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Differential effects of two prevalent environmental pollutants on host-pathogen dynamics

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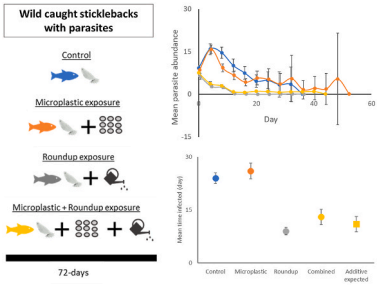
HIGHLIGHTS

- Investigated the impact of microplastic and Roundup® exposure on wild fish.
- Microplastic consumption significantly impacted disease resistance.
- Roundup® exposure significantly reduced infections but caused high mortality.
- Combined treatment of microplastic and Roundup® caused mass mortality in wild fish.

GRAPHICAL ABSTRACT



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ABSTRACT

Chemical pollutants are a major factor implicated in freshwater habitat degradation and species loss. Microplastics and glyphosate-based herbicides are prevalent pollutants with known detrimental effects on animal welfare but our understanding of their impacts on infection dynamics are limited. Within freshwater vertebrates, glyphosate formulations reduce fish tolerance to infections, but the effects of microplastic consumption on disease tolerance have thus far not been assessed. Here, we investigated how microplastic (polypropylene) and the commercial glyphosate-based herbicide, Roundup®, impact fish tolerance to infectious disease and mortality utilising a model fish host-pathogen system. For uninfected fish, microplastic and Roundup had contrasting impacts on mortality as individual stressors, with microplastic increasing and Roundup decreasing mortality compared with control fish not exposed to pollutants. Concerningly, microplastic and Roundup combined had a strong interactive reversal effect by significantly increasing host mortality for uninfected fish (73% mortality). For infected fish, the individual stressors also had contrasting effects on mortality, with microplastic consumption not significantly affecting mortality and Roundup increasing mortality to 55%. When combined, these two pollutants had a moderate interactive synergistic effect on mortality levels of infected fish (53% mortality). Both microplastic and Roundup individually had significant and contrasting impacts on pathogen metrics with microplastic consumption resulting in fish maintaining infections for significantly longer and Roundup significantly reducing pathogen burdens. When combined, the two pollutants had a largely additive effect in reducing pathogen burdens. This study is the first to reveal that microplastic and Roundup individually and interactively impact host-pathogen dynamics and can prove fatal to fish.

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1. Introduction

An expanding human population inevitably leads to an increase in the diversity of anthropogenic stressors that impact ecosystems (Piggott et al., 2015; Birk et al., 2020). When predicting biological responses to global stressors, one of the greatest challenges is the potential non-additive interactions between multiple abiotic and biotic stressors (Pimm et al., 2014; Dias et al., 2017; Birk et al., 2020). These interactions may be unpredictable, harming one species and benefitting another, either directly or indirectly (Koelmans et al., 2001; Crain et al., 2008; Piggott et al., 2015). Freshwaters are exposed to a cocktail of pollutants and their biota face high extinction rates, with freshwater fish and amphibians being lost at higher rates than all other vertebrates (Adams et al., 2014; Dias et al., 2017; Graf et al., 2020). Two factors implicated in this demise are pollution and infectious disease (Dudgeon et al., 2006; Collen et al., 2014; Tickner et al., 2020).

Parasites are the dominant biomass within freshwater and indeed in all ecosystems (Poulin, 1999; Kuris et al., 2008; Dunne et al., 2013). Interactions between parasites and their hosts influence the life-history traits of individuals (from metabolism to mortality; SeppÄnen et al., 2009; Masud et al., 2020) and these interactions are an essential part of maintaining ecosystem health (Marcogliese and Cone, 1997; Palm and Rückert, 2009; Lefèvre et al., 2009; Cable et al., 2017). This is supported by community level studies showing that habitats suffering environmental contamination have reduced parasite diversity (Marcogliese and Pietrock, 2011; Chapman et al., 2015). Although many investigations have assessed the impact of single environmental stressor impacts on host pathogen dynamics (e.g., Ackerman et al., 2006; Wysocki et al., 2007; Smallbone et al., 2016), few have taken a multi-stressor approach.

One of the most prevalent environmental stressors are microplastics (Moore et al., 2001; Fischer et al., 2016; Barboza et al., 2018; Rios Mendoza and Balcer, 2020). Increasing evidence shows the intrinsic impacts of microplastic consumption on aquatic organisms (e.g., dietary changes and metabolic stress: Watts et al., 2015; oxidative damage: Barboza et al., 2018) and the potential toxicological consequences associated with sorption of toxic chemicals (reviewed in Wang et al., 2018). In fact, microplastics are excellent transport vectors of persistent organic pollutants (POP's, e.g. PAHs, PCBs, PBDEs; Rochman et al., 2013; Wagner et al., 2014), toxic metals (Wang et al., 2018; Luís et al., 2015) and pharmaceutical residues (such as antibiotics, Wang et al., 2018; Li et al., 2018), all of which are common in freshwater bodies.

