Do polystyrene microplastics affect juvenile brown trout (*Salmo trutta* f. *fario*) and modulate effects of the pesticide methiocarb?

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Abstract

Background

During the last decade, there has been rising interest of the scientific community and the public in the environmental risk related to the abundance of microplastics in aquatic environments. Besides potential effects of the particles themselves, also their interaction with organic micropollutants is of particular concern. Up to now, however, scientific knowledge in this context is scarce and insufficient for a reliable risk assessment. This is especially true for data on microplastics in freshwater ecosystems.

Results

Against the background of this shortage, we investigated possible adverse effects of polystyrene particles (10 4 particles/L) and the pesticide methiocarb (1 mg/L) both alone as well as in combination in juvenile brown trout (Salmo trutta f. fario) after a 96 h laboratory exposure. PS beads (density 1.05 g/mL) were cryogenically milled and fractionated resulting in irregular shaped particles (<50 µm). Besides body weight of the animals, biomarkers for proteotoxicity (stress protein family Hsp70), oxidative stress (superoxide dismutase, lipid peroxidation) and neurotoxicity (acetylcholinesterase, carboxylesterases) were analysed. As an indicator of overall health histopathological effects were studied in liver and gills of exposed fish. Polystyrene particles alone did not influence any of the investigated biomarkers. In contrast, the exposure to methiocarb led to a significant reduction of the activity of acetylcholinesterase and the two carboxylesterases. Moreover, the tissue integrity of liver and gills was impaired by the pesticide. Body weight, the oxidative stress and the stress protein levels were not influenced by methiocarb. Effects caused by the mixture of polystyrene microplastics and methiocarb were the same as those caused by methiocarb alone.

Conclusions

Overall, methiocarb led to strong effects in juvenile brown trout. In contrast, polystyrene microplastics in the tested concentration did not negatively affect the health of juvenile brown trout and did not modulate the toxicity of methiocarb in this fish species.

1. Background

Public and scientific awareness for the problem of environmental pollution with microplastics (MP) has increased considerably over the last years. Nevertheless, the exact definition of the term microplastics itself is under discussion. A recent paper of Hartmann et al. (2019) suggests defining MP as particles or fibres between 1 µm and 1 mm. MP can be found ubiquitously in marine and freshwater ecosystems and even in remote regions (Dris et al. 2015, Bergmann et al. 2017, Horton et al. 2017). Despite most research focusses on the abundance and possible effects of MP in marine environments, the number of studies in freshwater ecosystems is also rapidly increasing (Horton et al. 2017). Nevertheless, many gaps in knowledge about abundance, toxicity and hazard of MP in freshwater systems still exist.

For an environmental risk assessment of MP, measured concentrations in the environment must be related to effect concentrations in exposed organisms. Whereas the presence of MP in surface waters and sediments is unquestionable in general, there are still considerable uncertainties with respect to their exact concentrations especially those of plastic particles smaller than 4 µm (Triebskorn et al. 2019). Possible effects of MP can be related to mechanical injuries caused by the particles (von Moos et al. 2012, Karami et al. 2016). This could lead for example to an increase of inflammatory processes or oxidative stress in organisms (e.g. Lu et al. 2016, Paul-Pont et al. 2016, Ding et al. 2018). Of particular interest are very small particles (mainly nanoplastics) for which uptake in cells were shown (e.g. Mattsson et al. 2017, van Pomeren et al. 2017, Ding et al. 2018). In addition to this physical damage MP can also potentially affect organisms due to the leakage of hazardous substances like residual monomers, polymerization solvents or additives (Lithner et al. 2009, Schiavo et al. 2018). A third process that should be considered in this context is that MP might ad/absorb hydrophobic organic pollutants like PAHs, PCBs or pesticides (reviewed by Wang et al. 2018) and transport them into organisms. Sorption of hydrophobic organic pollutants to MP is especially important for freshwater, since the concentrations of these pollutants are generally higher than in marine ecosystems (Dris et al. 2015). The sorption of organic pollutants to MP may alter the bioavailability of the pollutants. The bioavailability (and thereby effects) of pollutants can be decreased due to sorption to the polymer (Sleight et al. 2017, Kleinteich et al. 2018, Rehse et al. 2018). On the other hand, effects of pollutants on organisms can be enhanced by MP when they are ingested together with MP and desorb in the digestive track of the organism (Oliveira et al. 2013, Nematdoost Haghi and Banaee 2017, Guilhermino et al. 2018). Furthermore, Batel et al. (2016) showed the possibility of the transfer of organic pollutants adsorbed to MP along an artificial food chain. The biological relevance of MP as vectors for chemicals is still under debate. Several studies suggest the effect of MP to be negligible compared to other exposure pathways and the concentration of organic compounds in most MP to be in equilibrium with the surrounding media (e.g. Bakir et al. 2016, Koelmans et al. 2016, Beckingham and Ghosh 2017). To assess the risk of MP alone and in combination with organic pollutants more studies under controlled lab conditions are required.

Residues of pesticides are regularly found in freshwater ecosystems (e.g. Smith et al. 2012, Bundschuh et al. 2014, Köck-Schulmeyer et al. 2014). Pesticides enter surface waters mainly due to run-off from agricultural sites, drainage pipes, drift, atmospheric deposition and groundwater flow (Neumann et al. 2002, Peschka et al. 2006, Holvoet et al. 2007). Methiocarb (or synonym mercaptodimethur) is a carbamate pesticide, which is used as a molluscicide, herbicide, insecticide, acaricide and as repellent in seed treatment (Worthing et al. 1991, European Commission 2014, Loos et al. 2018). In the US products containing methiocarb are registered by the EPA for restricted usage (EPA 1994). In September 2019 the European Commisson did not renew the approval of methiocarb as active substance (European Commission 2019b). Prior to this decision generally products containing methiocarb were approved for usage in the EU, the application as a molluscicide is already prohibited since 2014 (European Commission 2014, European Commission 2019a). Methiocarb is on the 1st surface water Watch List under the Water Framework Directive of the European Union and is recommended for the 2nd Watch List (European Commission 2015, Loos et al. 2018). The average surface water concentration of methiocarb

in the EU wide monitoring campaign was between 6–40 ng/L - the maximal concentration 109 ng/L. The predicted no effect concentration (PNEC) of methiocarb is 2 ng/L (Loos et al. 2018).

The purpose of the present study was to investigate the effects of polystyrene (PS) MP alone and in combination with methiocarb in juvenile brown trout. To determine the amount of methiocarb sorbed on PS particles a separate sorption study would be necessary. Sorption of chemical contaminants to MP involves various mechanisms and depends on physicochemical properties of the sorbate, the sorbent as well as the medium characteristics (Tourinho et al. 2019). Brown trout (Salmo trutta f. fario) are sensitive test organisms, which are native to Germany. As top predator brown trout are ecologically relevant and, as popular food fish, also of commercial relevance (Dußling and Berg 2001, Klemetsen et al. 2003, Burkhardt-Holm 2009). The animals were exposed to either PS-MP alone (10^4 particles/L), to methiocarb alone (1 mg/L) or the combination (10^4 particles/L and 1 mg/L methiocarb) for 96 h. Besides apical endpoints (mortality and body weight), biomarkers for oxidative stress (activity of superoxide dismutase (SOD) and formation of lipid peroxidation (LPO)), proteotoxicity (stress protein family Hsp70) as well as neurotoxicity (acetylcholinesterase (AChE) activity and two protective carboxylesterases (CbE)) were examined. Furthermore, the histopathological status of gills and liver was analysed. Our purpose was to investigate whether PS-MP or methiocarb alone affect juvenile brown trout at sub-lethal level and whether PS-MP modulate the toxicity of methiocarb.

2. Materials And Methods

2.1. Test organism

Brown trout (Salmo trutta f. fario) belong to the family of Salmonidae and are native to most of Europe (Dußling and Berg 2001, Klemetsen et al. 2003). Brown trout prefer cool waters with high oxygen contents and are therefore typical inhabitants of upland streams (Dußling and Berg 2001). In the present study, approximately 11-month-old juvenile brown trout were used. Prior to the experiment, trout were acclimated in a 250 L tank to lab conditions (aerated filtered tap water – iron filter, particle filter, activated charcoal filter) for almost three months. Trout originated from a commercial fish breeder (Forellenzucht Lohmühle, D-72275 Alpirsbach-Ehlenbogen, Germany). In regular controls the breeding establishment is categorized as category I, disease free (EU 2006). The fish can be considered as close to feral forms and robust since they are also used for fishery restocking campaigns in German streams.

2.2. Test substances

PS-MP suspensions with a defined number of particles were produced according to the method of Eitzen et al. (2019). Briefly, transparent polystyrene pellets (Polystyrol 158 K, BASF, Germany) with a density of 1.05 g/mL were cryogenically milled (CryoMill, Retsch, Germany) resulting in irregularly shaped PS-MP particles. Subsequently, the particles were fractionated to a size < 50 µm. The number of PS particles in the pure suspension was determined with a particle counter (SVSS, PAMAS, Germany) by light extinction

in a laser-diode sensor (type HCB-LD-50/50). The particle size distribution is provided in the supplement (Figure S1).

Methiocarb (Fig. 1) was purchased from Sigma-Aldrich (product line: PESTANAL®; CAS number: 2032-65-7; purity 99.8%; molecular formula: $C_{11}H_{15}NO_2S$). The melting point of methiocarb is 119 °C, the vapour pressure 0.015 mPa (20 °C) and the octanol/water partition coefficient as log Pow 3.08. According to literature the solubility in water (20 °C) is 27 mg/L (Worthing et al. 1991, ILO 2012). Nevertheless, in our experiment it was not possible to solve methiocarb without a solvent. Therefore, dimethyl sulfoxide (DMSO) was used to solve methiocarb in water.

