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Avaliação da toxicidade aguda do Bisfenol A (BPA) em espécies marinhas e estuarinas
tropicais de diferentes grupos tróficos

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Biológicas (Biodiversidade Neotropical) da Universidade Federal do Estado do Rio de Janeiro como requisito para obtenção do grau de Mestre em Ciências Biológicas.

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Resumo

O Bisfenol A é um poluente químico recalcitrante de grande preocupação devido à sua toxicidade, tanto para o meio ambiente quanto para a saúde humana. Este composto está presente em efluentes humanos, o que acarreta em sua entrada nos ecossistemas aquáticos, ocasionando efeitos tóxicos (agudos e crônicos) para espécies de invertebrados e vertebrados. O presente trabalho consiste de um capítulo único, o qual foi publicado na revista *Environmental Pollution*. O objetivo deste artigo foi determinar a toxicidade aguda do BPA para espécies estuarino-marinhas tropicais de quatro níveis tróficos e integrar os valores de toxicidade do BPA usando a análise de distribuição de sensibilidade das espécies (SSD). Os organismos testados foram a microalga *Tetraselmis* sp., o herbívoro zooplanctônico *Artemia salina*, o invertebrado depositívoro *Heleobia australis* e o peixe onívoro *Poecilia vivipara*. A microalga apresentou a maior tolerância entre os organismos testados, sem uma resposta dependente da concentração. A sensibilidade ao BPA aumentou a partir de *A. salina*, seguido de *H. australis*, e *P. vivipara*. Embora tenhamos obtido uma hierarquia de toxicidade em relação aos níveis tróficos, a SSD não revelou um padrão entre os mesmos. O presente trabalho apresentou resultados quanto a toxicidade ao BPA a espécies ainda não testadas e evidenciou respostas distintas entre organismos de diferentes níveis tróficos.

Palavras-chave: BPA, toxicidade, teste de letalidade, LC₅₀, concentração-resposta, distribuição de sensibilidade de espécies

Abstract

Bisphenol A is a recalcitrant chemical pollutant of great concern due to its toxicity, both for the environment and for human health. BPA is a compound that is present in our effluents, which leads to its entry into aquatic ecosystems, causing toxic effects (acute and chronic) for invertebrates and vertebrates. The present work consists of a single chapter, which was published in the journal *Environmental Pollution*. This study aimed to determine the acute toxicity of BPA for tropical estuarine marine species of four trophic levels and to integrate the toxicity values of BPA using the species sensitivity distribution analysis (SSD). The tested organisms were the microalgae *Tetraselmis* sp., the zooplanktonic herbivore *Artemia salina*, the depositivorous invertebrate *Heleobia australis*, and the omnivorous fish *Poecilia vivipara*. The microalgae showed the highest tolerance among the tested organisms, without a concentration-dependent response. Species sensitivity have increased from *A. salina*, followed by *H. australis*, to *P. vivipara*. Although we obtained a hierarchy of toxicity about trophic levels, SSD did not reveal a pattern among them. The present study showed sensitivity results of not yet investigated species and evidenced distinct responses between organisms of different trophic levels.

Keywords: BPA, toxicity, lethality test, LC₅₀, concentration-response, species sensitivity distribution.

Lista de Abreviaturas

BPA – Bisfenol A

CI – Intervalo de confiança (confidence intervals)

EC₅₀ - Concentração de um composto efetiva que causa imobilidade em 50% dos organismos expostos

ECHA - Agência Europeia de Produtos Químicos (European Chemicals Agency)

EFSA – Autoridade Europeia para a Segurança dos Alimentos (European Food Safety Authority)

FA - Fração afetada (fraction affected)

FBW - água salobra filtrada (filtered brackish water)

FSW - Água do mar filtrada (filtered seawater)

HC5 – Concentração que afeta apenas 5% dos organismos expostos. (hazardous concentration)

LC₅₀ – Concentração letal de um composto que causa a mortalidade de 50% dos organismos expostos

SSD - Distribuição de Sensibilidade de Espécies (Species Sensitivity Distribution)

USEPA - Agência de Proteção Ambiental dos EUA (US Environmental Protection Agency)

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Introdução

Com o advento das novas tecnologias, e a demanda por diferentes produtos e serviços, a produção de novos poluentes tem se tornado inevitável. Muitos desses poluentes são ditos como poluentes emergentes, os quais consistem em produtos advindos de atividades industriais como resíduos químicos, medicamentos, cosméticos, pesticidas, plastificantes, surfactantes (Taheran et al., 2018). Muitas vezes esses poluentes estão presentes em nossos efluentes, fazendo com que sejam detectados em águas superficiais, em nossa água potável, assim como em vários outros corpos d'água (Liu et al., 2019). Poluentes emergentes podem ser caracterizados como compostos recalcitrantes, ou seja, que apresentam difícil degradação. As moléculas recalcitrantes podem ser de origem natural ou xenobiótica, isto é, estranhas ao ambiente, advindas de processos antropogênicos (Gaylarde et al., 2005).

O Bisfenol A (BPA) é um desses compostos xenobióticos recalcitrantes, apresentando alta produção, uma vez que seu uso está relacionado à fabricação de resinas epóxi, resinas fenólicas, poliacrilatos, policarbonatos, poliésteres e à fabricação dos revestimentos de latas de alimentos (Staples et al., 1998). Em ambientes aquáticos, ao ser despejado, o BPA pode causar inúmeros impactos, uma vez que seus níveis podem chegar a valores significativos, sendo em média (e valor máximo): 42,3 (63.640) ng L⁻¹ em água doce; 28,6 (5.100) ng L⁻¹ em água salobra; e 17,7 (1.918) ng L⁻¹ na água do mar (Wu & Seebacher, 2020). Além da possível presença em altas concentrações, a persistência deste composto em ambientes aquáticos é variável visto que seu tempo de degradação varia de menos de 5 dias (Kang et al., 2004; Kang & Kondo, 2005) a mais de 30 dias (Ying & Kookana, 2003; Kang & Kondo, 2005). Com isso, a ocorrência e persistência deste composto acarreta em toxicidade, tanto aguda quanto crônica, em organismos presentes em sistemas aquáticos contaminados (revisado em Kang et al., 2007; Mihaich et al., 2009). Alguns dos impactos causados pelo BPA em organismos aquáticos culminam na desregulação do sistema endócrino (revisado em Kang et al., 2007; Pinto et al., 2019); em problemas na reprodução de peixes e em seus estágios iniciais de vida, inibição de crescimento e alteração do comportamento (Lahnsteiner et al., 2005; Zha & Wang, 2006; Wang et al., 2019); e na inibição de processos fisiológicos de algas (Ji et al., 2014; Zhang et al., 2012, 2015; Ben Ouada et al., 2018).

Porém, mesmo com vários estudos a respeito do BPA, os efeitos do mesmo em invertebrados aquáticos e produtores primários ainda não são, de todo, conhecidos, especificamente em espécies marinhas e estuarinas. Os principais estudos e dados disponíveis atualmente para espécies aquáticas se refere a peixes de água doce (como em Metcalfe et al., 2001; Sohoni et al., 2001; Staples et al., 2002; Yokota et al., 2008; Kim et al., 2018, 2020; Pinto et al., 2019).

Alguns estudos com análogos do BPA já mostram que esses compostos podem biomagnificar e bioacumular em organismos aquáticos, o que sugere que o bisfenol A tenha a capacidade de biomagnificar ao longo dos níveis tróficos da cadeia alimentar aquática (Ji et al., 2014; Guo et al., 2017; Wang et al., 2017; Kim et al., 2020; Wu & Seebacher, 2020). No entanto, não se tem registros de estudos quanto aos impactos do BPA em sistemas marinho-estuarinos. Logo, o presente trabalho visou a determinação dos possíveis efeitos da toxicidade aguda do BPA em quatro espécies estuarinas e marinhas de diferentes níveis tróficos, bem como a comparação da sensibilidade das respectivas espécies quanto aos seus níveis tróficos.

