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Validation of an imaging FTIR spectroscopic method for analyzing microplastics ingestion by Finnish lake fish (*Perca fluviatilis* and *Coregonus albula*)☆

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ABSTRACT

Despite the ubiquitousness of microplastics, knowledge on the exposure of freshwater fish to microplastics is still limited. Moreover, no standard methods are available for analyzing microplastics, and the quality of methods used for the quantification of ingested microplastics in fish should be improved. In this study, we studied microplastic ingestion of common wild freshwater fish species, perch (*Perca fluviatilis*) and vendace (*Coregonus albula*). Further, our aim was to develop and validate imaging Fourier-transform infrared spectroscopic method for the quantification of ingested microplastics. For this purpose, enzymatically digested samples were measured with focal plane array (FPA) based infrared microscope. Data was analyzed with siMPle software, which provides counts, mass estimations, sizes, and materials for the measured particles. Method validation was conducted with ten procedural blanks and recovery tests, resulting in 75% and 77% recovery rates for pretreatment and infrared imaging, respectively. Pretreatment caused contamination principally by small <100 μm microplastics. The results showed that 17% of perch and 25% of vendace had ingested plastic. Most of the fish contained little or no plastics, while some individuals contained high numbers of small particles or alternatively few large particles. Perch from one sampling site out of five had ingested microplastics, but vendace from all sampling sites had ingested microplastics. The microplastics found from fish were mostly small: 81% had particle size between 20 and 100 μm, and most of them were polyethylene, polypropylene, and polyethylene terephthalate. In conclusion, the implemented method revealed low numbers of ingested microplastics on average but needs further development for routine monitoring of small microplastics.

1. Introduction

Microplastics (MPs) are emerging global pollutants in freshwater environments (Li et al., 2020). Nevertheless, knowledge of the ingestion of MPs by freshwater fish is still limited, compared to the data available from marine and brackish water environments (Budimir et al., 2018; Lusher et al., 2017; Neves et al., 2015; Pereira et al., 2020). However, the available data on the presence of MPs in fish suggests that the uptake and ingestion of MPs by freshwater fish is common.

Habitat and the level of MP pollution can affect MP uptake of fish. For example, higher numbers of MPs have been detected from the digestive tracts of fish in urban environments compared to more pristine areas in the Gulf of Mexico (Phillips and Bonner, 2015), in French rivers (Sanchez et al., 2014) and in a Central Texas River Basin (Peters and Bratton, 2016). However, in the tributaries of Lake Michigan, MP numbers in surface waters and the count of ingested MPs in fish did not correlate, but the feeding characteristics had a notable effect on MP uptake (McNeish et al., 2018). For example, the number of MP fibers in marine intertidal fish have been markedly higher in omnivorous than in herbivorous or carnivorous fish species (Mizraji et al., 2017). Moreover, Rummel et al. (2016) have suggested that demersal and pelagic fish with non-selective feeding strategies and a wide range of food sources are more likely exposed to MPs through their normal feeding habits than other fish, and may ingest more likely MPs.

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A recent experimental study on larval freshwater fish indicated that MPs are eaten with prey, and because fish larvae do not possess additive genetic variation, they probably are not able to adapt to the increasing MP pollution (Huuskonen et al., 2020). In addition to the harm caused to fish individuals, MPs can have negative effects in food chains (Wang et al., 2020). Seafood is also proposed to be a potential exposure route of MPs for humans (Barboza et al., 2018; Hantoro et al., 2019). However, the evidence for the importance of food or seafood in the exposure of humans to MPs and plastic-related chemicals is equivocal and the negative health effects are largely unknown (Rist et al., 2018). To assess the risks that MPs may cause to fish individuals or consumers of fish, more knowledge of the ingestion rates and types of ingested MPs is needed.

Regarding the analytical methodologies, Lusher et al. (2017), O'Connor et al. (2019), and Collard et al. (2019) have remarked that the quality of analytical procedures used for determining ingestion of MPs by fish should be improved. They propose wider use of quality control and assurance (QC/QA) measures, including negative controls (blanks), positive controls (recovery tests), use of non-plastic clothes and equipment, use of laminar flow cabinets, and adequate sample pretreatment and measurement methods. Additionally, Collard et al. (2019) have suggested that the number of samples analyzed should be representative, consisting of at least 50 individuals, and the MP particle size ranges should be reported according to true measured values instead of for example pore sizes of filters. Currently, most of the published studies have utilized methods, which include visual selection of particles before spectroscopic analysis (Collard et al., 2019). Instead of visual selection, which is prone to subjective bias and is not applicable for very small particles, imaging Fourier-transform infrared (FTIR) spectroscopy allows the automatic detection of every particle larger than approximately 20 μm (Löder et al., 2015).

The aim of this study was to develop and validate a method to analyze numbers and types of MPs from gastrointestinal tracts of two common freshwater fish species, vendace and perch. Both species are commonly used for human consumption in Nordic countries and are also popular for recreational fishing. Vendace is a planktivorous fish feeding almost entirely on zooplankton in the water column and surface (Czarkowski et al., 2007), while perch is a predatory species feeding on zooplankton, macroinvertebrates, and other fish (Jacobson et al., 2019). Currently, the discussion on the methods for MP monitoring are still ongoing, and the size classes are often determined by the sampling methods (Hartmann et al., 2019). While methods have not been harmonized yet, it is important to provide comparable datasets from different environments. For example Frias and Nash (2019) state that current and future monitoring programs will likely concentrate on >100 μm MP. However, in this study 20 μm–5 mm MP were qualified and quantified with imaging μFTIR spectroscopy, because the studied fish were relatively small, and small MPs have proposed to be more harmful.

