Microplastic vector effects: are fish at risk when exposed via the trophic chain?

Agathe Bour[1*], Joachim Sturve[1], Johan Höjesjö[1], Bethanie Carney Almroth[1]

[1] Department of Biological and Environmental Sciences, University of Gothenburg, Medicinaregatan 18A, Göteborg, Sweden
[*] Corresponding author: agathe.bour@bioenv.gu.se

Abstract

In aquatic organisms, trophic transfer is an important exposure route for microplastics (MPs). Despite their relevance, effect studies on fish exposed via trophic chains are currently very scarce. MPs are known to contain many chemicals that could be transferred to organisms and induce deleterious effects. However, there is currently no consensus on whether MPs represent a significant exposure pathway to chemicals in contaminated habitats. Here, we exposed three-spined stickleback (*Gasterosteus aculeatus*) to polyethylene MPs via prey ingestion, in a long-term experiment. MPs were either pristine or spiked with chlorpyrifos (CPF), and a CPF control was included to compare vector effects of MPs and natural prey. No effect was observed in fish exposed to pristine MPs. CPF accumulation was observed in fish exposed to CPF-spiked MPs (MP-CPF), confirming the vector potential of MPs. However, CPF accumulation was more important in fish exposed to CPF via prey. In fish exposed to MP-CPF, we observed significant AChE inhibition and hyperactivity, which could result in increased vulnerability to predation. CPF organ distribution differed between groups, suggesting that chemical exposure via MPs could alter organ distribution of chemicals. This can result in a change in the organs most at risk, likely increasing intestine exposure.

Key words: ecotoxicity; chlorpyrifos; behavior; uptake; stickleback

1. Introduction

Increasing numbers of field and laboratory studies have shown that most lower trophic level organisms are able to ingest microplastics (MPs)[1,2]. Ingestion of MP-contaminated prey by predator species is therefore very likely and trophic transfer has been identified as a relevant contamination pathway for MPs[3,4]. Despite their relevance, MP trophic transfer and its impacts on upper trophic level organisms are still poorly investigated, especially in studies involving fish species. MPs are known to contain many additives, such as plasticizers, flame retardants, stabilizers, surfactants and pigments[5], as well as environmental contaminants, such as PCB, PAHs and PBDEs[6]. A major concern is therefore their potential to act as vectors and to transfer chemicals to organisms[7]. However, modelling studies have questioned this hypothesis, arguing that the role of MPs in chemical transfer to organisms could be minor in a context of contaminated environments[8–10]. Especially, the ingestion of contaminated prey and/or natural particles could result in greater chemical uptake, compared to MPs. Despite the importance of comparing MPs to other natural vectors of contamination, such as natural prey, these alternative exposure pathways are still poorly investigated in MP ecotoxicity studies[6], especially those focusing on aquatic organisms.

To explore these major knowledge gaps, the present study aims to investigate i) the long-term effects of MPs on fish exposed via prey ingestion, ii) the potential of MPs to transfer chemicals (*i.e.* vector effect) to fish exposed via prey ingestion, iii) the consequences MPs' vector effect on organisms' performance, and iv) the relative importance of MPs' vector effect, in comparison with contaminated prey. For this purpose, we studied an experimental trophic chain comprising the three-spined stickleback (*Gasterosteus aculeatus*) as predator species, and brine shrimps (*Artemia sp.*) as natural prey. Three-spined stickleback naturally occupies a wide range of aquatic habitats[11]

and has been widely used in ecotoxicity and behavior studies[12–18]. For this study, stickleback individuals were fed during four weeks with brine shrimps previously exposed to pristine MPs, chemical-spiked MPs or chemical-contaminated water. To study the vector effect of MPs, we selected chlorpyrifos (CPF) as a model compound. CPF is a commonly used organophosphate pesticide that inhibits acetylcholine esterase (AChE)[19], an enzyme involved in neurotransmission, which can result in behavioral disorder in fish, as previously shown in *Gambusia*[20]. In addition to AChE inhibition, we assessed behavioral changes to study the effects of MPs and CPF on stickleback. Behavior is a sensitive endpoint likely to be affected at low contaminant concentrations, and behavior alterations can have consequences at the ecosystem level[21]. The study of behavioral endpoints is therefore very relevant in ecotoxicity studies. Here, we specially focused on feeding, locomotion, environment exploration and fish reaction to the introduction of a novel object.

2. Materials and Methods

2.1 Materials

Contaminants

Microplastics were purchased from Cospheric (Santa Barbara, USA; lot #120328-2--1). Opaque blue polyethylene microspheres were selected to ease quantification of exposure. According to the manufacturer, MPs are spherical (> 90% of particles), with a 27-32 µm diameter (> 90% of particles in the indicated size range) and a density of 1.00 g/cc. CPF was purchased from Sigma-Aldrich as powder (purity > 98%). High-purity methanol (99.89%) was purchased from VWR. All the chemicals used for the determination of AChE activity were of the highest purity available.

Fish

Three-spined sticklebacks (*Gasterosteus aculeatus*) were collected in a reference site with a hand-operated net, and immediately brought to the laboratory in aerated, thermally isolated boxes containing water from the sampling site. They were then acclimatized to and kept in artificial sea water for a month (13±1°C, 30 ‰, pH= 7.9) prior to start of the experiment. Fish were fed daily with red mosquito larvae. Continuous water flow and aeration ensured good water quality, and environmental enrichment was provided. Animal husbandry and experiments were conducted in compliance with ethical practices from the Swedish Board of Agriculture (Ethical permit number # 15986-2018).

2.2 Spiking of microplastics with chlorpyrifos

To spike MPs, a CPF solution was prepared in methanol at the concentration of 30 mg/ml. Glass material was used for the spiking to limit chemical sorption on the walls of the vials. CPF solution was added to MP batches of approx. 100 mg, to obtain a final ratio between MPs and solvent (methanol) of 1:1 (w:w)[22]. MPs were left in contact with CPF solution for 10 days, and gentle agitation was provided to ensure homogeneous spiking of MPs. To force the partitioning of CPF to the particles, milli-Q water was gradually added during the hole spiking process to eventually reach 90% of the final volume. After the last day of spiking, MPs were filtered using 10 µm nylon filters, rinsed with 50 ml of milli-Q water, and filtered again for recovery. Five filtration-recovery cycles were performed before storage (4°C).