2.3. Exposure of juvenile brown trout

Fish were exposed in a static three-block design for 96 h (18.11. – 23.11.2017). Each of the three blocks consisted of five tanks with the different treatment groups: a control group, a solvent control (0.01% DMSO), PS-MP (10^4 particles/L), methiocarb (1 mg/L) and a mixture (10^4 particles/L and 1 mg/L methiocarb). The test concentration of 1 mg/L methiocarb was selected due to published LC_{50} values for rainbow trout (Onchorynchus mykiss): The reported LC_{50} (96 h) values vary between LC_{50} = 0.198 mg/L (Johnson and Finley 1980) and LC_{50} = 4.82 ± 0.21 mg/L (Pesticide Action Network 2000–2019). Each aquarium contained 10 fish (in 15 L of the corresponding test medium) resulting in 30 fish per treatment (aquaria in triplicates) and 150 fish in total. To be able to consider potential confounding factors, the position of each treatment group within the blocks was randomized. Exposure took place in a thermo-constant chamber set to 7 °C with a light/dark cycle of 10 h/14 h (tanks were shaded from direct light). Aeration of the tanks was ensured by glass pipettes, which were connected to compressed air via silicone tubes.

In all treatment groups, filtered tap water was used. For the PS-MP treatments, the defined volumes of the stock suspensions (56.240 particles/mL) were added. To avoid a loss of particles in the glass vessels containing the PS-MP suspensions, the vessels were rinsed four times. For each tank containing methiocarb, a stock solution (15 mg/L methiocarb) was prepared as follows: 15 mg methiocarb were solved in 1.5 mL DMSO (resulting in a concentration of 0.01% DMSO in the tanks), and subsequently, 1 L water was added. Finally, the solutions were stirred for two days to ensure a complete solution of methiocarb. To provide comparability between the groups, 0.01% DMSO was also added to the tanks which contained only PS-MP.
Fish were fed daily a defined portion (4% of body weight) of commercially available fish food (0.8 mm, Biomar, Brande, Denmark). After 48 h and 72 h, 2.5 L of the test medium of each aquarium were removed to get rid of feces and remains of food. Water parameters were measured at the beginning and the end of the experiment. To avoid contamination of the measuring device, oxygen saturation and concentration as well as conductivity could not be measured in tanks containing methiocarb (average values of all measured tanks: temperature = 7.0 ± 0.3 °C, pH = 7.3 ± 0.5, oxygen concentration = 10.9 ± 0.4 mg/L,

oxygen saturation = 94.7 ± 2.4%; conductivity = 489.8 ± 15.8 µS/cm; for detailed information see supplement Table S1 and Table S2). Nitrite (NO_2^-) values did not exceed 0.2 mg/L.

After 24 h, four animals (one fish of block 1 methiocarb, two fish of block 3 methiocarb and one fish of block 2 mixture) were in very poor health conditions and had to be euthanized. Apart from these, no fish died during the experiment.

At the end of the experiment, fish were anesthetized and killed by an overdose of tricaine methanesulfonate (1 g/L MS222, buffered with $NaHCO_3$). Subsequently, death of the animals was ensured by severance of the spine. Body weight of each animal was recorded prior to dissection. For histological investigations samples of gills and liver were transferred into fixative (2% glutardialdehyde diluted in 0.1 M cacodylate buffer; pH 7.6) and stored at 4 °C until further processing. For biochemical analyses, samples of muscle (AChE and CbE activity), brain (LPO analysis), gill (stress protein analysis) and a part containing muscle and kidney (for analysis of SOD activity) were immediately frozen in liquid nitrogen. Subsequently, samples for biochemical analyses were stored at -80 °C.

Additionally, 30 fish (kept in a 250 L tank during the experiment) were sampled in an analogous manner as lab control to be able to recognize possible effects of the exposure procedure itself.

2.4. Chemical analysis

From each aquarium, 12 mL medium were taken at the beginning and the end of the experiment and frozen at -20 °C until further processing. Samples of the three replicate tanks were analysed separately by means of HPLC-ESI-MS/MS. Quantification was performed via liquid chromatography coupled to mass spectrometry (LC-MS/MS). As instrumentation a PerkinElmer Series 200 LC system (PerkinElmer, Waltham, USA), consisting of two Series 200 micro-pumps, a Series 200 vacuum degasser, and a Series 200 autosampler was coupled to a QqLIT mass spectrometer SCIEX QTRAP 3200 (AB SCIEX, Darmstadt, Germany) and ionization was achieved via electrospray ionization (ESI) in positive mode (further information are provided in the supplement).

Separation was performed at 23 °C using a XSelectTM HSS T3 reverse phase column (2.1 mm x 50 mm, 3.5 µm particle size) from Waters (Milford, USA). Injection volume was 10 µL and a gradient was run with a flow rate of 200 µL/min. Eluents used for chromatographic separation were mixtures of methanol and water (Eluent A: MeOH/H2O, 5:95, v/v; Eluent B: MeOH/H2O, 95:5, v/v) additionally containing 5 mM/L ammonium formate (NH_4HCO_2) and adjusted to pH 3 with formic acid (further information are provided in the supplement).

A multiple reaction monitoring (MRM) method was used for quantification. The individual transitions and optimized MS parameters are stated in Table S5 in the supplement.

Unless specified otherwise, all calculations were carried out via Microsoft Excel 2016. The developed method was validated regarding the limit of detection (LoD), the limit of quantification (LoQ), and the method precision and accuracy. LoD and LoQ were determined by application of the signal to noise (S/N) approach. The S/N ratios of two calibration standards (β = 0.25 ng/mL and 0.5 ng/mL) were measured in triplicate and their S/N ratio was determined. LoD was defined as S/N > 3; and LoQ with S/N > 9. With the determined S/N ratios the theoretical LoD and LoQ were calculated and are stated in Table S6 in the supplement

Concentrations of standards used for calibration ranged from 0.25–250 ng/mL and atrazine was used as internal standard. Measurement precision was determined by multiple (n = 5) of three standards and the calculation of their concentration on basis of the obtained calibration. Mean concentrations, relative standard deviation (RSD), and accuracy are stated in Table S7 in the supplement. For quantification calibration was measured in triplicate (before, in between, and after the samples). Samples were filtered via syringe filters (Carl Roth, SPARTAN® regenerated cellulose, 0.2 µm) after dilution (1:25; 1:100; V.V) with type 1 water and prior to their measurement.

2.5. Determination of oxidative stress level

2.5.1. Activity of SOD

The SOD activity (Cu/Zn SOD, Mn SOD and Fe SOD) in muscle/kidney samples was analysed with the Cayman Chemical superoxide dismutase assay kit (item no. 706002, Cayman Chemical Company, Ann Arbor, USA) in 96-well plates. To remove any blood residues, samples were rinsed in phosphate buffered saline (PBS; pH 7.4) before they were frozen in liquid nitrogen. The dilution of the final samples was 1:150. In the assay, superoxide radicals are generated with xanthine oxidase and hypoxanthine. Subsequently, the superoxide radicals are detected with tetrazolium salt. The absorbance at 450 nm was measured (Bio-Tek Instruments, Winooski VT, USA) after an incubation of 30 min at room temperature. All samples were analysed in duplicates.

2.5.2. Level of lipid peroxidases

The level of lipid peroxides was quantified with the ferrous oxidation xylenol orange (FOX) assay. The assay was performed in a slightly modified way for 96-well plates according to Hermes-Lima et al. (1995) and Monserrat et al. (2003). For homogenization, the brains were 1:2 diluted with HPLC grade methanol and subsequently centrifuged (15 000 rcf, 5 min, 4 °C). Supernatant was stored until the final assay at -80 °C. The assay was performed in 96-well plates. Into each well, 50 µL of 0.75 mM $FeSO_4$ -solution, 50 µL of 75 mM sulphuric acid, 50 µL of 0.3 mM xylenol orange solution, 40 µL supernatant and 10 µL bidestilled water were added successively. Triplicates of each sample were tested. Furthermore, a sample blank without $FeSO_4$ (replaced by bidestilled water) of each sample was measured to correct for potential Fe in the samples. After an incubation of 120 min at room temperature, the absorbance at 570 nm (ABS570) was measured (Bio-Tek Instruments, Winooski VT, USA). Afterwards, in each well 1 µL of 1 mM cumene hydroperoxide solution (CHP) was pipetted. The plates were incubated for another 30 min at room temperature and a second measurement at 570 nm was conducted. The data of both measurements were related to a master blank (200 µL bidestilled water). Cumene hydroperoxide equivalents (CHPequiv./mg wet weight) were calculated using the following equation:

$$CHPequiv. = \frac{ABS570\ sample\ -\ ABS570\ sample\ blank}{ABS570\ sample\ and\ CHP\ -\ ABS570\ sample\ blank\ and\ CHP} * volume\ CHP * \frac{total\ volume\ in\ well}{sample\ volume} * dilution\ factor$$

$$= \frac{ABS570\ sample - ABS570\ sample\ blank}{ABS570\ sample\ and\ CHP - ABS570\ sample\ blank\ and\ CHP} * 1 * \frac{200}{40} * 2$$

2.6. Stress protein analysis

Hsp70 quantification was performed as described by Dieterich et al. (2015). Samples were 1:3 diluted with extraction buffer (80 mM potassium acetate, 5 mM magnesium acetate, 20 mM HEPES and 2% protease inhibitor at pH 7.5) and homogenized on ice. The samples were centrifuged (20 000 rcf, 10 min, 4 °C) and the supernatant was divided into two samples. The first was used to quantify the total protein content via Bradford assay (Bradford 1976). Subsequently, the second sample (amount standardized to 40 µg of total protein content) was separated via SDS–PAGE (12% acrylamide, 0.12% bisacrylamide, 30 min at 80 V plus 90 min at 120 V). The proteins were blotted on a nitrocellulose membrane and immuno-stained with a monoclonal α-Hsp70 IgG (Dianova, Hamburg, Germany) followed by a secondary peroxidase-coupled α-IgG (Jackson Immunoresearch, West Grove, PA). The optical volume (area x average pixel intensity) of the protein bands was quantified (Image Studio Lite, 4.0.2.1, Li-Cor Biosciences, Lincoln, USA) and put into relation to an internal Hsp70 standard.

2.7. Analysis of neurotoxicity

For the analysis of the activity of AChE and two CbE, muscle tissue was diluted 1:5 in tris–LS buffer (20 mM $Tris_{base}$, 20 mM NaCl, inhibitor mix, pH 7.3) and homogenized. After centrifugation (5000 rcf, 10 min, 4 °C), 50% glycerol was added to the supernatant (1/4 of the amount of the supernatant), and the mixture was frozen at -20 °C. The protein content in the samples was determined with the Lowry method modified by Markwell et al. (1978). The AChE-activity was measured spectrophotometrically at 405 nm (Bio-Tek Instruments, Winooski VT, USA) according to Ellman et al. (1961) and modified by Rault et al. (2008). CbE activity was determined with the substrates 5 mM 4-nitrophenol acetate (pnpa) and 5 mM 4-nitrophenyl valerate (pnpv) described by Sanchez-Hernandez et al. (2009). In all assays, samples were analysed in triplicates. The data were related to the total protein amount (specific activity per milligram total protein content). One unit is described as one micromole substrate hydrolysed per minute.

2.8. Histopathology

Liver and gill samples were fixed in 2% glutardialdehyde diluted in 0.1 M cacodylate buffer (pH 7.6) and stored at 4 °C for at least 4 weeks. Prior to further processing, gill samples were decalcified in a 1:2 mixture of 100% formic acid and 70% ethanol. All samples were rinsed three times for 10 min in 0.1 M cacodylate buffer and subsequently three times for 15 min in 70% ethanol. Dehydration of the samples and embedding into paraffin was achieved in an automated tissue infiltrator (TP 1020, Leica Wetzlar, Germany). With a sledge microtome (SM 2000 R, Leica Wetzlar, Gemany), 3 µm thick histological sections were cut. Sections were stained (1) with hematoxilin-eosin (HE) which visualizes nuclei,

cytoplasm muscles and connective tissue and (2) alcian blue - PAS by which glycogen and mucus were stained. In a first step, the slides were evaluated qualitatively to identify pathologies. In a second step, the slides were examined semi-quantitatively in an observer blinded way. The samples were categorized into five classes (1: control; 2: slight reactions; 3: medium reactions; 4: strong reactions; 5: destruction) as suggested by Triebskorn et al. (2008).

2.9. Statistical analysis

Data analysis was performed with JMP®14.0.0 (SAS Institute Inc., North Carolina, USA). Standard distribution of the data was checked with the Shapiro Wilk test. Homogeneity of variance was checked with the Levene`s test. If necessary, data were transformed (AChE: root, weight: third root, SOD: fifth root, Hsp70: natural logarithm, Cbe-pnpa: 1/x, Cbe-pnpv: seventh root). Using a t-test for all investigated parameters we ensured that there were no significant differences between the control and the solvent control. Subsequently, the other treatments were compared to the solvent control. In addition to the comparison to the solvent control, the PS-MP and the methiocarb treatments were compared to the mixture treatment. Comparisons with the lab control were conducted only qualitatively and were not included in the mathematical analyses.

The base α-level was set to 0.05. Parametric data were analysed with a nested ANOVA including block as nesting factor. This allows considering potential confounding factors and avoiding pseudo-replication. The comparison between the single groups was made with Tukey HSD. The data of the activity of CbE with the substrate pnpa could not be transformed to reach homoscedasticity. Thus, a Welch ANOVA was performed to analyse the effect on the activity of CbE-pnpa mathematically. Frequency data were analysed with likelihood-ratio analysis and a post hoc Bonferroni-Holm correction.

2.10. Credibility of data

The information on the fulfilment of the criteria for reporting and evaluation of ecotoxicity data (CRED) proposed by Moermond et al. (2016) is provided in the supplementary information.

3. Results

3.1. Chemical analyses

At the beginning and the end of the experiment, in both control groups, the methiocarb concentrations were below the detection limit (LOD) of 0.25 µg/L (Table 1). In the PS-MP exposure, no methiocarb could be detected (< LOD). In both exposure groups with methiocarb (methiocarb and mixture), the measured concentrations at the beginning of the experiment were 50% of the nominal concentrations. After 96 h, in the treatment group with solely methiocarb, the concentration was further reduced by approximately 50%, in the mixture treatment by 34%.

Table 1
Nominal and measured concentrations of methiocarb in the different treatment groups. Displayed are the arithmetic means ± standard deviation of the three aquaria. The limit of detection was 0.25 µg/L methiocarb.

Treatment group	nominal concentration	measured concentration at start of experiment	measured concentration at end of experiment
control	0 µg/L	Below LOD	Below LOD
solvent control	0 µg/L	Below LOD	Below LOD
PS-MP (10^4 particles/L)	0 µg/L	Below LOD	Below LOD
methiocarb	1000 µg/L	463 µg/L ± 32 µg/L	237 µg/L ± 59 µg/L
mixture PS-MP + methiocarb	1000 µg/L	469 µg/L ± 133 µg/L	308 µg/L ± 76

3.2. Mortality and weight

After 48 h, three fish exposed to methiocarb and one fish exposed to the mixture had to be euthanized due to their poor health conditions. Apart from that, no mortality occurred during the experiment. Weight (overall mean: 2.96 ± 1.01 g) did not differ between the treatment groups (Table 2; DMSO- exposure groups: Nested-ANOVA: d.f.=3/104, F = 1.14, p = 0.3384).

3.3. Oxidative Stress

To assess the oxidative stress level SOD activity and the degree of LPO were investigated. No difference between SOD activity in the different treatment groups was found (Table 2; DMSO-exposure groups: Nested-ANOVA: d.f.=3/103, F = 0.77, p = 0.5136). The degree of LPO of the exposure groups were comparable to the solvent control (DMSO- exposure groups: Nested-ANOVA: d.f.=3/91, F = 0.66, p = 0.5770). For both tested endpoints neither PS-MP nor methiocarb nor the mixture caused oxidative stress.

Table 2
Summary of data for the investigated endpoints. All data are given as arithmetic means ± standard deviation.

	lab control	control	solvent control	PS-MP	methiocarb	mixture
Weight [g]	4.02 ± 1.09	2.49 ± 0.66	2.89 ± 0.87	3.21 ± 0.91	2.95 ± 1.27	3.30 ± 1.06
SOD [U/mL)	92.71 ± 26.12	105.29 ± 28.81	106.50 ± 24.22	106.66 ± 29.69	99.88 ± 31.59	99.43 ± 35.37
FOX [CHP-Equiv.]	2.17 ± 1.26	2.56 ± 1.09	1.97 ± 1.17	1.69 ± 1.21	1.83 ± 1.52	1.60 ± 1.39
Hsp70 [relative grey value]	1.71 ± 0.29	1.84 ± 0.29	1.78 ± 0.29	1.72 ± 0.26	1.77 ± 0.30	1.71 ± 0.27
AChE [mU/mg Protein]	69.32 ± 11.55	88.38 ± 24.98	77.37 ± 18.39	73.93 ± 20.28	31.48 ± 11.38	32.38 ± 11.64
CbE-pnpa [mU/mg Protein]	82.20 ± 9.16	79.13 ± 10.83	79.10 ± 11.38	76.68 ± 7.90	45.52 ± 5.74	44.48 ± 5.01
CbE-pnpv [mU/mg Protein]	60.60 ± 11.14	53.89 ± 12.38	54.01 ± 12.94	49.59 ± 10.46	9.10 ± 3.24	9.05 ± 1.91

3.4. Proteotoxicity

The analysis of the Hsp70 level did not reveal any differences between the control, solvent control and the exposure groups (Table 2; DMSO-exposure groups: Nested-ANOVA: d.f.=3/96, F = 0.35, p = 0.7922).

3.5. Neurotoxicity

No difference in the activity of AChE occurred between the solvent control and the treatment group with PS-MP (p = 0.8192). However, methiocarb and the mixture of methiocarb and PS-MP led to a significantly reduced activity of AChE in comparison to the solvent control and PS-MP alone (Fig. 1, DMSO-exposure groups: Nested-ANOVA: d.f. =3/101, F = 77.77, p < 0.0001). The activity of AChE in the methiocarb-exposed fish was 59% reduced compared to the solvent control (DMSO-methiocarb and PS-MP-methiocarb: p < 0.001). In fish exposed to the mixture of PS-MP and methiocarb the activity of AChE was reduced by 58% compared to the solvent control (DMSO-mixture and PS-MP-mixture: p < 0.001; Table 2). Between fish exposed to methiocarb alone and methiocarb plus PS-MP no differences were found (p = 0.9951).

Figure 1: Specific activity of acetylcholinesterase in muscular tissue of brown trout. The box plots display the median, the 25th and 75th quantiles as well as minimum and maximum values (whiskers), the dots indicate outliers. Different letters indicate significant differences. Methiocarb and mixture significantly reduced the AChE activity (DMSO-exposure groups: Nested-ANOVA: d.f.=3/105, F = 83.65, p < 0.0001).

The activity of CbE-pnpa was also inhibited by methiocarb and the mixture treatment (Fig. 2; DMSO-exposure groups: Welch ANOVA: d.f.=3/57, F = 192.15, $p < 0.0001$; DMSO-PS-MP: $p = 0.9357$; DMSO-methiocarb: $p < 0.001$; DMS0-mixture: $p < 0.001$; PS-MP-mixture: $p < 0.001$; methiocarb-mixture: $p = 0.8531$). The activity of the CbE-pnpa was not altered by PS-MP alone. Methiocarb alone reduced the activity compared to the solvent control by 42%, the mixture by 44%.

Even more pronounced was the inhibition of CbE with the substrate pnpv: Compared to the solvent control, the activity was reduced by 83% both in methiocarb exposed fish and in fish in the mixture treatment (Fig. 3; DMSO-exposure groups: Nested-ANOVA: d.f.=3/101, F = 726.11, $p < 0.0001$, DMSO-PS-MP: $p = 0.2175$; DMSO-methiocarb: $p < 0.0001$; DMSO-mixture: $p < 0.0001$; PS-MP-mixture: $p < 0.0001$; methiocarb-mixture: $p = 0.7609$). Again, PS-MP alone had no influence on the activity of CbE-pnpv. Beside the clear effect of the different treatments, the treatment nested in block had a significant effect (F = 2.22, $p = 0.0322$).

3.6. Histopathology

3.6.1. Liver

Healthy liver tissue consists of large bright cells containing high amounts of glycogen (Fig. 4A and 4B). In fish exposed to methiocarb alone or the mixture treatment, a reduction of the glycogen stores became obvious (Fig. 4D) resulting in atrophic cells and enlarged intercellular spaces. Furthermore, in both groups containing methiocarb vacuolization and inflammations occurred cumulatively, and in some cases, even small zones with karyopycnosis and focal necrosis were found (Fig. 4C). Semi-quantitative analyses showed a very similar condition of fish from the control, solvent control and exposure with PS-MP, whereas livers of fish exposed to methiocarb or the mixture treatment showed significantly stronger reactions (Fig. 5, DMSO-exposure groups: likelihood-ratio: n = 69, d.f.=9, $X^2 = 63.46$, $p < 0.0001$; DMSO-PS-MP: $p = 0.0577$; DMSO-methiocarb: $p < 0.0001$; DMSO-mixture: $p < 0.0001$; PS-MP-mixture: $p < 0.0001$; methiocarb-mixture: $p = 0.2396$).

Figure 5: Semi-quantitative analyses of liver sections of juvenile brown trout exposed for 96 h to PS-MP and methiocarb as well as a mixture of both. Category 1 represents an excellent health status, 3 a reaction status and 5 a destruction status. 2 and 4 are intermediary classes. No section was assigned to category 5. Statistical comparison showed significantly more reactions in liver samples of methiocarb or methiocarb plus PS-MP exposed fish. Different letters indicate significant differences (DMSO-exposure groups: likelihood-ratio: n=69, d.f.=9, $X^2=63.46$, $p<0.0001$; DMSO-PS-MP: $p=0.0577$; DMSO-methiocarb: $p<0.0001$; DMSO-mixture: $p<0.0001$; PS-MP-mixture: $p<0.0001$; methiocarb-mixture: $p=0.2396$).

3.6.2. Gills

Observed pathological alterations in gills of brown trout included hyperplasia and hypertrophy of chloride and pillar cells resulting, in some cases, in lamellar fusion (Figure 6). Furthermore, an increase of mucus secreting cells, lifting of epithelia, oedema and even necrosis in small areas occurred. Most of these

symptoms occurred in the methiocarb and the mixture treatment groups. Semi-quantitative analysis revealed a significantly worse condition of the two treatment groups containing methiocarb compared to the solvent control and fish exposed solely to PS-MP (Figure 7; DMSO-exposure groups: likelihood-ratio: n=115, d.f.=9, X^2=61.19, p<0.0001; DMSO-PS-MP: p=0.4562; DMSO-methiocarb: p<0.0001; DMSO-mixture: p<0.0001; PS-MP-mixture: p<0.0001; methiocarb-mixture: p=0.7266). Gill sections were classified in categories 1-4 of 5 categories in total. No difference was found between the control groups and the group containing solely PS-MP.

4. Discussion

In the present study, the effects of PS-MP and the pesticide methiocarb either applied alone or as their binary mixture were investigated in juvenile brown trout.

4.1. General considerations concerning the conducted experiment

The measured methiocarb concentrations corresponded to approximately half of the nominal concentration in the methiocarb and mixture groups at the beginning of the experiment and 24% (methiocarb group) and 31% (mixture group) at the end. Methiocarb has a comparable low persistence in laboratory aerobic soil-water systems (Arena et al. 2018). The degradation of methiocarb is pH dependent and higher under alkaline conditions. The main metabolites are methiocarb phenol and methiocarb sulfoxide phenol. The dissipation half-life (DT_{50}) of methiocarb in water at pH 7 is 24 days and 0.21 days at pH 9 (EFSA 2006). Thus, a degradation of methiocarb in this experiment was likely, but occurred to a greater extend as it could have been assumed in an environment with a pH of 7.3. During the experiment, beside the abiotic degradation, a metabolization by the fish may have contributed to the decreased methiocarb concentration after 96 h. In humans, mainly cytochrome p450 and flavin-containing monooxygenases in liver and kidney contribute to the metabolism of methiocarb (Usmani et al. 2004, Furnes and Schlenk 2005). For longer exposure to methiocarb regular exchange of the water with freshly prepared methiocarb solutions is recommended.

4.2. Do PS-MP affect juvenile brown trout?

In juvenile brown trout none of the investigated parameters were affected by 10^4 polystyrene particles/L. In the past, MP were found to cause oxidative stress in different organisms like in monogonont rotifers (Jeong et al. 2016), nematodes (Lei et al. 2018), mussels (Avio et al. 2015, Magni et al. 2018) or fish (Lu et al. 2016, Paul-Pont et al. 2016, Qiao et al. 2019). In contrast to these results, other studies found either no effects (Oliveira et al. 2013, Luís et al. 2015, Fonte et al. 2016) or effects in only some of the investigated endpoints for oxidative stress (Avio et al. 2015, Ferreira et al. 2016, Ding et al. 2018, Magni et al. 2018). The oxidative defense system is complex and consists of enzymatic (e.g. SOD, catalase (CAT), glutathione reductase) and non-enzymatic compounds (Lushchak 2016). In addition, the types of the investigated polymers, their concentrations, their potential additives as well as the size of the used MP

highly vary in the different studies. This complexity is reflected by the literature: as for example, the activity of CAT was found to be increased (Lu et al. 2016, Qiao et al. 2019) as well as decreased (Paul-Pont et al. 2016) or remained unchanged (Chen et al. 2017, Guilhermino et al. 2018) after exposure to MP. Jeong et al. (2016) analyzed the influence of the particle size on oxidative stress responses and showed that there is a clear connection to this parameter. In general, however, the database is still far too limited to decide on whether or not MP cause oxidative stress and, in case of any influence, which pathways are affected.

To the best of our knowledge, the present study is the first that investigated potential proteotoxic effects of MP. In the tested concentration, no effect of PS-MP on the stress protein level (Hsp70) was found in brown trout after 96 h exposure.

Also, the activity of AChE and two investigated CbE were not altered after exposure to PS-MP. In the past several studies reported significant reductions about 20% of AChE activity in common goby (Pomatoschistus microps) after exposure to 184 µg/L polyethylene (PE; 1–5 µm) for 96 h (Oliveira et al. 2013, Luís et al. 2015, Fonte et al. 2016). Under the same test conditions, Ferreira et al. (2016) only found a reduction of AChE activity by 13% in common goby. In red tilapia (Oreochromis niloticus) an even higher inhibition of AChE by 37.7% was found after exposure to PS nanoplastics (0.1 µm) in concentrations of circa 1.8×10^6, 1.8×10^7, 1.8×10^8 particles/L for 14 days (Ding et al. 2018). Chen et al. (2017) did not find any neurotoxic effect of PS-MP (45 µm, 20 particles/mL) in zebrafish (Danio rerio) but reported a significant reduction of AChE activity by 40% after exposure to PS nanoplastics (50 nm, 1.5×10^{10} particles/mL). Furthermore, a decrease of AChE activity in Amazonian discus fish Symphysodon aequifasciatus was found after exposure to 200 µg/L fluorescent PE (70–88 µm) for 30 days (Wen et al. 2018). Reduction of AChE was not only observed in fish: in the crab Eriocheir sinensis exposed to 40 µg/L PS microspheres (5 µm) for 21 days AChE activity was significantly reduced (Yu et al. 2018). An exposure of freshwater clam Corbicula fluminea to 0.2 mg/L caused an inhibition of 31% AChE activity, but a concentration of 0.7 mg/L only led to a non-significant reduction of 19% (Guilhermino et al. 2018). Avio et al. (2015) found no effect in the heamolymph of Mytilus galloprovincialis on AChE activity after exposure to PE and PS (< 100 µm 7 days 1.5 g/L) but an AChE activity decrease in gills. In peppery furrow shell Scrobicularia plana a reduction of the AChE activity in response to PS-MP exposure (20 µm, 1 mg/L) was not only found after 14 d of exposure but also still after a depuration period of 7 days (Ribeiro et al. 2017). In contrast, a study of Magni et al. (2018) on the neurotoxic effects of a mixture of PS microbeads (with 5×10^5 particles/L – 10 µm and 5×10^5 particles/L – 1 µm) on the zebra mussel (Dreissena polymorpha) showed no neurotoxic effect in 6 days despite of an increase in the dopamine amount. Other investigated parameters were serotonin, glutamate, AChE and monoamine oxidase. Magni et al. (2018) suggested that the increased dopamine level reduces the intake of MP or improves their elimination. In general, the mode of action how MP might cause neurotoxicity remains unclear. Besides the type of the polymer (including different additives) also the particle size seems to be of importance in this context, since neurotoxic effects were mainly found in studies using very small micro- or even nanoplastics. Furthermore, Ding et al. (2018) reported accumulation of nanoplastics in the brain of red

tilapia. These findings are supported by a study of Mattsson et al. (2017) who demonstrated that polystyrene nanoplastics are capable to penetrate the blood-brain barrier of Crucian carp (Carassius carassius). Since the plastic particles we used in the present study were < 50 µm with an unknown number of very small particles, possibly the larger particles were dominant in our experiment which might explain the lack of influences in neurotoxicity.

In the present study, also no alterations in the histopathological status of the liver and gills were found in fish exposed to PS-MP alone when compared to the solvent control group. Also Lei et al. (2018), did not find any effect of PS-MP (0.1, 1.0 and 5 µm up to 10 mg/L) on tissue integrity of intestine, gills, kidney and liver of zebrafish after 10 days of exposure. Furthermore, besides effects on the intestine Lei et al. (2018) did not find any alterations in gills, liver and kidney of zebrafish exposed to polyamide (PA), polypropylene (PP), polyvinyl chloride (PVC) and PE (each ~ 70 µm in a concentration up to 10 mg/L) for 10 days. Moreover, low-density PE (125–250 µm) caused no alterations in liver of zebrafish after 3 weeks of exposure (Rainieri et al. 2018). In silver barb fry (Barbodes gonionotus) no histopathological reactions were caused by PVC (0.2, 0.5 and 1.0 mg/L; 0.1–1000 µm) fragments besides a slight thickening of the intestinal mucosal epithelium (Romano et al. 2018). Rochman et al. (2017) exposed Asian clams (Corbicula fluminea) to polyethylene terephthalate (PET), PVC, PE and PS MP for 28 days. Subsequently exposed clams were fed to white sturgeon (Aciper transmontanus). In clams, the only histological reactions were mild or moderate tubular dilation in digestive glands, while no effect of MP were found in liver and gastrointestinal tract of white sturgeon. In contrast, Lu et al. (2016) observed early inflammation responses as well as lipid droplets in the liver of zebrafish after exposure to 5 µm polystyrene particles in a concentration of 2.9×10^4 particles/L for three weeks. In Japanese medaka (Oryzias latipes) severe glycogen depletion and fatty vacuolization in the liver occurred, but no alterations were found in gonads after exposure to PE for 2 months (Rochman et al. 2013, Rochman et al. 2014). Karami et al. (2016) exposed African catfish (Clarias gariepinus) to low-density PE (< 60 µm) for 96 h. They observed hyperplasia and sloughing and even necrosis in gills at a concentration of 50 µg/L and even more severe reactions like desquamation of cells at a concentration of 500 µg/L. Additionally, the degree of tissue damage in the liver of the fish was increased after exposure to low-density PE in a concentration of 500 µg/L (Karami et al. 2016). In general, there is no evidence for a uniform pattern under which conditions histopathological changes may occur after exposure to MP.

Reports of the environmental concentration of MP in surface waters of a size < 50 µm are rather scarce possible due to difficulties with the sampling and detection methodology (de Sá et al. 2018). Nevertheless, reported results of MP indicate higher concentrations of smaller MP compared to larger ones (Triebskorn et al. 2019). Karlsson et al. (2017) found a concentration of MP particles of 27 particles/L (> 30 µm) at the coast of the North Sea (Netherlands). In canals of Amsterdam even higher concentrations of MP (> 10 µm) between 48 and 187 particles are reported (Leslie et al. 2017). In the Chinese river Yangtze MP concentrations between 0.5 and 3.1 particles/L (> 20 µm) were found (Su et al. 2018). In the German river Elbe concentrations of MP particles as high as 1000 and 9000 particles/L were detected (Triebskorn et al. 2019). In the present study the concentration of PS particles was 10^4 and

therefore higher than the environmental concentration. Our study does not indicate a risk for brown trout at environmental concentrations. Nevertheless, to exclude potential negative effects of MP on brown trout other experiments with longer exposure time and other polymer types are necessary.

4.3. Does methiocarb affect juvenile brown trout?

Three fish exposed to methiocarb as sole pollutant showed strong behavioral reactions after 24 h and had to be euthanized. After 96 h, also all other fish exposed to methiocarb exhibit behavioral abnormalities like slower swimming and reduced escape behavior. To the best of our knowledge, this is the first study that investigated the effects of methiocarb on brown trout. In the present study weight of methiocarb-exposed fish were comparable to the control group. However, 96 h is a relative short time to observe changes regarding this endpoint in brown trout.

The observed reactions of fish can be seen as a consequence of the AChE inhibition by methiocarb. Acetylcholine (ACh) accumulates in the synaptic cleft leading to a cholinergic crisis (Rosman et al. 2009). In the present study, methiocarb led to a reduction of the AChE activity by 59% in the tested juvenile brown trout. The mode of action of carbamate pesticides is based on carbamylation of AChE and, thereby, inhibition of its ability to hydrolyze acetylcholine (Fukuto 1990). Therefore, it could have been expected that methiocarb reduces the activity of AChE also in brown trout. Comparable effects were observed by Essawy et al. (2008) in the land snail Eobania vermiculata in which AChE activity was reduced up to 69.3% by methiocarb. Carboxylesterases play an important role in pesticide detoxification (Wheelock et al. 2005). Sanchez-Hernandez et al. (2009) suggested that CbE might act as biochemical barrier for organophosphate pesticides in Lumbricus terrestris. Maymó et al. (2006) found that esterase activity is higher in western flower thrips (Frankliniella occidentalis) with increased resistance against methiocarb. To our knowledge no studies about the effect of methiocarb on AChE and CbE activities in fish were performed up to date. However, in the present study, CbE as well as AChE was inhibited by methiocarb and no protective effect became obvious. This might possibly be related to the fact that the methiocarb concentration was rather high and all three enzymes were inhibited and the protective effect of CbE ceased.

Samples of brown trout showed neither an increase of SOD nor an altered amount of LPO exposure to methiocarb. An increase of LPO and an alteration of reduced glutathione level was found in male Wistar rats fed with 2, 10 and 25 mg/kg methiocarb (Ozden and Alpertunga 2010). Ozden and Alpertunga (2010) found the highest malondialdehyde level in the brain and explained their finding by the comparably large amount of fatty acids in this organ. In another study, Ozden et al. (2012) found also an increase of glutathione as well as of the activities of SOD, CAT and glutathione peroxidase in male Wistar rats after administration of 25 mg/kg methiocarb.

To our knowledge no one has analyzed the effect of methiocarb on stress proteins so far, and rarely proteotoxic effects of carbamate pesticides were studied in general. Seleem (2019) found an increase of Hsp70 in Arabian toad (Bufo arabicus) after exposure of tadpoles to the carbamate pesticide methomyl. Bierkens et al. (1998) investigated the effect of carbaryl on the Hsp70 level of the microalga Raphidocelis

subcapitata. Hsp70 responded in a dose-dependent manner to carbaryl, with a lowest observed effect concentration of 10.3 µM. Moreover, the carbamate pesticides formetanate, methomyl and pirimicarb induced an overexpression of the chaperone GRP78 and reduced Hsp27, Hsp72/73 and Hsp90 levels in human cell cultures (Skandrani et al. 2006). However, in the present study, methiocarb did not cause any alteration of Hsp70 level of juvenile brown trout.

After exposure to methiocarb, prominent histopathological alterations became evident in livers and gills of the exposed fish. Altinok et al. (2006) observed similar effects in gills of rainbow trout exposed to 3.75 and 7.5 mg/L methiocarb. After 96 h, symptoms like lamellar edema, lifting of epithelia, telangiectasis, increased cytoplasmic granularity and lamellar fusion occurred. Effects were reversible in concentrations below 3.75 mg/L. In the fish livers Altinok et al. (2006) found necrosis. Altinok et al. (2006) assumed that the alterations are caused by ionic imbalance due to inhibition of AChE activity. In a follow up study, Altinok and Capkin (2007) found no histopathological alterations in liver, kidney, brain and spleen of rainbow trout after exposure to 2.5 or 3.75 mg/L methiocarb for 21 days. However, in gills of rainbow trout lamellar lifting occurred when fish were exposed to 3.75 mg/L methiocarb for 21 days (Altinok and Capkin 2007). Brown trout seem to be more sensitive to methiocarb than rainbow trout as indicated by the more severe effects in the present study. This was also shown in the past for other environmental stressors (Schmidt-Posthaus et al. 2001).

The tested methiocarb concentration (1 mg/L) was considerably higher than the average surface water concentration of 6–40 ng/L in an EU wide monitoring campaign (Loos et al. 2018). The study was designed to investigate acute effects of methiocarb and a potential modulation of these effects by PS-MP. Nevertheless, the strong acute effects of methiocarb in brown trout elucidate the need for experiments in which brown trout are exposed to methiocarb at environmentally relevant concentrations for a longer exposure time. Such experiments would allow to assess the current risk of methiocarb exposure for brown trout in the environment.

4.4. Do PS-MP modulate the effects of methiocarb?

When considering all investigated endpoints, fish exposed to the mixture of methiocarb and PS-MP showed the same reactions as fish exposed to methiocarb solely. Weight as well as the stress protein level and the level of oxidative stress remained unchanged. However, the activity of AChE and CbE were significantly reduced in the mixture to almost the same extent as caused by methiocarb alone. Furthermore, the observed histopathological alterations in liver and gills in fish of the mixture treatment were comparable to those found in fish exposed solely to the pesticide. Thus, the toxicity of methiocarb on brown trout was not modulated by PS-MP. To the best of our knowledge, the interaction of carbamate pesticides and MP have not been investigated before. In their review de Sá et al. (2018) identified only one out of 59 studies where no interaction between MP and another tested contaminant was found. Ferreira et al. (2016) reported, that the effects of gold nanoparticles were not modulated by PE particles (1–5 µm). Of course, de Sá et al. (2018) could not account in their meta-analysis for the probable bias against publishing negative results. Multiple studies show that effects of different pollutants were decreased in combination with MP. For example, PS-MP alleviated the effects of 17 α-ethinylestradiol

(EE2) on locomotion in zebrafish (Chen et al. 2017). Similar results were found after exposure of zebrafish larvae to EE2, phenanthrene and a mixture of both with PVC particles. In the presence of PVC particles, the expression of cytochrome P4501A and vitellogenin was reduced up to 48% for EE2 and by 33% for phenanthrene (Sleight et al. 2017). Furthermore, in Daphnia magna PA particles reduced the immobilization caused by bisphenol A (BPA) compared to an exposure of BPA alone. BPA in water appeared to be the most bioavailable fraction for daphnids compared to the BPA adsorbed to MP (Rehse et al. 2018). In general, a reduced toxicity of co-contaminants in the presence of MP might be explained by a decreased bioavailability of the chemicals due to sorption to the plastic particles. In the present study, chemical analyses revealed that the concentration of methiocarb was higher in the mixture treatment than in the groups exposed to methiocarb alone after 97 h. Thus, it is probable, that methiocarb did not sorb in considerable amounts to the plastic microparticles. In contrast to reports on a reduction of chemical-induced effects by MP, other studies conducted with MP in combination with chemicals found an intensification of such effects. For example, in common carp (Cyprinus caprio), MP increased the effects of paraquat on biochemical blood parameters. Thereby, higher concentrations of MP increased the toxicity (Nematdoost Haghi and Banaee 2017). Moreover, Fonte et al. (2016) found a significant interaction between PE-MP and cefalexin. In the mixture of both, the effect of cefalexin on the predatory performance of common goby was increased at 20 °C, but reduced at 25 °C. In the freshwater clam (Corbicula fluminea) the oxidative stress level and the AChE inhibition of the antimicrobial florfenicol was increased in a mixture with MP. Furthermore, the mixture led to a feeding inhibition and reduction of isocitrate dehydrogenase suggesting interactions of florfenicol and MP since a simple additive approach could not explain the observed effects (Guilhermino et al. 2018). In the present study the toxicity of methiocarb was not enhanced by MP. Compared to the uptake pathway via the water, PS-MP seemed to have a negligible effect on the uptake of methiocarb in juvenile brown trout.

5. Conclusion

Based on the results of our study, we conclude that methiocarb heavily impairs the health of brown trout whereas the studied PS-MP in a concentration of 10^4 particles/L do not. It can also be excluded that the studied PS-MP modulate methiocarb-induced effects in brown trout. In general, literature provides a diverse and inconsistent image with respect to the capacity of MP to modulate the toxicity of environmental chemicals. Although our study does not speak for an environmental risk related to the investigated polystyrene particles and their interaction with the pesticide methiocarb, this study provides only a very small piece of knowledge for a defined type and size class of plastics and a single pesticide, and emphasizes the need of further research in this field.

6. List Of Abbreviations

AChE	acetylcholinesterase	LC_{50}	lethal concentration 50%
ABS570	absorbance 570 nm	MiWa	microplastics in the water cycle
BPA	bisphenol A	MP	microplastics
CAT	catalase	PA	polyamide
CbE	carboxylesterase	PE	polyethylene
CHP	cumene hydroperoxide	PET	polyethylene terephthalate
EE2	17 α- ethinylestradiol	PS	polystyrene
Equiv	equivalents	PP	polypropylene
LOD	limit of detection	PVC	polyvinyl chloride
LPO	lipid peroxidation	SOD	superoxide dismutase

7. Declarations

7.1. Ethics approval and consent to participate

The experiment was approved by the animal welfare committee of the regional council of Tübingen, Germany (authorisation number ZO 2/16).

7.2. Consent for publication

Not applicable

7.3. Availability of data and material

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

7.4. Competing interests

The authors declare that they have no competing interests.

7.5. Funding

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7.6. Authors' contributions

HS wrote the manuscript except for section 4.2., which was written by SH. She contributed to the design of the experiment, conducted the experiment, analysed (or supervised analyses) of stress-proteins and oxidative stress. SH performed the chemical analysis and revised the whole manuscript. SK and TPK revised the entire manuscript. FR analysed the level of lipid peroxidation as well as the acetylcholinesterase and carboxylesterase activity. KR performed the histopathological analysis and parts of the analysis of the SOD activity; ASR provided the microplastic particles and revised the whole manuscript, HRK and RT designed the experiment, contributed to the interpretation of data and revised the entire manuscript

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Figures

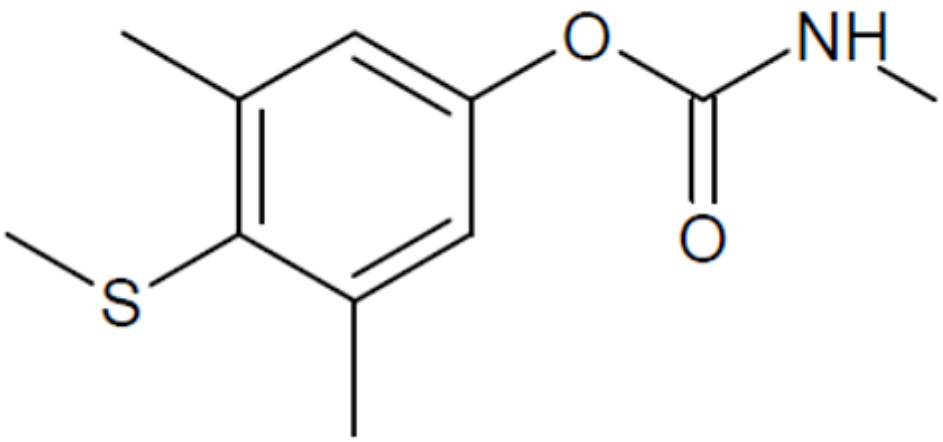

Figure 1

Structure of methiocarb

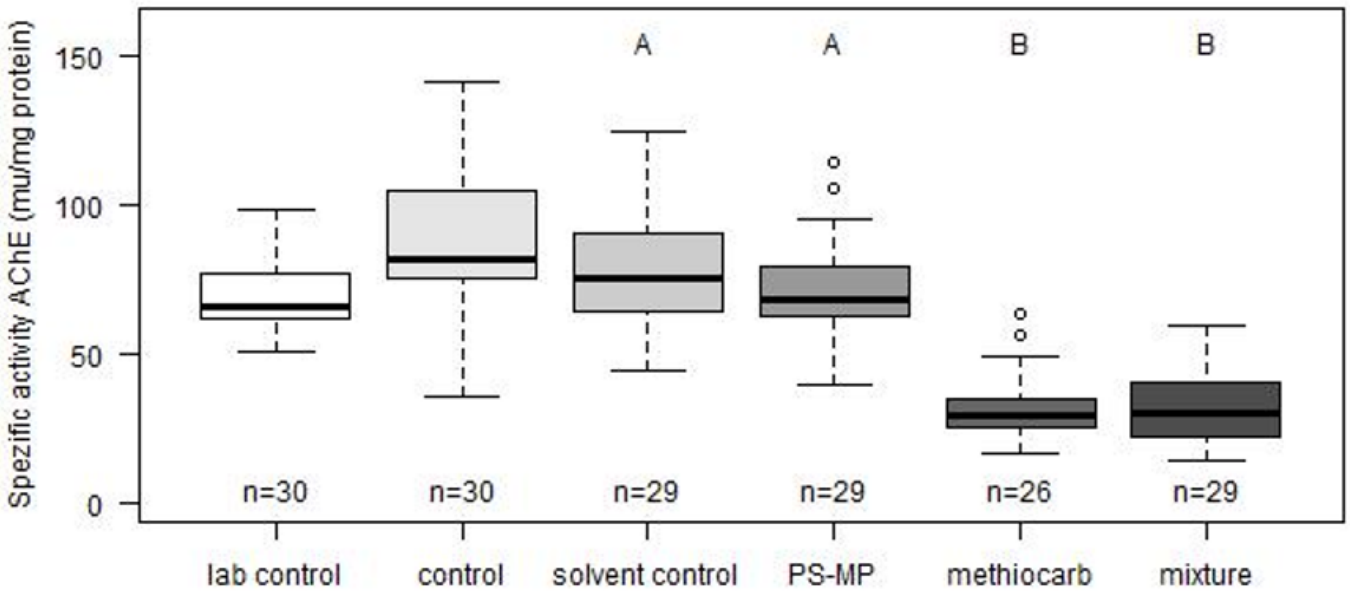

Figure 2

Figure 1: Specific activity of acetylcholinesterase in muscular tissue of brown trout. The box plots display the median, the 25th and 75th quantiles as well as minimum and maximum values (whiskers), the dots indicate outliers. Different letters indicate significant differences. Methiocarb and mixture significantly reduced the AChE activity (DMSO-exposure groups: Nested-ANOVA: d.f.=3/105, F=83.65, p<0.0001).

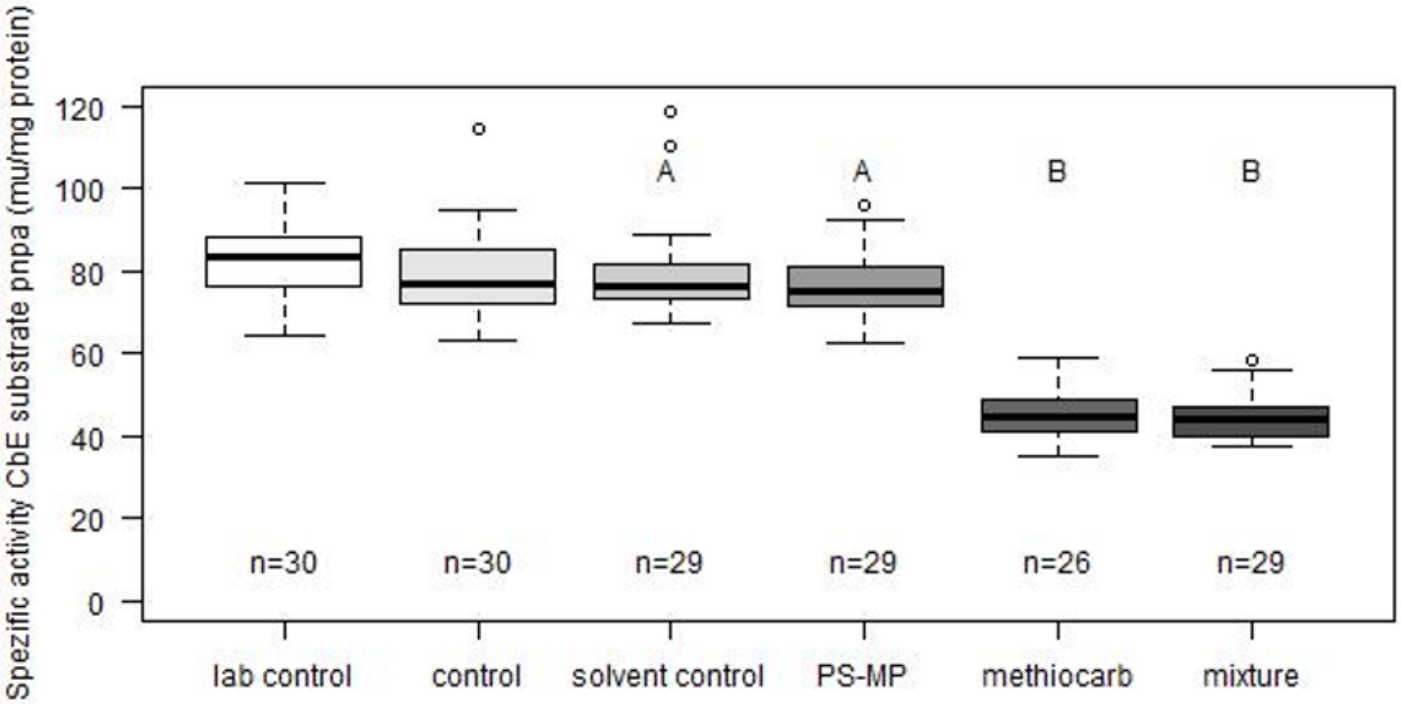

Figure 3

Figure 2: Specific activity of carboxylesterase with substrate pnpa in muscular tissue of brown trout. The box plots display the median, the 25th and 75th quantiles as well as minimum and maximum values (whiskers), the dots indicate outliers. Different letters indicate significant differences. Methiocarb and mixture significantly reduced the activity of the CbE-pnpa (DMSO-exposure groups: Welch ANOVA: d.f.=3/57, F=192.15, p<0.0001).

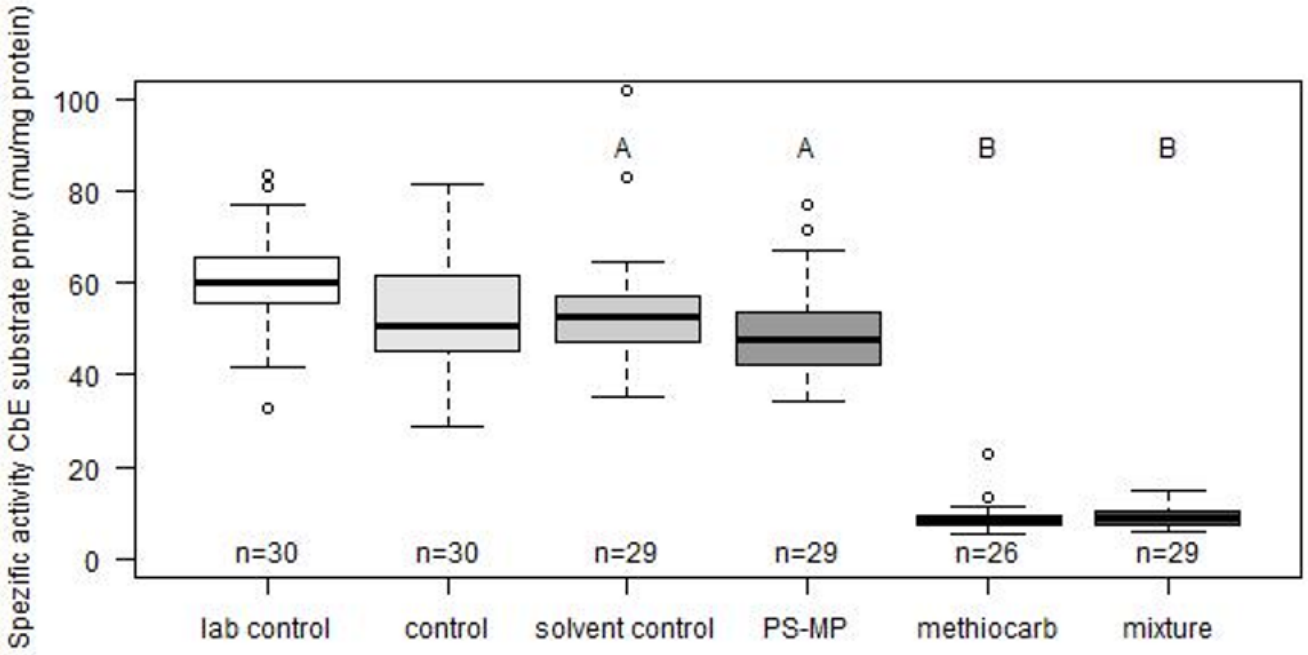

Figure 4

Figure 3: Specific activity of carboxylesterase with substrate pnpv in muscular tissue of brown trout The box plots display the median, the 25th and 75th quantiles as well as minimum and maximum values (whiskers), the dots indicate outliers. Different letters indicate significant differences. Methiocarb and mixture significantly reduced the CbE-pnpv activity (DMSO-exposure groups: DMSO-exposure groups: Nested-ANOVA: d.f.=3/101, F=726.11, p<0.0001).

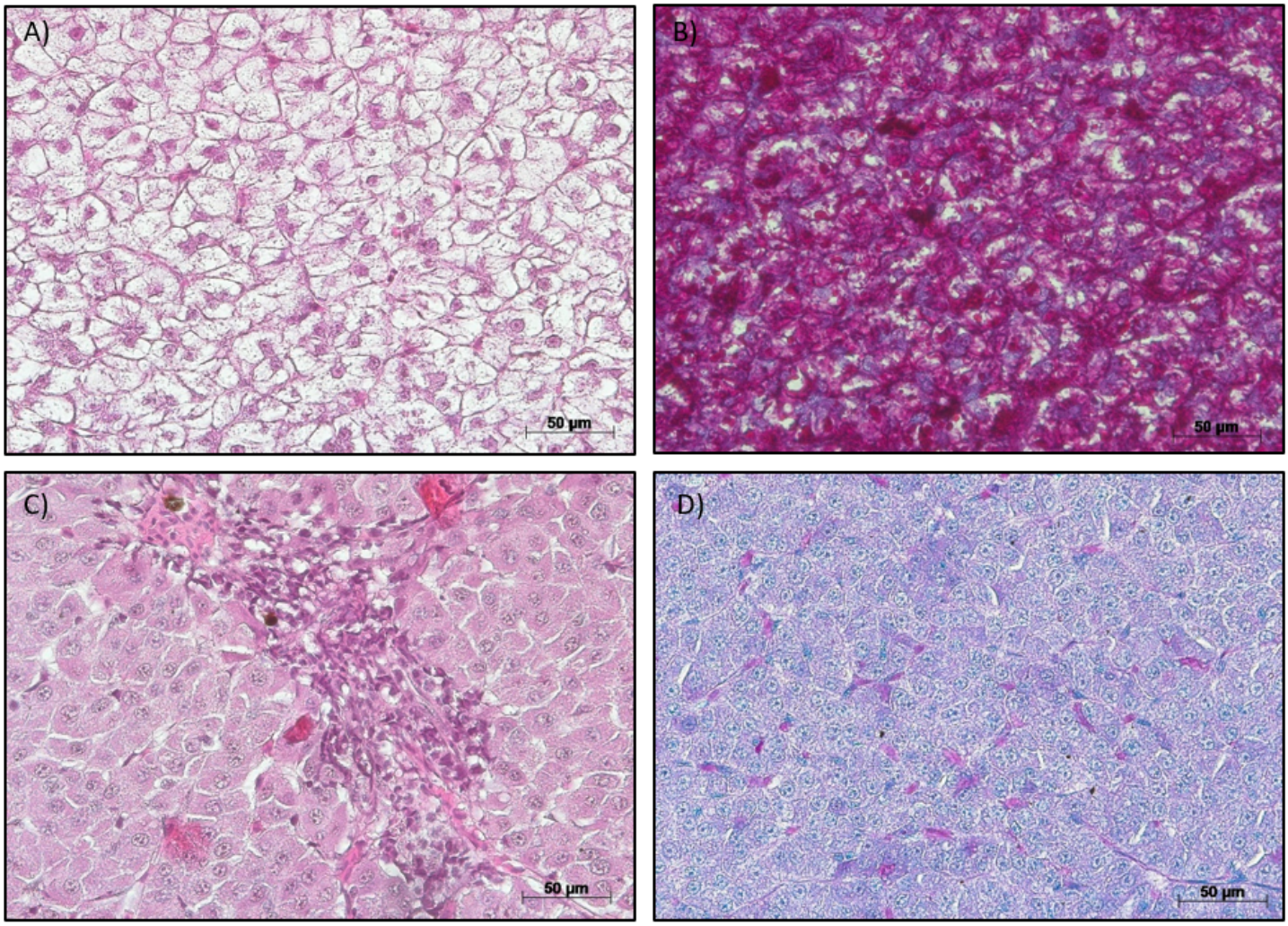

Figure 5

Figure 4: Control status of liver of juvenile trout with homogenous tissue with large hepatocytes (A). The cells contain a huge amount of glycogen (B). Occurring reactions were inter alia inflammations, vascular dilation and focal necrosis (C). Furthermore, reduced glycogen amount and increased intercellular spaces were found (D). A and C: haematoxylin-eosin staining; B and D: alcian blue-PAS staining.

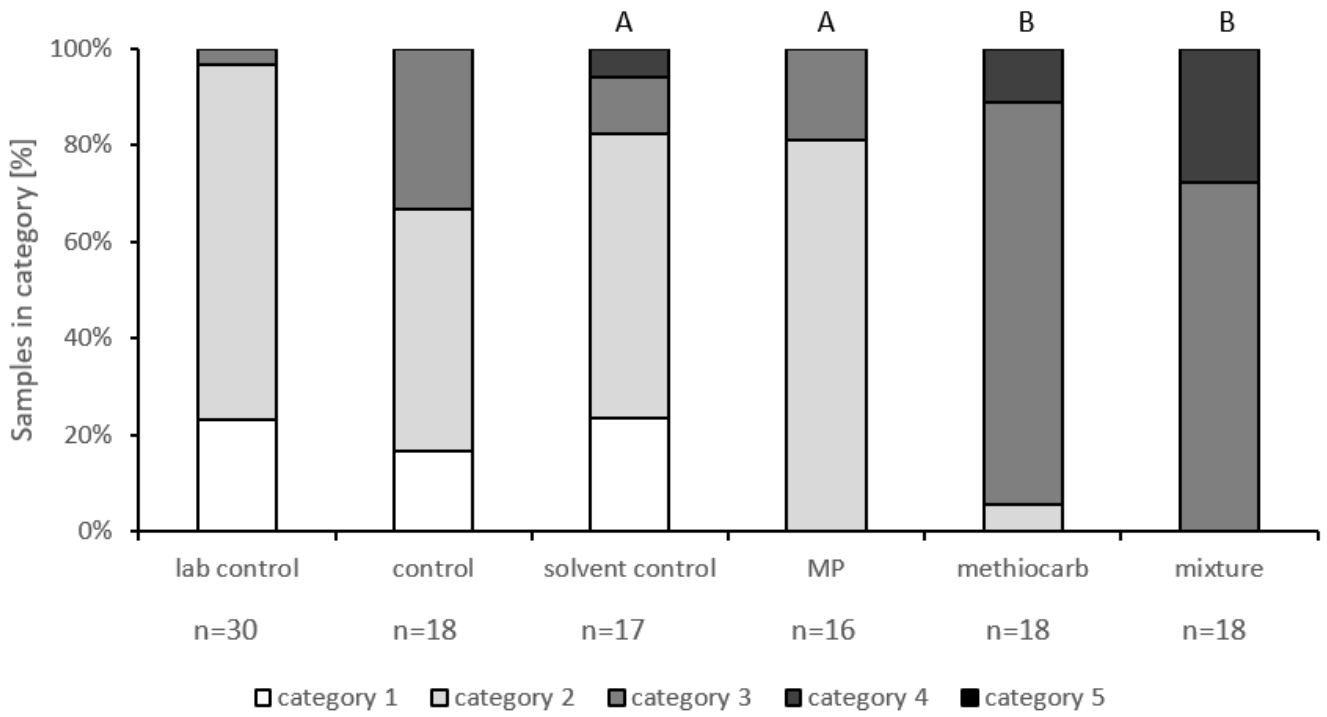

Figure 6

Figure 5: Semi-quantitative analyses of liver sections of juvenile brown trout exposed for 96 h to PS-MP and methiocarb as well as a mixture of both. Category 1 represents an excellent health status, 3 a reaction status and 5 a destruction status. 2 and 4 are intermediary classes. No section was assigned to category 5. Statistical comparison showed significantly more reactions in liver samples of methiocarb or methiocarb plus PS-MP exposed fish. Different letters indicate significant differences (DMSO-exposure groups: likelihood-ratio: n=69, d.f.=9, X2=63.46, p<0.0001; DMSO-PS-MP: p=0.0577; DMSO-methiocarb: p<0.0001; DMSO-mixture: p<0.0001; PS-MP-mixture: p<0.0001; methiocarb-mixture: p=0.2396)

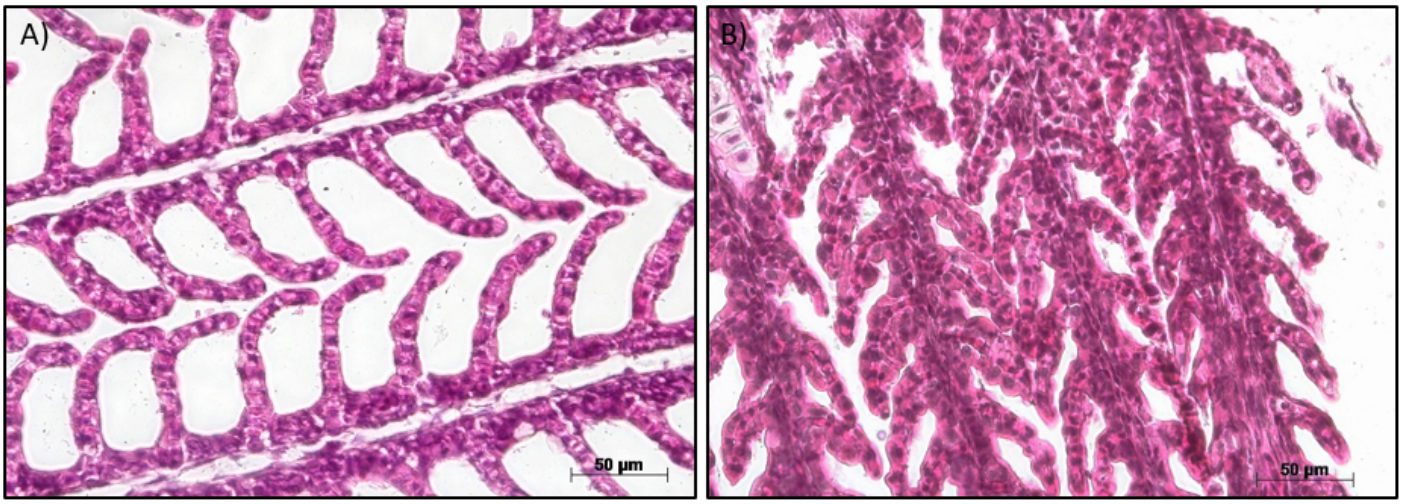

Figure 7

Figure 6: Control status of gills of juvenile trout with regular shaped secondary lamellae (A). Reaction status of gills showing hypertrophy and hyperplasia of cells, lamellar fusion reactions as well as oedema (B). A and B: haematoxylin-eosin staining.

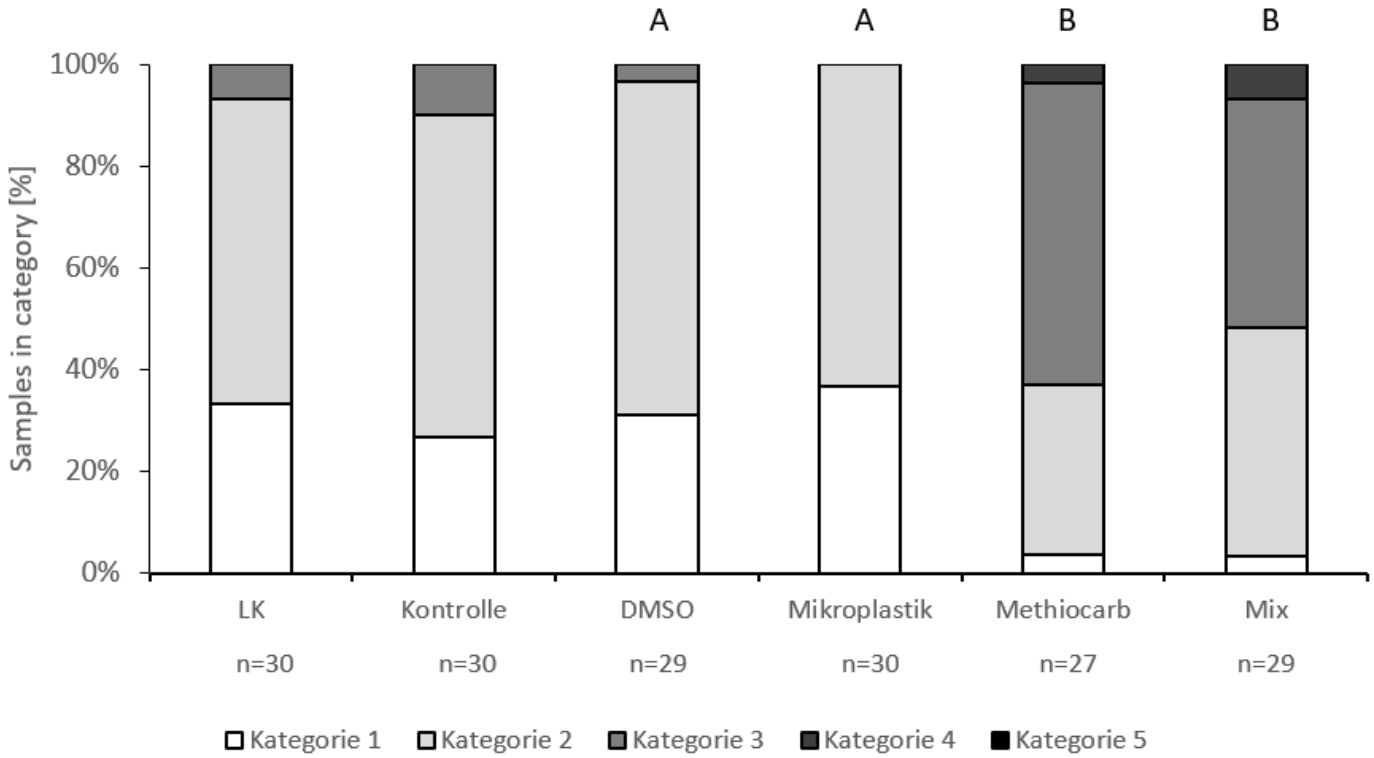

Figure 8

Figure 7: Semi-quantitative analyses of gills of juvenile brown trout exposed for 96 h to PS-MP and methiocarb as well as a mixture of both. Category 1 represents an excellent health status, 3 a reaction status and 5 a destruction status. 2 and 4 are intermediary classes. No section was assigned to category 5. Different letters indicate significantly differences. Statistical comparison showed a significant worse condition of both treatment groups containing methiocarb compared to solvent control (DMSO-exposure groups: likelihood-ratio: n=115, d.f.=9, X2=61.19, p<0.0001; DMSO-PS-MP: p=0.4562; DMSO-methiocarb: p<0.0001; DMSO-mixture: p<0.0001; PS-MP-mixture: p<0.0001; methiocarb-mixture: p=0.7266).

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