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Microplastic-stressor responses are rarely synergistic in freshwater fishes: A meta-analysis

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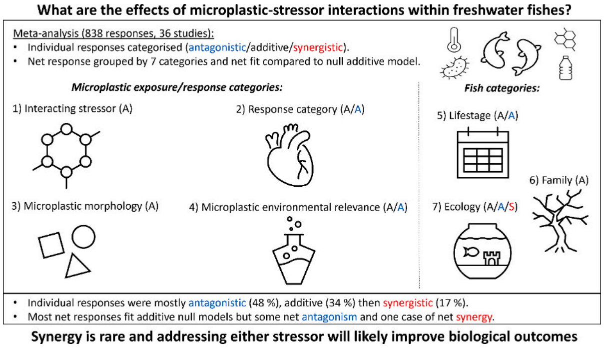
HIGHLIGHTS

- Responses to microplastic-stressor combinations are poorly understood.
- Studies investigating responses in freshwater fishes were reviewed.
- Responses were compared between fish and exposure category levels.
- Individual responses were mostly antagonistic and net effects additive.
- Targeting either stressor should produce positive management outcomes.

GRAPHICAL ABSTRACT



What are the effects of microplastic-stressor interactions within freshwater fishes?

Meta-analysis (838 responses, 36 studies):

- Individual responses categorised (antagonistic/additive/synergistic).
- Net response grouped by 7 categories and net fit compared to null additive model.

- Individual responses were mostly antagonistic (48 %), additive (34 %) then synergistic (17 %).
- Most net responses fit additive null models but some net antagonism and one case of net synergy.

Synergy is rare and addressing either stressor will likely improve biological outcomes

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ABSTRACT

Microplastic exposure can cause a range of negative effects on the biochemistry, condition and ecology of freshwater fishes depending on aspects of the exposure and the exposed fish. However, fishes are typically exposed to microplastics and additional multiple stressors simultaneously, for which the combined effects are poorly understood and may have important management consequences. Additive effects are those where the combined effect is equal to the sum, antagonistic where combined effects are less than the sum and for synergistic effects the combined effect is greater to the sum of the individual effects. Here, we performed a meta-analysis of studies recording freshwater fish responses to microplastic-stressor exposures to test if interactions were primarily non-additive (synergistic or antagonistic), and factors impacting the net response. Individual responses were classified (antagonistic/additive/synergistic) and the fit of net responses to a null additive model determined for 838 responses (36 studies) split by categorical variables for the microplastic exposure (environmental relevance, interacting stressor, microplastic morphology and response category measured), as well as the exposed fish (lifestage, ecology and family). Most responses were classified as antagonistic (48 %) and additive (34 %), with synergistic effects least frequent (17 %). Net responses fitted null additive models for all levels of interacting stressor, fish family and microplastic morphology. In contrast, net antagonism was present for biochemical responses, embryo lifestages, environmentally relevant microplastic exposures and fish with

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benthopelagic ecology, while synergism was identified for fishes with demersal ecology. While substantial knowledge gaps remain and are discussed, the data thus far suggest microplastic-stressor responses in freshwater fishes are rarely synergistic and, therefore, addressing either or both stressors will likely result in positive management and biological outcomes.

1. Introduction

Microplastics (plastics <5 mm in size) are an environmental contaminant of global freshwater systems (Li et al., 2020, 2018; Talbot and Chang, 2022). Particles may be ingested by aquatic organisms (Collard et al., 2019; Parker et al., 2021; Wootton et al., 2021) and cause a range of detrimental effects on the biology of affected organisms (Foley et al., 2018; Salerno et al., 2021; Wang et al., 2019). The exact consequences of microplastic contamination are highly variable depending on the features of the exposure and species, with effects ranging from cellular to population level (Cera et al., 2020; Parker et al., 2021). Meta-analyses on the impacts of microplastics provide contrasting findings, from mostly neutral and few negative impacts of exposure (Foley et al., 2018) to a range of negative impacts on feeding for juvenile and benthopelagic fishes (Salerno et al., 2021). These studies identify single effects for different responses, lifestages, species, and ecological traits within invertebrates and fishes (Foley et al., 2018; Salerno et al., 2021). However, microplastics are complex contaminants, with the impacts of contamination impacted by the exposure level, particle size, shape and polymer type, as well as the species and life-stage of the affected organism (Parker et al., 2021).

Freshwater organisms are simultaneously exposed to other anthropogenic stressors in addition to microplastic contamination, including climate change, pathogens and a variety of inorganic and organic pollutants, which may also induce concomitant biochemical and ecological consequences (Lange et al., 2018; Reid et al., 2019; Stendera et al., 2012). Freshwater organisms may be particularly susceptible to stressors due to their proximity to anthropogenic activities (Ormerod et al., 2010). The impacts of combined stressors on freshwater organisms are especially concerning, as multiple stressors may result in interactive effects where the actions of one stressor alters those of others, resulting in different interaction types (Khan and Thulin, 1991; Lange et al., 2018; Morgan et al., 2001), often in unexpected ways (Jackson et al., 2016; Morris et al., 2022). Interaction types include additive effects (combined effect is equal to the sum of the single effects) and the different multiplicative effects of synergistic (combined effect is greater than the sum of the single effects) and antagonistic (combined effect is lower than the sum of the single effects), however additional interaction types also exist (Jackson et al., 2016; Morris et al., 2022). Understanding the exposure to particular anthropogenic stressors, and their interactive effects, is critical for implementing effective remediation and/or mitigation steps. For example, with additive and synergistic multiple stressor effects, management can have positive outcomes when addressing either stressor; with antagonism, however, both stressors must be simultaneously addressed to produce a positive outcome (Brown et al., 2013; Piggott et al., 2015).

