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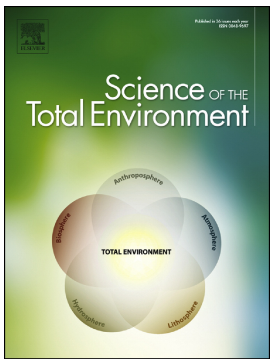

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PII: S0048-9697(19)31045-9
DOI: https://doi.org/10.1016/j.scitotenv.2019.03.067
Reference: STOTEN 31277

To appear in: *Science of the Total Environment*

Received date: 8 January 2019
Revised date: 5 March 2019
Accepted date: 5 March 2019

Please cite this article as: T. Vidal, J.I. Santos, L. Queirós, et al., Environmental benchmarks based on ecotoxicological assessment with planktonic species might not adequately protect benthic assemblages in lotic systems, Science of the Total Environment, https://doi.org/10.1016/j.scitotenv.2019.03.067

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Environmental benchmarks based on ecotoxicological assessment with planktonic species might not adequately protect benthic assemblages in lotic systems

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Abstract

Freshwater ecosystems face widespread diffuse and point-source contamination. Species sensitivity distributions (SSDs) have been used as a tool to determine chemical concentration benchmarks that represent protective levels for most species in the environment. Here we used a SSD approach to assess on the adequacy of standard planktonic organisms to reflect the response of benthic communities, critically supporting the structure and function of lotic ecosystems. For the purpose, SSDs reflecting non-lethal responses of standard planktonic and selected benthic organisms were built based on EC50 values (collected in the literature or estimated following testing herein) regarding three model contaminants: potassium dichromate (PD), 3,5-dichlorophenol (DCP) and lead chloride (LC). The derived HC5 estimates were discriminatory between chemicals and the uncertainty associated with the estimate was remarkably low. The HC5 estimates with corresponding uncertainty were generally within the same order of magnitude for the three chemicals tested, with better discrimination between chemicals regarding their hazardous potential being achieved

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for benthic organisms: DCP was clearly less hazardous than PD, but LC tends to be as hazardous as PD and DCP (assuming the confidence interval ranges). Moreover, benthic communities were more sensitive to both DCP and PD, in this later case the HC5 being lower by more than one order of magnitude than that found for planktonic communities; for LC, confidence intervals overlapped, preventing a feasible assumption regarding differential sensitivity of the compared communities. Microphytobenthos was highlighted as the most sensitive group to the three tested chemicals in SSDs covering the benthic compartment, while SSDs with planktonic organisms did not consistently show trends in sensitivity ordering. Overall, our results suggest that protective benchmarks retrieved from SSDs built with the responses of standard planktonic organisms (which are the most commonly used for regulation purposes) do not adequately protect benthic communities.

Keywords

Species Sensitivity Distribution; Benthic vs Planktonic organisms, Standard chemicals, Lead chloride

1. Introduction

Riverine ecosystems can often be exposed to contaminants such as pesticides derived from watershed runoff or urban discharges, or metals through e.g. industrial effluent discharge. The exposure to those stressors resulting from various human activities can cause adverse ecological effects. In this context, and supporting protective management actions, prospective environmental risk assessment (ERA) intends to evaluate the probability or likelihood that adverse ecological effects will occur (Forbes and Calow, 2002). The ecotoxicological line of evidence in most ERA frameworks relies in the

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derivation of a threshold concentration that can be used as a benchmark towards the protection of the structure and function of ecosystems. Such derivation is normally based on extrapolating the effects of contaminants noticed in bioassays using a limited number of test species representing those expected in the ecosystem. Hence, the reliability of the ecosystem-level effects that are transferred from the species-level effects measured in these bioassays ultimately depend on the assumptions made on how species-level and ecosystem level effects are linked.

Biological diversity undertakes a major challenge in such ecotoxicological assessment, since each species will respond differently to similar levels of exposure to toxic substances (Baird and van den Brink, 2007). By including in this assessment approach organisms representing different functional groups will, thereby, theoretically capture the community range of variability regarding toxicant sensitivity. The variation of species sensitivity within a community or an assemblage towards one or more toxicants can be expressed in terms of cumulative distributions known as Species Sensitivity Distributions (SSD). SSDs have been increasingly employed (Grist et al., 2006, 2002) in the determination of chemical concentration benchmarks that represent protective levels for most species in the environment. These usually match calculated HC5 values based on established SSDs, which represent the hazardous concentration for 5 % of species, thus expected to spare 95% of species in the assemblage. Sensitive endpoints are generally used in SSD building, such as single-species growth, reproduction or survival, depending on whether low, chronic contamination levels are focused or rather acute exposure. Therefore, and apart from its role as the path for protective benchmark definition, SSDs are an interesting tool to compare the sensitivity of several freshwater organisms, positioning them according to a sensitivity rank. This is the arena for the development of the present study, where we particularly challenged

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the representativeness of standard organisms widely used to build SSDs to reflect the responses and sensitivity of benthic communities in lotic ecosystems.

In the context of standard ecotoxicological assessment, widespread guidelines such as those by OECD (OECD, 2006a, e.g. 2004a, 2004b) recommend a limited number of standard test organism used as representatives to predict toxic effects. Bioassays using freshwater microalgae like *Raphidocelis subcapitata* apply in this context as representative of primary producers, but these planktonic species bear little ecological relevance on lotic habitats. As well, the zooplankter *Daphnia magna* is widely used as a model test organism but its degree of representativeness for invertebrate communities in general has been questioned (Koivisto, 1995; von der Ohe and Liess, 2004). Still, there are standard guidelines considering organisms from the benthic compartment, thus relevant in assessments focusing lotic systems. However, some have been receiving relevant criticism concerning the protective value of benchmarks generated, such as it is the case of standardized bioassays with chironomid larvae (OECD, 2010, 2004c, 2004a) and aquatic oligochaetes (*Lumbriculus variegatus*) (OECD, 2007). In fact, these organisms are known by their physiological tolerance to an wide array of stressors, including environmental contaminants, low oxygen availability, temperature changes and salinity (Colombo et al., 2016; Mantilla et al., 2018). Thus, they are likely to indicate safety threshold concentrations that underestimate the sensitivity of lotic communities. On the other hand, microphytobenthos plays an important role in the ecological dynamics of lotic freshwater ecosystems and bears high sensitivity to environmental changes (Vidal et al., 2014), but did not receive attention so far regarding the development of standardized ecotoxicological testing guidelines. On this basis, we hypothesised here that freshwater planktonic and benthic communities should have differential sensitivity to selected

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model chemical contaminants, translating into different protective benchmarks yield from dedicated SSDs.