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Capítulo Único

Acute toxicity of Bisphenol A (BPA) to tropical marine and estuarine species from different trophic groups

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Abstract

BPA is chemical pollutant of very high concern due to its toxicity to the environment and risks for human health. Environmental concern consists in BPA entrance into aquatic ecosystems due to acute and chronic toxicity to invertebrates and vertebrates. This study aimed to determine acute BPA toxicity to tropical estuarine-marine species of four trophic levels and integrate BPA toxicity values using species sensitivity distribution (SSD) analysis. Our hypothesis is that BPA toxicity increases towards higher trophic levels. Microalga (*Tetraselmis* sp.), zooplanktonic grazer (*Artemia salina*), deposit-feeder invertebrate (*Heleobia australis*), and omnivorous fish (*Poecilia vivipara*) were chosen as experimental models. *Tetraselmis* sp. showed the highest BPA tolerance, without a concentration-dependent response. Species sensitivity have increased from *A. salina* ($LC_{50,96h}=107.2 \text{ mgL}^{-1}$), followed by *H. australis* ($LC_{50,96h}=11.53.5 \text{ mgL}^{-1}$), to *P. vivipara* ($LC_{50,96h}= 3.5 \text{ mgL}^{-1}$). Despite the toxicity hierarchy towards trophic levels, which partially supported our hypothesis, SSD did not evidence a clear pattern among estuarine-marine trophic groups. Our study disclosed the sensitivity of not yet investigated species to BPA and, in an integrative way, highlighted BPA toxic effects at different trophic levels. Although estimated acute hazardous concentration ($HC5= 1.18 \text{ mg L}^{-1}$) for estuarine and marine species was higher than environmentally relevant concentrations, sublethal adverse effects induced by BPA exposure may lead to unbalances in population levels and consequently affect the ecological functioning of tropical coastal systems.

Capsule: Acute BPA toxicity was determined to tropical estuarine-marine species of distinct trophic levels, their LC_{50} s were integrated for SSD and $HC5$ analyses.

Keywords: concentration-response; endocrine disrupters; lethality test; LC_{50} ; Species Sensitivity Distribution (SSD).

1. Introduction

Bisphenol A (BPA), 2,2-bis(4-hydroxydiphenyl) propane, is one of the most used industrial chemicals worldwide that has been applied as a primary raw material for the production of polycarbonate plastics, epoxy resins, phenolic resins, and food lacquer coatings (Staples et al., 1998). World production of BPA reaches more than 7.3 billion of dollars annually and more than 100 tons year⁻¹ are released into the environment (Vandenberg et al., 2009; Corrales et al., 2015; Repossi et al., 2016). BPA is listed as a chemical compound of very high concern in the European Union (EU) due to its toxicity to reproduction (European Chemicals Agency - ECHA ED/30/2017) and risk of endocrine disrupting effects for the environment (ECHA ED/01/2017) and human health (ECHA ED/01/2018). Despite its toxicity, BPA is still permitted for general use in materials that are in contact with food; at least while the European Food Safety Authority (EFSA) reevaluates the risks of BPA in foodstuffs to public health. Due to the regulations on BPA (e.g., banned use in baby bottles), a variety of bisphenol analogues is being widely manufactured and applied; however, BPA is still one of the most predominant analogues that contaminates aquatic systems (Wang et al., 2017).

The major environmental risk source consists in BPA entrance into aquatic ecosystems through the release of effluents, sewage treatment wastewater, landfill leachate or natural degradation of other compounds (Yamamoto et al., 2001; Wintgens et al., 2003; Gatidou et al., 2007). Considering the inputs of human activities and local characteristics of aquatic ecosystems, BPA contamination may reach high levels with geometric mean (and maximum) recorded values of 42.3 (63,640) ng L⁻¹ in freshwater, 28.6 (5,100) ng L⁻¹ in brackish water, and 17.7 (1,918) ng L⁻¹ in seawater (meta-analysis performed by Wu & Seebacher, 2020). Moreover, the positive relationship between BPA contamination and microplastic particles in marine fish also suggests that plastic leachates may act as an important source of BPA contamination for aquatic species (Barboza et al., 2020). For this reason, BPA may lead to acute and chronic toxicity to aquatic invertebrate and vertebrate species in contaminated environments (reviewed in Kang et al., 2007; Mihaic et al., 2009). Several endocrine-disruptive effects of BPA have been detected in aquatic species (reviewed in Kang et al., 2007; Pinto et al., 2019). Chronic exposure to BPA was also related to some injuries in fish reproduction and early life stages, inhibition of male fish growth, and abnormal fish behavior (Lahnsteiner et al., 2005; Zha & Wang, 2006; Wang et al., 2019). Early life stages of invertebrates and vertebrates are highly

sensitive to BPA exposure, in which invertebrates and amphibians seem to be particularly affected (reviewed in Wu & Seebacher, 2020). In addition, BPA inhibition of algae physiological processes (e.g. cell division and photosynthesis) has been also detected (Ji et al., 2014; Zhang et al., 2012, 2015; Ben Ouada et al., 2018).

Despite the growing number of studies (e.g., Chen et al., 2002; Hill et al., 2002; Pascoe et al., 2002; Mihaich et al., 2009; Guo et al. 2017; Ben Ouada et al., 2018; Tato et al., 2018), the BPA effects on aquatic invertebrates and primary producers is still barely known, especially for marine and estuarine species. Most of the available effect data on aquatic organisms has been performed using freshwater fish (e.g., Metcalfe et al., 2001; Sohoni et al., 2001; Staples et al., 2002; Yokota et al., 2008; Kim et al., 2018, 2020; Pinto et al., 2019). Bioaccumulation and biomagnification of bisphenol analogues have been detected in aquatic organisms and their trophic magnification factors indicate that bisphenol is prone to biomagnify in aquatic food webs (Ji et al., 2014; Guo et al., 2017; Wang et al., 2017; Kim et al., 2020; Wu & Seebacher, 2020). However, the impacts of BPA within marine-estuarine systems context, i.e., populations from different trophic levels, have never been evaluated.

Therefore, the present study aimed to determine acute BPA toxicity to four estuarine and marine species from distinct trophic levels, as well as to compare species sensitivity regarding their trophic levels. For that, a species sensitivity distribution (SSD) curve was assembled and hazardous concentration (HC5) and fraction affected (FA) estimated based on our results and data available in the literature for marine and estuarine species amongst the four trophic groups. Our hypothesis is that BPA toxicity increases towards higher trophic levels. The primary producer, zooplanktonic grazer, invertebrate deposit-feeder, and omnivorous fish chosen as model organisms are representative species of tropical coastal ecosystems. In addition to disclose the sensitivity of not yet investigated species to BPA, the present study highlights, in an integrative way, the BPA toxic effects at different trophic levels providing evidences of the risks for estuarine and marine species and addressing their implications to ecological functioning of tropical coastal systems.

2. Material and Methods

2.1 Organisms

A unicellular primary producer, two invertebrates - one grazer and one depositivore, and one omnivorous fish were chosen for acute experiments to evaluate the risk of BPA exposure. Three of them are representative species of coastal aquatic systems that were already used for toxicity tests (US EPA, 1975; OECD, 2011; OECD, 2019): the chlorophyte microalga *Tetraselmis* sp. (Falcão et al., 2020; Li et al., 2017), the crustacean *Artemia salina* (Nunes et al., 2006), and the fish *Poecilia vivipara* (Almeida et al., 2019; Hawkins et al., 2003; Wester & Vos, 1994). The benthic gastropod *Heleobia australis* is a non-standard organism that was tested for the first time as candidate species for toxicity assays. This aquatic gastropod has been proposed as a bioindicator species in a tropical estuarine system (Neves et al., 2013a) and plays a crucial role for nutrient cycling in muddy compartments of aquatic environments (Figueiredo-Barros et al., 2006). Moreover, the snail *H. australis* comprises most of the recommended criteria for toxicity test species selection (Chapman, 2002)

Clonal culture of *Tetraselmis* sp. used in the present study was isolated from Guanabara Bay, Rio de Janeiro state (22°46'05.73" S, 43°10'04.31" W). Microalga stock culture was maintained in filtered seawater - FSW (glass-fiber filter, MilliporeAP-40, Millipore Brazil) supplemented with L2 enrichment medium. The culture was grown at exponential growth phase at a cell density of 1.14×10^6 cells mL⁻¹ in controlled conditions at Laboratory of Cultures and Experiments from the Federal University of the State of Rio de Janeiro (UNIRIO); for detailed culture conditions see Neves et al. (2019). Adult individuals of the brine shrimp *A. salina* were obtained from a specialized aquafarm and acclimated for experimental conditions 48 h-prior to acute toxicity test.

Adult individuals of *H. australis* and *P. vivipara* were collected at Rodrigo de Freitas Lagoon, Rio de Janeiro, Brazil (22° 58' 16.02" S, 43° 12' 42.18" W). Scientific research and collecting permit authorizing field studies were obtained from Chico Mendes Institute for Biodiversity Conservation (ICMBio), Brazilian Ministry of the Environment (permits numbers: 48201-2 and 56897-6). Rodrigo de Freitas Lagoon is an urban estuarine system (Vezzone et al., 2019), with no prior report of BPA contamination. Samples of superficial mud sediment (≈ 5 cm) were collected from shallow littoral sites (<30 cm depth) using a trowel and sieved (1 mm mesh net) to keep the adult snails. In the laboratory, *H. australis* was kept in a 1.5 L container filled with sediment

and water from the sampling site for 72 h of acclimatization to experimental conditions (24°C). Individuals of *P. vivipara* were caught in the same site of *H. australis* through a baited cylindrical trap (60 cm long × 25 cm height; 3 mm mesh) which was set in a shallow (<50 cm depth) area of the lagoon and retrieved after 2 h. After transportation to the laboratory, fish were acclimated to experimental conditions (24°C) in an aquarium (40 L) filled with aerated and filtered brackish water (FBW) for 14 days before experiments. Water was kept in the salinity measured at sampling site, thus experimental water salinity for gastropod and fish was adjusted to 6.87 using deionized water and seawater, after filtration (glass-fiber filter, MilliporeAP-40, Millipore Brazil) and sterilization by autoclaving. In addition, *P. vivipara* individuals were fed twice a day *ad libitum* with commercial fish food.