Other pollutants, such as organophosphates, are poorly studied in relation to microplastics, as they are less persistent in the environment than POP's (Walker, 2014). The most used organophosphate is the herbicide glyphosate (*N*-(phosphonomethyl)glycine), commonly found in Roundup. Glyphosate-based herbicides can reduce species richness in aquatic environments by up to 22% and cause reduced abundance within taxa of over 70% (Folmar et al., 1979; Rzymski et al., 2013). Glyphosate toxicity has also been shown in freshwater fish species (e.g., Java medaka: Yusof et al., 2014; zebrafish: Uren Webster et al., 2014; Sulukan et al., 2017), which is particularly concerning as freshwater fish are facing an extinction crisis (Dudgeon et al., 2006; Adams et al., 2014). Our understanding of the interactions between microplastics and glyphosate-based herbicides is extremely limited. While microplastics can modify the toxicity and bioavailability of glyphosate formulations towards invertebrates, reducing their survival (Yang et al., 2019; Zocchi and Sommaruga, 2019), to our knowledge, no one has investigated the interactions between microplastic and glyphosate formulations on vertebrate health. Under multi-stressor scenarios environmental contaminants can interact to have additive (sum of individual a + b stressor effects) or interactive effects (synergistic - greater than summed effect of individual a+b stressor; antagonistic - less than summed effect of a+b stressor; and reversal - opposite to summed individual a+b stressor effects) (reviewed by Crain et al., 2008 and Piggott et al., 2015).

Multi-stressors are likely to have a particularly negative impact on vertebrate immunity and, potentially, disease tolerance (Martin et al., 2010; Becker et al., 2020). For fish, microplastic exposure can impact immune responses (e.g., increasing neutrophil trap release, Greven et al., 2016; oxidative stress in leucocytes, Espinosa et al., 2018; immune gene upregulation, Chen et al., 2020). How this translates into changes to disease outcomes remains unknown. For fish exposed to glyphosate herbicides, lymphocyte apoptosis, reduced phagocytic activity and increased disease susceptibility has been demonstrated (Kreutz et al., 2010; Wang et al., 2020). Here, using a multi-stressor approach, we investigate for the first time how microplastic (sterile virgin polypropylene) and glyphosate-based herbicide (from Roundup) exposure impacts vertebrate host-pathogen interactions. The host is the established ecological and parasitological model, the three-spined stickleback (*Gasterosteus aculeatus*), and the pathogen is a common helminth ectoparasite, *Gyrodactylus gasterostei*. These monogeneans on wild stickleback populations are widely studied (reviewed by Stewart et al., 2017) and are known to influence fish behaviour including reduced shoaling tendencies (Rahn et al., 2015) and foraging efficiency (Anaya-Rojas et al., 2016). They have a direct transmission lifecycle via host-to-host contact and proliferate *in situ* on the host's skin and fins (Stewart et al., 2017). Typically for this infection, fish either achieve a disease-free status naturally or pathogen numbers increase till host mortality occurs and the duration of infection is hugely variable (Bakke et al., 2007). Here, to ensure that we report on natural infections that are ecologically relevant, we allowed infection trajectories to continue until all hosts either cleared infections or mortality occurred. Ultimately, we aim to understand the individual and interactive effects of two global contaminants on host-pathogen interactions.

2. Materials and methods

2.1. Fish collection, maintenance and pathogen detection

Wild adult sticklebacks (*Gasterosteus aculeatus*, n = 300) were collected by hand netting from a series of interconnected managed ponds at St Fagans National Museum of History, Cardiff, UK (51.488–3.270) in February 2020. The fish were transported to Cardiff University and acclimated by immediately isolating fish in 1L containers (at a density of 1 fish per 1L container) for 2 days at 14 ± 0.5 °C, with a 12 h light: 12 h dark cycle. An initial screening for *Gyrodactylus* spp. was then conducted by anesthetising individual fish using 0.02% tricaine methanesulfonate (MS-222), where standard length was measured using callipers (size range- 22–88.2 mm), and the presence or absence of gyrodactylids confirmed by placing the fish in dechlorinated water in a glass petri dish under a dissection microscope illuminated with a fibre optic cold light source (King and Cable, 2007; Stewart et al., 2017). Gyrodactylid prevalence was 58% on screened fish (i.e., 174 fish naturally infected with gyrodactylids). A sub-sample of worms was wet mounted on slides with a coverslip and viewed at x100 and x400 and confirmed as *G. gasterostei* according to the morphological description of Harris (1985).