The method was optimized for monitoring relatively large set of fish samples and was validated with blanks and recovery tests, consisting 90 μm polystyrene beads, which are standardized reference materials and enable the reproducible testing of different methods. The hypothesis was that method would perform well for the identification of MPs, but the efficiency of the pretreatment and possible contamination would affect sensitivity and recovery rate. It was further hypothesized that the studied fish species would ingest low numbers of relatively small-sized MPs of common plastic types. Because perch and vendace have differences in diet and habitat, they were expected to ingest different numbers of MPs. Moreover, fish were expected to ingest different amounts of MPs in different sites around the lake due to potential spatial difference in their exposure to MPs (Uurasjärvi et al., 2020). However, because vendace may move around the lake, they were presumed to represent the MP pollution of the lake generally instead of a sampling site.

2. Methods

2.1. Sampling

Juvenile first year class vendace (*Coregonus albula*) and perch (*Perca fluviatilis*) were sampled from Lake Kallavesi, located in Eastern Finland (Fig. 1). The sampling sites for perch were chosen based on previous observations on MP particle concentrations of surface waters (Uurasjärvi et al., 2020) to represent potential point sources. Vendace were caught from three transects in the open lake areas.

A beach seine was used to catch perch close to the shoreline. The beach seine was spread open approximately 10 m from the shoreline from a small motorboat and pulled to the shore by dragging from the side ropes of the seine. The fish were immediately terminated by decapitation, placed in clean plastic zip lock bags and transferred to the laboratory, where they were stored frozen −20 °C until further processing. Vendace were acquired from a local fisher, who caught them with a trawl from the open lake areas. Sizes and number of fish samples analyzed are shown in Table 1. The fish were chosen as similarly sized as possible (more information about the sizes is available in the Supplementary Material).

2.2. Sample pretreatment – enzymatic purification

Precautions for contamination. All laboratory equipment and materials were thoroughly rinsed with MilliQ water, and the solutions were filtered through 0.7 μm GF/F filters before use. Cotton lab coats were worn in the laboratory. The pretreatments were conducted in a laminar flow cabinet and the final filtrations before FTIR in a fume hood. To monitor the possible sample contamination and calculate limits of detection (LOD) for MP counts and masses, blank samples, bottles without fish organs (n = 10) were treated and analyzed similarly to fish samples. They represented in detail only the digestion and the FTIR measurement, but not the dissection. However, the dissection can be considered as a negligible source of contamination compared to the digestion.

Dissection of fish and digestion of tissues. The fish were thawed in room temperature and rinsed with Milli-Q water. The length and weight of each fish were recorded prior to dissecting. The fish were dissected by starting from the anus and cutting up to the gills with dissecting scissors. The gastrointestinal tracts (GIT) were carefully removed with tweezers and placed in 250 mL laboratory glass bottles, covered with glass lids. GITs of most of the fish were digested individually, but in 25% of all samples, concerning both vendace and perch, GITs from two small fish individuals of the same species were combined and digested together to reduce the time required for the analysis. In these cases, the results were calculated by dividing the results with the number of individuals per sample, or total mass of both individuals. The GITs were digested using the Universal Enzymatic Purification Protocol (UEPP) by Löder et al. (2017).

First, 100 mL of sodium dodecyl sulphate (SDS, 10%) was added and samples were incubated at 50 °C for 24 h. Then samples were divided into two size fractions using a 500 μm stainless steel sieve, as described by Löder et al. (2017). The fraction >500 μm was visually sorted for potential MPs under a stereomicroscope. The fraction <500 μm underwent the UEPP: Each sample was filtered through a stainless steel filter (47 mm diameter, pore size 20 μm). A single filter per sample was used throughout the whole digestion process. All equipment that contacted the samples were rinsed thoroughly with ethanol and Milli-Q water to avoid losing MPs. After each filtration, the filter with the residues was carefully placed in the glass bottle. Next, the respective enzyme or chemical solution was added and the samples were incubated as described by Löder et al. (2017). The following chemicals were used in this study: SDS 10%, protease, H_2O_2 (I), chitinase, and H_2O_2 (II), in this order. Lastly, samples were filtered and stored in glass bottles on the stainless steel filters, and approximately 50 mL of Milli-Q was added.

