QUANTIFICATION OF MICROPLASTIC IN FILLET AND ORGANS OF FARMED AND WILD SALMONIDS

-a comparison of methods for detection and quantification

SALMODETECT

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Summary

Microplastic (MP) is of growing concern to environmental and human health. This study investigated three analytical approaches to measure MP in tissues of salmonids. The study aimed to 1) determine and demonstrate the sensitivity of current analytical methods for MP in salmon tissues for the three different quantitative methods, 2) compare the utility of the different methods in terms of cost, time and sensitivity 3) quantify MP in a relevant selection of tissues of farmed and wild salmon in order to establish likely indicator organs for future documentation purposes. We here present the results, compare the methods and discuss uncertainties and needs for further method development.

Sammendrag

Mikroplast (MP) er av økende bekymring for miljøets og menneskers helse. Denne studien undersøkte tre analytiske metoder for å måle mikroplast i vev fra laksefisk. Studien hadde som mål å 1) bestemme og demonstrere følsomheten til dagens analysemetoder for MP i vev, med utgangspunkt i disse tre metodene, 2) å sammenlikne brukbarheten til metodene med hensyn til kostnader og tid i forhold til analysens følsomhet, og 3) å kvantifisere MP i noen utvalgte vev for å kunne evaluere om det var passende målorgan for senere bruk i dokumentasjonsarbeid og overvåkning. Rapporten presenterer resultatene, sammenlikner metodene og metodens usikkerheter og diskuterer behov for videre utvikling.

Acronyms

AS	Analytical sensitivity
BBP	Butyl benzyl phthalate
BEEP	Bis (2-ethoxyethyl) phthalate
BMEP	Bis (2-methoxyethyl) phthalate
BMPP	Bis(4-methyl-2-pentyl) phthalate
BnBP	Bi-n-butyl phthalate
DEHP	Bis(2-ethylhexyl) phthalate
DEP	Diethyl phthalate
DnBP	Di-n- butyl phthalate
DMP	Dimethyl phthalate
DHxP	Dihexyl Phthalate
DiBP	Dibutyl phthalate
DMP	Dimethyl phthalate
DOP	Di-n-octyl phthalate
DPP	Dipentyl phthalate
DEHP	di-2-ethylhexyl phthalate
EVA	Ethylene vinyl acetate
FPA	Focal plane array
FTIR	Fourier transform infrared spectroscopy
GC-HRMS	Gas chromatography/high resolution mass spectrometry
HDPE	High density polyethylene
HRLCMS	High resolution liquid chromatography/mass spectrometry
LDPE	Low density polyethylene
LLDPE	Linear low density polyethylene
NaTT	Sodium chloride, Tween®-20 and Triton™-X100
NQC	Norwegian quality cut
OPFR	Organic phosphorous flame retardants
PA	Polyamide (nylon)
PAN	Polyacrylonitrile
PC	Polycarbonate
PE	Polyethylene
PEEK	Polyetheretherketone
PET	Polyethylene terephthalate
PFA	Perfluoroalkoxy alkanes
PMMA	Polymethyl methacrylate
POM	Polyoxymethylene
PP	Polypropylene
PS	Polystyrene
PSUL	Polysulfone
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride
Py-GCMS	Pyrolysis-gas chromatography/mass spectrometry
RU	Rubber
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOP	Standard operating procedure

Contents

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Abstract

We investigated three analysis methods for the characterization of microplastic (MP) in fillet and liver of farmed and wild Atlantic salmon and wild mountain trout. Vibrational spectroscopy-microscopy and thermal degradation mass spectrometry were successfully applied to detect and quantify MP in the fish tissues. High-resolution mass spectrometry (HRMS-Orbitrap) was successful in quantifying phthalates and organophosphorus flame retardants pointing towards possible plastic contamination in tissues. Quantification by µFTIR and py-GCMS correlated significantly.

This study is the first to investigate the size distribution of MP in farmed and wild salmonids. MP in the size range from 10 to >200 µm were detected in both liver and fillet at all stations in all but one investigated individual. Smaller MP of <50µm were more frequent than larger ones. Quality controls indicated that these results were not due to external contamination of the samples although contamination was detected by control samples. There was no statistical difference in the measured overall concentrations of MP in muscle and liver. The present results indicate that muscle and liver may both be appropriate candidate tissues for MP analyses. This study is a step forward in methodological development. However, higher sample numbers with quality assurance and controls are needed to conclude on the exact levels of MP in the fillet and liver of salmonids, and the correlation between exposure to and concurrent levels of MP in edible tissues. No biological effects were evaluated within the study, and this study alone cannot conclude on health risks associated with MP exposure for fish or humans.

1. Introduction

Over the past decade, the widespread distribution of microplastics (MP) in the environment has been well established, including in the deepest abysses (Courtene-Jones et al., 2017, Pham et al., 2017), and in sediments of the Antarctic (Munari et al., 2017) and in the Arctic (Peeken et al., 2018, Bergmann et al., 2017). For the definition of MPs, the most commonly advocated threshold is <5 mm (EU Marine Strategy Framework Directive, Commission Decision 2017/848/EU). Sources of MP to the environment stem from several human activities on land and at sea using a range of synthetic materials. Fish farms benefit from the lightweight, strong and flexible plastics in permanent installations, net pens, rings, ropes, vessels, antifouling paints and varnishes and numerous other utilities. The wear and tear on plastic items in and around fish farms inevitably leads to the release of MP into the environment, as recently documented in the project TrackPlast - Tracking of Plastic emissions from the aquaculture industry" (Gomiero et al., 2020).

Farmed fish in such facilities are exposed to MP in the environment from local sources as well as to potentially long-range transported MP with the ocean currents. There is ample evidence of interactions of biota and microplastics (reviewed in Lusher et al., 2017a), and plastic ingestion by fish (Gall and Thompson 2015, Lusher et al., 2017), including those destined for human consumption (Neves et al., 2015, Rochman et al., 2015, Bessa et al., 2018, Ory et al., 2018, Barboza et al., 2019, Wu et al., 2019). However, the presence of MP in the gastrointestinal (GI) tract of fishes does not imply human exposure unless the GI tract is consumed (Lusher et al., 2017a). Understanding the potential for uptake and transfer of MP through the food chain is therefore of importance to understand the fate and distribution of MP and their potential environmental effects.

An increasing amount of plastic in the ocean and documentation of MP ingestion has led to growing concerns for trophic transfer and food safety (Lusher et al., 2017a). There is an immediate need for documentation of the occurrence and levels of MP, to be able to provide knowledge-based advice for authorities, consumers and food producers, including the aquaculture industry, to enable educated management. So far, the knowledge of exposure and effects is insufficient to establish proper risk assessment for MP for both the environment and human consumers (VKM 2019; Backaus et al., 2018).

Previous exposure studies in mammals and birds demonstrated that MP can be absorbed via the intestines and detected in liver and kidneys (Volkheimer et al., 1975, Deng et al., 2017). A pilot study has also identified MP in muscle and liver tissue of coastal animals from Norway (unpublished, NORCE report 06-2019). In the first study, to our knowledge, of the potential uptake and distribution of MP, Volkheimer (1975) found evidence for intestinal uptake of polyvinyl chloride (PVC) of an average size of 40 µm (10 – 110 µm) in rats, mice, guinea pigs, rabbits, chicken, and dogs. The uptake was described to happen through transcytosis, between the cells of the intestinal wall called enterocytes, and the MP were primarily transported via chyle and portal veins to the liver (Volkheimer, 1975). Minutes after the exposure MP were found in the bile, and over the following 24 hours post-exposure, MP were excreted via urine, lungs, milk and placenta, indicating multiple routes

of excretion. This also indicates that multiple body fluids and tissues may be reached by MP. The study by Volkheimer used histological sections inspected microscopically and identified PVC particles (~40 µm) in the tissues. Together with newer studies on mammals, these experiments point towards the need to assess the risk of MP exposure for human consumers (Deng et al., 2017, Xie et al., 2020).

Later experiments have shown that even smaller nanoplastic particles (<1µm) administered in high doses experimentally, reached the brain of fish and lead to altered behavior that may affect the fish capability of sustenance (Mattsson et al., 2015, Mattson et al., 2017, Chae et al., 2018). Such studies demonstrate that transfer of nano and microplastic into tissues through tissue barriers such as the intestinal wall and the blood-brain-barrier may occur and may cause effects in high doses. The smallest particles are more likely to cross tissue barriers (Deng et al., 2017, Chae et al., 2018), and remain in the animals (Kashiwada 2006, Browne al., 2008, Jeong et al., 2016, Critchell 2018). Smaller particles have a larger surface to volume ratio, are more reactive, and are of the highest concern in terms of negative health effects (Kögel et al 2020). Uptake and increasing levels upwards in the food chain, called bioaccumulation and biomagnification, is common for many contaminants such as the fat-soluble persistent organic pollutants. However, evidence for this is largely lacking for MP, except a few indications from exposure studies (Mattsson et al., 2017; Chae et al., 2018). Egestion of MPs larger than certain sizes seems to be efficient in fish and plankton (Peda et al., 2016, Grigorakis et al., 2017, VKM 2019). For MP quantification, a major limitation until now has been the available methodology, which has not been sufficient to detect and quantify the smallest MP below ~10µm while efficiently avoiding contamination. Several recent publications demonstrate the dominance of the smallest MP down to 10 µm in different matrices, such as snow, sediment, water and biota (Bergmann et al., 2019; Mintenig et al., 2017; Haave et al., 2019; Lorenz et al., 2019; Primpke et al 2019; van Alst et al., 2018; Mani et al., 2019; Gomiero et al., 2019 a, b). The number of particles may increase when moving beyond this current methodological size-limit of detection. This assumption is corroborated by the data for bottled drinking water, an easier matrix than biota (Ramsdoonk 2020). As larger plastic fragments degrade, the number of small MP will increase, unless they are degraded to the point of metabolizable components or mineralization. The presence of plastic particles in the nanometric size range has been documented qualitatively in the ocean (Ter Halle et al., 2017), but methods are currently inadequate to quantify their concentrations in any environmental matrix.

The increasing concerns about negative effects of MP on organisms and for food safety are attracting attention to this scientific field, and there is a demand to provide solid documentation. Knowledge of the size distribution, uptake and effects after exposure in natural habitats or aquaculture settings is currently lacking, even though investigations are required to document the current situation, as the Marine Strategy Framework Directive (MSFD; 2008/56/EC, decision 2017/848/EU) requires. This states that: “The amount of […] micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned. […] Micro-litter shall be monitored […] in a manner that can be related to point sources for inputs […] where feasible “. D10C3 specifies: “Litter and micro-

litter […] Assessed in any species from the following groups: […] fish or invertebrates". MSFD specified their needs as strategy development, matrix selection, method selection, size range selection, quality assurance and controlled and quantified dose-harm relationships. The seafood industry is accustomed to strict requirements for documentation of quality and food safety, and for that purpose, sensitive and reliable analytical methods as well as knowledge of the potential risks associated with the potential occurrence of MP need to be established.

At present, despite a large number of publications and reports, the concentrations of and the hazards associated with relevant environmental exposure to MP are unknown (Lusher et al., 2017). Due to the lack of sufficient quality-controlled data, a scientific environmental or human risk assessment cannot be made (VKM 2019). It is necessary to increase the sensitivity and reliability of MP analysis to be able to document the concentrations of MP in food, as was established for other undesirable substances, such as persistent organic pollutants, heavy metals and residues of drugs. To understand the risks and potential effects we must also understand the mode of uptake and excretion, the target organs, and study the potential effects.

