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The effects of Bisphenol A on the seagrass *Cymodocea nodosa*: Leaf elongation impairment and cytoskeleton disturbance

Ioannis-Dimosthenis S. Adamakis[a,1], Paraskevi Malea[b,*,1], Emmanuel Panteris[b]

[a] Department of Botany, Faculty of Biology, National and Kapodistrian University of Athens, 15784 Athens, Greece
[b] Department of Botany, School of Biology, Aristotle University of Thessaloniki, 54124 Thessaloniki, Macedonia, Greece

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ABSTRACT

Bisphenol A (BPA) is an emerging pollutant of environmental concern, classified as "moderately toxic" and "toxic", causing adverse effects on aquatic biota. Although information about BPA toxicity on aquatic fauna is available, the data about BPA effects on aquatic flora remain scarce, missing for marine macrophytes. The effects of environmentally relevant BPA concentrations (ranging from 0.03 to 3 μg L^{-1}) on juvenile leaf elongation and the cytoskeleton (microtubules, MTs and actin filaments, AFs) were studied in the seagrass *Cymodocea nodosa* for 1–10 days. The suitability of cytoskeleton disturbance and leaf elongation impairment as "biomarkers" for BPA stress were tested. The highest BPA concentrations (0.3, 0.5, 1 and 3 μg L^{-1}) affected significantly leaf elongation from the onset of the experiment, while defects of the cytoskeleton were observed even at lower concentrations. In particular, MTs were initially disrupted (i.e. "lowest observed effect concentrations", LOECs) at 0.1 μg L^{-1}, while AFs were damaged even at 0.03 μg L^{-1}. AFs appeared thus to be more sensitive to lower BPA concentrations, while there was a correlation between leaf elongation impairment and MT defects. Thus, AF damages, MT disruption and leaf elongation impairment in *C. nodosa*, in this particular order, appear to be sensitive "biomarkers" of BPA stress, at the above environmentally relevant BPA concentrations.

1. Introduction

Worldwide human development and urbanization have led to increased demand for food and beverage packaging, remedial equipment, electronics, flame retardants, adhesives, construction materials, plastic containers and paper coverings (among others see Corrales et al., 2015). The manufacture of the above products requires the substance known as bisphenol A (CAS No. 80-05-7) (BPA; 2,2-bis(4-hydroxyphenyl) propane). As a result, worldwide BPA production (e.g. 3.7 million metric tons per year) and consumption have steadily increased over the years (Corrales et al., 2015).

Similarly to other chemicals, marine environment is the major disposal site of BPA pollution. The major inputs of BPA enter the environment from production and processing industries, landfill wastes and effluents of wastewater treatment facilities. BPA discharges are percolated in rivers and streams towards estuarine and coastal systems (e.g. Yamada, 1999; Cousins et al., 2002; Kang et al., 2007; Mihaich et al., 2018). It has been recorded that BPA in municipal and industrial wastes can reach up to 23.02 μg L^{-1} (Al-Rifai et al., 2007), while in sewage pulps its concentration can be much higher 10-$>$ 100,000 μg kg^{-1} d.wt. (Corrales et al., 2015), and in some cases the reported values extended up to 3.2×10^7 μg kg^{-1} d.wt. (Harrison et al., 2006). The reported concentrations of BPA in aquatic environments vary, ranging from $<$ 0.0088–1.0 μg L^{-1} in marine water (Belfroid et al., 2002; Kawahata et al., 2004; Vethaak et al., 2005; Staniszewska et al., 2014), 0.0015–0.145 μg L^{-1} in estuarine and lagoon water (Fu et al., 2007), $>$ 1 μg L^{-1} in freshwater systems (Oehlmann et al., 2008; Flint et al., 2012), to elevated concentrations of BPA (100–1000 up to 20,136 and 13,392 μg kg^{-1} d.wt in Asia and Europe, respectively) in anaerobic sediments (see review by Corrales et al., 2015). Given its presence in the environment, earlier reports by the European Commission and the United States Environmental Protection Agency have classified this compound as "moderately toxic" and "toxic" to aquatic biota (Alexander et al., 1988).

