DEPARTAMENTO DAS CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA

UNIVERSIDADE DE COIMBRA

Is contaminant-driven avoidance independent of distance? An experimental demonstration of the spacelessness theorem at a realistic scale

David Martins Acúrcio

2014

DEPARTAMENTO DAS CIÊNCIAS DA VIDA

FACULDADE DE CIÊNCIAS E TECNOLOGIA

UNIVERSIDADE DE COIMBRA

Is contaminant-driven avoidance independent of distance? An experimental demonstration of the spacelessness theorem at a realistic scale

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob a orientação científica do Professor Doutor Rui Ribeiro (Universidade de Coimbra) e do Doutor Cristiano Araújo (Universidad Laica Eloy Alfaro de Manabí, Manta, Ecuador).

David Martins Acúrcio

2014

Agradecimentos

Em primeiro lugar gostaria de agradecer ao Doutor Cristiano Araújo pelos ensinamentos que me transmitiu e que permitiram que eu chegasse ao fim desta etapa tão difícil da minha vida académica. Por ser um orientador extraordinário, foi a luz ao fundo do túnel pela qual me guiei durante toda esta dissertação, incentivando, apoiando e moralizando, nunca deixando que me sentisse de algum modo sozinho. Mesmo não estando presente pessoalmente durante toda esta etapa, a sua ajuda nunca me faltou em qualquer situação. É um investigador fantástico, que tenho como exemplo.

Ao Professor Doutor Rui Ribeiro, por me ter aberto as portas para o mundo da investigação cientifica, orientando e aconselhando-me nesta viagem pelo mundo da ciência.

À Doutora Matilde Moreira Santos, pelas suas correcções, ensinamentos preciosos que permitiram que eu pudesse chegar ao fim desta etapa.

A todo o IMAR, que foi durante algum tempo uma primeira casa, em especial à Vânia Correia pela ajuda na pesca dos peixes para o ensaio, e à Gabi por conseguir pôr-me um sorriso nos momentos mais difíceis.

Ao Museu da Ciência da Universidade de Coimbra, pela crucial cedência de uma das suas galerias durante mais de uma semana, em especial ao seu director, Professor Paulo Gama Mota e à sua colaboradora Dra. Cristina Rufino.

Aos meus colegas pelo espírito de ajuda e camaradagem durante estes dois anos. Foi um orgulho partilhar estes dois anos com vocês.

Aos meus amigos por todo o apoio e compreensão nas alturas em que estive mais ausente.

Aos meus pais e irmã o meu muito obrigado pelos sacrifícios que fizeram para me darem a oportunidade e o privilégio de ter uma educação superior. Sem vocês nada disto seria possível.

Por último, mas nunca a menos importante, à minha namorada, Joana Gomes, pelo apoio incansável durante todo este percurso. Se cheguei ao fim desta etapa, em parte deve-se muito a ti.

A todos vocês, o meu mais humilde e sincero obrigado. Esta tese tem um bocadinho de todos vós.

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.

Sê todo em cada coisa. Põe quanto és
No mínimo que fazes

Assim em cada lago a lua toda
Brilha, porque alta vive

Ricardo Reis, in “Odes”

Index

Index

Abstract

Abstract

The detection of an avoidance response in several aquatic organisms led to an outdating of the majority of ecotoxicology and risk assessment aquatic assays standardized. Once standardized tests expose organisms to a contaminant in a confined compartment, the results are underestimated. On the other hand avoidance tests are generally performed in multi-compartmented small systems with a linear gradient of contamination. Even though these tests reproduce more reliably the contamination gradients that can occur in natural aquatic systems, they could lack ecologic relevance due to small spatial scale at which tests are performed, existing some uncertainties associated to results extrapolation. The goal of this study was to develop a realistic scale non-forced system with 30-meters, exposing, for 12h, the small fish *Danio rerio* to an acid mine drainage (AMD) gradient and to a non contaminated gradient (control) , expecting that AC_{50} values are equal in small and scaled-up system, thus demonstrating that avoidance intense is spaceless. Each 30-m system, with seventy compartments, was constructed gluing ten 3-m systems. In the absence of contamination, organisms were distributed uniformly along the compartments in both systems. *D. rerio* showed significant avoidance to AMD, being the mean AC_{50} 0.586 mg/L of AMD to 3-m system and 0.644 for 30-m system, confirming the logically deducted prediction/hypothesis – the spacelessness theorem. Therefore, avoidance assays are recommended in first tier levels in ecotoxicology and risk assessment, because they are more realistic, quickly and ethical than others tests.