As an enabling step towards a scientifically based risk assessment of MP in seafood this study aims to: 1) determine and compare the sensitivity of current analytical methods for MP in salmon tissues for three different quantitative methods, 2) compare the utility of the different methods in terms of cost, time and sensitivity, and 3) quantify MP in a relevant selection of tissues of farmed salmon to establish indicator organs for future surveillance purposes.

2. Materials and methods

Locations, fish collection and tissue sample preparation

Organs collected for MP analysis were muscle, liver and head kidney from farmed and wild salmon (*Salmo salar*) and wild brown trout (*Salmo trutta*). For farmed salmon, 20 individuals of approximately 4-5 kg were collected from an aquaculture facility located in Tysnes in the Hardanger fjord, and 20 individuals of approximately 2 kg were collected from an aquaculture facility located in Kjeahola in the Boknafjord. Additionally, 20 wild salmon of approximately 2-3 kg from Sørfjorden near Osterøy were delivered by the VOSSO scientific program as well as 15 brown trout of 90-250 grams collected from two regulated mountain lakes (Holmavatnet n=10, Vasstølsvatnet n=5) in Suldal, Rogaland. The lakes are regulated for hydropower production and are in an uninhabited mountain area. The fish were caught in August 2018 as part of the evaluation of the population of trout in the lakes (Lehmann & Velle, Uni research report 320-2018).

In this study, tissues from four to six individuals were pooled into three to four pools per location to maximize the representativity of the samples, while reducing the number of analyses done. The reasons for this were that the analyses are very time consuming and

expensive (Table 3), and pooled samples consisting of several individuals will give a better representation than of one individual if there is variation among individuals.

Tissue samples from liver, head kidney and muscle (Norwegian Quality cut (NQC) for salmon, general fillet for mountain trout) were dissected and prepared under plastic reduced laboratory conditions at the Institute of Marine Research (IMR, Bergen), minced by a stainless-steel meat mincer (TC-12/160 Elegant, Felleskjøpet) before packing and distribution. The pooled subsamples aimed for 100 g portions, if possible. For head kidneys, some of the livers, and fillets of mountain trout, sample sizes were smaller, due to a lack of material. Samples of 20 g NQC cut samples (Figure 1) and 1/3 of the livers were also sent to The Norwegian Institute of Air Research (NILU). The remaining part of the same fillet was minced, pooled, subdivided to 100 g portions and used for method development. Samples were processed through extraction for analysis with micro Fourier Transform Infrared Spectroscopy (µFTIR) at the IMR. After µFTIR analysis, the samples on filters were sent to NORCE Stavanger for analysis by py-GCMS. Since the samples on the filters were essentially unchanged between analyses, this functions as a comparison of the method of polymer identification and quantification by µFTIR and py-GCMS. Results from the head kidneys are not included in the report due to time-constraints in the analyses and will be published later.

Sample preparation for µFTIR and py-GCMS analyses

The method developed in this project aimed to extract MP from the investigated tissues and to apply a gentle and efficient purification step before chemical identification in a way that allows for a quantitative analysis. The main interferents for a reliable quantification are the organic components, proteins, and fats that may aggregate with the MP and reduce the efficiency of the extraction process. Organic material will also interfere with the chemical analysis and quantification process, causing an increase in the background signal and reducing the signal-to-noise ratio. The principle used for all the tissue samples was gentle degradation and removal of all biological tissues. This was done on homogenized tissues in a 500 ml Erlenmeyer flask by detergents (Tween®-20, Triton™ X-100), followed by alkali solution (KOH) and acid (HCl). The degradation was performed at 40°C. Fillet samples of mountain trout were post-treated with chitinase (according to Löder et al., 2017), as samples contained particles from fish scales after the initial digestion protocol.

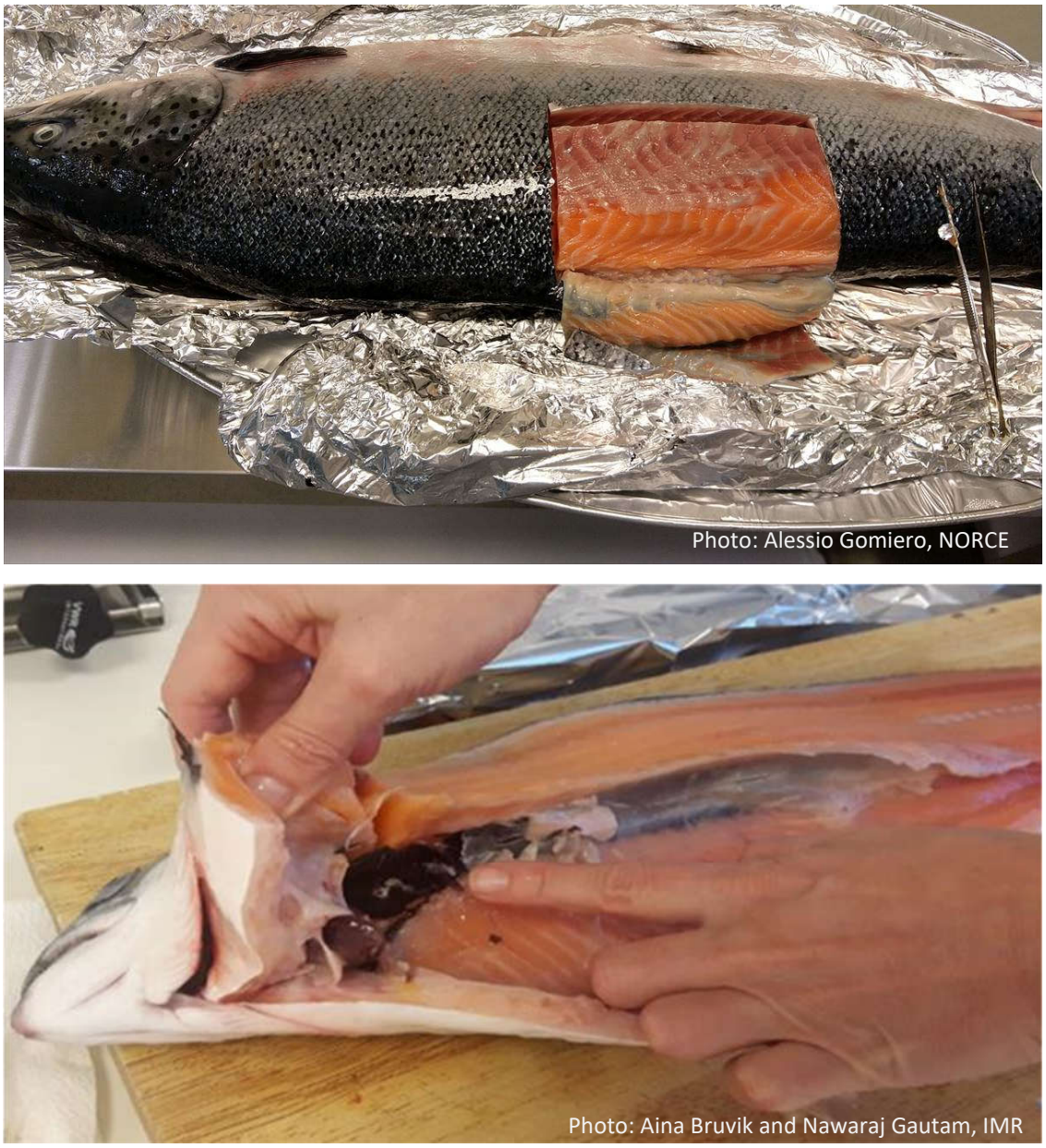

Figure 1. Sampling of farmed salmon. Muscle samples were collected from the area behind the dorsal fin, also called the Norwegian Quality Cut (NQC) (top). Collection of liver, fillet and head kidney (bottom).

Figure 2. Glass jars, food-grade, for storage of pooled head kidney (Hodenyre) and liver (Lever) samples (Photo: Aina Bruvik and Nawaraj Gautam).

Plastic reduced laboratories and contamination control

All tissue dissections were performed at the clean MP laboratory at IMR in Bergen. All the fish, both farmed and wild, were shipped whole to IMR and all dissections were performed in a MP clean room to avoid external contamination. Before incisions were made, the fish were wiped clean using paper towels and distilled water.

The laboratory is equipped with high efficiency ultra-low penetration HEPA filtration with an efficiency of over 99.9% for particles of 0.3-0.5 µm, overpressure and an airlock that prevents dust entry. The laboratory was entered with dedicated low-abrasion shoes and a cotton laboratory coat. Synthetic polymer fibers in clothing were avoided. Either no gloves or nitrile gloves were worn for the protection of personnel. Samples were handled under a laminar air-flow bench (LAF, Class II biological safety, Thermo Scientific SAFE 2020). To reduce sample contamination during sample digestion and analysis equipment in contact with the sample or solutions were made of glass or stainless steel, and perfluorinated polymers (PTFE and PFA) that are rare in the environment, when plastic equipment was unavoidable. Milli-Q water was used to prepare all solutions, and all final solutions were filtered using 0.7 µm glass fiber filters (VWR International) before use. All tools and glass equipment were covered with aluminum foil and burned in a muffle oven at 500°C for at least 5 hours to remove plastic contamination. Aluminum foil was used to cover samples during preparation and digestion when possible. Sampling of an individual was performed during the course of approximately 30 minutes to an hour.

Open glass jars of filtered Milli-Q water were placed in the working area in the laboratory and in the LAF bench during a full working day (8 hours) and analyzed for MP to control for potential airborne particles during a full day. 100 ml water was also run through the meat mincer and analyzed, in order to assess potential contamination from this procedure.

During the py-GCMS analyses, performed at NORCE's lab in Stavanger, similar dust trap collectors were used to evaluate possible contamination from airborne particles. Furthermore, the steel cups used for the pyrolysis were cleaned before use with a butane blow torch at 1400 °C to remove any plastic. The py-GCMS system was cleaned in between analyses by first running a clean empty cup containing a silylating agent to remove any polar, low-volatile contaminants from the GCMS system, followed by a second analysis performed on the same empty cup without the silylating agent to remove the excess of the derivatizing agent from the system.

The sample preparation for analysis of additives present in MPs was carried out in NILUs MP lab at the FRAM Centre in Tromsø, working either in a clean room or in a laminar flow cabinet facilitating particle filtration of the surrounding air.

Figure 3. NILU clean room at the FRAM Centre in Tromsø, and contamination prevention measures by particle free clean room clothing (Photo: Vegard Stürzinger, NILU).

All equipment used was plastic-free, rinsed and burned at 450°C before use to remove all plastic contamination. Together with the blanks (controls) provided from IMR covering the preparation of the samples there, additional blanks were included covering the sample preparation and extraction in the NILU MP laboratory.

Recovery tests

Recovery was tested on all three salmon matrices (fillet, head kidney and liver) spiking samples with approx. 100-150 particles of 100 µm red polystyrene beads (Sigma-Aldrich), as previously described (Liu et al., 2019b; Olesen et al., 2019; Simon et al., 2018). The recovery was around 83-89% for salmon fillet, 86-92% for liver and 93% for head kidney, with no discrimination between the matrices. This means that the method of extraction was acceptable for the tested size class, but some particles likely escaped extraction, which is common for all extraction procedures, including solvent extractions of chemical pollution. Recovery testing for smaller particles remains to be developed.