The interaction of microplastic exposure with another stressor is often predicted to have non-additive effects because of cumulative stress and also a similarity in biological responses to different stressors (Naqash et al., 2020; Parker et al., 2021). Additionally, differences in the binding and vectoring of harmful environmental contaminants by microplastic particles has been shown to sometimes enhance - but also sometimes reduce - their toxicity within freshwater organisms, with the overall response highly dependent on chemical interactions, the toxicity of the bound chemical and its subsequent release within the organism (Naqash et al., 2020; Parker et al., 2021). Consequently, the present study performed a meta-analysis of studies exposing freshwater fishes to microplastics and an additional stressor to determine the types of response. Individual responses were classified as additive, antagonistic or synergistic, and the fit of net (mean) effect sizes with null additive model predictions were examined grouping by categorical features of the exposure (environmental relevance, interacting stressor, microplastic morphology and response category measured) as well as the exposed fish (lifestage, ecology and family). We hypothesised that most of the individual responses and overall effect sizes would be non-additive (either antagonistic or synergistic) across all grouping categorical variables.

2. Methods

2.1. Data search and extraction

Research papers, accessible on the 2nd September 2022, were identified from research databases (Web of Science, JSTOR, SCOPUS and EBSCO, Table S1) using several different searches corresponding to microplastics (“Stressor 1”) and another interacting anthropogenic stressor (“Stressor 2”), including climate change, pollutants and pathogens (see Table S1 for full search terms and details). The initial 2986 hits were screened for duplicate articles and suitability so that they only included articles that: 1. included a freshwater fish as focal organisms (as identified by FishBase; Froese and Pauly, 2021), 2. Used living animals as experimental units (instead of cells/tissues), 3. Experimentally manipulated microplastics (hereby defined as particles ≥1 μm but <5 mm) and a second anthropogenic stressor, 4. The experimental design includes controls and stressor combinations with factorial design, 5. Used a laboratory setting and 6. Measured one or more biological responses to microplastic-stressor exposure (thereby excluding accumulation studies). This process generated a dataset of 36 original research articles (861 responses) for further screening each using a single fish species (Table 1).

Data were variously provided by the paper authors, available within the manuscript, or digitally extracted from manuscript plots using GetData Graph Digitizer (http://www.getdata-graph-digitizer.com/). Extracted data included all the responses measured, net responses with standard deviation, sample sizes, interacting stressor, exposure conditions (e.g. duration, microplastic size, type, concentration and environmental relevance), lifestage and species. A study was categorised as environmentally relevant where the manuscript based the exposure on stressor levels found in the environment. From the fish species, the family and the ecology (pelagic/demersal/benthopelagic) were assigned using FishBase (Froese and Pauly, 2021). Interacting stressors were categorised into groups: climate change, inorganic pollutants, organic pollutants and pathogens. All fish species were classified as benthopelagic except for *Salmo trutta* (pelagic) and *Misgurnus anguillicaudatus* (demersal) (Table 1).

Defined responses were first “cleaned” to identify instances of the same response given different names across studies. As there was a low level of replication at the individual response level, every response was additionally assigned a response category: 1) biochemical, defined as any chemical level or expression within the fish; 2) ecological, including behavioural measures and any interactions with the environment or other organisms; or 3) condition, which included all morphometric measures as well as measures of development and reproduction. This step was performed similarly to previous metanalyses (Foley et al., 2018; Salerno et al., 2021) and the assigned category for each response can be found within the supplementary material (Table S2). Where a response was measured over a time series, only data for the final timepoint were used. Metrics of survival and mortality (15 responses) were

Table 1

Experimental details of the studies investigating the effects microplastics and an interacting stressor in freshwater fishes. For each of the 36 experimental lab studies, the reference, study species (including the lifestage), details of the microplastic and second stressor exposure levels as well as the second stressor category, the exposure time (number of days), whether the environmental relevance of the exposures are justified within the manuscript (Y/N) and finally how the data were extracted M = manually with the aid of a plot digitiser, P = data present in or with the paper and A = data were provided from the author.

