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Original Research Paper

Investigating the ecotoxicological effects of dimethyl phthalate (DMP) and polyethylene (PE) on the floating aquatic plant, *Lemna minor*.

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Abstract: Freshwater ecosystems are increasingly threatened by microplastics and their associated leachates; as significant producers in the freshwater environment, aquatic plants are at the greatest risk of emerging contaminants, threatening biodiversity, supportive and regulative ecosystem services. Given this, the aim of the present study was to identify and evaluate the dose-response effects of both dimethyl phthalate and polyethylene (30µm fragments), on the frond number, surface area, dry weight and chlorophyll content of *Lemna minor*.

The results of this study indicated that while polyethylene did not consistently produce significant dose-response trends across the measured parameters, it did exhibit significant effects with respect to chlorophyll content (LOEC = 10 mg/L). Exposure to DMP caused significant ($p < 0.001$) effects on *Lemna minor* frond number and dry weight (LOEC = 10 mg/L). The combined exposure of DMP and PE revealed evidence of an interaction between the two contaminants, with notable effects on frond number and dry weights. Significant effects were also observed on chlorophyll content (LOEC = 50 mg/L of DMP when combined with both 10 and 100 mg/L of PE), supporting the hypothesis that a combination of the two amplify phytotoxicity.

The findings of this study highlight the potential for microplastics and their associated leachates to disrupt plant growth, underlining the need for improved ecotoxicity assessments. The outcomes of this work contribute to a growing wealth of literature which aim to inform ecological risk assessments, supplement freshwater management plans and improve the health of our freshwater ecosystems.

Keywords: Freshwater; Ecotoxicity; Microplastics; Phthalates

Introduction

1.1 An introduction to freshwater ecosystems

Aquatic ecosystems are crucial in maintaining ecological health, biodiversity, climate control and agricultural systems across the world (Prakash, 2021; Arya, 2021). Freshwater ecosystems, precursors to marine ecosystems and consequently interrelated, are particularly vulnerable to contamination and are exposed to higher levels of pollutants than most other environments (Bashir et al., 2020). Freshwater provides upwards of 20-30 ecosystem services including recreational, spiritual and agricultural services which benefit quality of life for humans in addition to climate services; namely microclimate regulation and carbon sequestration (Tomscha et al., 2017; Vari et al., 2022). Whilst comprising only 0.01% of the global water index, freshwaters have the highest estimated ecosystem value of all inland ecosystems (~ 38,100 x 1012 $/year in comparison to ~4166 x 1012 $/year for temperate forests and grasslands) (Costanza et al., 2014; Vari et al., 2022). As a habitat, freshwater systems are host to more than 25% of described vertebrates, 9.5% of described animal species and approximately 2600 macrophyte plants (IUCN, 2008;

Balian et al., 2008).

Ecological balance supports rich biodiversity, which in turn maintains genetic diversity and reinforces ecosystem stability (Verma, 2021; Arya, 2021); consequently, the ecological health of our freshwater systems is critical. Despite this importance, the impacts of anthropogenic activities on freshwater ecosystems remain under-researched and often overlooked (Lenton et al., 2019; Albert et al., 2021). On a wider scale, this contributes to deficits in policies and an inability to create specific management plans, fueling resistance and stakeholder conflicts (Schafft et al., 2021).

1.2 UK legislation

The Water Framework Directive (WFD) was introduced by the European Union to improve integrated water management however, under their 2018 review, the European Environmental Agency confirmed that less than 50% of aquatic resources were in good ecological status (Solheim et al., 2019; De Vito et al., 2020). Since leaving the EU, the UK has adopted The Water Environment (Water Framework Directive) (England and Wales) Regulations 2017; in which water quality is tested every three years as opposed to annually. In 2019, just 16% of English rivers and canals were reportedly in good ecological health compared to 20% in 2015 (GOV, 2023) – another report is not proposed until late 2025. Inconsistent assessments combined with incomplete datasets prevent a direct comparison of UK water quality both historically and globally (JNCC, 2023).

Recent evaluations by the Office for Environmental Protection (GOV, 2024) conclude that the UK is not on track to meet the WFD target of good ecological status by 2027, citing inconsistent monitoring, incomplete datasets, and the absence of a robust replacement for the EU Priority Substances watch list as key challenges. In addition to fundamental management failures, pollution remains a critical driver, with emerging contaminants such as microplastics and their leachates posing particular concern. These pollutants not only persist within aquatic environments but can also disrupt freshwater trophic dynamics, impair ecological function, and threaten food security (Cara et al., 2022).

1.3 Importance of aquatic plants

Given they form the base of the food chain as primary producers (Ceschin et al., 2021), terrestrial and aquatic plants are both direct and indirect authorities in any ecosystem (Naselli-Flores and Padisak, 2023). In addition to their role as producers, plants are responsible for a variety of supportive, regulative, provisioning and cultural ecosystem services (Ceschin et al., 2021). A systematic review conducted by Thomaz (2023) successfully identified up to 26 individual services of which they contribute – including: sediment formation, carbon sequestration, erosion prevention, water purification, nutrient cycling, and the provision of habitats, cultural, spiritual and educational services (Tomscha et al., 2023; Ceschin et al., 2021; Naselli-Flores and Padisak, 2023; Sridharan et al., 2021). Aquatic plants in particular have the ability to influence an ecosystem on a physio-chemical, hydrological or geomorphological level through interaction with biotic and abiotic factors (O'Hare et al., 2018). Thus, giving plants significant roles in ecosystem management plans and freshwater restoration projects; supporting threatened or endangered species whilst limiting the spread of invasives (Thomaz, 2023; O'Hare et al., 2018).

At present, ecotoxicology studies heavily focus on animals or unicellular algae, with a distinctive lack of research on freshwater plants (Blinova, 2004; Wang, 2018). The use of ecotoxicological tests on plants, in addition to animals and other heterotrophs, is vital towards a comprehensive evaluation of the effects of such contaminants (Ceschin et al., 2021; Bhatt and Chauhan, 2023).

1.3.1 Relevance of *Lemna* sp. as ecotoxicological models

The chosen focus as a bioindicator in the present study is *Lemna minor*; due to its clonal nature the plants have very little genetic diversity or complexity (Acosta, 2021), which makes measuring fitness consequences simplistic and eliminates the need to test multiple variables (Laurich and Candeias, 2023). The *Lemnaceae* family also have one of the fastest growth rates and are one of the smallest angiosperms, logistically making experimentation easier (Acosta, 2021; Jewell and Bell, 2023).

1.4 Microplastics and their fate

Amongst several anthropogenic activities which threaten aquatic ecosystems, microplastics (MPs) are one of the largest concerns despite being one of the least studied (Bashir et al., 2020). As emerging global contaminants, MPs (0.1-5mm) (Ceschin et al., 2022) are omnipresent in both marine and freshwater ecosystems, consumables, human biological samples and drinking water (Meng et al., 2020; Ding et al., 2019). MPs can enter the environment directly as the result of industrial and manufacturing processes, or form secondarily as the result of fragmentation and biodegradation of larger plastics (Ma et al., 2018). The presence of MPs in the environment poses several ecotoxicological challenges to biodiversity through ingestion and exposure to MP-associated contaminants (Ma et al., 2018). Their surface area provides an optimal site for the adhesion of biofilms and microalgae, affecting biodegradation processes and promoting the formation of hetero-aggregates, processes which have the potential to influence the transport, fate and bioavailability of MPs in aquatic ecosystems (Carson et al., 2013; McCormick et al., 2014; Nava and Leoni, 2021).

Microplastics pose a significant hazard not only in their physical presence, but also their physicochemical properties (Da Costa et al., 2016). Plasticisers incorporated into the polymer matrix of plastics during manufacturing have the ability to leech over a plastic's lifetime (Billings et al., 2021). Of these additives, phthalate acid esters (PAEs) are the largest class. PAEs are classed as non-persistent global organic pollutants because they do not naturally bioaccumulate in waterways however, their incessant discharge into the environment - during manufacturing, use and disposal of plastics - makes them pseudo-persistent (Tyler et al., 2018; Net et al., 2015; Zhang et al., 2020). The 2022 global production of plasticisers amounted to approximately 10.88 million metric tons, of which phthalates contribute ~ 70%, with a predicted compound annual growth rate between 4-6% until 2030 (Kumari et al., 2021; Statista, 2024; Technavio, 2024). Moreover, approximately 4.8-12.7 million tons of plastic is released into waterways annually (Berry et al., 2023).

