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ABSTRACT

Plastic pollution is continuously growing on a global scale and emerging as a major environmental hazard. Smaller-sized plastics, so-called microplastics (< 5 mm), are considered as being omnipresent throughout the aquatic environment, yet freshwater ecosystems have received little attention so far and are still largely unstudied. Present study aims to expand the current knowledge on microplastics in freshwater systems by documenting the occurrence in the digestive system of fish from 15 rivers at 17 locations in Flanders, Belgium. To increase inter-study comparability and identification accuracy, a more standardized protocol was combined with a conservative approach towards acceptance of microplastic particles. Four rivers were found to have fish containing microplastics. However, no significant differences could be established between the sampling sites. In total 78 specimens of gudgeon (*Gobio gobio*) have been investigated, 9% of which had ingested at least one microplastic item, thus showing that contamination appears to be limited. Microscopic and spectroscopic analysis showed the microplastics to be from various sources with a diverse range of physical characteristics. Out of the eight items identified as microplastics, seven different polymer types were identified. Although further detailed research is necessary, this preliminary study shows that gudgeons from several Flemish rivers are contaminated with microplastics.

Capsule: Preliminary research covers new scientific perspectives on the prevalence of microplastics in a wild freshwater fish species.

Key words: Microplastics; Freshwater; Fish; Gudgeon; Ingestion

INTRODUCTION

Plastic pollution is a widely recognized global issue and with worldwide plastic production growing to new heights, currently reaching over 330 million tons annually, so is the pressure and impact on natural ecosystems (Derraik, 2002, Eerkes-Medrano et al., 2015, PlasticsEurope, 2017). Plastics are synthetic polymers, compounds highly variable in their composition, a trait in common with the chemical additives used for optimizing their performance as multipurpose products (Andrady and Neal, 2009, Hidalgo-Ruz et al., 2012, Rahman and Brazel, 2004). While their lifespan is affected both by the environmental conditions and the properties of the plastic item itself, it is worryingly being estimated to be in a range from decades to centuries (Andrady, 2011, Barnes et al., 2009, Wolfe, 1987). Nowadays research has extended to a formerly overlooked part of this problem, being the smaller sized fraction of plastic items, the so-called microplastics (< 5 mm) (Andrady, 2011, Arthur et al., 2009, Eerkes-Medrano et al., 2015, Foekema et al., 2013, Sanchez et al., 2014). Microplastics can originate from larger plastics through fragmentation and degradation, but may also be associated with primary plastic production with utilizations ranging from industrial usage to cosmetic products such as facial cleaning scrubs (Andrady, 2011, Barnes et al., 2009, Gregory, 1996, Zitko and Hanlon, 1991). While there are many records on the adverse effects of larger sized plastics in biota (Laist, 1997), much less is known on the effect of microplastics. Alarmingly, reports suggest they would be able to enter other parts of the body, like the circulatory system, the muscles and the hepatic tissue (Akhbarizadeh et al., 2018, Avio et al., 2015, Browne et al., 2008, Collard et al., 2017, Farrell and Nelson, 2013, Kashiwada, 2006, Nemmar et al., 2003). Moreover, plastics are found to be adsorbing hydrophobic contaminants from their surrounding environment, therefore potentially acting as a vector for both item-specific plastic additives and environmental contaminants (Khan et al., 2017, Mato et al., 2001, Rahman and Brazel, 2004, Rochman et al., 2013, Teuten et al., 2007). Compared to macroplastics, microplastics would be ingested more frequently and be available to a wider variety of species due to their smaller dimensions (Barnes et al., 2009, Browne et al., 2007, Possatto et al., 2011). The scale of this environmental problem and the effects on biota are yet to be established (Dantas et al., 2012). Although the topic of microplastic pollution is receiving increasing scientific attention, efforts are largely focused on marine systems. The first record of plastic ingestion in fish was made by Carpenter et al. (1972) and the majority of subsequent studies portrayed pollution in fish from marine areas. Empirical data on the occurrence of microplastics in freshwater, estuarine and terrestrial environments are limited (Eerkes-Medrano et al., 2015, Horton et al., 2017, Sanchez et al., 2014, Vendel et al., 2017). However, rivers have been identified as major inputs of plastics into marine systems (Faure et al., 2015, Hermsen et al., 2017, Lechner et al., 2014). Initial studies have already shown microplastics to be polluting freshwater habitats in a similar magnitude to marine systems, as well as observing similar concentrations of adsorbed and plastic associated chemicals (Biginagwa et al., 2016, Eerkes-Medrano et al., 2015, Eriksen et al., 2013, Faure et al., 2015). For example, comparable numbers of microplastics have been observed in North American riverine sediments and similar microplastic prevalence in Chinese freshwater fish (Castaneda et al., 2014, Jabeen et al., 2017).

