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Chronic poly(L-lactide) (PLA)- microplastic ingestion affects social behavior of juvenile European perch (*Perca fluviatilis*)

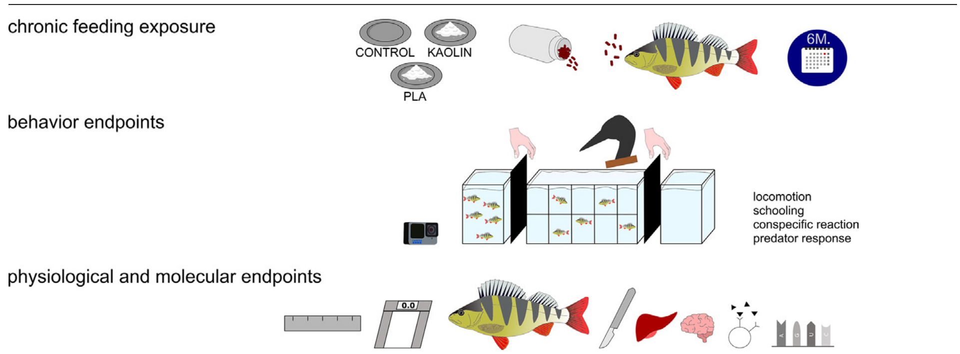
Azora König Kardgar [a,*], Dipannita Ghosh [b], Joachim Sturve [a], Seema Agarwal [b], Bethanie Carney Almroth [a]

[a] *Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden*
[b] *Macromolecular Chemistry II, University of Bayreuth, Bayreuth, Germany*

HIGHLIGHTS

- Chronic ingestion of PLA microplastics affected social behavior in juvenile perch.
- Chronic natural particle ingestion affected gene expression related to oxidative stress and androgen disruption.
- Both biobased polymer and natural particle exposure showed potential hazards in juvenile fish.

GRAPHICAL ABSTRACT



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ABSTRACT

Juvenile perch were exposed to 2 % (*w*/w) poly(L-lactide) (PLA) microplastic particles (90–150 μm) in food pellets, or 2 % (w/w) kaolin particles, and a non-particle control food over 6 months. Chronic ingestion of PLA microplastics significantly affected the social behavior of juvenile perch, evident as a significantly increased reaction to the vision of conspecifics. PLA ingestion did not alter life cycle parameters, or gene expression levels. In addition to reactions to conspecifics, fish that ingested microplastic particles showed tendencies to decrease locomotion, internal schooling distance, and active predator responses. The ingestion of natural particles (kaolin) significantly downregulated the expression of genes related to oxidative stress and androgenesis in the liver of juvenile perch, and we found tendencies to downregulated expression of genes related to xenobiotic response, inflammatory response, and thyroid disruption. The present study demonstrated the importance of natural particle inclusion and the potential behavioral toxicity of one of the commercially available biobased and biodegradable polymers.

1. Introduction

Aquatic environments are increasingly polluted with plastics. Larger plastic waste is visibly floating in oceans and freshwater systems, such as rivers and lakes, and microplastics are now known to be omnipresent on the planet. Once these materials are in the environment, they are considered to be complex contaminants made up of multitudes of substances, rather than a single pollutant (Rochman et al., 2019). Microplastics, small plastic particles, fragments, and fibers <5 mm, are found in all spheres (Hartmann et al., 2019) and consist of various polymers, shapes, colors, sizes, chemical plastic additives, and sorbed pollutants (Granek et al., in press; Rochman et al., 2019). Adverse effects on organisms exposed to microplastics are dependent on numerous factors including concentration, plastic characteristics (such as polymer type, shape, or size), exposure period, weathering condition, associated chemicals such as additives, and sorbed pollutants (Athey et al., 2020; Bucci et al., 2020; Choi et al., 2018;

* Corresponding author.
E-mail addresses: azora.konig@bioenv.gu.se (A. König Kardgar), dipannita.ghosh@uni-bayreuth.de (D. Ghosh), joachim.sturve@bioenv.gu.se (J. Sturve), seema.agarwal@uni-bayreuth.de (S. Agarwal), bethanie.carney@bioenv.gu.se (B. Carney Almroth).

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de Ruijter et al., 2020; Qiao et al., 2019a; Ziajahromi et al., 2017). Evidence of the hazards associated with microplastics in fish has accumulated. Jacob et al. (2020) found that 32 % of 782 investigated endpoints in fish on the organismal level of fish from embryonal to adult life stages were affected by exposure to virgin micro- or nanoplastics, including behavioral changes, reproductive success or life cycle parameters (Jacob et al., 2020). A study by Gove et al. (2019) investigated the ratio of prey-sized microplastic particles to natural prey in a larval fish nursery. In slick water (coastal surface layer of water), the ratio reached 1:55, which means almost up to 2 % of available prey for larval fish was microplastic (Gove et al., 2019).

To date, the vast majority of plastics produced, and consequently found in both marine and freshwater environments consist of petroleum-based polymer types such as polyethylene, polypropylene, polyethylene terephthalate, or polystyrene, which we refer to as 'conventional polymers' (Erni-Cassola et al., 2019; Lu et al., 2021). Concerns about the sustainability of these polymers lead to the attempt to replace them with so-called biobased and biodegradable polymers. The most prevalent biopolymer used today is poly(L-lactide) (PLA). The mechanical properties of PLA are comparable to those of polystyrene, which could allow PLA to be a substitute in applications such as packaging. Reasons for a still limited usage of PLA are the comparably higher price and a low deformation at break (low ductility) (Martin and Avérous, 2001). PLA is used in food packaging, textiles, and disposable cutlery, in medical applications, and for other plastic products on a small scale and in agriculture on a bigger scale, e.g., mulch films (Chen et al., 2016; Sreekumar et al., 2021). PLA is commonly used in industrial and desktop fused filament fabrication 3D printers (Yao et al., 2019).

