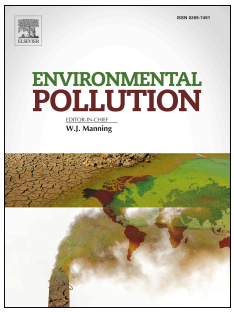

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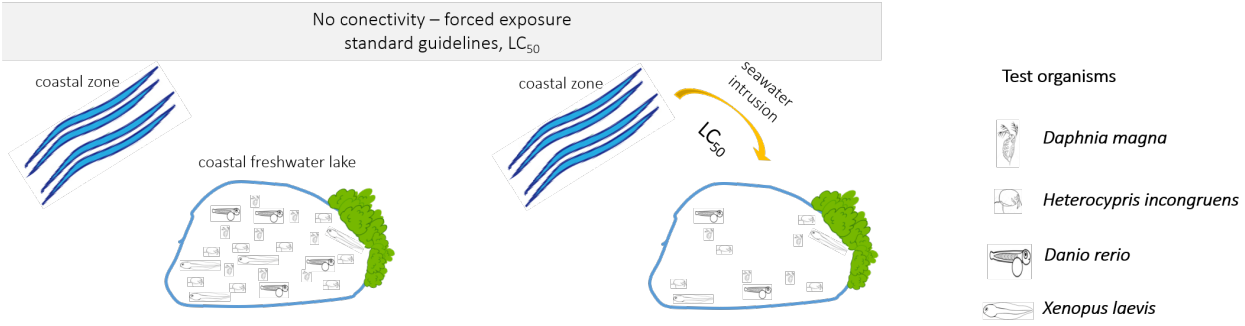

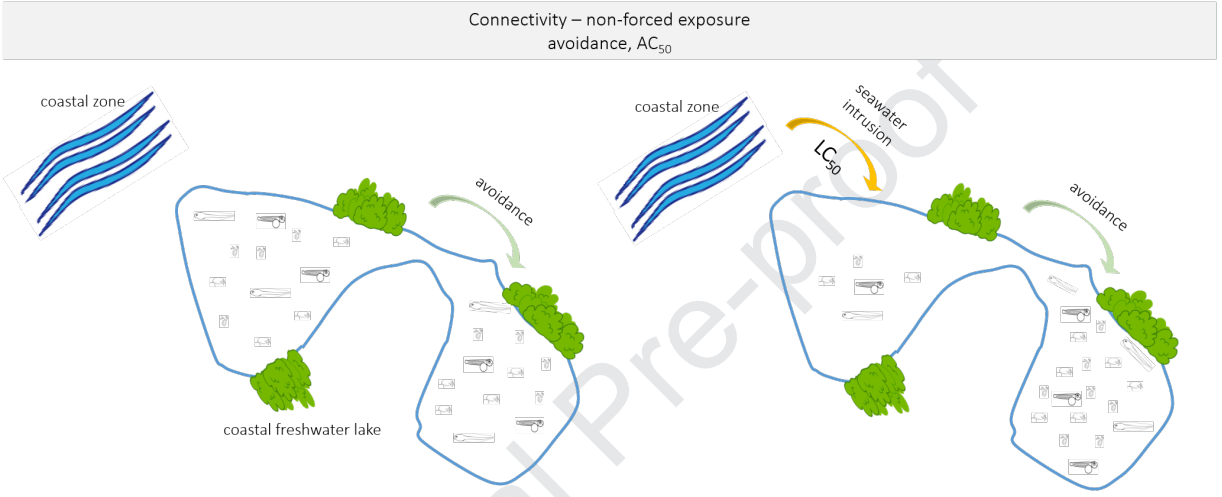

Active emigration from climate change-caused seawater intrusion into freshwater habitats