Keywords

Avoidance, *Danio rerio*, AMD, 30-m system, Spacelessness theorem

Resumo

Resumo

A observação de uma resposta de evitamento por parte de um elevado número de organismos aquáticos levou a uma desactualização dos testes padronizados em ecotoxicologia e avaliação de risco. A exposição dos organismos confinada a um contaminante leva a uma subestimação dos resultados provenientes destes testes. Por outro lado, a maioria dos testes de evitamento são realizados em pequenos sistemas multi-compartimentados, existindo um gradiente linear de contaminação. Apesar de estes testes reproduzirem com maior segurança o gradiente de contaminação que ocorre nos ambientes aquáticos da vida real, eles podem ter alguma falta de relevância ecológica devido à diminuída escala a que os testes são realizados existindo assim algumas incertezas na extrapolação dos resultados. O objectivo deste trabalho foi desenvolver um sistema realístico de contaminação não forçado com 30 metros, expondo por 12 horas peixes da espécie *Danio rerio* a um gradiente de drenagem ácida de uma mina (AMD) e a um meio não contaminado (controlo), esperando que a concentração que causa o evitamento de 50 % da população (AC_{50}) seja igual ao AC_{50} de um sistema igual mas de menor escala (3 metros) e concluindo que a resposta de evitamento é independente da distância. Cada sistema de 30 metros, com setenta compartimentos, foi construído através da colagem de dez sistemas de 3-metros. Na ausência de contaminação, os organismos apresentaram uma distribuição uniforme ao longo dos compartimentos em ambos os sistemas. *D. rerio* exibiu uma resposta de evitamento significativa face à contaminação com AMD, sendo a média da AC_{50} 0,586 mg/L para o sistema de 3-metros e 0,644 mg/L para o sistema de 30-metros, confirmando assim a hipótese deduzida logicamente – spacelessness theorem. Assim sendo, os testes de evitamento devem ser utilizados nas avaliações primárias de ecotoxicologia e de avaliação de risco, porque estes dispõem de um carácter mais realísticos, rápido e ético face aos testes padronizados.

Palavras-chave

Evitamento, *Danio rerio*, AMD, Sistema 30-m, Spacelessness Theorem

Introduction

Introduction

The avoidance concept is the act or practice of keeping away from or withdrawing from something undesirable, which in ecological context refers to temporally or spatially escaping from stressful conditions. Avoidance can be triggered by biotic factors e.g. presence of predators and competitors (Manríquez et al., 2014) or abiotic factors such as hypoxia and light (Pankhurst et al., 2013; Wang et al., 2014). In an ecotoxicological approach, some studies have shown that organisms can detect the presence of contaminants and spatially avoid them, escaping to most favorable areas (McNicol et al., 1993; Kravitz et al., 1999; Exley, 2000; Moreira-Santos et al., 2008; Rosa et al., 2012). Therefore, potential contamination harmful effects are underrated by the majority of laboratory studies because organisms are forcedly exposed to contamination, with no possibility to emigrate, which is probable in natural areas. In this way, if natural populations are able to escape from contaminated sites and avoid suffering lethal effects or even sub lethal effects, traditional toxicity tests lack realism and relevance (Dornfeld et al., 2009). Moreover, since spatial avoidance leads to population decline and, eventually, extinction, it can only be regarded as ecologically relevant as mortality, both leading to short term population decline (Lopes et al., 2004; Rosa et al., 2012; Ribeiro et al., 2013; Araújo et al., 2014a).

The higher sensitive shown by avoidance as a toxicity endpoint compared to lethality or and, frequently, to other sub lethal responses has already been demonstrated by some authors (Dornfeld et al., 2009; Rosa et al., 2012). Avoidance to contaminants has been observed in a large number of aquatic organisms, e.g. midge larvae, tadpoles, crustaceans, small and big fish (Wentsel et al., 1997; Araújo et al., 2014a,b; Lopes et al., 2004; De Lange et al., 2006; Vardhanan et al., 2002; Woodward et al., 1995; Henry et al., 2001) and the inclusion of the avoidance tests in risk assessment has been endorsed (Lopes et al., 2004; Moreira-Santos et al., 2008; Schafers et al., 2007). Although potentially being very relevant, contaminant-driven avoidance by small fish has scarcely been studied (Steele et al., 1990; Steele et al., 2004). Small fish exhibit a high range of advantages in toxicity studies, owing to their relevant trophic chain position in the aquatic environment (Cabral et al., 1998) and their easier handling, shorter life cycle, and less laboratory space requirements than large fish (Moreira-Santos et al., 2008). Regarding the avoidance response to contamination, zebra fish *Danio rerio*, when exposed to a gradient of copper and acid mine drainage, was able to detect and escape to lower concentrations (Moreira-Santos et al., 2008). Similarly, it was shown that fries of