Common polymers (PA, PC, PE, PET, PMMA, PP, PS and PVC) were also exposed to the digestion protocol without matrices, to investigate if the procedure might affect the MPs and reduce the credibility of the results. For this test, most of the MP were in the size range 400-500 µm, while PVC particles were around 150 µm. The recovery was between 72-95 % (PVC) and 90-102 % (PE), which was similar for most polymers, but down to 68-80 % for polymethyl methacrylate (PMMA) and 70-88 % for polycarbonate (PC). In other words, results are likely to represent most polymers fairly well, but are more likely to underestimate the levels of PMMA and PC, especially for smaller size classes, as with this amount of loss, 20 µm particles could be reduced in size sufficiently to then slip through 10 µm filters.

Identification of MPs by vibrational spectroscopy: µFTIR

Fourier Transform Infrared spectroscopy (FTIR) is an established, non-destructive technique in analytical chemistry using infrared (IR) radiation, also called vibrational spectroscopy. The FTIR instrument is equipped with a source emitting IR radiation of a spectrum of wavelengths at a known energy intensity level. The IR radiation is passed through a sample, and specific wavelengths match with atomic and molecular bond energies of a compound. The absorbed energy causes temporary charge changes within the chemical bonds and vibrating molecules and light with different wavelengths causes different vibrational patterns in the molecules of the polymers. Compounds, such as natural and synthetic polymers, absorb infrared radiation at different intensities. Transmission or absorption can be measured, and the resulting spectrum or "fingerprint" is used for molecule identification by comparison with reference databases of known compounds.

FTIR was performed in two ways depending on MP size. The particles over approximately 300 µm could be picked out using tweezers under a stereo microscope, measured, weighed and analyzed by Attenuated Total Reflectance FTIR (ATR-FTIR). If possible, three spectra would be acquired for each particle. However, there were no particles of these size classes in the extracted samples.

µFTIR imaging

A quantitative analysis of MP from 10-300 µm was done by µFTIR imaging (similar to Primpke et al., 2019). With this method, both polymers and particle size distribution of an extracted sample can be determined, down to approximately 10 µm. The lower size detection limit was also influenced by the 10-16 µm pore size of the glass filter crucibles (Por. 4, ROBU® Glasfilter-Geräte GmbH). Results for down to ~10 µm could be detected by the system, but the smallest size cannot be considered quantitative, as many particles below or close to the pore-size may have been lost.

Extracted environmental samples were initially divided into two size fractions 10-100 µm and above 100 µm. The separation into sizes below and above 100 µm did not lead to a clear distinction of size classes, as many particles below 100 µm were retained in the larger size fraction on the ceramic filters used. Therefore, this separation was not continued. The filtered sample was distributed on a Whatman® Anodisc Inorganic Membranes ceramic filter, to be imaged by the microscopic system. The filter was dried for 24 h at 40°C and stored in a glass petri dish with glass lid before analysis. µFTIR imaging was performed using an Agilent Cary 620 FTIR microscope coupled to a Cary 670 FTIR spectrometer with a liquid nitrogen cooled 128x128 Focal Plane Array (FPA) detector, allowing for imaging of 128x128 pixels in a single measurement, a MIR Source with a spectral range of 9000-20/cm, purged enclosure, 15x IR/Vis reflective objective (NA 0.62, WD: 21mm), 4x Vis glass objective (NA 0.2, WD: 38mm), motorized sample stage, 0.1x0.1 MCT as well as GladiATR for single particle analysis of larger MPs (Figure 4).

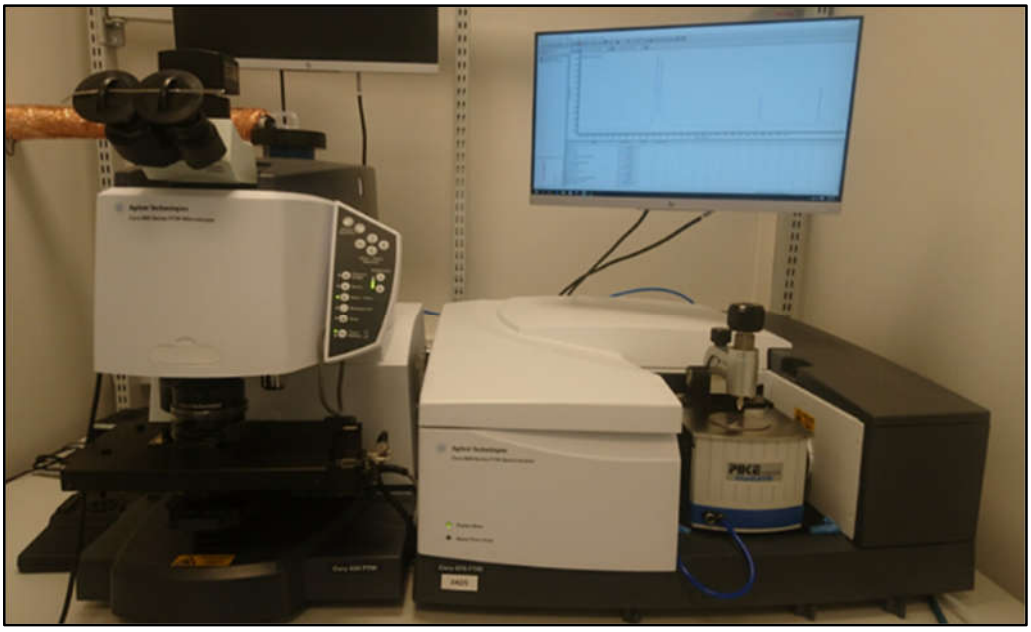

Figure 4. µFTIR equipment at the IMR microplastic laboratory (Photo: Ørjan Bjorøy, IMR).

The deposit area of the filter was imaged in transmission mode following the procedure of (Primpke et al., 2018; Simon et al., 2018). Each pixel was imaged for the whole spectrometric range, where different wavelengths (colors) are transmitted to a different extent. Simultaneous optical images give the size of the particles in two dimensions, usually the two larger dimensions, as the filtration process causes the particles to "lie down". Automatic image processing detects the size and position of the particles, smoothes edges and assigns a polymer group to the particles (Figure 5). For dataset analysis, data was processed by siMPLE (v.1.0.0; simple-plastics.eu) and spectra were compared to libraries from Bio-Rad and Agilent, the Alfred-Wegener Institute Helgoland (Gunnar Gerdts, Sebastian Primpke) and IMR.

The six groups of analytes of non-synthetic, organic materials (animal fur, plant fibers, sand, chitin, charcoal, coal), were omitted from this data-analysis, as they were not interesting for this study and would have prolonged analysis time considerably. The polymer groups included in the µFTIR analysis are shown in Figure 5. Of these polymers polyethylene (PE), oxidized polyethylene, chlorinated polyethylene, polypropylene (PP), polystyrene (PS), polycarbonate (PC), polyamide (PA, =nylon), polyvinyl chloride (PVC), chemically modified cellulose (Cellu), nitrile rubber (NBR), polyester (PES), acrylates/PUR/varnish (ACy), polyetheretherketone (PEEK), ethylene-vinyl-acetate (EVA), polyoxymethylene(POM), polyacrylonitrile (PAN) and rubber type 3 (EPDM, Rub3) were detected in at least one sample and are therefore listed in the results. The polymers that were not detected in any samples are excluded from graphs and tables.

1	Polyethylene
2	Polyethylene oxidized
3	Polyethylene chlorinated
4	Polypropylene
5	Polystyrene
6	Polycarbonate
7	Polyamide
8	Polyvinylchloride
9	Cellulose chemically modified
10	Nitrile rubber
11	Polyester
12	Acrylates/PUR/varnish
13	Animal fur
14	Plant fibers
15	Sand
16	Polysulfone
17	Polyetheretherketone
18	Polychloroprene
19	Chitin
20	Polyisoprene chlorinated
21	Polylactide acide
22	Polycaprolactone
23	Ethylene-vinyl-acetate
24	Polyimide
25	Polyoxymethylene
26	Polybutadiene
27	Polyacrylonitrile
28	Rubber 1
29	Rubber 2
30	Charcoal
31	Coal
32	Rubber 3

Figure 5: The polymer groups analyzed for using the µFTIR analysis.

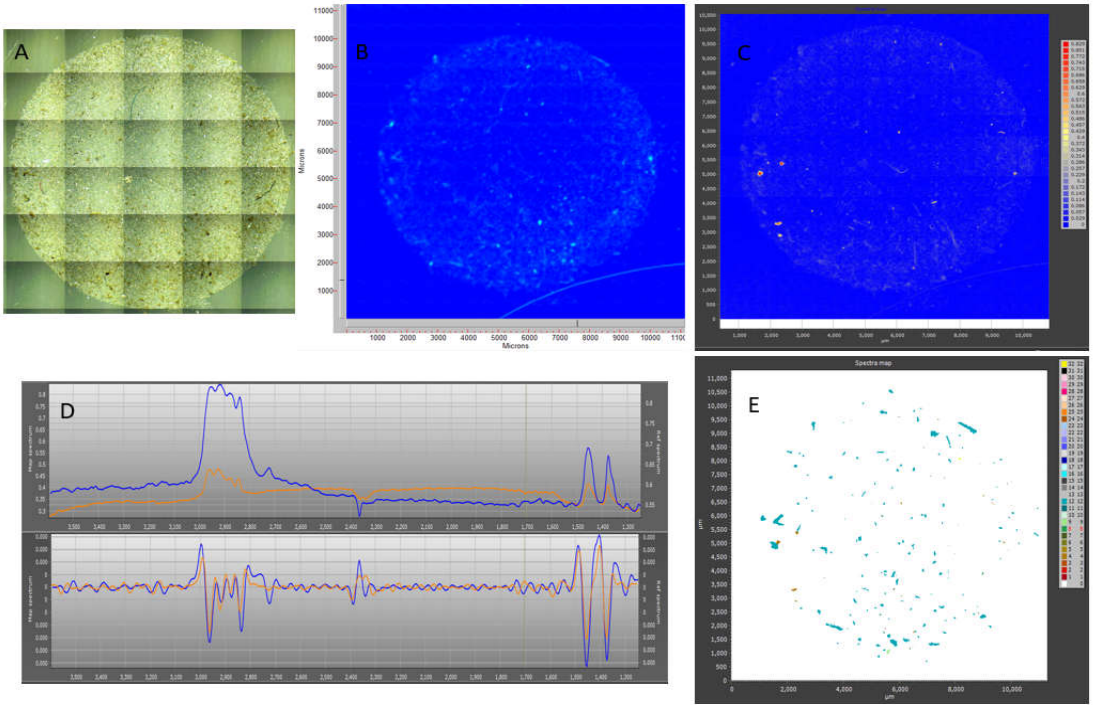

Figure 6. Visual image of the filter membrane (A). False color map showing relative intensity at 1725 cm-1 (B), from blue indicating no signal to red indicating high signal. Heat map showing the probability for polypropylene, from blue colors indicating low probability to red (C). Raw FTIR spectrum and derivative spectrum of polypropylene (blue = reference library spectrum, orange = sample spectrum) (D). False color overlay, plot (MP map) of identified synthetic polymers with information on major and minor dimensions. (E). Color codes for false color plots are shown in Figure 5.

Removal of polyamide from µFTIR dataset after quality control

In this study the identification of PA by µFTIR was considered uncertain due to technical issues and organic molecules that influenced the quantification of PA. After scrutiny of the results and the FTIR spectra, PA was excluded from the µFTIR results. The presence of PA was also documented by the py-GCMS analyses, where there were no technical issues. Py-GCMS identifies and quantifies the polymers based on indicator ions that are not overlapping with or influenced by the organic molecules that interfered with the µFTIR identification.