Three model contaminants were used to appraise this hypothesis: potassium dichromate ($K_2Cr_2O_7$ - PD), 3,5-dichlorophenol (DCP) and lead chloride (LC). The first two are standard substances used in OECD guidelines for the validation of ecotoxicological tests with standard freshwater species. Lead was chosen because it is a very common metal element contaminating freshwaters worldwide (Ali and Khan, 2018; Strungaru et al., 2018; Vidal et al., 2012). Contamination deriving from acid mine drainage (Vidal et al., 2012) or industrial activities (Ali and Khan, 2018) is a common source of lead in freshwaters. Lead and chromium (VI) have been given regulatory attention worldwide - threshold safety levels stated as 7.2 µg L^{-1} (E.U., 2008 Directive 2008/105) and 50 µg L^{-1} (Maycock et al., 2007), respectively – and 3,5-dichlorophenol is frequently used in the formulation of pesticides, disinfectants and as a chemical intermediates in the production of more complex chemicals (Xie et al., 2018). On the other hand, 3,5-dichlorophenol has no regulated safety thresholds.

The specific aim of the present study was to compare the ecotoxicological response of selected benthic organisms representing lotic ecosystems with that by standard planktonic organisms that can typically indicate on noxious effects in lentic ecosystems. SSDs were used to allow a broad, integrated perspective on the hypothesised differential sensitivity of benthic vs planktonic communities, a specific focus being put on periphyton, which is a significant benthos compartment that has been underlooked – generally, in ecotoxicological approaches, and more even specifically in the prospective risk assessment of chemicals. Furthermore, the comparison of HC5 and HC50 values estimated under this approach can support the need for the establishment of specifically tuned regulatory benchmarks for lotic ecosystems.

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2. Material and Methods

This study integrated data collected in the literature and data generated in our laboratory using an SSD approach, as detailed below. We targeted non-lethal median effect concentrations retrieved from short-term exposures to each of the three focused compounds (PD, DCP, LC) in all cases. This option covers for a more realistic scenario of putative exposure of natural communities once acute exposure to high contaminant levels is rarely felt, especially in lotic systems, where the water flow fosters the dilution of point-source and diffuse contamination.

2.1. Data collection from the literature

The search in the literature was directed to toxicity records (EC50 values) for each selected model chemical retrieved in tests with planktonic and benthic freshwater species. Given the reasoning of the study (see above), data referring to acute exposures reflecting in lethal endpoints were discarded. A primary search was performed on ISI-Web of Science® using the chemical name as a string for the topic field and then analysing each hit - title and abstract - for adequacy to the focus of the present study. The ECOTOX Knowledgebase also contributed as source of information following the same primary search string and qualitative analysis of all hits.

Given the overall scarcity of suitable data in the literature, we complemented the dataset by carrying out further tests with planktonic and benthic species for EC50 values estimation as required for building feasible SSDs (8 datapoints are normally understood as a minimum set to feed an SSD; TenBrook et al. (2009)). In some cases, regarding tests with planktonic species, literature was available (Erturk and Sacan, 2013; Kaiser and Palabrica, 1991) but we still decided to test within the present study to ensure

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consistency in test conditions, test design or endpoints evaluated among the data used to feed each SSD. Tests were always carried out by applying geometric concentration ranges of potassium dichromate (anhydrous salt 99.5% purity, Panreac); 3,5-dichlorophenol (99.7% purity, Aldrich); and lead(II) chloride (98% purity, Aldrich).

2.2. Ecotoxicological tests with planktonic organisms

Raphidocelis subcapitata and *Chorella vulgaris; Lemna minor* and *Lemna gibba* were selected for testing as representatives of green microalgae and macrophytes, both important producers in planktonic food webs. The freshwater cladoceran *Daphnia magna* was selected due to its ecological position (primary consumers) in the aquatic food web and its role as standard organism known to be very sensitivity to environmental stress (Hanazato, 2001). The bacteria *Aliivibrio fischeri* represent the important role in the degradation of organic matter by microorganisms; furthermore, the corresponding standard bioassay is of easy application, reproducibility, sensitivity and standardisation (Di Nica et al., 2017; Lambertson et al., 1992) with significant correlations between the toxicity data with that obtained for other species including fish, crustaceans and algae being commonly shown (Kaiser, 1998; Parvez et al., 2006).

2.2.1. *Aliivibrio fischeri* – Bioluminescence inhibition test

The *Aliivibrio fischeri* luminescence inhibition test was applied to stock solutions of PD, DCP or LC in distilled water, following the instructions of the manufacturer for the 81.9% basic test protocol (AE, 1998) and using the Microtox® Model 500 Analyzer. *Aliivibrio fischeri* was supplied lyophilized as part of the Microtox® test kit (AE, 1998) and reconstituted immediately before testing; and osmotic adjustment of test solutions was carried out before testing as recommended by the manufacturer. Light

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measurements were taken through the test and 30-min bioluminescence inhibition values were used in data analysis.