1.4.1 Polyethylene

Polyethylene (PE) is one of the most widely produced and utilised plastics globally, of packaging for both food and cosmetic products, alongside uses in manufacturing, engineering and clothing. The ecological effects of these interactions are not yet fully understood. Kalčíková et al., (2017) investigated the effects of two different types of polyethylene microbeads derived from cosmetic products (10-45µm). Despite significant adherence of PE to *Lemna minor*, the observations of this interaction displayed no significant impact to the growth rate or photosynthetic activity of the *Lemna minor* fronds. The authors attributed this to physical characteristics such as the shape and density of the microbeads, highlighting the importance of polymer morphology in these experiments.

Polyethylene terephthalate (PET) represents a major share of the global polymer market, estimated as high as USD $33 billion (Malpass, 2010). The production of PET commonly utilises phthalates as plasticisers, including dimethyl phthalate (DMP) and bi-butyl phthalate (DBP). Studies on rose-water packaged in PET (Hosseini et al., 2016) identified a significant increase in the migration of these leachates over the course of 8 weeks at a range of temperatures between 4-42°C. Given this information, the purpose of the current study was to identify if the combination of polyethylene, and a potential leachate with commercial relevance, such as DMP, could have an adverse effect on *L. minor* cultures in a toxicological experiment.

Studies on the River Kelvin, UK, found between 50-244 particles/kg2 (PE and polypropylene) (Shokunbi et al., 2024) and 161-432 particles/kg^2 (Blair et al., 2019). Similar studies on the River Thames – urban and downstream - and Medway, UK (Trusler et al., 2024) found levels between 170.8 particles/kg2 ± 46.64 (urban), 67 particles/kg2 ± 16.47 (downstream) and 167.4 ± 84.79 particles/kg^2, respectively. Of the MPs identified, PE was the most common making up ~42.4% across all sites. In similar studies (Horton et al., 2017; Rowley et al., 2020) all on the River Thames, PE and PET were the largest groups of identified polymers across all transects studied. Due to flow-dynamics and urbanisation, the Thames acts as a

perfect model as to the significance of rivers as MP-sinks, and the potential impacts of such bioaccumulation (Zhang et al., 2015; Horton et al., 2017; Rowley et al., 2020).

1.5 Phthalate acid esters and their ecotoxicity

Phthalates are esters of 1,2-dibenzene dicarboxylic acid, those with a high molecular weight (five or more carbon atoms) - including di-2-ethylhexyl phthalate (DEHP) - are typically utilized as plasticizers in polyvinyl chloride (PVC) products to improve flexibility (Sharma and Kaur, 2020a). Lower molecular weight (one to four carbon atoms) phthalate acid esters (PAEs) such as dimethyl phthalate (DMP), diethyl phthalate (DEP) or dibutyl phthalate (DBP) are often found in cosmetics, solvents, adhesives, pharmaceuticals and various other products (Tyler et al., 2018; Net et al., 2015).

Phthalates are largely unable to covalently bond to polymer products and readily enter environment (Kaur et al., 2017); low molecular weight (LMW) PAEs can also move through the polymer matrix more freely and leech from plastics at a faster rate (Hansen et al., 2013).

As known endocrine disruptors, carcinogens, mutagens and teratogens (Sharma and Kaur, 2020a; Ma et al., 2015); negative effects of PAEs on reproduction and mortality have been reported in a wide range of aquatic species (Seyhoum and Pradhan, 2019; Staples et al., 2009). Studies on terrestrial and aquatic plants (Ma et al., 2015; Sharma and Kaur, 2020b), as well as planktonic invertebrates and algae (Staples et al., 2009) have also demonstrated positive phthalate-mediated responses at lower concentrations.

1.5.1 Dimethyl phthalate

Pietrini et al., (2022) investigated the ecotoxicological and genotoxic effects of DMP on cultures on both *Lemna minor* and *Spirodela polyrhiza* at a range of concentrations. Results demonstrated that exposure to both 3mg/L and 30mg/L did not affect growth parameters for either species; at 600 mg/L however, both species showed symptoms of toxicity after just a few hours. Whilst this study attests that concentrations were chosen with regard to environmentally relevant concentrations - 2 mg/kg, 50 mg/kg and 250 mg/kg in sediment, soil and sludge, respectively (Net et al., 2015) - it is unclear where the justification for using 600 mg/L originated. Despite showing significant ecotoxicological effects, the time frame these effects were achieved in is unsustainable for the present study which aims to observe effects over a seven-day period.

With reference to DMP, there appears to be significantly less information in comparison to other phthalates; there are seldom studies on UK waterways, especially using modern quantification techniques, and those that did screen for DMP found it undetectable. Studies at Taihu Lake, China found levels ranging from undetectable to 1.32 ug/L (Gao et al., 2019); levels at the Songhua River, China and Chaohu Lake, China were 0.98 – 4.12 and 0.015 – 3.670 ug/L respectively (Gao et al., 2014; He et al., 2013); while levels in the Rhone River, France were measured between 0.003 – 0.005 (Palusselli et al., 2018) and 0.008 – 0.0024 ug/L (Schmidt et al., 2020). This is likely attributable to the higher affinity of DMP, as a LMW phthalate, to permissively move through polymer matrix and therefore disperse at a faster rate. It is of particular interest to the author why DMP therefore appears to have a lower ecotoxicity despite LMW phthalates generally being attributed to higher levels of toxicity, as a result of their ability to bioaccumulate in tissues (Giuliana et al., 2020; Staples et al., 2009).

The hydrolytic half-life of DMP is estimated at 3.2 years (Pietrini et al., 2022). With no current remediation or removal strategies in place, this raises questions about the potential for accumulation and impact of chronic exposure to PAEs in the environment (Takdastan et al., 2021; Billings et al., 2021; Net et al., 2015).

2.0 Methods

2.1 Materials, reagents and test species

Lemna minor fronds were originally obtained from Lincolnshire Pond plants and maintained at the University of the West of England facilities. Fronds were cultured prior to experimentation in a growth incubator (Eppendorf Innova S44i) (20 ± 2 °C; photoperiod 12hL:12hD) in Hoagland's solution. An adapted version of the OECD guidelines 221 (OECD, 2006) were used, which included alteration of the

growth temperature based on the findings from multiple culturing trials over an 18-month period. All chemicals and reagents used throughout the duration of this experiment were sourced from Sigma Aldrich (Merck, UK); polyethylene fragments were sourced from Goonvean Fibres Ltd.

2.2 Experimental design

This study consisted of a 7-day acute exposure experiment in which *L. minor* fronds were exposed to four differing treatments concurrently. These included Dimethyl Phthalate (C10H10O4, > 99% purity) at 10, 25, 50, 75 and 100 mg/L, polyethylene (PE) (30 µm) fragments (also at 10, 25, 50, 75 and 100 mg/L, and two distinct mixtures of PE at 10 mg/L and 100 mg/L with differing amounts of DMP (10, 25, 50, 75, 100 mg/L) (Figure 1).

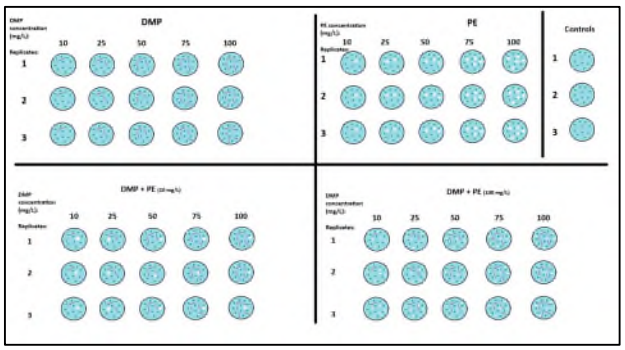

Figure 1. Experimental design of an acute exposure experiment of di-methyl phthalate (DMP) (10, 25, 50, 75, 100 mg/L), polyethylene (PE) (30µm) fragments (10, 25, 50, 75, 100 mg/L), and two mixtures of DMP (10, 25, 50, 75 and 100 mg/L) and PE (10 or 100 mg/L) on cultures of *Lemna minor*.

Prior to experimentation, 30 uniform, healthy green fronds were chosen for each mesocosm and thoroughly rinsed with DI water. All fronds were then transferred to a Laminar flow hood and mesocosm preparation took place in an aseptic environment. Excess moisture was dried off, roots were trimmed within 1mm, and fresh weight measurements were taken. Inert glass beakers (300ml) were chosen to prevent contamination from any plastics or their associated leachates, and these were filled with 150ml of the test media before fronds were added to each. Where treatments included polyethylene fragments, these were spiked prior to the addition of *L. minor* fronds.

The experiment was conducted under the same growth conditions outlined in the previous section and without renewal of the test media (static).