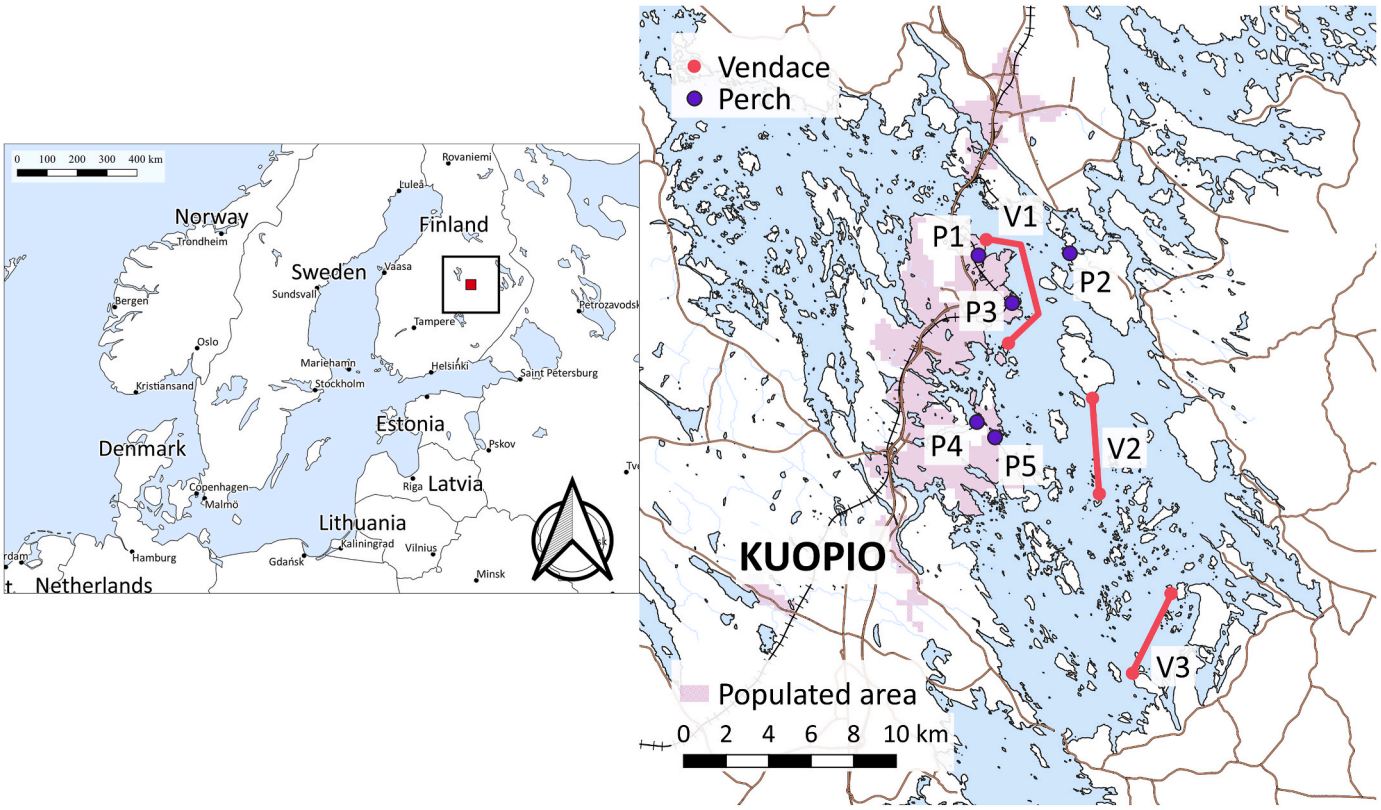

Fig. 1. Sampling sites of perch (P1, P2, P3, P4 and P5) and vendace (V1, V2 and V3) in the Lake Kallavesi, Eastern Finland.

Table 1
Numbers and sizes of analyzed fish. SD = Standard deviation.

Species	Vendace	Perch
Fish n	45	51
Mean ± SD weight (g)	6.3 ± 0.8	3.4 ± 2.8
Mean ± SD length (cm)	10 ± 0.6	6.8 ± 1.4

Preparations for infrared imaging. Samples were filtered to 5 μm silver membrane filters (Sterlitech Co) to circular area with 12 mm diameter for the FTIR imaging. Samples on the stainless steel filters and the container bottles were carefully rinsed to the filtration system to ensure, that all particles were filtered. To monitor contamination in the FTIR step, blank MilliQ samples (n = 3, V = 50 mL) were filtered and analyzed similarly.

2.3. Infrared imaging and data analysis

Fourier-transform infrared (FTIR) spectral maps were measured with an Agilent Cary 670/620 imaging FTIR spectrometer equipped with 128 × 128 focal plane array (FPA) detector. Measurements were done from the whole filter area (diameter 12 mm) in reflection mode using 15× cassegrain objective, 8 cm^{-1} spectral resolution, 4 scans, 3800–800 cm^{-1} spectral range, and 5.5 μm pixel resolution. The smallest detectable particle was therefore limited by the pore size of the metal filter used in the pretreatment (20 μm).

Data was analyzed with siMPle software (Primpke et al., 2020). Spectral libraries composed of the most common plastic polymers, including polyethylene (PE), polypropylene (PP), polyamide (PA), polyethylene terephthalate (PET), polystyrene (PS), acrylonitrile butadiene styrene (ABS), polyurethane (PU), polyvinyl chloride (PVC), polymethyl methacrylate (PMMA), polyacrylonitrile (PAN) and natural polymers (cotton, proteins) were used for material characterization. The library was composed from spectra provided with siMPle and in-house measured spectra (Talvitie et al., 2017). Reference materials were selected, because they have been found previously from the surface waters of the lake (Uurasjärvi et al., 2020).

The software calculates Pearson correlation coefficients between sample and reference spectra. The correlation thresholds for particle recognition in siMPle were set by interpreting the results according to Liu et al. (2019). To resolve the correct threshold for the future use, the thresholds were adjusted to be slightly too low, and after the automatic recognition, experienced spectroscopist manually examined that reference peaks were present and no extra peaks other than carbonyl peak from oxidation (at around 1700 cm^{-1}) or noise in the baseline was detected. Generally, particles which had higher than 60–70% correlation to reference were accepted. For the future, 60% threshold would work sufficiently, as the only problem with it was occasional partial mismatch of PS and ABS. It can be manually corrected easily if these types are not common in the sample. More details about the data analysis are provided in the Supplementary Material.

SiMPle analyses MPs qualitatively and quantitatively. It counts MPs, lists their polymer types, and measures their dimensions. The particle size reported in this study is the measured longest dimension of a particle. Particles were categorized to fibers or fragments by dividing the major (longest) dimension with the minor dimension. If the resulting ratio was >5, particle was counted as fiber, otherwise as fragment. Moreover, as the spectral image is two-dimensional, the thickness of a particle was estimated from the length and width to calculate the volume. The estimated mass was calculated from the volume of a particle and the density of the material (Liu et al., 2019).

2.4. QC/QA: determination of recovery rates and limits of detection

Preparation of the recovery rate samples. Recovery rate is the proportion of retrieved microplastics from a known addition after treatments. It was determined for the pretreatment and FTIR measurement separately. To determine the recovery rate of the pretreatment,

fluorescent polystyrene (PS) beads (Fluoresbrite® YG Microspheres 90.0 μm, Polysciences Europe GmbH, Germany, 57/43/50 items) were counted under a fluorescence microscope (Leica M165 FC) and transferred into three glass bottles each containing a fish GIT and 50 mL of SDS 10%. These three samples underwent the enzymatic digestion process described above. Finally, the recovered PS beads were counted under the fluorescence microscope, and the recovery rates were calculated.