In this study, we address the impacts of PLA on a common freshwater fish species, the European perch (*Perca fluviatilis*). Juvenile perch were chronically exposed to PLA microplastics, or natural particles (kaolin) via feed. The effects of two types of particles (natural and polymer) were compared with a non-particle feed treatment. The study was focused on adverse effects on fish behavior and biomarker responses measured via gene expression, to understand potential mechanisms underlying behavioral toxicity.

Microplastic exposure can have various adverse effects on fish behavior, such as swimming (locomotion), feeding, and foraging (de Sá et al., 2015; Limonta et al., 2019; Yin et al., 2018).

This study combined behavioral, physiological, and biochemical endpoints to maximize the output of the exposure experiment according to the 3R principles of animal research (Russell and Burch, 1959). The 3R principles aim at reducing the number of individuals in animal testing, refining test conditions for improved animal welfare and replacing animal model organisms (in vivo) with in vitro or in silico models (Russell and Burch, 1959). The challenge is to identify links between behavioral effects that are observed on the organismal level with molecular modes of action on the cellular level. An unknown number of molecular targets can be the cause of behavioral changes in fish. In this study we selected a battery of genes to cover potential biomarkers for different underlying pathways. We considered established pathways in response to microplastic exposure on oxidative stress or endocrine disruption as well as our own biomarkers of interest (inflammation and brain development). In adverse outcome pathways (AOPs) of microplastic exposure, a common initiating event is the formation of reactive oxygen species (ROS) (Jeong and Choi, 2020). Oxidative stress is studied via measurements of a number of involved genes and enzyme levels, including catalase, superoxide dismutase, glutathione reductase, or metallothionein (Khare et al., 2019; Xia et al., 2020), and is a frequently observed effect of acute and chronic microplastic exposure in laboratory-bred and wild fish (Barboza et al., 2020; Barboza et al., 2018; Solomando et al., 2020; Xia et al., 2020). Additionally, the established biomarker family of cytochrome P450 (cyp) to assess a xenobiotic response induced by diverse pollutants is a commonly investigated endpoint after exposure to microplastics (Jeong and Choi, 2020). Further, behavioral changes can be driven by changes in hormone levels resulting from e.g., exposure to endocrine-disrupting chemicals. Microplastics have been shown to be able to act as endocrine disruptors in fish (Wang et al., 2019). Here, androgen and thyroid pathways are interesting. The level of androgens is known to influence bold or aggressive behavior in fish (Dey et al., 2010; Miczek et al., 2002; Renn et al., 2012). The 17β-Hydroxysteroid dehydrogenase 3, is involved in maturation induction and spermatogenesis (Mindnich et al., 2005; Ozaki et al., 2006; Suzuki et al., 2020; Zhou et al., 2005; Zou et al., 2020), and aromatase cytochrome P450 (cyp19) (Callard and Tchoudakova, 1997; Cheshenko et al., 2008; Doering et al., 2019) are just two chosen potential biomarkers in androgen steroidogenesis, while thyroid hormone receptor beta (thrβ) is for thyroid disruption (Reinwald et al., 2021) in fish. Changes in nerve signaling can also underlie behavioral changes. Neurotoxicity is associated with microplastic exposure (Barboza et al., 2018; Bour et al., 2020; Jeong and Choi, 2020). The fish brain could be another organ affected by microplastic ingestion and is especially relevant for behavioral alterations. The growth factor angiopoietin-1 has been identified as an essential factor in the brain development of larval fish (Chen et al., 2015). In this study angiopoietin-1 was chosen as a possible biomarker in the brain of juvenile fish exposed to stressors, such as microplastics to investigate potential effects on the brain development linked to behavioral changes. As we exposed juvenile perch to PLA microplastics via food and hypothesized potential inflammatory responses, we selected hepcidin as another biomarker in the liver. The level of expression of the peptide hepcidin in the liver can reveal signs of (chronic) inflammation as it is involved in immune responses to xenobiotics, such as bacteria and viruses in fish (Cuesta and Meseguer, 2008). In humans, the level of hepcidin in the liver is studied due to its link to iron homeostasis and the induction by infection and inflammation (Fujita et al., 2007). This might be relevant for a potential inflammation caused by microplastic particles in the fish's gut.

2. Methods

2.1. Ethics statement

Animal husbandry and feeding experiments were conducted in compliance with ethical practices from the Swedish Board of Agriculture (Ethical permit number: 15984–2018). Fish were monitored daily. Two individual perch died during the start phase of the experiment: one in the control group and one in the PLA group. All other individuals were apparently healthy during the course of the experiment, after which time they were euthanized.

2.2. Fish and keeping conditions

Juvenile European perch (*Perca fluviatilis*) were obtained from Vadstena Fisk AB, Vadstena, Sweden. 60 fish were acclimatized in their later exposure tanks for 6 weeks before the start of the exposure in randomized groups of 5 fish per 45-L tank in an aerated flow-through freshwater system between 10 and 12 °C at a 14 h light: 10 h dark period. Water quality parameters, such as oxygen levels, temperature, nitrate levels were regularly monitored in the daily animal facility checks. Tanks were cleaned after each feeding from faeces or residual food. For each treatment 4 groups were tested. The European perch (*Perca fluviatilis*) is a shoaling fish species (Craig, 2000), and exhibited shy (potentially stressed) reactions when separated from its school in our observations, thus we attempted to reduce potential stress in our chronic experiment by keeping the perch in groups. Each fish group had no visual contact with other fish, as their tanks were shielded to prevent visual stress. All tanks were enriched with one half terracotta flowerpot as a shelter and a printed gravel ground underneath the tanks (this provides visual stimulation without potentially comprising water quality) (Birgersson, 2022). The perch were fed 3 % (*w*/w) food per fish body weight 3 times per week with commercial Amber Neptun ST 1.5 mm codfish food pellets (Skretting, Nutreco N.V., The Netherlands), and food rations were increased over the course of the experiment as the fish increased in weight.