Study	Freshwater fish species	MP exposure	Second stressor exposure	Time	Relevance	Data
1. Wen et al., 2018	Symphysodon aequifasciatus (juvenile)	200 μg L^{-1} PS spheres	Climate change: 31 °C (3 °C increase)	30	N	M
2. Banihashemi et al., 2021	*Oncorhynchus mykiss* (juvenile)	500 or 1000 mg Kg^{-1} HDPE	Pathogens: 5 or 10 % median lethal dose of *Yersinia ruckeri*	30	N	M
3. Karami et al., 2016	*Clarias gariepinus* (juvenile)	50 or 500 μg L^{-1} LDPE fragments	Organic pollutants: 10 or 100 μg L^{-1} of phenanthrene	4	N	M
4. Yang et al., 2020	*Danio rerio* (larvae)	50 ng mL^{-1} PS spheres	Organic pollutants: 10 ng mL^{-1} of 6:2 chlorinated polyfluorinated ether sulfonate	7	Y	M
5. Zhang et al., 2020	*Danio rerio* (embryos)	0.05, 0.1, 1, 5 or 10 mg L^{-1} PS	Inorganic pollutants: 1.01 mg L^{-1} of cadmium	$\leq$4	Y	M
6. Karbalaei et al., 2021	*Oncorhynchus mykiss* (juvenile)	30 or 300 μg L^{-1} PS pellets	Organic pollutants: 1 or 6 μg L^{-1} of chlorpyrifos	4	Y	M
7. Luo et al., 2021	*Danio rerio* (adult)	20 μg L^{-1} PS spheres	Organic pollutants: 100 μg L^{-1} of imidacloprid	21	Y	M
8. J. Zhang et al., 2021a	*Danio rerio* (adult)	10 or 40 mg L^{-1} PE powder	Organic pollutants: 5 or 500 μg L^{-1} of 9-Nitroanthracene	7	N	M
9. C. Zhang et al., 2021b	*Cyprinus carpio* (juvenile)	700 μg L^{-1} PS spheres	Organic pollutants: 1 μg L^{-1} of tetracycline	$\leq$4	Y	M
10. Chen et al., 2022	*Cyprinus carpio* (juvenile)	1.5, or 4.5 mg L^{-1} PE powder	Organic pollutants: 5 or 15 mg L^{-1} of glyphosate	60	Y	M
11. Li et al., 2022	*Danio rerio* (adult)	10 mg L^{-1} PS	Organic pollutants: 0.01, 0.5 or 1.0 mg L^{-1} of difenoconazole	8	Y	M
12. Zheng et al., 2022	*Danio rerio* (adult)	500 μg L^{-1} PS	Inorganic pollutants: 1200 μg L^{-1} of zinc oxide nanoparticles 500 μg L^{-1} dissolved Zn^{2+} from ZnSO4	30	Y	M
13. Zhu et al., 2022	*Danio rerio* (larvae)	1 mg L^{-1} PS	Inorganic pollutants: 1 μg L^{-1} of methylmercury	7	N	M
14. Qiao et al., 2019	*Danio rerio* (adult)	200 μg L^{-1} PS	Inorganic pollutants: 50 μg L^{-1} of copper	14	N	M
15. Zhao et al., 2020	*Danio rerio* (larvae)	2 mg L^{-1} PS	Organic pollutants: 1 mg L^{-1} of butylated hydroxyanisole	7	N	M
16. Sheng et al., 2021	*Danio rerio* (adult)	200 μg L^{-1} PP 200 μg L^{-1} PE 200 μg L^{-1} PVC	Organic pollutants: 300 μg L^{-1} of triclosan	28	N	M
17. Santos et al., 2022a	*Danio rerio* (embryos)	2 mg L^{-1} (unknown spheres)	Inorganic pollutants: 60 or 125 μg L^{-1} of copper	14	Y	M
18. Santos et al., 2022b	*Danio rerio* (adult)	2 mg L^{-1} (unknown spheres)	Inorganic pollutants: 25 μg L^{-1} of copper	30	Y	P
19. Santos et al., 2021b	*Danio rerio* (embryos)	2 mg L^{-1} (unknown spheres)	Inorganic pollutants: 60 or 125 μg L^{-1} of copper	14	Y	M
20. Santos et al., 2021a	*Danio rerio* (embryos)	2 mg L^{-1} (unknown spheres)	Inorganic pollutants: 60 or 125 μg L^{-1} of copper	14	Y	M
21. Santos et al., 2020	*Danio rerio* (larvae)	2 mg L^{-1} (unknown spheres)	Inorganic pollutants: 15, 60 or 125 μg L^{-1} of copper	4	Y	P
22. Huang et al., 2021	*Oreochromis niloticusas (juvenile)*	10 μg L^{-1} of aged PS 10 μg L^{-1} of virgin PS	Organic pollutants: 50 μg L^{-1} of propranolol 50 μg L^{-1} sulfamethoxazole	14	N	M
23. Cheng et al., 2021	*Danio rerio* (embryo)	1 mg L^{-1} of PET fibres 1 mg L^{-1} of PET fragments	Inorganic pollutants: 1 mg L^{-1} of cadmium	2	N	M
24. Qin et al., 2021	*Danio rerio* (larvae)	20 mg L^{-1} PS	Inorganic pollutants: 1 mg L^{-1} of cadmium	5	N	M
25. Hoseini et al., 2022a	*Cyprinus carpio* (juvenile)	0.5 mg L^{-1} PVC	Inorganic pollutants: 0.25 mg L^{-1} of copper	14	N	M
26. Hoseini et al., 2022b	*Cyprinus carpio* (juvenile)	0.5 mg L^{-1} PVC	Inorganic pollutants: 0.25 mg L^{-1} of copper	14	N	M
27. Qu et al., 2019	*Misgurnus anguillicaudatus* (adult)	50 mg L^{-1} PVC	Organic pollutants: 10, 100, 500 μg L^{-1} of rac-venlafaxine 10, 100, 500 μg L^{-1} of *S*-venlafaxine 10, 100, 500 μg L^{-1} of *R*-venlafaxine 10, 100, 500 μg L^{-1} of rac-*O*-desthylvenlafaxine 10, 100, 500 μg L^{-1} of *S*-*O*-desthylvenlafaxine 10, 100, 500 μg L^{-1} of *R*-*O*-desthylvenlafaxine	4	N	M
28. Banaee et al., 2019	*Cyprinus carpio* (juvenile)	250 or 500 μg L^{-1} PE	Inorganic pollutants: 100 or 200 μg L^{-1} of cadmium chloride	30	N	P
29. Schmieg et al., 2020	*Salmo trutta* (larvae)	10^5 or 10^6 L^{-1} PS pellets	Organic pollutants: 300 μg L^{-1} of amitriptyline	60	N	P
30. Hanachi et al., 2021	*Oncorhynchus mykiss* (juvenile)	30 or 300 μg L$^{-1 \text{ PS}}$ fragments	Organic pollutants: 2 or 6 μg L^{-1} of chlorpyrifos	4	N	A
31. Huang et al., 2023	*Danio rerio* (adult)	500 μg g^{-1} PS spheres	Organic pollutants: 20 or 200 μg g^{-1} of chlorpyrifos	14	N	A

(*continued on next page*)

excluded from the dataset as they are often found to follow a multiplicative null model (Schäfer and Piggott, 2018). All datapoints were extracted, including those where more than one stressor combination was utilised, resulting in 846 responses from 36 studies.

