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The Impact of Parabens and Their Mixtures on Daphnids

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Abstract: Parabens are recognized as emerging contaminants. Used in the pharmaceutical and cosmetic industries, they present significant ecological risks to aquatic ecosystems. This study evaluated the effects of two parabens—methyl 4-hydroxybenzoate (methylparaben) and propyl 4-hydroxybenzoate (propylparaben)—both individually and as a mixture on daphnids. Through a series of controlled exposure experiments, phenotypic endpoints, including mortality, feeding behaviour, and enzymatic activity, were measured. The results demonstrate that propyl paraben and the mixture of propyl and methyl paraben exhibit greater toxic potential compared to methyl paraben alone. We observed a dose-dependent impact on key enzymes, indicating significant metabolic disruption. These findings underscore the necessity of utilizing model systems to investigate the complex interactions and cumulative impacts of pollutant mixtures in aquatic environments. Furthermore, this study provides critical insights regarding the underlying toxicity mechanisms of parabens, highlighting the need for comprehensive water quality monitoring and risk assessment frameworks to address the challenges posed by emerging contaminants.

Keywords: *Daphnia magna*; parabens; toxicity; miniaturization; enzyme kinetics; mortality; feeding rate

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1. Introduction

Water pollution presents a serious challenge, driving the need for effective legislation and monitoring procedures that can support improved water quality through well-implemented strategies and regulations [1]. The discharge of emerging contaminants into aquatic ecosystems, fueled by rapid urbanization and population growth, is a pressing concern. Many of these contaminants, including pharmaceuticals, personal care products, and industrial chemicals, currently lack regulatory oversight in terms of monitoring or disclosure, leaving their impact largely unchecked [2]. As emerging contaminants accumulate, they pose unknown risks to human and environmental health, disrupt aquatic life, and have the potential to bioaccumulate in the food chain. This lack of regulation, combined with the increasing prevalence of emerging contaminants, highlights the urgent need for more research and proactive management strategies to mitigate their effects.

Water pollution arises from diverse sources, each contributing unique contaminants and ecological impacts. Industrial activities, including chemical discharges and oil spills, introduce toxic substances like heavy metals directly into waterways. These harm aquatic ecosystems and bioaccumulate in the food web. Agricultural activities, such as excessive pesticide and fertilizer use, lead to nutrient pollution, promoting algal blooms that deplete oxygen levels and disrupt species balance. Additionally, urban sewage and sludge contribute a wide array of emerging contaminants that persist in the environment and compound ecological challenges, particularly in densely populated regions [3].

Among various emerging contaminants, parabens—commonly found in cosmetics and personal care products—are of particular concern [4]. Parabens, such as propylparaben, have been used for their antibacterial properties for over half a century, resulting in their

widespread environmental presence [5]. Residual paraben levels in wastewater effluents contribute significantly to its detection in water supplies globally [4,6].

Although wastewater treatment plants (WWTPs) are capable of effectively removing target parabens, some WWTPs are still lacking on this front due to differences in design and removal processes [7]. For example, if a WWTP relies on ultrafiltration, the larger pore sizes of the filters relative to paraben molecules lead to poor paraben removal (only up to 9.9%). However, conventional methods like activated sludge systems can achieve approximately 91.8% of paraben biodegradation, while adsorption to sludge contributes to around 7.5% of removal. Depending on the type of paraben, the removal efficiency using conventional methods can vary, but for the common types such as propyl and methyl paraben, this can reach up to 97.6–99.8%. Advanced treatment methods such as ozonation may also be introduced for additional purification, where 100% removal can be achieved for most parabens [8]. Therefore, complete paraben removal is achievable; however, due to differences in WWTP systems, comprehensive removal is not possible. For example, a sampling site, Dassenhoek in South Africa, was recorded to have a removal efficiency of 48.3% and 49.7% for methyl and propyl paraben, respectively [7].

Paraben concentrations vary significantly globally, ranging from nanogram-per-litre to milligram-per-litre levels. For instance, in Africa, methyl paraben concentrations have reached up to 0.56 mg/L and 0.52 mg/L in Tunisian and Nigerian wastewater, respectively, while Nigeria has also reported high ethyl paraben levels (0.377 mg/L) in their surface waters. In Lake Khindsi, India, methyl paraben concentrations are up to 0.0182 mg/L [9]. This widespread presence of parabens reflects global disparities in product consumption rates and paraben regulation [6]. For instance, the European Union has enacted stricter regulations, with concentration limits of 0.14% for propyl and butyl parabens in cosmetics in relation to the total formulation in a product, as well as prohibitions relating to children's products. Methyl paraben and ethyl paraben are still allowed in certain food products and so are often found in confectioneries, decorations, coatings, cereals, and fillings. They may also be present in pharmaceuticals if they cannot be substituted in the medication. In the U.S., there are also regulations for parabens, and the Food and Drug Administration (FDA) allows the use of individual parabens in food and pharmaceuticals at concentration limits below a level of 1%, or 0.3% for mixtures. These are different from and notably higher than the allowances made by the EU regulations. Additionally, the FDA imposes no restrictions on the use of parabens in food packaging when they are listed as antimycotics, or in veterinary products. There is also a lack of information found regarding regulations for wastewater or drinking water [10,11].