2.3. PLA model microplastic particle preparation

PLA (Luminy LX175, Total Corbion, The Netherlands, 1 Kg) granulates were grinded and sieved to particles in a size range of 90–150 μm. The polymer shows high molecular weight having a bimodal curve with two peaks M_{p1} and M_{p2} around 290 KDa and 634 KDa respectively (see Appendix A Fig. S1). The thermal degradation temperature of the polymer has been observed at around 315 °C (see Appendix A Fig. S2). The polymer has a glass transition temperature (T_g) of around 60 °C and displays a melting point (T_m) of around 150 °C (see Appendix A Fig. S3). The polymer granulate was then mixed and cooled with liquid nitrogen (N_2) for about 10 min and then milled using an ultra-centrifugal mill (ZM 200, Retsch GmbH, Haan, Germany) (18,000 U/min, 1000 μm spacer sieve, 24 tooth rotor). The ground polymer was then dried under a high vacuum under Schlenk line for 4 h at 10^2 mbar, then at 40 °C at 5 mbar for 24 h. The dried polymer was then sieved using an airjet sieving machine with a 75 μm sieve (20 min, 2000 Pa) giving two fractions, (200–75) μm and 75 μm. The fraction of 200–75 μm was sieved once more and the PLA particles (a mixture of round and irregular shapes) in the size range of 90–150 μm were used for the experiments. The mean diameter of particles was around 117 μm, ± SD 49.08 (see Appendix A Fig. S4). The particle size was analyzed using Microtrac Flow Sync, Microtrac Retsch GmbH, Haan, Germany.

2.4. Food preparation and particle information

Commercial fish food, Amber Neptun ST 1.5 mm codfish food pellets (Skretting, Nutreco N.V., The Netherlands), were ground to a fine powder with a coffee grinder (purchased for this purpose, thoroughly cleaned and rinsed before use). 100 g ground food powder was mixed with a suspension of 6 g gelatine sheets (Dr. Oetker) in 96 mL heated Milli-Q water, and 5 tablespoons of red food colorant (Dr. Oetker). The red food colorant was added to mimic the appearance of natural prey, such as insect larvae, and worms (Domeneghini et al., 2008; Stejskal et al., 2020) since, in a pilot feeding experiment, the perch did not accept uncolored food. Additionally, either 3 % (*w*/w) 90–150 μm poly(L-lactide) (PLA) particles or 3 % (w/w) 20–35 μm (by definition) (Yahaya et al., 2017) kaolin particles, CAS: 1332-58-7 (Sigma-Aldrich) were added to the food mixture for two spiked feeds out of three feeds per week to achieve a total exposure of 2 % (w/w) of PLA or Kaolin. Based on prey-to-microplastic ratios in a study by Gove et al. (2019), the environmentally relevant concentration of 2 % (w/w) particles in the total amount of food and a chronic exposure period should give a better understanding of a real-life scenario of microplastic exposure in freshwater systems toward fish. The inclusion of non-polymer particles is essential for differentiating potential effects deriving from the physical nature of a particle, compared to the microplastic polymer particle (Doyle et al., 2022).The kaolin treatment should rule out particle effects on the fish. Whereas we found studies on potential toxicity of other natural particles, such as silica nanoparticles (Book and Backhaus, 2022), the application of kaolin as a reference particle seemed safe. Kaolin has been used in ecotoxicological studies on microplastics and has been shown to be less toxic than microplastics in *Daphnia magna* and *Chironomus riparius* (Scherer et al., 2020; Schür et al., 2020; Zimmermann et al., 2020). Food was prepared with a 3 % (*w*/w) concentration of PLA or kaolin, in order to achieve a 2 % PLA or kaolin exposure in the total ingested food in two feedings with spiked and one non-spiked feeding per week. The prepared control feed did not contain added particles. The food mixture was refrigerated for 1 h and pressed through a 10 mL syringe to shape elongated pellets.

2.5. Chronic feeding exposure

In the chronic feeding exposure experiment, 60 juvenile perch, with $n = 20$ fish per food treatment were used in groups of 5 fish. We defined the term ‘chronic’ based on the definition of an exposure of a minimum of 10 % of an individual's life-stage, in this case >10 % of the juvenile life-stage of perch (Environmental Technology Centre, 2005). The weight and length of each individual were measured before and after the exposure duration. The perch were fed over a 6-month period from March to August 2021 with 3 % of their body weight 3 times per week: 2 x/week with control food (no added particles), kaolin food (3 % *w*/w kaolin particles), or PLA food (3 % w/w 90–150 μm Polylactic acid particles), and 1 × /week all groups with control food, see 2.4.

2.6. Behavior recording and analysis

Before the start of the exposure (pre-exposure) and after the exposure period (post-exposure), the behavioral endpoints ‘locomotion’, ‘schooling’, and ‘reaction to conspecifics’ were documented with a GoPro HD Hero 2 camera (GoPro Inc). All behavior endpoints were tested in fish groups, not individual fish to reduce stress, therefore each treatment consisted of $n = 4$ groups. The video recordings took place on several days, randomized to avoid influences of the time of the day. For each test group, a single video was recorded. After introduction to the test aquarium, the groups were acclimatized for 1 h before recording. After each test group, the water in the aquarium was emptied and refilled. Video recording took place on a 45-L aquarium, marked with 10 equal squares on the front long side. Two-dimensional movement in the aquarium was documented in this experiment. Locomotion was counted as the total number of lines crossed by each individual group of 4 or 5 fish in the first 5 min of video recording. A crossing of a line vertically and horizontally was accepted after a fish's full head crossing. Locomotion was finally defined as the number of crossed lines per fish. Schooling was determined as the internal distance of the fish group as the total number of occupied squares in the marked aquarium in 11 consecutive counts in 30 s intervals of video recording, a total of the first 5 min of video recording. To measure Reaction to Conspecifics, a second tank with 5 perch (not involved in the exposure experiment) was placed beside the experimental tank so that these individuals were visible to the fish. We then measured the number of fish present in the 2 squares close to the conspecifics' tank, seen as a locomotive response to the vision of conspecifics, during 90 s. Following a 10-second delay after initiation (lifting of the dark plastic slide between tanks), a count was conducted every 10 s, with total possible reactions: 50 in 5 fish, 40 in 4 fish. A total possible reaction means that all individual fish of the respective test group, consisting of either 4 or 5 fish, would be present in the two squares in all 10 counts. Two predator response behaviors were analyzed after the chronic exposure period but not prior to the experiment, to avoid a learning effect in the perch. Escape and freezing reactions of the perch groups were recorded in seconds after the introduction of an artificial bird head (Johnsson et al., 2001) to the aquarium surface in a total of 15 s. All the video material was analyzed blindly so the observer had no knowledge of the treatment group.

