <sup>ns</sup>	8.33 (0.44) <sup>ns</sup>	8.29 (0.37)*
Alk.	101.6 (30.2)**	89.1 (32.10) <sup>ns</sup>	104.2 (20.30) <sup>ns</sup>	110.1 (30.4) <sup>ns</sup>	110.0 (10.0) <sup>ns</sup>	119.7 (22.10)*	79.0 (40.50) <sup>ns</sup>
NO <sub>2</sub>	0.60 (0.67)**	1.47 (0.86)*	0.33 (0.38) <sup>ns</sup>	0.20 (0.08) <sup>ns</sup>	0.55 (0.64)*	0.52 (0.74) <sup>ns</sup>	0.50 (0.30) <sup>ns</sup>
NO <sub>3</sub>	6.77 (12.11)*	28.46 (13.75)**	5.02 (9.26) <sup>ns</sup>	0.52 (0.66) <sup>ns</sup>	0.88 (1.14) <sup>ns</sup>	0.98 (0.96) <sup>ns</sup>	3.95 (4.30) <sup>ns</sup>
PO <sub>4</sub>	0.19 (0.28)***	0.53 (0.46) <sup>ns</sup>	0.10 (0.13) <sup>ns</sup>	0.10 (0.17) <sup>ns</sup>	0.10 (0.15) <sup>ns</sup>	0.15 (0.12)*	0.14 (0.16) <sup>ns</sup>
SiO <sub>4</sub>	0.63 (0.47)**	1.08 (0.62)*	0.75 (0.46) <sup>ns</sup>	0.54 (0.12) <sup>ns</sup>	0.57 (0.29)*	0.30 (0.37)**	0.47 (0.54) <sup>ns</sup>
Chl. <i>a</i>	7.25 (7.65)*	1.68 (2.36) <sup>ns</sup>	5.87 (6.46) <sup>ns</sup>	6.14 (6.90) <sup>ns</sup>	4.42 (3.38) <sup>ns</sup>	9.56 (4.05) <sup>ns</sup>	16.14 (11.10) <sup>ns</sup>
PDI	4.29 (3.15) <sup>ns</sup>	4.03 (3.72) <sup>ns</sup>	2.95 (1.50) <sup>ns</sup>	7.34 (5.45) <sup>ns</sup>	4.39 (1.98) <sup>ns</sup>	3.45 (0.48) <sup>ns</sup>	3.29 (0.49)*

**Discussion**

Salina Characterization

In general, one may say that the studied salina, still harbouring native *Artemia*, seasonally fluctuates from a brackish (during the non-production season) to a hypersaline

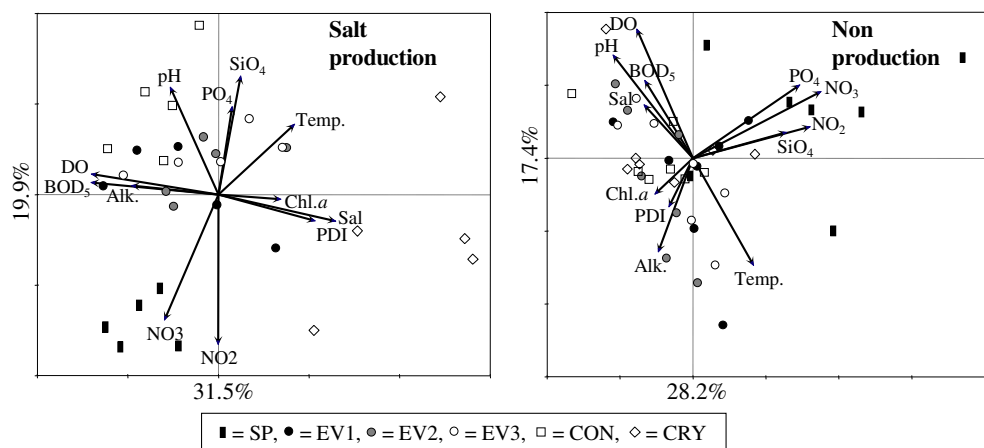
environment (during the salt production season), a change related to the salt production process. Since salt production is dependent on weather conditions, many of which temporal (production versus non-production), variations observed were predominantly seasonal (e.g. temperature), whereas others were clearly dependent on the salt production process (e.g. salinity), or both (e.g. DO which tends to be lower in