Of this dataset, 20 studies used organic pollutants as an interacting stressor (463 responses), 14 studies used inorganic pollutants (292 responses), 1 study used pathogens (80 responses), and 1 study simultaneously used warming (11 responses) and was thereby classified as "Climate change". Classifying responses into categories, 627 responses were designated "biochemical", 181 were assigned "condition" and 38 "ecological".

2.2. *Effect sizes and interaction types*

To assess interaction type for each single response, an additive null model was used (Folt et al., 1999) as a baseline for comparison, and calculated as per Morris et al. (2022):

$$\textit{Simple additive model} = S_1 + S_2 - C.$$

where S_1 is the stressor response to the microplastic singly, S_2 the response to the stressor 2 singly and C the control. Hedges' *d* was calculated as:

$$\text{Hedges}'\text{d} = \text{J}\frac{\text{X}_\text{O} - \text{X}_\text{P}}{\text{s}}$$

$$\text{Correction factor (J)} = 1 - \frac{3}{4(\text{n}_\text{o} + \text{n}_\text{p} - 2) - 1}$$

$$\text{Pooled standard deviation (s)} = \sqrt{\frac{(\text{n}_\text{o} - 1)(\text{s}_\text{o})^2 + (\text{n}_\text{p} - 1)(\text{s}_\text{p})^2}{(\text{n}_\text{o} + \text{n}_\text{p}) - 2}}$$

where X_o is the observed combined response to stressors 1 and 2, X_p the predicted combined response (from the null model), n_o and n_p the sample sizes for the observed (combined stressor treatment) and predicted responses (assigned to the minimum sample size of the two single-stressor treatments used to calculate the predicted response) respectively, *J* is a weighting factor to correct for small sample bias, and s_o and s_p are the pooled standard deviations for the observed and predicted responses respectively (Morris et al., 2022).

The variances used to provide inverse weights of Hedges' *d* (*d*) were calculated as:

$$\text{Inverse weight of Hedges}'\text{d (Vd)} = \frac{\text{n}_\text{o} + \text{n}_\text{p}}{\text{n}_\text{o}\text{n}_\text{p}} + \frac{\text{d}^2}{2(\text{n}_\text{o} + \text{n}_\text{p})}$$

For each response, the pooled sample size, its associated t-value and standard error were then used to calculate 95 % confidence limits. The individual interaction type was defined, following Jackson et al. (2016), as additive where the confidence intervals cross 0, antagonistic where both confidence limits were below and synergistic where both confidence limits were above 0.

2.3. *Dataset cleaning*

The dataset (846 responses, 36 papers) was subject to various cleaning steps which resulted in the loss of certain responses. First, responses were removed where the interaction type (additive, antagonistic, synergistic) and/or Hedges' *d* could not be determined (e.g. measured effects were 0 or sample numbers were absent). Secondly, responses were excluded where Hedges' *d* was considered an outlier ($<$ -30 or $>$ 30) as per Morris et al. (2022). After these steps, 8 responses were excluded to produce a final dataset of 838 responses from 36 studies, with this dataset then subjected to meta-analyses.

2.4. *Statistical analyses*

Analyses were carried out in RStudio version 3.5.1 (R Development Core Team, 2021). The mean (net) effect sizes were estimated using *rma.mv()* function models as part of the "metafor" package in R (Viechtbauer, 2010) separately for each of the exposure (secondary stressor, response category, microplastic morphology and environmental relevance) and fish (lifestage, family and fish ecology) categorical variables. Hedges' *d* (Hedges, 2016) was selected as a standardised net difference (with bias correction) between the additive null model and the observed responses to look at the overall deviation from additive interactions. Effect size directions were inverted to compare the strength of effects on an absolute scale (Jackson et al., 2016; Morris et al., 2022). These models used "ID" nested within "Study" as random effects to account for non-independence of observations from the same study and to allow the true effect sizes to vary across observations. We recognise that studies vary in their weighting/bias within models due to differences in the number of measured responses and also that responses within studies are not independent and therefore accounted for this using the random effects as per a previous study (Jackson et al., 2016). It was decided not to select a single response from each study for additional analyses within the categories investigated to avoid selection bias and reducing statistical power.

3. Results

3.1. *Microplastic exposure and response categories*

Most individual responses were antagonistic (48.3 %) or additive (34.2 %), with synergistic effects least frequent (17.4 %). However, when categorising responses by the second stressor, the mean net responses always fitted an additive null model (Fig. 1A).

Biochemical responses were antagonistic overall (Fig. 1B), while condition and ecological responses fitted null additive models. Net responses by microplastic morphology all fitted null additive models (Fig. 1C). Classifying by the environmental relevance of the microplastic exposure level, net responses were antagonistic for environmentally relevant but fitted additive models for non-relevant exposures (Fig. 1D).

Table 1 (*continued*)

Study	Freshwater fish species	MP exposure	Second stressor exposure	Time	Relevance	Data
32. Masud et al., 2022	*Gasterosteus aculeatus* (adult)	0.05 mg L^{-1} PP pellets	Organic pollutants: 3.6 mg L^{-1} of Roundup (glyphosate)	72	Y	A
33. Wang et al., 2022	*Danio rerio* (embryo)	0.1 ppm PVC fragments	Organic pollutants: 71 μg L^{-1} of di(2-ethylhexyl) phthalate	9	Y	M
34. He et al., 2021	*Danio rerio* (adult)	2 mg L^{-1} PS	Organic pollutants: 80 μg L^{-1} of triphenyl phosphate	21	N	A
35. Schell et al., 2022	*Danio rerio* (adult)	2 g L^{-1} PE fragments	Organic pollutants: 200 μg L^{-1} of chlorpyrifos 36 μg L^{-1} of hexachlorobenzene	14	N	A
36. Hanslik et al., 2022	*Danio rerio* (adult)	0.016 g L^{-1} PMMA beads 0.16 mg L^{-1} PS	Organic pollutants: 10 or 100 ng L^{-1} chlorpyrifos 0.78 or 50 μg L^{-1} benzo(*k*)fluoranthene	21	Y	M