2.2.2. *Raphidocelis subcapitata* and *Chlorella vulgaris* – Growth inhibition

Growth inhibition tests with *Raphidocelis subcapitata* and *Chlorella vulgaris* were conducted following the OECD guideline 201 (OECD, 2006a) adapted to 24-well microplates (Geis et al., 2000). Bulk algae cultures were maintained in the laboratory in Woods Whole MBL medium, under 20 ± 2 ºC and 16 h^L: 8 h^D photoperiod. The microalgae (10^4 cell mL^{-1} initial density) were then exposed in triplicate to PD, DCP or LC; a control treatment was conducted in all tests. The microplates were incubated for 96 hours under 23 ± 1ºC and continuous illumination (8000 lux). At the end of the test cell density was quantified spectrophotometrically at 440 nm based on previous established calibration curve. The biomass yield (cells/mL) was calculated as the difference between final and initial cell density. The growth rate (daily logarithmic increase in yield) was also calculated and used in data analysis.

2.2.3. *Lemna minor* and *Lemna gibba* – Growth inhibition

Growth inhibition tests with *Lemna minor* and *Lemna gibba* were performed following OECD guideline 221 (OECD, 2006b) adapted to the use of 6-well plates (Kaza et al., 2007). Bulk cultures were maintained in Steinberg medium at 23 ± 1 ºC and under continuous illumination. The macrophytes were exposed in triplicate to PD, DCP or LC. Each well was filled with 10 mL test solution and added three colonies of three fronds each. At the beginning of the test, three colonies of three fronds each in triplicate were oven dried for 24 hours at 60ºC to obtain the initial dry weight. The test plates were incubated for 7 days under 23 ± 1ºC and continuous illumination. At the end of the test,

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fronds in each well were counted and oven-dried. Yield and specific growth rates based on both frond number and dry weight records were calculated to feed data analysis.

2.2.4. *Daphnia magna* – Feeding inhibition

Assays with *D. magna* were conducted to evaluate feeding inhibition derived from exposure to PD, DCP or LC. Monoclonal bulk cultures have been maintained in the laboratory in ASTM hard water (ASTM, 1980) supplied with an organic additive extracted from *Ascophyllum nodosum* (Algea Fert Solid). Cultures were renewed three times per week, and the organisms were fed after renewal with 3.0 x 10^5 cells mL^{-1} of *R. subcapitata*. All cultures were kept under a $16h^L$:$8h^D$ photoperiod and temperature of 20 ± 2ºC. Neonates (<24 h old; born within 3^{rd}-5^{th} brood) were separated from the bulk culture and raised in the same conditions until reaching 4-5 days old (i.e. 4^{th} instar) as recommend by McWilliam and Baird (2002) and Allen et al. (1995). Groups of 5 juveniles were exposed to the test solutions or the blank ASTM control (10 mL) in borosilicate flasks, added food (*R. subcapitata* allowing a fixed ration of 3 x 10^5 cells/mL per vessel). Four replicates were used per treatment. Each treatment was added a microalgae control, structurally like the replicates but with no animals added to control for their growth during exposure. At the beginning of the experiment, groups of 5 daphnids were distributed randomly into each replicate and allowed to feed during 24 h in the dark. Individual feeding rates (number of algal cells ingested per animal per hour) were determined by following the change in cell density at the beginning of the test and after 24 h exposure, according to Allen et al. (1995). Cell density was estimated from absorbance measurements at λ = 440 nm (Schimadzu UV-VIS 1800) through a previously established calibration curve.

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2.2.5. *Thamnocephalus platyurus* – Post-exposure feeding inhibition

The test was performed using the TK37-RAPIDTOXKIT (MicroBioTests Inc.), following the manufacturer's protocol. Shortly, *T. platyurus* cysts were pre-hydrated under permanent illumination to hatch. The test was performed in glass tubes containing 9.5 mL of each test solution or Standard Freshwater for the controls, plus 0.5 mL of larval suspension. Each treatment and control were performed in duplicate and the test started with a 1 h incubation (dark; 25ºC), followed by the addition of a suspension of red beads to each test tube; *T. platyurus* larvae were allowed to feed on these beads for 15 min. The test was terminated by adding Lugol solution to each tube for organisms' fixation. The larvae bearing no red particles in their digestive track (thus, those that were affected by the exposure and did not feed on the beads) were counted under the stereoscope (Olympus SZX9). The results were expressed as the inhibition in mean percentage of particle uptake.

2.2.6. *Brachionus calyciflorus* – reproduction inhibition

The test was performed using the ROTOXKIT F Chronic (MicroBioTests Inc.) following the manufacturer's protocol. Shortly, *B. calyciflorus* cysts were transferred to a Petri dish with aerated Standard Freshwater and incubated under continuous illumination for hatching. Pre-test feeding was carried out soon after cysts hatching with Roti-rich food supplied in the kit, for 2 h. Disposable polystyrene 48-well microplates were used as the test support. Each well contained 1 mL test solution or Standard Freshwater as a control, added one rotifer, and each treatment had 8 replicates. A fixed amount of algal food suspension supplied in the kit was then added to each well. Microplates were covered with Parafilm M® and incubated at 25ºC, in the dark, for 48 h. At the end of the test, dead rotifers were counted and removed. Lugol solution was

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added to fixate the remaining living rotifers, facilitating counting under the stereoscope (Olympus SZX9). The results were expressed in terms of population growth rate inhibition (logarithmic increase in abundance per day).

2.3. Ecotoxicological tests with selected benthic organisms

Three benthic species (*Heterocypris incongruens, Corbicula fluminea* and *Navicula libonensis*) were selected for the ecotoxicological tests with PD, DCP or LC. *H. incongruens* is a cosmopolitan omnivorous epibenthic ostracod found in diverse freshwater habitats worldwide and commonly used as a bioindicator of contaminants for the benthos (Palma et al., 2016). *C. fluminea* is an invasive bivalve in Europe and USA, that can be considered as model to monitor contaminants due to its capacity to accumulate organic and inorganic contaminants. Moreover, *C. fluminea* are strong filter-feeders and feeding inhibition was already proven to be a relevant and sensitive sub-lethal endpoint rendering the bivalves good ecotoxicological models representing the benthos (Castro et al., 2018). *N. libonensis* is a ubiquitous benthic diatom representative of microphytobenthos in lotic environments, widely spread in Europe, North America and South America and was classified as sensitive to diffuse pollution (Cemagref, 1982), which potentially indicates sensitivity to environmental pollutants in general.