D. rerio avoided the fungicide pyrimethanil before any lethal effects were recorded (Araújo et al., 2014a).

Undoubtedly, non-forced avoidance assays reproduce more reliably the contamination gradients that can occur in natural aquatic systems. However, they could lack ecological relevance due to the small spatial scale at which tests are performed. To evaluate the uncertainty associated to extrapolations along a spatial scale, results from realistically large contamination gradients need to be compared with those from easy-to-use small systems. Theoretically, avoidance intensity should be spaceless – unaffected by or independent of distance – though not necessarily timeless, depending on the dispersal rate of each given species. This is so because, unless acclimation occurs, the proportion of avoiders – those detecting and escaping a stressor– is expected to be constant, through time, though the time to perceive and move away could be space-correlated. A first experimental demonstration of this logically deducted prediction/hypothesis – the spacelessness theorem – was provided by Rosa et al. (2012). The aim of that study was to compare AC_{50} (the concentration that causes avoidance by 50% of the exposed population) values of *Daphnia magna*, subjected to a dilution gradient of 18, 35, 70, 140, and 280 mg/L of atrazine, between a 1.1-m long system and a scaled-up three times longer. They concluded that AC_{50}, were similar- 49 mg/L for the 1-m and 41 mg/L for the 3-m long system. Nevertheless, this scaled up 3-m long system still kept a high lack of spatial realism unless a very abrupt contamination gradient occurs in nature. Therefore, the goal of the present study was to confirm that AC_x values derived from both a realistically large and an easy-to-use small system are, as expected, equal. Hence, small fish were exposed to a gradient of acid mine drainage (AMD) in a multi-compartmented avoidance assay system (Rosa et al. 2012) of two different sizes differing by one order of magnitude: 3 and 30-m long. This acid mine drainage gradient was chosen taking into consideration the study made by Moreira-Santos et al. (2008), using an identical AMD, where an AC_{50} for *Danio rerio* of around 1-2.5% of AMD was found. in a gradient of approximately 0/0.2 to 3%. Thus, the latter simulated the least contaminated extremity of a 1-Km long mixing zone, from 100 to 0% of AMD. The AMD was taken from the São Domingos mine (Southeast Portugal which is an abandoned open cast mine with serious environmental problems (Pereira et al., 2004). The AMD results from the continuous oxidation of the abandoned mine tailings, being characterized by its acidity (pH±2) and high dissolved metals concentrations (Fe, Al, Zn, Cu, Mn, Co, Ni, Cd, Pb, Cr), which cause serious impacts on the receiving aquatic system - The Chança reservoir (Ribeiro et al., 2012). The 1-Km AMD mixing zone simulated in the present study is, according to Pereira et al. (2004) and to Ribeiro et al. (2012), a spatially realistic scenario of contamination at the Chança River reservoir.

Materials and Methods

Test organisms

Individuals of *D. rerio* were provided by a commercial supplier and progressively acclimated in glass aquaria, filled with 50 L of dechlorinated tap water continuously aerated. Bearing in mind the welfare of the organisms under study and the European Union Directive 2010/53 on the protection of animals used for scientific purposes (EU 2010), glass aquaria were enriched with pebbles, dark walls and was located in a quiet place, isolated from noise and movement. Organisms were maintained in a climate room at 19 to 21ºC under an 8:16-h light:dark regime, for at least three weeks before use. During acclimation, fish were fed daily, ad libitum, with fish flake food (Tetramin; TetraWerk, Melle, Germany). All procedures with fish were conducted with maximum care to minimize their suffering, in order to comply to the European legislation in effect and to the three principles that encompasses it: replacement, reduction and refinement (EU 2010). All tested individuals were nonspawning fish with a mean (±standard deviation [SD]) standard length of 2.5 (±0.3, n=15) cm and body weight of 0.22 (±0.015, n=15) g. Fish were not fed for 24 h prior to avoidance tests.

AMD

Samples were collected, transported and stored at 4ºC till be used. The pH at the collection time was 1.93 and the conductivity was 4,700 µS cm^{-1}. Metal concentrations were determined by graphite furnace atomic absorption (Cd, Co, Ni, and Pb), by inductively coupled plasma atomic emission (method 3120; APHA, AWA, WPCF, 1995) (Al, Cu, Fe, Mn, and Zn), and by hydride generation atomic absorption (method 3113 B; APHA, AWA, WPCF, 1995) (As), in a certified laboratory (Instituto Superior Técnico, Lisbon, Portugal) (Sobral et al., 2013). Chemical data are described in Table 1. As with conductivity (Moreira-Santos et al., 2008), a strong relationship was found between AMD dilution and pH (r^2=99.1%) and, therefore, pH values were used to indirectly measure actual AMD concentrations.

Table 1- Total metal concentrations (mg L^{-1}) of the acid mine drainage (AMD) used in the avoidance tests.

AMD (%)	Metal (mg L^{-1})									
	Al	**Fe**	**Cu**	**Mn**	**Zn**	**Co**	**Ni**	**Cd**	**Pb**	**As**
100	440	353	41	28	21	2.5	0,800	0.31	0.24	0.0015
1	4.7	3.3	0.41	0.28	0.22	0.024	0.007	<0.001	<0.003	<0.001
0.1	0.45	0.3	<0.05	0.028	<0.050	0.003	<0.005	<0.001	<0.003	<0.001

3.0-m long system

A multi-compartmented system (Fig 1) developed by Araújo et al. (2014a) based on Rosa et al. (2012), with a total length of 3 m and a total volume of 4.2 L was constructed and replicated.