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Abstract

Ecological risk assessment associated with seawater intrusions has been supported on the determination of lethal/sublethal effects following standard protocols that force exposure neglecting the ability of mobile organisms to spatially avoid salinized environments. Thus, this work aimed at assessing active emigration from climate change-caused seawater intrusion into freshwater habitats. To specific objectives were delineated: first, to compute median 12-h avoidance conductivities ($AC_{50,12h}$) for freshwater species, and second, to compare it with literature data ($LC_{50,48\ or\ 96h}$, $EC_{50,6\ or\ 21d}$) to assess the relevance of the inclusion of stressor-driven emigration into risk assessment frameworks. Four standard test species, representing a broad range of ecological niches – *Daphnia magna*, *Heterocypris incongruens*, *Danio rerio* and *Xenopus laevis* – were selected. The salt NaCl was used as a surrogate of natural seawater to create the saline gradient, which was established in a 7-compartment system.

At each specific $LC_{50,\ 48\ or\ 96h}$, the proportion of avoiders were well above 50%, ranging from 71 to 94%. At each LC_{50}, considering also avoiders, populations would decline by 85 to 97%. Furthermore, for *D. magna* and *X. laevis* it was noticed that at the lowest conductivities eliciting mortality, the avoidance already exceeded 50%.

The results showed that the emigration from salinity-disturbed habitats exists and that can even be more sensitive than standard endpoints. Looking solely to standard endpoints involving forced exposure may greatly underestimate the risk of local population extinction, because habitat function can be severely disrupted, with subsequent stressor-driven emigration, before any adverse physiological effects at the organism level. Thus, the present study highlights the need to include non-forced exposure testing into ecological risk assessment, namely of salinity-menaced costal freshwaters.

Keywords: saline gradient; freshwater ecosystems; habitat disruption; avoidance; habitat function; risk assessment

Capsule: Salinity-driven emigration of considerable proportions of the populations may compromise freshwater habitat function.

Introduction

Grounded on climate change scenarios pictured for the coming years[1], the salinization of coastal freshwater ecosystems, either by superficial overtopping or groundwater intrusion, is an expected consequence. This leads to the degradation of these vulnerable habitats and subsequent severe diversity losses. The prospective effects of salinization usually rely on standard toxicity tests, which determine that organisms are forcedly exposed to the stressor of concern, thus without the option of moving away to less disturbed interconnected habitats. The spatial avoidance behavior from stress is a well-known response expressed by organisms under natural conditions, to exploit more prone areas. Examples are flying or running to escape from predators[2,3]; in estuarine environments organisms constantly move around or burrow to find the most suitable salinity, hypoxia and/or humidity conditions[4,5]; in organisms exhibiting negative phototaxis[6] or even in sessile organisms like plants, that are able to redirect their growth in order to avoid shading by neighbor plants[7]. Regarding ecotoxicology, several works showed that many aquatic species could detect and spatially avoid chemical gradients[8,9], such as snails[10], cladocerans[11,12], fish[13] and tadpoles[14]. However, water quality guidelines and ecological risk assessment frameworks, both prospective and retrospective, assume no risk if no adverse physiological effect is observed when organisms are forcedly exposed to the stressor. Therefore, organisms' ability to emigrate, long before severe suffering, is totally neglected, whilst passivity is assumed[9]. Results of Araújo et al.[14] are one of several very clear examples of the ecological relevance of spatial avoidance. The authors showed, with *Pelophylax perezi* and *Leptodactylus latrans* tadpoles, that a 200 µg L^{-1} copper concentration, which induced only 5% of mortality, was enough to cause a highly significant avoidance response of approximately 80%[14]. Therefore, in real situations of contamination and assuming the presence of interconnectivity among habitats, local population extinction would occur at much lower concentrations than the ones predicted by standard (forced-exposure) toxicity tests.