2.3.1. *Heterocypris incongruens* – growth inhibition

The test was performed using the OSTRACODTOXKIT F (MicroBioTests Inc.), following the manufacturer's protocol. Shortly, *H. incongruens* cysts were transferred to a Petri dish with aerated Standard Freshwater and incubated under continuous illumination for hatching. The pre-feeding with *Spirulina*-food supplied with the kit was

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performed immediately after hatching period that lasted for approximately 48 h. The freshly hatched ostracod length was measured under the stereoscope. The test was performed in 6 multiwell plates with reference sediment spiked with each of the test substances (DCP, PD, LC) and algal food suspension (*Scenedesmus sp.*). Each treatment and control were performed in triplicate and 10 ostracods were used for each treatment. Multiwell plates were covered with Parafilm M® and incubated at 25ºC, in the dark, for 6 days. At the end of the incubation period dead ostracods counted and removed. Living ostracods were recovered and Lugol solution was added to fixate them, facilitating length measurement under the stereoscope. The results were expressed as percentage of growth inhibition.

2.3.2. *Corbicula fluminea* – Post-exposure feeding inhibition

The tests were run with clams (shell length between 10 and 25 mm) collected in a shallow freshwater ditch near Mira (in the littoral centre of Portugal; 40º 24' 55" N, 8º 45' 04" W). Collected clams were transported to the laboratory in plastic buckets filled with local water, and gradually acclimated to dechlorinated tap water under permanent aeration under 20 ± 2ºC and a 16 h^L:8 h^D photoperiod. Twice a week, water was renewed, and clams were fed *ad libitum* with a *R. subcapitata* suspensions. A quarantine period of more than two weeks was always ensured prior to testing. Then, clams were randomly assigned to the test vessels containing 100 mL test solutions spiked with PD, DCP and LC (an equivalent control treatment with blank dechlorinated tap water was always set) to initiate the exposure period that lasted for 96 h, under permanent aeration and the same incubation conditions as described for clam stocks maintenance (adapted from mortality test design as established by e.g. Gomes et al.,

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2014; Silva et al., 2016). Each treatment held 20 replicates (1 clam per replicate), complying with the requirements of the feeding assay as described below.

At the end of the 96 h exposure period, a post-exposure feeding inhibition test was initiated following the protocol by Castro et al. (2018). For the purpose, 2 mL of concentrated microalgae suspensions were added to each test vessel, corresponding to ~3.5×10^6 cells mL^{-1}; OD_{440nm} = 0.141–0.179. A set of controls for microalgae growth during the test period (test solution with no clam) was always run in parallel (3 replicates). After adding the suspended algae to the vessels, a sample was immediately taken as the initial OD_{440nm} reading (t_0). OD_{440nm} measurements were also taken at the end of a 2-h (t_{120}) test period, held in the dark to avoid microalgae growth. OD_{440nm} measurements were converted to microalgae cell density following a previously established calibration curve, which allowed the calculation of the proportion of algae removed (Castro et al., 2018).

2.3.3 *Navicula libonensis* – growth inhibition

Growth inhibition tests with *N. libonensis* were carried out following the protocol by Vidal et al. (2014) to address the effects of LC. Bulk algae cultures were maintained in the laboratory in Chu 10 medium, under 22 ± 2 ºC and continuous illumination (8000 lux). The benthic diatoms initial (10^4 cell mL^{-1}) and final cell density measurements were made by microscopic (Olympus CKX 41) cell counting using a tubular plankton chamber (Hydro-Bios, Germany). The exposure was conducted in triplicate both for LC and control treatments, in glass tubes for 6 days (incubation conditions kept as described above for maintenance). At the end of the test, the benthic diatoms yield, in each replicate, was calculated as the difference between final and initial cell density.

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The growth rate (daily logarithmic increase in yield) was also calculated and used in data analysis.

Data analysis

The records obtained from the bioassays performed in the laboratory were used to estimate median effect concentrations (EC50) and the corresponding confidence intervals, for each chemical, by non-linear regression using least-squares method to fit the data to the logistic equation. The EC50 values collected from the literature and obtained from laboratorial testing were integrated in Species Sensitivity Distribution (SSDs) (Posthuma et al., 2002). These were built using the U.S.EPA's species sensitivity distribution generator (USEPA, 2005), fed by at least 8 EC50 values reflecting non-lethal endpoints. Validation of the quality of our EC50 estimates was done by following widely recommended guidelines (EC, 2007) to ensure the feeding of the SSD with a EC50 dataset holding tight confidence intervals, and consequently improving the overall model fitting of derived curves. SSDs were built for each chemical compound and separately considering each ecological compartment – planktonic and benthic organisms. SSDs allowed to feasibly estimate HC*p* (Hazard Concentration for *p%* of the species); we focused on HC5 and HC50, which are commonly used as benchmarks for environment protection purposes (Kefford et al., 2005; e.g. van den Brink et al., 2002).

Results

All the information gathered that was used to feed the SSDs with planktonic and benthic freshwater organisms is summarized in Tables S1 and S2, respectively. All the ISI-Web of Science® initial hits searched were narrowed down to 14 hits that fulfill all the

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established criteria. Regarding the ECOTOX Knowledgebase, 10 hits were used. The EC50 value collected for the ephemeropteran *Deleatidium sp.* (Table S2) was excluded from SSD building because it was considered extremely high. Ephemeroptera larvae are among the most sensitive taxa of insects used as bioindicator of good water quality in the Water Framework Directive (WFD) evaluation, challenging the feasibility of estimates positioning *Deleatidium* sp. as a very tolerant genus. Also, it was not possible to fully confirm the exposure conditions of the tests. These EC50 values found in the literature were complemented with those retrieved from selected tests carried out specifically for the present study to feed each specific SSD. In this way, the SSDs were built using generally 8 entries - 9 were used for the SSD on DCP with planktonic organisms (Tables S1 and S2).