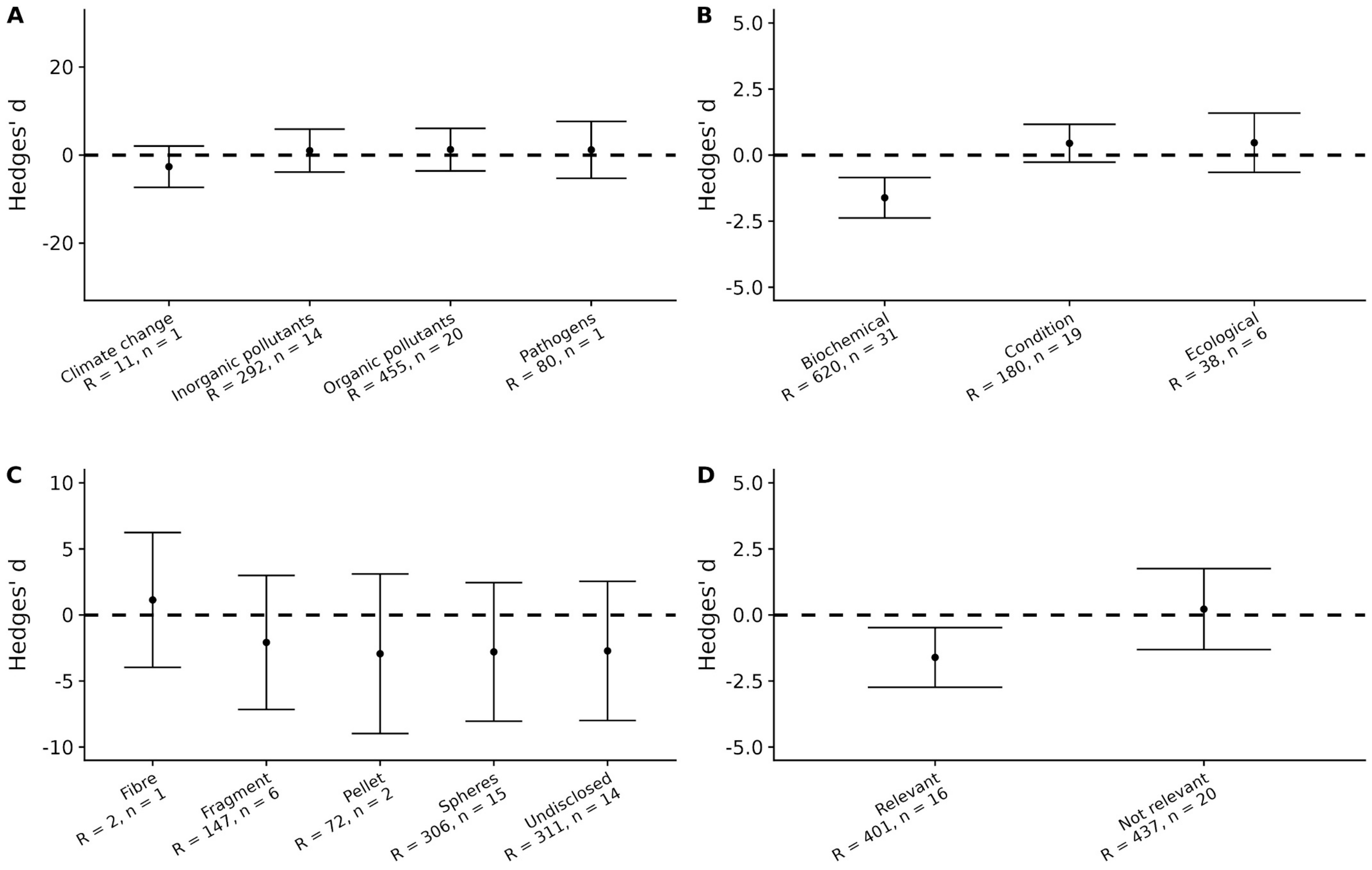

Fig. 1. Net Hedges' d parameter estimates grouped by microplastic exposure and response categories. Responses are given for: A) interacting stressor, B) response category, C) microplastic morphology and D) the environmental relevance of the microplastic exposure. Points represent mean effect size estimates and the confidence interval with additive estimates crossing the dashed line, synergistic estimates both above and antagonistic estimates both below the dashed line. The number of responses (R=) and studies (n =) are given for each category level.

3.2. *Fish categories*

Net responses fit null additive models for all lifestages except in embryos where there was overall antagonism (Fig. 2A). The mean effects for all families fitted null additive models (Fig. 2B). Grouping by fish ecology, responses for benthopelagic fishes were net antagonistic, net synergistic in demersal fishes and net additive in pelagic fishes (Fig. 2C).

4. Discussion

4.1. *General study findings*

The majority of individual responses were classified as antagonistic, followed by additive and synergistic. The overall net effect sizes mostly fitted null additive models, but with some exceptions: antagonism for biochemical responses, embryo lifestages, environmentally relevant microplastic exposure, and fishes with benthopelagic ecology, with synergism identified in fish with demersal ecology. Generally, the individual and net responses results support the findings of meta-analyses investigating multiple stressors in freshwaters where a majority of individually antagonistic responses also resulted in an additive overall combined effect in most cases (Jackson et al., 2016; Morris et al., 2022).