The lack of standardization of avoidance testing may be one disadvantage for its use. Nevertheless, two strengths can support and appeal to their application. First, the ecological relevance of avoidance is as important as mortality, i.e., if organisms can detect and avoid stressors, then effects at the ecosystem level are similar to those occurring if organisms have died, since in both situations the local population disappears[14,15,16]. Second, avoidance behavior

has been previously reported as an earlier response to stress comparatively to other sub-lethal endpoints, i.e., the median avoidance concentration (AC_{50}) was lower than other median effective concentrations (EC_{50})[14,16].

Results from avoidance tests may also contribute to tackle recovery dynamics of populations in restoring habitats. If organisms can detect and avoid spatially stressed environments, then they can posteriorly return when the habitat starts to recover. Indeed, it is deductible that a downsized population, due to emigration from its disturbed habitat, will regrow, due to immigration, as soon as the stressor level decreases fair enough, as postulated by the avoidance-recolonization theorem[17]. Therefore, studies on avoidance behavior can bring major contributions to understand habitat fragmentation and resilience/recovery of ecosystems under saline stress.

To date and to our knowledge, no information has been generated regarding self-propelled emigration by organisms inhabiting freshwater habitats at risk of seawater intrusion. Accordingly, this work aimed at assessing the effect of climate change-caused seawater intrusion in freshwater populations as a consequence of the emigration of organisms. To attain this main goal, two specific objectives were delineated. First, to compute median avoidance conductivities (AC_{50}) for species belonging to different trophic levels and/or with different life strategies when exposed to a saline gradient. Second, to compare and integrate these avoidance data with literature data for standard endpoints (LC_{50}, EC_{50}), aiming at addressing the relevance of including stressor-driven emigration into risk assessment frameworks. To tackle these objectives, four animal standard test species, representing a broad range of ecological niches, were exposed to a saline gradient: the primary consumers *Daphnia magna* (plankton) and *Heterocypris incongruens* (epibenthos) and the secondary consumers *Danio rerio* (fish) and *Xenopus laevis* (anuran).

Materials and Methods

Chemical

To carry out the avoidance tests, the salt sodium chloride (NaCl; Merck, St Louis, MO, USA) was used. This choice was based on previous works where this specific salt proved to be a suitable surrogate of natural seawater to assess the effects of salinization on freshwater organisms[18,19], since it induces a similar or slightly higher toxicity than natural seawater. Stock solutions of 10 g L^{-1} of NaCl (approximately 20 mS cm^{-1}) were prepared always fresh and by dissolving the salt directly into the medium according to the species. Conductivity (mS cm^{-1}) was used as a comparable measure of salinity between NaCl and seawater. The highest conductivity used for each species was the respective median lethal conductivity, from the literature, which was diluted by a factor of 1.2 to obtain the other conductivity (Table 1).

Avoidance tests

A non-forced exposure 7-compartment system[13] was used to perform the avoidance tests (Fig. 1). All tests were carried out in total darkness, with 125 mL of solution *per* compartment, for a period of 12 h, with different loadings and at different temperatures according to the species (Table 1). Small plasticine plugs wrapped in parafilm were used to close the connections between adjacent compartments prior to solutions and organisms being added. Plugs were then gently removed, to minimize excessive mixture between adjacent compartments. At the end of the tests, plugs were inserted again to allow counting the organisms present at each compartment. A total of five control replicates (culture medium only) and five test replicates (under a saline gradient – Table 1) were performed. Controls aimed at verifying that, in the absence of the contaminant, organisms preferred neither extremity of the system. Therefore, during the tests, any displacement towards one extremity would only be due to the contaminant. To check for the temporal stability of the NaCl gradient inside the system, conductivity was measured at the beginning and after 12 h, in triplicates in tests with no organisms added (Table S1).

Test organisms

Juveniles (4 days old) of *Daphnia magna* (BEAK clone) were obtained from laboratorial cultures of this cladoceran that were maintained under controlled conditions of temperature (20 ºC) and photoperiod (16:8 h L:D) in ASTM hardwater, containing vitamins and organic extract Marinure 25 (derived from the algae *Ascophyllum nodosum*; Pann Britannica Industries, Waltham Abbey, UK)[23]. Medium renewal was performed every 48h and organisms fed daily with the green algae *R. subcapitata* (at a concentration of $3.0x10^5$ cells mL^{-1}).