2.7. Euthanasia and tissue sampling

Each individual was anesthetized using 0.08 g/L MS222 (and 0.08 g/L sodium carbonate) and pit-tagged in their peritoneal cavity to allow us to track the weight and length. At the end of the experiment, fish were euthanized with a sharp blow to their head and final section through the cervical spine. Length growth [%], weight gain [%], and hepatosomatic index were determined for each individual. The brain and liver of each individual were sampled for further biomarker analysis.

2.8. Gene expression analysis

For the gene expression analysis, mRNA was extracted from liver and brain tissue with RNeasy Mini Kit (Qiagen) of 3 technical replicated of $n = 10$ fish per treatment, random choice of individuals. The reverse transcription of cDNA of isolated mRNA was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc.), and the respective protocol. The expression of genes related to xenobiotic response (cytochrome P450 1A: L-cyp1a), oxidative stress (metallothionein: L-mt, superoxide dismutase: L-sod, glutathione reductase L-gr, catalase: L-cat) brain development (angiopoietin-1: B-angpt-1), androgen steroidogenesis (aromatase cytochrome P450: B-cyp19,17β-Hydroxysteroid dehydrogenase 3: L-hsd17β3), chronic inflammation (hepcidin: L-hepc) and thyroid disruption (thyroid hormone receptor beta: L-thrβ) was measured in the liver (L) or brain (B) isolated mRNA using established RT-qPCR analysis methods (Bio-Rad Laboratories Inc.). Real-time polymerase chain reaction (RT-qPCR) was performed with the SYBR® Green Master Mix, Bio-Rad Laboratories Inc., using 20 ng cDNA in each 10 μL reaction and a final concentration of 500 nM forward/reverse Primer. Primer sequences and sources are listed in Appendix A Table S1. NTC (nontemplate) and NRT (no reverse transcription) controls were included for each analyzed gene to monitor primer quality and genomic DNA contamination. Expression levels of genes of interest were normalized against housekeeping genes' expression levels (ubiq, gapdh, and ef-1α). The following thermal protocol was conducted in the CFX Connect Real-Time System (Bio-Rad Laboratories, Inc.): Initial denaturation (2 min at 95 °C), 40 cycles of denaturation (5 s at 95 °C) and annealing (30 s at 60 °C), finalizing in a melting curve (5 s at 95 °C and 5 s from 65 to 95 °C in 0.5 °C increments).

2.9. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 9.4.0 for Windows, GraphPad Software, LCC, San Diego, California USA. A $p \leq 0.05$ was accepted as significance in all statistical analyses of this study. The pre- and post-exposure data were statistically compared with two-way repeated-measures ANOVA and a post-hoc Šídák's multiple comparisons test. Same time point data were compared with an ordinary one-way ANOVA and a post-hoc Dunnett's multiple comparisons test.

3. Results

3.1. Life cycle parameters

At the start of the experiment, before the food exposure phase started, two individuals died after the pit-tagging procedure. Over the course of the experiment no mortality or visual indications of sublethal effects occurred and water quality parameters were stable. The chronic food exposure to PLA-MP or kaolin natural particles had no significant effects on the growth in length or weight or on the hepatosomatic index (HSI) of juvenile perch in this study (see Appendix A Fig. S6).

3.2. Behavior

3.2.1. Locomotion

The locomotion of the three exposure groups control, kaolin, and PLA ($n = 4$) are presented in Fig. 1A. Before the exposure phase (pre-exposure), control fish showed mean locomotion of 18.34 ± 6.62 (SD) crossed lines in 5 min, with a minimum of 12.2 and a maximum of 27.75 lines. After 6 months of feeding on non-particle control food (post-exposure), locomotion was reduced to a mean of 11.68 crossed lines (± 1.5 SD). Fish of the kaolin natural particle food exposure group had mean locomotion of 18.3 (± 7.34 SD) before the exposure phase and a mean of 10.85 (± 17.35 SD) with a minimum 0 of and a maximum of 36.4 crossed lines in 5 min. Pre-exposure, we found a mean of 28.98 ± 7.64 (SD) crossed lines/fish in the PLA treatment group, with a minimum of 19 and a maximum of 35.4. After 6 months of feeding on PLA-spiked food, the fish showed a