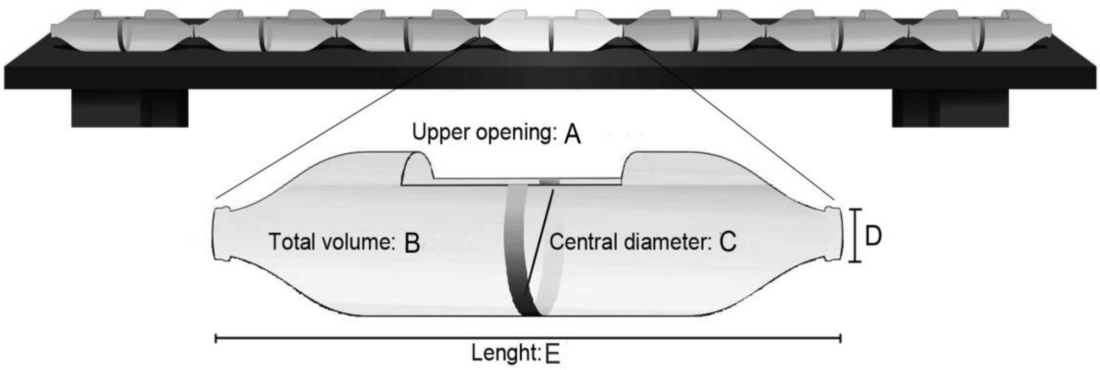

Fig.1: Representation of a multi-compartmented system, emphasizing one of the seven compartments. The measures of the compartment were: A = 8x14 cm, B = 650 mL, C = 6 cm, D= 2 cm and E = 40 cm.

The systems had seven compartments, which were obtained athwart from two plastic bottles, the basis were cut out and glued with polyurethane white glue (Sikaflex-11 FC, Baar, Switzerland). In order to achieve a 3-m system, each compartment was adjoined by gluing the mouth of the glued bottles, getting each compartment 40 cm of length by 6 cm of width and a volume of 650 mL. An opening was made in the surface, 14 cm length x 8 cm of width, so as to be able to poure the dilutions and the organisms into the system.

30-m long system

A scaled-up system, similar to previously presented but with 70 compartments, totaling a length of 30-m and capacity of 42 L, was built. Each compartment of this scaled-up system was equal to those of the 3-m long system.

Avoidance assays

Avoidance assays using the scaled-up system were always performed at the same time as the control, each system had, thus, one replicate. The assays were repeated twice for the 30-m system and three times for the 3-m system. The control systems, containing only culture water, aimed at verifying the absence of mortality and the presence of a random distribution of the organisms, with no preference for any compartment, in the absence of contamination. The avoidance tests with AMD, in the small system, were carried out with seven AMD concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%), which were poured in the compartments after isolating each from each other with spherical corks made of plasticine wrapped in parafilm. Then, the removal of the corks created a linear gradient of contamination. Since the 30-m system has 70 compartments, the same seven AMD concentrations plus 26 intermediate concentrations were prepared. The 0% concentration was placed in the first six compartments (#1 to #6) and the 3.0% concentration in the last six compartments (#65 to #70). The remaining concentrations were placed in two adjacent compartments each, with the concentrations 0.5, 1.0, 1.5, 2.0, and 2.5% being placed the compartments #15and #16, #25and #26, #35 and #36, #45 and #46, and #55 and #56, respectively. The number of fish used in each system was equal. In the small system, two fish were placed in each compartment, totaling 14 fish. Similarly, 14 fish were used in the scaled-up system, which were inserted, in pairs, in the compartments with the same AMD concentrations than in the small system (#1, #15, #25, #35, #46, #56 and #70). Organisms were never re-used. Assays lasted 12 hours in the dark at the temperature of 26 (±0.5) ºC. The initial AMD gradient (concentration varying from 0 to 3%) was very similar to the final ones, after 12 h, both in the small systems (from 0.55 to 2.78%) and in the scaled-up system (from 0.35 to 3%).

Data Analysis

The random distribution of the organisms in the control assays was assessed using the Chi^2 test comparing the number of expected *vs.* observed organisms in each compartment. For the avoidance tests with AMD, the procedure to calculate the number of avoiders per compartment was the same as that used by Araújo et al. (2014a,b). Avoiders were calculated as the number of expected organisms minus the number of the observed organisms. The expected number of organisms was determined from the exposed organisms (those introduced in a given compartment) plus potential immigrants (organisms introduced initially in adjacent compartments with higher concentrations). For the highest concentration, immigrant organisms were not expected, so the number of expected organisms was equal to the number of organisms initially introduced in that concentration. The avoidance percentage in each compartment was determined as the number of avoiders divided by the expected ones. Avoidance concentration of AMD that causes 20 and 50% of response and the respective 95% confidence limits, AC20 and AC_{50}, respectively, were calculated through a probit analysis, using the software PriProbit (Ver 1.63, 2000; Sakuma, 1998) and the AC_s differences between systems were compared using a t-test. The software STATISTICA 7.0 was used.