The adequacy of our options regarding the samples feeding the SSDs is corroborated by the model fitting (r^2) for the six SSD curves (3 chemicals x 2 environmental compartments; Fig. 1). It was very good in all cases, generally above 0.9: 0.954 (benthic DCP); 0.946 (planktonic DCP); 0.921 (benthic PD); 0.924 (planktonic PD), 0.944 (benthic LC) and 0.898 (planktonic LC). This translates into reliable prediction of HCx estimates with narrow confidence intervals (Table 1). The lowest r^2 found for the planktonic LC SSD translates a poorer model adjustment and therefore wide confidence intervals when comparing with the other curves with higher coefficients of determination.

SSDs with planktonic vs benthic organisms

Overall, the SSDs neither distinguished the hazardous potential of the tested chemicals nor could separate the benthic from the planktonic organisms as to sensitivity at the level of the HC50. Despite trends are apparent when focusing the absolute value of the

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benchmark – DCP seems more toxic than the metal salts and selected benthic organisms are more sensitive than planktonic ones, except for LC –, the confidence intervals always overlap (Table 1), preventing a robust assumption of the differences between estimates. However, the HC5 estimates were actually discriminatory and the uncertainty associated with the estimate was remarkably lower. The estimates at the lower and upper ends of predictive models such as those used in SSDs tend to bear higher uncertainty mostly because these are non-linear stages of the curves that are frequently less populated by experimental data (Wheeler et al., 2002), which was not the case in the present study (Fig.1). Although the HC5 estimates with corresponding uncertainty were generally within the same order of magnitude for the three chemicals tested, slightly better discrimination between chemicals regarding their hazardous potential was achieved regarding benthic organisms (for planktonic organisms, confidence intervals also overlap): DCP was clearly less hazardous than PD, but LC tends to be as hazardous as PD and DCP (assuming the confidence interval ranges). Moreover, benthic organisms were more sensitive to both DCP and PD, in this later case the HC5 being lower by more than one order of magnitude than that found for planktonic communities; for LC, confidence intervals overlapped, preventing a feasible assumption regarding differential sensitivity of the compared communities.

As shown in Fig.1, the SSDs with planktonic species exhibited no consistent pattern of response in terms of sensitivity ordering of the organisms. Conversely, SSDs with benthic species evidenced that the diatom *N. libonensis* was always the most sensitive species to the three chemicals substances.

Discussion

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The main hypothesis in this study was that benchmarks used for environmental protection of surface freshwaters are not interchangeable between lentic and lotic ecosystems. This hypothesis is reasoned by the assumption that the sensitivity of the benthic biota, which is the key biotic compartment in lotic ecosystems, should differ from that of the plankton, which is a critical component of lentic ecosystems. The necessary sensitivity comparison to tackle the established research hypothesis was made using SSDs specifically collecting the sensitivity of standard planktonic organisms or selected benthic species. Since these SSDs deliberately focused in non-lethal responses, the datasets feeding the models were relatively short, comprising 8-9 entries. SSD minimum sample size criteria have been matter of debate. For regulatory purposes, hence within a prospective environmental risk assessment framework, the US Environmental Protection Agency (its tools for SSD building were used in the present study) requires at least 8 species, while the European Union requires between 5 and 8 species, and Australia and New Zealand require 5 species (Del Signore et al., 2016; TenBrook et al., 2009). Overall, the number of data points used to feed SSD modelling can influence the uncertainty of the model output. Small sample size often translates into wider confidence intervals but, most importantly, it can lead to the estimation of unfeasible protection benchmarks (i.e. HC5) by reflecting the relevance of e.g. the slope of the model in predictions made over the tails of the fitted curve (Del Signore et al., 2016; Dowse et al., 2013; Kefford et al., 2005). Despite the relevance of using large sample sizes for SSD modelling within the context of retrospective environmental risk assessment or related specific research questions (e.g. Kefford et al., 2012), the size of our datasets is within the most common regulatory requests that typically respond to the challenge of producing benchmarks applying to the protection of widely distributed ecosystems bearing high variation in inhabitant communities.

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Despite the tails of the SSDs were always covered by experimental data, mitigating a putative lack of robustness in HC5 estimations, differences of more than one order of magnitude were found between HC5 and HC50 estimations, especially for PD and LC. This could raise questions on whether the inclusion of a particular species constrains the differences between HC5 estimates regarding benthic and planktonic organisms that are attenuated at the level of the HC50. Since more data are available for lethal endpoints regarding LC and specially PD, we provide the additional exercise of building SSDs for the benthos based on these data in Fig. S1 - S2. This confirmed the distance of more than one order of magnitude between the benchmarks, supporting further our interpretation on the differential sensitivity of benthic and planktonic organisms at relevant contaminant levels from a regulatory point of view (i.e. HC5). Still, our specific focus here was on so-called non-lethal or sub-lethal endpoints. These are more likely to reflect the most common scenarios of contamination in lotic ecosystems, which are related either (i) to diffuse sources translating in chronic, semi-continuous exposure of the organisms at low levels; (ii) or to point-source events that can be continuous in particular cases (e.g. acid mine drainage, Vidal et al (2012)), but where the contaminants are easily diluted and flowing with the water currents. Overall, it is widely recognised that chronic toxicity endpoints such as feeding, growth and reproduction are advantageous over acute (lethal) toxicity endpoints since they work as early warning signals denoting toxic effects (Del Signore et al., 2016; Jager, 2012; Posthuma and Suter, 2011). However, the building of the SSDs based on non-lethal endpoints is also recognised as somewhat limited by the scarcity of data available in the literature (Baird and van den Brink, 2007; Dowse et al., 2013; Sala et al., 2012), the present study contributing towards the overcoming of this constraint.