Five MP batches were prepared in total, each batch being sufficient for one week of organism contamination. A new batch of spiked MPs was prepared before every week of organismal exposure and used immediately for the exposure. Therefore, spiked MPs were stored for a

maximum duration of one week. Analysis of spiked MPs subsamples shows an average CPF concentration of 17.7 ± 8.4 µg/mg (46.6% of CPF sorption, on average).

2.3 Experimental setup

Fish were exposed to pristine MPs (further referred to as "MPs"), CPF-spiked MPs ("MP-CPF") or CPF only ("CPF") via prey ingestion during four weeks. Exposure was performed by feeding fish with pre-exposed prey every second day, three times a week. Every contamination day, live adult brine shrimps (*Artemia spp.*; approx. 1 cm) were placed for 15 minutes in Eppendorf tubes (2 ml; 15 *Artemia* individuals per tube) belonging to one of the experimental conditions (*i.e.* control, MPs, MP-CPF or CPF). Eppendorf tubes were prepared as follow, before the addition of *Artemia*: artificial seawater (ASW) only (control condition), approx. 2 mg of MPs or MP-CPF in ASW followed by strong manual shaking (MP and MP-CPF conditions, respectively), or CPF solution prepared in ASW (100 mg/L, solvent <10% total volume; CPF condition). After exposure, *Artemia* individuals were rinsed twice in ASW and fed to the fish from the respective experimental conditions less than five minutes later.

Every experimental condition, including control, was performed in triplicates, each replicate (randomly allocated aquarium; 20L) comprising eight fish. Fish size range was 3.3 – 5.6 cm, with random allocation to exposure groups and no significant size differences between groups. Exposure was performed under controlled temperature (14°C) and light (12:12, light:dark cycle) conditions. Half of the water was renewed every second day and aeration was provided in every aquarium, to ensure good water quality. Fish were individualized during the feeding to ensure equal prey ingestion between fish, then placed back together in their respective aquaria. Outside exposure days, red mosquito larvae were provided *ad libitum* without individualization of the fish.

After two weeks of exposure, significant mortality was observed in fish from the CPF group. The exposure was therefore stopped for this group, behavior tests performed on both control and CPF groups, then fish from the CPF group were euthanized. For MPs, MP-CPF and control groups, behavior assessment started after four weeks of exposure and lasted for a week. Exposure via prey continued during that week to prevent depuration of the fish and differing contamination levels between the first and last trial days. Behavioral trials were performed during the mornings and feeding in the afternoons, to prevent stress related to fish handling. After the last behavior trial, fish were euthanized, measured, weighed and organs (gut, liver, gonads, muscle, gills and brain) were sampled for further analysis. Samples intended for chemical analysis (CPF quantification) were stored at -20°C; samples intended for biochemical analysis (enzymatic activity, protein content) were snap-frozen in liquid nitrogen and stored at -80°C.

2.4 Determination of microplastic ingestion and chlorpyrifos quantification

MPs selected for this study are blue beads that could easily be seen through *Artemia* cuticle under microscopic condition. Therefore, *Artemia* individuals (n = 47) were observed and photographed (Leica EZ4HD stereomicroscope with integrated HD camera) without prior sample preparation. Images were further analyzed with ImageJ® software to count particles. Fish intestines (n = 17) were digested overnight in 10% KOH at 50°C. Extracts were then filtered on 10µm nylon mesh, filters observed under the stereomicroscope and particles counted.

CPF was quantified in MPs and organism samples by UHPLC-MS, using CPF-(diethyl-d10) as internal standard. Extraction and instrumental analysis were performed at the Swedish environmental research institute (IVL). Additional information concerning chemical analysis can be found in supporting information (SI).

2.5 Toxicity assessment

Acetylcholinesterase (AChE) activity:

AChE activity was determined in fish liver and brain, following an adapted procedure of Ellman's method[23,24]. Protein content was determined according to Lowry's method[25].

Behavior assessment:

Feeding, locomotion, environment exploration and reaction to the introduction of a novel object were assessed. For the feeding trial, fish were individualized, allowed to acclimatize for 10 minutes then fed two frozen mosquito larvae. The whole trial was video recorded and recordings were visually analyzed to determine the time required for each fish to ingest both larvae.

Locomotion, environment exploration and reaction to a novel object were assessed during a second trial, following the procedure described by Thompson et al.[18]. Fish were individualized in containers comprising one shelter (piece of tile) each, and allowed to acclimatize for 10 minutes. Video recording started after the acclimation period. After ten minutes of recording, a novel object (bolt attached to a transparent fishing line) was gently introduced in the center of the container and the fish reaction was recorded for 10 more minutes. Locomotion (immobility, total distance travelled, average speed, average acceleration, maximum speed, maximum acceleration) were determined over the first ten minutes of recording, using idTracker software. Environment exploration and fish behavior following the introduction of a novel object were determined by visual analysis. Fish containers were virtually divided between the area close to the shelter (half container) and the distal part of the container, to determine the total time spent inside the shelter, close to the shelter and far from the shelter, during the first 10 minutes. After introduction of the novel object, the assessed endpoints were (i) fish immediate reaction (*i.e.* freezing: immediate

immobility and slight curving of the tail; escape: fast swimming on the opposite direction of the novel object; no specific reaction), (ii) delay in returning to normal behavior after the immediate reaction, (iii) delay in active observation of the novel object (*i.e.* fish facing the novel object), (iv) delay in approaching the novel object after active observation and (v) whether the fish actively touches the novel object or not.

2.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism 8.1.1 software. Group comparisons of qualitative data (*i.e.* immediate reaction to a novel object and touching it or not) were performed with Chi-square tests. For quantitative data, normal distribution and homoscedasticity of residuals were verified with Shapiro-Wilk's and Bartlett's tests, respectively. Mann-Whitney tests were performed to compare CPF group and control group assessed after two weeks of exposure. One-way ANOVA tests were performed to compare MPs, MP-CPF and control group assessed after four weeks of exposure, when both normal distribution and homoscedasticity were verified. In cases of non-normal distribution, Kruskal-Wallis tests on rank were performed instead. Dunn's test was performed to compare groups when significant differences were detected. Detailed data on the performed statistical analyses is presented in supplementary information (table S1). Levels of significance were set at $p < 0.05$. For behavior endpoints, trends were considered from $p < 0.1$.

3. Results

3.1 Trophic transfer of MPs and CPF

In Artemia*:*

Artemia individuals ingested on average 204 ± 13 MPs before being fed to the fish. Based on MP ingestion and CPF concentrations in different MP batches, the expected (theoretical) CPF concentration in *Artemia* is 62.1 ng/individual. CPF quantification in *Artemia* samples from the MP-CPF group shows measured concentration of 97.3 ng/mg, equivalent to 57.8 ng/individual. Measured concentration in *Artemia* from the CPF group is 405.5 ng/mg, equivalent to 293.2 ng/individual.