A high incidence of antagonism suggests that, in most cases, microplastics did not amplify the effects of stressors such as warming, inorganic pollutants, organic pollutants or pathogens, and might suggest direct or indirect interactions between stressors, potentially through the binding of contaminants and/or the triggering of similar response pathways (Naqash et al., 2020; Parker et al., 2021). However, this result is biased by the number of responses for each study that are non-independent and likely correlated. By contrast, overall effects fitting null additive models suggest that the combined effects of microplastics and a second stressor is arguably “neutral” from a management perspective since the combined effect is no worse, but also no better than the sum of the individual stressors. Additive and synergistic interactions may be addressed by targeting either stressor, whereas in instances of antagonism, both stressors must be simultaneously addressed to produce a positive management outcome (Brown et al., 2013; Piggott et al., 2015). Meta-analyses provide critical and robust approaches for synthesising existing literature, describing general trends and addressing key management questions within environmental research, as demonstrated for previous multi-stressor meta-analyses (Jackson et al., 2016; Morris et al., 2022).

4.2. *Microplastic exposure and response categories*

Interacting stressor had no impact on the type of net responses, in contrast to other freshwater multiple stressor studies with comparable methods that demonstrate net antagonism between combinations such as temperature and contamination (Jackson et al., 2016; Morris et al., 2022). The present study found no such relationship between warming and microplastics, likely due to the present study including only a single study using warming (Wen et al., 2018) and/or since microplastics are a select group of contaminants. More research is therefore required. The literature suggests that microplastics can bind and accumulate various harmful chemicals, which are then vectored into organisms upon consumption, although this process depends on the types of chemical, microplastic and the physiological conditions (Naqash et al., 2020; Parker et al., 2021). Despite these known interactions, the net effect size

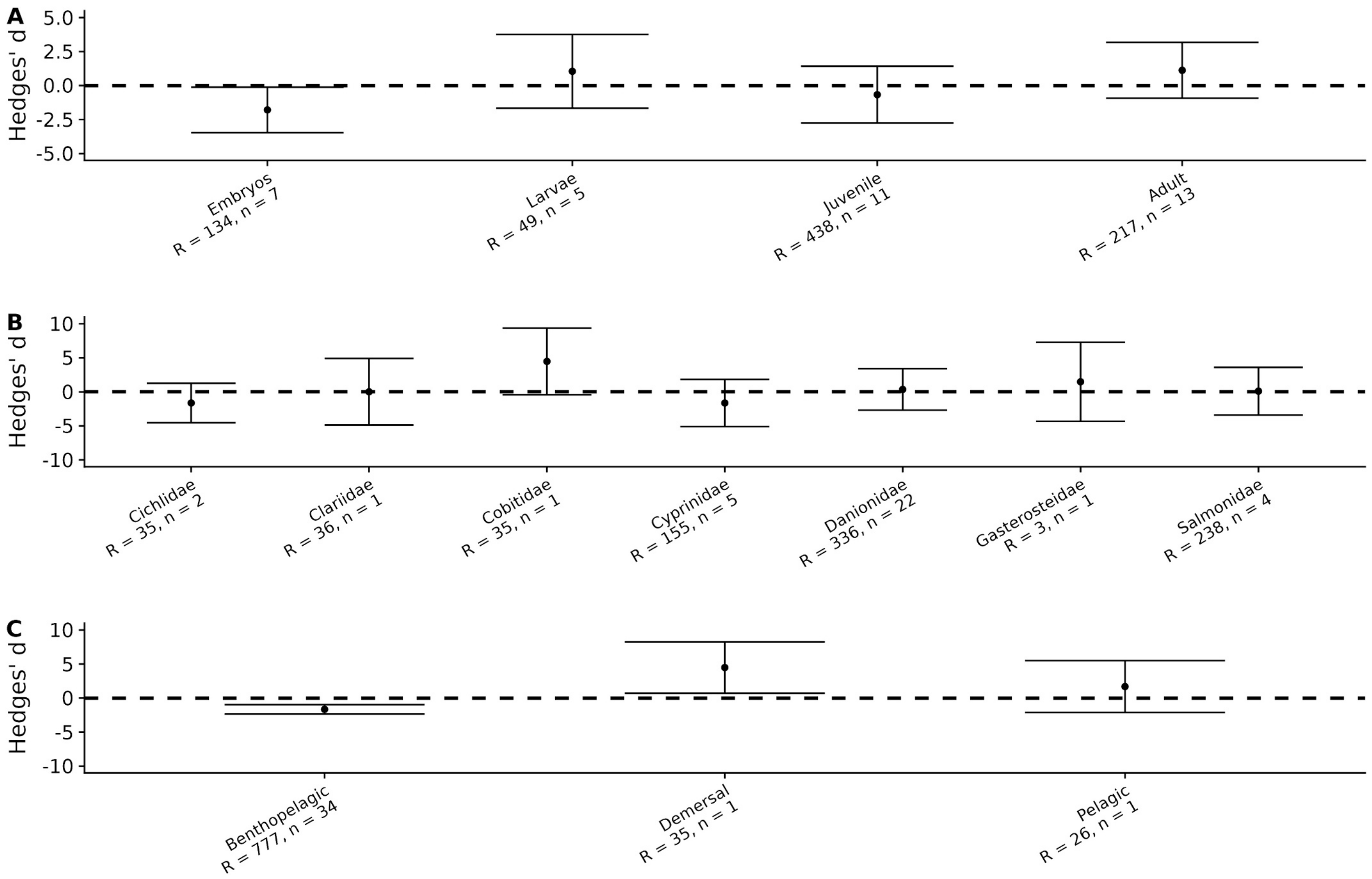

Fig. 2. Net Hedges' d parameter estimates grouped by fish categories. Responses are given for fish: A) lifestages, B) family and C) ecology. Points represent mean effect size estimates and the confidence interval with additive estimates crossing the dashed line, synergistic estimates both above and antagonistic estimates both below the dashed line. The number of responses (R=) and studies (n =) are given for each category level.

analyses indicated no difference from additive model predictions, suggesting either stressor may be targeted for management. Stressor interactions can, however, vary both spatially and temporally (Jackson et al., 2021), and dominant interactions also exist whereby a single stressor may drive the overall interactive effect as an extreme form of antagonism (Jackson et al., 2016; Morris et al., 2022). It is therefore possible that responses classified in the present study may be better classified as dominant, however this was outside of the scope of the present research.