Few studies in either laboratory or field settings have examined wildlife organisms' measurable responses to environmentally relevant BPA concentrations (0.08–12.5 μg L^{-1}; Flint et al., 2012) and their predicted no-effect concentrations have been determined at 0.06 μg L^{-1} (Wright-Walters et al., 2011). Most of the evidence derived mainly from studies on aquatic invertebrates, such as crustaceans and echinoderms,

* Corresponding author.
E-mail address: malea@bio.auth.gr (P. Malea).
[1] These authors contributed equally.

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with available data also from insects, fish, amphibians, reptiles and birds (Mihaich et al., 2009; Flint et al., 2012).

Plants, especially aquatic ones, have received far less attention (e.g. Mihaich et al., 2009). In particular, the research on BPA toxicity so far has been limited to the freshwater angiosperm *Lemna gibba* (Mihaich et al., 2009) and to some microalgae species (e.g. *Chlorella fusca, Stephanodiscus hantzschii, Chlamydomonas mexicana*, *Chlorella vulgaris*) (Alexander et al., 1988; Hirooka et al., 2005; Li et al., 2009; Ji et al., 2014), in which BPA induced defects in growth, antioxidant enzyme activity and photosynthetic pigments, respectively. In land plants, studies have examined BPA effects on vegetative growth (Ferrara et al., 2006) and some researchers have related the defects of microtubules (MTs) and actin filaments (AFs) induced by BPA treatment with the observed growth inhibition (Adamakis et al., 2013, 2016; Stavropoulou et al., 2018).

These potential disturbances, induced by BPA to the cytoskeleton and/or the vegetative growth, could be used as "biomarkers" for the evaluation of BPA contamination in specific marine "bioindicator" organisms. Marine macrophytes, especially the seagrasses, are of particular interest, given their well-known biological and ecological relevance and their sensitivity to anthropogenic disturbances (e.g. Malea, 1994; Malea and Haritonidis, 1995, 1999; Malea et al., 1995; Malea and Zikidou, 2011; Malea et al., 2013a, 2013b, 2013c, 2014). These characteristics mark them as excellent candidate "bioindicator" organisms (see review by Ferrat et al., 2003). Although some "biomarkers" have been determined for BPA toxicity, mainly in aquatic vertebrates and invertebrates, in some microalgae species and in one aquatic angiosperm (*Lemna gibba*) (Kang et al., 2007; Mihaich et al., 2009 and literature therein), none has been determined in marine macrophyte species and especially in seagrasses.

Cymodocea nodosa is a seagrass resilient to anthropogenic disturbances, displaying a fast growth rate (Cabaço et al., 2010). In seagrasses species, among which *C. nodosa,* the effectiveness of a number of "biomarkers" (e.g. photosynthetic activity, enzymatic activity, heat shock proteins, phenolic compounds, MT arrays, cell mortality, oxidative stress "biomarkers", biomarkers of detoxification) of various stressors (especially metals, metallic nanoparticles (NPs), pesticides, nutrients, etc.) has been tested and most of them appeared to be valuable and early warning signals for general or particular stress (see Ferrat et al., 2003 for review; Malea et al., 2013a, 2013b, 2014; Papathanasiou et al., 2015; Moustakas et al., 2016). Growth impairment has been examined as an assessor of the aquatic angiosperm health against various chemicals (e.g. Flores et al., 2013; Negri et al., 2015; Papathanasiou et al., 2015; Llagostera et al., 2016). However, the extent by which the effects on the cytoskeleton and ultrastructural and mitotic defects (Adamakis et al., 2013, 2016) may result in more obvious plant physiological and morphological responses (e.g. leaf elongation) remain unexplored.

Considering the above, in the present study we sought to investigate the effects of environmentally relevant BPA concentrations on the juvenile leaf growth (expressed as elongation), in relation to the defects in cytoskeleton organization in leaf cells of the seagrass *C. nodosa*. Moreover, this study contributes to the existing knowledge on the "lowest observed effect concentrations" (LOECs) and hence on "no observed effect concentrations" (NOECs) on this marine angiosperm, which can be used in risk assessment programmes. The suitability of MT and AF disturbance and of leaf elongation impairment as suitable "biomarkers" of BPA-induced stress is discussed.