To determine the recovery rate for the FTIR analysis, three samples containing a fish GIT were digested using the enzymatic purification protocol as described above. After the digestion process, fluorescent PS beads (size 90 μm, 45/60/45 items) were counted under the fluorescence microscope and transferred into three glass bottles containing the digested fish samples. The recovery samples were filtered, and the recovered PS beads were counted with FTIR imaging as described above. Additionally, the PS beads were counted with a stereo microscope (Zeiss Stemi 508; 6.3–50× magnification; Axiocam ERc 5s camera) to examine how fish residues affect the FTIR imaging compared to visual counting.

Recovery rate. The mean recovery rate for the pretreatment was 75.0 ± 10.0% (44/36/32 items). The recovery rate for the final filtration and FTIR imaging was 77 ± 26% (18/57/44 items) by particle count and 63 ± 21% by mass estimation. The mean particle size of PS beads was 90 ± 7.8 μm, which denoted that automatic FTIR imaging can analyze particle size distribution quite accurately and precisely. When the beads were counted with a stereomicroscope, recovery rate was 84 ± 1.3% (37/51/39 items), slightly higher than with FTIR imaging.

Therefore, the estimated overall recovery rate for particle count was 58% (0.75*0.77 = 0.58). No corrections were made to the results based on recovery, because the rate was only valid for PS beads and majority of the MPs in fish samples were neither PS nor spherical. More details about the recovery rate tests are provided in the Supplementary Material.

Background and limits of detection. The preparation of blank samples is reported in Section 2.2. The blank samples, representing both pretreatment and FTIR (n = 10) contained 9.3 ± 7.0 MPs/sample (mean ± SD). Correspondingly, the estimated plastic mass was 514 ± 945 ng/sample. Blanks for only FTIR contained 0.33 ± 0.47 MPs/sample.

The limit of detection (LOD) needs to be assessed for a measurement method to determine, whether analyte gives real signal from a sample (Brander et al., 2020). Both background/contamination and performance of the instrument affect LOD. Nevertheless, contamination from the pretreatment possibly increases LOD more than performance of imaging FTIR. To estimate the limit where samples contained statistically more analyte than blanks, LOD was calculated as

$$LOD(p) = \text{Mean} + 3*\text{SD} = 9.3 + 3*7.0 = 30$$

for MPs per sample and

$$LOD(m) = \text{Mean} + 3*\text{SD} = 514\ \text{ng} + 3*945\ \text{ng} = 3349\ \text{ng}$$

for estimated plastic mass per sample. All the samples in which particle counts and/or mass values were above LOD were considered as reliable detections of MPs. LOD for plastic mass fraction in fish (*LOD (mf)*) was calculated by dividing *LOD(m)* with the mean mass of all fish:

$$LOD(mf) = 3.35\ \mu\text{g} / 4.76\ \text{g} = 0.70\ \mu\text{g g}^{-1}$$

2.5. Statistical analyses

To test the significance of the difference in MP ingestion between the fish species, we used the *t*-test (Welch two sample *t*-test, x = 5%; p = 0.05). The significance of the difference between all sampling sites was tested with the Kruskal-Wallis test (x = 5%, p = 0.05), followed by pairwise Wilcoxon rank sum test (p = 0.05) if the difference was significant. The difference between sites among one species was tested similarly. Statistical analyses were done with RStudio (Version 1.1.463) (R Core Team, 2019).

3. Results

3.1. MP counts and mass fractions in fish

Because 25% of samples were analyzed as pooled, all results are not suitable for assessing the numbers of MP ingested by individual fish, but the focus is on the average numbers of MPs and differences between species and areas. From both species, 21% of fish contained higher counts of MPs than LOD, indicating that they had probably ingested plastic. The difference in the share of fish with ingested MPs between the two species was non-significant: 25% of vendace and 17% of perch had ingested plastic. Similarly, the count of ingested MPs was not statistically different between species (Welch *t*-test, t = −1.748, df = 40.004, p = 0.088). On average, vendace contained 25 ± 50 MPs/fish and 0.37 ± 1.0 μg g^{-1}, whereas perch contained 11 ± 16 MPs/fish and 0.38 ± 0.67 μg g^{-1}.

The highest count of ingested MPs (263 particles/fish) as well as the highest estimated mass of ingested MPs (5.8 μg g^{-1}) were found in vendace specimen from sampling site V3 (Fig. 2).

The difference in counts of MPs between all sampling sites was statistically significant (Kruskal-Wallis test: chi-squared = 36.768, df = 8, p < 0.001). Moreover, the difference in MP counts in perch between sites was significant (Kruskal-Wallis test: chi-squared = 33.523, df = 5, p < 0.001). In P4, perch contained significantly higher counts of MPs than in other sites (Wilcoxon rank sum test, p < 0.05). It was the only site, where counts of MPs were significantly higher than in blanks. Additionally, counts of MPs were below LOD in other sites P1, P2, P3, and P5, indicating that only perch from site P4 had probably ingested MPs. The situation of vendace was different: the difference in MP counts between sites, including controls, was not significant (Kruskal-Wallis test: chi-squared = 2.2349, df = 3, p = 0.525) but every site contained samples, in which MP counts were above LOD, implying that vendace from all sampling sites had ingested MPs.

Mass fractions were calculated by dividing the estimated plastic mass by fish mass. Because particle counts and sizes affect the overall plastic mass, the mass fractions at sampling sites were slightly different from the particle counts (Fig. 2).

The difference of microplastic mass fractions in fish between all sampling sites was significant (Kruskal-Wallis test: chi-squared = 36.333, df = 8, p < 0.001). In vendace, difference between sampling sites was not statistically significant (Kruskal-Wallis test: chi-squared = 4.4526, df = 3, p > 0.05), but in perch it was (Kruskal-Wallis test: chi-squared = 29.075, df = 5, p < 0.001). In perch, samples from site P4 contained higher mass fractions of microplastics than samples from other sites. Additionally, all samples from sites P1, P2 and P5 were below LOD, but at every vendace site, at least one sample was above LOD. Consequently, the patterns of plastic ingestion were the same when looking on counts or masses of MPs: perch from mainly one site (P4) had ingested MPs, but vendace from all sampling sites had ingested MPs. However, only a minority of individuals of both species had eaten MPs.