**Table 2** Means and standard deviations (SD) of physicochemical (Temp.: temperature in °C; Sal.: salinity in ppt; DO and BOD<sub>5</sub> in mg/l; pH at Sorensen scale; Alk.: alkalinity in ppt; NO<sub>2</sub>, NO<sub>3</sub>, PO<sub>4</sub> and SiO<sub>4</sub> in µgat/l) and biological (Chl.*a*: chlorophyll *a* in mg/l; PDI:

Pigment Diversity Index) data observed during salt production season in the different salina ponds (SP: supply pond, EV1: 1st evaporation pond, EV2: 2nd evaporation pond, EV3: 3rd evaporation pond, CON: condenser pond, CRY: crystallizer pond)

Parameter	Salina section						
	All Mean (SD)	SP Mean (SD)	EV1 Mean (SD)	EV2 Mean (SD)	EV3 Mean (SD)	CON Mean (SD)	CRY Mean (SD)
Temp.	21.78 (3.46)	19.80 (0.76)	20.10 (1.52)	22.00 (3.94)	22.60 (4.16)	21.70 (2.22)	24.50 (5.27)
Sal.	84.86 (93.28)	32.20 (14.29)	38.50 (10.48)	43.44 (14.27)	46.70 (16.03)	71.08 (25.40)	277.24 (71.07)
DO	3.21 (2.00)	4.39 (0.51)	3.39 (2.11)	2.82 (1.66)	2.76 (1.91)	5.08 (1.06)	0.22 (0.37)
BOD <sub>5</sub>	2.54 (1.71)	3.53 (1.09)	2.76 (2.06)	2.40 (1.40)	2.40 (1.81)	3.67 (0.73)	0.00 (0.00)
pH	7.93 (0.37)	7.62 (0.36)	8.12 (0.16)	7.98 (0.17)	8.06 (0.11)	8.22 (0.41)	7.58 (0.41)
Alk.	131.9 (50.9)	110.9 (18.7)	119.2 (19.30)	119.9 (20.4)	124.1 (17.9)	173.8 (27.4)	150.0 (21.21)
NO <sub>2</sub>	0.29 (0.32)	0.79 (0.18)	0.13 (0.24)	0.11 (0.09)	0.11 (0.09)	0.15 (0.13)	0.47 (0.37)
NO <sub>3</sub>	1.43 (2.21)	5.73 (1.79)	0.92 (1.24)	0.41 (0.48)	0.39 (0.33)	0.41 (0.47)	0.51 (0.85)
PO <sub>4</sub>	0.71 (0.80)	0.53 (0.25)	0.35 (0.31)	0.59 (0.74)	0.65 (0.80)	1.40 (1.31)	0.76 (0.91)
SiO <sub>4</sub>	1.03 (0.74)	0.48 (0.15)	0.58 (0.24)	0.90 (0.49)	1.11 (0.47)	1.88 (0.98)	1.26 (0.96)
Chl. <i>a</i>	18.35 (28.64)	4.45 (3.96)	20.69 (27.21)	13.36 (7.73)	10.51 (5.66)	11.86 (6.51)	49.23 (58.79)
PDI	4.85 (4.88)	3.02 (0.30)	3.45 (0.38)	3.71 (0.27)	3.83 (0.37)	3.63 (0.16)	11.43 (10.32)

**Fig. 3** First two axes of the principal component analyses (PCA) of physicochemical (Temp.: temperature, Alk.: alkalinity, Sal.: salinity) and biological (Chl.a: chlorophyll a, PDI: Pigment Diversity Index) data of samples from the salt production and from the non-production seasons; samples are marked according to the salina section and percentages of variance explained by the axes are given



warmer water, but showed extremely low values in the crystallizer pond, where high salinity further decreased oxygen solubility).