The main objective of the present study is to assess whether wild fish from Flemish rivers (Belgium) are found to be contaminated with microplastics. This preliminary study will provide a first perspective on the prevalence of microplastics in a freshwater fish species in Belgium and will broaden the very limited understanding of microplastic occurrence in freshwater ecosystems. Performing a parallel study to Sanchez et al. (2014), who found gudgeons (*Gobio gobio*) from French rivers to be

contaminated, could give more clues into the distribution and the extent of this problem in a different geographical region. Furthermore, the study aims to achieve a thorough quantification and characterization of the plastic particles found in fish from different rivers. To this end, a more standardized approach using techniques like spectroscopy and a qualitative, up-to-date protocol will be applied.

MATERIAL AND METHODS

Study area and sampling

Flanders is situated in the north of Belgium, it is the most densely populated region of the country with a large degree of urbanization (Bleys, 2013). A total of 17 different locations have been sampled at 15 different rivers across Flanders, sampling locations are all part of the fish reference network of the Research Institute for Nature and Forest (INBO) (Fig. 1 - Table 1). Site selection was based on the presence of gudgeons (*Gobio gobio*), a small rheophilic fish species that feeds on macroinvertebrate prey (Froese and Pauly, 2016). The average river width of the sampling locations ranges from 0.5 to 4 m; only the Bovenschelde (BE) is considerably wider at 50 m. All rivers are part of the Scheldt Basin with the exception of the rivers Merkske (ME) and Bosbeek (BA; BV) belonging to the basin of the Meuse. The majority of sampling sites are situated in the vicinity of roads, in lower urbanized and agricultural areas, with the rivers commonly passing through residential areas. Two to 10 individual wild gudgeons (mean total length 11.85 ± 1.13 mm; mean weight 16.02 ± 5.77 g) were caught by the INBO using fyke nets and electrofishing (Table 1). All fish were collected between 8th of April and 20th of November 2015, killed with MS-222 (Acros Organics, Geel, Belgium), immediately frozen and sent to the University of Antwerp (Wilrijk, Belgium) for further analysis. An approximate measure for anthropogenic pressure was assessed through the local municipal population, based on the site's location (ADSEI, 2013). Any geographical information, including positioning of wastewater treatment plants (WWTPs) was collected from VMM (2018).

Microplastic recovery

Microplastic extraction followed the method described by Avio et al. (2015) in combination with a higher density separation from the study of Nuelle et al. (2014) as to maximize extraction efficiency. To optimize the modified protocol, several procedural trials were performed in advance (Supplementary Information; SI1). Before the start of the dissection, the exterior of the defrosted fish specimens (stored at –20 °C) was meticulously rinsed with Milli-Q ultrapure water (MQ; EMD Millipore, Billerica, Massachussetts, USA) in order to remove any potential contamination from the plastic freezer bags in which fish were kept prior to analysis. Fish were weighed (Sartorius AG CP4202; accuracy – 0.01 g, Göttingen, Germany) and the total length was measured up to 1 mm (Table 1). Specimens were dissected, the sex determined and the entire digestive system from oesophagus to anal sphincter (including liver and gall bladder) was removed. Petri dishes with the digestive system were weighed, covered with aluminium foil and placed in a dry oven overnight at 60°C. Each dried sample was weighed again, ground using a mortar and pestle and added to at least 100 mL sodium iodide (NaI) solution of 1.6 – 1.8 g/cm³ (99.5% pure NaI, VWR chemicals prolabo, Leuven, Belgium) before being stirred for approximately 10 min and decanted. The floating phase was vacuum filtered over an 8 µm pore size cellulose nitrate filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and the remaining sedimented material was disposed of. Decantation and filtration were executed twice to have a higher