2.2 Stock and Test Solution Preparation

BPA stock solution (300 mg L⁻¹) was prepared by placing the weighed quantity of the commercial chemical BPA (Sigma-Aldrich, USA, purity 99%; CAS number: 80-05-7; EC number: 201-245-8) in a volumetric flask and bringing it to the appropriate volume with FSW at salinity 34 (for microalgae and crustacean assays) or FBW at salinity 6.87 (for gastropod and fish assays). The stock solution was stored at room temperature in the dark (covered volumetric flask). Exposure concentrations of BPA were prepared by serial dilutions (i.e., reducing its concentration by a fixed factor) of the stock solution using FSW or FBW according to the test organism.

2.3 Experimental design

Acute toxicity of BPA was assessed after 24, 48, 72 and 96 h of exposure, according to internationally recognized guidelines - Standard Evaluation Procedure guidelines (US EPA, 1975; US EPA, 1985). Aquatic organisms were exposed to eight decreasing concentrations of BPA. In controlled experimental conditions at 25°C, the recovery rate from chromatographic analysis for determining BPA concentrations in seawater is higher than 90% (Economou et al., 2019); thus, differences between nominal and detected concentrations are considered negligible in short-term assays. Moreover, since BPA degradation is not readily (>30 days) in seawater (Kang & Kondo, 2005) and marine sediments (half-life of 14.5 days; Ying & Kookana, 2003), BPA degradation was considered negligible in our short-term incubations (24-96 h). Negative controls were

performed by incubating the test organisms in FSW (microalga and crustacean) or FBW (gastropod and fish) without BPA. All the toxicity tests were carried out using sterilized glass laboratory supplies (e.g., bottles, tubes, flasks, pipettes) in a BOD incubator at 24 °C and a photoperiod of 12:12 h to simulate the environmental conditions. Moreover, preliminary tests were performed in order to set an appropriate range of BPA concentrations for acute assays.

2.3.1 Primary producer microalga

The microalga *Tetraselmis* sp. (1.5×10^5 total cells per replicate) were exposed to 15 mL of BPA solutions (2.34, 4.69, 9.38, 18.75, 37.5, 75, 150 and 300 mg L⁻¹) or FSW (negative controls) in glass culture tubes (23 mL). Three independent replicates of each BPA concentration and three replicates of negative control (without BPA) were performed. Homogenized aliquots of 2 mL were collected by experimental replicate and control using glass Pasteur pipette after 24, 48, 72 and 96 h of BPA exposure and then preserved in neutral lugol iodine solution. Microalga density was assessed by counting the lugol preserved cells on a Fuchs-Rosenthal counting chamber (three analytical replicates) using an inverted microscope (Primovert Zeiss). Finally, microalgal cell density was converted to growth rate (μ) according to the equation:

$$\mu \text{ (day}^{-1}\text{)} = \left(\frac{\text{Ln } D_1 - \text{Ln } D_0}{t_1 - t_0} \right)$$

where, D_1 is the cell density at the time 1, D_0 is the cell density at the beginning of incubation and t_1-t_0 is the exposure time interval evaluated in days.

2.3.2 Zooplanktonic grazer

Ten individuals of adult brine shrimp were placed in a glass Petri dish (90 x 15 mm) with 15 mL of each BPA solution (2.34, 4.69, 9.38, 18.75, 37.5, 75, 150 and 300 mg L⁻¹) or FSW (control). Experiments were performed using three replicates for each BPA concentration and three replicates for negative control (without BPA). Brine shrimp survival was monitored by counting the dead individuals after 24, 48, 72 and 96 h of BPA exposure using a stereomicroscope (Leica EZ4HD).

2.3.3 Deposit-feeder snail

Previously to experimental incubations, adult snails were sorted by size (> 2.0 mm) and their shells were gently washed with FBW to remove soft sediment particles. Ten individuals were placed in a glass Petri dish (90 x 15 mm) with 15 mL of each BPA solution (2.94, 3.67, 4.59, 5.73, 7.17, 8.96, 11.2 and 14 mg L⁻¹) or FBW (negative control). Three true replicates were applied for each BPA concentration and three replicates for the negative control. Gastropod survival was monitored by the counting dead individuals after 24, 28, 72 and 96 h after BPA exposure using a stereomicroscope (Leica EZ4HD).

2.3.4 Omnivorous fish

Adult individuals were weighed using a precision balance (0.001 g) and sexed by gonopodium presence (i.e., male) or absence (i.e., female) before incubations. Experimental replicates consisted in the incubation of one male fish ($\bar{X} \pm \text{SD}$; 0.53 ± 0.11 g wet weight) into 500 mL glass container filled with 300 mL of BPA solution (2.94, 3.67, 4.59, 5.73, 7.17, 8.96, 11.2 and 14 mg L⁻¹) or FBW (negative control). Three true replicates were performed by each BPA concentration and negative control. Individuals were fed twice a day with flake fish (Alcon Guppy®) food per replicate; however, in order to reduce feed effect in assays, the flake fish food was administered in amounts enough to guarantee that all food has been consumed immediately and without leftovers (as proposed by Salomão et al., 2020). Fish mortality was visually monitored at each exposure time interval (24, 48, 72 and 96 h) by counting the dead individuals.

2.4 Statistical analyses

Growth rate and survival proportion (i.e., number of alive cells/individuals by the initial number at t₀) was calculated by independent replicate for each exposure time. Arithmetic mean of independent replicates by BPA concentration and negative controls were used to calculate the cumulative percentage of growth inhibition (for microalga) or mortality (for invertebrates and vertebrate species). One-way analysis of variance (ANOVA) was carried out to evaluate the influence of BPA concentrations on microalga density or survival proportion of brine shrimp, gastropod and fish by the four exposure times (i.e., 24, 48, 72, and 96 h) independently (i.e., crossed factors). If necessary, data were transformed into square root (density data) or arcsine of square root (proportion

data) to conform the parametric test assumptions. Before the parametric analysis, Levene and Kolmogorov-Smirnov tests were applied to assess the homogeneity of variance and normality, respectively, of data distribution. Whenever parametric assumptions were not met, the non-parametric Kruskal-Wallis test was performed. Parametric and nonparametric tests were considered statistically significant if $p \leq 0.05$. Statistical analyses were performed using the software Statistica 8.0 (StatSoft).

The EC₅₀ or LC₅₀ (i.e., the BPA concentration that gives half-maximal response) and 95% of confidence intervals (CI) were determined for 96 h of exposure using the algal density or survival data normalized to the average of the negative controls. A concentration-response curve (variable slope model) with the least squares fitting method was applied after log-transformation of x-axis values (BPA nominal concentrations) using the equation:

$$Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + 10^{(\text{LogEC}_{50} - X) \times \text{Hill Slope}}}$$

where Top and Bottom are plateaus in mg L⁻¹ unit. Results were accepted if concentrationresponse curves had a $R^2 \geq 0.75$. Non-linear regressions and graphics were conducted using the software GraphPad Prism 8.02 (Graph Pad).

Effective and lethal concentration values (EC_{50s} and LC_{50s}) obtained in the present study were integrated with acute toxicity data of BPA to estuarine and marine species available in the literature to derive the Species Sensitivity Distribution curve (SSD's) and estimate Hazard Concentration for 5% of the species (i.e., HC5 – safety value that protect 95% of species from determined system) and the fraction affected (FA) at HC5 results. Data selection criteria from literature consisted in clearly reported EC/LC_{50s} values for marine and estuarine species representative of the following groups: primary producer microalga, zooplanktonic grazer, deposit-feeder snail, and omnivorous fish. The SSD model was constructed by fitting cumulative probability distribution of a full set of toxicity data, as proposed by Posthuma et al. (2002). SSD was generated using the US Environmental Protection Agency (USEPA) spreadsheet (SSD Generator V1). HC5 and FA were estimated using the software ETX 2.2 (RIVM) based on environmental BPA concentrations in surface waters of estuaries (n= 158) and marine systems (n= 202) (data compiled by Wu & Seebacher, 2020).

