The curious case of methylparaben: Anthropogenic contaminant or natural origin?

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Abstract

The widespread use of methylparaben as a preservative has caused increased exposure to natural aquatic systems in recent decades. However, current studies have suggested that exposure to this compound can result in endocrine disrupting effects, raising much concern regarding its environmental impact. In contast, methylparaben has also been found to be part of the metabolome of some organisms, prompting the question as to whether this compound may be more natural than previously assumed. Through a combination of field studies investigating the natural presence of methylparaben across different taxa, and a 54-day microcosm experiment examining the bioaccumulation and movement of methylparaben across different life stages of aquatic insects (order Trichoptera), our results offer evidence suggesting the natural origin of methylparaben in aquatic and terrestrial biota. This study improves our understanding of the role and impact this compound has on biota and challenges the current paradigm that methylparaben is exclusively a harmful anthropogenic contaminant. Our findings highlight the need for further research on this topic to fully understand the origin and role of parabens in the environment which will allow for a comprehensive understanding of the extent of environmental contamination and result in a representative assessment of the environmental risk that may pose.

Keywords: Trichoptera, Odonata, endocrine disrupting compound, *in situ*, microcosm

1. Introduction

The increased use of personal care products, pharmaceuticals, and pesticides over the past several decades has resulted in the widespread release of emerging contaminants into natural environments. Among these contaminants are parabens – alkyl esters of *p*-hydroxybenzoic acid – which are widely added to various cosmetics, food and pharmaceuticals as additives and preservatives due to their favourable physico-chemical and antimicrobial properties (Błędzka et al., 2014; Liao et al., 2013a; Soni et al., 2005). Although parabens are highly degradable through e.g., photo-transformation (Chuang & Luo, 2015; Foszpańczyk et al., 2018; Gmurek et al., 2015), ozone degradation (Tay et al., 2009) or microbial biodegradation (Amin et al., 2010; González-Mariño et al., 2011; Lu et al., 2018; Valkova et al., 2001; Zhu & Wei, 2019), with half-lives ranging from 0.79 to 35.2 h in aerobic aquatic environments (González-Mariño et al., 2011; Lu et al., 2018), they are still ubiquitously detected in natural ecosystems downstream of wastewater treatment plants due to their continuous input into aquatic environments (Haman et al., 2015; Yamamoto et al., 2011).

The presence of parabens has been confirmed in rivers, urban streams, marine environments and soils impacted by wastewater effluents and agricultural and storm water runoff, with maximum detected levels mainly remaining within the ng L^{-1} or ng g^{-1} range (Carmona et al., 2014; Kasprzyk-Hordern et al., 2008; Liao et al., 2013b; Núñez et al., 2008; Peng et al., 2014; Zhao et al. 2019). Although various parabens (e.g., methyl-, ethyl-, propyl-, and butylparaben) are often detected together in the environment, methylparaben generally exhibits the highest frequency of detection and measured concentrations due to its common use in skincare products (Błędzka et al., 2014; González-Mariño et al., 2011). In freshwater samples, maximum observed concentrations of methylparaben have been found to range between 3.4 and 920 ng L^{-1} (González-Mariño et al., 2009; Kasprzyk-Hordern et al., 2008;

Kimura et al., 2014; Li et al., 2016), apart from Evans et al. (2016) who measured concentrations of up to 13.78 μg L^{-1} in urban stormwater systems. Similar concentrations (4.87 to 104 ng L^{-1}) were also observed in studies measuring methylparaben levels in contaminated seawater (Kung et al., 2018; Xue & Kannan, 2016; Zhao et al., 2019).

Parabens were long considered to be non-toxic; however, recent studies highlighting the endocrine disrupting properties of these compounds have raised significant concern regarding their environmental impact (Nowak et al., 2018; Oishi, 2002; Routledge et al., 1998). As endocrine disrupting compounds, parabens can competitively bind to estrogen receptors, alter the production and degradation of endogenous steroids, and influence steroid sensitive tissues, in turn impacting the nervous system, immune system, and lipid homeostasis (Błędzka et al., 2014; Whitehead & Rice, 2006). Most studies on the estrogenic activity of parabens have focused on mammals (Lincho et al., 2021; Oishi, 2002; Vo et al., 2010), whereas those investigating the impacts on the endocrine system of insects are rare. There are, however, several studies that have examined the impacts of these compounds on aquatic and terrestrial invertebrates. For example, methyl-, ethyl- and propylparaben have been shown to affect the fecundity, growth, and development of the fruit fly *Drosophila melanogaster* (Chen et al., 2016; Li et al., 2014; Liu et al., 2014). Similarly, Dobbins et al. (2009) reported adverse effects of parabens on the growth and reproduction of the planktonic crustacean *Daphnia magna*. Evidence for the endocrine disrupting effects of parabens were also observed in the nematode *Caenorhabditis elegans* (Nagar et al., 2020) and the earthworm *Eisenia andrei* (Kwak & An, 2021). Furthermore, the negative impacts of parabens on hormone-dependent organs were observed in fish (Alslev et al., 2005; Bereketoglu and Pradhan, 2019; Bjerregaard et al., 2003; Dobbins et al., 2009; Merola et al., 2021; Yamamoto et al., 2011) and smaller mammals (Costa et al., 2017; Lemini et al., 2003;

Sun et al., 2016), emphasizing the estrogenic effects of these compounds across a variety of taxa.

Although parabens detected in natural environments are assumed to be of mostly synthetic origin, they have also been found to be part of the natural metabolome of some organisms. For example, the bacterium *Microbulbifer* sp. can biosynthesize and utilize butyl-, heptyl-, and nonylparaben for their antimicrobial and antifungal properties (Peng et al., 2006). Similarly, several species of plants have been shown to produce methylparaben (*Vanilla planifolia* [Zhang & Mueller, 2012]), as well as a combination of methyl- and propylparaben (*Rubus chamaemorus* L. [Baardseth & Russwurm, 1978]) and employ these compounds as antimicrobial aromatics. Furthermore, the honeybee queen (*Apis mellifera* L. [Keeling et al., 2003; Slessor et al., 1990]) and several families of beetles (Coleoptera [Dettner, 1993a; Dettner, 1993b; Evans & Schmidt, 1990]) have been observed to use methylparaben in their chemical communication system.