2.3 Test media preparation

Stock solutions of DMP were made up to 1000mg/L using ethanol as an auxiliary solvent; from this stock, two rounds of serial dilutions were conducted to produce DMP concentrations of 10, 25, 50, 75 and 100 mg/L diluted in Hoagland's solution (1.6g/L, w/v). This was repeated for the mixed exposure experiments and finalised by spiking beakers with either 10 mg/L or 100 mg/L PE fragments. To allow valid comparison, control triplicates were also produced using ethanol (4.6%, w/v).

For polyethylene, stock solutions of Hoagland's were made up before each respective beaker was spiked with PE fragments at 10, 25, 50, 75 and 100 mg/L before fronds were added.

2.4 Sample analysis

2.4.1 Physical growth parameters

L. minor growth was evaluated using three key parameters: dry weight, frond surface area and frond number; measurements were taken on t = 1 and t = 7 (where t = day) in addition to water temperature and pH of each mesocosm. From the results, the average specific growth rate, inhibition of growth and yield were calculated using the methods described in the OECD (2006) guidelines.

2.4.2 Chlorophyll content

Chlorophyll content was analysed using methods described by Xu et al., (2010). By removing the roots of samples; fronds were homogenised and mixed with 95% aqueous acetone. They were placed in a sonicator for 12 hours, then steeping for a successive 12 hours. The mixture was centrifuged and the supernatant separated and fixed. Absorbance was determined at 647nm and 664nm.

2.5 Statistical analysis

For each measured variable (frond number, frond surface area, dry weight, chlorophyll α and chlorophyll ϐ), the mean values, confidence intervals, and standard error of the mean was calculated. Normality was evaluated using Shapiro-Wilk's test, and homogeneity examined by Levene's test. A two-way ANOVA was then conducted between each dependent variable against the concentration level (10, 25, 50, 75 and 100 mg/L) and the treatment type (DMP, PE, DMP and PE10, and DMP and PE100). This was followed by post-hoc analysis on the estimated marginal means using Fisher's LSD to evaluate the significance of differences ($p < 0.05$) between the various exposure groups and control group and draw conclusions between their interaction.

3.0 Results

3.1 Frond number

3.1.1 Average specific growth rate

L. minor demonstrated an increase in the average specific growth rate (ASG) of frond number from day 1 to 7 in correlation with concentration between treatment groups DMP and PE; whilst the mixed treatment groups saw an overall decrease (Figure. 2). At 10 mg/L, the ASG of frond number ranged from -0.02125 ± 0.003 in the DMP treatment group to 0.04712 ± 0.006 fronds in the DMP + PE100 group, all values were compared to the control (0.01438 ± 0.005). DMP remained the lowest response group at 25 (-0.0181 ± 0.011), 50 (0.00191 ± 0.01), 75 (-0.01161 ± 0.01) and 100 mg/L (0.0044 ± 0.005). While PE had an observed increase in frond number from 10 mg/L (0.02952 ± 0.002) to 100 mg/L (0.03634 ± 0.005), both combined treatment groups saw a decrease; treatment group DMP (100 mg/L) + PE (10 mg/L) (0.01524 ± 0.005) was the closest to the control value.

A statistically significant difference was observed between the individual treatment groups (ANOVA: $F = 42.175$, $df = 3$, $p < 0.001$) and the interaction between treatment groups and concentration (ANOVA: $F = 2.757$, $df = 12$, $p = 0.007$). There was, however, no significant relationship between concentration of the test substance and the average specific growth rate of frond number. In addition, the only groups with a statistically significant difference observed at every concentration level were between DMP and the mixed treatment group, DMP + PE10 (at 10, 25, 50 and 75 mg/L $p < 0.001$, at 100mg/L $p = 0.021$) these were the only groups also significantly different to the control group ($p = 0.014$ and $p < 0.001$, respectively); significance between other groups is observed but not valid at every concentration tested. Based on the results observed in this experiment, the lowest effect concentration (LOEC) of DMP and DMP + PE10 were 10 mg/L and 50mg/L, respectively.

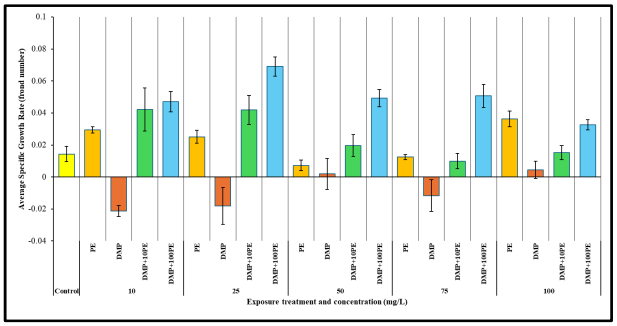

Figure 2. Average specific growth rate of *Lemna minor* frond number after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

3.1.2 % Reduction in yield

The only clear concentration-response relationships observed for the yield of *L. minor* frond number were in the DMP-only and DMP + PE10 exposure treatments (Figure 3). While the yield of DMP-only groups decreased linearly from 10 mg/L (316.67% ± 36.00) to 100 mg/L (50% ± 62.36), the DMP + PE10 demonstrated an increase in yield from 10 mg/L (-450% ± 184.09) to 100 mg/L (-66.67% ± 49.07). PE and DMP + PE100 both displayed a bell-shaped distribution, at 50 mg/L the PE-only was the only treatment group, at any concentration, other than DMP, to display a reduction in the yield of frond number (16.67% ± 36.00). These contrasts responses observed by the DMP + PE100 group at 25 mg/L (-983.33% ± 111.39) which has the greatest increase in yield across all concentrations and treatment types.

Statistical significance was observed both between

groups (Figure 4) (ANOVA: $F = 54.378$, $df = 3$, $p < 0.001$) and the interaction between groups and concentration (ANOVA: $F = 3.166$, $df = 12$, $p = 0.003$). This is further supported through post-hoc analysis where $p < 0.001$ for DMP across all concentrations in addition to comparison with other treatments.

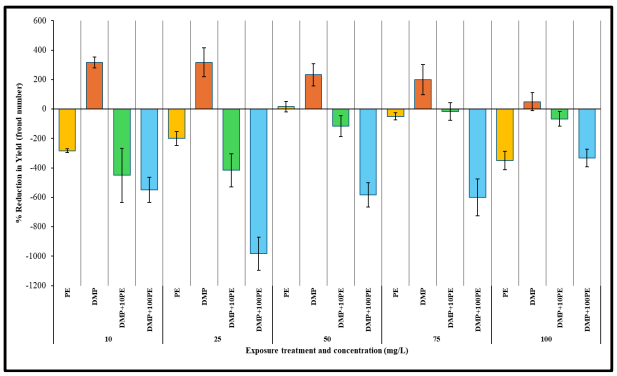

Figure 3. % Reduction in yield of *Lemna minor* frond number after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

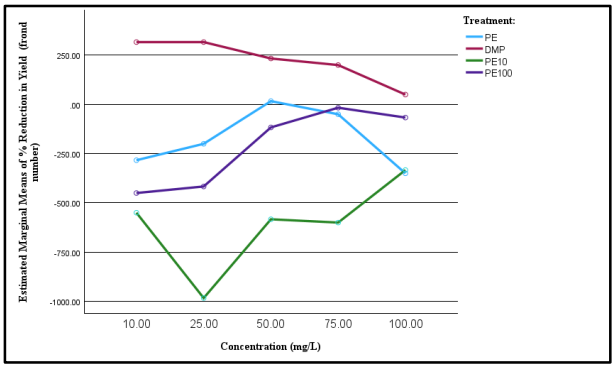

Figure 4. % Reduction in yield of *Lemna minor* frond number after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as estimated marginal means of triplicate mesocosms.

3.2 Surface area

3.2.1 Average specific growth rate

Across all exposure treatments a notable decrease in the ASG of *L. minor* surface area (mm^2) can be deduced when compared to the control group (0.03418 ± 0.005). There does not appear to be a clear concentration-response relationship for any of the treatment types. The treatment group with the lowest observed surface area across all concentrations was PE-only, which displayed an overall increase from 10 mg/L (-0.02917 ± 0.007) to 100 mg/L (-0.05033 ± 0.02) with a dip demonstrated at 75 mg/L (-0.01958 ± 0.006). Among the highest decrease in surface area was DMP-only at 25 mg/L (-0.07029 ± 0.015), DMP + PE100 at 50 mg/L of DMP (-0.0668 ± 0.01), and DMP + PE10 at 100mg/L of DMP (-0.07605 ± 0.01).