Six-days old ostracods (*Heterocypris incongruens*) were obtained from dried cysts. Hatching followed the procedures of the Ostracodtoxkit F (MicroBioTests, Ghent, Belgium). They were maintained at 26 ºC, 16:8 h L:D cycle, in ASTM hardwater[24]. Medium was partially renewed every other day and green algae was added as food (*Raphidocelis subcapitata* Korshikov, F. Hindák; formerly known as *Selenastrum capricornutum*), at a concentration of $1.5x10^5$ cells mL^{-1}.

Six-days old larvae of *Danio rerio* were provided by the facility established at the Biology Department (University of Aveiro, Portugal) (please see Domingues et al.[25] for detailed information on the maintenance of the breeding pairs). Briefly, eggs were collected, gently washed and inspected to discard dead and coagulated ones. Every other day, the culture water was renewed, and dead organisms removed. The larvae were not fed since the organisms used in the test were still feeding on the yolk sac.

Eggs from *Xenopus laevis* were obtained through reproduction in captivity (please see Alves, 2015[26] for detailed information on laboratorial procedure for reproduction of this species). Prior to the test and using a stereomicroscope, eggs with body abnormalities were discarded. Until they reached the desired state to be used in the avoidance test (NF 42-43[27]) they were maintained in FETAX medium (American Society for Testing and Materials; ASTM[28]) at 23ºC and 10:14 h light:dark period. Medium was renewed completely every 48 h and dead organisms (if any) were removed.

For both species of vertebrates (*X. laevis* and *D. rerio*), the larval stages used fall outside the scope of animal experimentation since they are not independently feeding forms; i.e., the yolk-sac from which they retrieve energy to develop is still present[27,28].

Data analysis and Calculations

The validity of the controls (no contaminant added) was checked with Spearman's correlation coefficients. Real conductivities during exposure (average between initial and final values in each compartment) never differed by more than 10% relatively to the nominal values, with two exceptions out of 24 (11 and 21%) (Table S1). These two exceptions occurred in compartments with low conductivities (most probably due to high differences in osmotic pressure) where both mortality and avoidance were negligible. Therefore, nominal values were used in all calculations.

The % of avoidance at each NaCl conductivity was calculated considering the number of avoiders as the difference between the number of expected organisms in each compartment (Ne) and the number of organisms observed in each compartment (No). Therefore, Avoiders=Ne-No and % of Avoidance=(Avoiders/Ne)×100. The calculation of Ne followed the procedure described in Moreira-Santos et al.[16]: for the highest conductivity compartment, it is considered that Ne is equal to the number of organisms introduced at the beginning of the assay; whilst, for the remaining compartments, the Ne includes the number of organisms initially included plus the organisms that are expected from the adjacent(s) compartment(s). As an example, in an assay where initially were introduced 3 organisms/compartment, in the highest conductivity compartment the Ne is 3; for the adjacent compartment the Ne is 6, and so on; for the least contaminated compartment the Ne is 21. This computation method was used instead of the refined formulae laid down in the recently published Standard Operating Procedure (SOP)[17] because test duration was only of 12 hours and some species (mainly ostracods and cladocerans) have a limited mobility. However, this latter option may lead to a slight overestimation of avoidance mainly by the most mobile species (tadpoles and fish). Contrarily, SOP formulae could lead to a slight underestimation of avoidance by ostracods and cladocerans. The calculation of the % of avoidance for each tested conductivity allowed to compute the median

avoidance conductivity (AC_{50}) and respective confidence limits at 95% (CL at 95%) through the probit transformation[29].