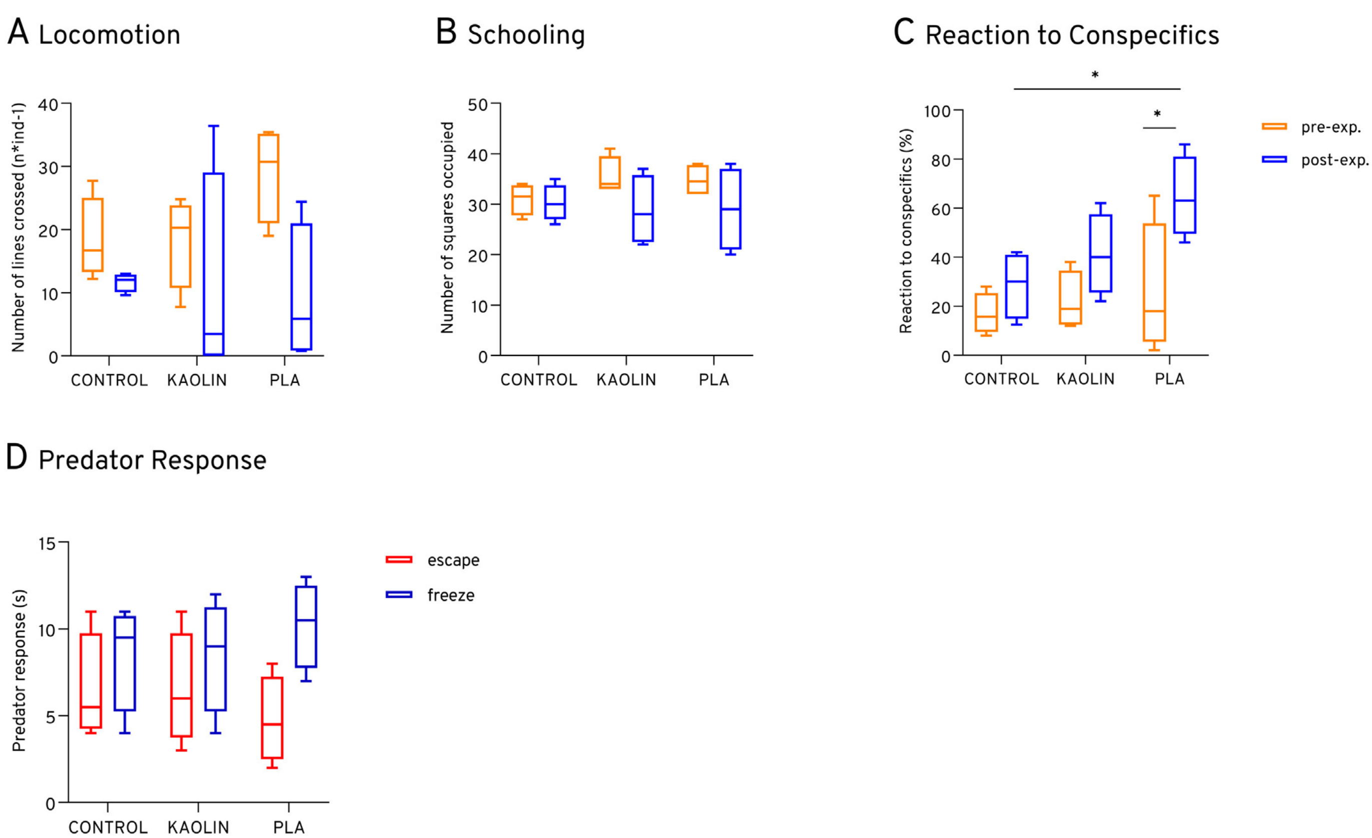

Fig. 1. Behavior analysis results of schools of juvenile perch pre-exposure and post-exposure to control food, kaolin food, or PLA food for 6 months ($n = 4$). A: Locomotion pre- and post-exposure, measured in the number of crossed lines/individual in 5 min, B: Schooling/internal distance pre- and post-exposure, measured in the number of squares occupied by the school in the tank in 11 counts of 30 s intervals, C: Reaction to Conspecifics pre- and post-exposure, measured in the percentage of possible total interactions after the vision of conspecifics, D: Predator Response post-exposure, measured as an escape or freeze reaction after predator introduction, in seconds. Data is presented in boxplots, min to max whiskers, and median. Statistical significance of same timepoint data was assessed by one-way ANOVA followed by a post hoc Dunnett's multiple comparison test and pre- and post-exposure data were compared by a two-way repeated-measures ANOVA and a post-hoc Šídák's multiple comparisons test based on 4 biological replicates. No notation: not significant, *: $p \leq 0.05$.

mean locomotion of 9.25 (± 11.13 SD), with a minimum of 0.8 and a maximum of 24.4 crossed lines/fish. There were no significant differences between treatment groups pre- or post-exposure. Regarding the mean values, all treatment groups showed a decreased locomotion after the 6-month feeding experiment. The average decrease of locomotion in the PLA microplastic exposure group was higher with a mean of −19.73 (*p*: 0.054) than for both the control and kaolin groups, with a decrease of −6.66 (*p*: 0.734) for the non-particle control and − 7.45 (*p*: 0.665) for the natural particle treatment.

3.2.2. Schooling

In Fig. 1B, schooling behavior results of fish groups ($n = 4$) pre-exposure and post-exposure to non-particle control food, kaolin food, and PLA MP food are presented as the value of internal distance, defined as the number of occupied squares. Pre-exposure, control fish groups occupied a mean of 31 squares (± 3.16 SD), while post-exposure the control fish showed a mean internal distance of 30.25 (± 3.69 SD). Fish schools of the kaolin exposure groups had a mean internal distance of 35.5 (± 3.79 SD) before the exposure and a mean occupation of 31 squares (± 6.38 SD) post-exposure. Pre-exposure the fish in the PLA groups showed a mean internal distance of 34.75 squares (± 3.20 SD), while post-exposure after 6 months of feeding on PLA MP spiked food their internal schooling distance was reduced to a mean of 29 (± 8.41 SD). There were no significant differences between treatment groups pre- or post-exposure. However, both particle spiked food exposure groups showed a higher reduction of internal distance, equal to a tighter perch school, than the non-particle control group, with a decrease of −6.75 (*p*: 0.175) in kaolin, −5.75 (*p*: 0.279) in PLA, and − 0.75 (*p*: 0.994) in the control.

3.2.3. Reaction to conspecifics

The results of the reaction of perch to the vision of conspecifics in percent of the total possible response, here called Reaction to Conspecifics, of the exposure groups control, kaolin, and PLA ($n = 4$) are presented in Fig. 1C. Perch of the control group pre-exposure showed a mean reaction to conspecifics of 16.88 % (± 8.39 SD), with a minimum of 8 % and a maximum of 28 %, post-exposure the mean reaction to conspecifics was 28.63 % (± 13.79 SD), with a minimum of 12.5 % and a maximum of 42 %. The fish in the kaolin food treatment groups had a mean reaction to conspecifics of 22 % (± 11.89 SD), with a minimum of 12 % and a maximum of 38 % before the feeding phase, and a mean of 41 % (± 16.69 SD), with a minimum of 22 % and a maximum of 62 % after 6 months feeding on kaolin spiked food. In both control and kaolin treatments, there were no significant differences in the reaction to conspecifics between the treatments (*p*: 0.888 pre-exposure, *p*: 0.463 post-exposure) or between pre-and post-exposure in the treatment group itself (*p*: 0.746 control; *p*: 0.404 kaolin). The perch schools of the PLA treatment showed a mean reaction to conspecifics of 25.75 % (± 27.28 SD), with a minimum of 2 % and a maximum of 65 % pre-exposure, while post-exposure the mean reaction was 64.5 % (± 16.6 SD), with a minimum of 46 % and a maximum of 86 %. The reaction to the friend perch group was significantly stronger after 6 months of feeding on PLA MP spiked food compared to the control (*p*: 0.019), while there was no significant difference before the exposure (*p*: 0.714). Exposure group internally, the reaction to conspecifics was significantly increased (*p*: 0.036) between pre-and post-exposure in the PLA group.

