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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.

55	Keywords separated by ' - '	<i>Artemia</i> - Salina - Ecology - Biological invasions
56	Foot note information

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.

Keywords *Artemia* · Salina · Ecology · Biological invasions

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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 aquacul-
 ture 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 Span-
 ish, 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 threat-
 ened 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, inva-
 sive species may alter the evolutionary trajectory of
 native species through competition, displacement, hy-
 bridization and even extinction (Mura et al. 2006).
 Because biological invasions are threatening global bio-
 diversity worldwide by altering the structure and func-
 tioning 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 inva-
 sive 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 gener-
 ally 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 poten-
 tiating invasions has also been addressed. For example, the
 spread of two species of algae along the Western Mediter-
 ranean 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 pres-
 sures (Occhipinti-Ambrogi and Savini 2003).

Several studies have been carried out on growth, survival,
 reproductive and life span characteristics of *Artemia* popu-
 lations 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 occu-
 pied by *A. franciscana*, have already been thoroughly char-
 acterised (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, Por-
 tugal). 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. salin-
 ity, temperature, pH, alkalinity, biochemical oxygen de-
 mand, dissolved oxygen availability or nutrient
 concentrations) would justify the observed distribution pat-
 tern 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 m²,
 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 exception-
 ally (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*

159 Water samples (2 l) were collected monthly, from April
160 2009 to March 2010, at six different sampling sites (Fig. 1b)
161 – in a supply pond (SP), three evaporation ponds (EV1,
162 EV2, EV3), a condenser pond (CON) contiguous to EV1
163 and a crystallizer pond (CRY). Water temperature (°C), pH
164 (Sorensen scale), dissolved oxygen (DO, mg/l), salinity
165 (ppt) and alkalinity (ppt) were measured *in situ* using a HI
166 9142 probe (Hanna Instruments) to determine the first two
167 parameters, a HI 98129 (Combo pH and EC) probe (Hanna
168 Instruments) for the dissolved oxygen and a refractometer
169 (model Zuvi, serie 300) to determine the salinity (ppt) and a
170 HI 755 Ckecker®HC Handheld Colorimeter (Hanna Instru-
171 ments) to determine the alkalinity (ppt). Given the small
172 water depth in most ponds during the salt production season,
173 water samples were obtained at the surface throughout the
174 year, to allow data comparisons between seasons. Immedi-
175 ately after the water sampling, 1 l of water was filtered
176 through a 4.5 cm of diameter glass filter (Whatman GF/C),
177 for pigment assay. Chlorophyll *a* (mg/l) was determined in
178 the laboratory according to the INAG protocol of phyto-
179 plankton analysis and sampling (INAG 2009) and estimated
180 according to the Lorensen equation (Lorensen 1967). The
181 Pigment Diversity Index (PDI) was also determined according
182 to Margalef (Margalef 1960). Nitrate (NO₃, µgat/l), nitrite
183 (NO₂, µgat/l), orthophosphate (PO₄, µgat/l) and silicate (SiO₄,
184 µgat/l) were determined according to the Standard Methods of
185 seawater analyses protocols (Strickland and Parsons 1972);
186 their concentrations were expressed as microgram-atoms
187 (µgat), where 1 µgat/l=1 µg/l=1×10⁻⁶g/l divided by the

atomic weight of nitrate, nitrite, orthophosphate and sili- 188
cate, depending on the compound. The biochemical oxy- 189
gen demand (BOD₅,mg/l) was determined by the Winkler 190
method (Strickland and Parsons 1972). 191

Data Analysis 192

Physicochemical and biological (pigments) data were sum- 193
marized and plotted. Data were analysed in time, comparing 194
the salt production (May to September) and non production 195
(October to April) seasons, and in space, comparing con- 196
ditions in the different salina sections. The variability and 197
distribution of values of each variable was presented using 198
Tukey's boxplots (Tukey 1977) with outliers (i.e. points that 199
differ more than 1.5 times the interquartile range from the 200
respective quartiles) presented as separate circles. Mean 201
values obtained from different seasons (salt production and 202
non-production) were compared using a Wilcoxon rank sum 203
test (Hollander and Wolfe 1973) since most variables failed 204
the Shapiro-Wilk normality test (Royston 1982). 205

Multivariate analyses were used to compare conditions 206
between samples from different seasons (salt production, 207
non-production) and sections (ponds). A correlation-based 208
Principal Component Analysis (PCA) of the physicochem- 209
ical variables, chlorophyll *a* and PDI, was carried out for 210
each salina pond. The distinction between seasons was 211
tested through Analysis of Similarity (ANOSIM) following 212
a 2D Non-Metric Multidimensional Scaling (NMDS) based 213
on standardized and normalized Euclidian distances. 214

215 All statistical tests considered the significance level $\alpha =$
 216 0.05. Multivariate analyses were performed with CANOCO
 217 4.5 (Ter Braak and Smilauer 1998) with the exception of
 218 NMDS and ANOSIM that were carried out in Primer 5
 219 (Clarke and Warwick 2001). All other statistical analyses
 220 were done in R (R Dev. Core Team 2009).