Taking into consideration that research has both shown parabens to be endocrine disrupting compounds, but also naturally produced in some organisms, it is valid to question whether levels detected in biota are solely due to the bioaccumulation of anthropogenically produced parabens, or whether certain amounts of these compounds are perhaps biosynthesized by the organisms themselves. As the natural production of parabens remains largely uninvestigated in freshwater invertebrates, and as these compounds are still widely considered to be anthropogenic contaminants, understanding their natural origin in biota is critical.

Our previous work which investigated the trophic cross-ecosystem transfer of emerging contaminants briefly hinted at the possibility of biosynthesis of methylparaben in aquatic insects (Previšić et al., 2021), prompting us to further examine the possible natural presence of this compound in various aquatic and terrestrial invertebrates. For this, we I)

111 gathered samples of invertebrates from sites both impacted and unimpacted by anthropogenic
112 pollution and determined levels of methylparaben present in their tissues, and II) conducted a
113 54-day microcosm experiment during which we measured concentrations of methylparaben
114 in samples of aquatic life stages (larvae, prepupae, pupae) and emergent adults of
115 Trichoptera, as well as in water and moss as a major food source for the insects.
116

2. Methods

2.1. In situ sample collection

Samples of water, biofilm, and aquatic and terrestrial invertebrates (Odonata, Trichoptera, Araneae and Amphipoda) were collected from seven representative freshwater locations in Croatia (Fig. 1; Table S1). Two of the sampling sites, the Krčić River spring reach and the Gacka River spring reach were chosen due to their pristine nature, located upstream of any anthropogenic impacts (Hrvatske vode, 2021). The remaining five locations were exposed to a gradient of pollution (wastewater effluents and agricultural runoff) and represented contaminated sites (Table S1) (Hrvatske vode, 2021). Water and biofilm collected from the polluted sites were taken in replicates; water was gathered in 1L bottles, and biofilm was gently scraped off from stones. Aquatic insect larvae were collected using a D-net, placed in 10L containers together with river water and transported to the lab, where they were left untouched for 24h to allow for gut clearance. All samples of biota were separated and identified to the lowest possible taxonomic level (Table S1), freeze-dried and stored at -80°C until further processing.

2.2. Microcosm experiment

We conducted the microcosm experiment with a simplified freshwater food web containing nonvascular macrophytes (or moss; Bryophyta) and larvae of the caddisfly *Micropterna nycterobia* (McLachlan, 1875) (Limnephilidae, Trichoptera) feeding mainly as shredders. Trichoptera larvae, water, sand and stones collected from the pristine Krčić River spring reach (N44.027321 E16.318936) in May 2019 were used for the experiment. Upon collection, aquaria (30x20x15 cm) were installed with 3 L of water each, along with equal amounts of sand (10 tablespoons), stones (3 stones >10 cm and 10 stones 2-5 cm), mixtures of moss species (total of 3 tufts 6-8 cm in diameter and plants up to 15 cm in length; *Cinclidotus aquaticus* [Hedw.] Bruch & Schimp and *Rhynchostegium riparioides* [Hedw.]

142 Cardot) and *M. nycterobia* larvae (cca 40 larvae per aquarium). Aquaria were placed in an
143 incubator (POL-EKO APARATURA, Poland) to ensure controlled temperature conditions,
144 i.e., the starting temperature was set at 9.5°C for 20 days and successively increased for
145 0.5°C every 15 days, mimicking the thermal regime of the Krčić River spring (Gottstein et al.
146 unpublished data). Daylight was used as a natural source of light for the aquaria. Two
147 treatment levels were created: control and experimental, with 3 replicates per treatment, 9
148 aquaria in total. The control treatment had no added methylparaben, while the experimental
149 treatments were exposed to methylparaben at a concentration of 500 ng L^{-1}, which is in the
150 upper level of environmentally observed concentrations (González-Mariño et al., 2009;
151 Kasprzyk-Hordern et al., 2008; Kimura et al., 2014; Li et al., 2016). Since our previous
152 finding suggests that 24 h after addition, methylparaben concentrations in experimental
153 enclosures drop below limits of detection (Previšić et al., 2021), methylparaben was added
154 daily. Water was aerated using aquaria air pumps, and each microcosm was covered with a
155 glass cover to minimize water evaporation. The volume of water was kept constant by adding
156 fresh dechlorinated tap water (cca 200 mL every two weeks). Prior to the beginning of the
157 experiment all microcosms were acclimatized for 7 days to allow the biota to adjust to the
158 new environment. As there is currently no standardized method available for quantitatively
159 determining the optimal acclimation period for lower trophic levels (i.e., moss and insects), a
160 7-day acclimation period was selected based on previous studies that worked with
161 invertebrates in micro- and mesocosms (Auffan et al., 2014; Brahim et al., 2019; Kefford et
162 al., 2007). Furthermore, Obernier & Baldwin (2006) and Hill et al. (2018) highlight that
163 shorter equilibration periods are preferred in limited systems, as these systems may move
164 towards disorder quicker than more complex systems that involve multiple trophic levels and
165 a larger number of species. We therefore selected an acclimation period in line with the

simplicity of our microcosm system which included only three species (Hill et al., 2018; Obernier and Baldwin, 2006).

Water, moss and Trichoptera samples were collected 5 times: after the acclimatization period – on day 0, day 15, day 30, day 45 and day 54 (hereafter referred to as T0, T15, T30, T45 and T54). Water samples were collected 24h after addition of methylparaben (i.e., prior to the new daily addition). At each sampling date, triplicate samples were taken from each aquarium (3x3 mL of water, 3x2 g of moss and 3x3 – 14 *M. nycterobia* larvae, prepupae and/or pupae), however, these were pooled to minimize variability between the aquaria, and analytical replicates for each sampling date were taken. All biota samples were freeze-dried and stored at -80 °C until further processing.

2.2. Analysis of methylparaben in water and biota samples

Water and biota samples were processed using a modified method of (Huerta et al., 2015) described in detail in our previous publications (Previšić et al., 2020; 2021). Briefly; 1.5 mL ice cold acetonitrile was added to 30 mg freeze-dried biota tissue. Methylparaben-D4 (C/N/D Isotopes, Canada) was added as a surrogate standard to the mixture. Tissues were lysed by bead beating in a home build bead beater with 2.3-mm-diameter chrome-steel beads at a frequency of 20 Hz for 5 min at 4°C. Samples were centrifuged at 20 000 x g for 10 min and the first supernatant was collected. The remaining pellet was re-suspended in 1.5 ml of ice-cold acetonitrile and additional lysis was done via ultrasonic probe (Sonoplus HD4050, Bandelin electronic GmbH, Germany) for 1 min at 50% intensity. Samples were vortexed for 5 min, centrifuged at 20 000 x g for 10 min and the second supernatant was collected. Both supernatants were evaporated to dryness and dissolved in 1 mL of water. Both water and biota samples were additionally cleaned with solid phase extraction using Waters Oasis HLB cartridges (60 mg, 3 mL). Cartridges were conditioned with 3 mL of acetonitrile followed by 3 mL of HPLC-grade water at a flow rate of 1 mL min^{-1}. 9 mL of water sample or 2 mL of

biota sample extracts were loaded at 1 mL min^{-1}. Samples were washed with 1 ml of water and consequently extracted with 1.5 ml of pure acetonitrile at a flow rate of 1 mL min^{-1}. Final extracts were evaporated to dryness under a gentle nitrogen stream and reconstituted in 300 μL methanol/water (50:50, v/v) and used for targeted analysis.

Chromatographic separation was performed on an ultra-performance liquid chromatography (UPLC) system (Waters Milford, USA) using the Waters ACQUITY BEH C18 (50 mm × 2.1 mm i.d., 1.7 μm particle size) column. The UPLC system was coupled to a hybrid quadrupole linear ion trap mass spectrometer Qtrap 5500 (Applied Biosystems, USA). Details regarding UPLC separation, ion source and MRM parameters can be found in Supplementary Information (Table S2). To unequivocally identify and quantify methylparaben, the detected substance had to meet the following performance criteria: I) the retention time of the substance matched that of the calibration standard and co-eluted surrogate standard II) identification of the precursor and two daughter ions leading to 4 identification points according to European Commission decision 2002/657/EC. The limit of quantification was defined as the lowest point of the calibration curve with a signal-to-noise ratio ≥10 and with a satisfactory number of ions to generate MS/MS fragmentation. The observed quantification limit, around 1 ng/g, was in agreement with the range reported during method development and was similar to the currently published procedures for quantification of methylparaben (Huerta et al., 2015; Jakimska et al., 2013), ranging from 0.01 to 1.4 ng/g. Analyst 1.5.1 software (Applied Biosystem) was used for instrument control, data acquisition and data analysis. Methylparaben was quantified using an internal standard method by the Bquant script for batch quantification of liquid chromatography mass spectrometry data using the procedure described in Rožman & Petrović (2016) and Rožman et al. (2018).

2.3. Quantification method performance and quality control

To minimise false positive results, method performances were additionally checked. The recovery of spiked samples (methylparaben standard at a final concentration of 100 ng g^{-1}) was 53% (±1.5%), which is in agreement with the range reported during method development (Huerta et al., 2015). Using information obtained via calibration curve, an apparent recovery of 95% (±7%) was observed. Accordingly, no recovery correction factor was applied to the reported values. An evaluation of the matrix effect of macroinvertebrate homogenate was performed by comparing the peak area of the methylparaben in macroinvertebrate extract spiked at 100 ng g^{-1} (after previous subtraction of the peak area of the analyte present in the extract) with the peak area of the analyte in the final solvent at the same concentration level. The observed matrix effect was -7.8% (± 0.7%). Test samples showed an accuracy of 5% (as the percentage value of the bias between the theoretical and calculated concentration) and precision of 12.6% (expressed as the coefficient of variation).

Blank samples were analysed every ten samples to check for traces of methylparaben or possible interference substances. To further eliminate the possibility of interference during solid phase extraction, extraction was tested on three different extraction cartridges Waters HLB Prime, Biotage PLD+ and Waters HLB. No statistically relevant difference between Waters HLB Prime and Waters HLB was detected. However, extraction with Biotage PLD+ (not used in the study) yielded ~ 22% higher concentrations ($F_{2,6} = 32.1877$, $P < 0.00341$).

2.4. Statistical analysis

Following confirmation of the normality of the data (Shapiro-Wilk test [$P < 0.05$] and Q-Q plot), univariate analyses were conducted to examine differences in methylparaben concentrations across sites, taxa, and life stages. For *in situ* data, one-way ANOVAs were used to test for overall differences in concentrations of methylparaben between water across different sampling locations, while three-way ANOVAs were used to test for differences in

methylparaben levels between Trichoptera life stages, collection sites and location types (i.e., pristine or contaminated). For microcosm data, repeated measures ANOVAs (rpANOVAs) were performed to evaluate the effect of different methylparaben treatments (control and experimental) over time (T0 to T54) on levels measured in water, moss and Trichoptera. All ANOVAs and pairwise comparisons for significant interaction effects were conducted using R 3.5.0 (R Core Team, 2018) and the rstatix package (v. 0.7.0; Kassambara, 2021). All ANOVAs were followed by multiple comparisons tests wherever results were found to be significant ($P < 0.05$). *P*-values were adjusted to control for experiment-wise error rate using the Bonferroni method.

3. Results and discussion

3.1. In situ collections put into question the current notion of methylparaben exclusively as an anthropogenic contaminant

Samples of various aquatic and terrestrial invertebrates (Odonata, Trichoptera, Araneae and Amphipoda) collected from pristine and contaminated freshwater sites in Croatia were screened for the presence of methylparaben. All water samples collected from the five contaminated freshwater locations indicated the presence of methylparaben at mean concentrations ranging between 0.58 to 3.70 ng L^{-1} (Fig. 1; Table S3), which were within the same range of those reported in literature (González-Mariño et al., 2009; Kasprzyk-Hordern et al., 2008; Kimura et al., 2014; Li et al., 2016; Núñez et al., 2008). No methylparaben was detected in either of the pristine sampling locations (the Krčić River spring reach and the Gacka River spring reach), confirming that the pristine sampling sites were unimpacted by anthropogenic exposure to methylparaben (Fig. 1). On the other hand, methylparaben was detected in all sampled biota, regardless of taxon, habitat, or sampling site, i.e., level of contamination (Fig. 2; Table S4). At contaminated sites, methylparaben was detected in various biota ranging from biofilm and freshwater crustaceans to different life stages of Odonata and Trichoptera (Fig. 2). Although the detection of methylparaben was not unexpected in contaminated sites, the presence of methylparaben in all sampled biota (moss, Trichoptera larvae and Trichoptera adults) across pristine sites unimpacted by anthropogenic contamination came as a surprise, suggesting that this compound may be naturally present in freshwater bryophytes and invertebrates. As virtually no studies have investigated the presence of methylparaben in pristine environments, it is difficult to compare levels measured here to other taxa. However, it is interesting to note that low levels (ng g^{-1}) of methylparaben detected in biota, both in pristine and contaminated environments, were within the range of those reported in literature across a variety of different taxa collected *in situ* from

contaminated environments (Jakimska et al., 2013; Pico et al., 2019; Previšić et al., 2021; Xue et al., 2015, Xue & Kannan, 2016; Zhao et al., 2019), Table S4. Furthermore, no statistical differences between methylparaben concentrations in Trichoptera from pristine versus contaminated environments were observed, further supporting the notion that methylparaben may be naturally produced in organisms at low levels (ng g^{-1}). Only the Krapina Kupljenovo sampling site differed in concentration, measuring higher levels of methylparaben compared to any other pristine or contaminated site (Table S5). This site is severely affected by untreated effluent and agricultural runoff, suggesting that heavy impacts of wastewater may play a role in impacting methylparaben accumulation in biota.

3.2. Microcosm experiment suggests a natural origin of methylparaben in moss and Trichoptera

The results of our microcosm experiment further support the possibility of the natural synthesis of methylparaben in aquatic biota (Fig. 3, Fig. 4; Table S6). Methylparaben was detected in moss in both our control and experimental treatments, however, concentrations were ~3-fold higher in the experimental enclosures than in the controls, increasing over time towards the final stages of the experiment (Fig. 4; Table S6, Table S8). Our findings suggest that nonvascular bryophytes may have the capability of bioaccumulation and/or sorption of methylparaben in environments exposed to elevated levels of this compound. Furthermore, the detection of methylparaben in moss within the control treatments offers further evidence for the natural origin of methylparaben in biota (Asakawa et al., 2012).

On the other hand, Trichoptera measured similar levels of methylparaben in tissues across both control (with no added methylparaben) and experimental treatments (Fig. 3; Table S7), indicating that methylparaben did not bioaccumulate in Trichoptera to any significant degree, despite chronic additions of methylparaben to experimental enclosures.

Although dietary and aqueous uptake within the microcosms was both possible and likely (Zhao et al., 2019), our findings suggest that any ingested methylparaben was rapidly metabolized and/or excreted from Trichoptera. As no research to date has examined the uptake and movement of this compound through aquatic insects, parallels may perhaps be drawn with other organisms, such as fish and mammals, where studies have confirmed the rapid metabolization and excretion of methylparaben (Moos et al., 2015; NICNAS, 2017; Renz et al., 2013; Tsukamoto & Terada, 1964; Wei et al., 2021; Ye et al., 2006). It is important to note, however, that current research on the bioaccumulation potential of parabens in biota is divided, as some authors have also observed the biomagnification of parabens in higher trophic levels (Xue et al., 2015; Xue et al., 2017; Xue & Kannan, 2016). Our results instead suggest that levels measured in Trichoptera may have been a result of natural origin, which is in line with our initial suggestion (Previšić et al., 2021) and reports on the natural presence of methylparaben in terrestrial insects (Dettner, 1993a; Dettner, 1993b; Evans & Schmidt, 1990; Keeling et al., 2003; Slessor et al., 1990). Since the data obtained from our *in situ* and microcosm experiment suggest that methylparaben may be biosynthesized in freshwater invertebrates, the question is raised regarding its physiological role in these organisms. Although no research to date has examined the natural production of methylparaben in aquatic insects, it is possible that any biosynthesized methylparaben has a similar role to that reported in other biota where this compound has been found to be produced naturally, e.g., as a defence mechanism (Bais et al., 2003; Dettner, 1993a; Evans & Schmidt, 1990; Peng et al., 2006; Zhang & Mueller, 2012) or semiochemical (Keeling et al., 2003; Slessor et al., 1990).

Methylparaben levels were higher in Trichoptera adults than in larvae (Fig. 3; Table S7). Although it is beneficial for aquatic larvae to utilize chemical signals in low-visibility, high-turbidity environments such as these (Crespo, 2010; Kats & Dill, 1998), it is not

unexpected to have measured higher concentrations of methylparaben in adult Trichoptera, which may possibly utilize methylparaben as a semiochemical. Comparable findings were reported in our previous work, in which methylparaben concentrations were higher in adults of the trichopteran *Drusus croaticus* Marinković-Gospodnetić, but differed from those found for *Potamophylax* sp., where levels were highest in larvae (Previšić et al., 2021). The reason for this discrepancy is likely due to variations in species' methylparaben production and/or bioaccumulation potential; however, may not be related to feeding behaviour as previously thought (Previšić et al., 2021), as both *M. nycterobia and Potamophylax* sp. are predominantly shredders, whereas *D. croaticus* is mainly a grazer (Graf et al., 2021). Nevertheless, this significant increase in *M. nycterobia* and *D. croaticus* adults suggests a potential insect origin and function in adult Trichoptera. Unfortunately, as virtually none of the studies examining the production and use of methylparaben as a chemical signal have quantified concentrations measured in insect tissues, it is challenging to draw comparisons with levels detected in our study.

It is interesting to note that the presence of methylparaben was detected in water 24 h after addition in both control and experimental enclosures (Fig. 5). While the control treatments should be free of any methylparaben present in water, when taking into consideration the findings of our previous experiment (Previšić et al., 2021), methylparaben concentrations in our experimental enclosures should have also been below limits of detection 24 h after addition of methylparaben. Although the presence of methylparaben in the control treatments may indicate possible contamination of the enclosures, all measures were taken to prevent any such contamination from external sources. Furthermore, if contamination had occurred, this would have likely been detected through a spike in methylparaben levels followed by a quick decrease in concentrations, as the methylparaben would have quickly degraded (Aristi et al., 2016) or accumulated in/adsorbed to the moss (as

seen in the experimental enclosures following continuous additions of methylparaben). Instead, we observed similar movement in methylparaben concentrations measured in water over time between the control and experimental enclosures, and no temporal change in methylparaben levels measured in moss in control treatments (Fig. 4, Fig. 5; Table S8, Table S9), suggesting that the elevated concentrations of methylparaben in the water may have been a result of the moss species (*C. aquaticus* and. *R. riparioides*) producing and releasing methylparaben into the surrounding water. Interestingly, levels of methylparaben in water showed a significant increase (~30 ng L^{-1}) towards the final stages of the experiment across both treatments (Fig. 5; Table S9). These elevated concentrations in water may have been due to the release of methylparaben from the moss in the enclosures, perhaps due to the stressful environment of the enclosures (Falik et al., 2011) or as a natural chemical defence mechanism of these bryophytes against bacteria, fungi or herbivores (Commisso et al., 2021; Glime, 2006; Ponce de León & Montesano, 2017). As *M. nycterobia* is predominantly a shredder that feeds on moss (Graf et al., 2021; Holzenthal et al., 2015), it is possible that the moss in both the control and experimental enclosures released methylparaben into the surrounding water as an anti-predatory response against damage caused by these herbivores – a common response seen among plants (Sánchez-Sánchez & Morquecho-Contreras, 2017; War et al., 2012).

Although laboratory studies have reported on the biotransformation of methylparaben from other parabens (transesterification from e.g., ethyl- and propylparaben) (Horiuchi et al., 1994; Wang et al., 2018), the likelihood of this occurring in a natural setting is fairly low – particularly in pristine environments with no anthropogenic input of parabens. In addition, transesterification often requires both the presence of microbes (e.g., *Pseudomonas*), a short-chained alcohol (e.g., methanol or ethanol), as well as a catalyst (Błedzka et al., 2014; Horiuchi et al., 1994; Lu et al., 2018; Wang et al., 2018), making any transformation

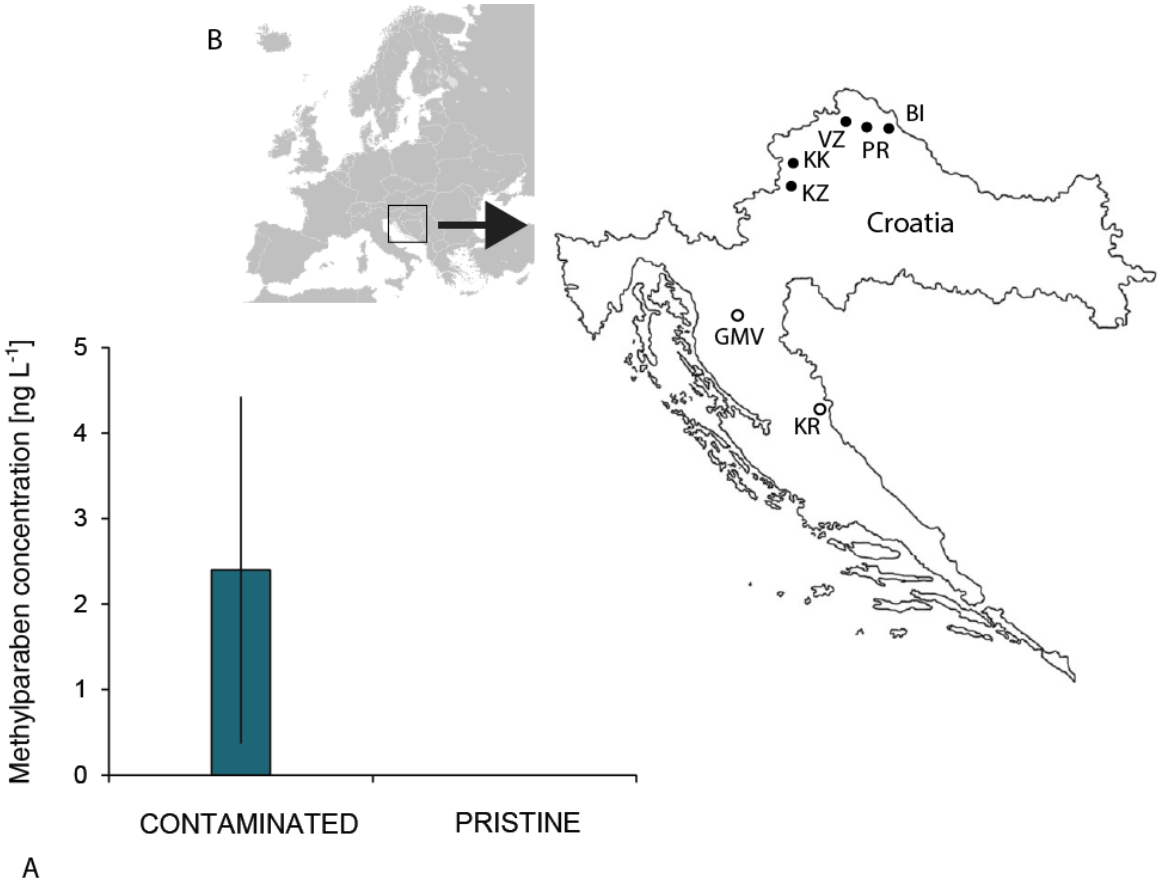
processes in the natural environment possible, but unlikely. Furthermore, no other parabens aside from methylparaben were added to the experimental enclosures in our microcosm experiment, and no parabens whatsoever to our control treatments, further supporting the notion that the formation of methylparaben from other parabens was possible in the contaminated sites, but highly unlikely in our pristine locations, as well as in our microcosm experiment.