Average specific growth rate of *L. minor* surface area (mm2) was statistically significant at the $p < 0.001$ level for every treatment group when compared to the control group (0.03418 ± 0.01098) (Figure 5). No significance could be drawn, however, between individual treatment groups or correlation to concentration, illustrated by the proximity of treatment groups observed in Figure 6. Due to a lack of statistical significance, LOEC estimates could not be made for the surface area of *L. minor* with respect to the exposure treatments used in this study.

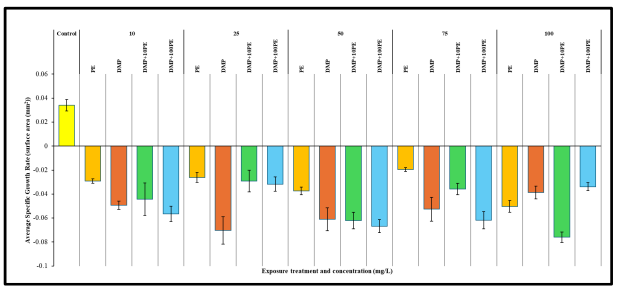

Figure 5. Average specific growth rate of *Lemna minor* frond surface area (mm2) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

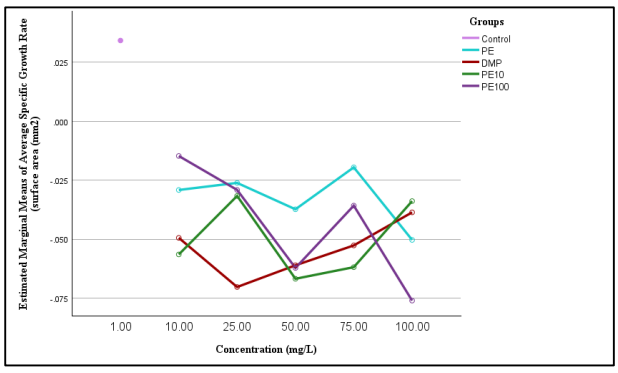

Figure 6. Average specific growth rate of *Lemna minor* frond surface area (mm2) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and

combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as estimated marginal means of triplicate mesocosms.

3.2.2 % Reduction in yield

The % Reduction in yield of frond surface area (mm2) in *L. minor* was positive across all treatment groups and concentrations (Figure 7), again in contrast to the Average Specific Growth rate observed in Figure 4. PE and DMP + PE10 both saw an overall increase in the reduction of yield from 10 mg/L (156.66% ± 16.22, 126.27% ± 4.32) to 100 mg/L (195% ± 41.02, 207.27% ± 10.65, respectively). In contrast, the opposite was observed of the other two groups; DMP + PE100 saw a decrease from 10 mg/L of DMP (188.73% ± 16.75) to 100 mg/L (151.83% ± 4.69), while a decrease was also observed in the DMP-only group from 10 mg/L (187.43% ± 7.99) to 100 mg/L (167.03% ±4.24), the deviation between each concentration interval is so insignificant, it cannot be assumed as the response to an increase in concentration. No relationships could be deduced from the results obtained, except that all treatment types had a negative impact on the yield of frond surface area. Additionally, no statistical significance could be determined between any of the treatment groups, concentration levels nor interaction of the two.

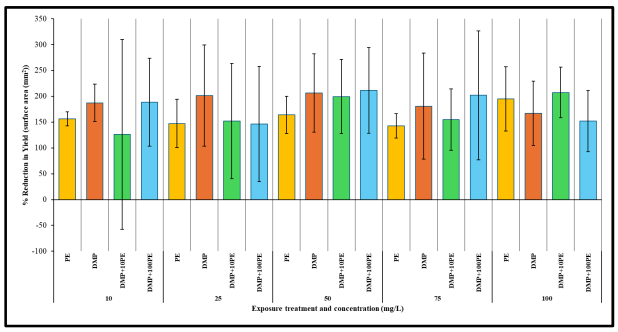

Figure 7. % Reduction in yield of *Lemna minor* frond surface area (mm2) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

3.3 Dry weight

3.3.1 Average specific growth rate

The Average Specific Growth Rate of *L. minor* dry weight (mg) decreased across all treatment groups at every concentration tested from day 1 to day 7 (Figure 8); at 100 mg/L, the group exposed to PE (0.06135 ± 0.015) was the only treatment that had an observed increase from the control (0.04365 ± 0.01026). While PE experiences a dip in ASG dry weight at 50 mg/L (0.05838 ± 0.037), results remain stable across all concentrations. Similarly, the DMP + PE100 group remained stable across 10 (0.07852 ± 0.015), 25 (0.08283 ± 0.004), 50 (0.0804 ± 0.012) and 75 mg/L of DMP (0.07459 ± 0.008) but experienced a sharp decrease at 100 mg/L (0.00783 ± 0.02). Conversely, the DMP + PE10 group saw a sharp decrease from 75 mg/L (0.02394 ± 0.013) to 100 mg/L (-0.0727 ± 0.009). The only group which had a negative ASG of dry weight across every concentration tested was DMP, with the least effect observed at 10 mg/L (-0.01661 ± 0.003).

Univariate analysis determined a statistical significance between treatment groups (ANOVA: $F = 37.424$, $df = 3$, $p < 0.001$) and concentration (ANOVA: $F = 3.94$, $df = 4$, $p = 0.008$) independently but not any interaction between the two categories. Where estimates of marginal means were compared in a post-hoc LSD test, only DMP ($p < 0.001$) and PE100 ($p = 0.03$) were significantly different from the control; PE100 was the only group significantly different from the other treatment groups. Other significant results were observed but no trends were observed in the other treatment groups or between varied concentrations, as a result the only LOEC estimates that could be made were for the DMP + PE10 and DMP + PE100 at 100 mg/L of DMP.

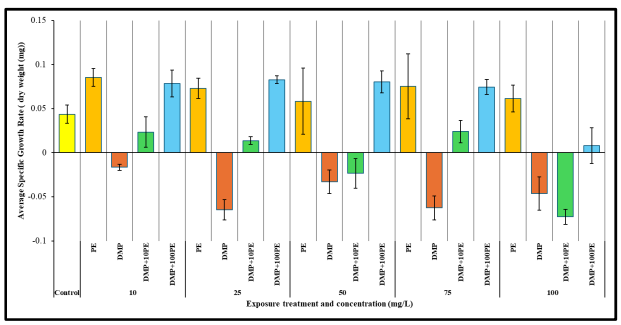

Figure 8. Average specific growth rate of *Lemna minor* frond dry weight (mg) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

3.3.2 % Reduction in yield

All treatments recorded had a discernible increase in reduction in yield of dry weight of *L. minor* fronds from 10 mg/L to 100 mg/L over the 7-day test period; of these, PE had the least effect on yield (Figure 9). At 10 (7.63% ± 28.3), 25 (40% ± 28.13), 50 (41.9 ± 100.3) and 75 mg/L (0% ± 103.18), the DMP + PE100 saw an increase in yield from day 1 to 7, contrasting the decrease observed at 100 mg/L (72.36% ± 50.55). DMP saw the greatest overall reduction in yield at 25 mg/L (209.53% ± 14.83), 50 mg/L (160% ± 21.37) and 75 m/L (205.7% ± 18.86) against all other treatment groups, and at 100 mg/L is overtaken by the DMP + PE10 group (220.93% ± 10.88).

Statistical significance was observed between all treatment groups (ANOVA: $F = 22.374$, $df = 3$, $p < 0.001$) and between differing concentrations (ANOVA: $F = 3.059$, $df = 4$, $p = 0.027$) – demonstrated by the significant distances in Figure 10. Significance was also determined between treatments at 10, 25, 50 and 75 when compared against 100 mg/L ($p = 0.002$, $p = 0.022$, $p = 0.034$ and $p = 0.011$ respectively).