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On the other hand, we were careful in complementing the data retrieved from the literature with actual testing towards improved coverage of typical functional roles. The choice of the taxa considered different trophic positions in the food web (i.e. producers, filter feeding consumers, detritivores and decomposers) of each focused compartment (planktonic, benthic). For example, the specific ecotoxicological tests with benthic species (ostracod, bivalve and benthic diatom) run in the laboratory were thought to complement the information already available in literature. The tested epibenthic crustacean ostracod (*H. incongruens*) provides a feasible direct insight on putative effects of contaminated sediments (Cieszynska-Semenowicz et al., 2018) in non-insect species. Also addressing a non-insect species, a sensitive endpoint tested in *C. fluminea* (Castro et al., 2018) was added to better representing bivalves and infaunal organisms, both uncommon in SSD approaches. In fact, the selection of species has been recognized relevant for the accuracy of the SSD outcome, since the overall sensitivity of a natural community depends on its species' individual sensitivity to each focused stressor (Del Signore et al., 2016).

In this context, an unbiased representation of the species that typically inhabit the focused ecosystems has been argued to be critical, covering e.g. the majority of the taxonomic groups therein (Forbes and Calow, 2002; Kefford et al., 2005). Despite covering the major functions in lotic ecosystems, our SSDs did not cover widely for insect species, which constitute a relevant fraction of benthic macroinvertebrates (Kefford et al., 2005). The reasoning for this is two-fold. First, although data regarding lethal toxicity were found for insect species in the literature (Fig. S1-S2), very few records of non-lethal endpoints were retrieved, and we could not find actually validated protocols (such as those used for testing with e.g. benthic diatoms and *C. fluminea*; Vidal et al. 2012 and Castro et al. 2018) for rapid and repeatable ecotoxicity testing of

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non-lethal endpoints with insect larvae. Second, evidences focusing on the sensitivity of insect larvae in short-term laboratory toxicity tests are somewhat inconsistent. For example, Buchwalter et al. (2007) found that short-term toxicity tests greatly underestimated the sensitivity of mayflies to metal contamination in natural ecosystems. Poteat and Buchwalter (2014) confirmed that the tolerance of aquatic insects to metals in laboratory toxicity tests could be more than one order of magnitude higher than that found in contaminated sites, this being likely due to a major route of toxicant uptake via contaminated food rather than contact with the waterborne chemical (Xie and Buchwalter, 2011). These particular features of insect toxicity responses could thus constrain the strength of comparisons between SSDs integrating their responses and SSDs collecting on well characterised toxicity responses by standard planktonic organisms, inherently hampering the conclusions that could be taken in the present study. Hence our option of disinvesting in e.g. testing with insect larvae to further populate the SSDs.

Despite lacking records on insect species, the SSDs collecting on benthic organisms in this study cover a largely under-represented, yet relevant biota group, the microphytobentos (Kefford et al., 2005; Vidal et al., 2014; Wood et al., 2014). In this context, it is worth noticing the sensitivity of the diatom *N. libonensis* evidenced by SSD benthic curves, which corroborated the high sensitivity to a broad array of contaminants claimed before by Vidal et al. (2014). This is remarkable since diatoms play an important role in benthic habitats as producers and biostabilizers of the sediments (Mendes et al., 2014) and are used as biological elements in the evaluation of the ecological status of rivers *sensu* WFD. Although macroinvertebrate assemblages are equally relevant biological elements for the evaluation of ecological status *sensu* WFD (see e.g. Hering et al., 2006a), their integration in SSDs with benthic organisms did not

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reveal a consistent trend regarding the sensitivity distribution when comparing the three tested chemicals.

In fact, benthic diatoms were already shown to respond more rapidly and/or more sensitively to changes in water chemistry, e.g. by nutrient load (Johnson et al., 2006b, 2006a), metal load (as synthetized by Roig et al., 2015) and acidification (Passy et al., 2004). Curiously, there is also evidence that the diatom communities do not respond sensitively compared e.g. with macroinvertebrates to organic contaminant compounds such as pesticides (Hering et al., 2006b; Passy et al., 2004) but this was not reflected in our results regarding DCP.

The specific toxic mode of action of a chemical can partly contribute to the variation in the sensitivity among different taxonomic groups. However, there are evidences of organisms that are generally amongst the most sensitive to a broad collection of toxicant stressors. For example, Wogram and Liess (2001) found that *D. magna* (and Cladocera, in general) is more sensitive to several organic and metal compounds (i.e. regardless the chemical group of the toxicant) compared to many other macroinvertebrate groups like Lamellibranchia, Hirudinea, Gastropoda, Oligochaeta, Isopoda, Tricladida, Coleoptera, Heteroptera, Odonata, Trichoptera, Diptera, Copepoda and Decapoda; while only Ephemeroptera, Ostracoda, Amphipoda and Plecoptera were found to be equally or more sensitive than *D. magna*. In our study, the response of *D. magna* was not so consistent, being this species the most tolerant of planktonic organisms to DCP while the rotifer *B. calyciflorus* was more sensitive to LC (Fig. 1); feeding inhibition could not be recorded at non-lethal concentrations of PD, hence no EC50 for *D. magna* was added to the corresponding SSD, but the corresponding immobilisation EC50 would locate the cladoceran as the most sensitive invertebrate, slightly more tolerant than the microalgae *Chlorella vulgaris* (Table S1; immobilisation

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EC50 for PD of 0.639 (0.577-0.696) mg/L by Loureiro et al., 2011). Wogram and Liess (2001) claim that in natural food webs, species at direct risk when exposed to the littlest levels of chemical stress must be identified and protected by adequately established protection limits, both benefiting from research considering SSDs. Our study suggests that periphyton should be better considered for the establishment of these limits in lotic ecosystems, and identified the diatom, *Navicula libonensis*, as a target for ecotoxicity analysis in this context as per its consistent position as the most sensitive benthic organism tested (Fig.1).