3.2. Plastic types and particle sizes

Altogether eight polymer types were identified from the studied fish. PE was the most common plastic polymer type found from vendace by particle count per fish (Fig. 3). In perch, PP was the most common by particle count. From all analyzed polymers types, PE, PP and PET were the most common.

However, the mass fractions of polymer types per fish showed different results than the MP counts. One vendace sample contained one very large PS particle, compared to the other MPs sizes. Further, PE and PP were common in vendace in terms of mass fraction. In both species, PE, PP and PS were the most common polymer types by mass.

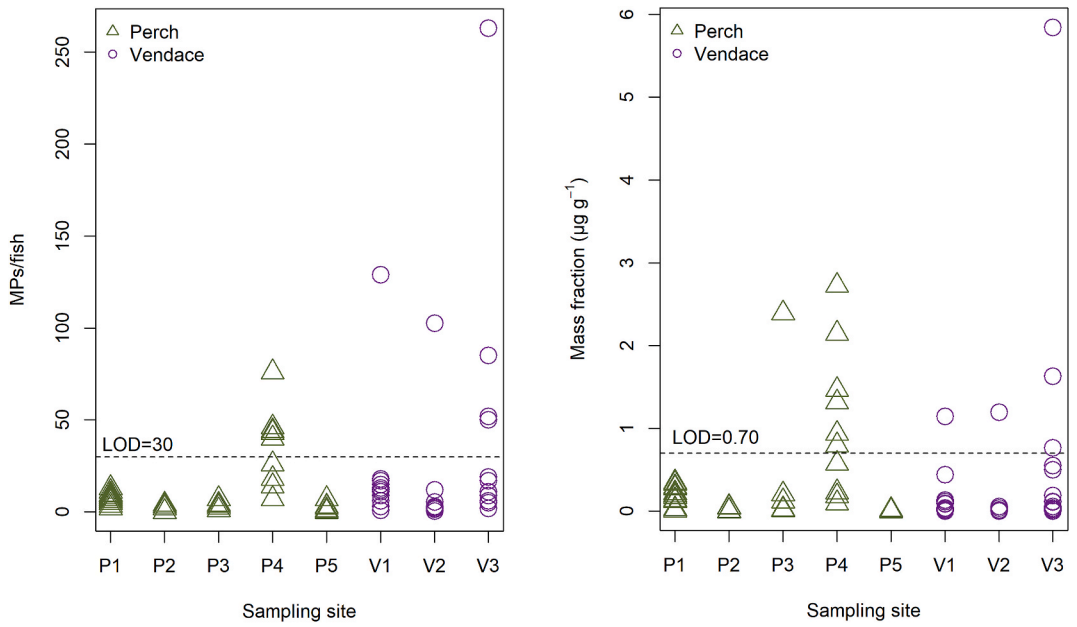

Fig. 2. MPs per fish (left) and mass fractions of plastic mass per fish mass (right) at sampling sites. See locations of the sites in Fig. 1.

The difference between the count and mass of MPs in fish results from different particle size distribution of polymer types, meaning fish contained many small PA and PET particles and few large PS particles. PE and PP were abundant in both particle counts and mass fractions; hence, fish contained many small PE and PP particles and few larger ones. Other polymer types, PVC, PMMA, and ABS were less common. Only a couple of fish individuals contained few small particles of these polymer types.

The average (mean ± standard deviation) particle size (major dimension) of MPs was 66 ± 63 µm in vendace and 81 ± 69 µm in perch. The largest particle found in vendace was 779 µm, and in perch 561 µm (Fig. 4).

To ease the comparison of the results with previous (and future) studies with different lower limits of particle size, MP counts are presented in Table 2 by the size fractions and corresponding lower limits. Small, < 100 µm particles were the most common, whereas large >300 µm were rare.

Besides the size, the shape was measured, and only 3% of MPs were defined as fibers, whereas the rest were categorized as fragments (See the Supplementary Material for more details).

4. Discussion

4.1. Method validity

4.1.1. Recovery rates – pretreatment and FTIR imaging

The hypothesis was that the efficiency of the pretreatment would affect the recovery rate, because fish residues on the filters may hinder the spectral recognition of MPs. Results supported the hypothesis, because the recovery rates of FTIR deviated more than the corresponding rates of visual counting. One recovery sample contained more fish residues than others did, which resulted in lower FTIR recovery rates. Especially perch were difficult to pretreat, because their GITs were less sensitive to enzymatic digestion or they contained high amounts of insoluble dietary residues. The residues occasionally formed white film to the filter surface, which prevented FTIR imaging of particles. Therefore, a minority of perch samples could not be analyzed reliably and were excluded from this study. However, for vendace the analytical methods worked well.

The recovery rate for the pretreatment was 75 ± 10%, which is acceptable. The recovery rate for the final filtration and FTIR imaging was excellent for particle size (90 ± 7.8 µm), acceptable for particle numbers (77 ± 26%) and tolerable for mass estimations (63 ± 21%). These recovery rates are only valid for spherical 90 µm PS particles, which are currently the best choice, because they are commercially available traceable standard materials, and easy to count under a fluorescence microscope. However, because environmental samples can contain MPs of various plastic types, sizes, and shapes, the recovery rates tested with PS beads are only indicative. In the future, more traceable standard materials with other polymer types, particle sizes and shapes are needed for reproducible testing of the analytical methods.

Primpke et al. (2020) have published similar methods than the one validated in this study. The main difference between the other methods and this method was that instead of depositing small aliquot to an IR transparent window (Liu et al., 2019; Primpke et al., 2020; Simon et al., 2018) or using Anodisc filters (Löder et al., 2015; Primpke et al., 2020) and measuring in transmission mode, we used silver membranes, filtered the whole volume and measured in reflection mode. Reflection mode and silver membrane filters were chosen for this study, because the filters are available in larger pore sizes, which allow faster filtration of samples and they do not limit the spectral range as Anodiscs do. Previously, we have tested gold-coated membrane filters, which produce slightly better signal to noise ratio, but were not available for >1 µm pore sizes (Uurasjärvi et al., 2021).