2. Materials and methods

2.1. Plant collection

Cymodocea nodosa (Ucria) Ascherson 1870 was collected from the eastern coast of the Gulf of Thessaloniki, Northern Aegean Sea at Viamyl site (40°33´N, 22°58´E), where it forms a continuous monospecific meadow. Leaf biomass and leaf blade length display an almost unimodal annual pattern attaining maximum values in July-August (e.g. Malea and Zikidou, 2011). Plant collection was done at 0.7–1.0 m depth in July 2017 with a 20 cm diameter acrylic corer, which penetrated to a depth of 30 cm. All plants were rinsed in seawater at the collection site and transported to the laboratory in plastic containers containing seawater.

2.2. Treatments and experimental conditions

Collected plants without epiphytes consisting of roots, plagiotropic (horizontal) and orthotropic (vertical) rhizomes and leaves were kept for 24 h in filtered seawater (0.45 μm Whatman GF/C) under laboratory conditions to equilibrate, under a constant 16 h day/8 h night regime at an ambient temperature of 21 ± 1 °C with light intensity set at 120 μmol m^{-2} s^{-1}. The seawater used for all experiments was collected from a nearby coastal area (Epanomi site, Thermaikos Gulf). All the chemicals and reagents used were purchased from Applichem (Darmstadt, Germany), Merck (Darmstadt, Germany) and Sigma (Taufkirchen, Germany), unless stated otherwise.

Two different sets of experiments were performed, both run in triplicates. In the first set about 24 orthotropic rhizomes attached to part of plagiotropic rhizomes with roots were incubated in non-BPA-based polypropylene (PP) copolymer (PPCO) aquaria covered with plastic foil to prevent evaporation. Plants were treated with 0.03, 0.1, 0.3, 0.5, 1 and 3 μg L^{-1} bisphenol (BPA) solutions in filtered seawater. For control, plants incubated in filtered seawater, were used. The solutions in the aquaria were changed every two days and were constantly aerated using aquarium air pumps. From each aquarium, four seagrass shoots (totally 12 shoots per BPA treatment) were randomly removed after 1, 2, 4, 6, 8 and 10 days and the meristematic region of the youngest juvenile leaf blade from each shoot was used for either tubulin immunostaining or AF staining.

In the second set of experiments, a similar experimental design, as above was generally applied. In each aquarium, in about four orthotropic rhizomes of *C. nodosa* attached to part of plagiotropic rhizomes with roots (about 12 shoots in each BPA treatment) the youngest accessible juvenile blades were marked by an inert sealant at their base and their initial lengths were measured (mean ± SE: 33.908 ± 2.034 mm). Leaf elongation (mm) was carefully measured as the newly formed leaf segments, hence the distance between the marker and the juvenile leaf base on the 2nd, 4th, 6th, 8th and 10th day (Mateos Naranjo et al., 2008). Leaf elongation rate (Negri et al., 2015) (expressed as mm day^{-1}) were also calculated as the leaf elongation (mm) among two consecutive days divided by the number of days that they mediate. The final length of each measured juvenile leaf, at the end of the experiment were also measured.

2.3. Imaging of MTs and AFs

Whole mount MT immunostaining was conducted in hand cut small juvenile leaf pieces, following the protocol of Katsaros and Galatis (1992) using anti-α-tubulin (YOL1/34, AbD Serotec, Kidlington, UK) and FITC-anti-rat secondary antibody (Invitrogen, Carlsbad, CA), both diluted at 1:80 in phosphate buffered saline (PBS). DNA was counterstained with 10 μgmL^{-1} DAPI (4′,6-diamidino-2-phenylindole) in PBS and the leaf pieces were finally mounted in an anti-fade solution. AF staining with TRITC-phalloidin was conducted according to Panteris et al. (2009), with some modifications as follows: Leaf pieces were firstly incubated with 300 μM m-maleimidobenzoyl- N-hydroxysuccinimide ester (MBS) in PBS + 0.5% (v/v) Triton X-100 for 30 min in the dark for F-actin stabilization. After AF stabilization, fixation was performed in 4% (w/v) paraformaldehyde (PFA) in the same buffer for 60 min. In the PFA solution 1% (v/v; diluted from a 10 μM stock solution in methanol) TRITC-phalloidin was added. The tissue sheets were then rinsed with PBS and extracted in 5% (v/v) DMSO