Mass estimates for µFTIR

µFTIR produces output in the form of particle numbers (counts) and particle size in two measured dimensions per polymer type. The particle numbers and dimensions can later be converted to a mass estimate using the following equation, according to the procedure of the SiMPle software developed by Aalborg University, Denmark and Alfred Wegener Institute, Germany:

$$\textbf{Length* width*(width*0.60) * } SG_p \qquad \textbf{Eq. 1.1}$$

where the length and width of the particle is measured by the software. The third dimension (height) of the particle is estimated as 60% of the width (minor dimension). The mass of each particle is thus calculated from the approximated volume per particle and typical density (specific gravity (SG_p) of the polymer type.

All masses and concentrations from both µFTIR and py-GCMS are given as µg/kg.

Thermal degradation analysis: Py-GCMS

Because the FTIR analysis method is non-destructive, the same samples could be analyzed by py-GCMS after FTIR, providing direct analysis of the mass per polymer group. For the py-GCMS, the limit of quantification (LOQ) in fish tissue is currently 1 µg/kg for most polymers, and 2 µg/kg for PMMA. The lower size for py-GCMS depends on the filter size, and for this study was ~10µm. Py-GCMS is a destructive method that uses thermal decomposition of materials at elevated temperatures in an inert (low-oxygen) atmosphere. Large molecules break at their weakest bonds, producing smaller, more volatile fragments. These fragments can be separated by gas chromatography and detected by a mass spectrometer. The output data can be used as a fingerprint to identify material. The obtained pyrograms, with peaks of ions appearing at different retention times, are compared with a customized database and cross-checked with literature to identify the chemical composition of the material using recommendations and selecting criteria from Fischer and Scholz-Böttcher (2017) and Gomiero et al., (2019). Standard curves with known concentrations are used to calculate the concentrations of target materials in the sample. Py-GCMS analyses were performed by NORCE (Stavanger) with a Shimadzu Optima 2010C GCMS controlled by GCMS solution V 4.45, equipped with a Rxi-5ms column (RESTEC, Bellefonte, PA) and coupled with Frontiers

lab's Multi-Shot Pyrolizer EGA/PY-3030D with auto-shot sampler (BioNordika, Norway, Figure 7).

Figure 7. - Py-GCMS equipment at NORCE PlastLab (Photo: Alessio Gomiero, NORCE).

Complementarity of results from py-GCMS and µFTIR

In contrast to µFTIR, which produces a count and size-measurements of particles, py-GCMS is a destructive method that irreversibly degrades the polymers and does not produce an image of the particles; however, it provides the total mass of the identified polymers independent of the particle size.
The methods FTIR and py-GCMS are therefore complementary and together increase the information gained from an extracted sample.

Orbitrap-Gas Chromatography-High Resolution Mass Spectrometry

The Orbitrap method (GC-HRMS) is based on chemical identification by high resolution mass spectrometry rather than optical/spectrometric identification. This is particularly relevant for particles smaller than 10 µm, which are difficult to detect. For Orbitrap, the samples were extracted with a suitable organic solvent after homogenization, followed by removal of lipids and other interferents. The method was tested as a supplement to µFTIR and py-GCMS that have a particle size limit above 10 µm. In contrast, the Orbitrap has the potential to detect signals from nanoparticles but will not identify the actual size of the particles. For this study we will therefore use the term microplastic (MP) and not nano/microplastic particles N/MP. We applied three different approaches for the determination of MP: 1) analyses of polymer signals detected in the fish tissue, 2) targeted analyses of additives commonly used in plastics, and 3) non-target analyses, in other words screening for unknown plastic related chemicals.

Target analyses of additives

We selected phthalates (softeners) and organic phosphor flame-retardant (OPFRs) as target additives. Both groups are commonly used in plastics in high amounts. They are not chemically bound to the polymer and can leach into the tissue of the organisms after uptake of the plastic (Koelmans et al., 2016). 14 phthalates and 19 OPFRs were analyzed in both the fish samples, the blanks and plastic material (feed pipe) from the fish farm.

Sample preparation for Orbitrap

In order to assess the presence of MP in salmonid tissues, we carried out several extraction procedures of fish tissue from farmed salmon and wild mountain trout for comparison together with material from feed pipes used in the fish farm supplied by the industrial partner (Lerøy). Abrasion from feed pipes has been suggested as a potential source of MP to aquaculture environments, which was also tested in the study Trackplast https://www.fhf.no/prosjekter/prosjektbasen/901519/. All extracts were analyzed together with the procedure blanks described above and then compared to relate the presence of polymer- and additive signals to the presence of MP in the tissues.

Statistical analyses and graphics

Data were analyzed and graphs made using IBM SPSS v.25.0 or Excel. Non-parametric correlation tests were used (Spearman's rho) to test the correlation between methods, and a non-parametric test (two samples Kruskal – Wallis) was used to test the difference in concentrations between tissues. Significance level was set at α=0.05.

3. Results

Microplastic detection and quantification by µFTIR and py-GCMS

The size distribution observed by µFTIR showed that the smallest particles (<50µm) dominated in numbers. µFTIR is able to detect particles down to ~10 µm, and it is highly likely that there are also smaller MP present, even if the current method cannot confidently quantify these particles. The majority of particles was below 50 µm but sizes up to 241 µm were also observed in samples from liver and muscle (Figure 8). The origin of the large particles may be from contamination, although the largest detected particles in controls were in the size class 151-175 µm. Based on this study we can however not conclude that transfer of such large particles into tissues occurs without closer inspection and explanation of the mode of uptake and transport.

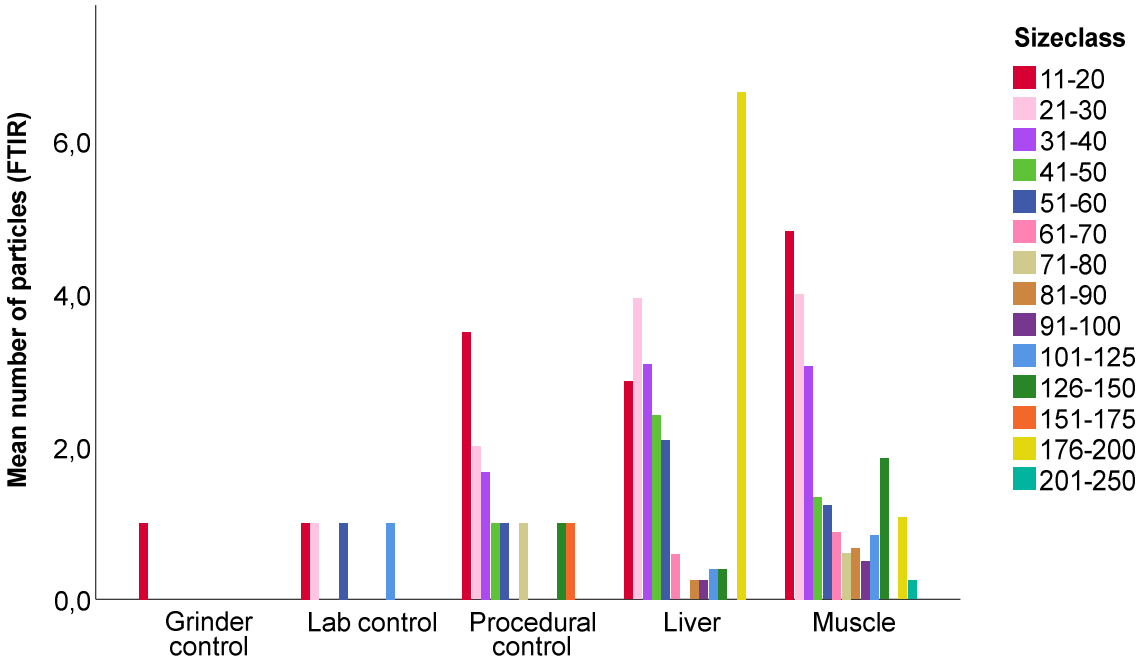

Figure 8. Mean count of MP particles per size class (µm) after µFTIR analysis, all locations combined. For tissues the numbers are normalized to 100 gram sample size.

Five of eight investigated polymer groups were identified by py-GCMS.

The most prevalent polymers detected by both methods (py-GCMS and µFTIR) were PP, PS and PE. According to py-GCMS analyses, PP and PS were found at all locations while PE, PA and PVC were found only at some locations (Figure 9).

14 of the 26 polymers groups analyzed by µFTIR were found in at least one sample. PA was removed from the µFTIR results due to technical difficulties (see materials and methods). When removing PA from the µFTIR-results, 13 of 25 investigated polymer groups were identified by µFTIR.

As FTIR screens for a library of thousands of polymers, several polymers in addition to those from py-GCMS were detected using µFTIR. In addition to the most found PP, PS and PE, µFTIR also found modified cellulose, Acrylates/PUR/varnish, PAN, and PES.

Tissue distribution of MP

Only one (mountain trout liver) of 28 pooled tissue samples did not contain any polymer particles when analyzed by µFTIR (Table 1). The positive samples contained three to 53 particles when normalized to 100 g tissue (PA excluded). Seven of 26 samples had MP concentrations below the LOQ for all polymers when analyzed by py-GCMS (Table 1). Note that also seven of eight procedural controls contained some polymer particles (nMP = 1-27) from µFTIR analyses, thus some of the detected MP in tissues are likely to be contamination. The procedural controls represent the extraction process for both µFTIR and py-GCMS, as both analyses were performed on the same extracts.

The number of analyses per tissue and location was low (2-4 pooled samples), and there was a considerable variation in results, shown by the large Confidence Intervals (Figure 10). Statistical analyses were hampered by the small data set and large variance. There were no statistically significant differences between MP concentrations in muscle and liver normalized to 100 g tissue. The variance was inequal (Levene's test for equality of variance), thus we used the non-parametric Kolmogorov Smirnoff independent samples test. There were no significant differences in MP concentrations between tissues or locations by either py-GCMS or µFTIR. Taking into account that a larger part of the fish consists of muscle than liver, and the analyzed sample sizes were comparable, the absolute amount of MP is likely higher in the muscle tissue than the liver per fish. The mean MP particles (from µFTIR) and mean concentration per tissue (py-GCMS) from wild trout, farmed and wild salmon is shown in Table 1. Median and spread of MP concentrations is shown by box-plot in Figure 10.

Contaminating polymers

For py-GCMS, a signal from PE was detected in one procedural blank and PP in two procedural blanks, but the concentrations of both polymers were of concentrations below the limit of quantification. PA was not found in the procedural blanks of py-GCMS. For µFTIR there was a higher number of control samples where contamination was observed. Especially for the procedural controls (n=8), the mean particle count (µFTIR) was high, indicating a level of contamination that may stem from reagents or equipment. The particles detected in the procedural blanks were among the smallest size classes 10-30 µm (Figure 8), and few particles per polymer type, which is probably why they were undetected by py-GCMS, whose LOQ corresponds to approximately one particle of 100 µm (depending on polymer density), and where larger particle derived signals dominate the results due to the particle mass.

Among the 26 polymers analyzed for by µFTIR and listed in figure 9, rubber type 3 (EPDM) was not seen in any tissue sample, and PC was found in one muscle sample only.