Parabens have the potential to exhibit synergistic toxicity, compounding their ecological impacts beyond their individual effects. While they are beneficial as preservatives, their combined toxicity can adversely affect aquatic ecosystems, as demonstrated by studies on *A. fischeri* [4]. However, further investigation, particularly using higher organisms, is crucial to understanding their broader ecological implications.

Conventional freshwater chemical assessments often lack preliminary toxicological testing, relying on chemical categories and analogue read-across methods. They are also insufficient to determine the probability that chemical contamination endangers aquatic ecosystems or human health [12,13]. These approaches focus on a number of chemicals which can be detected, making it impossible to determine the precise risk that mixtures of chemicals provide [14]. Hence, there is a considerable and growing chance that important dangers may be missed. Alternatively, New Approach Methodologies (NAMs) encompass a range of innovative technologies and approaches designed to provide insights into chemical hazards and risk assessment while minimizing or eliminating the need for animal testing [15]. These methodologies offer promising avenues for pollution assessment and contribute to the mechanistic understanding of testing and its relevance to humans, particularly in studies involving chemical mixtures that share similar modes of action with key species within freshwater ecosystems [14,16]. With the incorporation of NAMs, researchers gain valuable information on the effects of contaminants without relying on

traditional animal testing methods, enhancing both ethical considerations and scientific outcomes.

Daphnids are used extensively in molecular ecotoxicology as valuable indicators of chemical impacts on aquatic environments [17]. With sufficient and well-characterized molecular data, and being simple to culture in the laboratory, daphnids enable consistent assessments of toxicant effects, whilst being free from any genetic variability due to their parthenogenetic lifecycle [18]. As non-sentient species, daphnids provide an ideal model in ecotoxicology. When exposed to environmental stimuli, daphnids exhibit a range of morphological and behavioural changes that can significantly impact their survival and ecological interactions. These changes include alterations in growth rates, metabolic activities, defence responses (i.e., spines and helmets), and swimming, which can be indicative of stress responses and chemical signals to pollutants or environmental changes. It is also important to mention that daphnids are used extensively in toxicity testing in the context of the 3Rs principles (reduce, refine, replace) of higher evolved animals in research. Recent progress in evolutionary developmental biology and ecological functional genomics has highlighted the potential to reduce the time required for animal testing by utilizing simpler model organisms, such as daphnids [15]. Observations of these systems serve as the basis for established procedures used for chemical toxicity testing, including the EPA's chemical testing programme and the OECD's tests, with test 211 used for reproduction [19] and test 202 used for acute toxicity [20].

In this study, the impact of methyl 4-hydroxybenzoate (methylparaben) and propyl 4-hydroxybenzoate (propylparaben) on daphnids was assessed individually and in a mixture. These effects were quantified using phenotypic endpoints, such as mortality and feeding behaviour, alongside enzyme activity assays. The molecular data obtained provide valuable insights for water quality monitoring and contribute to a mechanistic understanding of paraben pollution in aquatic ecosystems.

2. Materials and Methods

2.1. Materials

All the chemicals used in this study were of the highest analytical quality. Methyl 4-hydroxybenzoate (CAS:99-76-3), propyl 4-hydroxybenzoate (CAS: 94-13-3), and *p*-nitrophenyl phosphate (CAS: 4264-83-9) were purchased from ThermoScientific. Dimethyl sulfoxide (CAS: 67-68-5) was purchased from Fischer Chemical. Reduced glutathione (CAS: 70-18-8) was purchased from MP Biomedicals. We purchased 2-nitrophenyl-β-D-galactopyranoside (CAS: 369-07-3) from Melford. L-Leucine-4-nitroanilide (CAS: 4178-93-2) was purchased from Alfa Aesar. We purchased 4-nitrophenyl butyrate (CAS: 2635-84-9), 1-chloro-2,4-dinitrobenzene (CAS: 97-00-7), and Brilliant Blue G (CAS: 6104-58-1) from Sigma Aldrich.