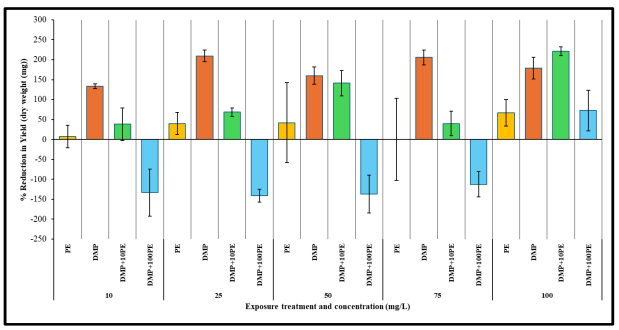

Figure 9. % Reduction in yield of *Lemna minor* frond dry weight (mg) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

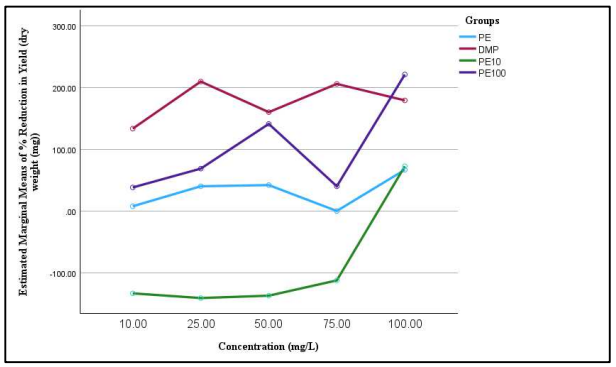

Figure 10. % Reduction in yield of *Lemna minor* frond dry weight (mg) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as estimated marginal means of triplicate mesocosms.

3.4 Chlorophyll a

L. minor had a notably lower difference in Chlorophyll a (ng/mL) concentration in the PE treatment group where compared to both the control (800.18 ± 15.6) and other treatments (Figure 11) - additionally, DMP + PE100 significantly peaks at 25 mg/L of DMP (1671.88 ± 181.766), decreasing again at 50 mg/L (727.37 ± 13.3) and linearly to 284.04 ± 12.43 at 100 mg/L. While the DMP only group remains relatively stable at all concentrations from 10 mg/L (766.32 ± 130.72) to 100 mg/L (636.5 ± 109.3), a clear concentration-response relationship can be observed in the DMP + PE10 group, decreasing at 50 (623.69 ± 67.95), 75 (267.02 ± 102.14) and 100 mg/L (53.04 ± 18.66).

Statistical significance was observed between treatment groups (ANOVA: $F = 49.558$, $df = 3$, $p < 0.001$), concentrations (ANOVA: $F = 20.217$, $df = 4$, $p < 0.001$) and the interaction between treatments and concentrations (ANOVA: $F = 9.07$, $df = 12$, $p < 0.001$). PE was the only treatment significantly different from all other groups ($p < 0.001$), and PE ($p < 0.001$) and DMP + PE100 ($p = 0.02$) were the only groups statistically significant from the control group. Based on the results obtained, the LOEC estimates for both combined exposure groups, DMP + PE10 and DMP + PE100, was 50 mg/L and 10 mg/L in the PE treatment group.

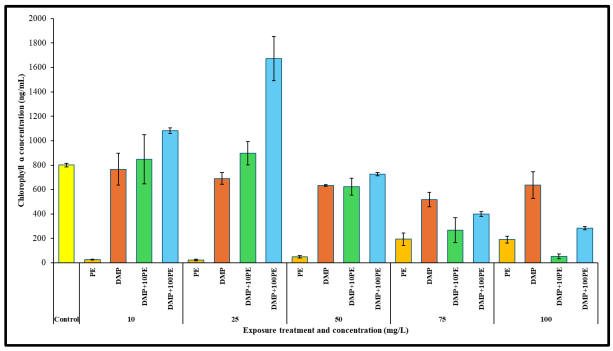

Figure 11. Chlorophyll α content of *Lemna minor* fronds (ng/mg) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

3.5 Chlorophyll ƃ

The Chlorophyll ƃ concentration (ng/mL) of *L. minor* fronds follows a similar pattern to Chlorophyll α, as expected. PE had the lowest response of all the treatment groups and decreased slowly from 10 mg/L (149.5 ± 60.23) to 100 mg/L (81.12 ± 4.22). The dose with the largest response was DMP + PE100 at 25 mg/L of DMP (897.17 ± 43.63) which after peaking, continued to decrease up to 100 mg/L at 213.03 ± 14.18. At 10 mg/L, the DMP treatment group (581.28 ± 101.36) was similar to that of the control (585.14 ± 35.62); a clear concentration-response relationship was observed in the DMP-only group, decreasing linearly to 225.77 ± 71.79 at 100 mg/L. A similar concentration-response was observed in the DMP + PE10 group, where eustress was observed at 10 (810.03 ± 135.71) and then again at 100 mg/L (644.03 ± 26.28). A comparable peak was observed in the DMP + PE100 treatment group at 25 mg/L (897.17 ± 43.63) contrasting the negative effects seen at 10 (493.86 ± 90.4), 50 (430.49 ± 73.22), 75 (328.98 ± 37.24) and 100 mg/L (213.03 ± 14.18).

Overall, chlorophyll ƃ concentrations (Figure 12) are lower than those of chlorophyll α (Figure 11). Moreover, all three variables – treatment (ANOVA: $F = 43.137$, $df = 3$, $p < 0.001$), concentration (ANOVA: $F = 9.937$, $df = 4$, $p < 0.001$) and interaction between these (ANOVA: $F = 2.185$, $df = 12$, $p = 0.031$) were all statistically significant. Both PE ($p < 0.001$) and DMP ($p = 0.018$) were significantly different from the control group, and PE was statistically significant from every treatment group ($p < 0.001$). While not significantly correlated with the control group, DMP + PE100 was significantly different to the other three treatment groups ($p < 0.001$). The LOEC estimates based on the results of this study for chlorophyll ƃ in the treatment groups PE, DMP + PE10 and DMP + PE100 were 10, 50 and 50 mg/L respectively.

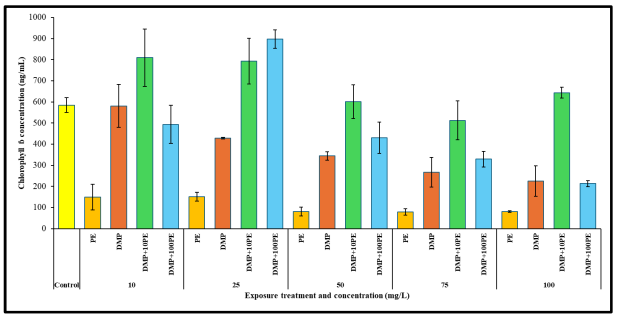

Figure 12. Chlorophyll α content of *Lemna minor* fronds (ng/mg) after seven days of exposure to 10, 25, 50, 75 and 100 mg/L of dimethyl phthalate, polyethylene, and combined treatments of dimethyl phthalate (10, 25, 50, 75, 100 mg/L and polyethylene (10, 100 mg/L). Data is displayed as mean ± SEM of triplicate mesocosms.

3.6 Physical observations

The highest visual levels of chlorosis were observed in the DMP and PE isolated treatment groups; necrosis, however, was only observed in the mixed treatment groups (DMP + PE10 and DMP + PE100). There was also notable aggregation of polyethylene particles on *L. minor* fronds in treatments which contained polyethylene at 75 and 100 mg/L (Figure 13). Substantial root growth was visually reported in the PE-only group when compared to both the control and other treatment groups however, as the focus of this study was primarily on the vegetative growth, this was not recorded. The choice was made by the author not to omit any of the triplicate values from statistical

analysis due to the natural variability of plant growth, and so those are included in the results, represented by standard error of the mean (SEM) bars. pH of the test media across all treatments and replicates at the beginning of the experiment was 4.9 ± 0.9, and at the end of the exposure period was 3.7 ± 0.65; temperatures of all replicates remained in the range of 20°C ± 2.2 throughout the experiment.

Figure 13. Aggregation of polyethylene particles (30µm) *on L. minor* fronds following seven days of exposure to 100 mg/L.

4.0 Discussion

4.1 Quantification of the dose-response effects of dimethyl phthalate on *Lemna minor*

4.1.1 Physical growth parameters and the significance of concentration

The first objective of this study was to identify and quantify the dose-response effects of dimethyl phthalate (DMP) on the growth of *Lemna minor*, assessed through frond number, surface area, dry weight and chlorophyll content. Analysis of the average specific growth rate (ASGR), defined as the logarithmic increase from day 1 to day 7, of *L. minor* frond number revealed an increase in the DMP treatment group. Despite a lack of significant concentration-response correlation, DMP and DMP + PE10 were the only groups which had a significant deviation from the control group, indicating that DMP did have a notable effect on *L. minor* tissues. These findings are consistent with Pietrini et al., (2022)'s, who observed DMP toxicity in *L. minor* at concentrations as low as 30 mg/L. Further research by Huang et al. (2006) on other phthalates revealed similar trends, which indicated that the relative frond number of *L. minor* decreased significantly with an increase in concentration while in the presence of DBP.