In contrast, classified biochemical responses were determined to be antagonistic overall, whereas condition and ecological metrics fitted null additive models in the present study. Interaction studies have demonstrated antagonism within community and organism responses to multiple stressors (Jackson et al., 2016), as well as negative physiological effects of multiple stressors in freshwater fishes (Lange et al., 2018). Additionally, microplastics have been shown to have greater negative effects on feeding and behavioural responses in fishes (Salerno et al., 2021), as well as taxa-dependent effects on consumption within fish and aquatic invertebrates (Foley et al., 2018). In contrast to other studies, the present study found no difference in response in consumption/feeding and behaviour metrics, hereby designated as “Ecological” in the present study. The net antagonism for biochemical metrics may be best explained by redundancy through similar biochemical responses being triggered by multiple different stressors, as suggested by Jackson et al. (2016). High levels of individual antagonism or synergism may indicate physical interactions such as adsorption of chemicals, known to occur for microplastics (Naqash et al., 2020). However, the present study found net additive effects suggesting that any physical associations between microplastics and co-contaminants did not increase or decrease the impacts on various freshwater fish responses relative to the sum of the single effects, in line with previous research (Jackson et al., 2016). Lange et al. (2018) looked at multiple stressor effects on freshwater fishes and demonstrated a negative multi-stressor effect for physiological metrics, defined as “Condition” or “Biochemical” in the present study. While the present study did not test the magnitude/direction of the combined effect, it demonstrated a reduction in the combined effect on biochemical/physiological metrics and suggested that the removal of either stressor singly will have a relatively minor impact on biological response measures compared to both simultaneously.

Net responses for microplastic morphologies all fit null additive models, however environmental relevance classification indicated an antagonistic effect for relevant compared to non-relevant microplastic exposures. Microplastic shape has been shown to impact responses within fishes and aquatic invertebrates (Foley et al., 2018) by impacting their ingestion and subsequent effects, though the present study found consistent net additive effects. The net antagonistic impact of environmental relevance, hereby classified where the study authors justified the microplastic exposure used, could suggest that studies using unjustified exposures may be overestimating the combined effects of microplastics and additional stressors. A previous meta-analysis on microplastic impacts demonstrated some publication bias towards the negative main results of microplastics on consumption, growth and reproduction, and also some differences between an inclusive dataset and a restricted dataset where outliers were removed (Foley et al., 2018). Future interaction studies using environmentally relevant exposures and subsequent meta-analyses will help to clarify the interactive effects and to further examine these potential biases. Nevertheless, this result has important management implications suggesting that management efforts to reduce environmentally microplastic exposure in fishes should simultaneously address the interacting stressor in order to maximise the positive impacts.

4.3. Fish categories

The present study demonstrated net antagonism in embryos, however larvae, juvenile and adult net responses all fitted null additive models. The literature has previously identified different responses between lifestages with Foley et al. (2018) finding no difference in response, however Salerno et al. (2021) found negative impacts for juvenile fishes but neutral effects for other lifestages. Antagonism within embryos may be considered a positive result since freshwater fishes may experience significant mortality at this lifestage, although there is some evidence that all freshwater lifestages are negatively impacted by multi-stressor effects (Lange et al., 2018). Studies on the impacts of microplastics identify generally neutral effects of microplastics on survival metrics (Foley et al., 2018; Salerno et al., 2021) and, therefore, microplastics appear to have largely sub-lethal impacts. However, some studies have detected negative multi-stressor effects on survival in freshwater fishes (e.g. Lange et al., 2018). The present study excluded survival metrics and so cannot comment on these impacts.

The present study found net responses were additive across all fish families. Previous research has suggested some species-specific differences in susceptibility to microplastics (Salerno et al., 2021) and some negative multi-stressor effects at the order level (Lange et al., 2018). Salerno et al. (2021) identified largely neutral effects for many of the families represented in the present study, notably *Danio rerio* (danionidae in the present study). However, Salerno et al. (2021) identified negative effects for *Cyprinus carpio* (the sole representative of Cyprinidae in the present study) for which the present study found net additive effects. The largely neutral effects of microplastics on most taxa likely explains the absence of antagonism and synergism at the taxa level. Salerno et al. (2021) additionally demonstrated some differences in responses within families. For example, microplastic exposure had neutral effects on *Carassius auratus* whereas negative effects on *C. carpio* (both Cyprinidae). These differences could originate from measuring different responses, as well as issues of sample size given both Salerno et al. (2021) and the present study were dominated by certain species - such as *D. rerio* - with a very low sample size for other taxa. Additionally, Lange et al. (2018) found negative overall multi-stressor effects within salmoniformes (Salmonidae in the present study), as well as cypriniformes (Cyprinidae in the present study). While stressor combinations may result in overall negative effects, the present study suggests the combined effect is likely additive.

It was identified here that overall antagonism was apparent within benthopelagic fishes, with net synergism for demersal fishes and additive effects for pelagic fishes. While the results for pelagic and demersal fishes are considered unreliable as the data are based on single studies, the antagonistic effects on benthopelagic fishes are interesting. Salerno et al. (2021) previously demonstrated negative impacts of microplastic exposure within benthopelagic and pelagic fishes. The negative effects of microplastics within benthopelagic fishes (Salerno et al., 2021) coupled with the antagonism demonstrated in the present study suggest that, while benthopelagic fishes may be more susceptible to microplastic exposure, an additional stressor may reduce the impact and/or dominate the interactive effect. Managers looking to enhance environmental quality for benthopelagic fishes in particular should therefore use management strategies that target both stressors at the same time for the largest positive impact.