extraction efficiency. The NaI solution was always recycled and preserved for later procedures. Before usage, NaI was prefiltered through a 0.45 µm cellulose nitrate filter (Sartorius Stedim Biotech GmbH, Göttingen, Germany). Due to NaI heavily reacting with H_2O_2 (Nuelle et al., 2014), filters were rinsed with at least 1.5 L of MQ-water under vacuum filtration before 20 mL of 15% H_2O_2 (30% w/w, Sigma-Aldrich, Diegem, Belgium) was added to each sample and put in a dry oven at 60 °C. Petri dishes were tightly covered with aluminium foil overnight and more loosely for several hours to allow filters to dry in advance of microscopic observation. Filters were checked with a stereomicroscope (Wild Heerbrugge; MFC-89000, Switzerland) for the presence of abnormal particles under a 32x magnification, after which a compound microscope (Standard 25, Zeiss, Zaventem, Belgium) with a magnification of up to 400x was used, to further visually identify possible microplastics. Suspicious particles were kept on wet filters and squeezed between two microscopic slides, until further analysis. Particles were marked as suspicious according to the following criteria: items with an unnatural, synthetic or manufactured appearance, following their shape or colour and lacking clear organic formations (e.g. absence of cellular structure), as described by Hidalgo-Ruz et al. (2012) and Norén (2007). As a preliminary screening, two gudgeons per location were checked for the presence of suspected microplastics considering the following shapes: pellet, bead, fragment, foam, film, line and fibre following previous studies (Faure et al., 2015, Free et al., 2014, Hidalgo-Ruz et al., 2012). Based on the screening results, following the number of suspected microplastics or the sampling location (governed by the proximity to larger urbanized areas), up to eight more fish were checked. All individual suspected microplastic particles were photographed using a macroscope (Nikon AZ100 Multizoom - Nikon fiber illuminator and Nikon Digital Sights DS-Ri1, attached to NIS-Elements D 3.2 imaging software; Nikon Instruments Europe B.V., Amsterdam, Netherlands) and measured at their largest cross-section as was previously performed by Foekema et al. (2013).

Polymer identification

To allow polymer identification of the microplastics a spectroscopic analysis was performed at the Royal Institute for Cultural Heritage (KIK-IRPA), Brussels, Belgium, using micro-Fourier Transform InfraRed spectroscopy (micro-FTIR - Bruker Hyperion 3000) and/or Raman spectroscopy (Renishaw inVia dispersive Raman spectrometer). Suspected particles were squeezed between a diamond compression cell before analysis with the micro-FTIR using transmission. Samples were compared to the commercial database from Renishaw for polymers (Raman), Hummel polymers and additives commercial database (micro-FTIR) and Nicolet/Aldrich condensed phase commercial database (micro-FTIR). Background interference was considered and removed from the analysis. The spectrum was analysed in the range 400 – 4000 cm^{-1} (micro-FTIR) or 100 – 3200 cm^{-1} (Raman; 785 nm (NIR) laser diode (Innovative Photonic Solutions, New Jersey, USA)) or to an adjusted range to focus on more characteristic and valuable regions of the spectrum. Only results of particles matching the databases above 60% were accepted, as previously applied in other studies (Avio et al., 2015, Lusher et al., 2013), or if obvious signs of a synthetic origin (both colour and shape) could visually be distinguished in combination with the spectroscopic analysis suggesting a synthetic background.

Quality Control

All steps were performed under a laminar flow cabinet and samples were always covered with aluminium foil in order to avoid aerial contamination. To further prevent contamination a 100% cotton lab coat and powder free nitrile gloves were worn at all times. Only laboratory glassware was used and

if plastic material (vacuum pump stoppers and ruler) usage could not be avoided, items were pre-checked under a stereomicroscope. Analogous, all dissecting tools were identified to be microplastic free. All materials were rinsed with MQ-water before usage and in-between usage to avoid cross contamination. To assess the magnitude of contamination, two types of controls were used. This included having three blanks per protocol run (of approximately 10 fish) and several petri dishes filled with MQ-water (randomly placed in the laminar flow cabinet) as had been formerly suggested by E. Foekema (pers. comm., 15th July 2015). White/transparent fibres were not accounted for in the analysis, since they could have originated from the cotton lab coat or the mandatory synthetic, white clothing (hair net, mouth mask and protective sleeves) needed when working in the laminar flow cabinet. Contaminating particles found in the samples that matched both shape and colour with the ambient background contamination (controls and petri dishes) were dismissed and not taken into account for further analysis.