Changing the measurement mode from reflectance to transmission can, however, enhance spectral quality for small particles. Transmission is not suitable for larger particles, because they do not transmit infrared radiation and saturate the spectra. Therefore, to use both transmission and reflection, particles should be size-fractioned and measured in two sets, which would have been too time-consuming for the purpose of this method. On the other hand, the quality of spectra and the recovery rate of FTIR imaging could be enhanced by measuring higher number of scans, but in this study scan numbers (measurement time) was limited, because the analytical method was optimized to be as fast as possible to enable the analysis of a large sample set.

4.1.2. Data analysis and duration of the analysis process

Presumably, the wider spectral library than included here would enable more comprehensive identification of plastic polymer types (Primpke et al., 2020). However, due to the high amount of samples and

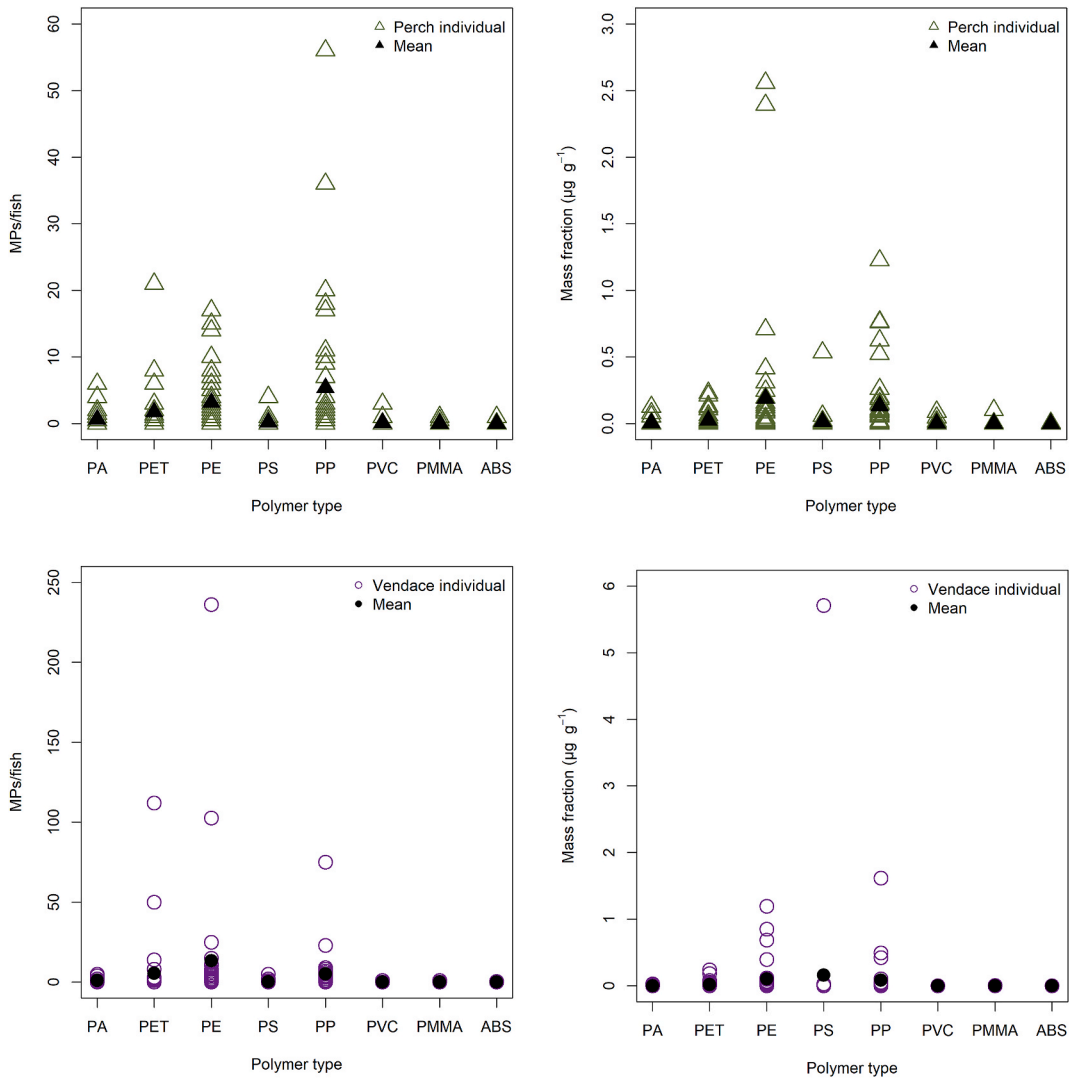

Fig. 3. Polymer types by MPs per fish and mass fraction, perch above and vendace below (PA = Polyamide, PET = Polyethylene terephthalate, PE = Polyethylene, PS = Polystyrene, PP = Polypropylene, PVC = Polyvinyl chloride, PMMA = Polymethyl methacrylate, ABS = Acrylonitrile butadiene styrene).

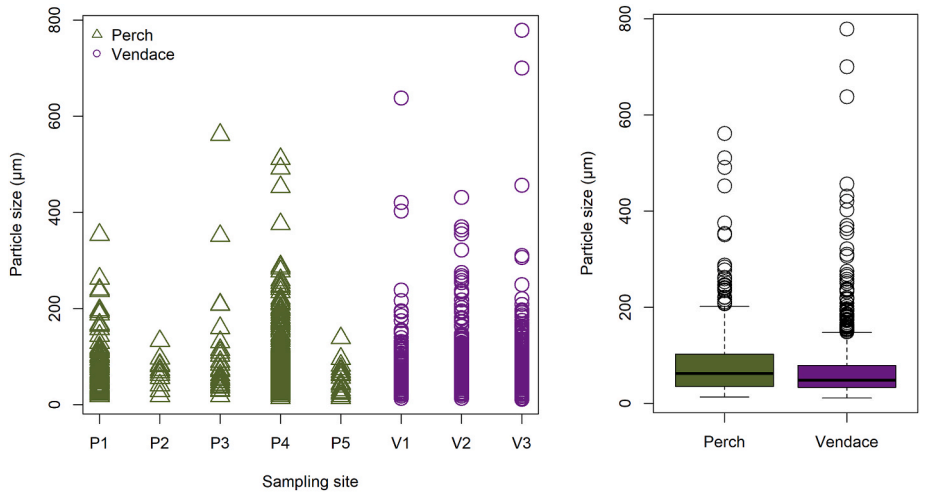

Fig. 4. Particle sizes (the longest dimensions) by sampling site (left) and species (right). See locations of the sites in Fig. 1.