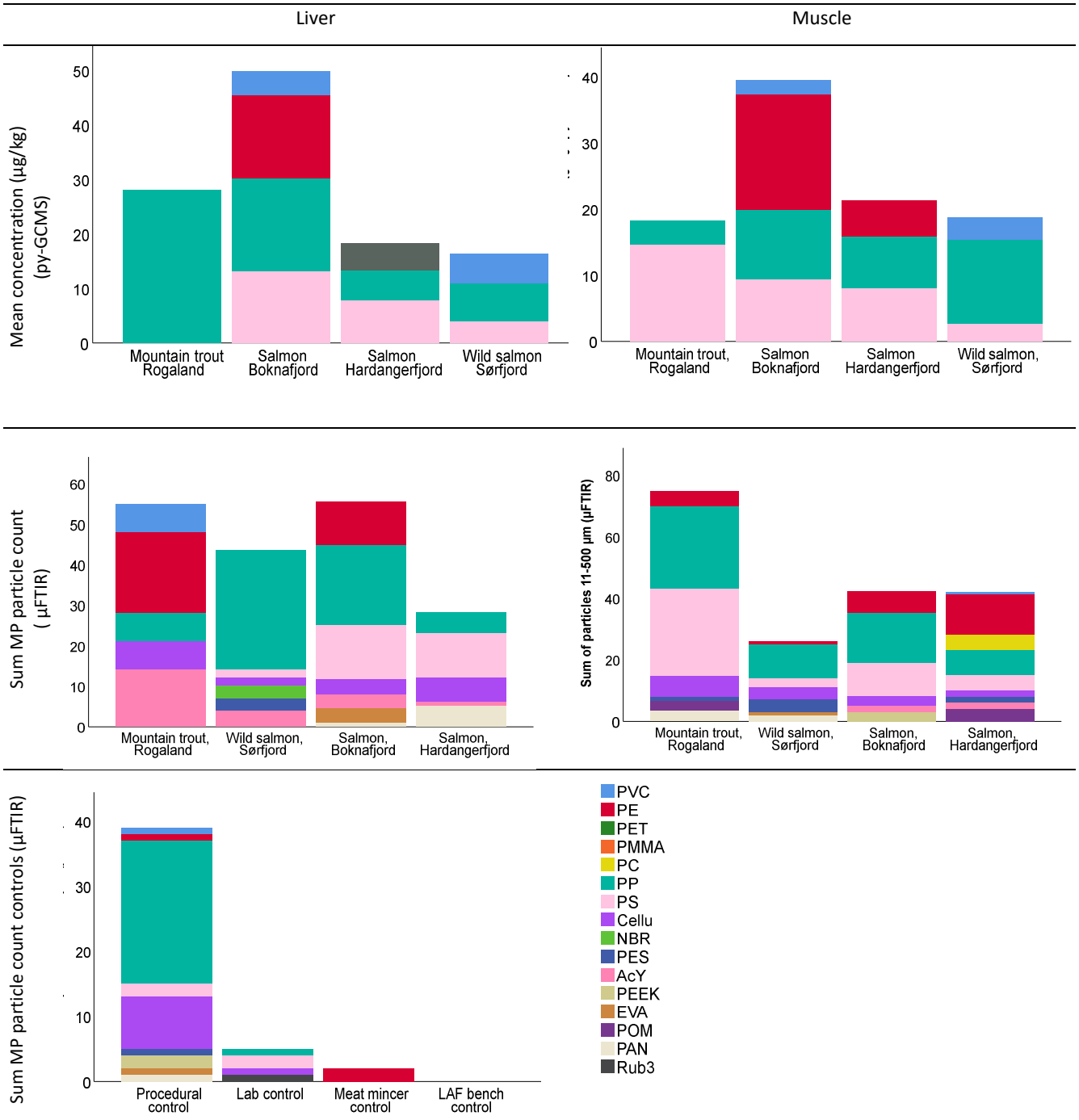

Figure 9. Polymers identified in salmonid liver (left column) and muscle (right column), per location and analytical method. Mean concentrations (µg/kg ww) following py-GCMS analyses of tissues and controls (top), and total sum of MP particles (nMP/100 g ww) (middle) after µFTIR analyses. Procedural controls and blanks from µFTIR analyses (bottom).

Table 1. Summary of direct results: concentrations of polymers (µg/kg (from py-GCMS) and number of MP particles/100g (from µFTIR) and in tissues of farmed and wild salmon and mountain trout.

		MP concentration (µg/kg) (Py-GCMS)			MP count (n) (µFTIR)		
		Positive N/Total N	Mean ± SD	Min-max	Positive N/Total N	Mean ± SD	Min-max
Salmon Boknafjord	Liver	3/4	49.9 ± 43.8	0-82.1	4/4	14 ± 10	3-27
	Muscle	4/4	39.5 ± 40.8	0-79.6	4/4	10 ± 9	3-23
Salmon Hardangerfjord	Liver	4/4	18.3 ± 12.5	0-28.0	4/4	7 ± 4	3-27
	Muscle	4/4	21.3 ± 8.7	10.9-32	4/4	11 ± 12	2-28
Wild salmon	Liver	2/2	16.5 ± 23.3	0-32.7	3/3	15 ± 10	3-22
	Muscle	3/3	18.7 ± 14.2	9.0-35	3/3	10 ± 4	6-13
Mountain trout	Liver	1/2	28.0 ± 39.6	0-56.0	2/3	18 ± 18	0-35
	Muscle	1/2	18.2 ± 22.7	0-43.7	3/3	25 ± 26	5-54
Controls	Procedural control	<LOQ	-	-	7/8	5 ± 5	0-15
	Lab air control	<LOQ	-	-	2/2	3 ± 2	1-4
	LAF bench air control	<LOQ	-	-	0/2	0	0-0
	Meat mincer control	<LOQ	-	-	1/1	1	-

The N value gives the number of samples with MP counts or concentrations above the LOQ. Numbers are standardized to 100 gram sample size and counts are rounded off to one decimal for py-GCMS and to whole numbers for µFTIR.

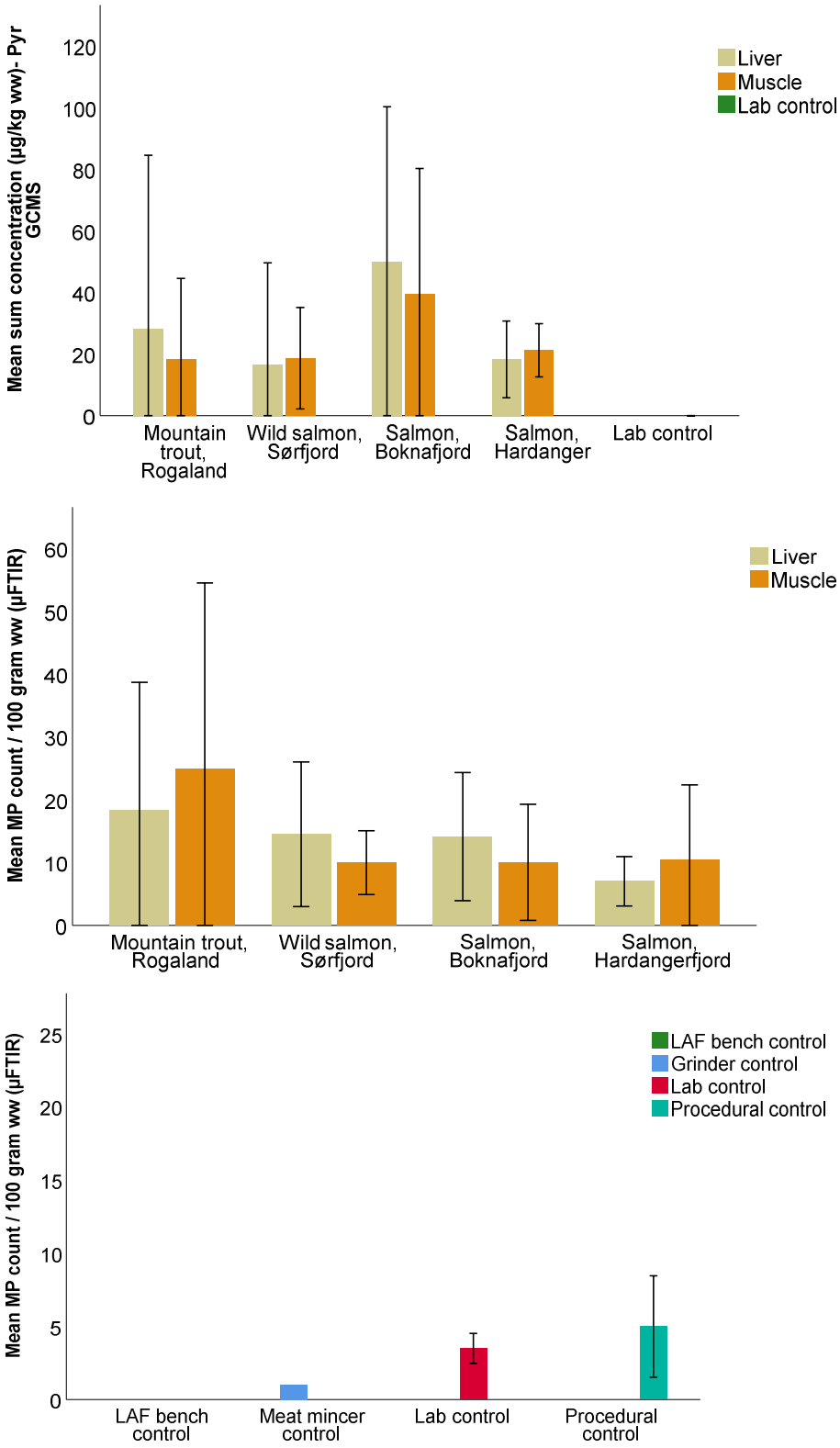

Figure 10. Bargraph showing the mean and 95% Confidence interval of MP in salmonid tissues and control samples. Concentrations as µg/kg ww from py-GCMS analysis (top), and mean sum of particles per 100 gram tissue from µFTIR analysis (middle). Procedural blanks (FTIR, bottom) are shown for comparison.

Correlation of results by py-GCMS and µFTIR analyses

MP concentration numbers resulting from py-GCMS analyses and particle numbers (MP counts) from µFTIR are shown in Table 1. After estimating the mass based on µFTIR counts (Equation 1.1), the concentration estimates by py-GCMS and µFTIR were compared and showed that py-GCMS tended to report higher concentrations than FTIR estimation (Figure 10, Annex 1, Table S1). Figure 11 shows the linear correlation between concentration estimates following FTIR and py-GCMS analyses. The figure shows the correlation after removing two extreme values from the FTIR results. The removed results were from one sample of farmed salmon muscle from Hardangerfjord (314.10 µg/kg), and one sample of mountain trout muscle (220.59 µg/kg). The result from the farmed salmon was influenced by four large particles in the size classes between 126 and 250 µm of PC, PP, PS, and the mountain trout had three large particles of PP in the size class 176-220 µm. When removing these two outliers, the linear correlation was $R^2 = 0.277$ (Figure 11). We see that py-GCMS in general finds higher concentrations than FTIR when analyzing the same samples. A perfect correlation would give an R^2 of 1.0. The correlation was weak, however, significant (Spearman rho = 0.679, $p<0.001$). Development and refinement of mass estimations by µFTIR as well as the inclusion of more polymer types for recognition in py-GCMS analysis may further improve the correspondence between the two methods. Increasing the analytical sensitivity of py-GCMS is also under development.