Statistical analysis

To investigate differences between the gudgeons that had ingested microplastics and the group without, the statistical program Graphpad Prism (Version 7.00) was used. Differences were considered as statistically significant if *p-value* < 0.05. The condition of the fish was also assessed by calculating the Fulton's Condition Factor (Nash et al., 2006, Ricker, 1975):

$$K = \frac{W}{L^3} x\ 100 \qquad \text{[K = condition index; W = fish weight (g); L = total length (cm)]}$$

To evaluate the differences between the condition index of microplastic contaminated fish and fish lacking microplastics, a student t-test was used. This was also applied to check for any dissimilarities between the two groups considering the length, weight of the fish, weight of the digestive system (both wet and dry) and the gender. Analogous, sites with and without microplastic occurrence were compared, based on the prior fish parameters (condition, fish length, fish weight, digestive system weight and gender), presence of WWTPs, the local human population, the municipal surface area, province and lastly the human population density (SI2 – Table 6). To assess for differences in background contamination between protocol runs, a Kruskal-Wallis test was performed.

RESULTS

Microplastic characterization

In total 16 particles were extracted following suspicions of being microplastics (Fig. 2), but after spectroscopic analysis, only eight of these particles were accepted as such (Table 2; SI3 – Fig. 3). From the discarded particles, four could not be clearly examined: one particle was lost in transfer to the diamond compression cell (Fig. 2; M), others either fragmented onto the cellulose nitrate filter (Fig. 2; J, N), leading to interference during the analysis, or were too small for the micro-FTIR (Fig. 2; I), not producing a clear hit. Only two suspected microplastics (Fig. 2; K, L) could formally be identified as non-microplastics, their primary nature being chipboard and quartz, respectively. The last couple of particles did not produce any clear hit or indication for a possible plastic source (Fig. 2; O, P). Overall, out of eight microplastics, seven different polymer types were found; ethylene-vinyl acetate copolymer (EVA), polypropylene (PP), polyethylene terephthalate (PET), polyvinylchloride (PVC), cellophane, polyvinyl acetate (PVA) and polyamide (PA) (Table 2). Only PET was detected twice.

Identified microplastics were highly variable in shape and colour, with green coloration being the most prominent. With the exception of two items, the average size was found to be below 500 µm (Table 2).

Microplastic prevalence

Among all the 78 investigated fish, 9% of gudgeons had ingested microplastic particles and only one individual was found to have two microplastics present in its digestive system. Gudgeons were found to be contaminated with microplastics in four rivers; the Dijle (DE), the Ijse (IN), the Wimp (WP) and the Velpe (VE) (Table 2; SI4 – Fig. 4). The highest plastic counts were found in the river Ijse (IN), where three out of ten individuals contained at least one microplastic particle. Although, it must be noted that the sampling size of fish was not similar in all locations. Data on the length, weight (fish and digestive system) and condition indices of all investigated gudgeons are presented in the supplementary information (SI5 – Table 7), along with their individual suspected microplastics. Usage of the VMM database (VMM, 2018) allowed to identify WWTPs directly upstream of eight sampling sites (data not presented). For the river Ijse (IN) one was located less than 1 km upstream, for the Dijle (DE), Velpe (VE) and Wimp (WP) a WWTP was found around 4 km upstream. Regarding the sites where no contaminated fish were found, only four sites had a WWTP input further upstream (± 1.5 km GK and WE; ≤ 100 m KN and ZN).

Controls

Negative controls revealed that background contamination was still present and almost solely came from fibres. Besides fibres, also fluorescent blue fragments were found in two of the water-filled petri dishes and in one sample. When observed, these particles were always found to be numerous in that particular sample and could be easily distinguished by their characteristic colour, which allowed for their disposal. Furthermore, contaminating fibres were found in several colours: red, black, blue, grey, purple and brown in a size range of 30 µm up to 6 mm. Blue fibres, followed by black and red fibres were found to be dominating in numbers in the fish samples, the blanks and the control petri dishes. In total 20 fibres were found in the blanks and 22 in the water filled petri dishes. Particles extracted from the fish samples that matched the background contamination as found in the controls, both in shape and colour, were excluded from the analysis. This led to discarding 86 out of a total of 88 fibres found in the fish samples and only two fibres were accepted, since their green colour was not found in the blanks or other. Fibres were consistently observed in the blanks, in contrast to the water filled petri dishes where contamination seemed to be very random in time, amount and positioning of the petri dish within the laminar flow cabinet.