Table 2
Size fractions of MPs found in GITs of two species of fish from the Lake Kallavesi, Finland.

Size fraction (μm)	<50	50–100	100–200	200–300	300–500	>500
MPs	753	522	240	38	15	5
Proportion (%)	48	33	15	2.4	0.95	0.32
Size limit (μm)	**>20**	**>50**	**>100**	**>200**	**>300**	**>500**
MPs	1573	820	298	58	20	5
Average MPs/ fish	16.4	8.5	3.1	0.60	0.21	0.05

extensive spectral map data, we focused on the most common plastic polymers, found from the surface waters of the lake (Uurasjärvi et al., 2020). With the reported measurement parameters, FTIR imaging took about 3 h per sample and data analysis with siMPle took about three to 5 h, when calculated with computers with 16 GB RAM memory. The total analysis time for one sample was approximately 9 days for pre-treatment and 8 h for FTIR imaging and data analysis. However, multiple samples can be pretreated at the same time, and the time mostly consists of incubation instead of active working. Similarly, FTIR imaging and data analysis do not require active working more than approximately 1–2 h per sample.

4.1.3. Contamination and the limits of detection

The hypothesis was that contamination would affect the sensitivity of the method, in addition to the recovery, and the results supported it. Only one MP was found from the FTIR blanks, whereas every blank for the pretreatment and FTIR contained multiple MPs of various polymer types and particle sizes. The blanks showed that most of the contamination arose during the digestion and filtrations. The proportion of fish containing at least one MP was 94%, whereas in only 21% fish samples the count of MPs was above the LOD. This highlights that LOD or other suitable similar measure must be reported with the results.

The visual selection of particles without any labels such as fluorescent dyes causes bias when MPs smaller than 100 μm are analyzed (Lenz et al., 2015; Maes et al., 2017). Especially when monitoring focuses on small fish, the proportion of small MPs is likely to be high. Consequently, imaging spectroscopy is a recommendable method for monitoring MP ingestion of fish, although contamination may affect it, too. The controls contained on average 9 MPs, which were mainly smaller than 100 μm, indicating that samples are particularly contaminated by small MPs.

However, it is difficult to decrease the level of contamination when analyzing fish or biota in general. Based on our long experience in microplastic analyses, laborious multi-step pretreatments including dissections, digestions and filtrations usually cause more contamination than simple treatments, which include only filtration or one digestion/filtration step. Procedural blanks have shown that for example biota and sediment samples that require heavy treatments are more prone to contamination, whereas clean water samples requiring only filtration are less or not at all contaminated (Finnish Environment Institute, Marine Research Center, unpublished results, University of Eastern Finland, SIB Labs, unpublished results).

4.2. MPs in fish GITs – Numbers, sizes, polymer types, and ecological implications

4.2.1. Numbers of ingested MPs per fish

The numbers of ingested MPs were hypothesized to differ between the two species, but this was not corroborated, because the difference was statistically insignificant. Further, as perch were more difficult to prepare for analysis than vendace, their MP ingestion can be slightly underestimated. Considering this, the differences between the species were very low. The differences in the diet and habitats did not cause difference in the ingestion of MPs.

Moreover, perch from different catching sites were hypothesized to ingest different numbers of MPs, because our previous results from surface waters of the same lake (Uurasjärvi et al., 2020) showed that MP concentrations in water were higher near the city and pollution hotspots compared to the open lake. However, vendace were not expected to show spatial variation, because they presumably move and feed around the lake, not only in the capture locations. The results supported the hypotheses, as perch caught from site P4 had ingested significantly more MPs than perch from other sites, whereas vendace caught from all sites had ingested similar numbers of MPs. Site P4 is close to a location where new houses are being built close to the shoreline, which indicates potential plastic input to the lake from the construction site. Contradictory to the water samples (Uurasjärvi et al., 2020), we did not find evidence that MP numbers would be higher in fish near the urban areas compared to the pristine areas of the lake. This result is in agreement with McNeish et al. (2018), who did not find correlation between microplastic numbers in fish and surface waters in river. Instead, they argue that both MP abundance in the environment and feeding behavior of fish affect how much MPs fish ingest, the latter being the most prominent.

This study is among the first to investigate MP ingestion in a northern lake fish with imaging spectroscopic methods. Comparison of the results on MP ingestion by fish is difficult between studies, because different analytical methods being applied. However, recent studies from Germany (Roch et al., 2019) and Poland (Kuśmierek and Popiołek, 2020), analyzed a comprehensive set of different freshwater fish species. The authors did not apply spectroscopic analysis, but used visual examination with a dissecting microscope and Roch et al. (2019) verified identification of plastics with hot needle test. In Germany, 16.5% of lake fish had ingested >40 μm MP, which is slightly smaller proportion than in this study. However, compared to lakes, in Polish river 54.5% of gudgeons and 53.9% of roaches had ingested MPs.