Besides the obvious seasonal component, spatial variability of the analysed parameters was also observed, reflecting the distinct conditions in the different salina ponds. Spatial patterns were determined by the different pond functions within the salt production process. Accordingly, they were more accentuated during salt production than during the non-production season. During salt production, the salina combines a spectrum of environments following the strong salinity gradient that ranges from a coastal lagoon environment in the

supply ponds to a hyper-saline environment in the crystallizers. This physicochemical diversity existing in the different salina ponds, which is mainly dependent on the gradual increase in salinity, is then reflected in the flora and fauna that colonizes each pond (Davis 2000).

#### What Makes this Refugium Singular?

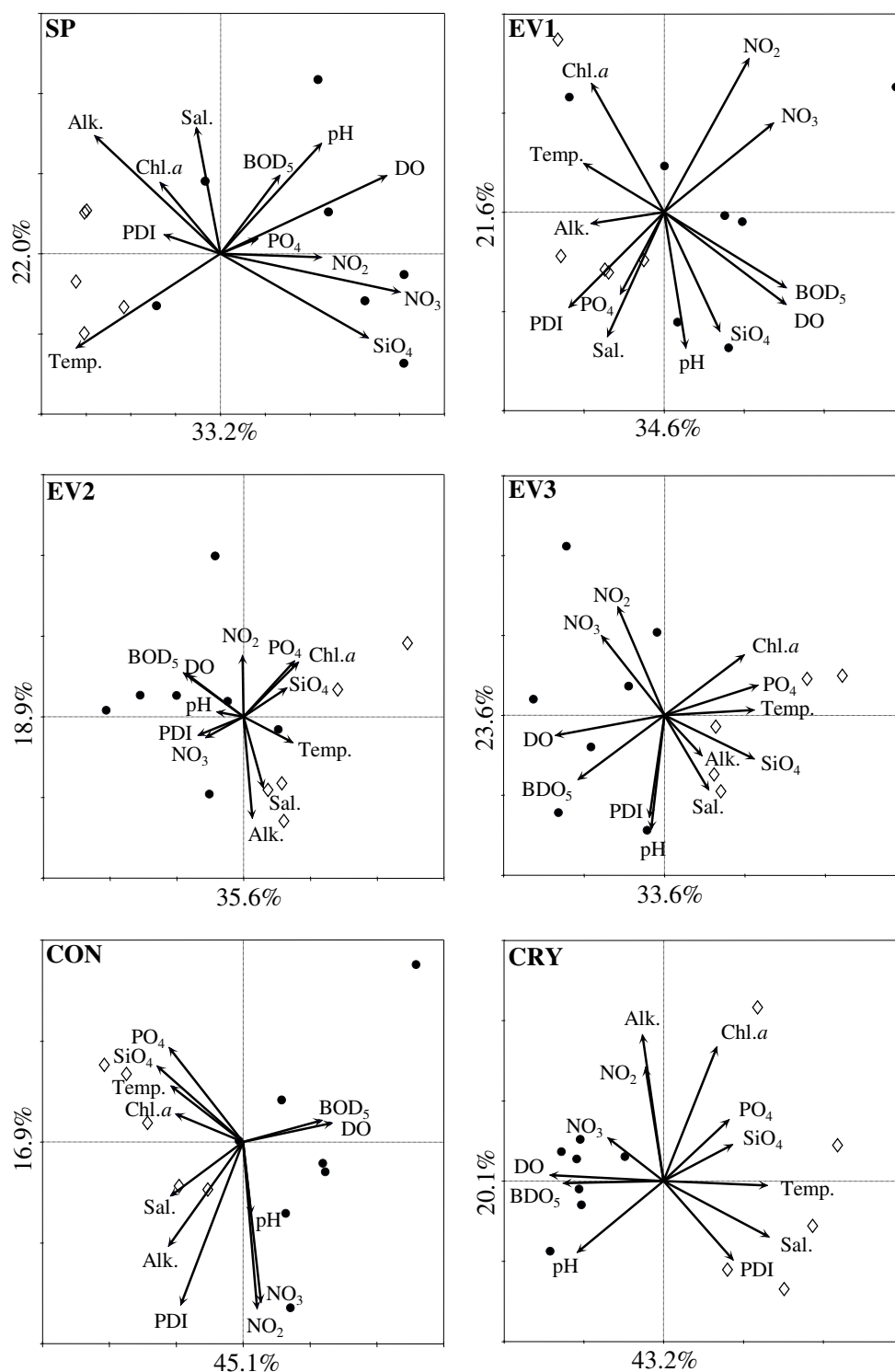
In the studied salina, native *Artemia* diploid parthenogenetic strains were found during the production season, in the evaporation ponds, which are the salina's compartments that offer the most propitious ecological conditions (food, salinity, temperature, oxygen levels and lack of predators) for their survival. During the non-production season, the salina is filled with water through the floodgates that stay open and remain so until March-April, at which time the preparatory works for salt production season begin (Rodrigues et al. 2011). Neither *Artemia* cysts nor the live brine shrimp can survive throughout the rainy season, because, at low salinities, the cysts hatch and the live animals are consumed by predatory fish and crustaceans (Persoone and Sorgeloos 1980).