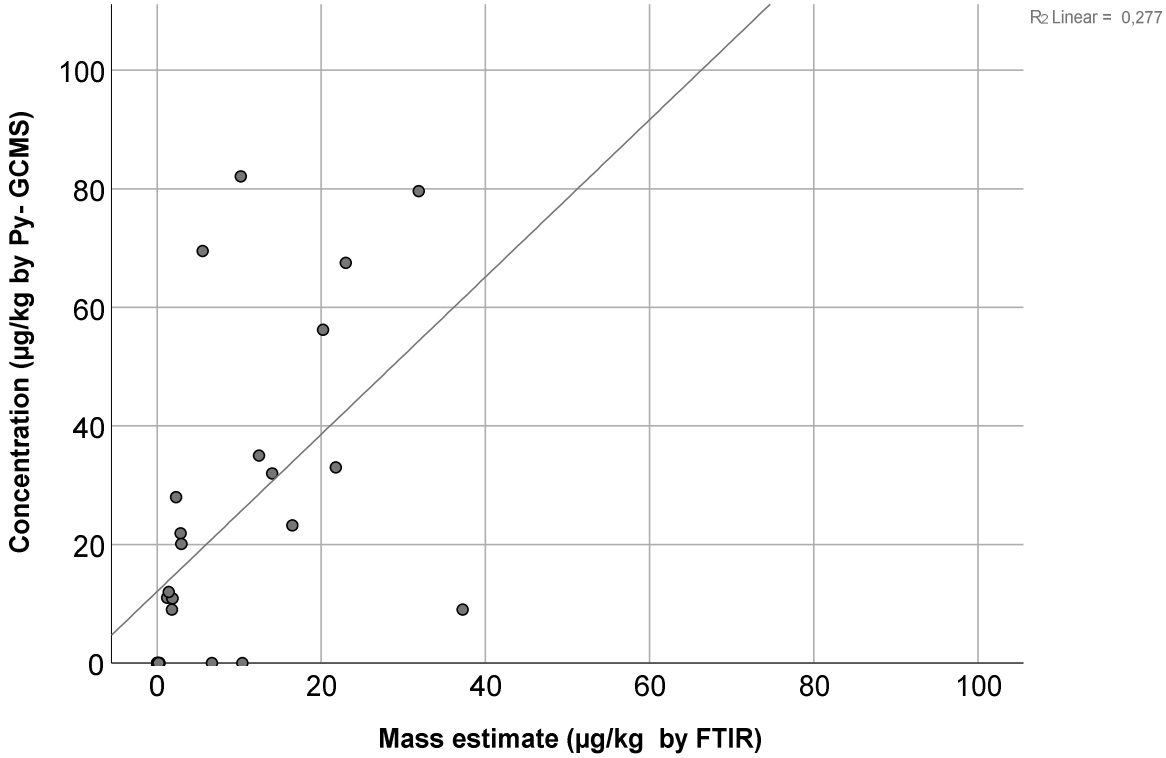

Figure 11: Linear correlation between py-GCMS analysis and µFTIR estimations of mass based on particle numbers and sizes. The correlation was significant at $p<0.001$.

Orbitrap High-Resolution Mass Spectrometry

Analyses of polymer signals

The analyses of plastic polymer related mass fragments resulted in inconclusive results, not specific enough to determine the presence of plastic polymers. This was caused by the similarity of the molecular signal produced by the traces of fish lipids still present in the sample and possible plastic signals from the polymer itself (Figure 12). Also, polymer particles will not dissolve completely with the organic solvents used, resulting in only non-specific degradation products/ unreacted monomers available for analyses.

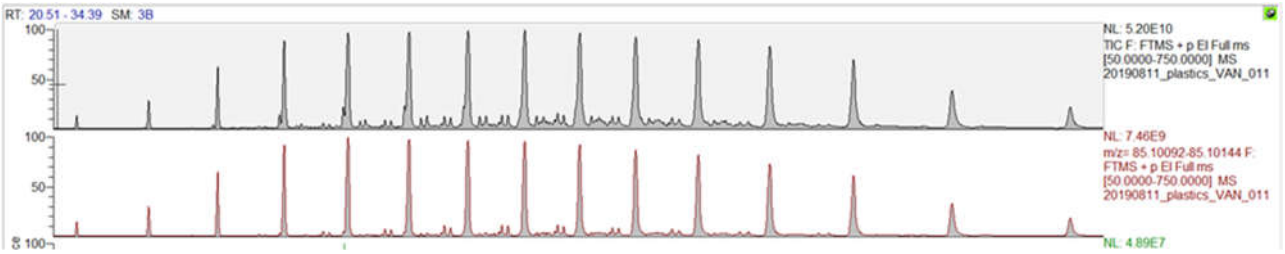

Figure 12. n-Alkanes found in extracts from fish samples

Phthalates

Both the muscle and the liver from farmed salmon contained higher phthalate concentrations than the mountain trout muscle (analyzed for comparison; Figure 13). All concentrations shown are blank corrected.

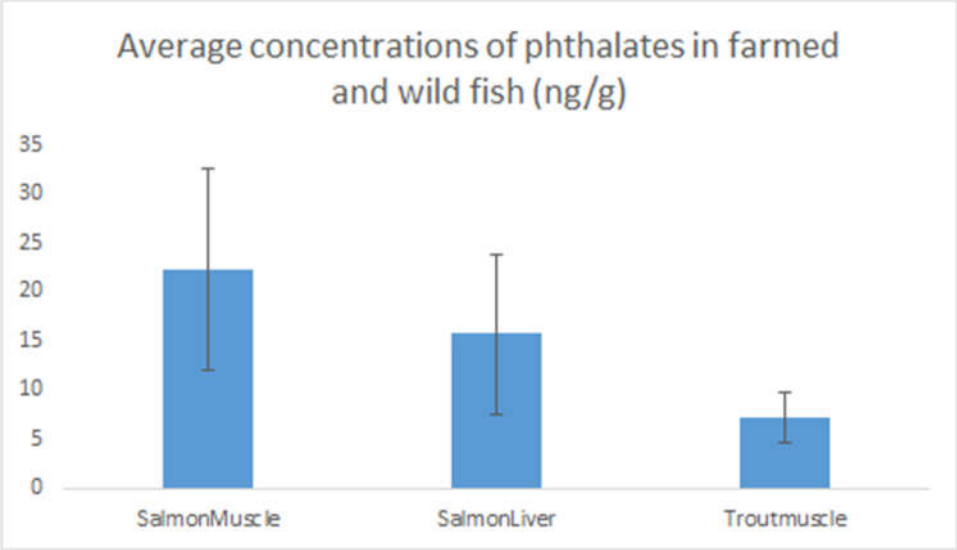

Figure 13. Comparison of the average of the sum of detected phthalates in ng/g with salmon muscle and salmon liver representing farmed fish, and trout muscle representing wild mountain trout. The given concentrations are blank corrected.

When evaluating the presence of the different phthalate compounds in the salmon and trout, we find one specific phthalate that is only present in the salmon muscle, as well as additive in the plastic of feed pipe, but not present in the reference samples from the wild trout (Figure 14). This phthalate is called bis(2-ethylhexyl) phthalate (DEHP) and its presence in muscle is a strong indicator of the presence of MP particles. Since this phthalate is metabolized when present in solution, the observation indicates continuous leaching from MP, acting as a continuous supply of this phthalate type. Thus, DEHP is detectable although it is quickly metabolized once it is absorbed by the fish. The presence of phthalates in all samples is likely due to the general environmental presence of the chemical group. This is also highlighted by the lower exposure in the remote lakes with less human activity and pollution.

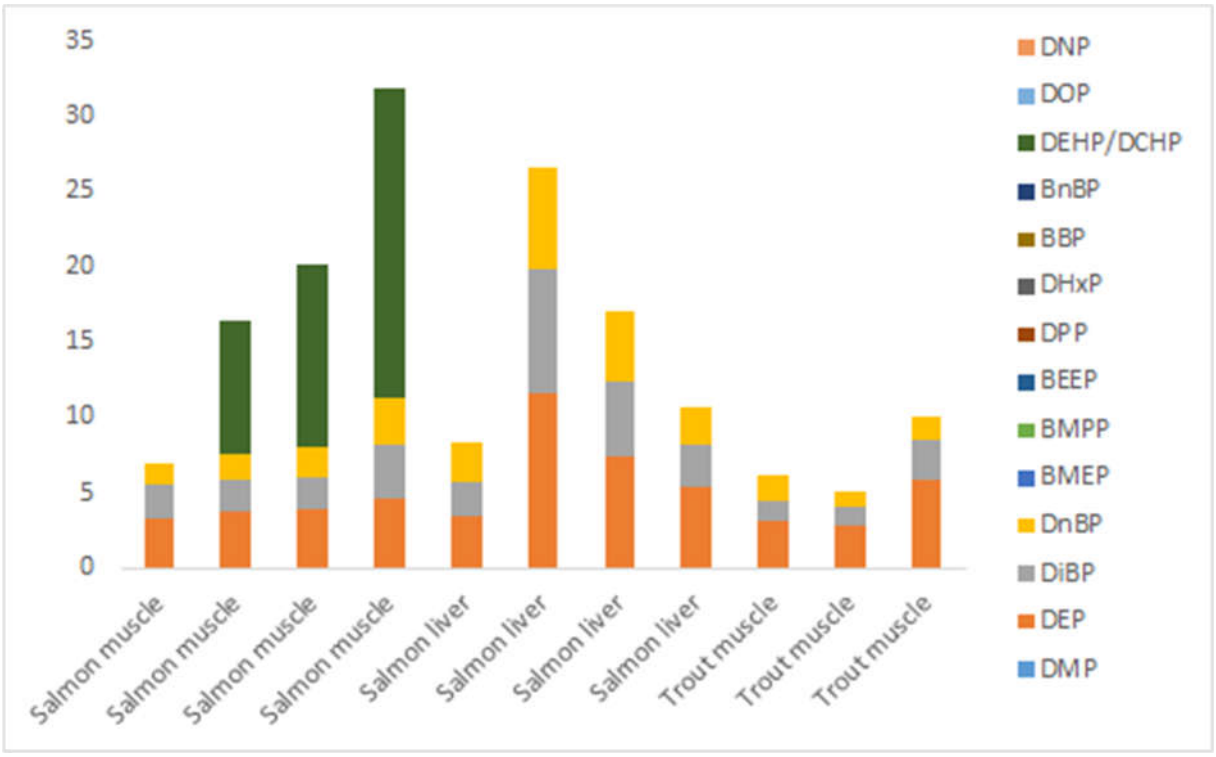

Figure 14. Phthalate concentrations in fish tissue in ng/g. All concentrations are blank corrected. (Abbreviations are listed in Acronyms and in Annex 4).

When comparing with the detected number of PPs in the same tissue samples, a similar polymer distribution was observed in all samples. Since the DEHP is only found in the muscle samples, and feeding pipes with abrasion were made of PE (Trackplast), PE could be the source for the detected phthalate concentrations.

Organohosphorous Flame retardants

For OPFRs no such relationship could be established with only limited detection in fish tissues in general.

Non-target measurements

Using the Orbitrap GC-HRMS for plastic additive analyses uncovered the presence of a multi aromatic phosphoric compound, most probably a UV stabilizer, in both plastic material obtained from the fish-farm, and to a lesser extent in the fish tissue. Additionally, a degradation product of the compound was found in both the fish and to a lesser extent in the pipe, indicating uptake of the original chemical by the fish followed by metabolization to the oxidized product.

As a secondary finding, the presence of chlorinated n-alkanes could also be proven (Figure 15).