3.2.4. Predator response

The two predator responses, escape and freeze, of the exposure groups control, kaolin, and PLA ($n = 4$) were recorded and analyzed after the chronic exposure phase and are presented in Fig. 1D. After introducing an artificial bird's head, perch schools of the control group had a mean escape reaction of 6.5 s (± 3.11 SD) and a mean freeze reaction of 8.5 s (± 3.11 SD). The perch, exposed to kaolin food, showed a mean escape reaction of 6.5 s (± 3.32 SD) and a mean freeze reaction of 8.5 (± 3.32 SD). PLA-exposed perch reacted with a mean escaping of 4.75 s (± 2.5 SD) and a freezing of 10.25 s (± 2.5 SD). No significant differences were found in the predator responses of the kaolin (*p*: >0.999) and PLA (*p*: 0.635) treatments and the control after the chronic food exposures. However, there was a visible tendency of PLA-exposed perch schools to demonstrate less escaping than freezing behavior, with a mean difference between escape and freeze of 5.5 s, compared to the control and the kaolin group with a mean difference of 2 s.

3.3. Gene expression

The relative change of expression of selected genes related to androgen steroidogenesis, thyroid disruption, inflammatory response, xenobiotic response, or oxidative stress was affected more potently by kaolin exposure than by PLA (Fig. 2). The expression of hsd17b3 (*p*: 0.0007), mt (*p*: 0.0248), and gr (*p*: 0.0158) in the liver was significantly downregulated by a chronic food exposure to kaolin particles in juvenile perch compared to the control groups' values. The expression of cyp19 and angpt1 in the brain or cyp1a, thrb, hepc, sod, or cat in the liver was not significantly affected by either kaolin or PLA exposure in juvenile perch, although a tendency of downregulation of the genes cyp1a with a mean difference of −0.253 (*p*: 0.099), −0.345 (*p*: 0.1573) in thrb, and − 0.822 (*p*: 0.058) in hepc in the liver was visible after kaolin exposure to the fish. The mean difference in these genes after PLA exposure to the control was lower with −0.053 (*p*: 0.881) in cyp1a, −0.036 (*p*: 0.976) in thrb and 0.121 (*p*: 0.922) in hepc.

4. Discussion

4.1. Effects of chronic PLA-MP ingestion on fish behavior

Most studies on hazards of microplastics in ecotoxicology are conducted on conventional polymers, as they are the most abundant in the environment (Erni-Cassola et al., 2019; Lu et al., 2021). Biopolymers, like PLA, catch more attention in recent times due to its purpose to replace conventional polymers, but knowledge about potential adverse effects and hazards in organisms is still lacking. To our knowledge the effects of a chronic PLA exposure on the behavior of perch have not been described previously.

The results of the chronic ingestion of PLA particles by juvenile perch showed significant effects of the microplastic particles on the social behavior of the fish. We found a significant increase in the reaction of MP-exposed fish toward conspecifics. We suggest that a chronic ingestion of microplastic particles causes physiological stress in the fish which could result in a search for conspecifics to form a bigger school. Locomotion was decreased after the exposure to both natural and PLA particles in comparison to the juvenile perch exposed to non-particle control food. This is in accordance with a study by Limonta et al., (2019) where exposure to high-density polyethylene (HDPE) and polystyrene (PS) microplastics for 20 days affected the daily rhythm, measured as locomotion in different times of day, of adult zebrafish. We observed similar effects for the schooling behavior after chronic ingestion of both particles. Juvenile perch tended to decrease internal distance, forming tighter schools. Fish form tight schools to defend themselves from predators and bigger fish and to increase feeding and swimming efficiency (Larsson, 2012). Schooling can especially be observed in risky situations, e.g. in presence of predators (Pavlov and Kasumyan, 2000). A fish school may mimic a larger fish as a confusing signal to the predators (Breder, 1959; Larsson, 2012; Pavlov and Kasumyan, 2000).

The ingestion of both natural and polymer particles over a chronic period might have induced stress in the perch in this study, leading to increased schooling and decreased locomotion. After 6 months of PLA microplastic ingestion, the juvenile perch showed a tendency toward a more passive predator response, where they escaped less and froze at the bottom of the tank when the predator was introduced. In addition, these fish showed a significantly increased reaction to conspecifics. This elevated social behavior is in accordance with the tendency of increased schooling behavior. Together, these endpoints describe the effect of the attempt at a tighter fish school. McCormick et al. (2020) showed that fish demonstrated