3. Results

The presence of BPA significantly affected microalgal growth and the survival of brine shrimps, gastropods and fish for all the exposure times tested - 24 h, 48 h, 72 h, and 96 h. All the specific results are presented in the following subtopics.

3.1 Primary producer microalga

Density of the microalga *Tetraselmis* sp. was significantly reduced by BPA at all the exposure times tested: 24 h (ANOVA, $F_{8,27} = 9.05$, $p \leq 0.001$), 48 h (ANOVA, $F_{8,27} = 4.16$, $p = 0.006$), 72 h (ANOVA, $F_{8,27} = 7.31$, $p \leq 0.001$), 96 h (Kruskal-Wallis, $H_{8,27} = 18.91$, $p = 0.015$). Negative growth rates were found for *Tetraselmis* sp. throughout the exposure time tested, even for the lowest BPA concentration (2.34 mg L^{-1}) (Fig. 1). A similar pattern in microalgal growth rate was found for treatments with the lower BPA concentrations (2.34 , 4.69 , 9.38 and 18.75 mg L^{-1}). In these BPA treatments, the growth rate of *Tetraselmis* sp. was similarly affected after 24-48 h of incubation and a reduction in BPA toxic effects (i.e., increase in growth rates) was noticed after 72-96 h of exposure (Fig. 1A-B). In contrast, a more pronounced effect in microalgal growth was shown after 48 h of exposure to 37.50 mg L^{-1} of BPA and the toxicity intensity was similar to those found at treatments with low BPA concentrations for the other incubation times tested (Fig. 1B). At the higher BPA concentrations (75 , 150 and 300 mg L^{-1}), a more severe effect in microalgal growth rate was evidenced after 24 h of exposure to the pollutant followed by a decreased tendency to growth inhibition from 48 to 96 h of incubation (Fig. 1C).

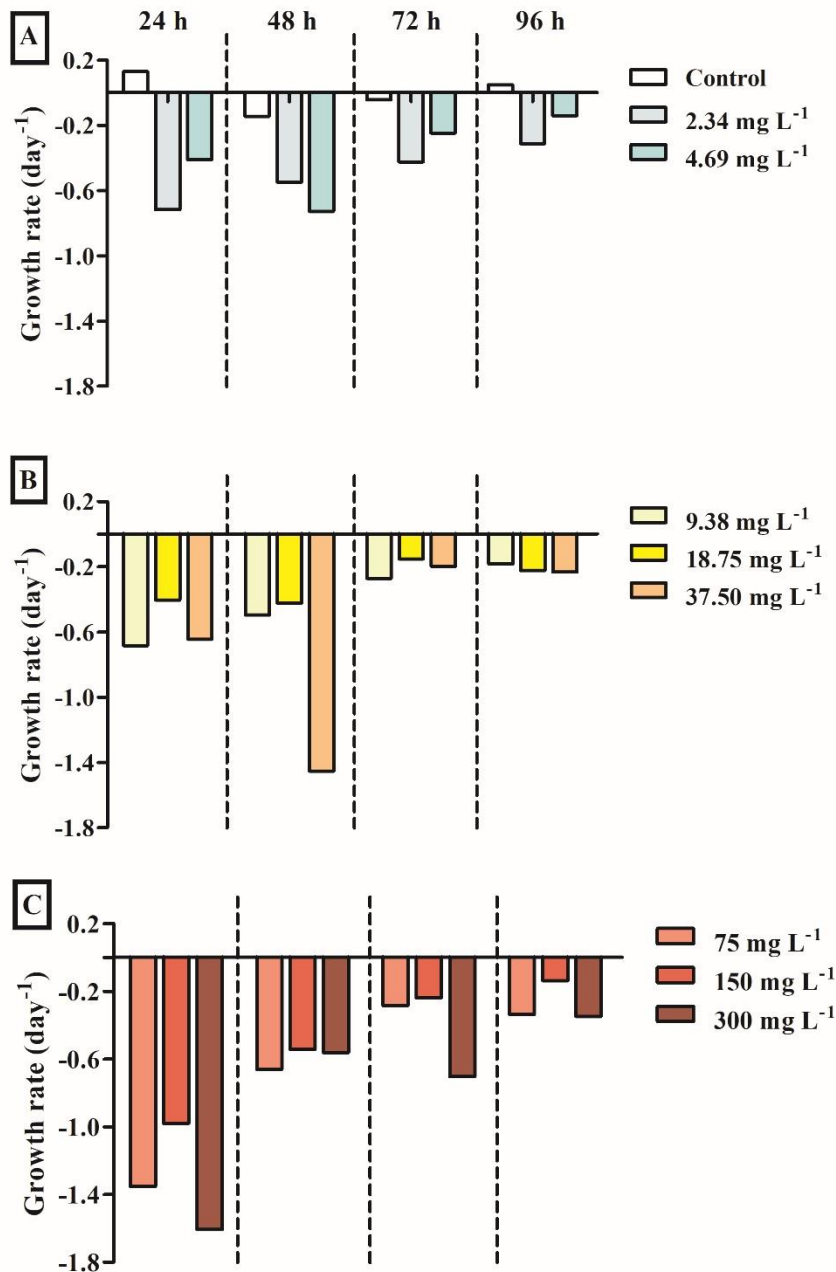


Fig. 1: Growth rate (day^{-1}) of the microalga *Tetraselmis* sp. after 24 h, 48 h, 72 h and 96 h of incubation with filtered seawater (control) and BPA solutions. (A) control, 2.34 and 4.69 mg L^{-1} ; (B) 9.38, 18.75 and 37.50 mg L^{-1} ; (C) 75, 150 and 300 mg L^{-1} . Data are presented as mean ($n=3$ per treatment and time).

The higher percentage in growth inhibition (33.3-79.97%) was induced after 24 h of BPA exposure (Table 1) and a concentration-dependent effect was shown from 18.75 to 300 mg L^{-1} . In contrast, algal growth inhibition was more pronounced after 48 h of exposure ($\sim 42\%$) at treatment with BPA concentration of 4.69 mg L^{-1} . A reduction in

BPA toxicity in growth rates of *Tetraselmis* sp. was observed after 48-72 h at treatments with higher concentrations, and after 72-96 h at treatments with medium to low BPA concentrations. Cumulative growth inhibition (%) showed great variability amongst BPA concentrations from 48 to 96 h of exposure. Both the lowest and highest tested concentrations (2.34 and 300 mg L⁻¹, respectively) showed similar cumulative values after 96 h of BPA exposure (Table 1).

Table 1. Cumulative growth inhibition (%) for the microalga *Tetraselmis* sp. exposed to BPA solutions and negative control.

BPA (mg L ⁻¹)	Growth inhibition (%)			
	24 h	48 h	72 h	96 h
0	-13.77*	37.50	11.88	-1.25*
2.34	51.23	66.85	72.08	71.25
4.69	33.73	75.60	52.50	43.13
9.38	49.57	69.95	55.63	100.00
18.75	33.30	51.85	36.88	59.17
37.50	47.47	93.67	44.58	60.42
75	74.13	64.35	57.29	73.96
150	62.47	67.5	51.25	42.29
300	79.97	73.75	87.92	75.21

*negative values mean positive algal growth

3.2 Zooplanktonic grazer

A significant decrease in the survival of the planktonic crustacean *Artemia salina* was evidenced at all the exposure times tested: 24 h (Kruskal-Wallis, $H_{8,27} = 18.52$, $p = 0.018$), 48 h (Kruskal-Wallis, $H_{8,27} = 20.39$, $p = 0.009$), 72h (Kruskal-Wallis, $H_{8,27} = 19.25$, $p = 0.014$), and 96 h (Kruskal-Wallis, $H_{8,27} = 18.01$, $p = 0.021$). Moreover, time and concentration-dependent effect of BPA on *A. salina* mortality was detected (Table 2). There was a tendency to increase in toxicity with exposure time, as well as a significant increase in mortality at the two highest concentrations (i.e., 150 and 300 mg L⁻¹ BPA).

Table 2. Cumulative mortality (%) of *Artemia salina* individuals exposed to BPA solutions.