In fish:

Each fish was fed 51 *Artemia* in total, therefore ingesting theoretically 10 404 MPs in total (equivalent to 140 µg of MP). However, after four weeks of exposure MPs were found in only two fish (samples contained two and three particles, respectively).

CPF concentrations were measured in gonads, viscera (*i.e.* intestine, liver and gall bladder), body muscle, gills and brain (table 1). For fish from the MP-CPF group, samples were either pools of three fish found dead before the end of the exposure, or samples from single fish euthanized at the end of the exposure. Daily checks ensured that dead fish were removed from the water and stored in less than 18 hours. CPF concentrations were below the limit of detection (LOD; 0.194 ng/ml) for most samples of single fish, while CPF was detected in every sample of pooled fish. Values below LOD could be due to very limited uptake in euthanized fish and higher uptake in dead fish. But as it cannot be excluded that that this difference results from experimental procedure (fish pooled or not), both results are presented in table 1. For CPF, MPs and control groups, CPF was quantified on pooled fish.

Table 1: CPF concentrations measured in fish organs from different exposure groups. LOD: Limit of Detection (0.194 ng/ml).

	CPF (ng/mg)				
	Ctrl	MPs	MP-CPF[1]	MP-CPF[2]	CPF[3]
Gonads	< LOD	< LOD	0.21	0.05 ± 0.05	3.51 ± 0.21
Viscera	< LOD	< LOD	0.51	0.13 ± 0.13	1.05 ± 0.20
Muscle	< LOD	< LOD	0.14	0.03 ± 0.03	0.67 ± 0.06
Gills	< LOD	< LOD	0.07	0.02 ± 0.02	0.49 ± 0.10
Brain	< LOD	< LOD	0.27	0.07 ± 0.07	1.66 ± 0.09

[1]Values from pooled samples only. [2]Average (± SE) of values from pooled and non-pooled samples. [3]Average values (± SE).

CPF transfer to fish via *Artemia* ingestion, both with and without the inclusion of MPs, was quite low (figure 1), with values ranging from 0.2 to 2% of total CPF ingested, depending on exposure conditions and organs. The relative distribution of CPF in the internal organs differed between MP-CPF and CPF groups (figure 2). While the organs showing the highest concentrations were the viscera and gonads for the CPF group (33 and 30%, respectively), most CPF was detected in the viscera (70%) in fish from the MP-CPF group. Compared to the CPF group, gonad samples from the MP-CPF group showed low percentage of CPF uptake (9%).

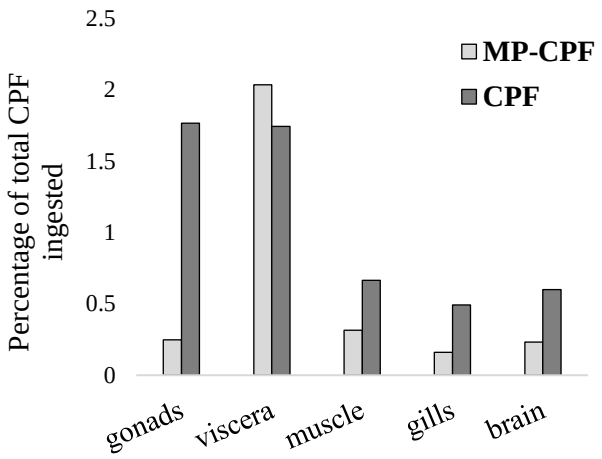

Figure 1: CPF transfer to fish, expressed as the ratio (x100) between the total amount of CPF measured in organs (ng) and the total amount of CPF ingested via *Artemia* (ng; theoretical values).

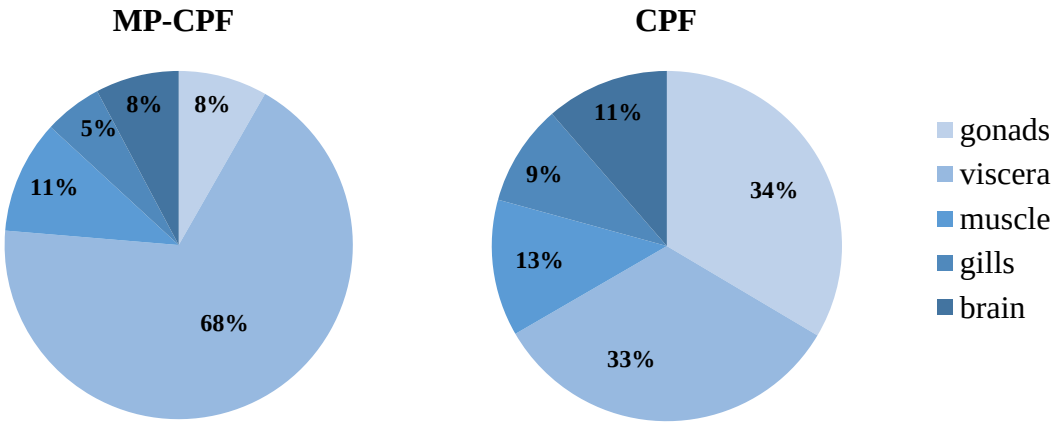

Figure 2: CPF relative distribution in different fish organs, expressed as the percentage of total CPF in fish.

3.2 Effects on fish

Mortality:

Significant mortality (58%) was observed in fish from the CPF group after two weeks of exposure. The exposure was therefore stopped for this condition after 16 days. The mortality recorded over the four weeks of exposure for control, MPs and MP-CPF groups (5, 7 and 8 fish, respectively) was attributed to natural sensitivity of the species and handling stress, and considered non-significant.

AChE activity:

Brain AChE inhibition was observed in fish exposed to MPs, MP-CPF and CPF (30.6%, 46.9% and 85.5% inhibition, respectively, compared to control). However, the difference compared to the control group was statistically significant only for fish exposed to CPF alone ($p < 0.01$). In liver, 10% and 55% inhibition were observed for the MP-CPF and CPF groups, respectively. An increase in liver AChE activity was observed in fish exposed to MPs (28.2% increase, compared to control). However, the differences observed between groups were not statistically significant ($p > 0.05$). AChE activities are presented in figure 3.