Interestingly, the toxicity of DMP in relation to frond number peaked at 25 mg/L, then decreasing up to 100 mg/L, suggesting a potential for adaptive endurance. This result was reflected in the frond yield, where DMP showed a significantly greater effect than all other treatment groups and was the only one to exhibit a linear decrease as concentration increased ($p < 0.001$). These findings contrast with those of Gao et al., (2019), who studied the effects of DMP on the marine diatom *Phaeodactylum tricornutum* and found no significant changes in cell density until concentrations reached 100 and 200 mg/L.

To the best of the author's knowledge, the only other study examining the effects of DMP on *L. minor* was conducted by Pietrini et al., (2022). They reported no significant correlation between DMP concentrations at 3 and 30 mg/L and frond surface area, while at 600 mg/L, fronds displayed significant effects of toxicity within just a few hours of exposure. A limitation of this study was the lack of intermediate concentrations tested between 30 mg/L and 600 mg/L, leaving a substantial gap. In the present study, no significant relationship could be found between DMP concentration and frond surface area at any of the tested concentrations (10, 25, 50, 75, 100 mg/L). For these reasons, it is therefore recommended that future studies include a broader range of concentration intervals, particularly between 100 and 600 mg/L, to identify an appropriate Lowest Observed Concentration Effect (LOEC) (OECD, 2006)

4.1.2 Biomass and the impact of unsustainable growth

Based on the information available to the author, no other studies have investigated the effects of DMP on the dry weight of *L. minor*. In post-hoc LSD analysis, DMP was the only treatment that exhibited a negative growth rate at all concentrations and was one of only two groups (the other being DMP + PE100) that differed significantly from the control ($p < 0.001$ and $p = 0.003$, respectively). This indicates that the presence of DMP negatively impacted the dry weight of *L. minor* both when applied alone and in combination with 100 mg/L of microplastics.

These results are consistent with Kumari and Kaur (2022) who examined the effect of DBP on Hordeum vulgare and observed a decline in dry weight across all concentrations (800, 1600, and 2400 mg/kg) and exposure periods ranging from 15 to 120 days. Studies on terrestrial plants suggest that phthalates may disrupt germination and reproduction by interfering with protein synthesis and

transfer, as well as the communication of auxins. The results of the present study demonstrate a positive correlation between frond number and increasing DMP concentration, while a negative correlation is observed with dry weight. This suggests that, in response to DMP-induced toxicity, the plants may attempt to produce more fronds to maximize nutrient absorption, but these new fronds are unable to grow adequately in higher phthalate conditions. This interpretation is further supported by the overall negative response observed across all treatment groups in relation to surface area; indicating that although frond number increases, surface area and dry weight decrease due to the plants' inability to sustain this growth alongside disrupted protein synthesis.

Since this is the only paper of its kind and no direct comparisons to dry weight are available, the underlying mechanisms behind this behaviour can only be hypothesised at this time. Further research is necessary to confirm or refute the presence of this phenomenon. As a clonal plant, *L. minor* exhibits much lower genetic diversity compared to that of terrestrial or rooted plants; variability between colonies may partially account for the results. To address this, future studies would benefit from using a greater number of replicates, from different cultures, to ensure that the observed effects are not driven by genetic variation within the population. Additionally, the static nature of this study may have resulted in reduced nutrient availability within the test media (Mateos-Cárdenas et al., 2019). Further research should consider employing a semi-static or dynamic approach, incorporating more frequent sampling (e.g. measurements every 3 days) in addition to endpoint data, particularly if the study aims to examine longer-term effects. The results of yield indicated that concentrations of 10, 25, 50 and 75 mg/L were significantly different from results at 100 mg/L ($p = 0.002$, $p = 0.022$, $p = 0.034$ and $p = 0.011$ respectively). These findings further support the hypothesis of a dose-concentration response of *L. minor* dry weight in the presence of DMP.

4.1.3 The use of chlorophyll content as an indicator of oxidative stress

The study by Pietrini et al., (2022) found no significant evidence of phytotoxic effects of DMP at 3 mg/L or 30 mg/L in either *L. minor* or *Spirodela polyrhiza*, and although a reduction in chlorophyll content of *L. minor* was observed at 600 mg/L, it was not statistically significant. In contrast, Gao et al., (2019) reported a significant decrease in chlorophyll α in *P. tricornutum* with increasing DMP concentration. A reduction of 20.4%, 26.1%, and 45.0% at 50, 100, and 200 mg/L, were observed respectively. The findings of these studies suggest that to accurately estimate the EC50 value for DMP in *L. minor*, a broader range of concentration intervals should be examined. Given that only one other study has investigated the effects of DMP on *Lemna minor*, and considering the wide discrepancies in tested concentrations, it is difficult to determine whether these results can serve as a reliable model for future experiments.

Huang et al., (2006) found that when *Lemna minor* and *Spirodela polyrhiza* were exposed to DBP concentrations below 2.5 mg/L, their chlorophyll content remained unchanged, but at concentrations above 7.5 mg/L, chlorophyll content decreased significantly. A possible explanation is that the phthalate interferes with the plants' ability to synthesise carotenoids, which are essential for photoprotection of chlorophyll pigments and therefore reducing their ability to absorb light. These findings were consistent with Xu et al., (2010) who reported that the inhibition of protein synthesis caused by DEHP on *L. minor* lead to reduced photosynthesis as a result of reduced enzyme production. To assess oxidative stress, they measured malondialdehyde (MDA) content, an indicator of lipid peroxidation. Several studies have reported significant correlations between increased MDA levels and concentrations of phthalates (Xu et al., 2010; Huang et al., 2006) and microplastics (Gao et al., 2019). As no studies have yet examined the effect of DMP on *L. minor* in relation to MDA content, it would be valuable for future research to investigate this, particularly in conjunction with carotenoid and chlorophyll content, to better explore potential links between oxidative and photokinetic stress mechanisms.

There was a statistically significant decrease in chlorophyll content with correlation to increasing DMP concentration ($p < 0.001$), and from 10 mg/L to 100 mg/L chlorophyll b content was almost halved (Fig 9). In contrast, no clear relationship could be determined between DMP concentration and chlorophyll α content. This could be explained by considering the role of chlorophyll b, which has the ability to transfer photokinetic energy to chlorophyll α under stress, leading

to further damage to chlorophyll b pigments. This transfer might be occurring between the 75 mg/L and 100 mg/L intervals, where a decrease in chlorophyll b was followed by an increase in chlorophyll a at 100 mg/L. However, given the inconsistency of these results and the lack of data above 100 mg/L, further research with a broader range of concentrations is needed to confirm this hypothesis.

4.2 Evaluation of the dose-response effects of polyethylene on *Lemna minor*

4.2.1 The influence of specific polyethylene characteristics on physical growth

In this study, exposure to polyethylene (PE) was associated with an overall increase in the ASGR of frond number. The most pronounced decrease in frond number occurred at 50 mg/L in contrast with results obtained at 100 mg/L where frond number was approximately twice that of the control group. While no statistical significance between concentration and response could be established in this experiment, the results observed suggest that greater PE concentrations have the potential to promote *Lemna minor* growth. These findings align with those of Mateos-Cárdenas et al., (2019) who reported that despite substantial microplastic coverage of *L. minor* fronds, their presence had no significant effect on frond number or surface area over a 7-day period.

A potential explanation for the results observed in this study is that similar to findings in studies reporting increased root growth as a stress response to enhance nutrient absorption (Kalčíková et al., 2017), by increasing frond number and therefore the surface area they covered, plants were able to absorb more light. This hypothesis is supported by the increase in chlorophyll a pigment at 75 and 100 mg/L (Fig 8). Similar responses have been reported by Košpić et al. (2025), who observed that polystyrene microplastics enhanced frond growth and chlorophyll content in *L. minor*, suggesting that mild oxidative stress can sometimes stimulate growth.

Although not actively measured in this experiment, visual observations of root growth indicated that treatments containing PE, especially at higher concentrations, showed notable increases in root length when compared to both DMP and the control group. Meanwhile, Kalčíková et al., (2017) found that PE microbeads (30-600µm) adversely affected root growth of *L. minor* through adsorption onto the root surface, blocking auxin activity and causing mechanical damage. In the current study, PE fragments aggregated on *L. minor* fronds rather than the roots, which would suggest that this difference is a result of density of the microbeads versus PE fragments, which primarily float on the water surface, even when agitated. Similarly, Mateos-Cárdenas et al., (2019)'s research using (10-45um) PE fragments found that MPs adhered to all surfaces of *L. minor* fronds, negatively correlating with increasing relative growth rate of frond number. This suggests that PE microplastics elicit a stress response in *L. minor* which could be attributed to the plants to reach further from the source of microplastics in an effort to maximize nutrient uptake while avoiding physical obstruction (Ceschin et al., 2022). Future studies would benefit from including root growth analysis to facilitate direct comparisons between studies and better elucidate the impact of particle morphology on plant growth.