Results

Results

Avoidance response

In the absence of contamination, organisms presented a random distribution in both systems. No significant statistical difference was found (Chi^2 test, $p>0.05$) among the organisms observed in each compartment. No mortality was found in any of the controls.

Percentages of avoidance for the small system and for the scaled up system are presented in Figs 2 and 3, respectively, and AC_{50} and AC_{20} values in table 3. For both systems, organisms were able to avoid all concentrations of AMD, and that response was concentration-dependent. From the concentration of 1% of AMD the avoidance response was 100%. No mortality was recorded in the small system; however, in the scaled-up system, 1 and 3 organisms were found dead in assay #1 and #2, respectively. The AC_{50} values did not differ by more than 1.06 and 2.02 fold in the small and scaled-up system, respectively. Ascertaining the AC_{50} means (n=3 and n=2 for 3-m and 30-m systems, respectively) for the both systems, the difference between the two systems was merely 1.12 fold, which statistical analyzed, not represent a significant difference (ρ =0.74). Just as AC50, the AC_{20} values were similar in both systems, differing in average 1.58 fold.

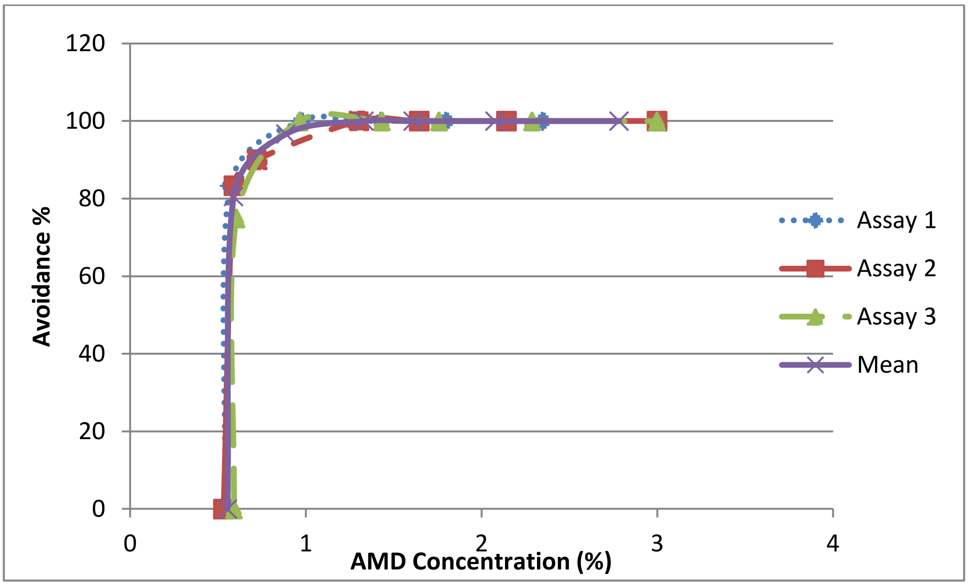

Figure 2: AMD concentrations and avoidance percentage for the 12 h assay avoidance in the 3 m system.

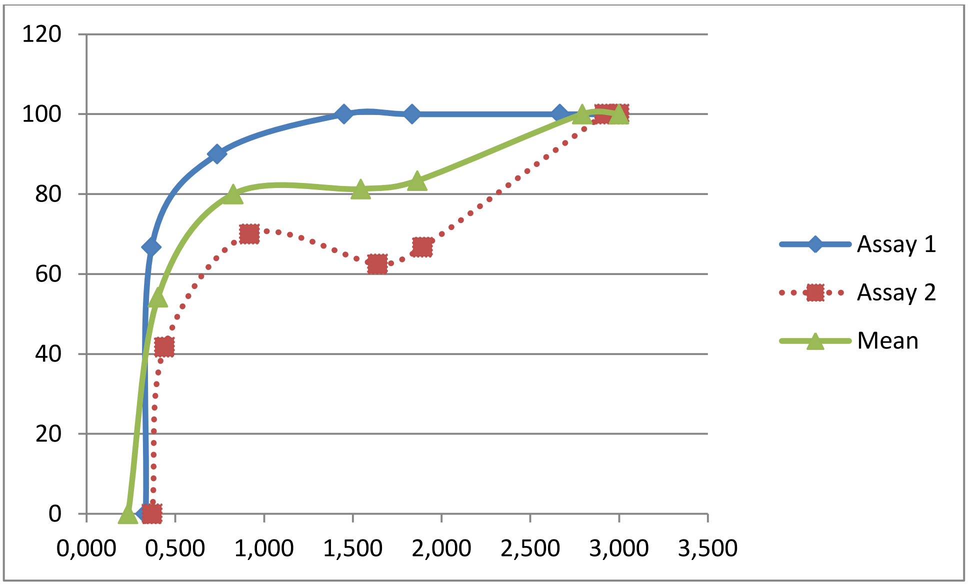

Figure 3: AMD concentrations and avoidance percentage for the 12 h assay avoidance in 30 m scaled-up system.