Fish obtained from the managed ponds may not have been microplastic free prior to commencing the experiment. To obtain hosts that were *G. gasterostei* free, 126 fish were gyrodactylid free upon arrival from St. Fagans, Cardiff, and 7 additional fish with 2 worms each had their worms manually removed with watchmaker forceps (Stewart et al., 2017), to obtain enough fish for the uninfected stressor treatments. The pathogen free fish served as infection controls and to survey mortality in response to multi-stressors. Control fish (isolated as single fish in 1L containers) were confirmed parasite free after three consecutive screens (Schelkle et al., 2009) conducted over seven days. Any hosts with other parasites or visible signs of pathogenesis (e.g., clamped fins, distended abdominal cavity, external cysts/growths; Stewart et al., 2017) were excluded from the subsequent experiment. Fish with gyrodactylids were distributed to infected treatment categories according to pathogen number, such that there was no significant difference in starting point pathogen number (χ^2 = 2.8, d.f. = 3, p = 0.4) between all infection

treatment (mean starting pathogen intensity for all infected treatments = 2 worms, range 1–6).

After screening, all fish (including uninfected controls) remained in individual 1L containers to acclimatise for a further 4 days before exposure to treatments. Throughout the experiment the fish were maintained in these containers, with a 100% water change performed every other day with dechlorinated water (14 ± 0.5 °C) and were fed daily a diet of AQUARIAN® flake food (4% bodyweight).

2.2. Microplastic and glyphosate preparation

To avoid potential confounding effects of eco-toxins, we used sterile virgin polypropylene pellets (Sigma-Aldrich, ground into microplastics using pestle and mortar). All equipment utilised for microplastic grinding and sieving was rinsed with acetone followed by ethanol to avoid procedural contamination, and all preparation of diets conducted under a fume hood. Immediately before grinding, pellets were placed in cryogenic vials (STAR LAB Ltd) and subsequently dipped in liquid nitrogen using stainless-steel forceps (Rochman et al., 2013). After grinding, plastics were sieved in stainless-steel metal sieves with 0.3 mm aperture to collect fragments. From this stock, 0.05 mg of microplastics were weighed out and applied to each 1L container. Roundup with a glyphosate concentration of 7.2 gL^{-1} was used, with 500 μL pipetted into each litre of water to give a concentration of 3.6 mgL^{-1}. The concentration of microplastics represents levels found in certain freshwater sediments and rivers near urban settlements (Fischer et al., 2016). Roundup levels applied correspond to pollution events observed in freshwater systems (Skeff et al., 2015). To ensure that fish were consuming microplastic, a preliminary feeding and behavioural trial on n = 20 sticklebacks lasting 1 week was conducted. Here, the same concentration of microplastic was administered every alternate day to half the fish (0.05 mg L^{-1}). Controls were fed only flake food. A researcher clearly observed sticklebacks consuming the microplastic every alternate day during feeding and microscopic faecal examination conducted every 24 h (from the commencement of feeding) detected their presence (in the microplastic group only) for the duration of the trial.

2.3. Exposure to multiple stressors and disease surveillance

Experimental fish were divided into eight treatment categories and four stressor groups, all fed the same volume of flake food but with exposure to different stressors: 1) a stressor control group without microplastic or Roundup exposure; 2) microplastic exposure with no Roundup but with the same amount of flake food to ensure nutrition was not a confounding variable; 3) Roundup exposure with no microplastic addition; and 4) the combined stressors of microplastic and Roundup exposure. From each of these stressor groups, approx. 56% of hosts (i.e., 167/300 fish) were infected with naturally acquired pathogens and served as the basis on which impacts of individual and combined abiotic stressors on host-pathogen dynamics were assessed (see Fig. 1). Stressor treatments were re-applied every other day in conjunction with water changes, and in combination with host screening this lasted a total of 72 days. Infected hosts were screened every fourth day until all lost their infections, and the number of pathogens present on the fish was recorded at each screening.

2.4.1. Animal ethics statement

All work was approved by the Cardiff University Animal Ethics Committee and conducted under UK Home Office licence PPL 303424.

2.5. Statistical analysis

All statistical analyses were conducted within R Studio Version 1.3.1073. The relationship between the biological response measured (pathogen metrics and host mortality) and the individual and combined abiotic stressors was investigated using Generalised Linear Model (GLM) analysis. A Kaplan-Meier survival analysis could not be conducted as the time of death for all hosts could not be determined. All mortality counts, however, were recorded and therefore a GLM with a Poisson error family and log link function was utilised to analyse the association between percentage mortality (i.e., percentage fish death) and treatment type respectively, where percentage of fish deaths was a response variable. For illustrating mortality data, the control fish (uninfected and not exposed to any pollutants) were used as a baseline (zeroed for visualisation purposes-see Fig. 2) in the GLM. Furthermore, to visualise the