Several authors stress out that only the most sensitive taxonomic groups should be used for hazard assessment based on SSD and the species selection should be preferably according to the substances specific mode of action e.g., primary producers preferred for herbicides and arthropods preferred in case of insecticide testing (Maltby et al., 2009, 2005; van den Brink et al., 2002; von der Ohe and Liess, 2004). By using only the most sensitive groups, the HC5 should decrease, thus allowing more protective benchmarks. In this context, Maltby et al. (2005) reinforced the need to address the representativeness of the species assemblages used in SSDs generation considering the ecosystem to be protected and the chemical being assessed. They concluded that (i) arthropods are indeed the organisms that should be used to generate protective benchmarks for insecticides as the corresponding HCx estimates were significantly lower than those found when non-arthropod communities were tested; (ii) the habitat (freshwater vs saltwater or lotic vs lentic in freshwater) of selected arthropod species can hardly constrain the HCx estimate for insecticides; (iii) SSDs generated based on the responses of sensitive species and standardized monospecific assays indeed reflect the responses of natural functioning assemblages (see in addition Emans et al., 1993; Maltby et al., 2005; Schroer et al., 2004; van den Brink et al., 2002), which frames the

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SSD approach using sensitive laboratory species as a (ecologically) relevant ecotoxicological tool to assist environmental protection.

While Maltby et al. (2005) focused on insecticides, i.e. toxicant chemicals with a well-defined mode-of-action and with inherently known mechanisms of toxicity facilitating the identification of the most sensitive non-target species for environmental assessment, this is not the case of the toxicant chemicals selected for the present study. DCP unspecific toxicity relates with hydrophobicity of the individual compounds and free radical formation (Michalowicz and Duda, 2007). It causes growth inhibition in primary producers by disrupting energy metabolism (Xie et al., 2018), inhibits respiration through the impairment of electron transport in thylakoids and mitochondria (Escher et al., 1996) and photophosphorylation (Berden-Zrimec et al., 2007). DCP has been used as a recommended reference toxicant in standard guidelines for toxicity testing with green microalgae and the duckweed (OECD, 2006a, 2006b). PD (Cr (VI)) has generalist toxic effects in humans (Straif et al., 2009), other animals (Venkatramreddy et al., 2009), plants (Shanker et al., 2005) and microorganisms (ATSDR, 2012; Yao et al., 2008). Cr (VI) penetrates the cell membrane, being then reduced into the more stable form Cr (III) with the production of reactive intermediates (Prabakaran et al., 2006). Cr (III) is an essential element for the energy metabolism (Prabakaran et al., 2006) but the reactive intermediates cause histological and morphological alterations in gills, liver, kidneys, as well as may induce genotoxic effects, including micronucleus proliferation, nuclear abnormalities, DNA damage; several other physiological and metabolic alterations, as well as behavioural disturbances have been mentioned (e.g. Domingues et al., 2010). Pb, dosed as LC here, is not naturally involved in any physiological function, but it induces diverse toxic effects that derive e.g. from primary impairment of the antioxidant defence (through the inhibition of key antioxidant enzymes) and consequent

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oxidative damage of cell membranes (Aouini et al., 2018). The broad and unspecific mechanism of toxicity of the chemicals selected for testing in the present study justifies our focus on covering natural assemblages with representatives of different trophic levels and functional roles, rather than on focussing specific, putatively more sensitive groups. The variation observed in species distribution as to relative sensitivity supports this option. It is worth reinforcing that the exception to this trend was the microphytobenthos, and specifically *N. libonensis*, denoting that this group should be always included in integrative studies appraising the establishment of protective benchmarks in lotic ecosystems.

Conclusion

The SSD approach is widely accepted as a useful method in decision-making regarding the establishment of environmental quality criteria and in the estimation of ecological risks (Del Signore et al., 2016). Our results evidence that *N. libonensis*, a benthic diatom from the microphytobenthos community was the most sensitive to the three tested chemicals in SSDs addressing the benthic compartment, while SSDs with standard planktonic organisms did not consistently show trends in sensitivity ordering. Overall, our results suggest that protective benchmarks (HC5) retrieved from SSDs built with the responses of standard planktonic organisms (which are the most commonly used for regulation purposes) may not adequately protect benthic communities. This highlights the need to revisit benchmark establishment to appropriately cover lotic ecosystems, where structure and function are critically supported by benthic assemblages.

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Acknowledgements. Thanks are due for the financial support to CESAM (UID/AMB/50017 - POCI-01-0145-FEDER-007638), to FCT/MCTES through national funds (PIDDAC) and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. This study was funded by PTDC/ATP-EAM/0063/2014; JI Santos, L Queirós and A Ré are recipients of individual research grants by FCT (SFRH/BD/121341/2016, SFRH/BD/129871/2017 and SFRH/BD/112210/2015, respectively). JL Pereira and T Vidal are funded by national funds (OE), through FCT, in the scope of the framework contract foreseen in the Decree-Law 57/2016 (article 23) changed by Law 57/2017, of July 2017. N Abrantes acknowledges FCT for the contract IF/01198/2014. The authors are grateful to an anonymous reviewer, whose challenging comments greatly contributed to the improvement of the manuscript.