221 Results

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

228 With the exception of pH and Pigment Diversity Index
 229 (PDI), temporal (or seasonal) differences were significant (p
 230 < 0.05) for all of the determined parameters (Tables 1 and 2).
 231 The variation of the studied physicochemical parameters
 232 was more accentuated during the period in which the salina
 233 was active, i.e. producing salt (Table 2; Fig. 2). Next to the
 234 seasonal differences, there were also spatial variations of the
 235 determined parameters, reflecting the distinct conditions that
 236 exist in the different salina ponds (Tables 1 and 2, Figs. 2
 237 and 3). Considering the several parameters individually, it
 238 seems clear that temperature and salinity clearly reached
 239 higher values in all salina sections during the salt production
 240 period. These parameters showed an increasing trend from
 241 the supply to the crystallizer ponds, with particularly high
 242 salinities in the condenser and crystallizer ponds.

243 The pH values were relatively stable during the non-
 244 production seasons, although more variable in the condenser
 245 and crystallizer ponds. During salt production there are
 246 some lower pH values in the supply pond and lower and
 247 more variable pH values in the crystallizer, which are asso-
 248 ciated with very variable alkalinity. Alkalinity was, in gen-
 249 eral, higher during the production season, with significantly
 250 different values between seasons in the condenser pond,
 251 where the highest mean values were achieved. Though
 252 statistically non-significant, alkalinity inside the crystallizer
 253 is much higher during salt production (150 ppt; Table 2)
 254 than in the non-production season (79 ppt; Table 1).

255 Dissolved oxygen (DO) values were lower during the salt
 256 production, with poorly oxygenated water in the different
 257 salina sections. During this season, the condenser exhibited
 258 the highest DO values and the crystallizer the lowest values,
 259 including some situations of anoxia. The BOD₅ values were,
 260 in general, higher during the non-production period, with
 261 quite significant seasonal differences between the condenser
 262 and crystallizer ponds.

263 Nitrate (NO₃) and nitrite (NO₂) concentrations were gen-
 264 erally higher during the non-production season. Seasonal

differences are significant for the supply pond (especial-
 ly in terms of nitrates), which presented very high NO₃
 and NO₂ values during the non-production season. On
 the contrary, the amount of orthophosphate (PO₄) 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 PO₄ values during the produc-
 tion season.

In the evaporators 2 and 3, the condenser and crystallizer,
 the silicate concentration (SiO₄) was higher during salt
 production, whilst in the supply pond and evaporator 1 the
 values were higher during the non-production season. Sea-
 sonal 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 rela-
 tively stable in both seasons. However, during the produc-
 tion period, the crystallizer pond exhibited higher and very
 variable values for both pigment parameters.

The principal components analysis (PCA) of the stud-
 ied parameters measured during salt production (Fig. 3),
 showed that variability between samples was mainly
 characterized by a salinity/PDI and inverse DO/BOD₅
 gradient (on the 1st axis), and by a NO₂/NO₃ and
 inverse SiO₄/PO₄/pH gradient (2nd axis). In terms of
 spatial patterns, samples from the supply pond (SP) and
 samples from the crystallizer pond (CRY) are well sep-
 arated 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 increas-
 ingly 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 $R \geq 0.720$. Compari-
 sons 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 corre-
 spond to the supply pond appear segregated, character-
 ized 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, NO₃ and SiO₄ versus alkalinity and temperature in SP; BOD₅, DO and NO₃ versus PDI in EV1; PO₄, Chl. *a*. and temperature versus DO and BOD₅ in EV2; PO₄, SiO₄ and temperature versus DO and BOD₅ in EV3; SiO₄ versus BOD₅ and DO in CON. In the CRY, the first

axis is characterized by temperature and salinity versus DO and BOD₅ 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 BOD₅ in mg/l; pH at Sorensen scale; Alk.: alkalinity in ppt; NO₂, NO₃, PO₄ and SiO₄ 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 super-scripts (ns: non significant; *: $\alpha=0.05$; **: $\alpha=0.01$; ***: $\alpha=0.001$)