BPA (mg L ⁻¹)	Cumulative mortality (%)			
	24 h	48 h	72 h	96 h
0	26.67	50.00	50.00	56.67
2.34	26.67	33.33	36.67	46.67
4.69	26.67	43.33	56.67	66.67
9.38	10.00	20.00	26.67	33.33
18.75	16.67	40.00	50.00	56.67
37.5	20.00	33.33	46.67	56.67
75	26.67	43.33	50.00	60.00
150	46.67	83.33	96.67	100.00
300	96.67	100.00	100.00	100.00

3.3 Deposit-feeder snail

BPA exposure significantly affected the survival of the benthic snail *H. australis* at all tested times: 24 h (Kruskal-Wallis, $H_{8,27} = 18.92$, $p = 0.015$), 48 h (Kruskal-Wallis, $H_{8,27} = 21.82$, $p = 0.005$), 72 h (Kruskal-Wallis, $H_{8,27} = 23.43$, $p = 0.003$), and 96 h (Kruskal-Wallis, $H_{8,27} = 23.61$, $p = 0.003$). No death was recorded at negative controls and

the lower tested BPA concentrations (i.e., 0.063, 0.125, 0.25, and 0.5 mg L⁻¹) (Table 3). At the concentration of 7.17 mg BPA L⁻¹, the same mortality percentage (30%) occurred at all exposure times (Table 3). No significant time effect on snail's mortality was detected at BPA concentration of 1.0 mg L⁻¹. However, time- and concentration-dependent effects were shown in *H. australis* mortality at higher BPA concentrations (i.e., 2.0 – 8.0 mg L⁻¹) (Table 3).

Table 3. Cumulative mortality (%) of *Heleobia australis* snails exposed to BPA concentrations.

BPA (mg L ⁻¹)	Cumulative mortality (%)			
	24 h	48 h	72 h	96 h
0	0	0	0	0
0.063	0	0	0	0
0.125	0	0	0	0
0.25	0	0	0	0
0.50	0	0	0	0
1.0	30.00	30.00	30.00	30.00
2.0	10.00	13.33	20.00	30.00
4.0	23.33	43.33	46.67	60.00
8.0	66.67	73.33	93.33	100.00

Moreover, *H. australis* snails presented excessive relaxation and numbness of the mantle before their death (e.g., 1-8 mg L⁻¹) or as a sublethal response at concentrations that did not cause death (e.g., 0.125 mg L⁻¹) during acute exposure.

3.4 Omnivorous fish

The survival of *P. vivipara* individuals was significantly affected by the exposure to BPA (Kruskal-Wallis, H_{8,27} = 22.92, p = 0.0035). There was no fish mortality at the lower tested concentrations (2.94 – 7.17 mg BPA L⁻¹) (Table 4). In addition, no time-effect of

BPA exposure was recorded for fish mortality (Table 4); thus, BPA concentration effect on individual's survival was the same for all exposure times tested. Fish mortality was just recorded at BPA concentrations higher than 2.0 mg L⁻¹ (Table 4) and the same mortality response (100%) was detected at the highest BPA concentrations (i.e., 4.0 and 8.0 mg L⁻¹).

Table 4. Cumulative mortality (%) of *Poecilia vivipara* individuals exposed to BPA concentrations.

BPA (mg L ⁻¹)	Cumulative mortality (%)			
	24 h	48 h	72 h	96 h
0	0	0	0	0
0.063	0	0	0	0
0.125	0	0	0	0
0.25	0	0	0	0
0.50	0	0	0	0
1.0	0	0	0	0
2.0	66.67	66.67	66.67	66.67
4.0	100.00	100.00	100.00	100.00
8.0	100.00	100.00	100.00	100.00

Abnormal behavior was detected in exposed fish just before its death at BPA concentrations of 2-8 mg L⁻¹. Fish showed some signs of intoxication such as rapid gill movement, erratic swimming pattern, circular swimming, and swimming upside-down or in vertical position. No stress signal was detected in fish from control or exposed to nonlethal BPA concentrations (i.e., 0.063 – 1 mg L⁻¹).

3.5 BPA acute toxicity among trophic groups

Only data obtained after 96 h of BPA exposure showed a proper fit to concentrationresponse curve for both invertebrates and vertebrate, allowing to compare BPA toxicity among the different trophic groups tested (Fig. 2). Except for the microalga *Tetraselmis* sp., LC₅₀ values for invertebrates and vertebrate species were validated

according to validation criteria presented in section 2.4 ($R^2 \geq 0.75$). Validated LC_{50} values obtained for tested organisms are presented in Table 5. For the microalga, a tendency of concentration-dependent effect in algal growth was shown after 24 h of exposure to BPA from 18.75 to 300 $mg L^{-1}$; however, EC_{50} value did not meet validation criteria. For the other exposure times (48-96 h), algal growth data did not adjust to concentration-response model for the range of BPA concentrations tested. In general, microalgal growth was similarly affected by increasing BPA concentrations without a marked concentration-response effect (Fig. 2).

Table 5. Concentration-response LC_{50} (95% CI) results ($mg L^{-1}$) after 96 h of BPA exposure for the tested organisms from distinct trophic groups. All the assays shown met the validation criteria.

Trophic group	Species	LC_{50} ($mg L^{-1}$)	Confidence intervals (95%)	R^2
Filter feeder	<i>Artemia salina</i>	107.2	19.9 - 579.1	0.76
Deposit feeder	<i>Heleobia australis</i>	11.5	2.9 - 44.9	0.90
Omnivore	<i>Poecilia vivipara</i>	3.5	0.9 - 13.3	0.79

Distinct species sensitivity to BPA has been found according to the trophic group evaluated (Table 5), in which a rank toxicity hierarchy was evidenced towards the species tested as follows: microalga *Tetraselmis* sp.* < brine shrimp *A. salina* < gastropod *H. australis* < fish *P. vivipara*

*Although microalgae EC_{50} were not validated, growth inhibition response was similar for entire BPA concentration range tested (2.94 - 300 $mg L^{-1}$).

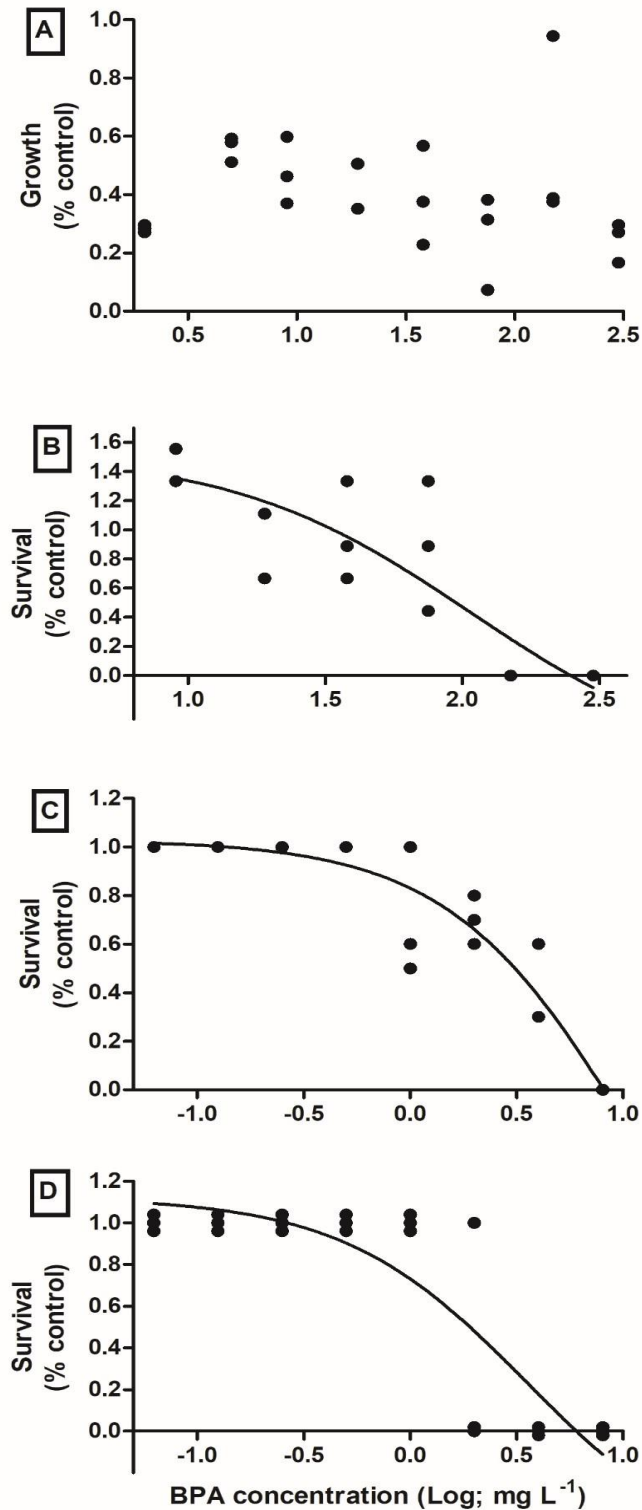


Fig. 2: Dose-response curves between BPA concentrations (Log-transformed) and growth or survival at 96 h exposure (normalized by controls) for each tested organism. A. primary producer microalga *Tetraselmis* sp.; B. filter feeder crustacean *Artemia salina*; C. deposit feeder mud snail *Heleobia australis*; D. omnivorous fish *Poecilia vivipara*.