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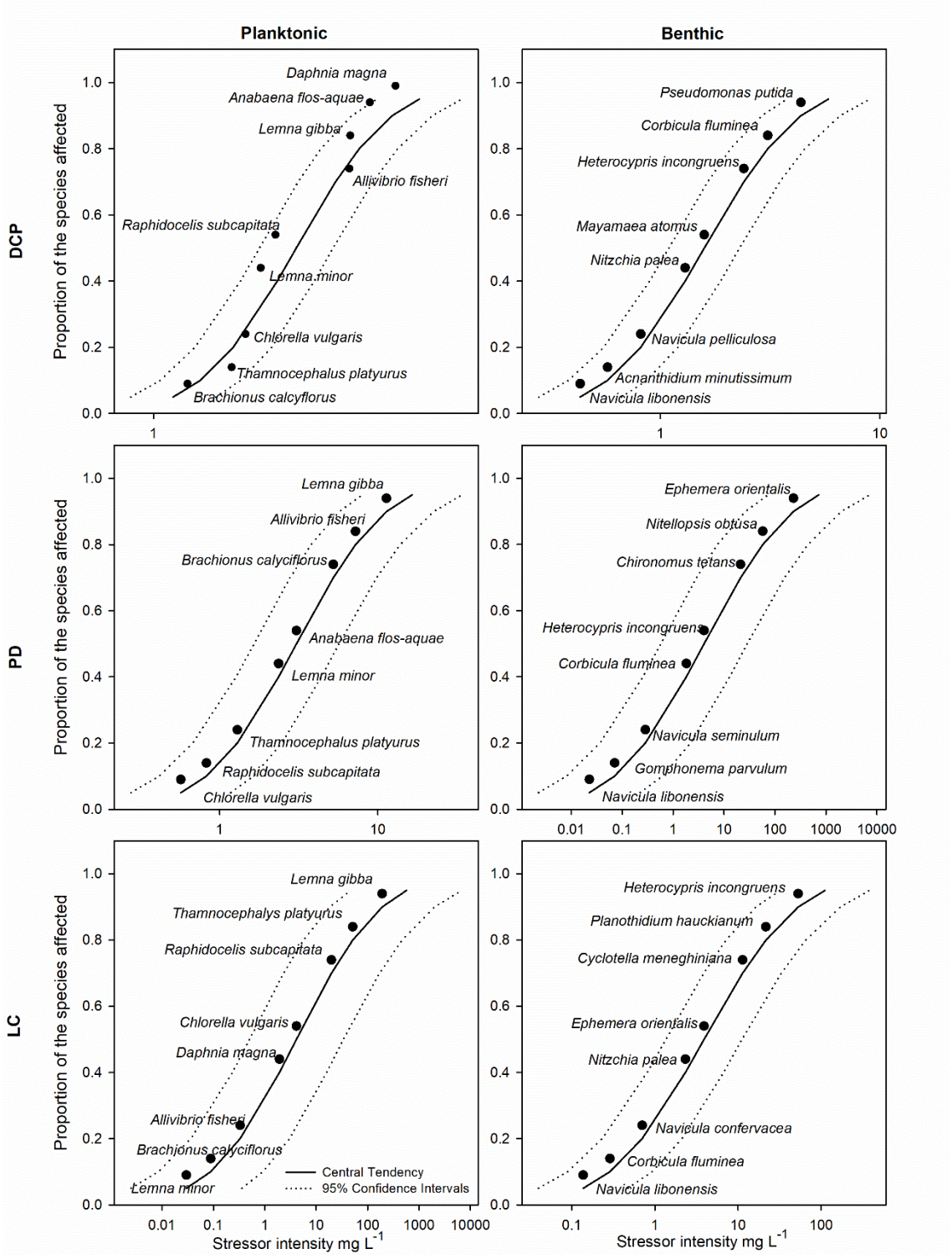

Fig.1. Species Sensitivity Distribution (SSD) plots for DCP (3,5 – dichlorophenol), PD (potassium dichromate) and LC (lead chloride), built on the basis of the non-lethal EC50 values collected from literature and estimated following targeted toxicity tests carried out in the present study, for standard planktonic (left-hand panel) and selected

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benthic (right-hand panel) species. The line expresses the SSD model fitted to the EC50 data and the dotted lines define the corresponding uncertainty.

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Table 1. HC5 and HC50 (Hazard Concentration for 5 and 50% of the community) estimation from the fitted SSDs curves obtained from ecotoxicological bioassays with 3,5-dichlorophenol (DCP), potassium dichromate (PD) and lead chloride (LC), in mg L^{-1}, for selected benthic organisms and standard planktonic freshwater organisms.

	DCP (mg L^{-1})	**PD (mg L^{-1})**	**LC (mg L^{-1})**
Benthic HC_5	0.430 (0.278-0.664)	0.023 (0.002-0.226)	0.137 (0.040-0.472)
Benthic HC_{50}	1.581 (1.091-2.293)	4.056 (0.577-28.520)	3.899 (1.359-11.187)
Planktonic HC_5	1.117 (0.883-1.411)	0.567 (0.274-1.174)	0.030 (0.002-0.358)
Planktonic HC_{50}	2.199 (1.795-2.694)	3.055 (1.645-5.672)	4.126 (0.503-33.824)

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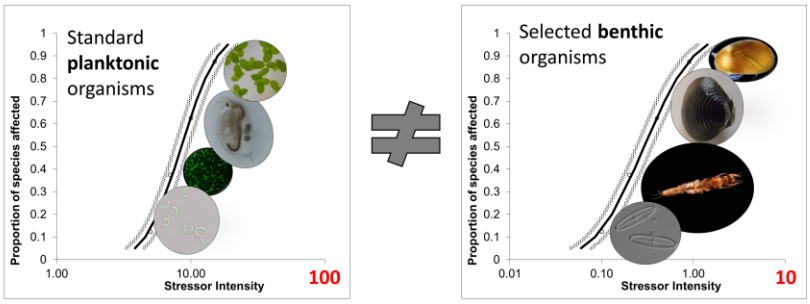
Graphical Abstract



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Highlights

Sensitivity to reference chemicals was assessed regarding standard planktonic species

Sensitivity to reference chemicals was also assessed for selected benthic species

The HC5 discriminated sensitivity between planktonic and benthic organisms

Planktonic species exhibited no consistent pattern in sensitivity ordering

N. libonensis was the most sensitive benthic species to the tested chemicals

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