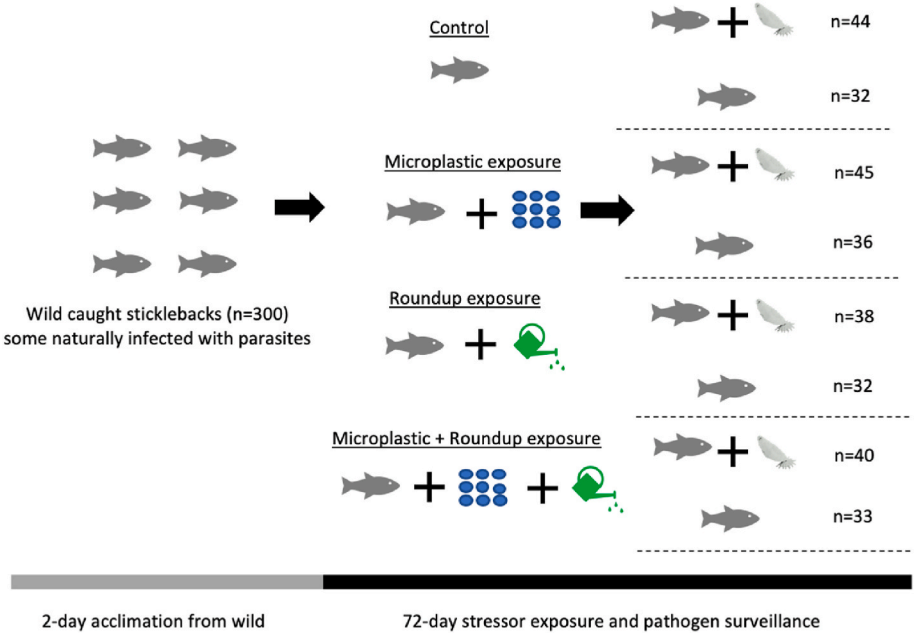

Fig. 1. Experimental design showing how wild caught sticklebacks (*Gasterosteus aculeatus*) were exposed to individual and combined abiotic stressors. Each treatment group was subdivided into naturally infected and uninfected cohorts with *Gyrodactylus gasterostei* monitoring occurring every fourth day, while the uninfected fish were sham handled in the same manner.

multi-stressor effects (i.e., interactive as opposed to additive expected) on peak pathogen burden and peak day, data were normalised relative to controls by zeroing the control fish's peak pathogen burden and peak day and calculating increases or decreases for each response variable (i.e., anything above zero being an increase and anything below being a decrease). Under multi-stressor scenarios we would expect additive interactions to equal the sum of the individual stressor effects and a deviance from this additive expected would indicate, under multi-stressor scenario, an interactive effect (see Crain et al., 2008 for detailed review).

We used the following pathogen metrics as response variables in the GLM analysis: peak pathogen burden, area under curve (AUC), peak pathogen day and time spent infected. For all GLM models analysing pathogen metrics, independent variables were controls, individual stressor treatment (microplastic + Roundup), combined stressor treatment (microplastic:Roundup) and fish standard length. The combined treatment allowed us to distinguish between interactive and additive expected effects (additive expected plotted in Figs. 2–4). When analysing all pathogen metrics, control infected fish (not exposed to any pollutants) were used as the baseline in our GLM's for comparisons. For obtaining AUC values we integrated under the data for each treatment utilising the trapezoid rule (White, 2011). For analysing AUC, we had to log transform our raw data as no error family produced normalised residuals for the GLM analysis. Post transformation, we used a GLM with a gaussian error family and identity link function for AUC values. For analysing peak day and time spent infected we utilised a GLM with an inverse gaussian error family and square root link function. For peak pathogen burden, we utilised a GLM with a negative binomial error family and log link function within the 'MASS' package in R software. Standard length was a non-significant independent variable for our GLM models when analysing peak pathogen burden (Std. Error = 0.01, Z = −1.31, p = 0.08), peak day (Std. Error = 0.01, T value = 1.45, p = 0.14) and AUC (Std. Error = 0.005, T value = 0.26, p = 0.78) and therefore was removed from our models to ensure model refinement (Thomas et al., 2017).

When analysing peak pathogen burdens, we utilised the odds coefficients and extracted the incident rate ratio (exponential of the odds coefficient) from the GLM to predict the direction (increase or decrease) and extent of change in pathogen numbers. To determine if there was a survivorship bias on peak pathogen burdens, whereby the selective removal of hosts due to mortality was affecting pathogen loads, we analysed the association between host mortality and peak pathogen burdens. To this effect we used a GLM with a negative binomial error family, chosen based on the lowest theta dispersion parameter with a log link function with individual and combined stressor treatment and mortality status of fish (dead/alive) treated as independent variables. For GLM's using count data (i.e., peak pathogen burden and mortality count) we selected between Poisson and negative binomial error families based on the lowest AIC values (Thomas et al., 2017) and the same procedure was implemented for continuous data (peak day, time under infection and AUC).