2.2. Culturing Daphnids and Acute Exposures

Daphnids were cultured in aqueous media in a 16 h:8 h of light/dark photoperiod at 20 °C. Media were renewed once a week. The cultures were fed with algae (*Chlamydomonas rheinhartii*) daily and with an organic seaweed extract at the point of media renewal, as described previously [21]. Briefly, media were prepared that contained salt dissolved in the final concentration (0.29 g $CaCl_2.2H_2O$/L, 0.123 g $MgSO_4.7H_2O$/L, 0.065 g $NaHCO_3$/L, 0.0058 g KCl/L, 2 µg Na_2SeO_3/L). Media were aerated for 24 h and the pH was adjusted to a pH of 7.7. For experiments, neonates (<24 h) were collected from the third brood of their mothers, as this is the accepted practice among the research community for acute toxicity exposures, which according to observations have better yield and reproducibility. Neonates were chosen for this study as they are more sensitive to environmental stimuli compared to daphnids in adult life stages, making them particularly vulnerable to the effects of pollutants and toxicants. Fifteen daphnids were exposed to methyl paraben (M), propyl paraben (P), and their 1:1 mixture (MP) at a range of concentrations (1–100 mg/L) for 24 h (OECD, 2004), and toxicity curves were plotted with their concentrations in a logarithmic scale. Using the Hill four-parameter model, the EC_{50} values were calculated

using GraphPad software (version 12) with the Hill slope; the EC1 and EC5 were also estimated to determine non-lethal concentrations. Finally, 0.01, 0.1, and 1 mg/L were selected for all experiments as these measurements were far lower than any lethality observed (with 0.01 being 500 times lower than EC_1) and had environmental relevance. Approximately 200 animals were used for each replicate, with four replicates used for each concentration and the non-exposed control. Parabens were initially dissolved in DMSO, and the final concentration of paraben was maintained to a minimum of 0.05% for all exposures.

2.3. Enzyme Activity and Feeding (Ingestion) Assays

In the assessment of enzyme activities, all animals were alive following exposure and daphnids were collected immediately, homogenized, and diluted in the appropriate buffer (phosphate pH 7.2, or boric acid pH 9.4 or acetic acid pH 4.5, depending on the enzyme assay) with an Eppendorf pestle homogenizer. The homogenates were cleared by centrifugation (12,000× *g* at 4 °C for 5 min) and the supernatants were collected and assayed for enzyme activity and protein levels in order to normalize all results based on previous methods [22].

Phosphatase activity was measured based on the synthesis of p-nitrophenol in 50 mM boric acid, pH 9.8 for alkaline phosphatase or acetic acid buffer pH 4.5 for acid phosphatase. We catalyzed the conversion of 1.6 mM *p*-nitrophenyl phosphate for 30 min at room temperature. The reactions were alkalized with the addition of NaOH (0.66 M) and the absorbance of *p*-nitrophenol was measured at 405 nm. It was first converted to nmoles using a standard curve and then expressed as the rate or units of activity. Similarly, the activity of β-galactosidase was quantified based on the synthesis *o*-nitrophenol, using *o*-nitrophenyl-β-galactoside as a substrate, while the lipase was estimated based on the synthesis of *p*- from *p*-nitrophenyl-butyrate. Kinetic assays for β-galactosidase and lipase were performed in 50 mM phosphate buffer, pH 7.2, and measured at 405 nm.

Using L-leucine-4-nitro-anilide as the substrate, the activity of peptidase was measured at 418 nm in 50 mM phosphate buffer, pH 7.2 from the released 4-nitro-aniline. Absorbance was converted into nmoles 4-nitro-aniline using a standard curve, and then into units. Finally, glutathione-S-transferase activity was quantified as the S-DNP-GS product of the conjugation of reduced glutathione and 1-chloro-2, 4,dinitrobenzene (CDNB). The rate was monitored at 340 nm for 10 min and was expressed as a rate in units using the extinction coefficient of the complex [23]. Finally, the protein was assessed using a sensitive method based on the Coomassie Brilliant Blue [23] and all enzyme activities were reported as units (in nmoles product per minute) per protein.

For feeding, following exposure five daphnids were incubated with 26 mg/L carboxyl-functionalized microparticles in a 1 mL volume. The selection of these parameters was based on the optimized method of Rowan et. al. [24]. Samples (200 µL) were collected after every 10 min. As a reference starting point, wells without animals were used to represent the initial amount of available microparticles. The difference in fluorescence (at Ex/Em 560/590 nm), measured with a TECAN plate reader, was converted into the ingested microparticles using the corresponding standard curve.