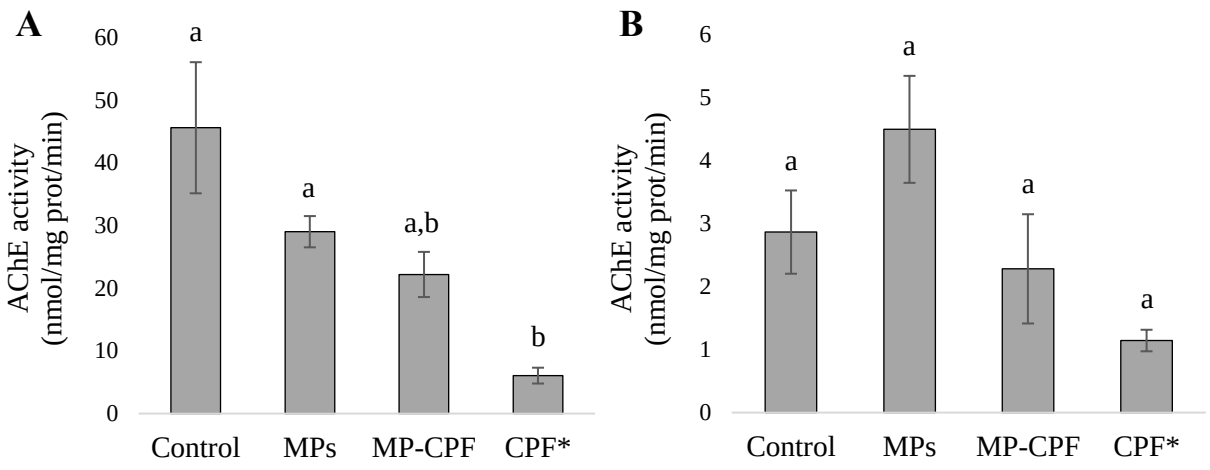

Figure 3: AChE activity (nmol/mg protein/min, mean values ± SE) in fish A) brain and B) liver. Different letters (a,b) indicate statistically different groups ($p < 0.05$). *Fish from the CPF group were euthanized and sampled after two weeks of exposure.

Behavior:

Behavior endpoints were grouped under four categories: feeding, locomotion, environment exploration and reaction to a novel object. Fish exposed to MPs did not present any behavioral changes compared to control fish. Fish exposed to MP-CPF exhibited slight changes in environment exploration and in their reaction to the introduction of a novel object. They spent less time in the shelter, compared to control fish ($\Delta t = -54$ %), and returned faster to a normal behavior after their first reaction following the introduction of the novel object ($\Delta t = -47$ %). Stronger behavioral changes were observed with fish exposed to CPF via prey, with changes in all four endpoint categories. CPF fish exhibited significantly longer feeding time and immobility ($\Delta t = +309$ % and + 97%, respectively), they spent less time in the shelter ($\Delta t = -73$ %) and active looking at and proximity with the novel object were delayed ($\Delta t = +138$ % and 205 %, respectively). These

results are summarized in table 2 and detailed results are presented in supporting information (table S2, figures S1 and S2).

Table 2: Summary of behavior assays results.

	MPs	MP-CPF	CPF
Feeding	no effect	no effect	slower feeding** (Δt = + 309 %)
Locomotion	no effect	no effect	longer immobility** (Δt = + 97 %)
Environment exploration	no effect	less time spent in shelter* (Δt = - 54 %)	less time spent in shelter** (Δt = - 73 %)
Reaction to novel object (NO)	no effect	faster return to normal behavior* (Δt = - 47 %)	delayed NO observation** (Δt = + 138 %) delayed approach of NO** (Δt = + 205 %)

*$p < 0.1$; **$p < 0.05$

4. Discussion

4.1 Experimental trophic chain

Trophic transfer of MPs has been identified as a most relevant contamination pathway for predators[1]. In the present study, the selected trophic chain allows to control MP ingestion by preys and therefore fish exposure to MPs. We observed a consistent number of MPs in *Artemia* individuals throughout the experiment (204 ± 13 MPs per individual on average) and CPF concentrations measured in *Artemia* from the MP-CPF group were very close to the expected concentrations. These results indicate that fish exposure to MPs and CPF was consistent throughout the experiment and validate the use of the present trophic chain as an appropriate method for controlled fish exposures. The absence of MPs in most fish samples is explained by the short particle retention time in three-spined stickleback (< 48h; data not shown): the last

contamination before fish were sampled was performed more than 48h and total egestion of MPs could be expected.

Chlorpyrifos is widely used in agriculture and in urban areas, and this compound and/or its metabolites are present in waters and sediments of streams, rivers, ponds, lakes and estuaries[19]. In the present study, the high concentrations used to spike MPs is not representative of environmental concentrations[26–28] but was chosen to compensate for potential loss of chemical during the spiking process[22]. For the same reasons, a high CPF concentration was used to contaminate *Artemia* (CPF group), as they were exposed in CPF solution for only 15 minutes. Pre-test were performed to ensure that these exposure conditions did not alter *Artemia* survival and swimming behavior (data not shown). Altered swimming could have indeed influenced fish predation and resulted in fewer prey ingested.

4.2 Chlorpyrifos uptake in fish and vector effect of microplastics

CPF uptake varies importantly between organs (table 1), with bioaccumulation factors ranging from 0.001 (gills) to 0.009 (gonads) for fish from the CPF group. A previous study showed much higher bioaccumulation of CPF in *Aphanius iberus* exposed via contaminated *Artemia*, with a bioaccumulation factor of 0.3[29]. These results are not contradictory since CPF accumulation is highly dependent on fish species: other studies have investigated CPF accumulation in fish exposed via water and observed values ranging from 0.004 to 380 ng/mg[20,30,31]. In their study, Varó et al. observed that after exposing fish to CPF via the trophic route, CPF was eliminated after one day of feeding with non-contaminated preys[29]. This quick elimination could explain that no CPF was detected in euthanized fish from the MP-CPF group, since their last feed with contaminated *Artemia* was provided at least four days before. Conversely, fish found dead received

their last feed less than 48h before, which was potentially too short for the fish to eliminate CPF enough so it would not be detected. The detection of CPF in fish from the MP-CPF group shows that MPs can act as a vector for organic contaminants when ingested via the trophic chain. As ingested CPF quantities were initially different between MP-CPF and CPF groups, CPF transfer was expressed as the percentage of ingested CPF (figure 1). These values show that except in viscera, CPF accumulation in fish was much lower in the MP-CPF group. This result shows that although MPs can as vector of contamination, contaminant transfer is limited compared to other exposure routes. The same phenomenon has been observed in most studies comparing contaminant uptake (PCBs, BFRs, PFCs, PBDEs, PAHs and organic contaminants) via MPs and other matrices[32–34], although one study showed higher contaminant transfer in fish when exposed via MPs, compared to spiked food[35].