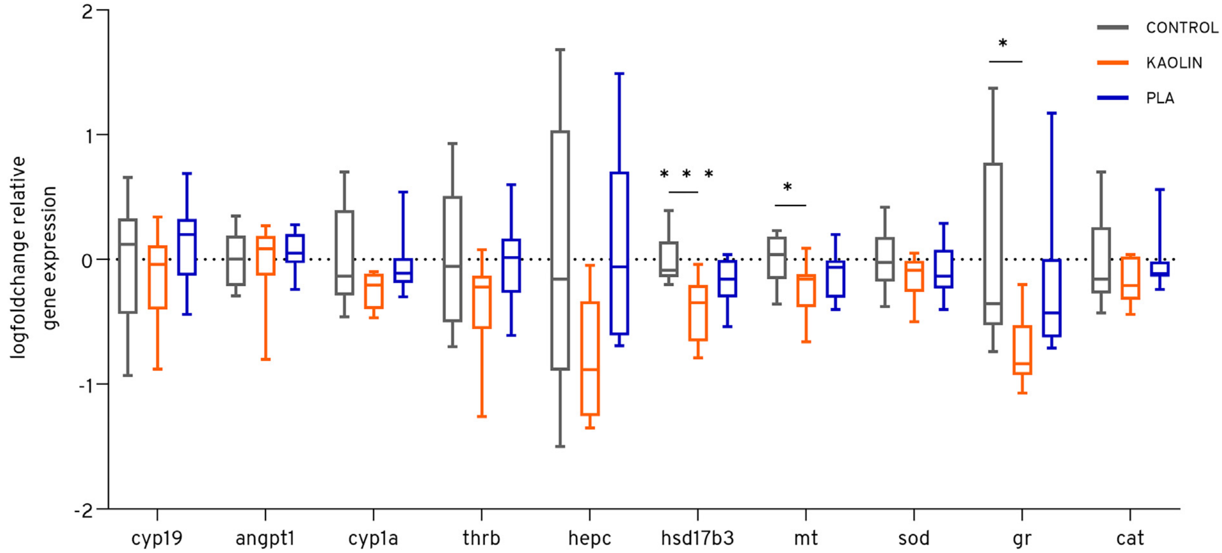

Fig. 2. RT-qPCR analysis of genes related to androgen steroidogenesis, thyroid disruption, inflammatory response, xenobiotic response, or oxidative stress upon chronic exposure to non-particle control, kaolin particles, or PLA MPs. The expression of cyp19 and angpt1 was measured in brain tissue, and the expression of cyp1a, thrb, hepc, hsd17b3, mt, sod, gr, and cat in liver tissue of perch upon exposure to the indicated food after 6 months ($n = 10$). Log2-fold change as compared to the non-particle control means are in the boxplots, min to max whiskers, and median. Statistical significance was assessed by one-way ANOVA followed by a Dunnett's multiple comparison test based on ten biological replicates and three technical replicates. No notation: not significant, *: $p \leq 0.05$, ***: $p \leq 0.001$.

increased boldness, and exploratory and risky behavior after feeding on polystyrene microplastic. Risky behavior in the exposed fish was associated with increased mortality (McCormick et al., 2020). Whether the increased reaction to conspecifics, schooling and decreased active response to predators in our study can be interpreted as boldness or exploratory behavior and ultimately have negative effects on the population's survival or can be a sign of a defense response to stress and fear to form tighter schools requires further investigation.

In addition to PLA microplastics, we also exposed fish to kaolin, representing natural, non-polymer particles. These would induce effects dependent upon physical interactions with solid matter, so called 'particle effect'. The results in the kaolin-exposure group indicate tendencies toward changes in fish behavior after chronic ingestion. While kaolin could induce particle-mediated effects on the fish, the effect of PLA ingestion on behavioral endpoints was stronger, arguing for a combined particle and polymer effect. The PLA used in this study was commercial and did not show the presence of additional additives in any significant amount based on nuclear magnetic resonance (NMR) characterization after application of chosen solvents (see Appendix A Fig. S5).

However, after internal degradation in the gut of the fish, altered PLA particle properties could play a role here. Following ingestion of the particles, degradation processes in the gastrointestinal tract of the fish, chemicals could be released and taken up via the gut. It has been previously demonstrated that PLA microplastics can cause gastrointestinal damage in zebrafish and altered the diversity of intestinal microbiota (Duan et al., 2022). Our hypothesis is that these degradation products could have caused the elevated effect compared to kaolin particles. It was shown that internal detoxification processes of degraded PLA were slower which led to increased developmental disruption in zebrafish larvae (Zhang et al., 2021a, 2021b). In general, PLA, being a polyester, undergoes ester hydrolysis leading to the lowering of pH in the vicinity (Agrawal and Athanasiou, 1997). Duan et al. (Duan et al., 2022) showed that degrading PLA particles decreased the pH of simulated fish intestinal fluid, while PET particles did not exhibit this effect. Knowledge on the mechanisms of the internal degradation of PLA in the fish intestinal tract and how a potential decrease in pH could impact the fish gut is lacking. Therefore, a precise reason leading to the different effects of PLA and kaolin remains unknown.

We measured a number of endpoints, including life cycle parameters (length growth & weight gain), and assessed gene expression of considered relevant genes, but these could not clearly explain the behavioral effects caused by PLA particles.

However, there were significant changes in the gene expression of two genes relevant to oxidative stress and one gene relevant to androgen steroid genesis after exposure to the natural particles (kaolin). The enzyme 17β-Hydroxysteroid dehydrogenase 3 which is involved in the androgen steroidogenesis, more specifically was shown to be one of the maturation-induction factors in juvenile fish (Mindnich et al., 2005; Ozaki et al., 2006; Suzuki et al., 2020; Zhou et al., 2005; Zou et al., 2020) and was significantly downregulated in juvenile perch after chronic kaolin ingestion. Interference in androgen steroidogenesis could result in severe adverse effects for the fish population if the maturation of the young is either premature or delayed. In Japanese medaka (*Oryzias latipes*) it was shown that after the exposure to benzophenone-3 a downregulation of the 17β-Hydroxysteroid dehydrogenase 3 transcription accompanied lower average egg reproduction and decreased growth of second-generation fish (Kim et al., 2014). In addition to steroidogenesis, the transcription of two genes that serve as biomarkers for oxidative stress was also affected. These chosen biomarkers involved in the oxidative stress pathway were metallothionein and glutathione reductase, and both were significantly downregulated by chronic kaolin ingestion, while not by PLA microplastics ingestion. Oxidative stress is often connected to microplastic exposure in various fish species (Barboza et al., 2020; Barboza et al., 2018; Solomando et al., 2020; Xia et al., 2020). Here we have shown that natural particle exposure can induce oxidative stress in fish, which argues for a particle effect over a potential chemical effect of PLA after degradation in the fish gut. Due to the smaller size range of the natural particles, a translocation to the liver cannot be excluded. The mechanisms behind the negative impacts of the natural particles remain unknown.