4.4. Future research and recommendations

Evaluating the studies used within the present analysis, 20 of the 36 studies did not justify the level of microplastic and second stressor exposures within the manuscript while 14 studies did not include details on the microplastic morphology exposed to (Table 1). All of the studies investigated the combined effects of microplastics with one other stressor in a single species with 22 studies using zebrafish *Danio rerio*. Some categories were represented by a single study with single studies for warming (Wen et al., 2018) and pathogens (Banihashemi et al., 2022) as well as for pelagic (Schmieg et al., 2020) and demersal fishes (Qu et al., 2019). Consequently, we support the recommendations put forward by Matthaei and Lange (2016) on multiple stressors in freshwater fishes, such as including novel fish species as model organisms (especially pelagic and demersal species), investigating the combined effects of three or more stressors, and investigating responses within multiple species in the same study and/or at the community level. Experimentally, we also support the suggestions to select pervasive stressors in future works, investigating the mechanisms of action for the combined effects seen, and assessing the role of genetic diversity and its impacts on responses to multiple stressors (Matthaei and Lange, 2016). Considering the publications used in the present study, the research field is relatively new and we argue these recommendations remain valid today.

The sample sizes for the number of studies and interactions in the present study may be considered low compared to other studies (Jackson et al., 2016; Morris et al., 2022). However, we argue they accurately reflect the literature base. As such, more interaction studies are required to increase the level of replication, especially for interacting stressors such as climate change (Wen et al., 2018) and pathogens (Banihashemi et al., 2022), for which there was only a single study. Additionally, knowledge gaps exist for the potential interactions of stressors such as eutrophication and microplastics in freshwater fishes, where interactions have already been investigated in freshwater *Daphnia* (Hiltunen et al., 2021; Sadler et al., 2019). Additionally, microplastics and habitat degradation have been shown to have a combined effect on behaviour and survival within marine fishes (McCormick et al., 2020), although more studies are needed. Understandably, most of the interaction studies in the present study were for inorganic and organic contaminants, since microplastics are assumed to bind these chemicals and/or increase their toxicity (Naqash et al., 2020; Parker et al., 2021). However, future studies might also focus on other stressors that could result in cumulative stress (Parker et al., 2021). Similarly, the use of different microplastic morphologies (beads were most common) and increasing the use of alternative study species in multi-stressor research rather than using zebra fish almost exclusively, will improve the generalisation of the study findings. There are >15,000 freshwater fish species globally (Froese and Pauly, 2021) and the present study echoes the call of Lange et al. (2018) for more studies on underrepresented fish taxa, such as pelagic and benthic fishes, as well as those that are more piscivorous. This balance is crucial to truly understand the responses of global freshwater fishes to microplastics and interacting stressors in order to effectively manage their threats. Salerno et al. (2021) already demonstrated negative effects of microplastic exposure for both common carp *Cyprinus carpio* and European perch *Perca fluviatilis*, whereas Lange et al. (2018) demonstrated negative effects for multi-stressor effects within Cypriniformes, Salmoniformes and Esociformes and, therefore, these freshwater taxa are excellent candidates for future works as alternatives to zebrafish, as well as being facultative and obligate piscivores.

A number of studies initially considered were excluded during screening on the basis of methodology, with many studies lacking a factorial study design that included blank controls and all combinations of single and combined treatments. It is important that the microplastic exposure level used is justified by the author and ideally uses current or predicted microplastic exposure conditions that are justified in the text, as this enables better evaluation by reviewers and the reader. However, we do recognise that the span of freshwater microplastic levels globally can justify most levels of microplastic exposure (Li et al., 2020). Finally, it is recommended that journal editors, reviewers and researchers are especially wary of pseudo-replication and non-factorial study designs, while also ensuring all exposure details are present and that raw data are submitted together with the associated article. Several meta-analyses on the effects of microplastics in fishes (Salerno et al., 2021), fishes and invertebrates (Foley et al., 2018) as well as multi-stressor studies in freshwater fishes (Lange et al., 2018) have provided important insights into the impacts of microplastics and multi-stressors. These previous studies and the present study synthesise the available literature and are thus highly dependent on accessible data and transparent study designs in order to describe overall patterns in the data and to make suitable future suggestions to advance the research field.

5. Conclusions

The present study completed a meta-analysis to examine the types of interactive effects in freshwater fishes between microplastics and additional stressors. The individual and net responses were summarised by different categories and determined that: i) overall, the majority of individual responses were classified as antagonistic, then additive with synergistic responses least frequent, and ii) net responses fitted null additive models for most categories and category levels, however, net antagonism was found for biochemical responses, environmentally relevant exposures, embryos and benthopelagic fishes, whereas synergism was detected for demersal fishes from a single study. The dominance of antagonistic individual interactions is most likely due to comparable responses to different stressors and the frequency of net additive responses likely reflect the average of many weaker antagonistic and some stronger additive effects. Taken together, the current literature and study results indicate that, while microplastic exposure can undoubtedly have negative impacts on freshwater fishes, the combined effects are often equal to or less than the sum of the individual stressor effects. However, the literature is still developing and requires further studies, with the addressing of confounding issues such as underrepresentation in fish taxa, ecologies and interacting stressors in addition to experimental design limitations and sometimes a lack of critical information. Nevertheless, the rarity of synergism between stressors in the present study is a positive for multiple stressor management and suggests that freshwater fish managers may continue to address microplastic contamination and additional stressors singly or in combination to produce positive management outcomes.

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CRediT authorship contribution statement

Ben Parker: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **J. Robert Britton:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Iain D. Green:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization. **Michelle C. Jackson:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Demetra Andreou:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Conceptualization.

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.

Data availability

The research data are submitted with this manuscript. Please refer to the supplementary material for a Readme section on the data.

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Appendix A. Supplementary data

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