The SSD curve assembled for estuarine and marine species exposed to BPA is presented in Fig. 3. Data obtained in the literature resulted in 14 values (Supplementary Material S1), consisting in eight different species, that were integrated with the three effective concentrations determined in the present study; thus, data from eleven different species (N) were included in SSD analysis. Compiled toxicity data of BPA showed a good adjustment and SSD curve presented high explicability value ($R^2= 0.968$). However, considering the scarcity of BPA concentration effective data for marine and estuarine species with similar ecological traits, few species were representative of each trophic group (e.g., two species of microalga, five species of zooplankton grazer, one species of depositfeeder snail, and five species of omnivorous fish).

The integrated EC/LC₅₀ values ranged from 1.0 mg L⁻¹ for the diatom *Skeletonema costatum* to 107.2 mg L⁻¹ for the crustacean *Artemia salina*. No clear pattern of BPA sensitivity was evidenced among the different trophic groups evaluated (i.e., primary producer microalga, zooplanktonic grazer, deposit-feeder snail, and omnivorous fish). *Artemia* species were the most tolerant organisms, occupying the top of the curve. While the most sensitive species were represented by the microalga *S. costatum* and two guppy fish (*P. reticulata* and *P. vivipara*). The estimated HC5 value (i.e., the value that protects 95% of the species) was of 1.18 (0.38-2.49) mg L⁻¹, in which lower and upper values of fraction affected (FA) at HC5 results were 1.10 and 15.22.

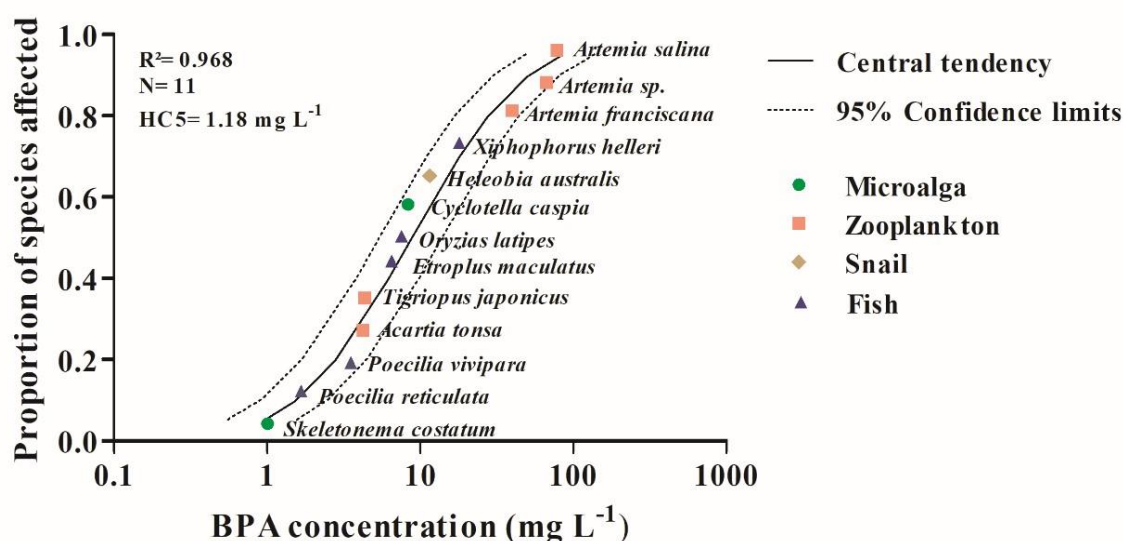


Fig. 3: SSD curve for acute toxicity of BPA (x-axis in Log₁₀) with respect to estuarine and marine species. Individual points represent the acute EC/LC₅₀ values discriminated by microalga (●), zooplanktonic grazer (■), deposit-feeder snail (◆) and omnivorous fish

(▲). Lines represent the central tendency (full line) and 95% confidence limits (dotted lines) results of the logistic regression on these data.

4. Discussion

The present study was designed to provide insights of BPA effects to tropical marine and estuarine organisms by evaluating its acute toxicity on representative species of different trophic groups: primary producer microalga, zooplanktonic grazer, depositfeeder snail, and omnivorous fish. Data of acute BPA toxicity to marine and estuarine biota barely known and, so far, is focused on few model species. Despite the limited available data of BPA effective concentration effects on marine and estuarine organisms, we have combined the available EC/LC₅₀ values distinguished by trophic groups using an SSD analysis as a tool to rank species sensitivity and estimate HC5. To our knowledge, a similar approach integrating toxicity data of BPA for marine and estuarine species has not been done before. Specific topics of each tested group and the integrated toxicity comparison are discussed in the following subtopics.

4.1 Primary producer microalga

Growth inhibition of marine microalgae is one of the most used parameters (Van Wezel & van Vlaardingen, 2004) to assess toxicity of emerging pollutants, including the plastic-derived compounds (Casado et al., 2013; Besseling et al., 2014; Zhang et al., 2012; Ben Ouada et al. 2018). In the present study, the exposure of *Tetraselmis* sp. to increasing BPA concentrations (2.34 – 300 mg L⁻¹) have induced growth inhibition (i.e., negative growth rates) during short-term experiment (24-96 h). Acute exposure to BPA has inhibited the growth of many Chlorophyceae microalgae at different concentrations and exposure times. For example, significant reductions in microalgal growth were detected for *Chlorella pyrenoidosa* (24 h: 50 mg L⁻¹; 48 h: 25 and 50 mg L⁻¹; 72 h: 10-50 mg L⁻¹; and 96 h: 1-50 mg L⁻¹) and *Scenedesmus obliquus* (24 h: 10-50 mg L⁻¹; 48-96 h: 25 and 50 mg L⁻¹) (Zhang et al., 2012), and *Picocystis* sp. (24-144 h: 25-75 mg L⁻¹) (Ben Ouada et al., 2018).

In the present study, the higher intensity in growth inhibition of *Tetraselmis* sp. was induced after 24 h of exposure to BPA. A reduction in BPA toxic effects on microalgal growth were evidenced just after 48 h of exposure to higher concentrations (i.e., 75 and 300 mg L⁻¹). Moreover, the same tendency was noticed after 72-96 h of

exposure to medium (i.e., 4.69-37.50 mg L⁻¹) and low (i.e., 2.34 mg L⁻¹) BPA concentrations. The reduction in BPA toxicity to *Tetraselmis* sp. growth with exposure time suggests a rapid decrease in the number of less resistant microalgal cells, mainly at higher concentrations, and a selection for more resistant cells which may contribute to increase the tolerance of this algal strain to BPA. A gradual decrease with time in BPA harmful effects on growth rate of microalgae at concentrations of 1-50 mg L⁻¹ was already detected during chronic exposure (5-30 days) (Zhang et al., 2012).

Despite the wide range of BPA concentrations tested (two orders of magnitude) in the present study, *Tetraselmis* sp. exhibited high variability in growth response. These results suggest the testing of other toxicity biomarkers to detect a concentration-dependent response of *Tetraselmis* sp. BPA inhibitory effect on microalgal growth may be related to damages in many physiological processes, including photosynthesis. Parameters directly related to primary production have been already applied as proxies to assess BPA toxicity for microalgae, such as chlorophyll content and photosynthetic activity (Gattullo et al., 2012; Mao et al., 2018). Moreover, the disruption of photosynthesis process induced by growth inhibitors leads to excess production and accumulation of reactive oxygen species (ROS) (Sies, 1997); thus, antioxidant activity markers (e.g., lipid peroxidation, ascorbate peroxidase and catalase activity) can also be used as an indicator of BPA toxicity to primary producers (Ben Ouada et al., 2018). In addition, metabolic activity and cytoplasmic membrane potential were sensitive parameters for acute toxicity evaluation of emerging compounds related to personal care products on *Tetraselmis suecica* cells (Seoane et al., 2017), which makes them potential proxies to assess pollutants toxicities on *Tetraselmis* species.

Tetraselmis sp. showed high tolerance to acute exposure (96 h) to BPA concentrations up to 300 mg L⁻¹. High tolerance of other chlorophytes has already been demonstrated to BPA. For example, *Monoraphidium braunii* was just negatively affected at BPA concentration of 10 mg L⁻¹ (Gattullo et al., 2012), whereas *Picocystis* sp. were affected at higher BPA concentrations (Ben Ouada et al., 2018). Both *M. barunii* and *Picocystis* sp. are proposed as promising species for the phytoremediation of waters contaminated with BPA. Thus, in the view of the great tolerance of *Tetraselmis* sp. detected for high concentrations of BPA, future studies should evaluate its ability as a BPA sequestrant in contaminated effluents before their arrival in marine systems.