2.4. Statistical Analysis

Data were presented as average ± standard deviation (SD) and were analyze and plotted with the GraphPad Prism software. Working from the unexposed control, significant differences were identified by one-way ANOVA (feeding) and two-way ANOVA (enzyme activity), followed by Dunnett's post hoc test. This was followed by comparisons wit the DMSO, which was used as the carrier solvent. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3. Results

The acute toxicity of individual parabens and their (1:1) mixture was evaluated in daphnids with toxicity curves in neonates following 24 h of exposure (Figure 1), and EC values were calculated (Table 1). The EC_{50} values of methyl and propyl parabens were significantly different than the EC_{50} values of 45.31 mg/L and 11.02 mg/L, respectively, while their mixture displayed an EC_{50} of 11.01 mg/L. This highlights the increased toxicity of propyl paraben in comparison to methyl paraben, and show that propyl paraben is the main driver of toxicity in the mixture.

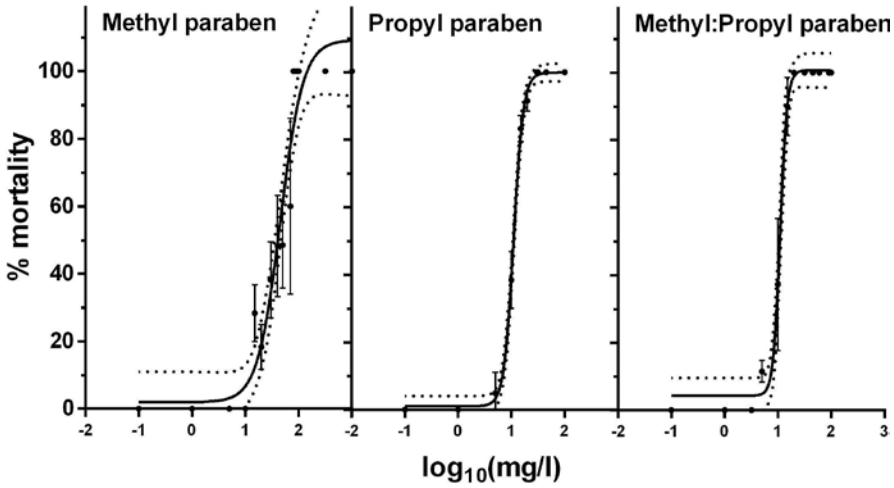


Figure 1. The acute toxicity of parabens. Data represent average ± SD (N = 4 replicates per concentration). The four-parameter logistic (4PL) model was used to calculate EC_{50} values.

Table 1. EC values (in mg/L) for acute toxicity in neonates.

Chemical	EC_{50}	Hill Slope	EC_1	EC_5
Methylparaben	45.31	2.032	4.72	10.63
Propylparaben	11.02	4.751	4.19	5.93
Methyl propyl mixture	11.01	6.664	5.52	7.08

Feeding is a non-invasive endpoint for daphnids, reflecting energy demands and performance. It was assessed with a novel protocol that monitored the ingestion of fluorescent microparticles (Figure 2). In this assay, both methyl paraben and propyl paraben were tested at the highest concentration of 1 mg/L. Additionally, the paraben mixture (1:1) was also tested at the same concentration of 1 mg/L. This concentration was chosen because it was expected to produce a measurable effect. Methyl paraben exhibited a 27% decrease and propylparaben was the only toxicant to show a significant increase of 126% in the feeding rate. On the other hand, the paraben mixture did not show any notable change compared to the DMSO carrier solvent, which was expected as both chemicals had opposing individual effects.

The impact of parabens on key enzyme activities reflects on the physiology of daphnids, and enzymes were impacted differently by the exposure to individual parabens and their mixture (Figures 3 and 4). For phosphatase activity (Figure 3), methylparaben exposure led to a significant decrease in the activity of both alkaline phosphatase and acid phosphatase, ranging from 28% to 46% across exposure concentrations. In contrast, propylparaben decreased the activity of acid phosphatase by 25% at both 0.1 and 1 mg/L concentrations but had no effect on the activity of alkaline phosphatase. The mixture of parabens resulted in a 29% decrease in the activity of alkaline phosphatase at 1 mg/L, while acid phosphatase activity was reduced by 27% at 0.1 mg/L and by 46% at 1 mg/L.