Table 2: AC_{50} and AC_{20} values for the 3-m systems and for the scaled up systems, with respectively confidence limits (95%CL) and means.

	AC50		**AC20**	
Assays	**3 m**	**30 m**	**3 m**	**30 m**
#1	0.566(0.563-0.570)	0.426 (0.356-0.551)	0.563(0.560-0.566)	0.316(0.209-0.375)
#2	0.588 (0.555-0.626)	0.863 (0.560-1.28)	0.541(0.483-0.570)	0.406(0.184-0,587)
#3	0.604 (0.603-0.606)		0.603(0.601-0.604)	
Mean	0.586	0.644	0.569	0.361

Discussion

Discussion

Data obtained from the controls support that, in the absence of a toxicant, organisms have no preference for any compartment; fish distribution was similar among all compartments. Thus, the scaled up system test was suitable to perform avoidance assays with fish once avoidance response was not influenced by possible external confounding factors and no social attraction occurred. The presence of the organisms and their mobility could be cause for concern, since they could jeopardize the contamination gradient and its linearity, however that not occurred. Hereupon, it is recommended that the number of organisms per assay should not only respect the maximum loads accepted for testing but must also take into account the maintenance of the gradient.

This study was successful in the maintenance of the 3 and 30-m systems contamination gradients for 12 hours. Thus, the use of pumps to maintain the gradient, as done by Lopes et al. (2004), Moreira-Santos et al. (2008), and Rosa et al. (2008 and 2012) is not necessary. This is not a novelty since avoidance tests have been performed without the use of pumps, being the gradient successfully maintained (Araújo et al., 2014a,b).

Danio rerio avoided significantly the acid mine drainage (AMD), as already reported in literature (Moreira-Santos et al., 2008), being the AC_{50} values from the scaled up system very close to the small system.

To simulate the contamination gradient with a series of linked modular mesocosms was suggest by Peterson et al. (2005) as the best alternative approach to compressed systems, once the gradient of interest may be too large to compress within a single mesocosms, which may cause unrealistic dynamics. The conditions in each compartment are maintained relatively constant but differ among compartments. This discrete approach can supply a more realist model to understand how ecosystems respond to a contamination scenario (Peterson et al., 2005). The studies involving estuarine salinity gradients benefited a lot with these discrete compartments (e.g., Margalef 1967; Doering et al. 1995). Nevertheless, the majority of the tests used as tools to assess the quality and risk in the ecosystems in ecology are enclosed in a very small scale (mesocosms, microcosms and microecosystems), being the communities, with their physical environment, containerize and isolated with the intent of experimentation (Ives et al., 1996; Peterson et al., 2005). Smaller scale in aquatic experiments can be very practical due to its high replicable but the answers given by them can be spurious (Schindler, 1998) and scale dependent (Bergström et al., 2004), since the reduced scale length can repress the interactions that occur in diverse environments (Peterson et al., 2005). Thus, the designing of a scaled up system might be encouraged to cease the results uncertainties of small systems tests.

In order to reinforce the validity of the scaled-up system, the results obtained were compared with the results of Moreira-Santos et al. (2008) using a 1-m avoidance system, the same organisms and contaminant that were used in the present work. The number of organisms per compartment used by Moreira-santos et al. (2008) was different, well as the duration of the assay, at least 24 h, and the gradient of contamination, from 1.21 to 2.06%. Nevertheless, and despite the differences mentioned, the AC_{50} in both studies are similar. Would be expected, that avoidance had been more intense, i.e. a lower AC_{50}, in the scaled-up system due to lack of time. Nevertheless, this has not been verified and 12h are sufficient to achieve an identical distribution of organisms. Moreira-Santos et al. (2008) compared, the AC_{50} obtained after 24 and 96 hours for *Danio rerio* exposed to an acid mine drainage and the results were similar. This appears to indicate that the AC_{50} for these organisms is attained quickly and remains constant. Based on the above mentioned results, the type of fish in study appears not acclimatize over the 4 days of study and thus, cause the increasing of the AC_{50}. It is highly probable that for small organisms and with less mobility, the duration for the scaled system would need to be higher and, if the AC_{50} is close to the LC_{50}, which could compromise the comparison between the avoidances systems cause the fish would die or occur moribundity due to the time required to their detect and avoid the contamination. Moribundity can decrease or even lead to a loss of the ability to avoid contamination, having already been recorded in tadpoles exposed to cooper and to pyrimethanil (Araújo et al., 2014b,c), stream macroinvertebrates exposed to crude oil (Araújo et al., 2013) and in cladocerans and copepods exposed to metals and the insecticide endosulfan (Gutierrez et al., 2012). Even with all this, the 3-m system proved, so far, to be easy to handle and very relevant, which is a plus not only because incorporates the spatial avoidance (nonforced exposure) but also because it can replace, at first tier levels, the use of lethal assays, thereby respecting the animal experimentation directive (DIRECTIVE 2010/63/EU). For being an "humane" endpoint, assays with avoidance, should be recommended along side of other assays, even though with the others sublethal endpoints, cause the last ones, cause more suffering to the test organisms, since they are forced to remain exposed and to suffer physiologic effects. These conditions that the organisms are subjected, violate, somehow, the three “R’s” specified in the DIRECTIVE 2010/63/EU, once the organisms have no form of escaping to contaminant, thus suffering from possible sore/pain, anguish or physiologic damages. Therefore, to perform first avoidance assays should be the most legitimate and ethical procedure option, because it is the most satisfying the requirements of the in force Directive.