CPF accumulation varies between organs in both exposure conditions (figure 1). Previous studies showed CPF distribution patterns in fish similar to what was observed here in fish from the CPF group, gonads, brain and viscera being the most exposed organs[30,31]. In fish exposed via MPs, most of the CPF was detected in the viscera. This is likely due to the presence of MPs in the intestine when organs were sampled, resulting in the quantification of CPF sorbed on MPs rather than actual uptake. However, the presence of CPF in other organs shows that some CPF desorbed from the MPs and was taken up in the organism. Batel et al. came to same conclusion after exposing zebrafish via the trophic route to benzo[a]pyrene (BaP) sorbed on MPs: some BaP was found in fish liver, showing its partial desorption from MPs[36].

In other organs, initial sorption of CPF on MPs not only decreases CPF uptake, but also changes CPF distribution among organs (figure 2). While gonads seem to be the most exposed organ in the CPF group, the relative CPF concentration in gonads decreases in the MP-CPF group

and reaches values below viscera and body muscle. This result suggests that when chemical exposure occurs via MPs, the organs most at risk can be different compared to water exposure, decreasing gonads exposure in the case of CPF and dramatically increasing intestine exposure.

4.3 Effect of contaminants

Although no effects were observed in fish exposed to pristine MPs, individual response distributions within groups show patterns differing from the control group, for the distance travelled, the average speed and average acceleration (supplementary information, figure S1). Therefore, it cannot be excluded that symmetrical distributions of values around means mask changes in fish behavior following MP exposure. Similarly to our results, studies assessing the ecotoxicity of PE MPs on fish exposed via trophic chains also reported no effects[37–39]. Moreover, most studies exposing fish via water showed either no effects[34,40–43] or mild effects, which include inhibition of AChE activity, impaired energy reserves, decreased swimming and predatory performances, changes in plasma biochemistry and histological changes in gills[44–48]. The adverse effects observed with pristine PE MPs in these studies could be due to the presence of monomers or additives[5] sorbed on MPs but that were not reported, either because no chemical analysis was performed or because concentrations were below limits of detection. Different hypotheses could explain the absence of effect following exposure via trophic chain, reported both in the scientific literature and in the present study. First, it could be explained by a faster elimination of MPs, directly related to gut retention time, while MPs could have a longer retention time when directly ingested or could get stuck in the gills when present in water. Another explanation could be that in trophic chain experiments, the additives potentially present on pristine MPs affect the preys but not the fish; the prey would therefore “protect” the predator species against the effects of MPs.

One hypothesis is that the prey partially metabolizes the contaminant, thereby limiting the toxicity for predators.

In contrast with fish from the pristine MPs group, strong effects were observed on fish from the CPF group. As most organophosphate pesticides, CFP is a neurotoxic that inhibits AChE activity. Fish metabolize CPF to multiple metabolites, including CPF-oxon, which is the most efficient AChE inhibitor among the activated forms of CPF[19]. It has been shown to be acutely toxic to fish, with 96h LC_{50} ranging from 300 to 650 µg/L[31]. The significant mortality observed here shows that CPF trophic transfer was important enough (up to 3.5 ng/mg; table 1) to induce acute toxicity in stickleback. In a previous study, authors reported no acute toxicity in Tilapia despite CPF accumulation values exceeding 100 ng/g[30], which highlights important differences in species sensitivity to CPF. Unsurprisingly, significant brain AChE inhibition (84% decrease in activity) was observed in fish from the CPF group (figure 3). This strong inhibition is likely to be a cause of the important behavior impairment observed[49]. Taken together, the behavior changes observed show a dramatic hypoactivity, in comparison to control fish. Slow feeding is a sign of decreased predatory performance that could result in population changes for fish and their major prey[50]. Increased immobility and time spent in the open field (the shelter being considered as a safe location) increase fish susceptibility to predation[51,52]. Finally, fish behavior following the introduction of a novel object is indicative of their interaction with their environment. Previous studies observed changes in inspection of a novel object by fish, including stickleback, following exposure to chemicals[15,53]. Here, the decreased curiosity, highlighted by the delay in observing and approaching the novel object, is likely to be a result of the severe hypoactivity in fish from the CPF group. These results show that CPF can severely impact stickleback behavior, with potential

consequences at the population level. The reported behavior results should be interpreted with caution, given the small number of individuals left in the CPF condition.

While strong effects were observed in the CPF group with a general decrease in movement patterns, suggesting hypoactivity, fish exposed to CPF via MPs (MP-CPF) exhibited altered behavior to a lower extent, and in the opposite direction. Indeed, their faster reaction following the introduction of a novel object, combined with decreased time spent in the shelter, suggests hyperactivity. CPF has previously been shown to induce hyper excitability in exposed fish, increasing their vulnerability to predation[54–56]. It has also been shown that different behavior alterations occur at different thresholds of AChE inhibition[56]. This explains the differences in behavioral responses between CPF and MP-CPF exposed fish, since different levels of AChE inhibition were observed between the two groups (figure 3). These results show that the quantities of CPF transferred from MPs to fish are high enough to induce behavior impairment, potentially resulting in increased exposure to predation and increased energy expenditure, which should be considered when evaluating consequences at the ecosystem level[21].

Acknowledgements:

This research was supported by the Swedish Research Council FORMAS (2016-00895). The authors are grateful to Dr. Georgios Giovanoulis (IVL) for his help with chemical characterization. We also acknowledge Dr. Tobias Lammel for his help with fish collection, and Magnus Lovén Wallerius for his help with setting up the video recording.

Supporting Information: Detail of the statistical tests performed and p-value results, detailed behavior assays results, distribution of individual behavioral responses of exposed fish, detailed methodology of CPF quantification.

References:

(1) Lusher, A. Microplastics in the Marine Environment: Distribution, Interactions and Effects. In *Marine Anthropogenic Litter*; Springer, Cham, 2015; pp 245–307. https://doi.org/10.1007/978-3-319-16510-3_10.