The existence of native *Artemia* strains in the studied salina is of particular interest since *Artemia franciscana* has been found in adjacent salinas (Amat et al. 2007). The Aveiro's salinas complex is the country's second largest salt pond area. It is part of a large lagoon, the Ria de Aveiro, and has an important function as a breeding and feeding site for a large number of resident and migratory aquatic birds (Rodrigues et al. 2011). Many salinas in the Aveiro complex (37 %) have been converted into aquaculture units (Morgado et al. 2009; Rodrigues et al. 2011). This specific situation (presence of migratory birds and aquaculture) leads us to believe that inoculation of exotic *Artemia* species by human intervention or dispersion via water birds must have occurred repeatedly. Nevertheless, the studied salina seems to remain still as a sanctuary for native *Artemia*, suggesting that one or more factors may prevent *A. franciscana* from completing the ongoing invasion/eradication process.

**Table 3** Global and pairwise ANOSIM test results (R and p-values) comparing samples from different ponds, i.e. variability in space (ns: non significant, \*:  $\alpha=0.05$ , \*\*:  $\alpha=0.01$ , \*\*\*:  $\alpha=0.001$ ). The 2D MDS stress was 0.12 for the non-production and 0.11 for the production season data

Groups	Non-production		Salt production	
	R	p-value	R	p-value
Global	0.103	0.006**	0.369	0.001***
SP, EV1	0.259	0.011*	0.532	0.008**
SP, EV2	0.324	0.008**	0.692	0.008**
SP, EV3	0.280	0.004**	0.788	0.008**
SP, CON	0.343	0.011*	0.864	0.008**
SP, CRY	0.260	0.009**	0.934	0.008**
EV1, EV2	0.016	0.374ns	-0.148	0.952ns
EV1, EV3	-0.080	0.792ns	-0.112	0.897ns
EV1, CON	-0.001	0.462ns	0.252	0.040*
EV1, CRY	0.150	0.030*	0.720	0.008**
EV2, EV3	-0.091	0.864ns	-0.184	0.889ns
EV2, CON	-0.007	0.500ns	0.248	0.079ns
EV2, CRY	0.089	0.116ns	0.887	0.008**
EV3, CON	-0.122	0.864ns	0.236	0.095ns
EV3, CRY	0.124	0.066ns	0.960	0.008**
CON, CRY	0.041	0.256ns	0.840	0.008**

**Fig. 4** First two axes of the principal components analyses (PCA) of physicochemical (Temp.: temperature, Alk.: alkalinity, Sal.: salinity) and biological (Chl.a: chlorophyll a, PDI: Pigment Diversity Index) data, for each salina section (SP: supply pond; EV1: 1st evaporator; EV2: 2nd evaporator; EV3: 3rd evaporator; CON: condenser e CRY: crystallizer). The samples are marked according to season (white diamond = salt production, black circle = non-production of salt) and percentages of variance explained by the axes are given