The comparison of the AC_{50} values of the two systems with different scales used in the present study confirms the avoidance spacelessness theorem. This confirmation is an important step in risk assessment and ecotoxicology, once it allows the complementary of standardized tests, thus obtaining a high ecological relevance.

Conclusion

Conclusion

In conclusion, developing a scaled-up system permitted validate the extrapolation of the 3-m system results. Therefore, the small scale systems, as the used in this study, are suitable to perform avoidance tests with small fish, like *Danio rerio*, toward contaminants, providing realistic results. The 30-m system AC_{50} results came to confirm the spacelessness theorem, that means avoidance intensity is spaceless - unaffected by or independent of distance. This is a relevant development in ecotoxicology and risk assessment, since it allows for fasting, economic and ethical tests.

References

References

APHA, AWA, WPCF (American Public Health Association, American WaterWorks Association, Water Pollution Control Federation). 1995. Standard Methods for the Examination of Water and Wastewater. American Public Health Association, 19th edn, Washington DC, USA.

Araújo CVM, Moreira-Santos M, Sousa JP, Ochoa-Herrera V, Encalada AC, Ribeiro R. 2013. Active avoidance from a crude oil soluble fraction by an Andeanparamo copepod. Chemosphere (under review).

Araújo CMVM, Shinn C, Mendes LB, Schneider DD, Sanchez AL, Espíndola ELG. 2014a. Avoidance response of *Danio rerio* to a fungicide in a linear contamination gradient. Science of the total environment 484:36-42.

Araújo CVM, Shinn C, Moreira-Santos M, Lopes I., Espíndola ELG, Ribeiro R. 2014b. Cooper-driven avoidance and mortality in temperate and tropical tadpoles. Aqua toxicology 146:70-75.

Araújo CVM, Shinn C, Vasconcelos AM, Ribeiro R, Espíndola ELG. 2014c. Preference and avoidance responses by tadpoles: the fungicide pyrimethanil as a habitat disturber. Ecotoxicology 23(5):851-60.

Ares J. 2003. Time and space issues in ecotoxicology: population models, landscape pattern analysis, and long-range environmental chemistry. Environ Toxicol and Chem 22:945 – 957.

Bergström ULF, Englund G. 2004. Spatial scale, heterogeneity and functional responses. Journal of animal ecology 73:487-493.

Cabral JA, Mieiro CL, Marques JC. 1998. Environmental and biological factors influence the relationship between a predator fish, Gambusia holbrooki, and its main prey in rice fields on the lower Mondego River valley (Portugal). Hydrobiologia 382:41–51.

De Lange HJ, Sperber V, Peeters ET. 2006. Avoidance of polycyclic aromatic hydrocarbon-contaminated sediments by the freshwater invertebrates *Gammarus pulex* and *Aselius aquaticus*. Environ Toxicol Chem 25:452–457.

DIRECTIVE 2010/63/EU OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 22 September 2010 on the protection of animals used for scientific purposes. 2010. OJ L276/33.

Doering PH, Oviatt CA, Nowicki BL, Klos EG, Reed LW. 1995. Phosphorus and nitrogen limitation of primary production in a simulated estuarine gradient. Mar Ecol-Prog Ser 124:271-287.

Dornfeld CB, Moreira-Santos M, Espíndola ELG, Ribeiro R. 2009. Do Larvae and Ovipositing *Chironomus riparius* (Diptera: Chironomidae) Females Avoid Copper-Contaminated Environments? Human and Ecological Risk Assessment: An International Journal 15:63-75.

Exley C. 2000. Avoidance of aluminum by rainbow trout. Environ Toxicol Chem 19:933–939.

Gutierrez MF, Paggi JC, Gagneten AM. 2012. Microcrustaceans escape behavioras an early bioindicator of copper, chromium and endosulfan toxicity. Ecotoxicology 21:428-438.

Henry TB, Irwin ER, Grizzle JM, Brumbaugh WG, Wildhaber ML. 2001. Gill lesions and death of bluegill in acid mine drainage mixing zone. Environ Toxicol Chem 20:1304-1311.

Ives AR, Foufopoulos J, Klopfer ED, Klug JL, Palmer TM. 1996. Bottle or big-scale studies: how do we do ecology? Ecology 77:681–685.