Parameter	Salina section						
	All	SP	EV1	EV2	EV3	CON	CRY
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	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) ^{ns}	32.62 (8.20) ^{ns}	29.16 (7.75) ^{ns}	30.84 (8.87) ^{ns}	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 ₅	5.86 (2.98)***	4.88 (1.40) ^{ns}	4.57 (3.03) ^{ns}	6.04 (3.94) ^{ns}	5.85 (3.66) ^{ns}	7.08 (2.87)*	6.75 (2.60)**
pH	8.12 (0.39) ^{ns}	7.95 (0.48) ^{ns}	7.98 (0.40) ^{ns}	8.15 (0.35) ^{ns}	8.01 (0.17) ^{ns}	8.33 (0.44) ^{ns}	8.29 (0.37)*
Alk.	101.6 (30.2)**	89.1 (32.10) ^{ns}	104.2 (20.30) ^{ns}	110.1 (30.4) ^{ns}	110.0 (10.0) ^{ns}	119.7 (22.10)*	79.0 (40.50) ^{ns}
NO ₂	0.60 (0.67)**	1.47 (0.86)*	0.33 (0.38) ^{ns}	0.20 (0.08) ^{ns}	0.55 (0.64)*	0.52 (0.74) ^{ns}	0.50 (0.30) ^{ns}
NO ₃	6.77 (12.11)*	28.46 (13.75)**	5.02 (9.26) ^{ns}	0.52 (0.66) ^{ns}	0.88 (1.14) ^{ns}	0.98 (0.96) ^{ns}	3.95 (4.30) ^{ns}
PO ₄	0.19 (0.28)***	0.53 (0.46) ^{ns}	0.10 (0.13) ^{ns}	0.10 (0.17) ^{ns}	0.10 (0.15) ^{ns}	0.15 (0.12)*	0.14 (0.16) ^{ns}
SiO ₄	0.63 (0.47)**	1.08 (0.62)*	0.75 (0.46) ^{ns}	0.54 (0.12) ^{ns}	0.57 (0.29)*	0.30 (0.37)**	0.47 (0.54) ^{ns}
Chl. <i>a</i>	7.25 (7.65)*	1.68 (2.36) ^{ns}	5.87 (6.46) ^{ns}	6.14 (6.90) ^{ns}	4.42 (3.38) ^{ns}	9.56 (4.05) ^{ns}	16.14 (11.10) ^{ns}
PDI	4.29 (3.15) ^{ns}	4.03 (3.72) ^{ns}	2.95 (1.50) ^{ns}	7.34 (5.45) ^{ns}	4.39 (1.98) ^{ns}	3.45 (0.48) ^{ns}	3.29 (0.49)*

Discussion 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

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

Table 2 Means and standard deviations (SD) of physicochemical (Temp.: temperature in °C; Sal.: salinity in ppt; DO and BOD₅ in mg/l; pH at Sorensen scale; Alk.: alkalinity in ppt; NO₂, NO₃, PO₄ and SiO₄ 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	SP	EV1	EV2	EV3	CON	CRY
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	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 ₅	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 ₂	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 ₃	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 ₄	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 ₄	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	<i>p</i> -value	R	<i>p</i> -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

t4.1 **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

t4.2	Section	R	<i>p</i> -value
t4.3	SP	0.263	0.040*
t4.4	EV1	0.053	0.279ns
t4.5	EV2	0.265	0.029*
t4.6	EV3	0.410	0.040*
t4.7	CON	0.699	0.002**
t4.8	CRY	0.529	0.003**

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

417 Given that the initial steps of eradication of native *Artemia*
418 from a particular location logically imply a variable
419 period of co-existence with the non-native species, we might
420 rule out any mechanism (either direct or indirect) related
421 with interspecific agonistic interactions that results in *A.*
422 *parthenogenetica* survival and *A. franciscana* eradication.
423 Following Occam’s razor principle, if such a mechanism
424 existed, many more *foci* of native *Artemia* would be appar-
425 ent throughout the Atlantic and Mediterranean coasts. Thus,
426 a distinct set of causes, neither directly related to the sea-
427 sonally fluctuating environment nor to interspecific interac-
428 tions, may be playing a decisive role in the preservation of
429 this native *Artemia* refugium. Although apparently contra-
430 dicting the most obvious common sense, we believe that this
431 set of causes is linked to pollution levels.

432 The presence of pollutants, such as heavy metals in the
433 water that feeds the salina, may play a decisive role in the
434 prevention of the invasion if we consider that different
435 *Artemia* species and strains have been demonstrated to have
436 distinct sensibilities when exposed to the same toxicants
437 (e.g. Bagshaw et al. 1986; MacRae and Pandey 1991; Go
438 et al. 1990; Sarabia et al. 1998, 2002, 2003, 2008). The Ria
439 de Aveiro lagoon is one of the most mercury-contaminated
440 systems in Europe, due to the continuous mercury dis-
441 charges of a chlor-alkali plant, during more than four deca-
442 des (1950–1994), into an inner bay of 2 km² called Laranjo
443 Bay (Pereira et al. 1998). Although the anthropogenic sour-
444 ces of mercury into the aquatic systems have been consid-
445 erably reduced through legislation (Pereira et al. 2009),
446 mercury concentrations in the surface sediments of some
447 areas of the Ria are still higher than pre-industrial levels,
448 namely in the above-cited Laranjo bay (Coelho et al. 2005).
449 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 μ 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 μM 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 μM 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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