Data analysis

As a consequence of low microplastic numbers and limited sampling size per location, statistical analysis was not able to portray any significant differences between locations with contaminated fish and without, for biometric parameters, WWTPs and anthropogenic pressure. In addition, no significant differences were found between the fish that ingested microplastics and the fish with microplastics absent, considering gender, weight of the digestive system (wet and dry), biometric parameters and condition index. The amount of background contamination during the different procedural runs did not differ significantly.

DISCUSSION

Protocol

Modification

The analysis of organisms for microplastics has stumbled across several problems, from preventing background contamination to utilizing a standardized protocol (Foekema et al., 2013, Hidalgo-Ruz et al., 2012). The most efficient methods involve a density separation, a digestion step along with microscopic observation and spectroscopic analysis (Avio et al., 2015, Lusher et al., 2017). Besides differences in the methodology, different parts of the fish's digestive system are being checked, a practice which limits the possibility of comparison between studies (Jabeen et al., 2017). Microplastics could translocate in other organs as well (Avio et al., 2015, Collard et al., 2017), therefore it is critical to take the entire digestive system into account (including the epithelial lining, liver and gallbladder) as performed in this research, rather than solely using the stomach contents. While this study closely followed the methodology from Avio et al. (2015), having shown the highest extraction efficiency compared to other protocols, a modification was also made. The high density saline solution (1.2 g/cm^3) suggested would not cover the entire density range of plastic polymers that could be encountered. Considering that bottom dwelling benthivores such as the gudgeon (Kottelat and Freyhof, 2007) could have an increased potential of encountering higher density plastics, an improved density separation was desirable. By utilizing a high density NaI solution (1.6 – 1.8 g/cm^3) as used by Nuelle et al. (2014), a larger range of different polymers could be checked.

Protocol limitations

While the protocol from Avio et al. (2015) shows great potential as a standardized methodology, there are still drawbacks. Even when it has displayed high extraction efficiencies, it is still unable to recover a full 100% of microplastics, therefore still underestimating the level of plastic particles present (Avio et al., 2015). Having run through the entire procedure, occasionally heavier sand particles were found on the filter. These could be falsely suspected of being microplastics if only visual identification would be performed, e.g. one of the suspected particles being silicate (Fig. 2; L). Avio et al. (2015) also found plastics denser than suspected from the density separation and argued that the items could have stuck to less dense organic matter, allowing them to float. A similar event could have happened to the sand particles found in the present study. Even though modifying the protocol to include a higher density solution is believed to have increased the extraction efficiency, it also entailed several problems. Firstly, NaI and H_2O_2 react violently with one another (Nuelle et al., 2014). Therefore, an extra step is needed to wash away the NaI from the filter and organic material before the digestion step. With the entire protocol already being labour intensive, this was by far the most time-consuming step, needing at least 1.5 l of MQ to flow through the 8 µm filters. The speed of which was highly dependent on the amount of organic material present. In addition to this, from time to time residues of the interaction between the NaI solution and H_2O_2 were found as brown crystalline structures on the filter, complicating visual detection of microplastics. The brown colour likely originates from the recycled NaI solution turning brownish after multiple uses. A more expensive polytungstate solution (Nuelle et al., 2014), could be a valuable alternative, possibly further increasing the extraction efficiency and being more time-saving. Furthermore, while the digestion and cleaning steps are believed to have increased the extraction efficiency far beyond that of a direct visual identification, it is still possible that levels of contamination in the fish are underestimated. Microscopic observation as part of many protocols, still

remains the most "subjective" section in current methods. In particular, very small and transparent particles can be overlooked.

Spectroscopic analysis

Micro-FTIR spectroscopy was found to be effective in analysing the polymer type of the suspected microplastics, although particles below 40 µm could not be detected by the micro-FTIR. A similar range to Rummel et al. (2016), who were unable to measure items below 20 µm, whereas Biginagwa et al. (2016) found the limit to be already at 500 µm. Analysis of the very small particles is still possible using Raman spectroscopy (Collard et al., 2015, Löder and Gerdts, 2015, Löder et al., 2015, Rummel et al., 2016). Both micro-FTIR and Raman where used in the present study. Raman usage occurred only to identify and/or double-check particles for which the initial micro-FTIR-spectrum was not entirely clear. The observation of 16 potential microplastic particles of which only 50% were accepted by a combination of visual appearance and FTIR/Raman spectroscopy, further supports the general belief that only visual identification is not as reliable (Avio et al., 2015, Eriksen et al., 2013). This result is in line with other studies finding around 60% of the suspected particles to be of a synthetic nature (Brate et al., 2016, Karami et al., 2017).