The GI tract is probably the most important exposure route for fish, but MPs have been shown to be egested from the gut in 24 h, making the exposure time via GI tract rather short (De Sales-Ribeiro et al., 2020). However, the exposure via the GI tract has been suggested to correlate with higher contaminations of plastic-associated harmful chemicals in fish (Barboza et al., 2020; Gassel and Rochman, 2019; Rochman et al., 2013). Hence, chemicals can migrate from MPs in GI tract, though MPs go through it. Moreover, particle size affects the toxicity and migration of chemicals in the GI tract (Hartmann et al., 2017; Jeong et al., 2016). Therefore, future studies should focus on how frequently individual fish ingest MPs and what properties of MPs and exposure periods are harmful, not only on how much MPs has an individual ingested at a time.

In addition to the risks caused for the primary consumer level fish, also secondary level predatory fish may be exposed to MPs when they feed on small vendace and perch, and MPs may be transported along the food web. If the exposure to MPs on the base of the trophic food web is high and continuous, harmful effects such as leaching of hazardous substances may occur.

We also conclude that such a low presence of MPs in the studied size range in the GITs does not pose a hazard to human consumers, who commonly remove these parts from the fish before preparing them for food. However, the studied fish are common human food in the area and small vendace are typically consumed whole without removing the GITs. This could be one but presumably negligible route of exposure to humans.

4.2.2. Particle sizes and polymer types of ingested MPs

The results supported the hypothesis that small particles would be the most abundant among the ingested MPs: only 1.3% of MPs had particle size larger than 300 μm and 18.9% larger than 100 μm. Instead, nearly 50% of MPs were in a range of 20–50 μm. Roch et al. (2019) estimated mathematically that the size of 95% of MPs in fish would be below 40 μm, and our results support the estimation that smaller particles are more abundant.

Fish undergo ontogenetic diet shifts and smaller prey items often dominate during the early life stages. Further, the gape size of fish and

season affects their prey selection. The studied fish were small on average (perch 7 cm and vendace 10 cm) and caught in early autumn, thus their diet consists mainly of zooplankton (Jacobson et al., 2019). Interestingly, the share of ingested MPs smaller than 50 μm was higher than the share of larger size fractions, and together the MPs <100 μm comprised 81% of all ingested particles. This is most likely due to the higher encounter rate with small particles, which are more numerous than larger particles in the water (Uurasjärvi et al., 2020). Because vendace migrate vertically in the water column in search of selected prey zooplankton (Mehner and Kasprzak, 2011), they may encounter MPs in various parts of the water column. Fish may also ingest MPs indirectly, for example when feeding on benthic invertebrates, incidentally ingesting sedimented MPs, or potentially also by trophic transfer (Batel et al., 2016; Nelms et al., 2018).

Common zooplankton in Finnish lakes includes rotifers, copepods and cladocerans, ranging in size from approximately 50 μm up to 800 μm. From the two fish species studied, vendace feeds on zooplankton for its entire life, and the size of the prey increases with fish growth. Overall, vendace possess a high number of gillrakers with narrow spacing (Northcote and Hammar, 2006), which enables the ingestion of zooplankton from the lower size range. The vendace in this study represented the first year class (0+), which may partly explain the small average size of the ingested MPs. MPs are possibly ingested together with real prey and captured passively by the filtering apparatus. However, the average size of the ingested particles in perch was also smaller than 100 μm. Finally, MPs from the environment can be fragile and fragment to smaller particles during the sample pretreatment, though the reference PS beads in recovery tests did not show any decrease of size.

The hypothesis that the most produced polymer types would be the most abundant in fish GITs was corroborated, as PE, PP, PET and PS were the most common types found in this study. They have also been found from freshwater fish in other studies worldwide (Biginagwa et al., 2016; Phillips and Bonner, 2015; Yuan et al., 2019). However, because the spectral library did not contain rare plastic types, the result can be biased towards the common polymers, if some samples would have contained numerous rare MPs. A large majority of the particles were fragments while fibers were infrequent. This is in disagreement with many previous studies from marine and freshwater fish, which have reported a prominent proportion of MPs to be fibrous (eg. Beer et al., 2018; Roch et al., 2019; Yuan et al., 2019). However, Pereira et al. (2020) reported fragments to be the most common shape of microplastics in fish. Again, the different methods and analyzed particle sizes hinder the comparisons. Imaging FTIR and siMPle are not very good at quantifying synthetic fibers according to our practical experience, because sometimes fibers are not properly attached to the filter surface (Uurasjärvi et al., 2021). Therefore, the number of fibers is more likely to be underestimated than overestimated in the results.

5. Conclusion

Approximately one fifth of small perch and vendace had ingested MPs, which are contaminants in food chains. Thus, perch and vendace can transfer MPs from plankton and/or water to larger predatory fish along the food web. Moreover, they are common human food, which could be one, but presumably a minor exposure route of humans to MPs. The mean number of MPs in fish was rather low in this study, but more research of the levels and effects of microplastic contamination in fish is needed to assess the potential threats to fish with different feeding habits. For example, in areas with high particle concentrations of small MPs in water, species which can feed on small prey throughout their whole lifespan, such as vendace, may potentially be more vulnerable to harm from MPs.

Imaging FTIR followed by automatic spectral analysis is a promising and developable method for monitoring MP ingestion by fish. However, the methodology for analyzing low particle concentrations and/or small <50 μm microplastics needs still development, because current dissection and digestion methods are prone to contamination of the samples by small MPs. Contamination increases uncertainty of the analysis, leading to higher limits of detection. Moreover, the pretreatment method must efficiently remove the matrix, which can hinder the spectroscopic identification of MPs, leading to lower recovery rates. Hence, to compare and evaluate the suitability of different analytical methods, we encourage reporting the limits of detection and recovery rates or other suitable validation parameters for other methods as well.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data is available upon request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2021.117780.

Credit author statement

Emilia Uurasjärvi: Formal analysis, Investigation, Validation, Methodology, Software, Visualization, Writing – original draft, Erika Sainio: Investigation, Validation, Methodology, Writing – original draft, Outi Setälä; : Conceptualization, Investigation, Writing – original draft, Maiju Lehtiniemi: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – original draft, Arto Koistinen: Conceptualization, Funding acquisition, Investigation, Project administration, Writing – review & editing.

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