The proportion of the population that can disappear is only perceived when we integrate the avoidance and mortality percentages. This endpoint is denominated as Population Immediate Decline (PID) and was computed through equation 1[12]:

$x = (1 - (1 - y/100) \times (1 - w/100)) \times 100$, Equation 1.

x being the percentage of PID, y the mortality (in %) and w the avoidance (in %). The Equation 1 can be decomposed in the following elements:

$(1 - y/100)$ is the proportion of organisms that do not die

$(1 - w/100)$ is the proportion of organisms that do not escape

$(1 - y/100) \times (1 - w/100))$ is the proportion of organisms that do not die and do not escape

$(1 - (1 - y/100) \times (1 - w/100))$ is the remaining proportion of organisms: those that died or escaped.

Percentages of mortality and of PID were calculated for all tested conductivities because raw data from all mortality tests, except fish, were obtained from the respective authors (Table 1).

Results

All four avoidance tests were valid because, in the absence of NaCl (controls), organisms did not move preferentially to one of the extremities ($r_s \leq |0.02|$, $p>0.05$, $n=35$). Sodium chloride elicited avoidance by the four tested species (Fig. 2), with no mortality during the 12-hours exposure periods. Even though avoidance tests lasted for only 12 h, at the condcutivity of NaCl causing a 50% of mortality in 2- to 4-days long forced exposure tests, the proportion of avoiders largely surpassed 50% (from 71 to 94%, Fig. 2) in all tested species. The AC_{50} values were attained at conductivities 64 to 76% lower than the respective LC_{50} values (Table 2). Worth noting, the cladoceran *D. magna* and the anuran *X. laevis* avoided the lowest level of salinity and, at the lowest conductivities eliciting mortality, avoidance already exceeded 50% (Fig. 2b, 2c). For these two species and also *D. rerio*, taking only into account the LC_{50} would severely underestimate the probability of local population extinction because the respective Population Immediate Decline was 95% or higher (Fig. 2).

Discussion

This research intended to appraise the need to include non-standard ecotoxicological approaches (tests with non-forced exposure allowing active migration) in ecological risk assessment frameworks, by presenting, as far as we are aware of, first data regarding specifically scenarios of seawater intrusion into freshwater systems. So far, avoidance studies

with freshwater organisms already tackled many contaminants[8,9], though avoidance along a saline gradient was lacking. In the present work, all selected species presented a median avoidance NaCl conductivity ($AC_{50,12h}$) clearly lower than the standard endpoint of mortality ($LC_{50,48\ or\ 96h}$), with the AC_{50}/LC_{50} ratio varying from 64 to 76% (Table 2). Considering the proportion of both avoiders and dead, at each specific median lethal conductivity, resulted in a population decrease by 85 to 97%. Furthermore, it should be emphasized that if avoidance tests lasted as long as mortality tests, then this ratio could be even lower. This means that, if the possibility of emigration to less disturbed habitats exists, then standard mortality tests underestimate the risk of local populations' extinction, in the short-term, due to salinization. This is inline with previous published works showing this trend for fungicides[13,30], herbicides[31], copper[11,14,16], mixture of metals[10], acid mine drainage[16], pulp mill effluents[32], and other[9]. Moreover, short-term (12 hours) avoidance by *D. magna* was even more sensitive than long-term (21 days) reproduction inhibition after 21 days, with an AC_{50}/EC_{50} ratio of 71% (with no 95% confidence limits overlapping; Table 2), meaning that ignoring salinity-driven emigration by looking solely at physiological sub-lethal endpoints may still result in an ecological risk underestimation. The opposite happened with the ostracod *H. incongruens* with the highest ratio AC_{50}/EC_{50}; 6-long growth inhibition being more sensitive than avoidance (with no 95% confidence limits overlapping; Table 2). The high value of this ratio was due to a relatively low value of the EC_{50} and not to a high value of the AC_{50}. No empirical anticipation was possible since no data were ever published on the latter response by ostracods, as far as we are aware. Although this ostracod species being endowed with swimming silks that aid in locomotion[33], its small size and its preference for inhabiting the sediment surface and/or burrowing in the upper layer could restrict its dispersal capacity and, therefore, restrain its avoidance intensity[34].