(2) Scherer, C.; Weber, A.; Lambert, S.; Wagner, M. Interactions of Microplastics with Freshwater Biota. In *Freshwater Microplastics : Emerging Environmental Contaminants?*; Wagner, M., Lambert, S., Eds.; The Handbook of Environmental Chemistry; Springer International Publishing: Cham, 2018; pp 153–180. https://doi.org/10.1007/978-3-319-61615-5_8.

(3) Farrell, P.; Nelson, K. Trophic Level Transfer of Microplastic: Mytilus Edulis (L.) to Carcinus Maenas (L.). *Environmental Pollution* **2013**, *177*, 1–3. https://doi.org/10.1016/j.envpol.2013.01.046.

(4) Nelms, S. E.; Galloway, T. S.; Godley, B. J.; Jarvis, D. S.; Lindeque, P. K. Investigating Microplastic Trophic Transfer in Marine Top Predators. *Environmental Pollution* **2018**. https://doi.org/10.1016/j.envpol.2018.02.016.

(5) Lambert, S.; Wagner, M. Microplastics Are Contaminants of Emerging Concern in Freshwater Environments: An Overview. In *Freshwater Microplastics : Emerging Environmental Contaminants?*; Wagner, M., Lambert, S., Eds.; The Handbook of Environmental Chemistry; Springer International Publishing: Cham, 2018; pp 1–23. https://doi.org/10.1007/978-3-319-61615-5_1.

(6) Koelmans, A. A. Modeling the Role of Microplastics in Bioaccumulation of Organic Chemicals to Marine Aquatic Organisms. A Critical Review. In *Marine Anthropogenic Litter*; Bergmann, M., Gutow, L., Klages, M., Eds.; Springer International Publishing, 2015; pp 309–324. https://doi.org/10.1007/978-3-319-16510-3_11.

(7) Rochman, C. M. The Role of Plastic Debris as Another Source of Hazardous Chemicals in Lower-Trophic Level Organisms. In *Hazardous Chemicals Associated with Plastics in the Marine Environment*; Takada, H., Karapanagioti, H. K., Eds.; The Handbook of

Environmental Chemistry; Springer International Publishing: Cham, 2019; pp 281–295. https://doi.org/10.1007/698_2016_17.

(8) Gouin, T.; Roche, N.; Lohmann, R.; Hodges, G. A Thermodynamic Approach for Assessing the Environmental Exposure of Chemicals Absorbed to Microplastic. *Environ. Sci. Technol.* **2011**, *45* (4), 1466–1472. https://doi.org/10.1021/es1032025.

(9) Koelmans, A. A.; Besseling, E.; Wegner, A.; Foekema, E. M. Plastic as a Carrier of POPs to Aquatic Organisms: A Model Analysis. *Environ. Sci. Technol.* **2013**, *47* (14), 7812–7820. https://doi.org/10.1021/es401169n.

(10) Teuten, E. L.; Rowland, S. J.; Galloway, T. S.; Thompson, R. C. Potential for Plastics to Transport Hydrophobic Contaminants. *Environ. Sci. Technol.* **2007**, *41* (22), 7759–7764. https://doi.org/10.1021/es071737s.

(11) Bell, M. A.; Foster, S. A. *The Evolutionary Biology of the Threespine Stickleback*; Oxford University Press: Oxford; New York, 1994.

(12) Dingemanse, N. J.; Wright, J.; Kazem, A. J. N.; Thomas, D. K.; Hickling, R.; Dawnay, N. Behavioural Syndromes Differ Predictably between 12 Populations of Three-Spined Stickleback. *Journal of Animal Ecology* **2007**, *76* (6), 1128–1138. https://doi.org/10.1111/j.1365-2656.2007.01284.x.

(13) Fürtbauer, I.; Pond, A.; Heistermann, M.; King, A. J. Personality, Plasticity and Predation: Linking Endocrine and Behavioural Reaction Norms in Stickleback Fish. *Functional Ecology* **2015**, *29* (7), 931–940. https://doi.org/10.1111/1365-2435.12400.

(14) Girvan, J. R.; Braithwaite, V. A. Population Differences in Spatial Learning in Three–Spined Sticklebacks. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **1998**, *265* (1399), 913–918. https://doi.org/10.1098/rspb.1998.0378.

(15) Jutfelt, F.; Bresolin de Souza, K.; Vuylsteke, A.; Sturve, J. Behavioural Disturbances in a Temperate Fish Exposed to Sustained High-CO2 Levels. *PLoS One* **2013**, *8* (6). https://doi.org/10.1371/journal.pone.0065825.

(16) Marchand, A.; Porcher, J.-M.; Turies, C.; Chadili, E.; Palluel, O.; Baudoin, P.; Betoulle, S.; Bado-Nilles, A. Evaluation of Chlorpyrifos Effects, Alone and Combined with Lipopolysaccharide Stress, on DNA Integrity and Immune Responses of the Three-Spined Stickleback, Gasterosteus Aculeatus. *Ecotoxicology and Environmental Safety* **2017**, *145*, 333–339. https://doi.org/10.1016/j.ecoenv.2017.07.025.

(17) Sturm, A.; Wogram, J.; Segner, H.; Liess, M. Different Sensitivity to Organophosphates of Acetylcholinesterase and Butyrylcholinesterase from Three-Spined Stickleback

(Gasterosteus Aculeatus): Application in Biomonitoring. *Environmental Toxicology and Chemistry* **2000**, *19* (6), 1607–1615. https://doi.org/10.1002/etc.5620190618.

(18) Thompson, R. R. J.; Paul, E. S.; Radford, A. N.; Purser, J.; Mendl, M. Routine Handling Methods Affect Behaviour of Three-Spined Sticklebacks in a Novel Test of Anxiety. *Behavioural Brain Research* **2016**, *306*, 26–35. https://doi.org/10.1016/j.bbr.2016.03.015.

(19) Ware, G. W. *Reviews of Environmental Contamination and Toxicology: Continuation of Residue Reviews*; Springer New York : Imprint : Springer: New York, NY, 1999.

(20) Rao, J.; Begum, G.; Pallela, R.; Usman, P.; Rao, R. Changes in Behavior and Brain Acetylcholinesterase Activity in Mosquito Fish, Gambusia Affinis in Response to the Sub-Lethal Exposure to Chlorpyrifos. *International Journal of Environmental Research and Public Health* **2005**, *2* (3), 478–483. https://doi.org/10.3390/ijerph2005030013.

(21) Galloway, T. S.; Cole, M.; Lewis, C. Interactions of Microplastic Debris throughout the Marine Ecosystem. *Nature Ecology & Evolution* **2017**, *1* (5). https://doi.org/10.1038/s41559-017-0116.