Bhattacharya et al., (2010)'s concluded that positively charged microplastics have a higher electrostatic attraction to cellulose molecules which could explain their increased adherence to plant tissues. The present findings combined with preexisting knowledge reinforces that adhesion, and the impact that occurs as a result, is likely significantly influenced by the microplastics' physicochemical characteristics, including shape and size of particles (Xu et al., 2010). Future studies would therefore benefit from the use of FTIR or GC-MS techniques to analyse particles prior to their inclusion in experimental treatments.

In the present study, the ASGR across all treatment groups was significantly lower than the control group (0.03418 ± 0.01098 mm2) although no significant differences were drawn between treatment types or concentrations. These findings are consistent with Ceschin et al., (2022), who also reported no significant differences between PE treatments and the control group in *L. minor*. Similarly, all treatment groups in the current study showed a reduction of yield; the absence of a clear dose-response trend, however, suggests that the concentration alone is not responsible for this pattern but were instead influenced by additional external factors. These findings support the hypothesis of a biphasic eustress response in addition to highlighting the complexity of concentration-

response relationships in plant-microplastic interactions.

To better understand these mechanisms, future research would benefit from comparison of various shapes and sizes of the same plastic at equal concentrations to isolate this influence. Such insights would be particularly valuable at the community level, as primary producers like plants have a bottom-up influence on heterotrophic communities. This interaction therefore had the ability to facilitate contaminant transfer through the food chain, potentially impacting on a wide range of species, with implications for food security (Cara et al., 2022). This was demonstrated in the study by Mateos-Cárdenas et al., (2019), who reported that despite no negative effects of PE microplastics being determined on *L. minor*, the ingestion of treated plants by *Gammarus duebeni* led to significant bioaccumulation of microplastics in their tissues.

4.2.2 Effects observed to biomass and recommendations for further understanding

While the ASGR of *L. minor* dry weight was highest at 10 mg/L, no significant correlation between dry weight and PE concentration could be established; these results were similarly reflected in the yield. These results are consistent with Mateos-Cárdenas et al., (2019) which observed a reduction in dry weight in plants exposed to PE microplastics (20% w/v). Interestingly in this study, a significant difference was also found at 100 mg/L when compared independently with 10, 25, 50 and 75 mg/L. Overall, the data support the hypothesis that *L. minor* growth was not adversely affected by PE microplastics and may even have been stimulated.

Comparative results were reported by Ceschin et al., (2022). Significant effects of polystyrene (50mg/L and 100mg/L) microplastics on *L. minor* biomass could not be detected during the first 14 days of exposure however, at 28 days, negative impacts were observed at 50 and 100 mg/L, highlighting the importance of exposure duration. It remains to be determined whether PE microplastics would elicit a similar response in *L. minor* over a longer exposure period. In the present work, PE was the only treatment that consistently showed a significant increase in growth compared to the control group across all tested concentrations. Košpić et al., (2025) proposed that while PS microplastics induced oxidative stress, the level was insufficient to cause severe damage, instead stimulating peroxidase enzyme activity. The promotion of this enzyme helps to maintain cellular growth including an increase in photosynthetic capacity, which can enhance frond area and growth.

The mechanisms behind oxidative stress pathways warrant attention of further studies. Reactive oxygen species (ROS) are established indicators of ecotoxicity, accumulation of intracellular ROS can result in a reduction of chlorophyll content and biomass, while MDA levels reflect the extent of membrane lipid peroxidation damage (Gao et al., 2019; Ma et al., 2018). Future studies would benefit from incorporating the measurement of these variables as more specific endpoints to better elucidate the underlying mechanisms driving the differences observed across parameters. This is of particular importance in the study of microplastics, especially for floating aquatic plants, where the impacts of microplastics may depend not only on internalisation or adsorption onto roots, but also on the adaxial leaf surface. This will provide a more comprehensive understanding of a microplastic's specific impacts based on a plant's position in the water column, with relation to microplastic density.

4.2.3 Chlorophyll content and the influence of polyethylene microplastics

In this study, chlorophyll content in PE was significantly lower when compared to all other treatment groups, including the control ($p < 0.001$). Ceschin et al., (2022) similarly reported that total chlorophyll was affected by PS microplastics (50, 100 mg/L) after 7 days of exposure, with significant decrease compared to the control after 14 and 28 days ($p < 0.001$). The relationship between chlorophyll content and microplastic concentration suggests a possible dose-response pattern, although further analysis is necessary to clarify this link. Conversely, similar studies (Kalčíková et al., 2017; Mateos-Cárdenas et al., 2019) found no significant effects of PE microbeads on chlorophyll α or ϐ in *L. minor*, highlighting inconsistencies across plastic types.

An inverse relationship was observed between chlorophyll α and chlorophyll ϐ in this study, with PE consistently demonstrating the lowest values across all treatment groups, with significant deviations from the control ($p < 0.001$). These results support the hypothesis of energy transfer from chlorophyll b to chlorophyll a,

was most evident under increasing PE concentrations. These results could be attributed to light blockage caused by microplastic particles adsorbed onto the adaxial leaf surface, thereby limiting photosynthetic activity. This interpretation is supported by the reduced frond surface area observed here, where an increase in frond number did not translate to functional growth or photosynthetic capacity.

4.3 Determination of the significance of effects produced by a combined exposure of polyethylene and dimethyl phthalate on *Lemna minor*

4.3.1 The impact of dualistic effects on physical growth parameters

In this experiment, the ASGR and yield reduction varied considerably depending on the measured variable (frond number, surface area, or dry weight). The results indicated a significant difference in the ASGR of frond number across all treatment groups. However, while there was a significant correlation between PE and DMP when each was compared individually to the control, no significant differences were observed between the combined treatment groups (DMP + PE10 and DMP + PE100). Reduction in the yield (frond number) also demonstrated a statistically significant difference between treatment types, and the interaction between treatment and concentration, although no significant results were observed based on concentration alone. Similar conclusions were reported by Prata et al., (2018) on the marine microalgae *Tetraselmis chuii*, who found that mixed-polymer microspheres alone did not cause significant differences in growth rates ($p = 0.24$). Though populations exposed to procainamide alone displayed significant deviations from the control, combined microplastic-procainamide treatments resulted in a more notable decrease in ASGR ($p < 0.001$). Despite the lack of significant correlation in the present study, it can be inferred that the combination of PE and DMP influenced the effect on the ASGR of frond number, but without significance to concentration of the mixtures.

Although this study is the only one of its kind which aims at specifically identifying the combined ecotoxicological effects of DMP and PE on *Lemna minor*, similar research like Gao et al., (2019) investigated the effects of di-butyl phthalate (DBP) (5 mg/L) and PE (23μm; 0.25 mg/mL, 0.5, and 1 mg/L) on *Lactuca sativa*. Their findings showed that, compared to their DBP-only treatment, the combined exposure resulted in all growth parameters being reduced after 14 days (reduction of leafy dry weight = 16 .68%, reduction of leaf number = 2.92%). Furthermore, higher microplastic concentrations led to a greater inhibition of growth rate.

Positively charged particles, including PE and various other microplastics, exhibit stronger electrostatic attraction to the cellulose components of the plant cell walls. (Bhattacharya et al., 2010). In the broader context, toxicological interactions between combined substances, in addition to their interactions with the test media and the cellular walls of the *L. minor*, has the potential to amplify the toxic effects of DMP, even if such interactions do not produce significant results when substances are tested individually.

Overall, the results obtained using DMP in combined exposure experiments at the tested concentrations (10, 25, 50, 75, and 100) was not strong enough to establish a definitive dose-response relationship. Further investigations at higher concentrations or using a similar experimental design to Gao et al., (2019), for example, fixed DMP levels with varying microplastic concentrations (e.g., DMP10 + PE (varied) versus DMP100 + PE (varied)), could provide more insight.

Analysis of surface area parameters, including ASGR and yield, did not reveal statistically significant results between treatment groups, concentrations, or their interactions. The only consistent relationship observed was a decrease across all treatments and concentrations when compared to controls. Moreover, when dry weight analysis was conducted, the ASGR decreased across all treatment groups with statistical significance related to the treatment type and concentration. When compared to that of the control, DMP-only and the mixed measure group 'DMP + PE10' were the only two which showed reductions compared to the control, but these results were inconsistent across concentration intervals. For DMP-alone, the highest observed effect was at 25 mg/L, decreasing again with an increase in concentration, potentially indicating a tolerance response from *L. minor*. Conversely, in the DMP + PE10 group, results decreased in a non-linear pattern, peaking at 100 mg/L. The differences between the mixed treatment groups might be explained by the greater bioavailability and surface area

to microplastic ratio at lower PE concentrations, leading to increased adhesion to tissues and reduced clumping, which might enhance the mechanical and physiochemical damage compared to DMP + PE100.