Mild seawater intrusion events (~10% v/v; seawater conductivity ≈ 52 mS cm^{-1}), increasing the salinity freshwater habitats to only half of LC_{50} values (that ranged from 9.98 to 20.6 mS cm^{-1}), would not be lethal at all to *D. magna* (Fig. 2b) and *X. laevis* (at Fig. 2c), and only slightly lethal to *H. incongruens* (Fig. 2a), but avoidance would already be noticeable. Regarding the former two species, cladocerans and fish, at the lowest salinities causing expressive mortality (>10%), clearly more than half of organisms already moved away to less salty habitats. Therefore, the present study reinforces previous findings that even non-lethal concentrations of chemicals can trigger very adverse effects and interfere with the ecosystem as a whole[8,9,17]. Contaminants/stressors cannot be regarded solely as potential poisons causing lethal or sublethal effects at the individual level, but also as habitat disturbers[8]. Non-forced exposure testing allows appraising the habitat function (i.e., its ability to sustain the biological diversity). Furthermore, just as important changes in the ecosystem from which organisms escape can occur, contamination driven migration will certainly trigger also important changes in the ecosystems into which organisms move, namely at intra and inter-specific relationships[9].

Here again, a fundamental key point emerges that would help in more appropriate risk derivation frameworks. However, these extrapolations and forecasts are, as with all results from ecotoxicity tests, oversimplified since organisms' behavior is also influenced by many other factors besides the contaminant/stressor itself. For instance, when exposed to a dilution gradient of a plant treatment effluent, tilapia fish were able to actively avoid contamination, moving to the lowest contamination compartments; but when food was provided only where contamination was present, the avoidance behavior pattern previously registered was altered, with fish intermittently visiting the most contaminated compartments[35].

A major challenge to a widespread use of non-forced exposure testing is, in our opinion, its standardization. Besides the physical characteristics of the testing system, also life stages of organisms to be used need to be consensually accepted. Organisms age selection and inclusion of this information in materials and methods description is a fundamental step. This because the selection of age, despite the organism, might influence their response towards salinity, which might difficult the interpretation and/or comparison of these results with future data sets. For instance, in the present study, the larval stages of both vertebrate species were selected to maximize sensitivity[36,37,38] but guaranteeing mobility and, thus, responsiveness.

Conclusions

Ecological risk assessment or water quality criteria frameworks are based on standard methodologies where forced exposure is mandatory. Findings of the present work are a major contribution in what concerns appropriate risk derivation for freshwater coastal ecosystems at risk of salinization as they are (to our knowledge) the first results on active avoidance behavior of freshwater species when confronted with a saline gradient. The results reveal that even small saline intrusions may compromise habitat function leading to the emigration of considerable proportions of the populations inhabiting these freshwater systems. Considering the results obtained, severe habitat disruptions in freshwater ecosystems at risk of salinization are expected.

Moreover, avoidance responses, currently viewed as a non-mandatory or simple complement to standard guidelines, should be evaluated and considered in the near future as a mandatory standard approach, broadening the traditional prospective site-specific risk assessment a to prospective risk assessment at the landscape level.

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Additional Information

Authors Contribution

Venâncio C. wrote the paper, prepared and executed the experiments and analyzed the data. Ribeiro R. and Lopes I. conceived and designed the analysis. All authors edited the manuscript, contributed intellectual expertise and approved its submission.