(22) Smedes, F.; Booij, K. Guidelines for Passive Sampling of Hydrophobic Contaminants in Water Using Silicone Rubber Samplers. *TIMES* **2012**, No. 52, 24.

(23) Ellman, G. L.; Courtney, K. D.; Andres, V.; Featherstone, R. M. A New and Rapid Colorimetric Determination of Acetylcholinesterase Activity. *Biochemical Pharmacology* **1961**, *7* (2), 88–95. https://doi.org/10.1016/0006-2952(61)90145-9.

(24) Sturve, J.; Scarlet, P.; Halling, M.; Kreuger, J.; Macia, A. Environmental Monitoring of Pesticide Exposure and Effects on Mangrove Aquatic Organisms of Mozambique. *Marine Environmental Research* **2016**, *121*, 9–19. https://doi.org/10.1016/j.marenvres.2016.05.005.

(25) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein Measurement with the Folin Phenol Reagent. *J. Biol. Chem.* **1951**, *193* (1), 265–275.

(26) Arain, M.; Brohi, K. M.; Channa, A.; Brohi, R. O. Z.; Mushtaque, S.; Kumar, K.; Samuee, A. Analysis of Chlorpyrifos Pesticide Residues in Surface Water, Ground Water and Vegetables through Gas Chromatography. *Journal of International Environmental Application and Science* **2018**, *13* (3), 167–173.

(27) F. Müller, J.; Duquesne, S.; Ng, J.; R. Shaw, G.; Krrishnamohan, K.; Manonmanii, K.; Hodge, M.; K. Eaglesham, G. Pesticides in Sediments From Queensland Irrigation Channels and Drains. *Marine Pollution Bulletin* **2000**, *41* (7–12), 294–301. https://doi.org/10.1016/S0025-326X(00)00095-3.

(28) Marino, D.; Ronco, A. Cypermethrin and Chlorpyrifos Concentration Levels in Surface Water Bodies of the Pampa Ondulada, Argentina. *Bull Environ Contam Toxicol* **2005**, *75* (4), 820–826. https://doi.org/10.1007/s00128-005-0824-7.

(29) Varó, I.; Serrano, R.; Pitarch, E.; Amat, F.; López, F. J.; Navarro, J. C. Bioaccumulation of Chlorpyrifos Through an Experimental Food Chain: Study of Protein HSP70 as Biomarker of Sublethal Stress in Fish. *Archives of Environmental Contamination and Toxicology* **2002**, *42* (2), 229–235. https://doi.org/10.1007/s00244-001-0013-6.

(30) Thomas, C. N.; Mansingh, A. Bioaccumulation, Elimination, and Tissue Distribution of Chlorpyrifos by Red Hybrid Tilapia in Fresh and Brackish Waters. *Environmental Technology* **2002**, *23* (11), 1313–1323. https://doi.org/10.1080/09593332308618324.

(31) Tilak, K. S.; Veeraiah, K.; Rao, D. K. Toxicity and Bioaccumulation of Chlorpyrifos in Indian Carp Catla Catla(Hamilton), Labeo Rohita (Hamilton), and Cirrhinus Mrigala (Hamilton). *Bull Environ Contam Toxicol* **2004**, *73* (5), 933–941. https://doi.org/10.1007/s00128-004-0516-8.

(32) Browne, M. A.; Niven, S. J.; Galloway, T. S.; Rowland, S. J.; Thompson, R. C. Microplastic Moves Pollutants and Additives to Worms, Reducing Functions Linked to Health and Biodiversity. *Current Biology* **2013**, *23* (23), 2388–2392. https://doi.org/10.1016/j.cub.2013.10.012.

(33) Grigorakis, S.; Drouillard, K. G. Effect of Microplastic Amendment to Food on Diet Assimilation Efficiencies of PCBs by Fish. *Environmental Science & Technology* **2018**, *52* (18), 10796–10802. https://doi.org/10.1021/acs.est.8b02497.

(34) Rainieri, S.; Conlledo, N.; Larsen, B. K.; Granby, K.; Barranco, A. Combined Effects of Microplastics and Chemical Contaminants on the Organ Toxicity of Zebrafish (Danio Rerio). *Environmental Research* **2018**, *162*, 135–143. https://doi.org/10.1016/j.envres.2017.12.019.

(35) Granby, K.; Rainieri, S.; Rasmussen, R. R.; Kotterman, M. J. J.; Sloth, J. J.; Cederberg, T. L.; Barranco, A.; Marques, A.; Larsen, B. K. The Influence of Microplastics and Halogenated Contaminants in Feed on Toxicokinetics and Gene Expression in European Seabass (Dicentrarchus Labrax). *Environmental Research* **2018**, *164*, 430–443. https://doi.org/10.1016/j.envres.2018.02.035.

(36) Batel, A.; Linti, F.; Scherer, M.; Erdinger, L.; Braunbeck, T. Transfer of Benzo[a]Pyrene from Microplastics to Artemia Nauplii and Further to Zebrafish via a Trophic Food Web Experiment: CYP1A Induction and Visual Tracking of Persistent Organic Pollutants:

Trophic Transfer of Microplastics and Associated POPs. *Environmental Toxicology and Chemistry* **2016**, *35* (7), 1656–1666. https://doi.org/10.1002/etc.3361.

(37) Jovanović, B.; Gökdağ, K.; Güven, O.; Emre, Y.; Whitley, E. M.; Kideys, A. E. Virgin Microplastics Are Not Causing Imminent Harm to Fish after Dietary Exposure. *Marine Pollution Bulletin* **2018**, *130*, 123–131. https://doi.org/10.1016/j.marpolbul.2018.03.016.

(38) Mazurais, D.; Ernande, B.; Quazuguel, P.; Severe, A.; Huelvan, C.; Madec, L.; Mouchel, O.; Soudant, P.; Robbens, J.; Huvet, A.; et al. Evaluation of the Impact of Polyethylene Microbeads Ingestion in European Sea Bass (Dicentrarchus Labrax) Larvae. *Marine Environmental Research* **2015**, *112*, 78–85. https://doi.org/10.1016/j.marenvres.2015.09.009.

(39) Rochman, C. M.; Kurobe, T.; Flores, I.; Teh, S. J. Early Warning Signs of Endocrine Disruption in Adult Fish from the Ingestion of Polyethylene with and without Sorbed Chemical Pollutants from the Marine Environment. *Science of The Total Environment* **2014**, *493*, 656–661. https://doi.org/10.1016/j.scitotenv.2014.06.051.

(40) Batel, A.; Borchert, F.; Reinwald, H.; Erdinger, L.; Braunbeck, T. Microplastic Accumulation Patterns and Transfer of Benzo[a]Pyrene to Adult Zebrafish (Danio Rerio) Gills and Zebrafish Embryos. *Environmental Pollution* **2018**, *235*, 918–930. https://doi.org/10.1016/j.envpol.2018.01.028.

(41) Ferreira, P.; Fonte, E.; Soares, M. E.; Carvalho, F.; Guilhermino, L. Effects of Multi-Stressors on Juveniles of the Marine Fish Pomatoschistus Microps: Gold Nanoparticles, Microplastics and Temperature. *Aquatic Toxicology* **2016**, *170*, 89–103. https://doi.org/10.1016/j.aquatox.2015.11.011.

(42) Karami, A.; Groman, D. B.; Wilson, S. P.; Ismail, P.; Neela, V. K. Biomarker Responses in Zebrafish (Danio Rerio) Larvae Exposed to Pristine Low-Density Polyethylene Fragments. *Environmental Pollution* **2017**, *223*, 466–475. https://doi.org/10.1016/j.envpol.2017.01.047.

(43) Malinich, T. D.; Chou, N.; Sepúlveda, M. S.; Höök, T. O. No Evidence of Microplastic Impacts on Consumption or Growth of Larval *Pimephales Promelas*: No Microplastic Impacts on Consumption or Growth of Larvae. *Environ Toxicol Chem* **2018**, *37* (11), 2912–2918. https://doi.org/10.1002/etc.4257.

(44) Choi, J. S.; Jung, Y.-J.; Hong, N.-H.; Hong, S. H.; Park, J.-W. Toxicological Effects of Irregularly Shaped and Spherical Microplastics in a Marine Teleost, the Sheepshead Minnow (Cyprinodon Variegatus). *Marine Pollution Bulletin* **2018**, *129* (1), 231–240. https://doi.org/10.1016/j.marpolbul.2018.02.039.

(45) Karami, A.; Romano, N.; Galloway, T.; Hamzah, H. Virgin Microplastics Cause Toxicity and Modulate the Impacts of Phenanthrene on Biomarker Responses in African Catfish (Clarias Gariepinus). *Environmental Research* **2016**, *151*, 58–70. https://doi.org/10.1016/j.envres.2016.07.024.

(46) Luís, L. G.; Ferreira, P.; Fonte, E.; Oliveira, M.; Guilhermino, L. Does the Presence of Microplastics Influence the Acute Toxicity of Chromium(VI) to Early Juveniles of the Common Goby (Pomatoschistus Microps)? A Study with Juveniles from Two Wild Estuarine Populations. *Aquatic Toxicology* **2015**, *164*, 163–174. https://doi.org/10.1016/j.aquatox.2015.04.018.

(47) Oliveira, M.; Ribeiro, A.; Hylland, K.; Guilhermino, L. Single and Combined Effects of Microplastics and Pyrene on Juveniles (0+ Group) of the Common Goby Pomatoschistus Microps (Teleostei, Gobiidae). *Ecological Indicators* **2013**, *34*, 641–647. https://doi.org/10.1016/j.ecolind.2013.06.019.

(48) Wen, B.; Zhang, N.; Jin, S.-R.; Chen, Z.-Z.; Gao, J.-Z.; Liu, Y.; Liu, H.-P.; Xu, Z. Microplastics Have a More Profound Impact than Elevated Temperatures on the Predatory Performance, Digestion and Energy Metabolism of an Amazonian Cichlid. *Aquatic Toxicology* **2018**, *195*, 67–76. https://doi.org/10.1016/j.aquatox.2017.12.010.

(49) Peakall, D. B.; Thompson, H.; Baatrup, E. Relationship Between Behavior and the Biochemical/Physiological Biomarkers of Expoure to Enviromental Pollutants. In *Behavioural Ecotoxicology*; Dell'Omo, 2002; pp 187–208.

(50) Weis, J. S.; Smith, G.; Zhou, T.; Santiago-Bass, C.; Weis, P. Effects of Contaminants on Behavior: Biochemical Mechanisms and Ecological ConsequencesKillifish from a Contaminated Site Are Slow to Capture Prey and Escape Predators; Altered Neurotransmitters and Thyroid May Be Responsible for This Behavior, Which May Produce Population Changes in the Fish and Their Major Prey, the Grass Shrimp. *BioScience* **2001**, *51* (3), 209–217. https://doi.org/10.1641/0006-3568(2001)051[0209:EOCOBB]2.0.CO;2.

(51) Lafferty, K. D.; Morris, A. K. Altered Behavior of Parasitized Killifish Increases Susceptibility to Predation by Bird Final Hosts. *Ecology* **1996**, *77* (5), 1390–1397. https://doi.org/10.2307/2265536.

(52) Seppälä, O.; Karvonen, A.; Tellervo Valtonen, E. Parasite-Induced Change in Host Behaviour and Susceptibility to Predation in an Eye Fluke–Fish Interaction. *Animal Behaviour* **2004**, *68* (2), 257–263. https://doi.org/10.1016/j.anbehav.2003.10.021.

(53) Maximino, C.; de Brito, T. M.; da Silva Batista, A. W.; Herculano, A. M.; Morato, S.; Gouveia, A. Measuring Anxiety in Zebrafish: A Critical Review. *Behavioural Brain Research* **2010**, *214* (2), 157–171. https://doi.org/10.1016/j.bbr.2010.05.031.

(54) Little, E. E. Behavioural Measures of Injuries to Fish and Aquatic Organisms: Regulatory Considerations. In *Behavioural Ecotoxicology*; Dell'Omo, 2002; pp 411–431.

(55) Halappa, R.; David, M. Behavioural Responses of the Freshwater Fish, Cyprinus Carpio (Linnaeus) Following Sublethal Exposure to Chlorpyrifos. *Turkish Journal of Fisheries and Aquatic Sciences* **2009**, *9* (2). https://doi.org/10.4194/trjfas.2009.0218.

(56) Tierney, K.; Casselman, M.; Takeda, S.; Farrell, T.; Kennedy, C. The Relationship Between Cholinesterase Inhibition And Two Types Of Swimming Performance In Chlorpyrifos-Exposed Coho Salmon (Oncorhynchus Kisutch). *Environ Toxicol Chem* **2007**, *26* (5), 998. https://doi.org/10.1897/06-459R.1.