4.2. Importance of natural particle inclusion

Significant downregulation of several genes related to oxidative stress and androgen steroidogenesis in the liver occurred in the perch chronically exposed to the natural particle kaolin. These effects were not shown for the PLA exposure. Most studies on the hazards caused by microplastic lack natural particle controls (Doyle et al., 2022; Waldschläger et al., 2022). Natural particles in experiments are important to understanding the mechanisms behind the adverse effects of microplastic exposures. It has to be ruled out whether observed effects are based on the polymers, the chemicals associated with microplastics or solely on the physical properties of the particle, e.g. mechanistic damage in the gut of fish (Qiao et al., 2019b). A recent meta-analysis on particle effects in aquatic animals showed that there are some indications that, compared to natural particles, microplastics might induce more reproductive effects, changes in growth and development and mortality, though effects were not consistent between endpoints, and that there are differences in sensitivity between taxonomic groups (Doyle et al., 2022).

While some studies did find impacts of exposure to natural particles (Book and Backhaus, 2022), studies on *Daphnia magna* and *Chironomus riparius* found no effects of kaolin (Scherer et al., 2020; Schür et al., 2020; Zimmermann et al., 2020). Based on our results of gene expression analysis, we cannot confirm these findings in fish. Rather, we find that a chronic 2 % (*w*/w) kaolin exposure had stronger, significant effects on gene expression of chosen genes related to oxidative stress and androgen steroidogenesis in the liver of juvenile perch than the exposure to 2 % PLA microplastics. It has to be noted that there was a size difference between the kaolin and PLA particles; we exposed the perch to PLA microplastics in the size range between 90 and 150 μm. The size of commercial kaolin particles is between 25 and 35 μm by definition (Yahaya et al., 2017). However, kaolin naturally forms agglomerations in humidity and possibly in our prepared fish food. There is therefore a degree of uncertainty concerning the size or shape of kaolin agglomerates the fish were exposed to when ingesting the spiked food. The size and shapes of microplastics can determine the level of toxicity in organisms, likely similar to natural particles (Waldschläger et al., 2022; Wang et al., 2020). A smaller size of particles can lead to greater inflammation and interaction in the digestive tract (An et al., 2021; Zhang et al., 2021a, 2021b). In fact, a meta-analysis revealed that fish species are among the most sensitive species to natural silica nanoparticles and that surface chemistry (e.g. ionic charge, hydroxyl groups) is an important factor in the determination of the level of toxicity (Book and Backhaus, 2022). In this study, we decided to limit our exposure scenario to the weight ratio in food. Future studies should investigate the toxicity of natural particles in fish species, in comparison with other biobased/biodegradable and conventional polymers. It must be further investigated to which extent the particle itself induces toxicity.

4.3. New behavior testing methods in European perch

Most established ecotoxicology testing in OECD guidelines is conducted on endpoints such as mortality or physiological malformations in fish. Newer research tends to investigate more sensitive and sublethal endpoints such as endocrine disruption, measured on gene expression and different biomarkers. However, only a few studies focus on behavioral toxicity in fish.

The relative lack of standardized tests for behavioral effects has led to exclusion of this kind of research from regulatory toxicology and risk assessment, but also to a call for increased research as behavioral impacts can have important community and ecosystem-level effects (Ford et al., 2021).

Behavioral ecotoxicology can be a sensitive first “early warning” signal to assess environmental quality (Hellou, 2011). Peakall (1996) linked behavioral changes in wildlife to pollutants and discussed whether these changes cause risks to wildlife populations. Generally, effects on behavior are more difficult to quantify and less reproducible than biochemical changes (Peakall, 1996). As early as 1966 Warner et al. (1966) argued for the application of behavioral endpoints as pieces of evidence in sublethal pesticide toxication in fish. They stated that investigating a behavioral endpoint could be beneficial over physiological or biochemical endpoints as it includes a multitude of physiological or biochemical processes that lead to the behavioral effects in a sublethal manner, while the investigation of behavior is usually less invasive to the fish (Ford et al., 2021; Peakall, 1996; Warner et al., 1966).

Especially in microplastic research, little is known about potential adverse effects on the behavior of fish. Potential behavioral effects after microplastic exposure, especially biobased polymers, have not been described in European perch (*Perca fluviatilis*) schools before. We observed European perch as shy individuals, which are dependent on their school. According to the 3R principles (Russell and Burch, 1959) animals' welfare must be ensured, so stress should be minimized. Separating perch would result in stress reactions and falsify behavioral endpoints. Therefore, we argue for behavioral observations and testing where perch are kept in their school. And we have demonstrated here that it is possible to record environmentally relevant behavioral observations using this approach.

5. Conclusion

We have demonstrated that a chronic ingestion of the biobased polymer poly(L-lactide) (PLA) had significant effects on the social behavior of juvenile European perch. This gives indications on the possibility that biobased polymers can pose hazards to the environment just like petroleum based polymers do. Therefore, more studies on direct comparisons of biobased polymer microplastics to conventional polymer microplastics should give more insights into the levels of toxicity of conventional and biobased polymers and whether biopolymers are less hazardous for organisms such as fish, therefore a beneficial alternative to conventional polymers. Another important finding of our study is that the chronic ingestion of natural particles (kaolin) can potentially cause endocrine disruption on the androgen axis and oxidative stress on the molecular level. These effects were not observed in the PLA treatment. This points out the importance and relevance of the inclusion of natural particles in microplastic studies. The size, shape, and surface effects of natural and polymer particles have to be considered.

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CRediT authorship contribution statement

Azora König Kardgar: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Dipannita Ghosh:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Joachim Sturve:** Resources, Supervision, Writing – review & editing. **Seema Agarwal:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. **Bethanie Carney Almroth:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Data availability

The datasets generated and analyzed during the current study are available in the Zenodo repository, doi:https://doi.org/10.5281/zenodo.7541297.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163425.

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