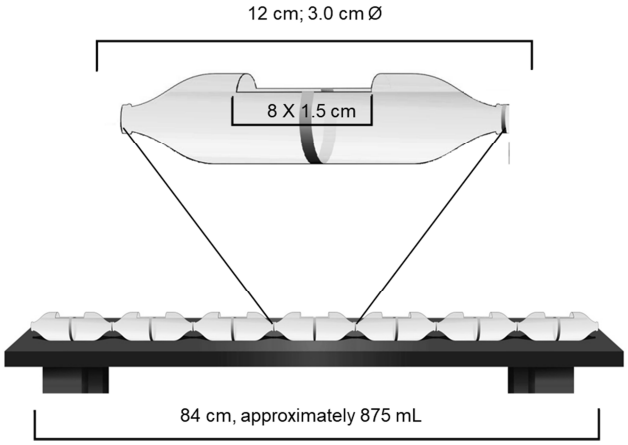
Competing interest

The authors declare no competing interests.

Data Availability

Correspondence and requests for materials should be addressed to catiavenancio@gmail.com.

Figures and Tables



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412 Figure 1: Schematic representation of the non-forced exposure 7-compartment avoidance system (adapted from
413 Araújo et al.[14]).

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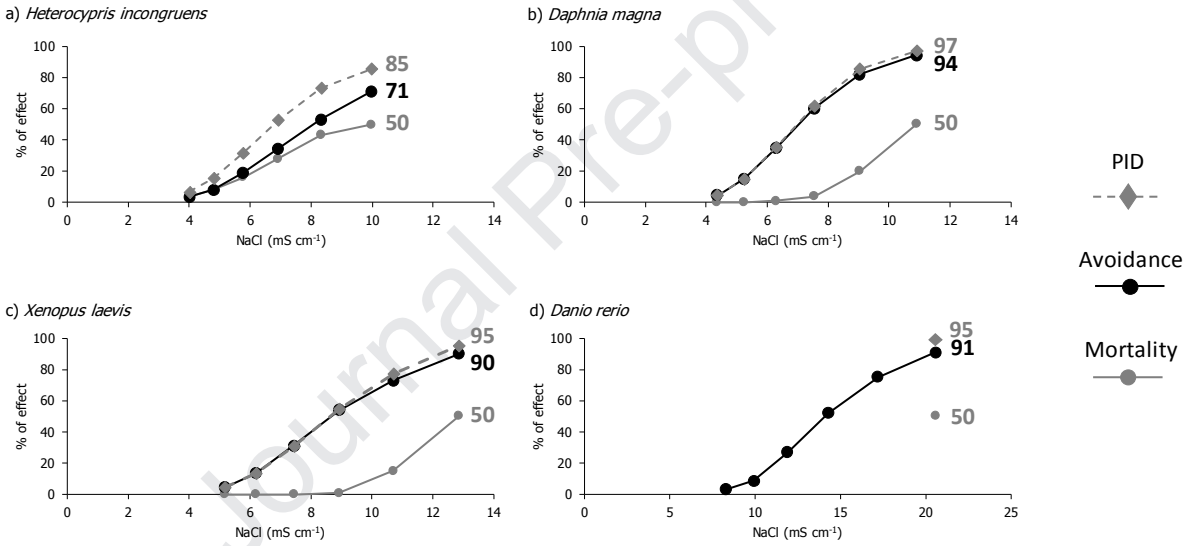

416

417 Figure 2: Effects of sodium chloride on mortality, avoidance and Population Immediate Decline (PID_x) of the four
418 tested species. For *Danio rerio*, only the median lethal conductivity and the respective PID could be displayed in the
419 graph since no further information was available.

420

Table 2: Comparison of the median lethal or effective conductivity after days (d) or hours (h) of exposure ($LC_{50,x}$ or $EC_{50,x}$; in mS cm^{-1} and corresponding concentration to g L^{-1}) to sodium chloride (NaCl) with the median avoidance conductivity (AC_{50}) after 12 h of exposure to NaCl. The Ratio AC_{50}/LC_{50} gives indication on how much further apart the endpoints are. CL 95% corresponds to the confidence limits at 95%. N.d. – not determined.