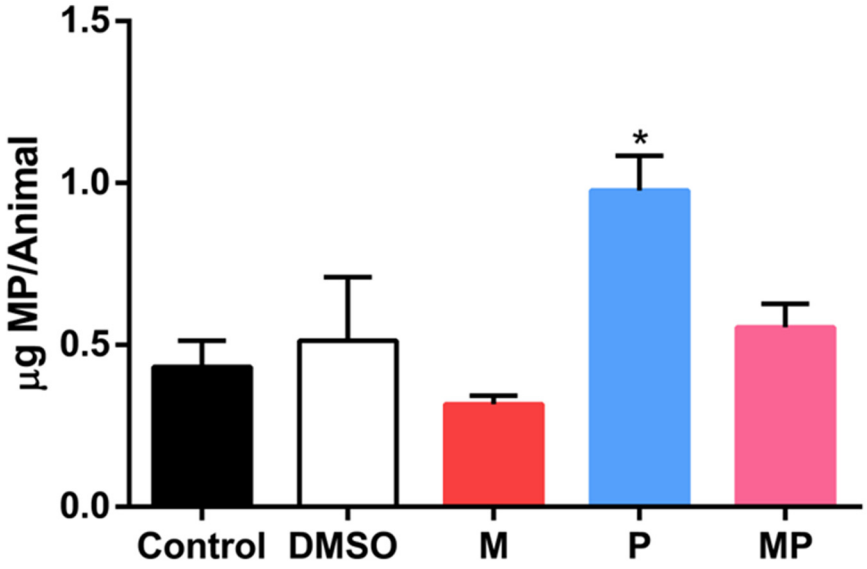


Figure 2. The impact of parabens on the feeding rate of daphnids. Data represent average ± SD (N = 4 replicates for each condition). Statistically significant differences were identified with one-way ANOVA followed by comparison with the DMSO carrier solvent with a Dunnett's post hoc test. Asterisks indicate statistical significance: * $p < 0.05$.

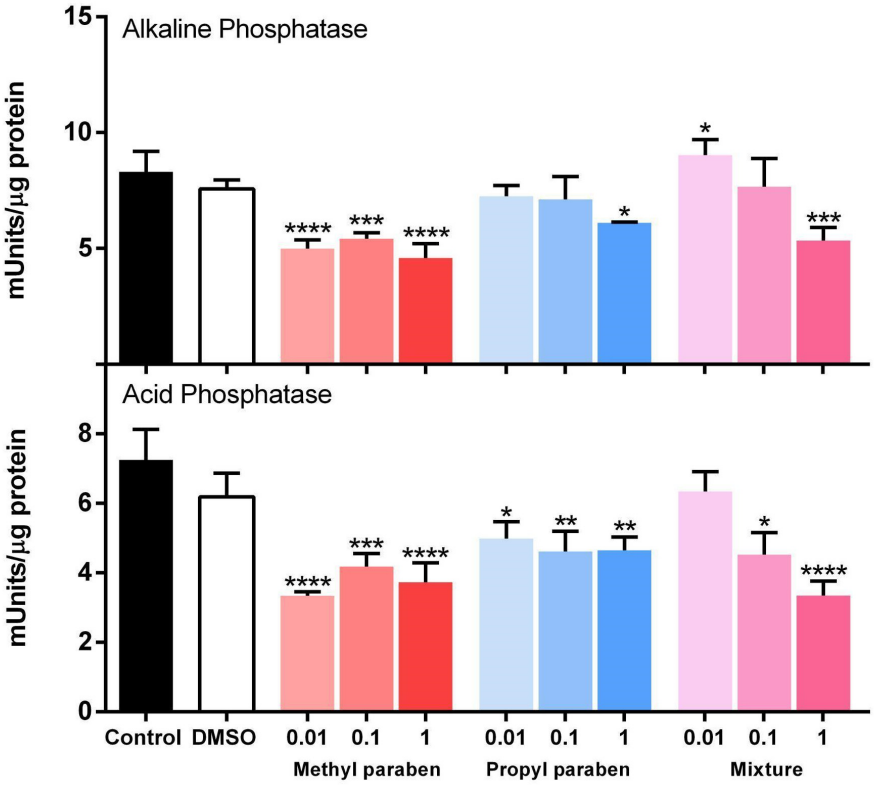


Figure 3. The impact of parabens on the activities of phosphatases. Data represent average ± SD (N = 3 replicates for each condition). Statistically significant differences were identified with two-way ANOVA, followed by comparison with the DMSO carrier solvent with a Dunnett's post hoc test. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