Since environmental parameters, such as salinity, temperature, food or oxygen availability decisively influence the response of different populations in terms of biological fitness and life span (Browne et al. 1984, 1988, 1991; Barata et al. 1995, 1996a, b), we have tried to exhaustively depict the seasonal and spatial dynamics of one of the last refuges of native *Artemia* within Northern Portugal. Given the speed

and success rate of the ongoing *A. franciscana* invasion process, we hoped that the proposed multifactorial analysis, encompassing the biotic and abiotic parameters of this specific salina, would have highlighted which factors are slowing down or even impairing the final steps of colonization. Surprisingly, the physicochemical and biological parameters studied in the Troncalhada salina showed similar values to



**Table 4** ANOSIM test results (R and *p*-values) comparing samples from the salt production and non-production seasons, i.e. variability in time, for each of the salina compartments (ns: non significant, \*:  $\alpha=0.05$ , \*\*:  $\alpha=0.01$ ). The 2D MDS stress values ranged between 0.09 and 0.13

Section	R	<i>p</i> -value
SP	0.263	0.040*
EV1	0.053	0.279ns
EV2	0.265	0.029*
EV3	0.410	0.040*
CON	0.699	0.002**
CRY	0.529	0.003**

those presented by Vieira and Bio (2011) for another Aveiro salina (Tanoeiras) invaded by *A. franciscana*. Thus, our initial hypothesis, stating that some explicit differences in environmental factors would justify the distribution of native and invasive species, seems to have no direct support.

Given that the initial steps of eradication of native *Artemia* from a particular location logically imply a variable period of co-existence with the non-native species, we might rule out any mechanism (either direct or indirect) related with interspecific agonistic interactions that results in *A. parthenogenetica* survival and *A. franciscana* eradication. Following Occam's razor principle, if such a mechanism existed, many more *foci* of native *Artemia* would be apparent throughout the Atlantic and Mediterranean coasts. Thus, a distinct set of causes, neither directly related to the seasonally fluctuating environment nor to interspecific interactions, may be playing a decisive role in the preservation of this native *Artemia* refugium. Although apparently contradicting the most obvious common sense, we believe that this set of causes is linked to pollution levels.

The presence of pollutants, such as heavy metals in the water that feeds the salina, may play a decisive role in the prevention of the invasion if we consider that different *Artemia* species and strains have been demonstrated to have distinct sensibilities when exposed to the same toxicants (e.g. Bagshaw et al. 1986; MacRae and Pandey 1991; Go et al. 1990; Sarabia et al. 1998, 2002, 2003, 2008). The Ria de Aveiro lagoon is one of the most mercury-contaminated systems in Europe, due to the continuous mercury discharges of a chlor-alkali plant, during more than four decades (1950–1994), into an inner bay of 2 km<sup>2</sup> called Laranjo Bay (Pereira et al. 1998). Although the anthropogenic sources of mercury into the aquatic systems have been considerably reduced through legislation (Pereira et al. 2009), mercury concentrations in the surface sediments of some areas of the Ria are still higher than pre-industrial levels, namely in the above-cited Laranjo bay (Coelho et al. 2005). This mercury is transported, primarily via tide action

(Pereira et al. 1998; Ramalhosa et al. 2001; Monterroso et al. 2003) and can enter into the salinas when they are supplied with new seawater. Not surprisingly, due to its geographical location, the studied salina is one of the first locations to receive the contaminated water, which will inheritably grant it higher contamination levels.

Another study (Martins et al. 2010) analysing sediment surface samples from channels belonging to Ria de Aveiro, revealed the presence of “hot spots” of pollution from past industrial activities, which, next to mercury, have high available concentrations of other toxic heavy metals such as aluminium, cadmium, copper, cobalt, iron, lead, manganese and zinc, as well as high concentrations of total organic carbon. As the town channels are used for navigation and recreational purposes they are subjected to routine maintenance dredging to prevent siltation and to maintain the hydrodynamics features of the lagoon system. Work on the channel can cause re-suspension of polluted sediments and the dredging of contaminated sediments may dramatically modify their physicochemical and biochemical properties. Relatively slight alterations in these conditions (e.g. pH, Eh, etc.) could induce rapid changes in the mobility and availability of heavy metals (Martins et al. 2010). The analysis of pollutants, especially heavy metals, in the salinas of Aveiro where native and non-native *Artemia* exist, and the testing of pollutant effects on the life history traits, demographic and competitive interactions will provide valuable insights into whether pollutants may be i) preventing *A. franciscana* from directly colonizing particular spots where native strains remain isolated or ii) modifying the hatching success of *A. franciscana* cysts.