4.2 Filter feeder invertebrate

Acute exposure to BPA has induced time- and concentration-dependent responses of brine shrimps. An increase in the mortality of *Artemia salina* individuals was evidenced towards high BPA concentrations, as well as cumulative lethality effect throughout exposure time. Brine shrimps have been often used in toxicity studies (Kalčíková et al., 2012; Shaukat et al., 2014; Neves et al., 2017; Silva & Abessa, 2019), due to its relative sensitivity and easy maintenance (i.e., cultivation, short life cycle, resistance to manipulation). The sensitivity of *Artemia* nauplii and adults was already detected for different chemicals (e.g., organic solvents, industrial chemicals, inorganic compounds and metals) (Kalčíková et al., 2012), phenolic compounds (Shaukat et al., 2014), harmful dinoflagellates (Neves et al., 2017), nanoparticles (Arulvasu et al., 2014; Kumar et al., 2017) and emerging contaminants (Castritsi-Catharios et al., 2013; Silva & Abessa, 2019). In our assays, there was a slight tendency to a decrease in lethality intensity at treatments of intermediate BPA concentrations tested (i.e., 9.38-37.50 mg L⁻¹) in comparison to lower (2.34-4.69 mg L⁻¹) and higher (75-300 mg L⁻¹) concentrations. This response is possibly a hormetic effect (i.e., overcompensation response to a disruption in homeostasis; Calabrese, 1999). The occurrence of hormetic concentration response was demonstrated for many organisms exposed to several chemicals and physical agents (reviewed in Calabrese & Blain, 2005). In addition, hormesis is a highly frequent phenomenon independently of tested stressor, biological endpoint, and experimental model system (e.g., microbe, plant, invertebrate and vertebrate) (reviewed in Calabrese & Baldwin, 2001). Hormesis is characterized as an evolutionary-base adaptive response to disruptions in organism homeostasis induced by an environmental stressor (reviewed in Calabrese & Baldwin, 2001), in which the response depends on organismal physiological system. Thus, it is not expected that a determined intensity of specific stressor (e.g., a chemical concentration) could induce similar hormetic responses in different biological systems. In the view of our results using lethality as endpoint, the acute exposure to BPA at concentrations of 9.38 to 37.50 mg L⁻¹ only seemed to induce hormetic effect in *A. salina* individuals.

In the present study, the LC_{50,96h} value obtained for *A. salina* exposed to BPA was higher (i.e., lower sensitivity) than values previously found for *Artemia* species to the same emerging pollutant (Table 6). In contrast to previous studies, for the first time, we have assessed BPA toxicity in adult individuals of *A. salina* after 96 h of exposure using

mortality as endpoint. Evidenced differences in *Artemia* sensitivity (LC/EC₅₀) among studies may be related to assay conditions (e.g., biological endpoint, exposure time, physical and chemical factors), life stages tested, variability in populational resistance and, possibly, interindividual variability. Sublethal indicators of toxicity have already been applied for *Artemia* as biological endpoints, such as dysfunctions in reproduction and growth (Hirano et al., 2004), alterations in swimming speed (Morgana et al., 2018), behavioral changes (Neves et al., 2017), immobilization (Kalčíková et al., 2012) and genotoxicity (Kim et al., 2019). Since some of the sublethal indicators seem to be useful to detect *Artemia* effects at low toxicant concentrations, these indicators could be applied in a complementary way or as an alternative endpoint for mortality assessment, mainly for subtle responses (i.e., all-or-nothing).

Table 6. Acute toxicity of BPA to *Artemia* species.

Species	Life stage	Endpoint	Exposure	LC/EC ₅₀	Reference
			(h)	(mg L ⁻¹)	
<i>Artemia franciscana</i>	Nauplius	Mortality	24	44.8	Castrits-Catharios et al., 2013
			48	34.7	
<i>Artemia</i> sp.	Nauplius	Mortality	24	74.77	Silva & Abessa 2019
			48	59.4	
<i>Artemia salina</i>	Adult	Mortality	96	107.2	Present study
<i>Artemia salina</i>	Nauplius	Immobilization	24	56.1	Kalciková et al. 2012

4.3 Deposit feeder invertebrate

In the present study, the non-conventional model gastropod *Heleobia australis* exposed to BPA exhibited mortality increase in a time- and concentration-dependent manner at the highest tested concentrations; except for the concentration of 1.0 mg L⁻¹ in which 30% of snail's mortality was shown for all exposure times. No snail mortality was observed for BPA concentrations of 0.063-0.5 mg L⁻¹. Higher resistance to BPA was expected for *H. australis* that is known as a highly tolerant species under adverse conditions and several pollutants, such as hydrocarbons (e.g., petroleum and diesel) (Neves et al., 2011; Egres et al., 2012; Sandrini-Neto et al., 2016), salinity stress (Neves et al., 2011), and eutrophication (Neves et al., 2013a).

Acute toxicity test of BPA to *H. australis* (LC_{50,96h} = 11.5 mg L⁻¹) was the first assessment for hydrobiid species, as well as for estuarine snails. This benthic species shares similar ecological traits with other hydrobiids (e.g., *Hydrobia ulvae*, *Potamopyrgus antipodarum*) (Fretter & Graham, 1994). Moreover, the sublethal responses (e.g., excessive relaxation and numbness) shown in the present study by *H. australis* snails at non-lethal concentrations or before snail's death have never been described for snails exposed to BPA. These sublethal responses may be consequence of neurotoxic effects on snails. Other aquatic taxa (e.g., mollusc, ascidia, fish) have demonstrated disorders of nervous system through various cell signaling pathways induced by BPA exposure (Messinetti et al., 2019; Olsvik et al., 2019; Kim et al., 2020). Neurological disturbances caused by pollutants exposure in natural systems may decrease individual's health status, negatively affect its survival and defense ability to predators and parasite infestation (Alda et al., 2011), leading to long-term alterations at population level.

Among the invertebrates and considering their endocrine systems, prosobranch aquatic snails were proposed as test organisms for toxicity assessment of endocrine active compounds (Oehlmann et al., 2000; Duft et al., 2003, 2007). Previous studies have evaluated the chronic effects of xeno-estrogenic compounds (e.g., BPA) on hydrobiid snail's reproduction (Duft et al., 2007; Sieratowicz et al., 2011). A stimulation in the number of embryos produced by *P. antipodarum* females was shown after two to eight weeks of BPA exposure (Duft et al., 2003, 2007; Sieratowicz et al., 2011). Moreover, the freshwater prosobranch snail *Marisa cornuarietis* chronically exposed to BPA have shown a syndrome referred as "superfemale" (i.e., formation of additional female organs), which resulted in higher female mortality and stimulation of oocyte and spawning mass

production (Oehlmann et al., 2000; Duft et al., 2007). Despite no previous assessment of chronic impacts of xeno-estrogenic compounds on *H. australis*, environmental impacts of xeno-androgen endocrine disruptors (e.g., tributyltin) was detected in female snails with the record of imposex (i.e., superimposition of male sexual characters) for the species (Neves et al., 2013b). Chronic reproductive impacts on hydrobiids at low BPA concentrations (e.g., $EC_{50,2-8 \text{ weeks}} = 0.004 - 24.5 \mu\text{g kg}^{-1}$ dry sediment; Duft et al., 2003), as well as for other prosobranch snails, highlight the potential effects of environmentally relevant concentrations on snails from contaminated aquatic systems. Further studies focusing on acute toxicity tests with deposit-feeder snails and other macroinvertebrates are needed considering their functional importance in nutrient cycling and food webs of estuarine and marine systems. The mud snail *H. australis* was effective to assess BPA toxicity in acute assays, but its effectiveness as a tropical model species must be tested for chronic toxicity assessment at environmentally relevant BPA concentrations.

4.4 Omnivorous vertebrate

In the present study, acute exposure to BPA have induced *Poecilia vivipara* mortality in a concentration-dependent manner. However, all individuals exposed to low and intermediate BPA concentrations have survived (i.e., 0.063-1.0 mg L⁻¹) and no timeeffect was detected in fish response. Similarly, acute lethality in the omnivorous fish *Pimephales promelas* was detected only at the higher BPA concentrations tested (e.g., 5.613.3 mg L⁻¹) without time-effect (Alexander et al., 1988). As evidenced in the present study, the fast-lethal response of fish may be explained by the hydrophobic nature of BPA that permits its readily transport to the cell cytoplasm of fish inducing chemical cellular damages (Fei et al., 2010). Previous studies have evidenced that BPA action mechanism seems to be efficient to induce histological injuries and changes in fish transcriptional response even after short-term exposure. Kim et al. (2018) have found that BPA exposure significantly upregulated mRNA expression of lipid metabolism and downregulated genes involved in several biochemical and physiological processes, as well as relevant genes for vertebrate immunity. In addition, acute BPA exposure may also alter dorso-ventral patterning and brain development in fish during early embryogenesis (Tse et al., 2020) and induce cellular injuries in liver and gills of adults (Asifa & Chitra, 2015).