3. Results

In this study, the baseline mortality level was that of control uninfected fish not exposed to any pollutants (11/32 fish-34% mortality), which is expected for adult sticklebacks (Barber, 2013). For the GLM analysing percentage mortality of hosts, the null deviance was $6.0251e^{+01}$ on 7 degrees of freedom and the residual deviance being $1.1102e^{-14}$ on 0 degrees of freedom. As a single stressor, Roundup had no significant effect on mortality of uninfected fish compared with baseline levels, with the Roundup exposed group having the same proportion of deaths as control uninfected fish (GLM: Std. Error = $2.425e^{-01}$, Z = 0, p = 1). For uninfected fish exposed to microplastic interestingly, mortality rates were much lower than control uninfected fish (GLM: Std. Error = $2.864e^{-01}$, Z = −2.03, p = 0.04). When combined, microplastic and Roundup had a very clear interactive reversal effect leading to significantly increased mortality of uninfected fish (GLM: Std. Error = $2.076e^{-01}$, Z = 3.68, p = 0.0002, Fig. 2). For infected fish, the individual pollutants had contrasting effects on host mortality with microplastic having no significant effect (GLM: Std. Error = $2.666e^{-01}$, Z = −1.3, p = 0.19) and Roundup had a significant effect (GLM: Std. Error = $2.182e^{-01}$, Z = 2.2, p = 0.02, Fig. 2). The combined stressors had a moderate interactive effect, specifically synergistic, as the observed impact on mortality was slightly greater than the additive expected (i.e., +11 dead fish above the control uninfected treatment which is zeroed for illustrating multi-stressor effects; GLM: Std. Error = $2.197e^{-01}$, Z = 2.02, p = 0.04, Fig. 2).

Regarding analysis on pathogen metrics, infection trajectories were monitored until either hosts cleared their infections naturally or host mortality occurred and the duration of infections for this study ranged from 4 to 72 days. However, fish exposed to microplastic as a single stressor maintained their infections for significantly longer than control infected fish not exposed to any pollutants ($p < 0.001$, see Table 1 and Fig. 3). In contrast, Roundup alone significantly reduced peak pathogen burdens, AUC and time spent infected and peak pathogen day (63% reduction in overall pathogen burden, GLM $p < 0.05$ for all metrics; see Tables 1 and 2 and Figs. 3 and 4). The combination of microplastic and Roundup had an additive effect in significantly reducing peak burdens, AUC and time under infection compared to infected controls (54% reduction in overall pathogen burden, GLM $p < 0.001$ for all pathogen metrics; see Tables 1 and 2 and Figs. 3 and 4). However, for peak pathogen day the combined treatment had a non-significant synergistic effect (p = 0.22; see Fig. 4 and Table 1). It is also notable, though expected, that mortality of hosts had a marginally significant association with peak pathogen burdens (Std. Error = 0.14, Z = −1.92, p = 0.054).

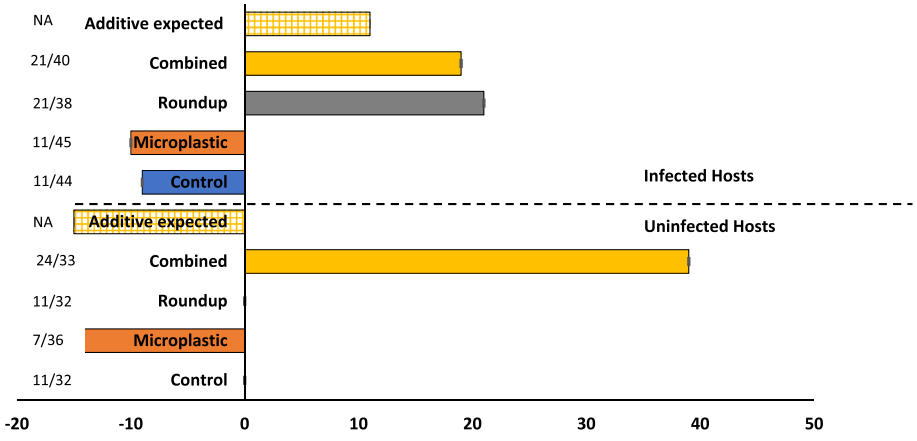

Fig. 2. Stacked columns depicting percentage of hosts (*Gasterosteus aculeatus*) dying in each treatment, including additive expected. Additive expected is the theoretical sum of the individual stressor effects. Here, combined treatment = microplastic + Roundup exposure. All infected treatments are shown in the top panel and uninfected treatments in the bottom panel. Control treatment (fish not infected or exposed to any stressor) serves as a baseline against which all other treatment mortalities are analysed in the GLM (see methodology above). Note, mortality numbers in Roundup exposure treatments were identical to those in controls. Also included to the left of each treatment plot is the number of deaths/total number of fish. Standard error bars are shown but are all <0.08.