Kravitz MJ, Lamberson JO, Ferraro SP, Swartz RC, Boese BL, Specht DT. 1999. Avoidance response of the estuarine amphipod *Eohaustorius estuarius* to polycyclic aromatic hydrocarbon-contaminated, field-collected sediments. Environ Toxicol 18: 1232–1235.

Lopes I, Baird DJ, Ribeiro R. 2004. Avoidance of copper contamination by field populations of *Daphnia longispina*. Environ Toxicol Chem 23:1702–1708.

Manríquez PH, Jara ME, Mardones ML, Torres R and others. 2014. Ocean acidification affects predator avoidance behaviour but not prey detection in the early ontogeny of a keystone species. Mar Ecol Prog Ser 502:157-167.

Margalef R. 1967. Laboratory analogues of estuarine plankton systems. American Association for the Advancement of Science 515-521.McNicol RE, Scherer E. 1993. Influence of cadmium pre-exposure on the preference-avoidance responses of Lake Whitefish (*Coregonus clupeaformis*) to cadmium. Environ Contam Toxicol 25:36–40.

McNicol RE, Scherer E. 1993. Influence of cadmium pre-exposure on the preference-avoidance responses of Lake Whitefish (*Coregonus clupeaformis*) to cadmium. Environ Contam Toxicol 25:36–40.

Moreira-Santos M, Donato C, Lopes I, Ribeiro R. 2008. Avoidance tests with small fish: Determination of the median avoidance concentration and of the lowest-observed-effect gradient. Environ Toxicol Chem 27:1576–1582.

Pankhurst NW, Hebert NA. 2013. Fish Physiology and ecology: contribution of the Leigh Laboratory to the collision of paradigms. New Zealand Journal of Marine and Freshwater Research 47:392-408.

Pereira R, Ribeiro R, Gonçalves F. 2004. Plan for an integrated human and environmental risk assessment in S. Domingos Mine area (Portugal). Human and Ecological Risk Assessment 10:543-578.

Peterson J.E, Englund G. 2005. Dimensional approaches to designing better experimental ecosystems: a practitioners guide with examples. Oecologia 145: 216-224.

Ribeiro R, Baird DJ, Soares AMVM, Lopes I. 2012. Contaminant driven genetic erosion: A case-study with *Daphnia longispina*. Environmental Toxicology and Chemistry 31:977-982

Ribeiro R, Lopes I. 2013. Contaminant driven genetic erosion and associated hypotheses on alleles loss, reduced population growth rate and increased susceptibility to future stressors – an essay. Ecotoxicology 22:889-899.

Rosa R, Moreira-Santos M, Lopes I, Picado A, Mendonça E, Ribeiro R. 2008. Development and sensitivity of a 12-h laboratory test with *Daphnia magna Straus* based on avoidance of pulp mill effluents. Bull Environ Contam Toxicol 81:464–469.

Rosa R, Materatski P, Moreira-Santos M, Sousa JP, Ribeiro R. 2012. A scaled-up system to evaluate zooplankton spatial avoidance and the population immediate decline concentration. Environ Toxicol and Chem 31(6), 1301–5.

Sakuma M. 1998. Probit analysis of preference data. Appl Entomol Zool 33:339–347.

Schafers C, Kloppel H, Takahashi Y. 2007. Zooplankton avoidance behavior following spray drift exposure to fenpyroximate. Hum Ecol Risk Assess 13:527–534.

Schindler DW. 1998. Replication Versus Realism: The Need for Ecosystem-Scale Experiments. Ecosystems 1:323-334.Sobral O, Marin-Morales MA, Ribeiro R. 2013. Could contamination induced mutations lead to a genetic diversity overestimation? Ecotoxicology 22:838-846.

Steele CW, Scarfee AD, Owens DW. 1991. Effects of group size on the responsiveness of zebrafish, *Brachydanio rerio* (Hamilton Buchanan), to alanine, a chemical attractant. J Fish Biol 38:553– 564.

Vardhanan YS, Radhakrishnan T. 2002. Acute toxicity evaluation of cooper, arsenic, and HCH by paddy fish field crab, *Paratelphusa hydrodromus* (Herb.). J Environ Biol 23:387-392.

Wang J, Liu C, Ma F, Chen W, Liu J, Hu B, Zheng L. 2014. Circadian clock mediates light/dark preference in zebrafish (*Danio rerio*). Zebrafish 11:115-121.

Wells JB, Little EE, Calfee RD. 2004. Behavioral response of young rainbow trout (*Oncorhynchus mykiss*) to forest fire-retar- dant chemicals in the laboratory. Environ Toxicol Chem 23:621– 625.

Wentsel R, McIntosh A, McCaffertyW, Atchison G, Anderson V. 1977. Avoidance response of midge larvae (*Chironomus tentans*) to sediments containing heavy metals. Hydrobiologia 55:171–175.

Woodward DF, Hansen JA, Bergman HL, Little EE, DeLonay AJ. 1995. Brown trout avoidance of metals in water characteristic of the Clark Fork River, Montana. Can J Fish Aquat Sci 52:2031– 2037.