Background contamination

Some studies completely dismiss fibres in the overall assessment of microplastics, finding them to be the prevailing form of contamination (Avio et al., 2015, Foekema et al., 2013, Hermsen et al., 2017), whereas others only exclude fibres that resemble the contaminating particles (Campbell et al., 2017, Faure et al., 2015, Guven et al., 2017, Rummel et al., 2016). It is still unclear to what degree including, partially excluding (through careful attention to background contamination) or completely excluding fibres altogether will lead to an over- or underestimation of the results (Foekema et al., 2013, Rummel et al., 2016). For instance, Rummel et al. (2016) only excluded fibres with the diameter or the length matching background contamination. Our approach took into account only items that did not resemble background contamination both in microplastic shape and in colour. When studies lack a strict quality control or a clean workplace, they may be subject to biased results following contamination, often finding high microplastic concentrations with the bulk of the items consisting of fibres (Hermsen et al., 2017). For this reason, a conservative approach was followed in the present study, excluding almost all fibres, even though this could have led to underestimations of microplastics that could have been present in the fish's digestive system. Taking rigorous precautions to avoid background contamination is essential, but while Foekema et al. (2013), Wesch et al. (2017) and Hermsen et al. (2017) mention a clean air flow cabinet to be helpful in minimizing or even preventing aerial contamination, this practice did not seem to be the case in the present study. Fibres were still frequently encountered in most samples, even when using all possible precautionary measures. However, the appropriate usage of multiple control blanks per run and water-filled petri dishes, was found to be sufficient to differentiate between background contamination and particles present in fish digestive systems. Care also has to be taken when using fume hoods as "clean air environments" (Mizraji et al., 2017, Roch and Brinker, 2017); while they can limit the amount of aerial contamination, they are not as effective compared to a laminar flow cabinet (Wesch et al., 2017). The unfiltered air flow could possibly draw more contaminating particles onto the samples (E. Foekema, pers. comm., 15th July 2015).

Microplastic characterization

Microplastic sizes found in gudgeons from Flemish rivers are comparable to the most common size range of microplastics in fish, found to be below 2 mm (Avio et al., 2015, Bellas et al., 2016, Dantas et al., 2012, Lusher et al., 2013, Rummel et al., 2016). While in general black and blue are frequently encountered colours (Akhbarizadeh et al., 2018, Alomar et al., 2017, Karlsson et al., 2017), green microplastics were more abundant in the present study. This could be due to the low microplastic numbers found overall. Moreover, the shape of microplastics was diverse, in contrast to other studies where microplastics almost solely consisted out of fibres (Jabeen et al., 2017, Lusher et al., 2013, Pazos et al., 2017, Peters and Bratton, 2016, Peters et al., 2017, Silva-Cavalcanti et al., 2017). The majority of polymer types observed, belonged to the main plastic polymers produced worldwide (Lithner et al., 2011, PlasticsEurope, 2017). All polymers detected have already been previously encountered in marine or freshwater fish (Alomar et al., 2017, Biginagwa et al., 2016, Brate et al., 2016, Jabeen et al., 2017). The particles found in the gudgeons likely fragmented from larger items, hence our study did not find any indication of primary microplastics, such as microbeads (Eriksen et al., 2013, Zitko and Hanlon, 1991). Also no direct relationship between polymer types and their possible point sources from business or industrial activities were observed upstream to the sampling locations (VMM, 2018). The most likely inputs are therefore similar to Biginagwa et al. (2016), where urban waste and discarded consumer products are considered as the main culprits (Table 2), particularly when taking into account that the location of sampling was often in more rural areas downstream of human settlements.