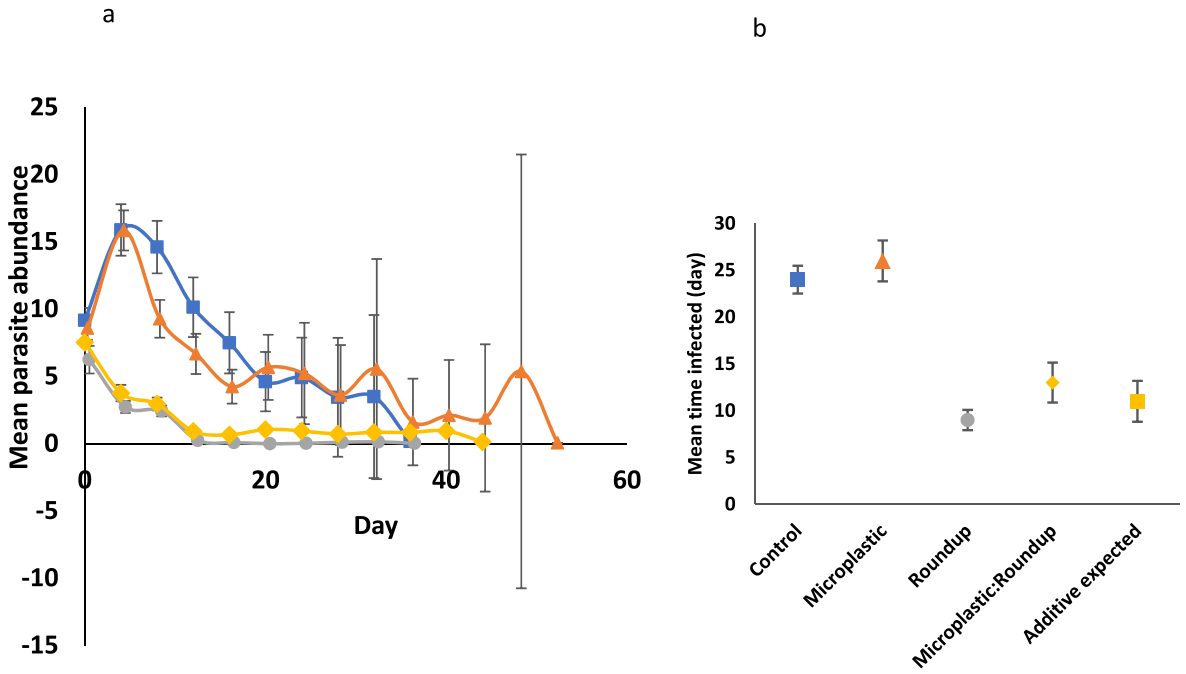

Fig. 3. (**a**) Smoother curve of observed mean parasite (*Gyrodactylus gasterostei*) abundance for individual and combined stressors and (**b**) mean time hosts were infected from each treatment category including the additive expected (summed effect of individual stressors microplastic and Roundup). Shown here, standard error bars with negative y-axis for observed mean parasite abundance to accommodate large error bars, due to increased host mortality and parasite clearance as infections progressed.

4. Discussion

The Anthropocene has been scarred by pollution, with freshwaters now recognised as the most affected ecosystem on the planet (Dudgeon et al., 2006; Persson et al., 2013; Collen et al., 2014). All freshwaters are now exposed to a plethora of pollutants (Crain et al., 2008; Piggott et al., 2015; Birk et al., 2020) and investigating the potential interactive impacts of these multiple stressors on organism health is essential to provide a realistic assessment of animal welfare. Despite this, few studies have investigated how multiple stressors impact host-pathogen dynamics. Here, using a multi-stressor approach, we reveal the impacts of two prevalent pollutants, microplastic and the glyphosate formulation Roundup, on vertebrate infectious disease and mortality.

When analysing mortality levels in the current study, for infected fish there was an increased number of host deaths when exposed to Roundup (55%) and a synergistic effect on mortality of the combined treatment (microplastic and Roundup, 53%). More concerningly, for uninfected fish, the combined stressors led to a very clear reversal effect on mortality whereby 73% of fish died over the 72-day exposure period. The interactive effects of microplastic and Roundup suggests microplastic can chemically interact with Roundup, thereby modifying toxicity. This is not unexpected considering microplastics are excellent vectors for chemical contaminants (reviewed in Wang et al., 2018). Though insignificant, 20% fewer deaths occurred within the infected controls compared to the uninfected controls, which was unusual. However, while control uninfected fish were gyrodactylid free, like all the fish in this study they may have acquired bacterial or viral infections prior to arrival in the lab.

Thus far, other invertebrate studies have revealed that when glyphosate formulations are combined with polyethylene terephthalate and polyamide fibres, the toxicity of glyphosate is amplified, with increased mortality seen in *Daphnia magna* (see Zocchi and Sommaruga, 2019). However, the mechanism behind the interactive effects on elevated mortality remains unknown. The buffering effect of infection on mortality levels seen in our combined stressor treatments was not, however, seen among infected fish exposed to just Roundup as this increased mortality levels by 21% compared with baseline levels, indicating that Roundup was reducing host tolerance to infections. In other studies that investigated glyphosate toxicity, induced genotoxic damage was seen in fish erythrocytes and gill cells (Shiogiri et al., 2012), and glyphosate also caused ionic imbalance, stress responses and liver histopathologies (Langiano and Martinez, 2008). Similarly, microplastic consumption alone can induce stress responses in fish (Tang et al., 2018), as well as hepatic lesions and necrosis (Rochman et al., 2013) and for fish larvae and juveniles, elevated mortality (Naidoo and Glassom, 2019; Pannetier et al., 2020). In our current study, microplastic consumption was not associated with any significant impacts on mortality for adult fish regardless of infection status, consistent with other investigations suggesting that once fish reach maturity, microplastic consumption alone does not have a pronounced effect on growth or survival (Oliveira et al., 2013; Rochman et al., 2017), and this includes three-spined sticklebacks used for this study (Environment Agency et al., 2015; Bunge et al., 2021), though longer term exposure of microplastics across life histories may reveal measurable effects on development and growth.