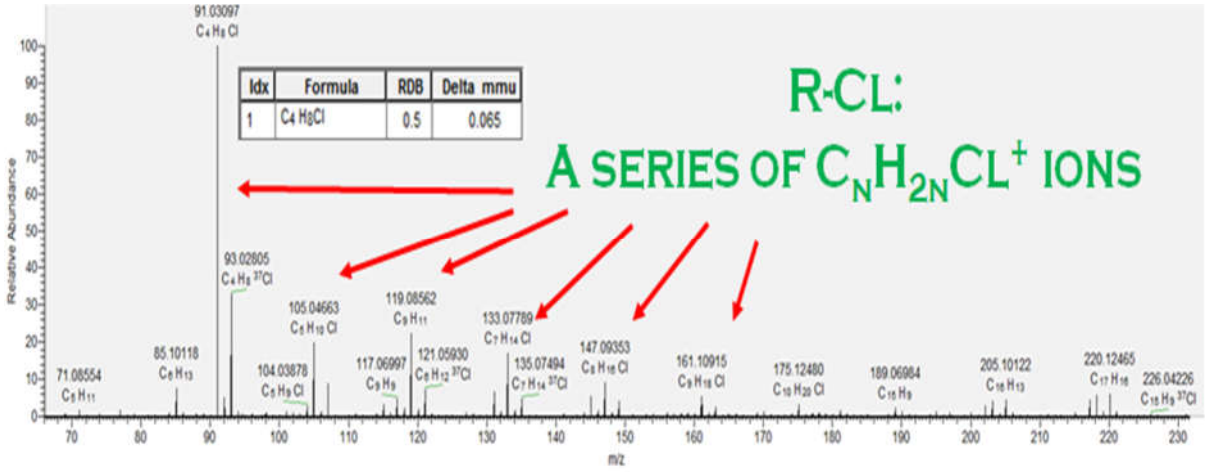

Figure 15: Mass spectra showing relative abundance of identified chlorinated alkanes in the samples.

Since high concentrations of both chemical groups could be found in the plastic from the fish farm, we see the potential to use them as a tracer for plastic uptake into fish tissues at very low concentrations, impossible for py-GCMS or FTIR to detect. The utility of this potential tracer will be followed up in future work.

4. Discussion

This study has detected MP of <10 to >200 µm in muscle and liver from farmed and wild salmonids, comparing three different methods of quantification. The study is the first to report both chemical characterization, size classes and tissue distribution of MP as well as the correlation of results between the quantitative methods in fish tissue. Published studies so far have focused on the ingestion of MP or attachment to surfaces and appendages of MP in marine fishes, but not on incorporation into the consumed tissues. Furthermore, it is important to collect information on the size of the plastic particles, as this influences the potential uptake, retention and effects for animals and consumers.

In this study, we have observed the presence of MP particles from the smallest size classes that are detectable by µFTIR (~10µm) to fibers of considerable size (>200µm), in both wild salmon, mountain trout and farmed salmon. Two of the methods tested (py-GCMS and µFTIR) were able to identify and quantify the polymers in the samples, while the HRMS

(Orbitrap) method was able to detect and identify chemical additives at very low concentrations.

Polymers and size classes

Observations of the higher prevalence of the smallest size classes of MP, mainly below 50 µm, corresponds to previous findings of higher levels of small particles in the environment (Bergmann et al., 2017, Mintenig et al., 2017, Haave et al., 2019, Lorenz et al., 2019). The size distribution in tissues suggests easier uptake of smaller particles, supporting previous experimental findings (Deng et al., 2018).

Both FTIR and py-GCMS determined PP, PE and PS to be the most prevalent polymers. These polymers are extensively used in both aquaculture and society at large.

The findings of MP in tissue samples reflect the omnipresence of MP in the environment. This suggests uptake and transfer of MP particles of 10-50 µm into edible tissues of both farmed salmon and wild fish that have been exposed in their natural habitats. The actual tissue concentrations must be interpreted with care, but the observation of higher levels of MP in tissue samples than in control samples cannot be disregarded.

Tissue distribution

There were no significant differences in MP concentrations or particle counts when standardized to 100 gram samples, between liver and muscle with either of the methods tested. However, significant differences might exist, but higher sample numbers and/or better methods may be necessary to detect real differences. This study can therefore not conclude a definite target organ for MP. Muscle tissue could however be suggested as a future primary target tissue for monitoring MP concentrations in aquaculture and fishery products, since 1) muscle samples are abundant and are routinely sampled for documentation of quality, levels of pharmaceuticals and other undesired chemicals, 2) the total amount of MP in muscle will be higher than the total amount in liver, if the amounts per 100 g are similar. However, liver would also be suitable, and may also be combined with the studies of biomarkers of toxicity in liver. Further studies are needed to confirm the relationship between exposure and uptake, and the suitability of muscle as a tissue for monitoring purposes. Extraction protocols require further development. In particular, extraction from fatty tissues is more demanding, as both fatty tissue and plastics are lipophilic. When compared to the controls, MP concentrations extracted from tissue, especially fatty tissue, are more likely to be underestimated due to loss during extraction from complicated matrices. Future upgrade of the methods might therefore also change these conclusions.

Comparison of methods

One major aim of the study was to test and evaluate the different methods for quantification of MP in biological tissues. The methods are still under development, and the results must be interpreted with the current methodological limitations in mind, and not considered to be sufficiently validated or accurate for standardized monitoring at this point,

however they do provide a better understanding of the situation, and may as such provide useful information. Both methods have been tested in comparative experiments with artificial simple matrices, and the laboratories have gained considerable expertise and insight into the method possibilities and limitations. A few of the method considerations are discussed in the following section.

Both methods determined PP, PS and PE to be the most dominant polymers in tissues. In some cases, some differences between py-GCMS mass estimates and µFTIR particles characterization analysis were observed. Such phenomena were mainly observed when MP levels were very low in the sample, indicating that the sensitivity of Py-GCMS may cause a "blind spot" for small particles of low mass, which will affect the correlation. On the other hand, mass estimation through theoretical conversion from particle sizes, assuming an ellipsoid of regular ratio between the dimensions as performed by the µFTIR approach, will necessarily lead to inaccurate results as a consequence of the deviation among the expected and the actual volume and shape of the observed two-dimensions of the particles. Looking at samples with higher concentrations, the mass estimates correlate significantly, showing that the same three polymers dominate the samples by both methods. Therefore, these polymers are likely to be present in the tissues of the analyzed samples.

Differences in the chemical identification

Remnants of organic matter may interfere with the identification of polymers, and a good purification procedure is key to successful identification in both methods. Additionally, standardization to 100 g probably affected small samples more than large samples, as multiplying the results from small samples to represent 100 g magnifies measurement errors. Therefore, the results for mountain trout are affected with higher level of uncertainty as compared to those for wild and farmed salmon.

Certain identification of several of the polymers and sensitive quantification of polymers in biological samples continues to be an area of development.

Differences in the mass estimates by FTIR

The tissue concentrations calculated from FTIR particle numbers depends on how the mass is estimated from the 2D microscopy-spectroscopy data and the mass calculation based on an idealized ellipsoid particle with a height that is always estimated as 60% of the minor dimension (Eq. 1.1). The height likely varies from 60% of the width, which may contribute to both under and over-estimation of mass. Compared to the direct concentration output by py-GCMS the mass estimates from FTIR were slightly lower.

The influence of particle shape

Fibers twist and often do not lay flat on the filter surface. Thus, several parts of a fiber are usually not in focus for µFTIR imaging. Also, other particles may partly cover the fibers, which cause them to be identified as several smaller particles (Figure 19). These two analytical challenges render parts of large fibers invisible to FTIR, which leads a single fiber

to appear as several particles, and thereby contributes to the lower MP mass and higher particle estimates by FTIR.

Using the µFTIR microscope one can provide an accurate description of fibers in the >100 µm fractions, providing information of polymer identity, color, and sizes. However, this would be slow, manual, and labor-intensive work. Due to instrument capacity and volume of the project, a qualitative description of fibers was not performed. However, in a follow up study, allocating more time, such an analysis would probably deliver valuable information. Another possibility is to "sandwich" the sample between two plates, pressing the fiber flat. That is possible for µFTIR analysis, but precludes subsequent py-GCMS, as particles are lost when disassembling the "sandwich".

Differences in the polymer selection

While µFTIR detects polymers of many kinds based on comparison to a reference library of thousands of polymers, py-GCMS quantifies a selection of polymers based on purchased standard polymers, and where standard curves have been made. As an exploratory tool for rare or unexpected polymers, py-GCMS may therefore give a limited picture compared to µFTIR.

Limits of Quantification

The LOQ for py-GCMS is currently at 1-2 µg for the investigated polymers, corresponding approximately to one particle of 100 µm, depending on polymer density. Py-GCMS depends on the presence of polymers in concentrations over the LOQ, which means that a single small particle may not be detected by py-GCMS, whereas it may be detected and classified using µFTIR. µFTIR visualizes single particles down to ~10 µm. However, the mass estimate for µFTIR will depend on both estimated area and theoretical polymer density. For such reason, the analytical sensitivity for µFTIR is not directly comparable to the LOQ for py-GCMS.

Comparison of methods – cost, time and sensitivity

It is at this point not possible to say that one of the methods of quantification is more correct than the other, and the methods continue to be under development to increase sensitivity and reduce measurement uncertainty, time and costs. Ring tests targeting MP in organic matrices, and the validation of results and quality assured Standard Operating Procedures are still needed and require time and effort. This has been started by Quasimeme for several matrices and the by the EU commission/Joint Research center /Bundesumweltamt, Germany, for water and sediments.

A summary of the evaluation of the methods is presented in Table 2.

Table 2: Summary of methods in terms of costs and time, result output and limitations.

Method	Hours per sample	Cost including basic data analysis (NOK) (personnel time + consumables)	Output, possibilities and limitations
µFTIR analysis of synthetic polymers	16	~16 hours + consumables = ~17000 per sample + time and costs for control samples	- The output is number of particles and size range of particles per polymer - Lower size limit ~10µm, 1 particle = ~0.5 ng - Misidentification of polymers can be caused by matrix-effects - Polymers are identified based on a reference library of several thousand spectra, including natural polymers (hair, cellulose, chitin) - Mass estimates depending on accuracy of volume estimate - Fibers are more laborious to identify, as they do not lie flat on the filter - Possibility to co-analyze additives, requires method development
Py-GCMS of synthetic polymers	13	~13 hours + consumables = ~ 14000 per sample + time and cost for control samples	- The output is given as mass per sample weight (µg/kg ww) - Limit of Quantification ~1µg/kg in biota, - Low mass single particles ~ 0.5 -1.0 µg are hard to detect - A selection of polymers that are actively looked for will be quantified, while other polymers will not be identified - Particle size is not shown. The method is destructive. - The sample can be physically separated into size fractions before analysis to gain information on size class - Possibility to co-analyze additives within the same pyrogram
HRMS (Orbitrap) Target analysis of additives	3,5	~3.5 hrs per sample + consumables ~ 4500 NOK per sample + time and cost for control samples, QC/QA	- Sensitive to very low levels of target compounds (pg/g) - The presence of polymer and particle size cannot be identified - Additives indicating parent compound (non-metabolized plastic) can be found. - Metabolized plastic additives indicating previous exposure can be found. - The output is given as concentration (pg/g)

The costs are shown per tissue sample, not including necessary control samples. One procedural control (no matrix) should be run for every five samples. Air and other contamination controls should also be run, number depending on time and complexity of the procedure.

HRMS - Orbitrap

The application of high-resolution mass spectrometry combined with gas chromatography proved to be a useful supplemental tool to link the detected MP to the presence of additives in the fish tissue at ultralow concentrations at the low pg/g range. This low concentration range is not accessible for the other methods applied in this project for measuring MPs and could be further developed to assist with the detection of MPs and potentially also NPs in farmed fish. However, the concentrations found of BBP, DEHP and DIDP below 50 µg/kg of fillet will not exceed the Tolerable Daily Intake (TDI) for five phthalates of 50 µg/kg of body weight (µg/kg bw) per day based on their effects on the reproductive system, as estimated by the European Food Safety Authority (EFSA, 2019) at normal consumption. DBP and DINP remain to be analyzed. So far, a good chemical tracer for the presence of MPs has not been found, since MPs are too diverse and the tracer should not be already distributed in the environment as a pollutant. The approach tested here uncovered several candidates, which are worthwhile developing further. If successful, this alternative approach would allow the determination of a relevant subgroup of MPs in tissues in a very fast and sensitive manner.

All tested methods are highly specialized and require skills, training, time and, not least, specialized equipment. Each method has benefits and limitations, and further development is needed to make them more cost-efficient. To date, the methods are complementary, giving slightly different information that together provides a better picture than any of the methods do alone.

In the following, we will discuss some of the uncertainties that are recognized for this study.

Possible contamination of samples

The presence of MP was found in control samples from µFTIR, suggesting contamination during the procedure, or from reagents or equipment.

The room and LAF bench controls as well as procedural controls showed generally lower levels of MP than the pooled tissue samples. No air-borne plastic contamination was detected in two 100 ml samples of water left open in the LAF bench the entire working day. The lab control contained two fragments of PP in size classes 21-30 µm and 101-125 µm, and one PVC particle of 51-60 µm. The PVC likely stems from the lid coating of the glass jar used.

The eight procedural controls (blank samples without matrix) showed a higher number of polymers and particles than airborne controls and meat mincer control. PP was the most dominant polymer, with 13 of 17 particles, including one particle of size class 151-175 µm. Chemically modified cellulose was also among the large particles. The range of polymers indicates that the procedure may contribute to results in terms of number of particles, although the mass contribution from the small particles is low. However, looking at the results from py-GCMS, the blanks were under the LOQ. This points out the potential of µFTIR as a sensitive technique to assess the sources of contamination during sample preparation and analyses.

The main polymer found from the 100 ml water sample run through the stainless-steel meat mincer was PA. This type of polymer was unexpectedly present in a part of the mincer, thus leading to potential contamination of the samples during processing. For this reason, PA was removed from the outcomes of the present report, and the meat mincer is to be replaced.

In this context, presence of contamination in blank analyses is common in all chemical analyses and implies that the results must be considered to have an inherent uncertainty. Subtracting blank corrections from procedural controls for µFTIR – analyses based on total particle numbers, disregarding type and size categories, would in this study lead to negative results for many samples. Therefore, a blank correction was not performed, but the inherent uncertainty is considered in the interpretation. However, it is worth mentioning that the salmonid samples contained polymer types that were not present in the procedural controls. For the py-GCMS method the blanks were zero and would have been unchanged by a blank-correction.

Loss of material during the process

In this study the smallest size class (10-20 µm) had lower numbers than the size class 21-30 µm. The glass filtration crucibles had a pore size of 10-16 µm, which may have caused partial loss of particles below 16 µm. Observations also indicate that the smallest steel filter of 10 µm did not retain all particles of 10 µm. Similarly, few particles of 100 µm were found to pass through the 100 µm filter, but they would end up on the smaller filter and be included in the final sample. Particles smaller than 100 µm also frequently remained on the 100 µm filter. For these reasons we combined all size classes per sample and did not analyze the filter size classes separately.

Potential detrimental effects of microplastic in tissues

It was not within the scope of this study to assess any physiological effects of MP, uptake mode or histological localization within the tissues and organs. However, the presence of MP particles in muscle and liver, as well as previous findings of MP in gills (Gomiero et al., 2020, https://www.fhf.no/prosjekter/prosjektbasen/901519/) indicates that future effect-studies are warranted.

Conclusion

- This study has analyzed 14 pooled samples of muscle and 14 pooled samples of liver from 75 salmonids altogether, and analyzed MP in the tissues, testing three different state of the art methods.
- The results show that the methods are sufficient to detect and identify synthetic polymers in salmonid tissues.
- The results suggest that MP is present in both wild and farmed salmon as well as wild mountain lake trout. The levels of MP in both muscle and liver was higher as compared to control samples.
- Particles of 10-50 µm are the most prevalent sizes.

- The most frequently detected polymer types are PS, PP and PE, some of which are also detected in wild trout from remote mountain lakes. Thus, the results supports the omnipresence of MP, and the possibility of distribution by atmospheric fallout. The dominant polymers are not found in control samples to the same degree or with the same size distribution as in tissue samples, and for py-GCMS the control samples had MP below LOQ.
- The observation of MP occurrence is considered reliable, yet the actual concentrations still carry uncertainty.
- The methods are more likely to underestimate than overestimate the concentrations, based on loss of material during extraction/filtration and low analytical sensitivity.
- The presence of MP was found in control samples for µFTIR analyses suggesting contamination during the procedure, or from reagents. For py-GCMS the level of contamination in controls were below LOQ.
- Py-GCMS and µFTIR are methods that show different aspects of the same situation, and one cannot be considered more accurate than the other. There was a significant correlation between the results, and the methods are currently complementary.
- The application of GC/HRMS (Orbitrap) supplements existing methods by its potential to determine additives originating from MPs.
- There are no observed differences between MP in liver and muscle tissues, per 100 grams, and as target tissue for MP exposure it is not possible to recommend one over the other. For monitoring purposes, muscle samples are routinely obtained, and may therefore be a suitable candidate for further investigation of the occurrence of MP in fish. Muscle samples may also be used for investigation of the correlation between exposure and uptake of MP.

The observation of MP particles in tissues warrants further investigations of negative physiological effects in exposed fish and consumers.

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Annex 1 - Full results

Table S1: Concentration means and SD, maximum, number of analysed pooled samples. Sum MP particles from µFTIR, sum mass py-GCMS and calculated total massfrom µFTIR . All results are normalized to 100 gram sample size.

		Sum MP particles (µFTIR)				sum Mass py-GCMS µg/kg				Stated total mass (FTIR) µg/kg			
		Mean	SD	Maximum	N	Mean	SD	Maximum	N	Mean	SD	Maximum	N
Salmon Boknafjord	Liver	15.0	11.1	29.0	4	49.9	43.8	82.1	3	11.1	9.3	23.1	4
	Muscle	26.5	28.5	66.7	4	39.5	40.8	79.6	4	25.3	14.7	41.4	4
Salmon Hardanger-fjord	Liver	9.1	4.6	14.0	4	18.3	12.5	28.0	4	7.3	6.8	17.1	4
	Muscle	16.8	9.6	29.1	4	21.3	8.7	32.0	4	86.8	152.3	315.1	4
Wild salmon, Sørfjord	Liver	14.5	9.9	21.8	3	16.5	23.3	33.0	2	9.2	11.2	21.8	3
	Muscle	12.0	5.3	16.0	3	18.7	14.2	35.0	3	5.4	6.5	12.9	3
Mountain trout, Rogaland	Liver	27.3	23.7	42.1	3	28.1	39.7	56.0	2	29.2	33.7	66.1	3
	Muscle	25.0	25.6	53.8	3	18.2	22.7	43.7	3	74.0	126.9	220.6	3

Annex 2 - Biometric data

Table S2: Average (±Standard deviation) of fish size, and liver weight of livers used for analyses of microplastics. All results for pooled samples were normalized to 100 gram sample size.

	Total fish	Pooled fish	N	Average lenght	Average weight	Average liver weight	Gender	
				(cm)	(g)	(g)	Male	Female
Farmed salmon (small) Boknafjord	20	fish 1-5	5	45.8±3.4	1375.2±275.7	12.9±2.9	1	2
		fish 6-10	5	44.6±1.8	1170.6±206.2	11.2±2.5	2	3
		fish 11-15	5	45.6±1.8	1338.3±200.9	10.9±1.6	5	0
		fish 16-20	5	46.5±0.8	1349.4±76.8	11.1±0.6	3	2
Farmed salmon (large) Hardangerfjord	20	fish 1-6	6	66.9±3.3	3767.2±317.1	38.1±8.6	2	4
		fish 7-11	5	68.8±2.7	3861.0±119.7	36.6±5.8	3	2
		fish 12-16	5	69.8±4.0	4107.6±426.1	42.1±7.6	3	2
		fish 17-20	4	69.7±1.6	4060.2±406.8	38.1±2.8	3	1
Mountain trout, Rogaland	15	Trout Vasstølvatnet 1-5	5	18.4±2.2	86.4±30.2	0.84±0.17		
		Trout, Vasstølvatnet 6-10	5	23.3±1.0	149.8±13.6	1.08±0.23		
		Trout Holmavatnet 11-15	5	26.2±1.0	234.8±116.4	3.1±2.3	2	2
Wild salmon Sørfjorden	15	fish 1-5	5	52.0±4.6	1370±517.6	13.2±5.7	5	0
		fish 6-10	5	70.6±7.0	4025±866.4	56.6±17.7	2	3
		fish 11-15	5	75.6±7.0	4209±1017.3	46.3±14.6	2	3

Annex 3 - Statistical analyses

Table S3. Correlation analysis between concentration from py-GCMS compared to sum of all particles identified by µFTIR.

			Sum Mass µFTIR (µg/kg)	Sum Mass Py-GCMS (µg/kg)
Spearman's rho	Sum Mass µFTIR (µg/kg)	Correlation Coefficient	1.000	0.679**
		Sig. (2-tailed)	.	0.000
		N	40	25
	Sum Mass Py-GCMS (µg/kg)	Correlation Coefficient	0.679**	1.000
		Sig. (2-tailed)	0.000	.
		N	25	29

**. Correlation is significant at the 0.01 level (2-tailed). After removal of two outliers in the FTIR analyses, influenced by large fragments.

Annex 4: analytes for Orbitrap

DEHP	Bis(2-ethylhexyl) phthalate
DEP	Diethyl Phthalate
DHxP	Di-n-hexyl phthalate
DiBP	Phthalic acid diisobutyl ester
DiDcP	Di-iso-decyl phthalate
DiNP	Di-iso-nonyl phthalate
DMP	Dimethyl Phthalate
DMPP	Bis(4-methyl-2-pentyl) phthalate
DnBP	Di-n-butylphthalate
DNP	Di-n-nonyl phthalate
DOP	Di-n-octyl phthalate
DPP	Dipentyl Phthalate
EHDP	2-Etylhexyldiphenylphosphate
T2IPPP	Tris(2-isopropylphenyl) phosphate
T35DMPP	Tris(3,5-dimethylphenyl) phosphate
TBEP	Tris(2-butoxyethyl) phosphate
TCEP	Tris(2-chloroethyl)phosphate
TCPP	Tris(2-chloroisopropyl) phosphate
TDCPP	Tris(1,3-dichloro-2-propyl) phosphate
TEHP	Tris(2-ethylhexyl) phosphate
TEP	Triethyl phosphate
TMTP	Tri-m-tolyl phosphate
TNBP	Tri-n-butyl phosphate
TOTP	Tri-o-tolyl phosphate
TPHP	Triphenylphosphate
TPrP	Tripropyl phosphate
TPTP	Tri-p-tolyl phosphate