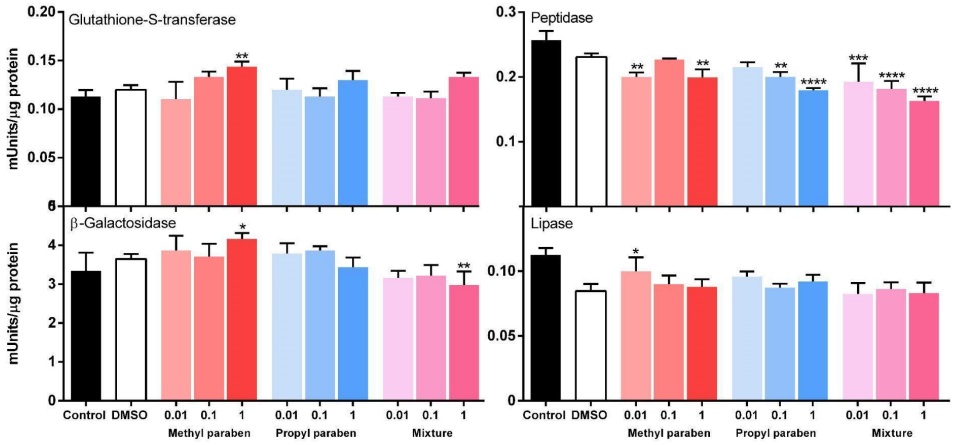


Figure 4. The impact of parabens on the activities of catabolic enzymes and glutathione-S-transferase. Data represent average ± SD (N = 3 replicates for each condition). Statistically significant differences were identified with two-way ANOVA, followed by comparison with the DMSO carrier solvent with a Dunnett's post hoc test. Asterisks indicate statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

The activity of glutathione-S-transferase (GST) showed an increase of 19%, but only at the 1 mg/L concentration of methylparaben. For peptidase, methyl paraben caused a decrease in activity, with reductions of 28% at 0.01 mg/L and 43% at 1 mg/L. Similarly, propyl paraben reduced peptidase activity by 1% at 0.1 mg/L and 35% at 1 mg/L. The mixture increased the activity of peptidase by 38% at 0.01 mg/L, but then led to decreases of 9% and 48% at 0.1 mg/L and 1 mg/L, respectively. Lastly, no significant changes were observed in the activities of lipase or β-galactosidase in any of the conditions.

4. Discussion

Our study used similar concentrations to capture the reality of the effects of these pollutants. Mixtures of pollutants are equally important as the relevant components of freshwater environments. In polluted ecosystems, numerous toxicants often coexist, leading to additive, antagonistic, or synergistic interactions [25]. Certain mixtures display combined synergistic effects that exceed predictions based on current effect models. This substantial uncertainty complicates the ability to accurately forecast the biological impacts of toxicant mixtures. Many studies fall short of capturing every class of chemical mixture found in freshwater and, consequently, do not assess the possibility of combined toxicity. Our study investigates this and obtains significant results with environmentally relevant concentrations and mixture compounds.

4.1. The Effect of Parabens on the Ingestion Rate of Daphnids

The feeding rate of daphnids is a key physiological endpoint for environmental analysis, as food intake directly influences development, growth, fecundity, survival, and overall population dynamics [26]. Additionally, compared to traditional mortality assessments, the feeding rate offers a highly sensitive and rapid assay, allowing for the detection of pollutants at environmentally relevant levels. This sensitivity makes it a valuable tool in ecological risk assessment, emphasizing the importance of understanding how various pollutants, including paraben mixtures, affect feeding behaviours.

In our study, we found that propylparaben exposure elevates the ingestion rate of daphnids, which could be due to a mix of stress-induced metabolic demands, hormonal disruptions, and compensatory feeding behaviours. Propylparaben, as an endocrine disruptor, appears to influence neurochemical pathways related to appetite, potentially increasing food consumption [27]. Moreover, the toxic stress from propylparaben exposure may heighten metabolic rates, leading to increased energy demands and thus higher food intake [28]. Additionally, pollutants like propylparaben can impair nutrient absorption

efficiency, prompting organisms to consume more to meet their nutritional needs [29]. Together, these factors likely explain why daphnids exhibit increased ingestion rates when exposed to propylparaben.

Although the impact of parabens on the ingestion rate of daphnids is not well understood, some studies have evaluated the effects of parabens on other phenotypic endpoints, which can either affect or be affected by the ingestion rate. For instance, Eghan [30] evaluated the effects of methyl and propylparaben on the thoracic limb activities of *D. magna* and noted a dose-dependent decrease for both parabens. As filter feeders, daphnids feed on small, suspended particles in their environment and are highly dependent on their thoracic limbs for basic needs such as food intake [31]. However, the observed decrease in the activity of the thoracic limb, shown by Eghan [30], differs from our findings regarding propylparaben in relation to feeding behaviour, which was increased in our study, but decreased in the study of methylparaben.

The implications of parabens on *D. magna* have not been extensively studied; however, another model freshwater organism from the family, *C. dubia*, has been investigated for phenotypic endpoints. Terasaki [32] observed a dose-dependent decrease in the number of offspring and time to first brood for *C. dubia* when exposed to methyl and propylparaben at concentrations of 1.56–12.5 mg/L and 0.63–10 mg/L, respectively. Reproductive cycles of daphnids only occur under optimal conditions, such as when there is sufficient food supply, and any changes in these factors could inhibit this cycle [33]. This link between reproduction and ingestion is similar to the thoracic limb activities of daphnids, in that any changes in these biological factors could be due to the inhibition of feeding.