	NaCl (CL 95%)		**AC_{50} (CL 95%)**		Ratio
	mS cm^{-1}	g L^{-1}	mS cm^{-1}	g L^{-1}	AC_{50} (mS cm^{-1}) / $L(E)C_{50}$ (mS cm^{-1})
Daphnia magna	$LC_{50,48h}$ @ 20ºC: **10.9** (10.7 – 12.9)	5.50 (5.40 – 6.50)	@ 20ºC:	3.53 (2.97 – 4.42)	0.64
(Cladocera)	$EC_{50,21d}$ (reproduction) @ 20ºC: **9.90** (9.70 – 10.1)	5.00 (4.90 – 5.10)	**6.99** (5.89 – 8.75)		0.71
Heterocypris incongruens	$LC_{50,48h}$ @ 26ºC: **9.98** (8.91 -11.1)	5.04 (4.50 – 5.59)	@ 26ºC:	3.85 (3.07 – 9.59)	0.76
(Ostracoda)	$EC_{50,6d}$ (somatic growth) @ 26ºC: **4.86** (4.38 – 5.34)	2.45 (2.21 – 2.70)	**7.62** (6.09 – 19.0)		1.57
Danio rerio (Cypriniformes)	$LC_{50,96h}$ @ 28ºC: **20.6** (n.d.)	10.4 (n.d.)	@ 28ºC: **14.2** (13.4 – 15.2)	7.17 (6.77 – 7.68)	0.69
Xenopus laevis (Anura)	$LC_{50,96h}$ @ 23ºC: **12.8** (11.3 – 17.7)	6.49 (5.68 – 8.94)	@ 23ºC: **8.91** (8.51 – 9.54)	4.50 (4.30 – 4.82)	0.70

Table 1: Temperature (ºC – equal in the avoidance and the lethal tests, from the literature), number of organisms per compartment and age of the organisms used in the avoidance tests with sodium chloride. All avoidance tests were run for 12 h, in total darkness. The $LC_{50,h}$ indicates the median lethal conductivity after *h* hours of exposure, from the literature. CTR – control.

Species	**Temp** (ºC)	**Organisms/compartment** **Age of organisms**	**$LC_{50,h}$** (mS cm^{-1})	**Saline Gradient** Dilution factor x1.2 (mS cm^{-1})	**Reference**
Daphnia magna (Cladocera)	20	3 4-days old	48h: 10.9	CTR, 4.38, 5.26, 6.31, 7.57, 9.08, 10.9	Gonçalves et al., 2007[24]
Heterocypris incongruens (Ostracoda)	26	7 6-days old	48h: 9.98	CTR, 4.01, 4.81, 5.77, 6.93, 8.31, 9.98	Venâncio et al., 2018[22]
Danio rerio (Cypriniformes)	28	5 6-days old	96h: 20.6	CTR, 8.25, 9.9, 11.9, 14.3, 17.1, 20.6	Doleželová et al., 2009[25]
Xenopus laevis (Anura)	23	3 NF stage 42-43	96h: 12.8	CTR, 5.17, 6.20, 7.44, 8.93, 10.7, 12.8	Gabriel, 2016[26]

Highlights

- ✓ Freshwater ostracods, cladocerans, tadpoles, and fish larvae spatially avoided salinity.
- ✓ Median avoidance conductivities ($AC_{50,12h}$) were 64 to 76% the standard $LC_{50,48\ or\ 96h}$.
- ✓ At each LC_{50}, considering also avoiders, populations would decline by 85 to 97%.
- ✓ Standard tests underestimate ecological risk of seawater intrusion into freshwaters.

Additional Information

Authors Contribution

Venâncio C. wrote the paper, prepared and executed the experiments and analyzed the data. Ribeiro R. and Lopes I. conceived and designed the analysis. All authors contributed on the writing and editing the manuscript, contributed on intellectual expertise and approved its submission.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: