Microplastics and Nanoplastics

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Combined proteomic and gene expression analysis to investigate reduced performance in rainbow trout (*Oncorhynchus mykiss*) caused by environmentally relevant microplastic exposure

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Abstract

The pollution of the environment with microplastics (MPs) is affecting aquatic organisms worldwide, and yet intensive research, has thus far failed to deliver an adequate understanding of the detrimental effects of MP ingestion by fish. Investigations using established health and performance parameters are often insufficient to determine MP toxicity, especially when considering MPs in environmentally relevant concentrations. In the present study, label-free quantitative (LFQ) proteomics of liver tissue was combined with gene expression analysis in order to investigate the long-term effects of MP exposure on rainbow trout (*Oncorhynchus mykiss*). With the help of a specially designed diet, two groups of fish were exposed for 120 days to environmentally relevant concentrations of MPs (on average 13 particles per fish, every 2 days) and to slightly increased levels representing those expected in the near future (on average 73 MP particles per fish, every 2 days). Both groups were compared to a control. The results provide evidence that long-term exposure to MPs has a dose-dependent negative effect on the performance of rainbow trout. No differences in blood glucose level, hematocrit level or lipid peroxidation were observed between treatments. The proteomic analysis revealed 6071 unique proteins, but no significant change in hepatic protein concentrations compared to their matching controls, although certain proteins appear to have been up- or down-regulated multifold and should be considered in continuing experiments. When comparing highly regulated proteins with the levels of their respective mRNA transcripts, a good correlation was observed just for "differentially regulated trout protein 1", encoded by *drtp1*. This may therefore be a suitable biomarker for future studies with trout. Several hypotheses were put forward to explain the observed differences in growth: nutrient dilution, caused by increased amounts of non-digestible material in the diet, and growth effects due to differences in diet quality could be excluded. Physical interference of MPs with the gastrointestinal tract are also unlikely, as fish are regularly exposed to particulate matter in natural environments and previous studies did not find evidence of such interferences. Instead, indirect detrimental effects of MPs, either due to their hydrophobic surface properties or the presence of certain additives, could cause allergic reactions, microbiota dysbiosis or general stress responses. Although no clear cause for the reduced growth was identified, the current study demonstrates the potential utility of omics approaches when dealing with such a complex question. Future

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studies should extend analyses to the gastrointestinal tract and associated tissues. It should be ensured that the MP exposure is realistic and that the duration of the experiments covers several months. Direct evidence of a significant negative influence of long-term exposure to realistic and near-future MP concentrations on fish highlight the importance of measures to prevent a further increase of MPs in the environment.

Keywords: Liver proteome, Omics, Health effects, Freshwater, Specific growth rate, Food conversion rate, Oxidative stress

Introduction

Pollution with microplastics (MPs) is widely recognized as an emerging threat to the global environment [1], with longevity in the environment and an exponential increase in plastic production over the last eight decades combined [2], resulting in a rapid accumulation of MPs in rivers, lakes and the ocean [3, 4]. The myriad pathways by which these pollutants enter the environment are not completely understood but are known to include direct inputs via wastewater [5] and via the mechanical and chemical degradation of larger debris [6, 7]. MPs are usually defined as particles of plastic smaller than 5 mm [8], and are therefore small enough to be ingested by a wide range of aquatic organisms [9]. Numerous studies have shown that MPs are taken up by a variety of fish species in both freshwater and marine systems, where they are primarily detected in the gastrointestinal tract [10, 11]. MPs are ingested either accidentally when foraging or indirectly with other food already containing plastic particles [12]. In some cases, MPs are also mistaken for natural food [13]. Research has addressed the detrimental effects of MPs on fish early in the history of MP pollution studies. However, the results obtained so far are often contradictory, with some studies failing to demonstrate any negative impact, while others highlight a variety of adverse health effects for fish (for a review, see: [10, 14]).

A major issue when evaluating potential adverse health effects of MP in laboratory experiments is that the concentrations of MPs applied tend to be much higher than those occurring routinely in the environment. Often the discrepancy is greater than two orders of magnitude, sometimes as many as seven, making it difficult to extrapolate the results and inferences of such studies to natural conditions are potentially leading to biased conclusions [15, 16]. The size of the particles also plays a decisive role. Very small particles, for example can pass through the intestinal wall, migrate into organs like the liver [17, 18] and therefore cause a different pathology to larger particles restricted to the intestinal passage. These and other factors make it difficult to assess the actual harmfulness of microplastics to fish [19].

A variety of different approaches have been applied to study the detrimental effects of pollutants on aquatic organisms. Generic baseline parameters such as growth and mortality [20], are often considered alongside other established stress parameters such as levels of cortisol or heat shock proteins or evidence for lipid oxidation [21, 22]. However, conflicting findings to date suggest that these parameters might not be sensitive enough to indicate the physiological effects of MP concentrations currently found in the environment. For this reason, recent studies have begun to adopt more sophisticated molecular biological approaches, including omics techniques [23, 24]. A striking advantage of mass spectroscopy when searching for a needle in a haystack is that the technique permits simultaneous identification of thousands of proteins in a sample, up to the analysis of the entire proteome [25]. The identities and abundances of the various proteins may then suggest pathways by which, for example, particular concentration differences, have come about, and may thus provide important insights into the effects of short- and long-term MP exposure.

To date, proteomic approaches have been used only sporadically when investigating the effects of MP exposure on aquatic organisms. A recently conducted proteomic analysis of blue mussels (*Mytilus edulis*) exposed to MPs for 52 days identified 40 differentially regulated proteins in the haemolymph compared to a control group [26]. These were involved in immune regulation, detoxification and other vital biological processes. Another proteomic study on zebra mussels (*Dreissena polymorpha*) suggested a threshold exposure level beyond which stimulation with MPs began to modulate the proteome in the short-term and furthermore pointed to high sensitivity of proteomic analyses [27]. So far, this approach has not been used to address potential MP impacts in fish. However, several studies have evaluated transcriptomic alterations in aquatic organisms exposed to MP and produced greatly varying lists of differentially regulated genes [28–30].

In the present study, a proteomic approach was trialed alongside established methods in an investigation of fish experiencing long-term exposure to environmentally relevant concentrations of MPs. Two groups of rainbow trout (*Oncorhynchus mykiss*), a widely used model organism for ecotoxicological studies [31], were exposed to MP concentrations and sizes currently encountered in wild fish and an increased concentration, expected to occur

in the near future [3, 32]. These groups were compared to a control group maintained in MP free conditions. The plastic was administered in a controlled manner using a specially designed diet. In addition to a selection of typical performance and stress parameters, the liver proteome was examined using a label-free quantification (LFQ) approach [33]. Proteins that exhibited obvious shifts in regulation were selected, and gene expression analysis was used to confirm and extend the results at the transcript level. Based on the results, different hypotheses on the impact of microplastics on fish were developed and possible biomarkers for future studies were considered.

Materials and methods

Husbandry, MP particle characteristics and experimental setup

Juvenile rainbow trout were acclimatized for 3 weeks in nine tanks (100 fish per tank, tank size: $50 \times 55 \times 55$ cm) and fed twice a day with a commercial dry feed (Inicio, BioMar, Brande, Denmark) and an experimental diet (Gesellschaft für Marine Aquakultur mbH, Büsum, Germany; for more details, see: [34]). All fish were held in a flow-through system (8 L/min water exchange rate) with an average water temperature of 8 °C and oxygen levels between 8 and 12 mg/L. Light intensity was around 300 lx, with 12 h illumination and 30 min transition periods simulating dawn and dusk. Water parameters were monitored continuously, and tanks were cleaned several times a day.

In the MP exposure experiment the fish were divided into three groups, two of which received the experimental diet modified to contain different concentrations of polymethylmethacrylate (PMMA) particles. PMMA is a widely used plastic with a variety of applications and is routinely present in the environment [35–37]. In order to simulate a realistic exposure situation in which irregular shaped fragments are a frequently occurring particle shape and the number of particles increases exponentially with smaller size [38], fluorescent PMMA granules (Glow-Side, Kretz, Germany) were crushed in a mortar grinder (Pulverisette 2, Fritsch, Germany) and then wet sieved to exclude particles smaller than 20 µm and larger than 1000 µm. The resulting fragments (Fig. 1 (A)) were then incorporated into the experimental feed matrix.

Two pellet sizes of the diet were used in the exposure experiment. The MP concentration was 2729 ± 1639 particles per gram diet (particle weight: 0.004 ± 0.003 g per gram diet) for the 2 millimetre pellets and 3526 ± 966 particles per gram diet (particle weight: 0.010 ± 0.009 g per gram diet) for the 4 millimetre pellets. Particle sizes ranged from 27 to 778 µm (mean ± standard deviation (SD) = 149 ± 128 µm, Fig. 1 (B)). The MP dose delivered

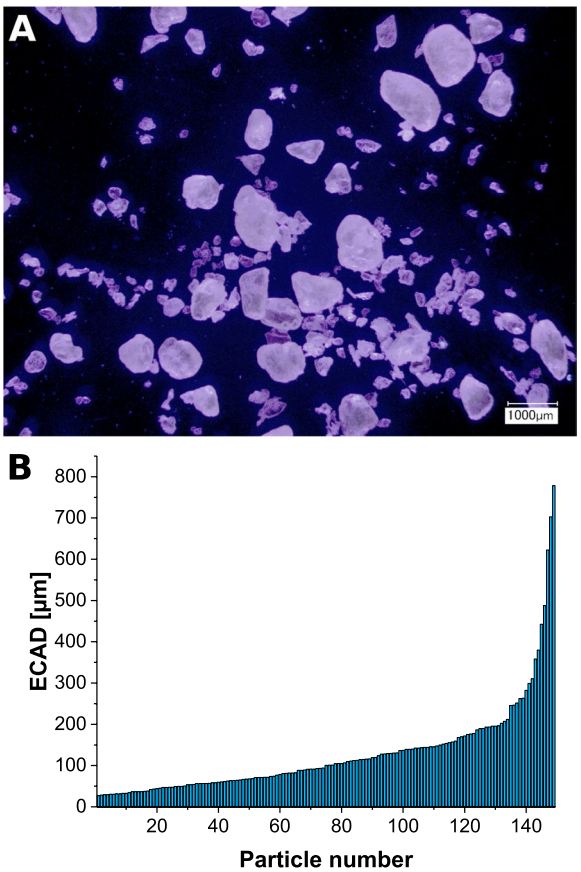


Fig. 1 Shape and size distribution of microplastic particles used in the exposure experiments. **(A)** Microscopic image of fluorescent polymethylmethacrylate (PMMA) particles. **(B)** Accumulative histogram of the equivalent circular area diameter (ECAD) of all PMMA particles detected in 10 pellets of the experimental diet

to the low exposure group was 19 mg MP diet per fish, while the high exposure group received feed dosed with 85 mg MP per fish respectively. The portion of the experimental diet containing MP particles (hereafter: MP diet) was thus modified after each sampling event in order to maintain the same dose. The MP diet was provided separately before the untreated diet every 48 h to ensure an even uptake of the MP and to allow the majority of particles to pass the gastrointestinal (GI) tract prior to the next exposure, as shown in previous exposure experiments with rainbow trout [34]. Generally, the quantity of food supplied was equivalent to 1.5% of fish body weight and was dispensed within the same hour each day, 6 days a week. After 10 weeks, the pellet size of the diet was changed from two to 4 mm and the food ration was increased to 1.8% of fish body weight. A third group served as a control and fed the experimental diet without MPs. For all treatments three replicates were performed, resulting in nine experimental groups.

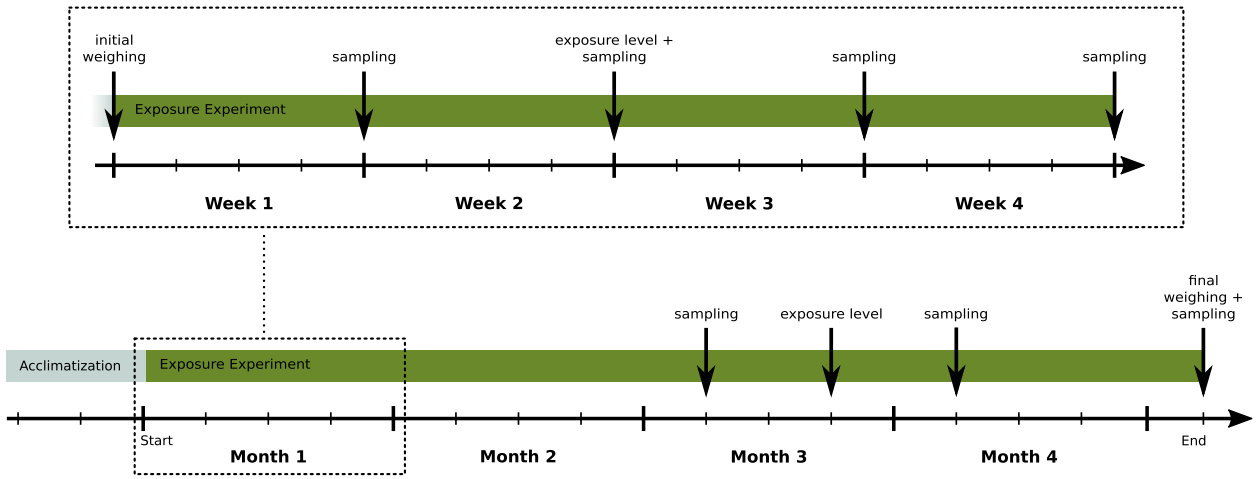


Fig. 2 Overview of the sampling scheme in the exposure experiment (additional weighing of all experimental fish was conducted at the beginning and the end of the experiment)

Sampling and tissue preparation

The experimental fish were treated for 17 weeks and sampled on a regular basis. At the start and the end of the experiment, all individuals were weighed to the nearest 0.1 g and total length was measured to the nearest 0.1 cm. During the exposure experiment, sampling took place on three consecutive days, with three tanks being sampled per day. An overview of the sampling time points is given in Fig. 2. Five individuals per time point and treatment were anaesthetized with clove oil (0.1 mL per L water, Euro OTC Pharma, Boenen, Germany), the wet weight and total length was measured and the caudal fin was ablated. A blood sample was taken for further analysis (see below). Subsequently, the fish were euthanized with a cut at the gills and stored on ice until further examination.

To prepare tissue samples for further analysis, for each individual the liver and intestine was dissected and cleaned with 1x PBS (phosphate-buffered saline). The liver was also weighed to calculate the hepatosomatic index (see below). The tissues were then dried with a cloth and stored in 1.5 mL Eppendorf tubes at − 80 °C.

Additional sampling events took place after two and 7 weeks of exposure, in order to compare MP concentrations in experimental fish fed the 2 mm MP diet und 4 mm diet respectively (Fig. 2). After anaesthetization and euthanization, the whole gastrointestinal tract was removed and MP concentration was determined according to [34]. Briefly, the tissue samples were digested using a combination of sodium hydroxide (NaOH, 1 mol/L, Chemsolute, Th. Geyer, Germany) and nitric acid (HNO3, 65%, Chemsolute, Th. Geyer, Germany), and filtered on a cellulose-nitrate filter (47 mm, 8 µm pore size, Sartorius Stedim Biotech, Germany). MP particles recovered on the filter were counted and measured using a digital microscope (Keyence VHX700, Keyence, Japan), a black-light lamp and the on-board software of the microscope.

All experiments were conducted according to the German Animal Welfare Act (TierSchG) and approved by Referat Tierschutz of Regierungspräsidium Tübingen (LAZ 2/16, AZ 35/9185.81–4).

Measures to prevent contamination during the experiment

Fish were kept in a flow-through system to reduce the risk of exposure to microplastics through contamination. The tanks were covered with a lid, which was only opened for feeding and cleaning. Since the water used in the experiments is natural lake water, pre-treatment was performed by default to ensure that no relevant contamination is introduced. Cleaning the tanks several times a day ensured that the chance of re-exposure to MP from the faeces was low. The fluorescent properties of the PMMA particles ensured that these could be clearly distinguished from possible background contamination when determining the MP concentration in the experimental fish. During digestion of the tissue samples, established protective measures to reduce contamination (as described in [39]) were followed.

Calculation of performance and health parameters

To determine the performance of the experimental fish, all fish at the beginning of the experiment ($n = 300$ for all three treatments) and all remaining fish at the end of the experiment (control: $n = 127$, low exposure: $n = 122$, high exposure: $n = 125$) were considered. Specific growth rate

(SGR, in % per day) for fish in each treatment was calculated using the mean weight of all fish at the start and end of the experiment according to the formula:

$$SGR = \frac{\ln\left(mean\ initial\ weight\ [kg]\right) - \ln\left(mean\ final\ weight\ [kg]\right)}{length\ of\ the\ experiment\ [days]}. \quad (1)$$

The feed conversion rate (FCR) describes the amount of food needed in order to produce 1 kg of fish. It was calculated at the end of the experiment, using the formula:

$$FCR = \frac{amount\ of\ feed\ [kg]}{weight\ gain\ [kg]}. \quad (2)$$

The hepatosomatic index (HSI) was calculated for each sampling event, using the formula:

$$HSI = \frac{liver\ weight\ [g]}{fresh\ weight\ [g]}. \quad (3)$$

The following health parameters were determined individually at each sampling point for five randomly selected fish in each treatment. In order to determine haematocrit levels, two haematocrit capillaries (sodium heparinized; 75 mm; Hettich, Tuttlingen, Germany) were filled with blood, centrifuged for 10 min at 14000 ×g in a haematocrit centrifuge (HAEMATOCRIT 210, Hettich, Tuttlingen, Germany) and the mean haematocrit value of both capillaries was determined. Blood glucose concentrations were measured using a standard blood glucose meter (ACCU-Chek Aviva, Roche, Mannheim, Germany).

A TBARS (thiobarbituric acid reactive substances) assay kit (TCA method; Cayman Chemical, Ann Arbor, USA) was used to test for increases in lipid peroxidation (LPO) resulting from oxidative stress [40]. The assay detects malondialdehyde (MDA), which is generated by membrane oxidation and damage [22], and was performed according to the manufacturer instructions. Briefly, 100 mg of liver tissue was blended using a tissue homogenizer (Bead Ruptor 4, OMNI International, Kennesaw, USA). The homogenized sample was centrifuged at 1600 ×g for 10 min and the supernatant mixed with thiobarbituric acid (TBA). After boiling for 1 hour, the TBARS formation was quantified using a plate reader (BioTek ELx 800, BioTek Instruments GmbH, Germany) at a wavelength of 540 nm.

Protein extraction and digestion using pressure cycling technology (PCT)

The samples were prepared by measuring 0.2 to 1.1 mg of tissue into a 150 µL FEP (fluorinated ethylene propylene) MicroTube (Pressure BioSciences Inc., South Easton, USA) with 30 µL of freshly prepared lysis buffer (8 M Urea; Sigma-Aldrich, St. Louis, USA). 0.1 M ammonium bicarbonate (Sigma-Aldrich, St. Louis, USA), 1x complete protease Inhibitor (Roche, Basel, Switzerland) were then added and the tubes sealed using a PTFE (polytetraflouroethylene) MicroCap (50 µL, PBI). Tubes were placed in the Barocycler (NEP2320; Pressure BioSciences Inc., South Easton, USA) and pressure alternated for 60 cycles according to the following plan: high pressure (45,000 psi) for 50 s followed by ambient pressure for 10 s. The temperature of the reaction chamber was maintained at a steady 33 °C using a circulating water bath. The MicroTubes were sonicated for 20 s in a water bath sonicator (VWR, Radnor, USA). The MicroCap was removed and Tris(2-carboxyethyl) phosphine hydrochloride (TCEP; Sigma-Aldrich, St. Louis, USA) and iodoacetamide (IAA; Sigma-Aldrich, St. Louis, USA) were added to a final concentration of 10 mM and 40 mM, respectively. The solutions were mixed by pipetting up and down and the tubes were then sealed with the MicroCap and incubated for 30 min at 25 °C in a Thermo Shaker (Eppendorf, Hamburg, Germany) at 600 rpm. The samples were diluted to 6 M Urea with 0.1 M ammonium bicarbonate and Lys-C (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) was added in an enzyme to protein ratio of 1:40 w/w. The tubes were placed in the Barocycler and pressure in the tube was alternated for 45 cycles of high pressure (20,000 psi) for 50 s followed by ambient pressure for 10 s, again at 33 °C. The samples were diluted to 1.6 M Urea with 0.1 M ammonium bicarbonate and Trypsin (Promega) in an enzyme to protein ratio of 1:50 w/w. The tubes were sealed using a 150 µL MicroCap and placed in the Barocycler. Pressure in the tube was alternated for 90 cycles of high pressure (20,000 si) for 50 s followed by ambient pressure for 10 s, at 33 °C. The samples were transferred to a fresh 1.5 mL Eppendorf tube and acidified with trifluoroacetic acid (TFA; Honeywell, Charlotte, USA) to a final concentration of 0.5%. Peptides were cleaned with C18 Stagetip (selfmade). The samples were dried and re-solubilized in 3% acetonitrile (ACN; Merck, Darmstadt, Germany) and 0.1% formic acid (FA; Sigma-Aldrich, St. Louis, USA) for MS analysis and the peptide concentration was normalized by with Nanodrop (DS-F11 FX+; DeNovix, Wilmington, USA; Abs at 280 nm).

Liquid chromatography-mass spectrometry analysis

Mass spectrometry analysis was performed on a Q Exactive HF-X mass spectrometer (Thermo Scientific, Waltham, USA) equipped with a Digital PicoView source (New Objective, Littleton, USA) and coupled to a M-Class UPLC (Ultra-Performance Liquid Chromatography; Waters, Milford, USA). Solvent compositions in the two channels were 0.1% formic acid for channel

A and 0.1% formic acid, 99.9% acetonitrile for channel B. For each sample, 3 µL of peptides were loaded on a commercial MZ Symmetry C18 Trap Column (100 Å, 5 µm, 180 µm × 20 mm; Waters, Milford, USA) followed by nanoEase MZ C18 HSS T3 Column (100 Å, 1.8 µm, 75 µm × 250 mm, Waters, Milford, USA). The peptides were eluted at a flow rate of 300 nL/min by a gradient from 8 to 27% B in 85 min, 35% B in 5 min and 80% B in 1 min. Samples were acquired in a randomized order. The mass spectrometer was operated in data-dependent mode (DDA), acquiring a full-scan MS spectrum (350–1400 m/z) at a resolution of 120,000 at 200 m/z, after accumulation to a target value of 3,000,000, followed by HCD (higher-energy collision dissociation) fragmentation on the 20 most intense signals per cycle. HCD spectra were acquired at a resolution of 15,000 using a normalized collision energy of 25 and a maximum injection time of 22 ms. The automatic gain control (AGC) was set to 100,000 ions. Charge state screening was enabled. Single, unassigned, and charge states higher than seven were rejected. Only precursors with intensity above 250,000 were selected for MS/MS. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1012 and 445.1200.

The mass spectrometry proteomics data were handled using the local laboratory information management system (LIMS; [41]) and have been deposited to the ProteomeXchange Consortium via the PRIDE [42] partner repository with the dataset identifier PXD033331.

Protein identification and label-free protein quantification

The acquired raw MS data were processed using MaxQuant (version 1.6.2.3), followed by protein identification using the integrated Andromeda search engine [43]. Spectra were searched against a Uniprot *Oncorhynchus mykiss* reference proteome (taxonomy 8022, version from 2018 − 09-03), concatenated to its reversed decoyed fasta database and common protein contaminants. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation and N-terminal protein acetylation were set as variables. Enzyme specificity was set to trypsin/P allowing a minimal peptide length of 7 amino acids and a maximum of two missed-cleavages. MaxQuant Orbitrap default search settings were used. The maximum false discovery rate (FDR) was set to 0.01 for peptides and 0.05 for proteins. Label-free quantification was enabled and a 2 min window for matches between runs was applied. In the MaxQuant experimental design template, each file is kept separate in the experimental design to obtain individual quantitative values.

Gene expression analysis

Based on the proteomics analysis, proteins exhibiting substantial up- or downregulation (> |10|) were selected to determine whether their different expressions was also evident at the transcript level. Additionally, four genes involved in general immune and stress responses were analysed: complement component C3 (isoform 3; *c3–3*); immunoglobulin mu (heavy chain, membrane-bound form; *ighm*); interleukin-1β (*il1b*); and hepcidin antimicrobial peptides (*hamp*). Finally, the gene 60S ribosomal protein L7 (*rpl7*) was added to the gene expression analysis as an additional reference gene, which was also upregulated in the proteomic analysis. The primer design was performed using the Pyrosequencing Assay Design software v.1.0.6 (Biotage, Uppsala, Sweden), subject to the condition that either the sense or antisense primer was placed on an exon–exon boundary. The oligonucleotide primers listed in Table S1 were tested via standard PCR and the resulting PCR products were sequenced. The quality of the PCR products was assessed by gel electrophoresis and melting-curve analysis.

RNA was isolated from the liver samples of treated and control rainbow trout ($n = 4$ per treatment and time point) in separate tubes using TRIzol (Thermo Fisher Scientific, Bremen, Germany) and subsequently purified with the ISOLATE II RNA Micro Kit (Bioline/Meridian Bioscience, Luckenwalde, Germany). RNA concentration and quality was assessed using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies/Thermo Fisher Scientific, Wilmington, USA). RNA specimens from week 4 (low exposure) did not meet the criteria and were excluded from further investigations. For cDNA synthesis, 1 µg of RNA was reverse-transcribed in a total volume of 150 µL using the SensiFAS cDNA Synthesis Kit (Bioline/Meridian Bioscience, Memphis, USA).

Quantitative real-time expression (qPCR) analysis was performed using the LightCycler 96 System (Roche, Basel, Switzerland). The LightCycler 96 protocol was optimized for a 12-µL-reaction volume using 6 µL SensiFAST SYBR No-ROX Mix (Bioline/Meridian Bioscience, Memphis, USA), 1 µL primers and a total cDNA equivalent of 50 ng RNA. The qPCR program included an initial denaturation (95 °C, 5 min.), followed by 40 cycles of denaturation (95 °C, 5 min.), annealing (60 °C, 15 s) and elongation (72 °C, 15 s) steps and fluorescence measurement (72 °C, 10 s).

Statistical analysis

A series of linear models were utilized to analyse MP concentrations in fish stomachs, as well as performance and health parameters obtained throughout the

exposure experiment. The models were generally constructed as follows:

$$y_i \sim \beta_0 + \beta_1 x_1 + \cdots + \beta_i x_i + \varepsilon_i, \quad (4)$$

where y_i is the dependent variable, β_0 is the intercept, β_i is the regression coefficient, x_i is the predictor variables, and ε_i is the random residual error. Further details of the models used and associated model effects are summarized in Table S2. Data for fish weights at the start and end of the exposure experiment, FCR, SGR and mortality for each tank were tested for differences between treatments using a contrast test [44]. The statistical analyses were performed using JMP Pro 15 (Vers. 15.1.0, SAS Institute Inc., Cary, USA).

In order to analyse the results of the LFQ, protein fold changes were computed based on the peptide intensity values reported by MaxQuant, using linear mixed-effects models [45]. The reported peptide intensities were pre-processed as follows: all proteins with only one identified peptide and intensities equal to zero were removed. Non-zero intensities were $\log_2$ transformed and modified using robust z-score transformation (using the median and median average deviation). For each protein, a linear mixed-effects model was fitted to the peptide intensities:

$$medpolish \sim Sampling\ time * Exposure\ level \quad (5)$$

Fold changes and p-values for each contrast were computed using the R package lmerTest [46] and filtered for Benjamini-Hochberg false discovery rate (FDR [47];) to adjust p-values (hereafter referred to as q-value). An overview of all computed contrasts is provided in Table S3.

To estimate fold-changes for proteins to which a mixed-effects model could not have been fitted because of an excess in missing measurements, the mean intensity of all peptides for each condition was computed first. For proteins with no measurements in that condition, peptide intensities were imputed using the mean of the 10% smallest average peptide intensities calculated in step one. Afterward, the contrasts (differences between conditions) for each peptide were computed. Finally, the median of the peptide estimates was used to provide a per protein fold change estimate (pseudo estimate). Only proteins with a conservative fold-change value > |2| were considered as "differentially expressed". This value is an established threshold in proteomic and transcriptomic studies and was also selected because the sample size was small, the variability in the data was expected to be high and the aim was to identify (robust) biomarkers to examine the effects of MP exposure [48–51]. The selected proteins (with unique UniprotIDs) were re-annotated using the Basic Local Alignment Search Tool for amino-acid sequences (BLASTP) tolerating only > 80% sequence coverage and identity.

QPCR data for the target genes was analysed using the LightCycler 96 analysis software v.1.1 (Roche, Basel, Switzerland) and normalized against the geometric mean of the *eef1a1* and *rps5* transcript values [52, 53]. One sample (week 1, low exposure) was excluded from any further statistical analysis, as the normalization factor (0.120) deviated extremely from the expected value of 1.0. A linear model was then utilized to investigate factors influencing RNA concentrations in experimental fish (Table S2). If statistically significant differences between treatments were detected, a Dunnett's test [54] was performed to compare the mean of the low and high exposure groups against the mean of the control group. Finally, a regression analysis [55] was conducted and an analysis of variance (ANOVA) was performed in order to evaluate the correlation between differential protein concentrations and gene expression.

Results

Microplastic exposure level

The number of particles in the gastrointestinal tract of the experimental fish, and thus the average exposure level for each treatment, is summarized in Table 1. The linear model ($p < 0.0001$; Table 2) revealed statistically significant differences between treatments ($p < 0.0001$). However, differences between food pellet sizes were not apparent ($p > 0.05$) and there was no significant influence of the interaction between exposure level and pellet size ($p > 0.05$).

General performance and health parameters

An overview of the relationship of total length to wet weight of all sampled fish shown in Fig. 3 (A). When considering all fish at the start and the end of the

Table 1 Mean microplastic concentration in the experimental fish for each treatment. n = number of examined fish. SD = Standard deviation

Food pellet size	Treatment	n	Mean particle concentration ± SD [number per fish]
2 mm	Control	15	0
	Low exposure	15	15 ± 16
	High exposure	15	61 ± 47
4 mm	Control	15	0
	Low exposure	15	12 ± 16
	High exposure	15	86 ± 47

Table 2 Results of utilized linear models to analyse the microplastic concentration in fish, as well as the performance parameters, health parameters and gene expression, obtained from the exposure experiment. n = number of observations. df = degrees of freedom

Parameter	n	df	Model effect	F-value	*p*-value
Microplastic concentration	90	5, 84		0.5113[a]	**< 0.0001***
			Exposure level	54.863	**< 0.0001***
			Pellet size	0.0	1.0000
			Exposure level x pellet size	2.0846	0.1312
Start weight	900	2, 897		0.1073	0.8983
End weight	374	2, 371		4.2307	**0.0152***
			Exposure level	8.2067	**0.0044***
Specific growth rate (SGR)	9	2, 6		4.9084	0.0546
			Exposure level	9.7445	**0.0205***
Food conversion rate (FCR)	9	2, 6		6.41268	**0.0232***
			Exposure level	88,148	**0.0250***
Mortality	9	2, 6		0.8571	0.4705
Blood glucose level	315	6, 308		0.1033[a]	**0.0201***
			Exposure level	1.1565	0.3243
			Sampling time	4.2788	**0.0394***
			Fish total length	0.6181	0.4324
			Exposure level x fish total length	2.1262	0.1213
Hematocrit	315	6, 308		-0.1403[a]	0.2310
			Exposure level	0.6617	0.5212
			Sampling time	0.0264	0.8710
			Fish total length	0.0580	0.8099
			Exposure level x fish total length	1.0487	0.3518
Hepatosomatic Index	315	6, 308		0.3366[a]	**< 0.0001***
			Exposure level	0.1808	0.8352
			Sampling time	0.4921	0.4835
			Fish total length	8.3918	**0.0040***
			Exposure level x fish total length	0.9471	0.3891
TBARS Assay (liver)	133	4, 128		0.0945[a]	0.0914
			Exposure level	0.0919	0.7707
			Sampling time	7.8470	**0.0059***
			Fish total length	8.0589	**0.0053***
			Exposure level x fish total length	0.0156	0.9008
TBARS Assay (intestine)				0.2483	**0.0010***
			Exposure level	0.2423	0.6374
			Sampling time	8.5565	**0.0041***
			Fish total length	4.5318	**0.0352***
			Exposure level x fish total length	0.1893	0.6642

[a] = adjusted r^2-value. * = statistically significant differences ($p < 0.05$)

experiments, it is revealed that start weight did not differ statistically between the treatments ($p > 0.05$, Table 2). Overall mean wet weight (± SD) and total length (± SD) were 21.1 ± 3.6 g and 12.6 ± 0.7 cm respectively. In contrast, for end weight, SGR and FCR, contrast tests revealed statistically significant dose dependent differences between treatments (end weight: $p = 0.0044$; SGR: $p = 0.0205$; FCR: $p = 0.0250$; Fig. 3 (C-E); Table 2).

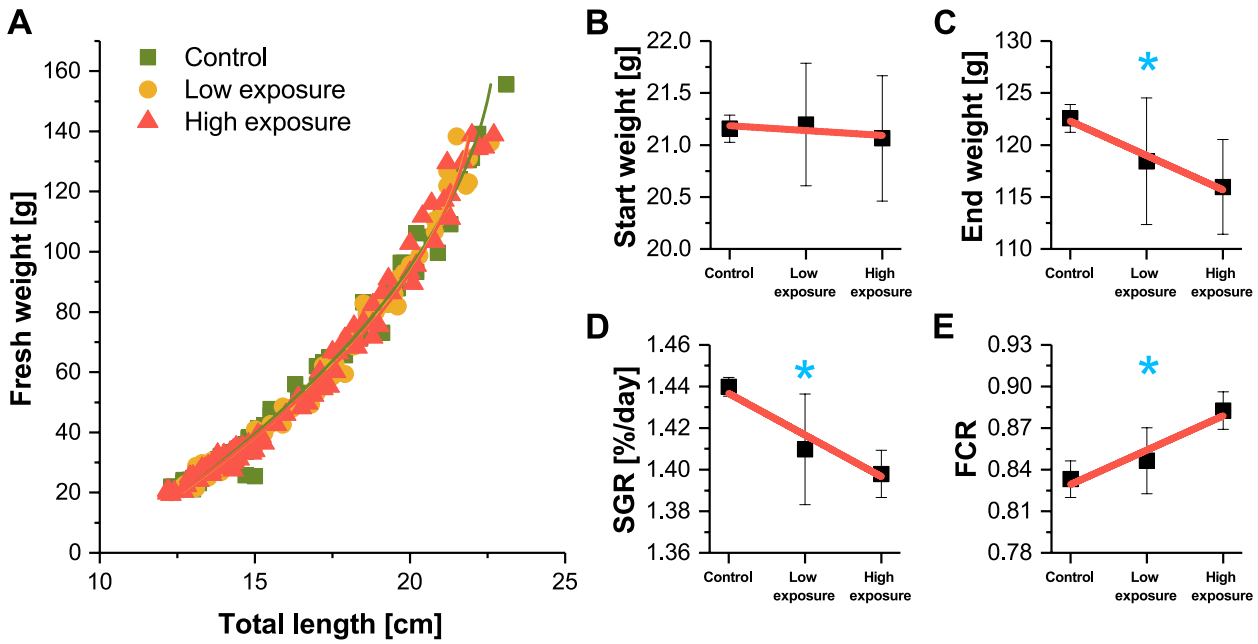


Fig. 3 Growth and performance parameters of rainbow trout for each treatment in the exposure experiment. **(A)** Relationship between total length and wet weight. **(B)** Mean weight (± standard deviation; SD) of fish at the beginning of the experiment. **(C)** Mean weight (± SD) of fish at the end of the experiment. **(D)** Mean specific growth rate (SGR ± SD) of fish. **(E)** Mean feed conversion ratio (FCR ± SD) of fish. Asterisks indicate statistically significant dose dependent effect between treatments (contrast test, $p < 0.05$; for more details please refer to Table 2)

Mortality ranged from two to five individuals per tank during the course of the experiment but did not differ between treatment groups ($p > 0.05$, Table 2).

For the blood glucose levels, the utilized linear model ($p = 0.0201$; Fig. 4 (A); Table 2) revealed significant differences over the course of the experiment ($p = 0.0394$). However, there were no statistically significant differences between treatments ($p > 0.05$), for fish total length ($p > 0.05$) or the interaction of fish total length and treatment ($p > 0.05$). Haematocrit levels showed no statistically significant change over time, between treatments and for fish total length ($p > 0.05$; Fig. 4 (B); Table 2). For the HSI ($p < 0.0001$; Table 2), statistically significant differences were revealed between fish of differing total length ($p = 0.0040$; Fig. 4 (C)), but not for any of the other variables (treatment: $p > 0.05$; sampling time: $p > 0.05$; fish total length x exposure level: $p > 0.05$).

Analysis of liver MDA concentrations (Fig. 4 (D)) revealed statistically significant differences with sampling time ($p = 0.0041$) and total length of fish ($p = 0.0352$). However, model effects did not reveal a statistically significant influence on the overall response ($p = 0.0914$; Table 2). MDA concentrations in the intestine were strongly elevated during the first week compared to other sampling occasions ($p = 0.0010$; Fig. 4 (E); Table 2). There were statistically significant differences for the factors total length ($p = 0.0352$) and time ($p = 0.0041$), but not for treatments ($p > 0.05$).

Proteomic analysis

In total, 6071 unique proteins were identified in the proteomic analysis. An overview of the LFQ results are presented as a heat map based on protein intensity correlation among samples (Fig. 5 (A)). The number of statistically significant up- or downregulated proteins (q-value < 0.05) for each computed contrast is summarized in Fig. 5 (B). When considering protein intensities in general, the variability in the dataset was relatively high (Fig. S1).

The pairwise comparison of the datasets from low or high MP exposure versus control groups revealed no proteins with a statistically significant regulation ($q < 0.05$) and a fold change > |2| respectively (Fig. 6 (A)). In some cases, however, considerable differences in concentration were apparent (Fig. 6 (B)). For instance, in livers of rainbow trout exposed to the lower MP concentration for 1 week, the level of otopetrin-3 (*otop3*) was 44-fold higher ($\log_2$ fold change = 5.47, 95% CI [3.06, 7.88]) than in control fish (Fig. 6 (B)). Concomitantly, the hepatic level of the bile salt export pump *abcb11* was 68-fold ($\log_2$ fold change = −6.09, 95% CI [−7.19, −4.99]) reduced. After 1 week of exposure to the higher MP concentration, levels of collagen (*col1a1*) increased by 30-fold

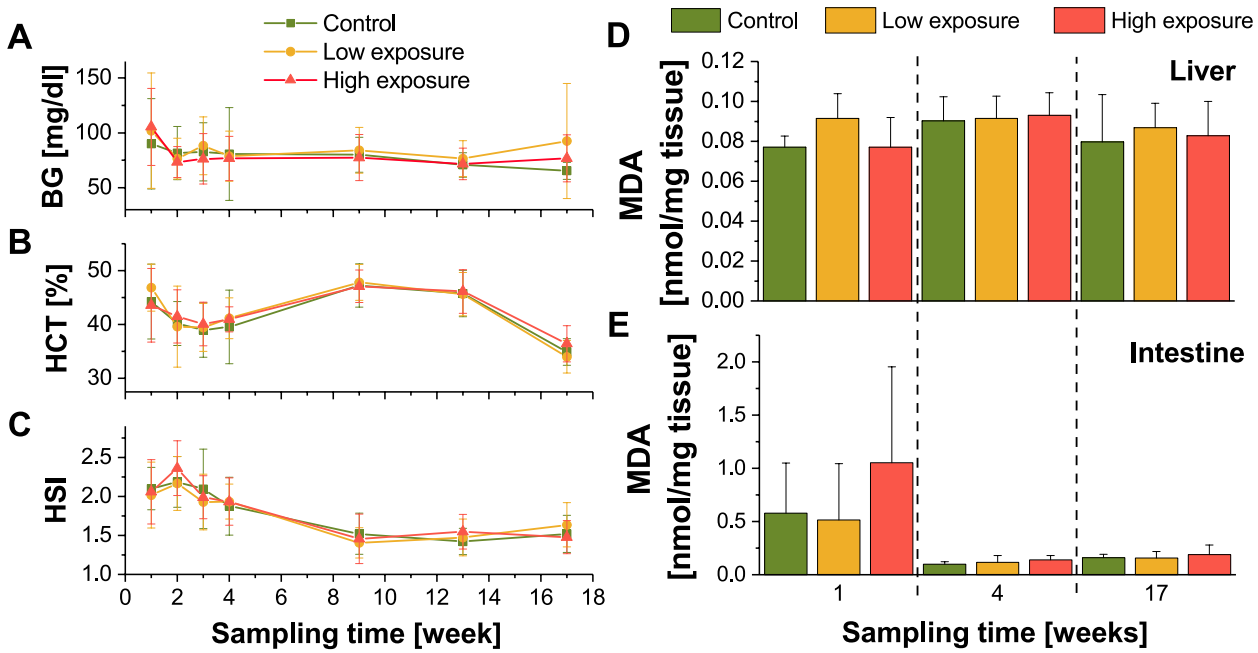


Fig. 4 Health parameters of rainbow trout for each treatment in the exposure experiment. **(A)** Mean blood glucose concentration (±SD) for each sampling. **(B)** Mean haematocrit levels (±SD) for each sampling. **(C)** Mean hepatosomatic index (±SD) for each time point. **(D)** and **(E)** Results of the TBARS Assay for three time points. The mean malondialdehyde (MDA) concentration (±SD) in the liver and intestine is shown. For statistically significant differences, please refer to Table 2

($\log_2$ fold change = 4.90, 95% CI [−3.08, 12.89]), while those of the *mhc2* antigen decreased by 13-fold ($\log_2$ fold change = −3.68, 95% CI [−6.47, −0.89]) relative to the controls. Likewise, levels of other proteins were markedly altered after 4 and 17 weeks of exposure, when comparing both low and high MP treatments with the control (Fig. 6 (B)).

In contrast to the pairwise comparisons between treatments, comparisons between sampling points identified a number of statistically significantly regulated proteins. Of particular note were the pairwise comparison of datasets from week 17 versus week 1, which highlighted statistically significant ($q < 0.05$) differences in concentration for the low MP exposure (180 proteins, −7.0 to 3.9-fold) and high MP exposure (239 proteins, −12.5 to 4.9-fold) groups (Fig. 6 (B)). Comparisons between Week 17 and week 4 revealed significantly different concentrations for 11 proteins ($q < 0.04$, −3.5 to 1.7-fold) between the control and low-exposure group only. These three datasets share significantly modulated levels of the enzyme formimidoyltetrahydrofolate cyclodeaminase and the hormone insulin-like growth factor 2 (Fig. 7 (A)). However, if only variable proteins with a $\log_2$ fold change > |2| are considered, the large majority of candidates are eliminated leaving just two in the low exposure group and three proteins in the high exposure group exhibiting significant differences in concentration over time, in both cases when comparing week 17 and week 1 (Fig. 6 (B)).

Gene expression analysis

Based on the list of proteins exhibiting differential regulation in response to low or high MP exposure (Fig. 5 (B)), the transcript levels of 11 genes were profiled in the liver samples of respective treatment groups. The gene *mthfd1* was also analysed, as it was upregulated 12-fold in the proteomic analysis (week 1: low exposure vs. high exposure). However, it was not found among the top three regulated proteins and therefore does not appear in Fig. 6 (B). This list was extended to four genes (*c3–3*, *ighm*, *il1b*, and *hamp*) with vital roles in stress responses and immunity. The concentrations of three transcripts coding for *krt13*, *mthfd1* and *otop3* (derived from the list of differentially regulated proteins), as well as *il1b* and *hamp* (derived from literature research), were very low and were therefore excluded.

The results of the linear models used to analyse individual effects on gene expression are summarized in Fig. 8 and Table S5. The model effect "exposure level" had a statistically significant effect on the expression of *col1a1*, *ighd*, *rpl7* and *c3–3* (Fig. 8). Dunnett's tests showed that for *col1a1* ($p = 0.0227$), *ighd* ($p = 0.0159$) and *rpl7* ($p < 0.0001$), the low exposure group differed significantly

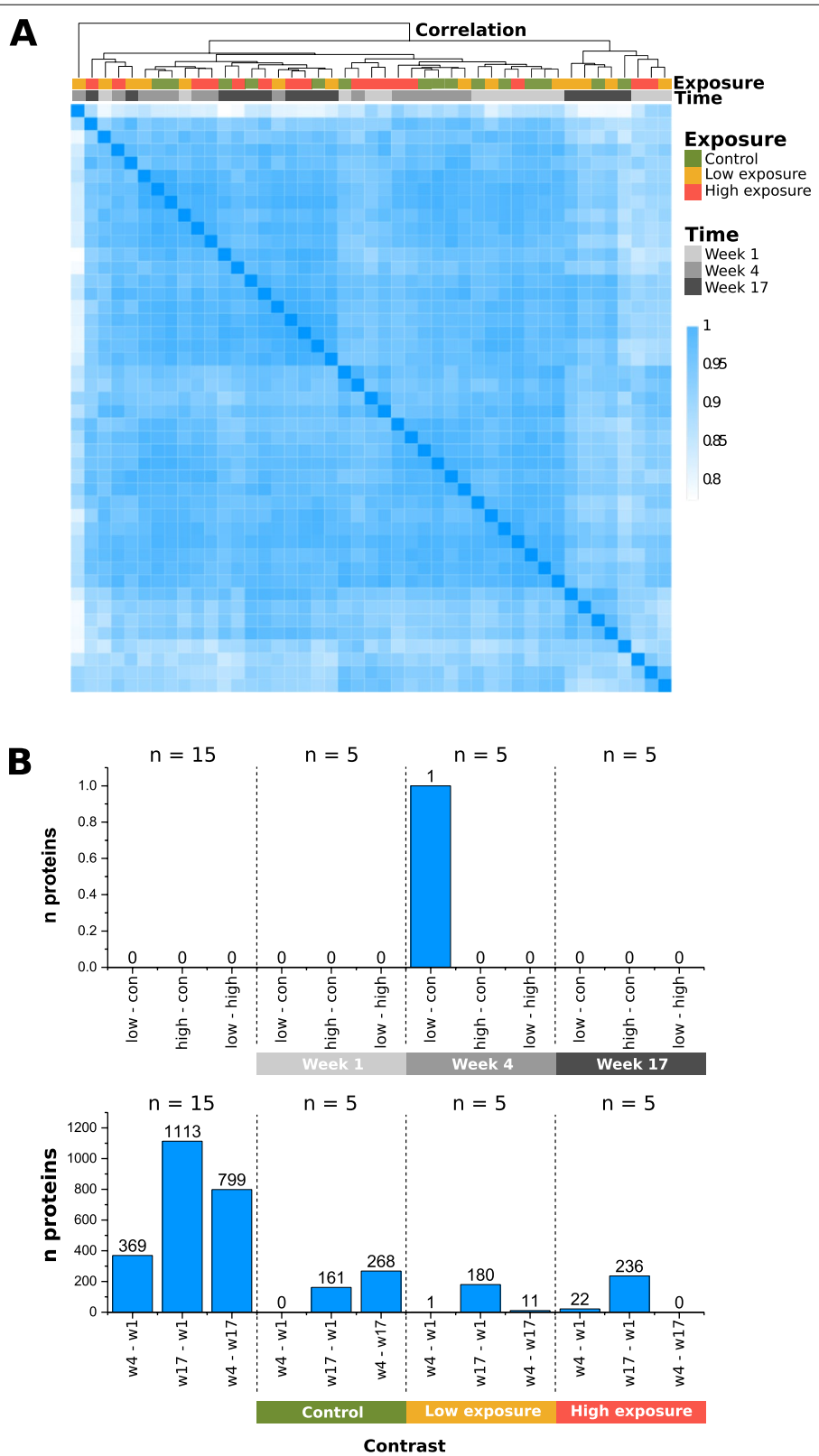
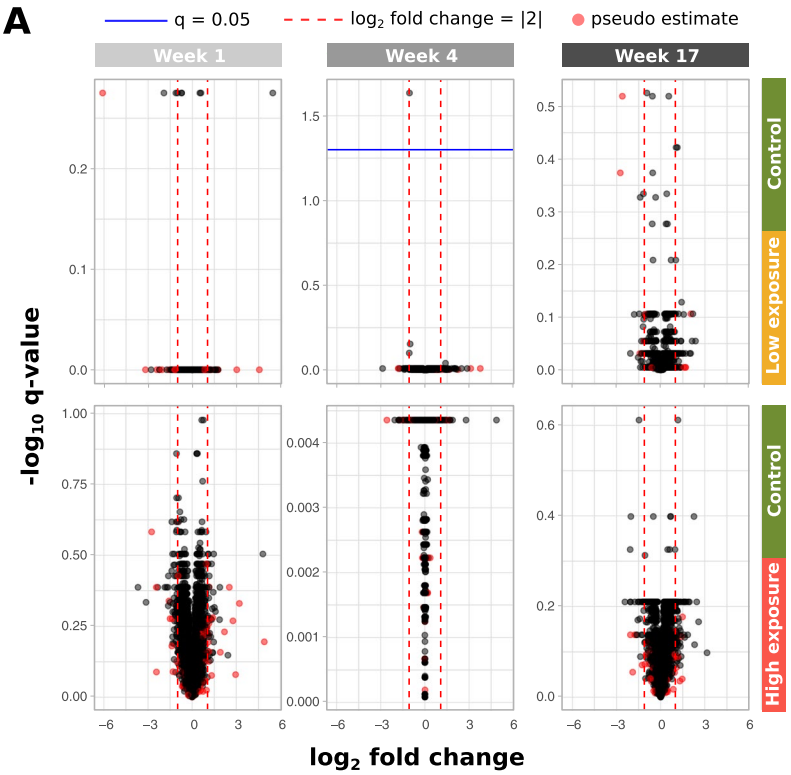


Fig. 5 Overview of the results of the label-free proteomics quantification (LFQ). **(A)** Heat map based on protein intensity correlation among samples. **(B)** Number of statistically significantly regulated proteins ($q < 0.05$) for each analysed contrast (w = week, con = control, low = low exposure, high = high exposure). n = number of samples in each condition

A



B

Comparison			UniProt accession	Protein name	Protein/LOC symbol	Fold change	Reg	q
Week 1	Low	Control	A0A060Z4M4	Bile salt export pump	abcb11b*	68.2	↓	0.53
			A0A060XWM0	Otopetrin-3	otop3*	44.3	↑	0.53
			Q9DFD5	Differentially regulated trout protein 1	drtp1*	23.5	↑	1.00
	High	Control	A0A060XQS9	Collagen alpha-1(I) chain	col1a1*	29.9	↑	0.64
			A0A060XWM0	Otopetrin-3 X1	otop3*	28.0	↑	0.31
			Q2XPB1	MHC class II antigen, beta-chain	mhc2*	12.8	↓	0.41
Week 4	Low	Control	A0A060XQS9	Collagen alpha-1(I) chain	col1a1*	64.3	↓	1.00
			Q9DFD5	Differentially regulated trout protein 1	drtp1*	23.5	↑	1.00
			A0A060ZHD6	Membrane-bound immunoglobulin delta heavy chain, isotype D	ighd*	13.5	↑	0.98
	High	Control	A0A060WLW3	Keratin, type I cytoskeletal 13	krt13*	29.2	↑	1.00
			A0A060X1S1	Chymotrypsin-like protease	ctrl*	16.0	↑	1.00
			A0A060ZJV2	Mitochondrial dnaJ homolog subfamily A member 3	dnaja3	6.9	↑	1.00
Week 17	Low	Control	A0A060X1S1	Chymotrypsin-like protease	ctrl*	19.7	↓	1.00
			A0A060VRU7	CSC1-like protein 2	tmem63b*	12.8	↑	1.00
			A0A060Z4M4	Bile salt export pump	abcb11b*	12.1	↑	1.00
	High	Control	A0A060X1S1	Chymotrypsin-like protease	ctrl*	19.0	↑	1.00
			O76009	Keratin, type I cuticular Ha3-I	krt33a	8.9	↑	0.80
			A0A060W9D0	Plectin a	plec	6.0	↑	0.68
Low	Week 17	Week 1	**A0A060ZBW0**	**Calponin-2**	**cnn2**	**7.0**	↓	**0.02**
			A0A060WL80	**Fatty acid amide hydrolase**	**faah**	**5.3**	↓	**0.01**
			A0A060Z1T2	**Microfibril-associated glycoprotein 4**	**LOC110491216**	**3.7**	↓	**0.04**
	Week 17	Week 4	**A0A060W3Z2**	**Calcium-binding and coiled-coil domain-containing protein 2**	**calcoco2**	**3.5**	↓	**0.005**
			A0A060XIN0	**Translation initiation factor eIF-2B, subunit alpha**	**eif2b1**	**2.8**	↓	**0.02**
			A0A060XW28	**SEC61 translocon, subunit alpha**	**sec61a**	**2.2**	↓	**0.03**
High	Week 17	Week 1	**A0A060YXK5**	**Catechol-O-methyltransferase domain containing protein 1**	**comtd1**	**12.5**	↓	**0.01**
			A0A060YPA0	**ATP-citrate synthase**	**acly**	**4.9**	↑	**0.04**
			C1BGT4	**Mid1-interacting protein 1**	**mid1ip1**	**4.6**	↑	**0.007**
	Week 17	Week 4	A0A060YXK5	Catechol-O-methyltransferase domain containing protein 1	comtd1	9.1	↓	0.49
			A0A060Z4U9	RAB GTPase-activating protein 1-like protein	rabgap1l	7.6	↓	1.00
			A0A060X3U2	Plexin D1	plxnd1	6.4	↓	1.00

* = Analysed on trancript level (fold change > |10|). Reg = Up- or downregulation.
q = FDR adjusted p-value. **BOLD** = statistically significant regulation (q < 0.05).

Fig. 6 Results of the linear mixed-effects models of the label-free proteomics quantification (LFQ). **(A)** Volcano plot showing q-values plotted against $\log_2$ fold changes. **(B)** Table of the top three regulated proteins identified by contrast analysis for each contrast. Information about the general function of the listed proteins can be found in Table S4

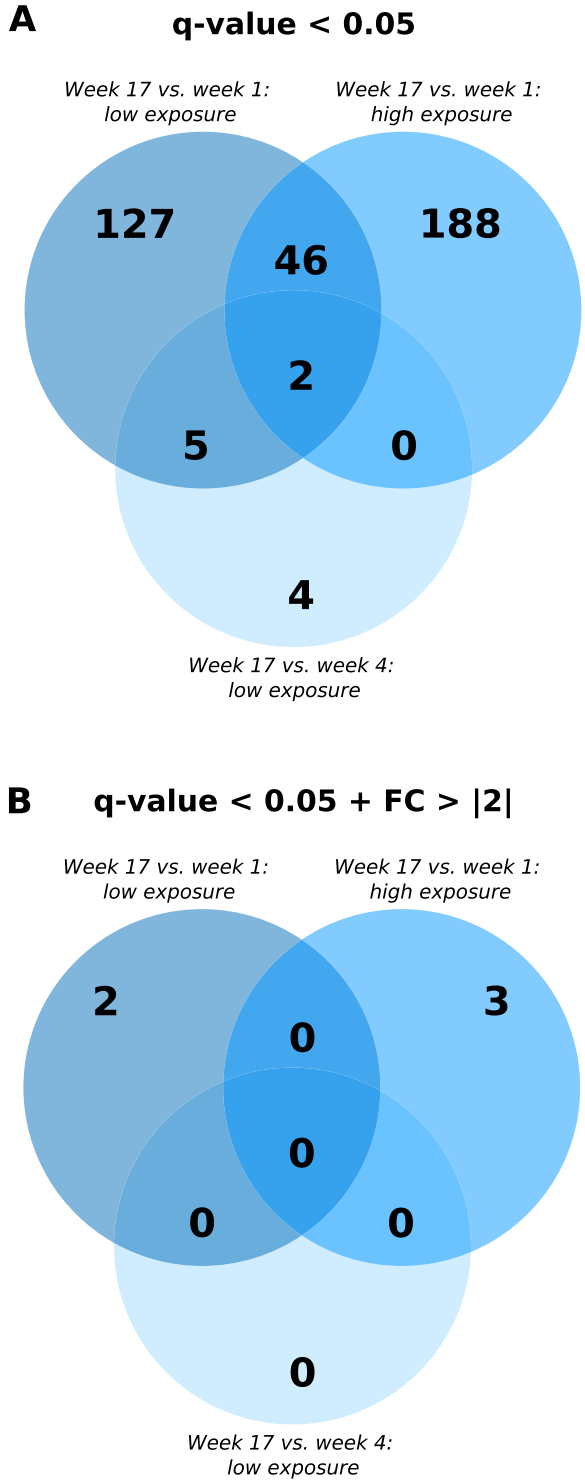


Fig. 7 Venn diagram illustrating the number of individual and shared significantly different protein concentrations for three selected contrasts. **(A)** Number of proteins with a *q*-value < 0.05. **(B)** Number of proteins with a q-value < 0.05 and a fold change (FC) > |2|

from the control, whereas for *c3–3* ($p > 0.05$) no differences were apparent between the control and either of the exposure groups. The model effects "sampling time" and "total length" had a statistically significant effect on the expression of *tmem63b* only (Fig. 8). Finally, the interaction of sampling time and exposure level had a statistically significant effect on *ctrl*, *tmem63b* and *c3–3* (Fig. 8).

The modulation of protein levels correlated only marginally with that of transcription (df = 1, 12, F = 11,339, $p > 0.05$), with an r^2 of 0.0863 (Fig. 9). However, particular changes in transcription did reflect those observed at protein level. After 1 week of exposure to high MP concentrations, the levels of *drtp1* increased 8.6-fold ($\log_2$ fold change = 3.1; $p = 0.2$), corresponding with a 7.8-fold ($\log_2$ fold change = 2.9) higher concentration of *drtp1* proteins. After 4 weeks' exposure to high MP concentrations, the levels of *abcb11b* and *ctrl* decreased approximately 3-fold ($p < 0.05$) matching with the below 6-fold elevated levels of the respective proteins.

Discussion

The negative effects of MPs on fish are part of a complex issue that, despite intensive research, remains far from fully understood [19]. The many confounding factors, including a lack of standardization in experimental setups (fish species, age, length) and the sheer variety of different types, sizes, concentrations of plastic and secondary contaminants involved, have led to widely varying results [16]. A major shortcoming of several studies is the obvious discrepancy between concentrations of MP seen in the environment and those applied in exposure experiments [15, 16]. In the present study, the average concentration of 13 particles in the gastrointestinal tract per fish (equivalent to an MP particle weight of 0.14 mg per diet portion) in the "low exposure" group are comparable to those frequently detected in environmental fish samples [10, 56, 57]. Furthermore, the use of consistent MP concentrations, and a reduced exposure schedule (every 2 days), ensured that the present experiments reflected natural conditions as closely as possible. In the "high exposure" group, MP values were increased to an average of 73 particles per fish (equivalent to an MP particle weight of 0.66 mg per diet portion) in order to examine dose dependent effects and simulate increases in environmental MP concentrations expected in the near future. The global volume of plastic waste is expected to triple by 2060, assuming no drastic measures are taken in waste reduction [58]. Everaert et al. [3] calculated that the risk to marine ecosystems of floating MPs at the ocean surface layer will increase almost 10-fold by the year 2100, assuming a worst-case scenario of plastic discharge. A study of preserved fish samples from the Chicago region (USA) between 1900 and 2017 revealed a

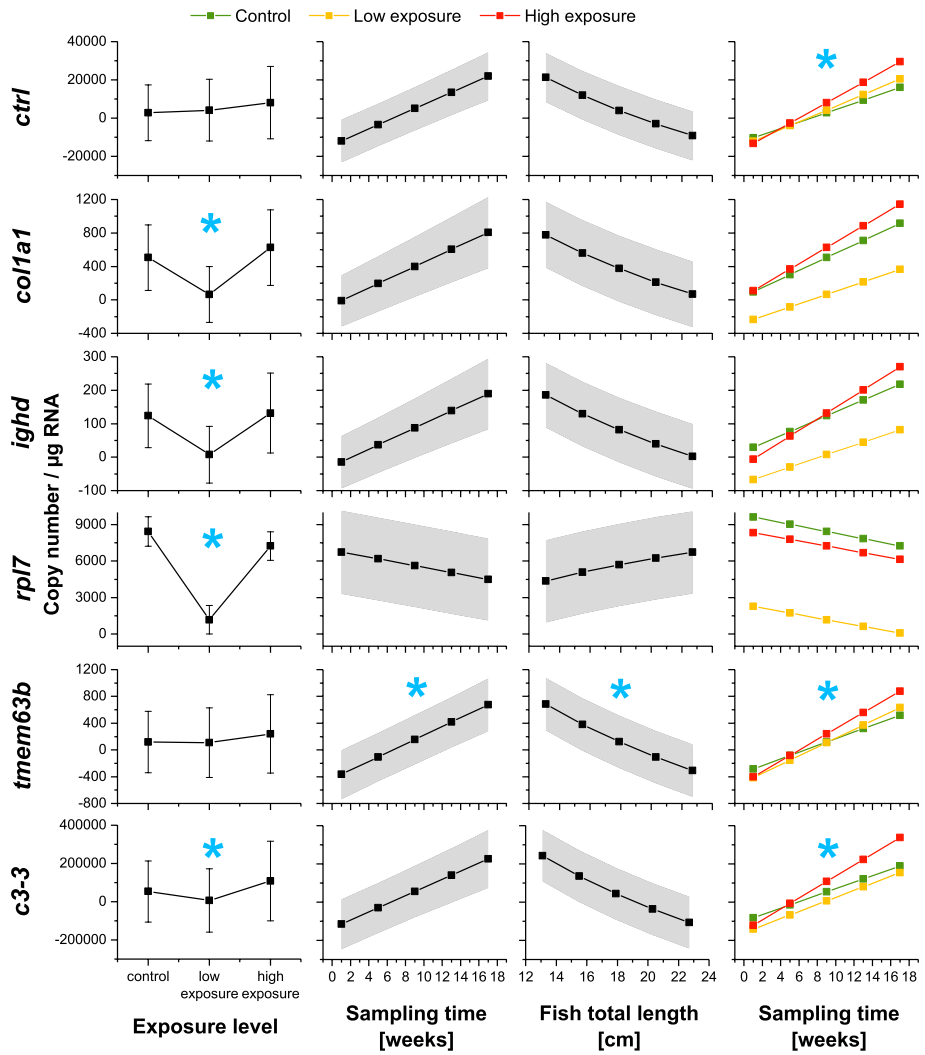


Fig. 8 Grand marginal means of selected linear models, examining statistically significant model effects on transcript concentrations of selected features derived from the proteom analysis and established stress genes from the literature. Grey bands indicate standard deviation. Asterisks indicate statistically significant differences between model effects. The last column illustrates the interaction of sampling time and exposure level in the utilized models (for more details, please refer to Table S5). Information about the general function of the analyzed proteins can be found in Table S4

correlation between plastic production and microplastic contamination of fish [59]. It is therefore to be expected that the contamination of fish with microplastics will continue to increase in line with plastic production in the near future [32].

There are many different approaches to studying the impact of pollutants on fish. Performance parameters, such as growth and mortality, are regularly considered in ecotoxicological studies, as they serve as an integrative proxy for the fitness or stress exposure of an individual [60]. While the present study agrees with other investigations in suggesting MP exposure for 120 days has no significant effect on mortality [10, 61], it does reveal a significant negative effect on fish growth. There was a dose dependent effect between MP concentration and weight at the end of the experiment, with the "high exposure" group being 3.5% underweight compared to the control group. These differences were mirrored in significantly reduced SGR values and an increased FCR for this group. Exposure experiments carried out in other studies have revealed significantly reduced growth rates but the scale of the effect varies, presumably as a result of differences in the duration of studies, in the age of fish and the type of MP particle used [62, 63], while some failed to record any effect at all [64, 65]. This lack of consistency

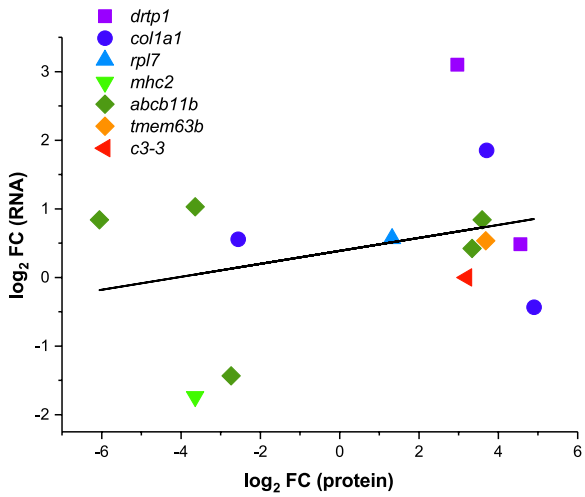


Fig. 9 Regression analysis of the correlation between differential protein concentrations and differential gene expression ($F(1,12) = 1.1339$, $p > 0.05$, $r^2 = 0.0863$). Some proteins occur more frequently if they have been detected more than once in the contrast analysis of proteomic data. FC = fold changes. Information about the general function of the analysed proteins can be found in Table S4

in the literature reinforces the urgency of establishing a sound experimental framework for such studies [66].

In principle, there are several possible mechanisms of action by which increased MP concentrations can lead to the observed impairments in performance. Nutrient dilution, caused by increased amounts of non-digestible material in the diet, can affect growth in fish [67]. However, such an effect due to the presence of MP can be excluded in the present study, as MP particles constituted only a fraction of the weight of the diet (see calculations in Table S6). Even at the beginning of the experiment, when the proportion of microplastic in the diet was highest, it accounted for less than 0.15% of the total diet weight. Previous studies on non-digestible feed supplements clearly show that those supplements, when applied below 0.3% weight, have no influence on the growth of salmonids [68]. Similar results have been reported in experiments where markers such as chromic oxide, have been used in digestibility studies [69].

Growth effects due to differences in diet quality are also rather unlikely in the current context, as the diet used was produced specifically for this study and subjected to strict quality controls (for more details about the diet composition, see [34]). Furthermore, the MP diet was provided alongside the untreated experimental diet supplied to all groups. At the beginning of the exposure experiments, when the proportion of the MP diet was highest, the proportion in the "high exposure" group comprised on average < 22% of the total diet weight. As the experiment progressed, the proportional weight of the MP diet decreased continuously, as MP concentrations were kept steady throughout the experiment.

Another potential factor could be physical interference with the gastrointestinal tract by MPs. The available literature on the topic is ambivalent with regard to MPs. Lei et al. [70] demonstrated significant histopathological damage in the gut of zebrafish (*Danio rerio*) and Ahrendt et al. [71] found an impact of MPs on gut integrity in *Girella laevifrons*. However, comparable studies failed to confirm such physical damage and attributed the findings to incorrect sample preparation and post-mortem autolytic processes which can easily lead to misinterpretation of histopathologic results [18, 72, 73]. Generally, a direct physical effect seems implausible in this respect, as fish are exposed to a variety of different non-digestible inert materials in the wild, which they routinely ingest [74, 75]. Similarly, wild fish experience regular exposure to large quantities of fine sediment, e.g. due to flooding [76] which does not appear to cause physical damage, e.g. to the gills [77, 78].

A more likely scenario seems to be an indirect detrimental effect of MP, either due to their hydrophobic surface properties or the presence of certain additives in the particular plastic used in the experiments. In humans, the addition of bran-like plastic particles to the diet led to a laxative effect and a higher water content in the stool [79, 80]. It was suggested that it was not the amount of particles that was responsible for the observed changes, but that certain sizes and shapes may cause a tactile stimulation of the enteric nervous system [79]. Similar effects are also observed with plant-based diets in salmonid fish and result in reduced lipid digestibility [81]. Furthermore, the gastrointestinal tract of fish can react very sensitively to certain substances, potentially leading to an allergic reaction and subsequently reduction in lipid digestibility [82]. Such reactions are regularly observed with the alternative plant-based protein sources increasingly used in diets for carnivorous fish. For example, saponins in soybean meal trigger inflammation processes in the intestine in several fish species [83, 84]. No allergic reaction or histamine-mediated inflammation was observed in different human cell types exposed to several polystyrene concentrations (0.5–1000 µg/mL) and sizes (0.46–100 µm). However, there was some evidence of local inflammation caused by small MPs in high concentrations [85] and exposure to polypropylene particles (size: 20–200 µm) may cause hypersensitivity at elevated concentrations [86]. Transposing mammalian results to fish is problematic, since there are fundamental differences in the nature of intestinal immune responses, especially regarding associated antibodies and cell types [87, 88]. However, the influence on the intestinal microbiota is of interest, as such disturbances are known to impact the immunity and health

of fish [89, 90]. For example, Jin et al. [91] reported that in adult zebrafish when exposed to polystyrene particles (concentration: 1000 µg/L, size: 0.5 and 50 µm) for 14 days, the abundance of *Bacteroidetes* and *Proteobacteria* decreased significantly, while the abundance of *Firmicutes* increased. A similar microbiota dysbiosis was found in a further study where zebrafish were exposed to three different shapes (fibers, fragments, beads) of MP [92]. Lastly, some additives, including plasticizers, flame retardants and antioxidants routinely used in the production of plastics are known to be harmful to fish [93, 94]. Although the leaching of such substances from MPs has so far been considered a rather minor issue for fish compared to other sources of environmental pollution [95, 96], additives remain a potential hazard for aquatic organisms [97]. Notably, the majority of studies conducted to date use virgin plastics, and rarely is a distinction made between the effects of MP particles alone versus potentially associated substances [66]. In the present study, the influence of specific additives on the performance of the fish cannot be excluded, as the plastic particles used were not tested for the presence of such compounds.

Finally, it may be that the presence of MPs in the diet could trigger a general stress response that influences the performance of exposed fish, for example by reducing feed conversion efficiency via increased energy consumption or the disruption of metabolic processes [98–100]. Various stress markers are regularly used as indicators of fish health status, as they provide clues to the presence of a disturbance [101, 102], which may be chemical, physical and perceptual [103]. So far, stress markers have been used only sporadically in studies on the impact of MPs on aquatic organisms. In adult water fleas (*Daphnia magna*), expression levels for two stress response genes (heat shock protein (HSP) 60 and 70) were significantly altered after exposure to a mixture of different MP types (around 30 particles per individual, size: 40 µm [104];). Cortisol, another frequently used biomarker for stress in fish, was detectable in early life stages of sea trout (*Salmo trutta*) exposed to polystyrene particles but not in the control group [105]. However, the number of examined samples was low, so results must be treated with caution.

Beyond these theoretical considerations, the investigation of established health parameters in the present study does not offer direct indication of the causes of the observed differences in growth. The parameters blood glucose, hematocrit and HSI varied significantly during the course of the experiment, but differences between the treatments were not apparent. A significant decrease in HSI, with increasing fish length was also observed, in line with previous studies [106]. No differences in LPO in the liver and intestine were found between treatments, although MDA levels were significantly elevated in the intestine after 1 week. Oxidative stress is often reported as a response to MP exposure in fish, however, it seems to be associated with high concentrations of MP: significant increases in reactive oxygen species (ROS) levels in male and female zebrafish gonads and livers were observed after a 21-day exposure to polystyrene particle concentrations > 100 µg/L [107]. Iheanacho & Odo [108] found significantly increased LPO levels in the liver of juvenile *Clarias gariepinus* after exposing them to polyvinyl chloride particles for 45 days (concentration: 0.5–3% of total diet weight). Since the strong increase in the present study was observed in both treatment groups and the control, a connection with MP exposure seems unlikely. Instead, it may be a general reaction to the experimental diet [109]. In summary, the present results strengthen the argument that more sensitive approaches are needed to study the negative effects of MP exposure at environmentally relevant concentrations [23].

Omics approaches are becoming a preferred method by which to understand the complex interactions of MPs and aquatic organisms [24, 110], but to our knowledge have not yet been applied to salmonid fish in this context. The rainbow trout data presented here indicates that long-term exposure to both environmentally realistic and increased MP concentrations did not induce any significant change in hepatic protein concentrations compared to their matching controls, although certain parameters were regulated many times up or down. However, it must be noted that the sample size in the current proteomic analysis was generally small and the variability of protein intensities between replicates was rather large. As comparable experiments do not yet exist, transcriptomic studies may indicate the proteins and associated pathways affected. It is worth noting that a transcriptomic study on the liver of adult zebrafish reported differential expression in 147 and 201 genes, after 20-day exposure to 100 µg/l or 1000 µg/l polystyrene and high-density polyethylene respectively [111]. Regulated features in common with the present list of differentially concentrated proteins include members of the abc transporter family (*abca1b/*abcb11b), transmembrane channel proteins (*tmem106bb/*tmem63b) and keratins (*krt96/*krt13, krt33a). The first two groups are associated with transport processes and their altered regulation might indicate a pollution response [112]. Keratins provide barrier functions [113] similar to the complement components shown to exhibit differential expression in other fish exposed to MPs [30, 114, 115]. Although the statistical analysis does not suggest significantly different regulation between treatments, the highly regulated proteins found

in the present study should be considered as potential biomarkers in future studies. When the changes in protein regulation over time were analyzed, a large number of statistically significant changes were found. However, most of these exhibited a fold change < |2| and therefore their biological relevance is questionable [48, 49]. In the present study, liver tissue was chosen for proteomic and subsequent transcriptional analysis, since MP exposure has previously been demonstrated to induce toxicity [116], metabolic disorders [117] and lesions [118, 119] in the livers of several fish species. Furthermore, it has been shown that the fish gut and liver act in collaboration during intestinal inflammation [120]. As there was no clear evidence of inflammation or stress in the liver tissue and considering the above mentioned hypotheses on the impact of microplastics on fish, an investigation of the gut proteome would be a logical next step.

The small number of highly up- or down-regulated proteins identified in the current study were selected and examined at the transcript level. Generally, the correlation between protein and RNA regulation was very low. Still, a clear correlation was found for the differentially regulated trout protein 1 encoded by *drtp1* when comparing the high exposure group and control group at week one of the experiment. This protein has been previously identified as a suitable biomarker for acute stress in fish [121, 122]. Further studies should clarify whether this marker is a suitable candidate for studying the effects of MPs on trout. One reason for this otherwise low correlation between protein and RNA regulation could be the fact that the variations in levels of transcripts and their associated proteins do not necessarily coincide in time and also can vary dynamically in their association with other factors [123]. Additionally, the small sample size, and strong individual differences between sampled fish [124, 125], could further disguise possible correlations. No evidence of a negative influence of MPs on fish were seen when analyzing several of the "established" genes regularly used to investigate fish health [126, 127]. For example, Il1b, an indicator of inflammation [128, 129], was not detected in relevant concentrations. However, the gene expression analysis did suggest that the duration of exposure may be a key factor governing the effects of MPs on fish. For example, the data indicates that for *ctrl*, *tmem63b*, and *c3–3*, gene expression increased significantly more over time in the "high exposure" group than in the other two groups. This should be a consideration in future studies, as previous investigations have rarely used exposure times longer than 2 months [16, 130]. Interestingly, the gene expression results for *col1a1*, *ighd* and *rpl7* showed significantly lower expression in the "low exposure" group, an observation for which there appears to be no obvious biological explanation.

Conclusion

The results presented here provide clear evidence that long-term exposure to environmentally relevant MP concentrations has a dose-dependent negative effect on the performance of rainbow trout, and highlight the importance of measures to prevent further increases of MPs in the environment. With increasing concentrations, growth was clearly inhibited, indicating a reduced feeding efficiency. Several hypotheses could explain the observed differences, of which nutrient dilution, quality differences in the provided diet and physical interference MP particles in the intestine were considered improbable. More likely explanations include an allergic reaction due to the hydrophobic properties of MP or the presence of additives. Furthermore, it is suggested that MP exposure could cause a general stress reaction and thus lead, for example, to disruption of metabolic processes. Unfortunately, neither established approaches nor the combined proteomic and gene expression analysis provide support for these presented theories. One reason for this could be the use of liver tissue in the analysis. An analysis of the intestine proteome may provide further insights in the observed effects. Despite these limited results, the study found several highly regulated proteins, which should be considered in future studies. Furthermore, *drtp1* was identified as a potential biomarker for salmonid fish, demonstrating the potential value of omics approaches in this complex topic. Future studies should combine histological examinations and proteomic and transcriptomic analyses of intestinal tissue in order to better understand the detrimental effects of MPs in fish. Furthermore, targeted markers for hypersensitivity reactions and changes in energy uptake and lipid digestibility should be considered. Finally, for environmentally relevant MP concentrations, the duration of exposure must be taken into account.

Abbreviations

MP: Microplastic; LFQ: Label-free quantification; PMMA: Polymethylmethacrylate; PBS: Phosphate-buffered saline; SGR: Specific growth rate; FCR: Feed conversion rate; HSI: Hepatosomatic index; TBARS: Thiobarbituric acid reactive substances; LPO: Lipid peroxidation; MDA: Malondialdehyde\; TBA: Thiobarbituric acid; PCT: Pressure cycling technology; FEP: Fluorinated ethylene propylene; PTFE: Polytetraflouroethylene; TCEP: Tris(2-carboxyethyl) phosphine hydrochloride; IAA: Iodoacetamide; TFA: Trifluoroacetic acid; ACN: Acetonitrile; FA: Formic acid; UPLC: Ultra-Performance Liquid Chromatography; DDA: Data-dependent mode; HCD: Higher-energy collision dissociation; AGC: Automatic gain control; MS: Mass spectrometry; LIMS: Local laboratory information management system; FDR: False discovery rate; PCR: Polymerase chain reaction; BLASTP: Basic Local Alignment Search Tool for amino-acid sequences; QPCR: Quantitative PCR; SD: Standard deviation; ROS: Reactive oxygen species.

Supplementary Information

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Additional file 1.

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Authors´ contributions
SR and AB designed the study. SR, AR and WW conducted the investigation. All authors contributed to the statistical analysis. All authors contributed to writing and editing the manuscript. AB supervised the study. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD033331.

Declaration

Ethics approval and consent to participate
All experiments were conducted according to the German Animal Welfare Act (TierSchG) and approved by Referat Tierschutz of Regierungspräsidium Tübingen (LAZ 2/16, AZ 35/9185.81–4).

Competing interests
The authors declare that they have no competing interests.

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