To our knowledge, there have been no studies showing the effects of a paraben mixture on the ingestion rate of daphnids and there is not much literature on parabens and daphnids. However, Lee [4] showed that a mixture of methylparaben and propylparaben exhibited acute toxicity to another freshwater organism, *Aliivibrio. fischeri*, by inhibiting bioluminescence by about 40%, suggesting an additive toxicity effect. Contrary to these findings, our study on feeding did not observe an additive effect in our assay, highlighting a potential difference in the response mechanisms between ingestion and bioluminescence endpoints.

4.2. The Effect of Parabens on Key Enzyme Activities of Daphnids

Alterations in daphnid enzyme activity have been shown to be good predictors of environmental pollution and are used in an array of ecotoxicological studies due to their high sensitivity and the ease of carrying out the experimental assays. Changes in enzyme biomarkers in daphnids occur when exposed to pollutants such as heavy metals and common pharmaceuticals. These changes in enzyme activities have been used to interpret the impact of therapeutic agents, including analgesics, antipsychotics, antihypertensives, anticonvulsants, nonsteroidal anti-inflammatory drugs, and more. The alteration in enzymes used in the study and changes in phosphatases, in lipase, in peptidase, in β-galactosidase, and in GST have been explored in previous studies on environmental contamination. Some other enzymes used as biomarkers for daphnids are lactate dehydrogenase, glutathione reductase, cellulase, amylase, trypsin, catalase, cholinesterases, glutathione-peroxidase, and esterase [21,34,35]. As with measuring the ingestion rate, studying changes in enzyme activities allows for pollution to be detected at sub-lethal concentrations. Thus, it provides a more realistic insight into the natural environment where contaminants would persist but be diluted in the aquatic ecosystem.

Acute exposure to methylparaben and propylparaben resulted in changes in enzyme activity in daphnids (Figures 3 and 4). Following exposure to methylparaben, the enzyme activities of phosphatases were decreased in all three concentrations. Changes in alkaline phosphatase exposure indicate that methylparaben does not have a dose-dependent effect for the enzyme. Changes in the activity of peptidase and acid phosphatase also support this conclusion. GST saw an increase in the highest concentration. From the results, it is evident that methylparaben can impact markers of physiology upon initial exposure.

Propylparaben also alters enzyme activity in daphnids. As observed in Figures 3 and 4, it was able to affect acid phosphatase and peptidase, but changes were only significant at the higher concentrations. The enzyme activity of the daphnids exposed to propylparaben also points towards a dose-dependent effect, unlike what is seen with methylparaben. Although both contaminants are parabens, they exert very different effects on daphnids. Overall, propylparaben generated a decrease in enzyme activity, but in contrast to methylparaben, it had a smaller impact on enzyme activity despite displaying a lower EC_{50} value. Propylparaben's greater toxicity may be due its higher lipophilicity and cell permeability due to its longer alkyl chain [36]. On the other hand, the difference in permeability and chain length potentially causes different interactions with phosphatases. For methylparaben, this may mean it can impact enzyme activity without causing toxicity. The two chemicals also produce different minor metabolites [37,38] this can also be another factor that can influence how enzymes are altered. For example, methanol, a metabolite from methylparaben, has been shown to be able to affect phosphatase activity in prawn *(Penaeus penicillatus)* in the past literature [39]. However, these explanations need further research if they are to confirm the findings, as it is unlikely that these metabolites were concentrated enough to have played a significant role in this study.

Their combined effect was also evaluated because in realistic scenarios, compounds are not singled out but exist in mixtures. However, there is value in predictions of their actions with concentration addition (CA) or independent action (IA) models; therefore, we carefully used term synergy as predictions were not the aim of the study. For the mixture, although it elicited fewer changes in enzyme activity in daphnids when introduced as an individual chemical, once it was present in a cocktail of methylparaben and propylparaben, propylparaben was shown to be a stronger driving force regarding the alterations observed for enzyme activity. Alterations in daphnids exposed to the mixture are similar to the alterations seen in samples exposed to propylparaben, highlighting the fact that it has a greater influence on enzyme activity. This is also seen in the toxicity of the parabens (Figure 1), where the mixture displays an EC_{50} similar to that of propylparaben. Additionally, it similarly has a dose-dependent effect in peptidase and acid phosphatase, where enzyme activity decreases as the concentration increases. The difference between the alterations in the two examined parabens and their mixture compared to the results of the study also show the importance of assessing different compounds, even those from the same class of chemical group. This variation in changes means that different processes or pathways may be affected.