Although the information on the response of cysts, nauplii or adults of *Artemia* populations to all these toxicants is incomplete, previous studies suggests that *A. parthenogenetica* is more resistant to this particular kind of stress than *A. franciscana*. As an example, Go et al. (1990) reported a reduction in the hatching of *A. franciscana* cysts exposed to mercury at a concentration as low as 0.01  $\mu\text{M}$  Hg. However, Sarabia et al. (1998) did not find any mercury-related effect on the emergence and hatching of *Artemia* diploid parthenogenetic strain, within the same range of concentrations. This pattern of results can be ported to zinc exposure where a lack of response in the emergence and hatching in *A. parthenogenetica* (Sarabia et al. 2008) clearly differs from the high sensitivity exhibited by *A. franciscana* (Bagshaw et al. 1986; MacRae and Pandey 1991). This variability in responses to heavy metals may be the result of differences among species in cyst's structure, metabolism and physiology among species (Amat et al. 2005; Varo et al. 2006). Vanhaecke et al. (1980) have already considered the importance of all these factors in *Artemia* hatching success. Rafiee et al. (1986) reported drastic effects of cadmium on the emergence and hatching of *A. franciscana* at concentrations

of 1  $\mu\text{M}$  whereas Sarabia et al. (2003) did not find any effect of cadmium exposure in *A. parthenogenetica*, even when the assayed concentrations were very high (44.5  $\mu\text{M}$  of Cd).

Sarabia et al. (2002) studied the lethal responses to cadmium of instar II nauplii from several strains and species of the genus *Artemia*. The variability found in cadmium sensitivity of nauplii corresponding to the several *Artemia* populations and species studied, supports differences in physiology and metabolism among species in relation to the mechanism for metal detoxification (Sarabia et al. 2008). The two populations of *A. franciscana* were the most sensitive to cadmium toxicity whereas parthenogenetic *Artemia* evidenced a reduced toxicity after cadmium exposure (Sarabia et al. 2002). A generalization of these results should be done with extreme caution as the effects of heavy metal exposure on *A. franciscana* hatching reported by several authors are not entirely consistent. For example, whereas Bagshaw et al. (1986), MacRae and Pandey (1991) and Rafiee et al. (1986) reported hatching being highly inhibited by copper, cadmium and zinc, Brix et al. (2006) reported that the hatching success is not particularly sensitive to cadmium and zinc. However, a preliminary study (Almeida et al. unpubl.data) conducted specifically on *Artemia* populations from Aveiro, clearly showed that the nauplii from the native *Artemia parthenogenetica* strain proved to be more resistant to mercury (at concentrations between 1 and 100 mg/l) than those from the invasive competitor (*A. franciscana*).

More research on the mechanisms underlying the biological invasion processes is needed. The role of the environment in the spreading of non-native *Artemia* must be better understood in order to control bioinvasions and prevent native populations from disappearing. At the moment, it is difficult to propose any particular measure, similar to measures usually applied to other types of invaders, to prevent the settlement of exotic brine shrimp species. Local populations can be informed about the negative consequences of exotic *Artemia* inoculation in salinas and the advantages of culturing native *Artemia* parthenogenetic strains can be stressed, which are: i) the native strains are sustained and their genetic diversity is preserved, ii) these strains are perfectly adapted to the area, not prompting any unpredictable modifications to the ecosystem and iii) the diploid parthenogenetic strains existing in the Aveiro salinas have biometrical, chemical and hatching characteristics that allow their successful use in the feeding of fish and crustacean during their larval states (Vieira 1989). If, on the other hand, *A. parthenogenetica* has indeed resisted complete obliteration as a consequence of a higher tolerance to contaminants, the message to the local populations, or even to the policy makers, will be much harder to convey.

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