4.3.2 Impact of polyethylene aggregation on photosynthesis

This perceived tolerance phenomenon was also observed in Martins et al., (2024) chlorophyll measurements of *L. minor*. This study recorded that while there was no significant difference in Chlorophyll ɑ between treatments between treatments or the control, Chlorophyll ɓ showed a significant reduction in both the Low-Density PE (LDPE)-isolated group and the LDPE + medroxyprogesterone acetate group. Plant stress can lead to the photokinetic energy transfer to Chlorophyll ɑ pigments from Chlorophyll ɓ, resulting in damage to the ɓ pigments. These findings suggest that the presence of microplastics can amplify ecotoxicological effects by disrupting the transport of photosynthetic products and increasing the adsorption of phthalates (Bhattacharya et al., 2010).

Interestingly, in the DMP + PE100 treatment group, Chlorophyll ɑ and ɓ pigments peaked at 25 mg/L of DMP, before significantly decreasing up to 100 mg/L. A significant difference was not observed, however, between DMP + PE100 and the DMP isolated group at a maximum concentration of 100 mg/L, nor the control group. In contrast, Prata et al. (2018) found significant differences in chlorophyll content between treatments, in particular they reported lower EC_x values in the presence of microplastics, suggesting that the interaction between the two contaminants intensified their effects.

These results suggest that while DMP had a statistically significant effect on chlorophyll production in *L. minor*, the combination of DMP + PE did not exacerbate symptoms of toxicity. One possible explanation is that DMP, being a plasticizer, may have caused polymerization of the microplastic when in powder form, resulting in fewer free particles to interact with *L. minor* fronds. This could account for the differences between the mixed exposure groups (DMP + PE10 and DMP + PE100) especially at 100 mg/L (Fig 9). At lower MP concentrations combined with higher DMP levels, there would be fewer PE particles available for DMP to act upon. Additionally, some studies suggest that microplastic particle size influences ecotoxicity, implying that larger aggregates formed at higher particle counts might exert less effect on plant tissues (Bhattacharya et al., 2010).

4.4 Wider implications

The OECD (2006) provides guidance for conducting ecotoxicological tests of contaminants on vegetative plant growth; some issues exist with the vigour of these procedures. Results indicate that the phytoxicological effects of these contaminants typically inhibit both photosynthetic and oxidative processes. The limited literature on this topic typically focuses on the adsorption of plastics on external tissues by providing a physical barrier to light (Kalčíková et al., 2017; Ceschin et al., 2021). By improving ecotoxicological studies to include more sensitive endpoints, (i.e. Chlorophyll ɑ, ɓ, carotenoids, ROS and MDA); it enhances the value of data on photosynthetic activity and oxidative damage in plant tissues (Bejarano et al., 2023; O'Hare et al., 2018). Whilst a handful of papers exist to explore the concepts introduced in this study, to the best of the author's knowledge, none compare these two specific contaminants, PE-MPs and DMP, side by side.

At present, there is a substantial lack of EC_x values, especially with respect to microplastics and phthalates on *Lemna Minor* specifically. In order to provide a more diverse index of values, the OECD recommends the introduction of a wider range of EC_x values, including EC10 (OECD, 2006; Pietrini et al., 2022). Development of EC and LOEC values is essential in developing species sensitivity distribution (SSD) models that can be used in risk assessments, wastewater management and remediation programmes to further improve the health of our aquatic ecosystems (Staples et al., 2002, Staples et al., 2008). The criteria for producing SSDs requires robust and reproduceable data (Bejarano et al., 2023). As a result, there is currently a lack of compounds with sufficient data to produce SSDs, additionally there are a number of existing SSDs which are statistically unreliable due to the small dataset (Posthuma et al., 2019).

Improvements to the list of Potentially Toxic Elements (PTE)s by the UK government puts the responsibility of wastewater infrastructure on producers, which could lead to a significant impact on the UK's ability to meet and surpass the WFD objectives in coming years (GOV, 2024; Whitehead et al., 2021). However, in order to supplement these policies, substantial evidence must be collated on a range of contaminants. As a result, an improvement in the robustness and reproducibility of

ecotoxicological studies regarding microplastic leachates, including phthalates, will improve the bank of knowledge in addition to increasing awareness to really drive the efforts to improve and maintain our freshwater systems both in the UK, and globally.

4.4.1 Environmentally relevant recommendations

Given the limited research combining microplastics and leachates side by side, it is recommended that further studies are conducted. By investigating a range of microplastic types, shapes and sizes, and various different plastic-associated leachates, this would help to enhance the current understanding of how these factors influence toxicity, adhesion and bioavailability. The use of a variety of aquatic plants could also provide insight into species-specific responses and enhance current understanding of stress-response mechanisms. Additionally, future studies could investigate water samples collected from freshwater ecosystems, where phthalates had been previously detected, to simulate environmentally relevant concentrations.

Conclusion

The purpose of this study was to investigate the ecotoxicological effects of both dimethyl phthalate and polyethylene, isolated and combined, on the floating aquatic plant *Lemna minor*. To explore this, three specific objectives were addressed.

With respect to the first objective, to evaluate the toxicity of dimethyl phthalate, was the most clearly achieved. Dimethyl phthalate exposure resulted in significant effects on frond number ($p < 0.001$) and chlorophyll ϐ content ($p = 0.018$); with the most significant concentration-response decline evident in chlorophyll ϐ concentration. While frond number increased with rising concentration, this appeared to be a tolerance response rather than stimulation, as surface area and dry weight either remained stable or decreased. These findings, in combination with the significant negative concentration-response behaviour, are consistent with previous studies, indicating that phthalates act as metabolic disruptors, impairing protein synthesis and limiting sustainable growth mechanisms.

The second objective, to determine the dose-response effects of polyethylene, did not consistently produce concentration responses across all endpoints. A clear significance was observed in relation to chlorophyll content across all (10, 25, 50, 75, 100 mg/L) polyethylene treatment groups, which deviated significantly from both the control and other treatments ($p \prec 0.001$), showing a steady decline from the LOEC (= 10 mg/L). Across both chlorophyll α and ϐ concentration, polyethylene, at every concentration, had the strongest influence out of all treatments tested, indicating disruption to photosynthetic mechanisms. In the context of the literature, these findings suggest that polyethylene microplastics can exert physiological stress on *L. minor* through physical obstruction as well as affinity to plant tissues, resulting in adherence, aggregation, and potential internalisation.

The final aim, to determine the effect of combined exposure treatments; involved keeping polyethylene static at 10 mg/L and 100 mg/L, whilst varying the dimethyl phthalate concentration (10, 25, 50, 75, 100 mg/L). This revealed evidence of an interaction between polyethylene microplastics and dimethyl phthalate. Although not all endpoints revealed significant concentration-response trends, the combined treatments frequently displayed more significant effects, particularly for frond number (DMP + PE10, LOEC = 50 mg/L) and chlorophyll (α and ϐ) concentration (LOEC = 50 mg/L, for both combined exposure groups). Such results suggest that the combination of microplastics and associated leachates have the potential to intensify ecotoxicological impacts, even when individual substances produce minimal responses.

Overall, this research offers valuable insights for the wider community and contributes to a growing wealth of evidence that microplastics and their associated leachates threaten freshwater ecosystems, with implications for biodiversity. Limitations of the current study include the use of clonal *Lemna minor* cultures, which limits the genetic variability, in addition to the use of a restricted range of biochemical markers. Future studies would benefit from incorporating further physiological markers and a greater number of replicates from varying *Lemna sp*. cultures. In conclusion, this study addressed it's aims and provides experimental evidence that both

polyethylene microplastics and dimethyl phthalate independently impair *L. minor* growth, while their combined effects can further exacerbate toxicity. These results indicate the ecological relevance of investigating not only single contaminants, but also the complex interactions between them and their associated leachates.

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Author's Contributions

This paper and its contents were executed and produced by L-M. Reid as the principal designer and student; overseen by S. Sargeant (supervisor) and F. Radford (secondary supervisor).

Ethics

Ethical approval for this study was waived due to a lack of human and animal participation.

The Authors declare that there is no conflict of interest.

The datasets used during the current study are available from the corresponding author on reasonable request.

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