It is important to note that any risk naturally produced methylparaben may pose on the ecosystem is likely very low, as the concentrations detected in our microcosm experiment were a factor of 10^{-6} to 10^{-8} lower than levels reported in literature that have caused endocrine disrupting effects in both invertebrates and vertebrates (Chen et al., 2016; Dobbins et al., 2009; Yamamoto et al., 2011). The significance of our findings instead lies in the reconsideration of methylparaben solely as an anthropogenic contaminant through examining the possibility of its biosynthesis in aquatic biota. It is necessary, however, to put into perspective the relative production of methylparaben in organisms, compared to the vast amounts that originate from anthropogenic sources, i.e., the concentrations of naturally synthesized parabens are very low in most cases. We therefore cannot, under any circumstances, neglect the monitoring of parabens in the environment, especially in populated areas prone to anthropogenic contamination.

5. Conclusion

Through a combination of *in situ* field surveys across pristine and contaminated freshwater habitats and a 54-day microcosm experiment, this study examined the presence of methylparaben in various aquatic and terrestrial invertebrate taxa. In our field studies, methylparaben was detected in all sampled biota, regardless of taxon or sampling site (i.e., pristine or contaminated). Our microcosm experiment gave similar results to our field study,

with measurable levels of methylparaben in Trichoptera and moss detected across both control and experimental enclosures, suggesting that methylparaben may be naturally present in freshwater bryophytes and macroinvertebrates. However, in contrast to Trichoptera, concentrations in moss were significantly higher in the experimental treatments than in the controls, suggesting that moss may have the capacity to bioaccumulate or adsorb methylparaben when exposed to elevated concentrations of this compound. Our findings put into question the current paradigm that all parabens detected in biota result from the accumulation of anthropogenically produced parabens. Instead, we must consider the possibility that certain amounts of this compound, in addition to bioaccumulation, may be naturally produced in organisms. As our study partly demonstrated that some aquatic biota may produce methylparaben, further explorations into this topic are needed to better understand the presence and impacts of parabens in the environment.



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415 **Fig. 1.** Mean concentrations (± SD) of methylparaben measured in water samples across
416 contaminated (PR: Prelog, BI: Bistrec, VZ: Varaždin, KK: Krapina Kupljenovo and KZ:
417 Krapina Zaprešić) and pristine sampling sites (KR: Krčić and GMV: Gacka River spring) in
418 Croatia.
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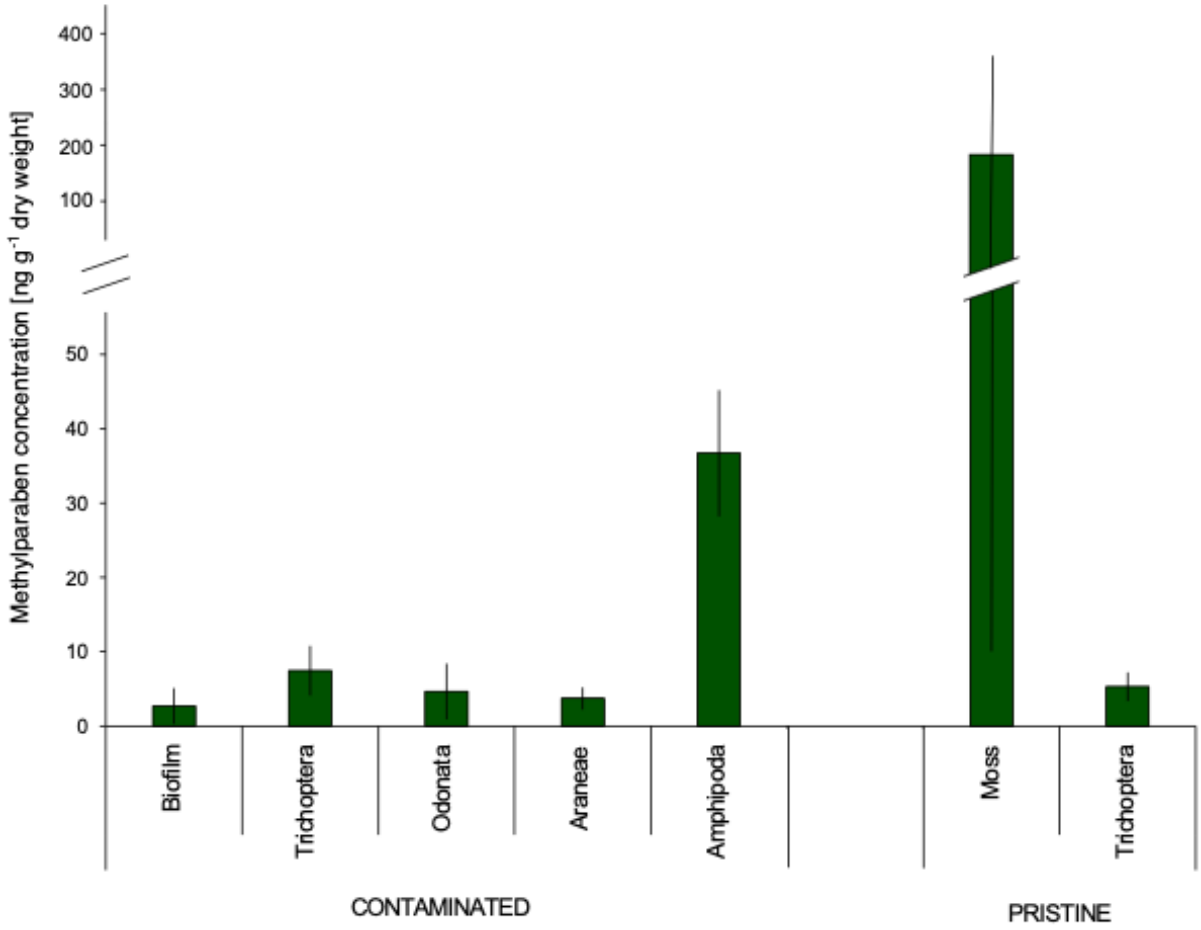
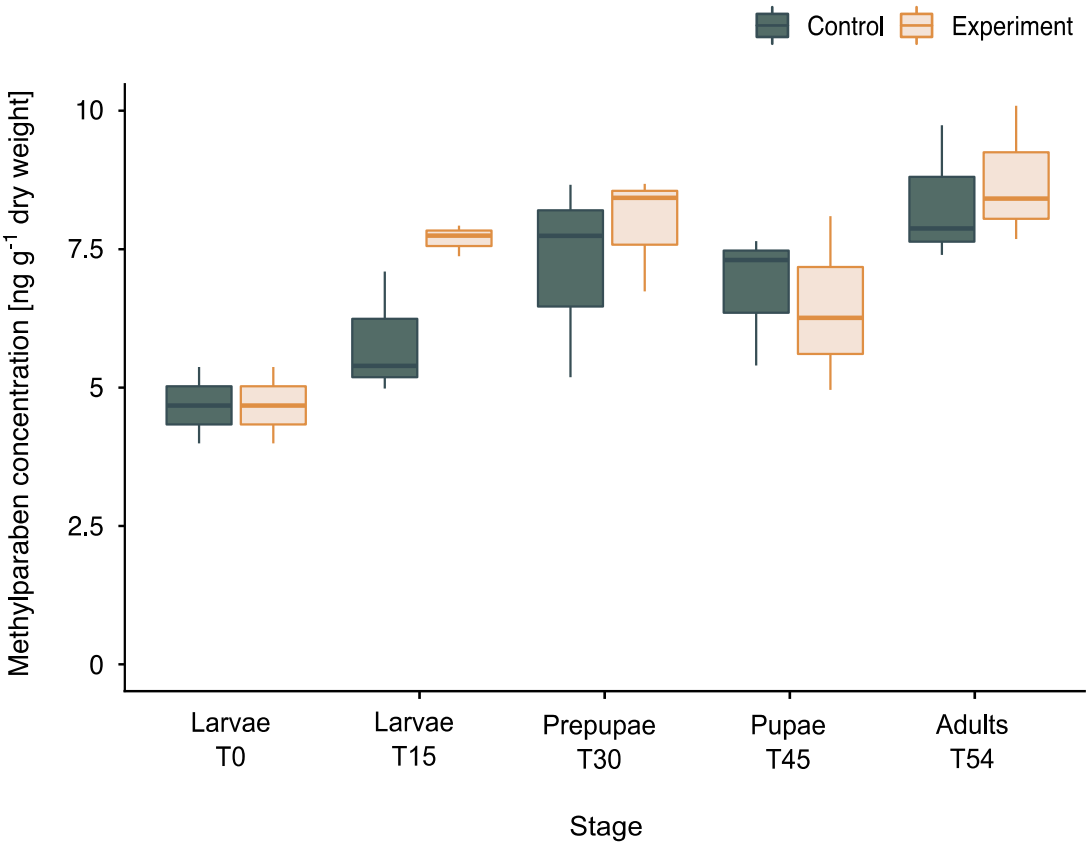


420
421
422 **Fig. 2.** Mean concentrations (± SD) of methylparaben measured in biota (biofilm, moss,
423 Trichoptera, Odonata, Araneae, Amphipoda) across contaminated (Prelog, Bistrec, Varaždin,
424 Krapina Kupljenovo and Krapina Zaprešić) and pristine sampling sites (Krčić and Gacka
425 River spring) in Croatia.



426
427 **Fig. 3.** Mean concentrations (± SD) of methylparaben measured in various life stages of
428 Trichoptera (larvae, prepupae, pupae and adults) from 0 to 54 days in control and
429 experimental treatments.
430

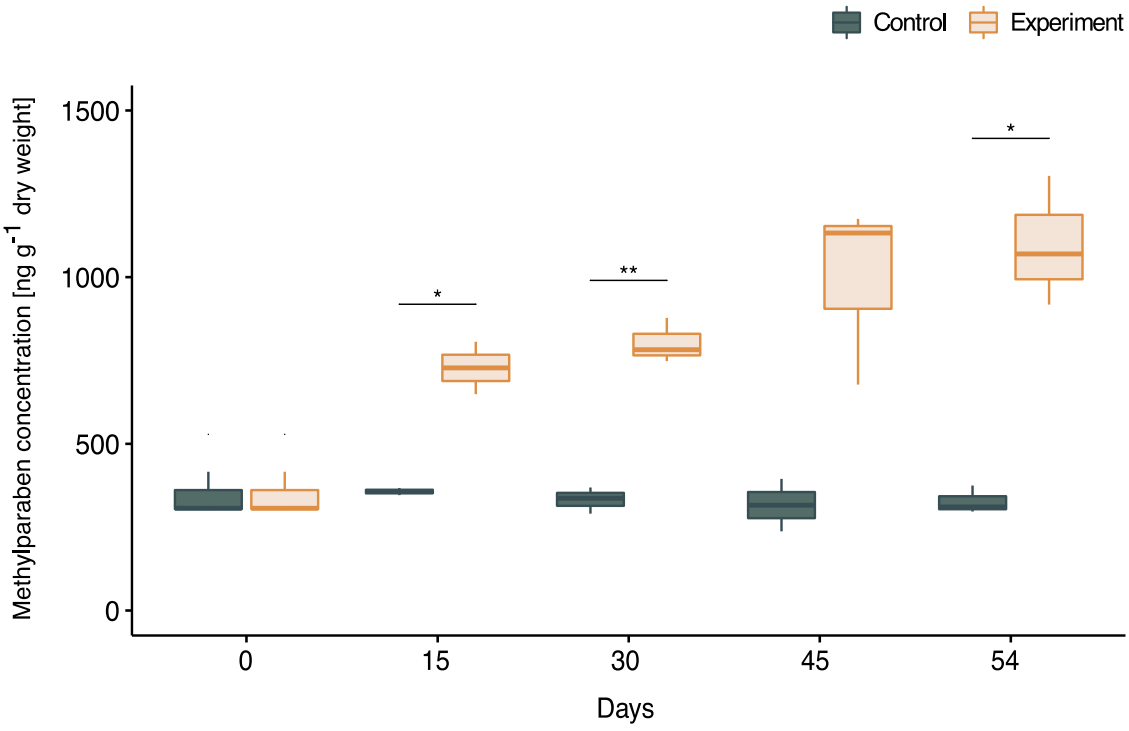


Fig. 4. Mean concentrations (± SD) of methylparaben measured in nonvascular macrophytes (moss) from 0 to 54 days in control and experimental treatments. *P*-values in the range of 0.01 – 0.05 are summarized with one asterisk (*), and *P*-values in the range of 0.001 – 0.01 are summarized with two asterisks (**).

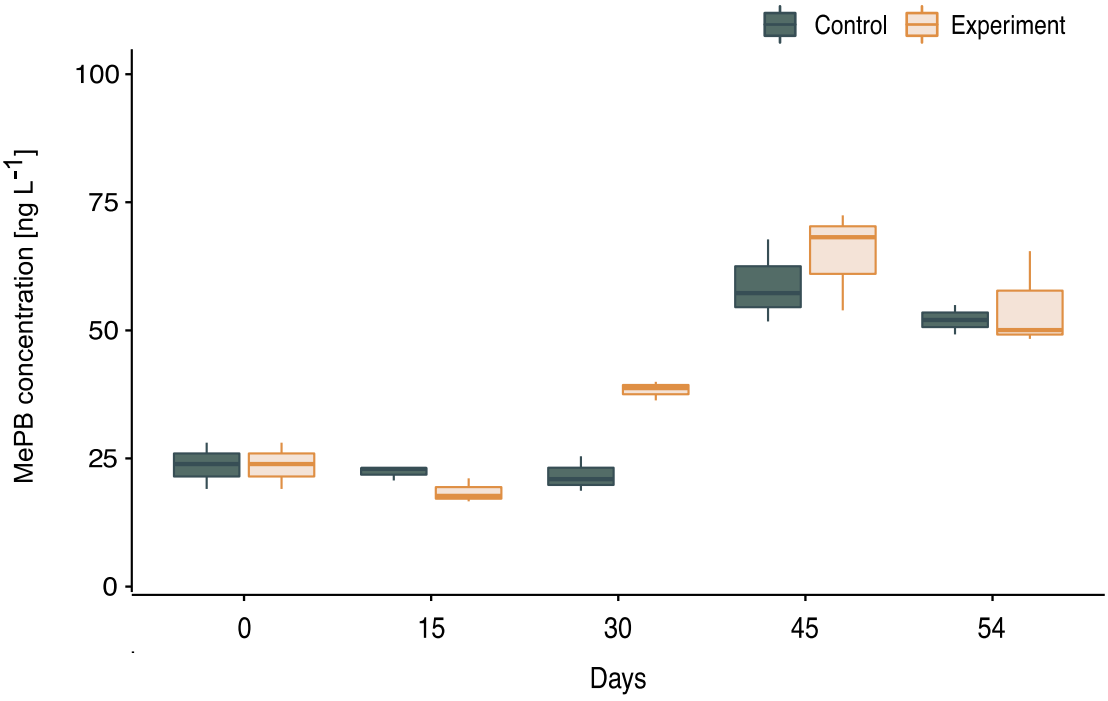


Fig. 5. Mean concentrations (± SD) of methylparaben measured in water from 0 to 54 days in control and experimental treatments.

Supporting Information

Supplementary information 1.xlsx – Table S1

Supplementary information 2.pdf – Tables S2 to S9

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CRediT Authorship Contribution Statement

Katarina Cetinić: Data curation, Investigation, Visualization, Writing – original draft, Writing – review & editing. Ivana Grgić: Investigation, Methodology, Visualization. Ana Previšić: Conceptualization, Funding acquisition, Investigation, Methodology, Writing – review & editing. Marko Rožman: Data curation, Conceptualization, Funding acquisition,

Investigation, Methodology, Software, Visualization, Supervision, Writing – review & editing.

Competing Interests

The authors declare no competing interests.

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