In the present study, alterations in fish behavior have been detected as an early stress response for BPA concentrations that induced lethality (i.e., 2-8 mg L⁻¹), while no

behavioral change was detected in fish exposed to non-lethal BPA concentrations (i.e., 0.0625-1 mg L⁻¹). Most of the aberrant fish behavior shown was erratic swimming pattern (e.g., circular) and fish position (e.g., turned upside-down or in vertical position). Fish behavior has been applied as indicator of sublethal toxicity for several toxicant stressors (e.g., Little and Finger, 1990; Ajuzie, 2008; Cazenave et al., 2008; Neves et al., 2020; Qiu et al., 2020). Acute BPA exposure have also induced abnormal behavior in cichlid fish (*Etroplus maculatus*) that showed erratic activity followed by restricted movements and loss of equilibrium (Asifa & Chitra, 2015). A reduction in swimming performance of the zebrafish *Danio rerio* was also demonstrated after chronic exposure to BPA (Little & Seebacher, 2015). The action mechanism of xeno-estrogenic chemicals (e.g., bisphenols) affects estrogen receptors, such as the brain of adult teleost fish that exhibits intense activity and expression of estrogen receptors and steroidogenic enzymes (Diotel et al., 2011). The rapid permeability of BPA in blood-brain barrier (e.g., 1-2 h at BPA concentration of 10 mg L⁻¹) seemed to lead to several changes in neurochemical pathways, which induced acute effects on fish larvae such as impaired behavioral patterns (e.g., swimming distance and velocity, altered color-preference), reduced heart rate and developmental deformities (Kim et al., 2020). Therefore, in the present study, the aberrant behavior described for exposed *P. vivipara* individuals may be consequence of rapid neurological dose-dependent effects only induced by BPA concentrations that led to fish mortality.

Several studies have evaluated chronic impacts of BPA on fish. Sublethal physiological responses were demonstrated for guppy fish, among them some alterations in male testicular structures (Kinnberg & Toft, 2003), changes in fish sex ratio, sperm quantity and quality, and reproductive success (Chen et al., 2015). Several other sublethal effects were described for teleost fish, which includes injuries in embryonic-larval development, higher incidence of morphological abnormalities, reduction in female body size, effects on blood flow, heart rate and muscles, cardiac edema, effects on hair cell survival and regeneration, alterations in gene expression, and disturbs in hormonal and molecular pathways (e.g., Duan et al., 2008; Chen et al., 2015; Hayashi et al., 2016; Little & Seebacher, 2015; Murata & Kang, 2018; Olsvik et al., 2019; Kim et al., 2020). Moreover, chronic exposure to environmentally relevant BPA concentrations may trigger fish avoidance behavior (Silva et al. 2018), changes in recognition memory and color preference (Li et al., 2017; Naderi et al., 2020), and adverse effects on population

reproduction (e.g., changes in the percentage of ovulated females and ovulation period) (Lahnsteiner et al., 2005). Taking into account that deleterious effects of BPA on fish may be irreversible and strongly affect population structure and dynamics, the impacts of BPA at environmentally relevant concentration on marine and estuarine fish must be further assessed.

Despite the wide application of guppy fish as a vertebrate model for acute toxicity tests (e.g., Yilmaz et al., 2004; Hafez et al., 2016; Vajargah et al., 2020), the LC₅₀ value of BPA was obtained, for the first time, for *P. vivipara*. Our study is only the second acute toxicity test of BPA for a guppy fish (*Poecilia reticulata* LC_{50,72h}= 1.66 mg L⁻¹; Silva et al., 2018). *Poecilia vivipara* showed high sensitivity to BPA acute exposure, in which its LC₅₀ value are within the lower values determined for teleost fish species of brackish and marine systems (e.g., 1.66 - 6.48 mg L⁻¹; Asifa & Chitra, 2015; Silva et al., 2018). The guppy *P. vivipara* has been applied in toxicity tests of several compounds (e.g., biocides, metals, personal care products) regarding its effectiveness as a sensitive model (Ferreira et al., 2012; Escarrone et al., 2016; Zebral et al., 2018; Lopes et al. 2020) and its neotropical distribution (Ferreira et al., 2012). This species is also a valuable model considering its tolerance to a wide range of salinity (i.e., euryhaline) and temperature (i.e., eurythermal) (Ferreira et al., 2012). Therefore, in the view of species traits and the present results, the guppy *P. vivipara* seems to be a promising model organism for the evaluation of BPA impacts on brackish-marine systems.

4.5 BPA toxicity among trophic groups

In the present study, marked differences in model organism's sensitivities to BPA were detected. The primary producer microalga (*Tetraselmis* sp.) showed high tolerance to BPA, without a concentration-dependent response. Species sensitivity have increased from the zooplanktonic grazer (*A. salina*), followed by the deposit-feeder snail (*H. australis*), to the omnivorous fish (*P. vivipara*).

Despite our experimental data could suggest a species sensitivity tendency towards distinct trophic levels, and partially supported our hypothesis (i.e., that BPA toxicity increases with the increase in trophic levels), the integration of BPA toxicity data using SSD analysis did not evidence a clear toxicity hierarchy for estuarine and marine species from different trophic levels. In addition, SSD curve have evidenced a great variability in species sensitivity within the groups tested, except for *Artemia* species that

showed the higher tolerance to BPA. Variability in species sensitivity within trophic groups seems to be related to interspecific responses, probably related to the action mechanism of BPA, in addition to assay conditions (e.g., possible differences in exposure time, physical and chemical factors among the studies). Considering the chemical compound nature (i.e., estrogenic endocrine disruptor) and endocrine system complexity of taxa tested, differences in the action mechanism of BPA and its toxicity are expected among species, even for organisms trophic and/or with taxonomically similar.

SSD analysis methodology assumes that the acceptable effect level (i.e., sensitivity) of different species in the environment follows a probability function (Dowse et al., 2013). Considering the scarcity of BPA concentration effective data for marine and estuarine species of similar ecological traits, few species could be retrieved for each trophic group which may have affected a fully evaluation of species sensitivity among trophic position levels. Moreover, our results reinforce the assumption that a limited number of tested species is a random sample of the whole biological system (Posthuma et al., 2002), as well as the importance to assess acute and chronic toxicity of compounds (especially the emerging ones) for marine and estuarine species including the non-conventional ones with ecological relevance at aquatic ecosystems.

Although the LC_{50} values and estimated HC5 (1.18 mg L^{-1}), with lower and upper values of FA from 1.10 to 15.22, for marine and estuarine species were higher than environmentally relevant concentrations (Deblond et al., 2011; Gavrilesco et al., 2015; Lima et al., 2017), sublethal adverse effects induced by BPA exposure may lead to unbalances in population levels. Evaluated species covered important groups for brackishmarine system functioning (i.e., primary producer and consumer, secondary consumer, and detritivore), therefore the impact of BPA on these key groups may have underlying and less perceptible ecological, commercial and human health impacts.

5. Conclusion

Differences in species sensitivities to BPA were detected, in which the primary producer microalga showed the highest tolerance (i.e., no concentration-dependent response), and species sensitivity have increased from the zooplanktonic grazer, followed by the depositfeeder snail, to the omnivorous fish. Despite the toxicity hierarchy towards distinct trophic levels evidenced by our assays, which partially supported the study hypothesis, the novel approach used to integrate BPA toxicity values (i.e., from the present study and

available in literature) using Species Sensitive Distribution (SSD) analysis did not evidence a clear pattern towards higher trophic groups. Model organisms tested in the present study were effective to assess BPA toxicity in acute assays and covered important groups for brackish- marine system functioning, therefore the impact of BPA on these key groups may have ecological, commercial and human health impacts.

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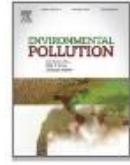
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Conclusões Gerais

- Foram detectadas diferenças quanto a sensibilidade das espécies ao composto.
- A microalga apresentou a maior tolerância entre os organismos testados, sem uma resposta dependente da concentração.
- A sensibilidade das espécies aumentou do herbívoro zooplancônico *Artemia salina*, seguido do gastrópode *Heleobia australis*, para o peixe *Poecilia vivipara*.
- Apesar da hierarquia de toxicidade em relação aos níveis tróficos, a SSD não revelou um padrão entre os mesmos.
- Organismos modelo testados no presente estudo foram eficazes para avaliar a toxicidade do BPA em ensaios agudos.
- O impacto do BPA nesses grupos-chave pode acarretar em impactos ecológicos, comerciais e na saúde humana.



Acute toxicity of Bisphenol A (BPA) to tropical marine and estuarine species from different trophic groups ☆

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