These enzymatic alterations, occurring due to exposure to methylparaben, propylparaben, and their mixture, deserve attention as exposure will likely disrupt key metabolic and detoxification pathways. The analyzed enzymes are integral to cellular processes like detoxification, phosphate metabolism, lipid metabolism, lactose metabolism, and protein digestion and modification. Alkaline phosphatase is essential for bone mineralization and energy regulation [40]. Acid phosphatase is involved in lysosomal processes and cellular digestion [41]. GST is involved in signalling pathways and protects cells from oxidative stress and supports cellular detoxification [42]. Lipases are crucial for energy production, cell membrane synthesis and signalling pathways due to their role in in lipid metabolism [43]. Peptidases degrade peptide bonds and are needed for protein digestion and cellular recycling [44]. β-galactosidase is required for lactose metabolism [45]. These enzymes contribute to a broad range of physiological processes and take part in basic metabolic functions; hence, the ability of parabens to alter the activity of these substances can hinder and impact the health and survival of affected organisms and their environments.

Exposure to parabens was proven to influence enzyme activity in this study, and this is supported by the previous literature. Aside from influencing GST activity, they are capable of impacting the activity of other antioxidant enzymes, such as glutathione peroxidase and superoxide dismutase [46]. They can also be responsible for endocrine disruption. They can interfere with estrogen metabolism by inhibiting estrogen-activating enzymes [47], are a

suspected cause of infertility in girls [47], can impact the thyroid, and can cause an increase in birth weight [48].

Other organisms can also be impacted, such as aquatic creatures like the zebrafish (*Danio rerio*). The Nile tilapia fish (*Oreochromis niloticus*) also experienced changes in enzyme activity after paraben exposure [49,50]. Methylparaben, propylparaben, and butylparaben are also toxic to zebrafish, the common carp (*Cyprinus carpio)*, and African clawed frogs (*Xenopus laevis*) at high concentrations. They can cause significant developmental issues, such as cardiovascular malformations like heart edemas that develop in the embryo, and delay hatching rates in fish and amphibians. The downregulation of certain genes also suggests that there is an impact on stress response and detoxification pathways [51,52]. Common parabens can also lead to signs of narcosis in fathead minnows (*Pimephales promelas)* [53]. From the acute results shown in Figures 3 and 4, it can be seen that the pollutants are capable of impacting daphnids too. Research conducted by Eghan et al., which assessed the neurotoxicity and cardiotoxicity of acute paraben exposure in daphnids, also found they can impact behaviour, heart rate, and limb activity [30]. Exposure can also influence mortality and growth in daphnids [53].

5. Conclusions

This study provides evidence that methylparaben, propylparaben, and its mixtures pose significant ecological risks to daphnids, and in extension, to aquatic ecosystems, expanding and confirming the findings of the previous literature. The novel insights demonstrate that both individual and combined exposure to these parabens result in alterations in phenotypic endpoints, including mortality rates and feeding behaviours, alongside synergistic disruptions in enzymatic activities critical to biological processes. Additionally, propylparaben and the mixture exhibited a greater toxicity when compared to methylparaben, highlighting the necessity of considering the ecological ramifications of pollutant mixtures rather than evaluating chemicals in isolation. The dose-dependent impacts observed also indicate that even low concentrations of these contaminants can disrupt key physiological functions in daphnids, raising concerns about their broader implications for freshwater food webs and ecosystem health. However, although alterations were observed in all acute experiments, the lack of data on long-term effects is a limitation of this study and most of the previous literature on this topic. For future research, chronic or even transgenerational exposure experiments should be conducted on daphnids. By diverging from previous research and analyzing long-term effects, this research would reveal if the species would adapt to the pollutants, or if the impact would be exacerbated. This would also give a more realistic view of how the chemicals would interact with the natural environment, as many organisms would have a fast generation time like *Daphnia*, and because pollutants are rarely removed from the contaminated ecosystem in short periods [54,55]. Overall, this study highlights the critical need for mitigation measures in regard to the impacts of emerging contaminants like parabens in our waterways. Given the widespread prevalence of parabens in the environment, which is due to insufficient wastewater treatment and regulatory gaps, these findings underscore the urgent need for enhanced monitoring and risk assessment strategies.

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Institutional Review Board Statement: Having the confirmation of the university (Mr. Damian O'Donohue from the Biological Research Unit who oversees the use of biological agents in the School of Biotechnology) research on daphnids does not require ethical review or approval from the university or the authorization from the Health Products Regulatory Authority (HPRA). This is

because daphnids are not regarded as animals used for scientific purposes in the regulation SI543 of 2012 on the protection of animals in research. Additionally, daphnids are in line with context of the 3Rs strategy and are commonly used in studies as replacements for more advanced species.

Informed Consent Statement: Not applicable.

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