When analysing pathogen metrics, the current study revealed that individually, microplastic and Roundup had contrasting outcomes for infections. Microplastic consumption significantly increased the amount of time fish remained infected while also marginally increasing peak pathogen burdens compared with control infected fish (i.e., not exposed to any pollutants). Microplastics have already been shown to negatively impact immune responses in freshwater invertebrates and fish (e.g., Chinese mitten crabs, Liu et al., 2019; Zebrafish, Limonta et al., 2019; Marine medaka, Chen et al., 2020) but to our knowledge this laboratory study is the first example of microplastic impacting infection dynamics. With pathogens influencing the structure of food chains by influencing host life history traits (e.g., growth, Soler et al., 2003; longevity, Libert et al., 2006), changes to host-pathogen interactions may have detrimental consequences for the survival of populations, particularly if infections are persisting beyond normal ranges. Compared to microplastic exposure, Roundup significantly decreased host pathogen burdens and infection duration. Increased mucous production was observed in these Roundup exposed fish (personal observations), providing a possible

Table 1
GLM analysis of individual and combined stressors, microplastic and Roundup, on peak pathogen burdens (*Gyrodactylus gasterostei*), area under curve (AUC), time under infections of hosts (*Gasterosteus aculeatus*) and peak pathogen day. Infected controls (no microplastic or Roundup exposure-not shown) are the reference point against which all other treatments are compared. Model formula on top with error family below. Significant explanatory variables are highlighted by asterisks *. Est = Estimates; Std. Error = Standard Error.

GLM: individual and combined stressor impacts on peak pathogen burden	Est.	Std. Error	Z value	P value
(Intercept)	2.89	0.36	7.98	<0.001
Microplastic	0.11	0.18	0.63	0.52
Roundup	−0.97	0.19	−5.03	<0.001*
Microplastic:Roundup	−0.77	0.18	−4.08	<0.001*
Error family = negative binomial				
Null deviance = 225.26 on 166 degrees of freedom				
Residual deviance = 178.69 on 163 degrees of freedom				
GLM: individual and combined stressor impacts on AUC			T value	
(Intercept)	2.12	0.07	28.28	<0.001
Microplastic	0.07	0.10	0.67	0.50
Roundup	−0.86	0.11	−7.59	<0.001*
Microplastic:Roundup	−0.68	0.11	−6.12	<0.001*
Error family = gaussian				
Null deviance = 69.253 on 164 degrees of freedom				
Residual deviance = 41.864 on 161 degrees of freedom				
GLM: individual and combined stressor impacts on time under infection				
(intercept)	3.74	0.14	26.36	<0.001*
Microplastic	0.33	0.11	2.90	<0.01*
Roundup	−2.03	0.08	−25.01	<0.001*
Microplastic:Roundup	−1.28	0.08	−14.3	<0.001*
Standard length	0.03	0.003	9.3	<0.001*
Error family = inverse gaussian				
Null deviance = 133.910 on 3182 degrees of freedom				
Residual deviance = 95.691 on 3178 degrees of freedom				
GLM: individual and combined stressor impacts on peak pathogen day				
(intercept)	2.84	0.34	8.21	<0.001*
Microplastic	−0.04	0.48	−0.09	0.92
Roundup	−1.20	0.36	−3.27	<0.01*
Microplastic:Roundup	−0.52	0.42	−1.23	0.22
Null deviance = 58.448 on 166 degrees of freedom				
Residual deviance = 52.865 on 163 degrees of freedom				
Error family = inverse gaussian				

Table 2
Incident rate ratio of individual and combined abiotic stressors with direction of observed change and the percentage change in pathogen (*Gyrodactylus gasterostei*) burden compared with control infections.

Treatment	Incident rate ratio	Direction of change	% Change in pathogen burden
Microplastic	1.12	+	12
Roundup	0.37	–	63
Microplastic: Roundup	0.46	–	54

mechanism for the impact on parasites, as fish mucous contains anti-microbial peptides such as pro-inflammatory cytokines that are implicated in killing gyrodactylids (Lindenstrøm et al., 2006). Indeed, it is notable that glyphosate formulations have elevated the expression of inflammatory factors in Carp (Wang et al., 2020), and therefore the increased mucous production seen in the current study paired with results from Wang et al. (2020) does suggest that an inflammatory response may be implicated in infected fish clearing their ectoparasitic burdens. However, this parasite clearance of ectoparasites may be a double-edged sword as studies have also shown that glyphosate formulations at sublethal concentrations (10% of the LC_{50}-0.73 mgL^{-1}) decreases phagocytic index that is associated with decreased resistance to *Aeromonas hydrophila* infections (Kreutz et al., 2010). Of course, while the inflammatory immune response seems a plausible explanation, there is a possibility of direct mortality of ectoparasites from Roundup exposure as this herbicide is toxic to many aquatic invertebrates (e.g., Folmar et al., 1979; Gaupp-Berghausen et al., 2015) and may also impact the fish skin microbiome having knock on consequences for immunity (e.g., Friberg et al., 2019).

When microplastic was combined with Roundup, it displayed an additive effect leading to parasite clearance (as seen in decreased peak burdens, peak days, and time under infection) at a slightly reduced rate when compared to Roundup alone. If Roundup toxicity was responsible for parasite clearance (as opposed to an inflammatory immune response), the effect of Roundup alone was so strong in reducing pathogen burdens that this was not offset by microplastic's potential to interact with the herbicide, as was evident in the multistressor interaction that impacted fish mortality. Considering glyphosate formulations comprise the most commonly used herbicides globally and are still marketed as ecologically safe, our results support the mounting evidence that this is not the case (Folmar et al., 1979; Gaupp-Berghausen et al., 2015).

5. Conclusion

Our study has shown how microplastic and a commercial glyphosate-based herbicide, Roundup, individually and as combined stressors, impact host-pathogen interactions and mortality for wild fish populations. We reveal that for uninfected fish, microplastic and Roundup had contrasting effects on mortality, with microplastic reducing and Roundup increasing mortality in relation to control fish not exposed to any stressors. However, when the two stressors were combined, they interacted to cause mass mortality (73%) of uninfected fish. For infected hosts, while microplastic had no significant impact on mortality compared with baseline levels, Roundup exposure did see an increase in the number of fish dying indicating its potential to reduce infection tolerance. The two stressors, when combined, also had an interactive effect in increasing mortality levels of infected fish from baseline levels. When analysing infection dynamics, microplastic consumption significantly increased persistence of infections and marginally increased overall pathogen burden, while Roundup significantly reduced

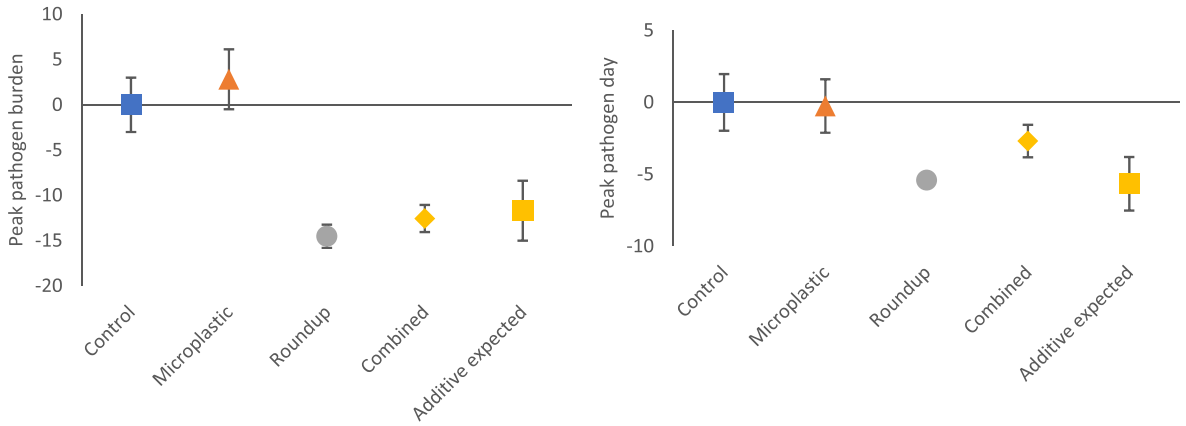

Fig. 4. Wild caught *Gasterosteus aculeatus* naturally infected with *Gyrodactylus gasterostei.* Shown here, the individual abiotic stressors microplastic and Roundup, the combined stressors and the additive expected affect on (**A**) predicted peak pathogen burdens and (**B**) predicted peak day of infection derived from GLM models (see methodology above). Data is normalised relative to control conditions (dotted line = 0 mg L^{-1} microplastics + 0 mg L^{-1} Roundup). Additive expected is the summed effect of the individual stressors. Predicted standard error bars shown derived from the GLM.

pathogen burden and infection persistence. Furthermore, the two pollutants had an additive effect in reducing pathogen burdens for infected fish compared to controls, but at a slightly reduced rate than Roundup alone, suggesting that for infected hosts, Roundup was a more potent acute stressor than microplastic with regards to impacts on mortality and pathogen dynamics. With reports indicating freshwater fish are facing higher rates of extinction than any other group of vertebrates (Magurran and Phillip, 2001; Adams et al., 2014; Dias et al., 2017), this study provides novel welfare assessments for two global pollutants.

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Authorship contribution statement

NM, AD-J and JC designed and wrote up the study. The work was conducted and analysed by NM and AD-J. BG contributed to data collection. All authors approved the final manuscript version.

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.

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