Contamination in fish

Microplastic ingestion

The prevalence of microplastics in 9% of the gudgeons in Flemish rivers compared to 12% in the study of Sanchez et al. (2014) would suggest that gudgeons found in French rivers are subject to higher contamination pressures. These moderate to low levels of microplastic prevalence are paralleled by results found in a variety of fish species in different geographical locations (Table 3), such as lake Victoria, lake Geneva, freshwater rivers along the Gulf of Mexico and the Rhine river, with microplastic ingestion frequencies of 20%, 7.5%, 8% and 24% respectively (Biginagwa et al., 2016, Faure et al., 2015, Phillips and Bonner, 2015, Roch and Brinker, 2017). In contrast, several studies have also reported much higher plastic prevalence in freshwater fish, ranging from 45% to almost 96% (Campbell et al., 2017, Jabeen et al., 2017, Peters and Bratton, 2016, Silva-Cavalcanti et al., 2017). Besides plastic ingestion, some studies have also reported the presence of translocated microplastic particles in fish livers (Avio et al., 2015, Collard et al., 2017). The low prevalence with which microplastics were found in the present study, would suggest that gudgeons are not readily accumulating microplastics. Although, it has to be noted that no differentiations were made between specific tissues during microplastic extraction. As for now, differences in the protocol and sufficiently controlling background contamination impede a clear comparison of the results. Literature on microplastic ingestion in the marine environment is far more expansive, but is faced with similar difficulties as in freshwater, by not being able to readily correlate results. The problem with incomparable studies leads us to only speculate about the differences in the prevalence between species and regions.

Feeding behaviour

Plastic items are not always uniformly spread throughout marine and freshwater systems, they rather aggregate in certain areas following prevalent water currents, bottom profile or source proximity (Moore et al., 2001, Peters and Bratton, 2016, Possatto et al., 2011, Ryan et al., 2009, Wang et al., 2017). Thus, it seems likely that microplastic prevalence does not only depend on the location. Several studies have tried to link microplastic ingestion with the influence of species feeding habit, although encountering contradicting results (Biginagwa et al., 2016, Campbell et al., 2017, Guven et al., 2017, Jabeen et al., 2017, Mizraji et al., 2017, Peters et al., 2017, Vendel et al., 2017). Besides species-specific differences that could influence the exposure to plastics, the particle's characteristics, including density, could make them more prevalent in certain layers of the water column (Eriksson and Burton, 2003, Song and Andrady, 1991, Teuten et al., 2007). In our study, 75% of the microplastics had a density higher than freshwater (Table 2), which could either suggest that heavier polymers are more prominent in Flemish rivers and/or benthivorous fish such as gudgeon are prone to ingestion of higher density plastics. The remaining lower density particles could be ingested after biofouling, increasing the overall density or were attached to heavier organic materials resulting in ingestion (Mattsson et al., 2015, Peters and Bratton, 2016, Song and Andrady, 1991). Large portions of the gudgeons gut contents often consisted of sandy sediment with similar dimensions to most of the microplastics encountered. It has been hypothesized that mechanical effects (e.g. obstructions, abrasion,…) may lead to adverse effects in the organisms (Foekema et al., 2013, Rummel et al., 2016). Although in the present study, it seems unlikely that the few microplastics exerted adverse mechanical effects in larger measure than that of the ingested sand particles. Freshwater macroinvertebrates are also known to consume microplastics (Hurley et al., 2017, Imhof et al., 2017, Scherer et al., 2017). Following ingestion of macroinvertebrate prey, microplastics could have entered the gudgeon's digestive system (Campbell et al., 2017, Eriksson and Burton, 2003, Farrell and Nelson, 2013).

Microplastics in Flemish rivers

The river Ijse showed the highest amount of contaminated fish, which could be explained by the higher degree of urbanization further upstream and a closer vicinity to towns (VMM, 2018), similar to findings from Peters and Bratton (2016) and Silva-Cavalcanti et al. (2017). The study of Sanchez et al. (2014) reported seven sites (out of a total of 11) in six different rivers to have contaminated fish. This higher degree of contamination could be due to differences in pollution or because in the present study, not all rivers have been as intensively studied as others. Due to practical reasons, only two individuals were checked for microplastics in the initial screening, which might be too low a number for a proper representation of the actual contamination levels per site. Since no other studies have ever been performed on a similar subject in the region, the screening provided a basic indication towards more interesting sites where more individuals were analysed. Nevertheless, the combined data of 78 individuals fulfils the quality criteria on the recommended sampling size (> 50 ind.) for microplastic research as discussed by Hermsen et al. (2018). Consequently, the entire dataset still provides an insight into the contamination levels in Flanders. While no actual conclusions on the differences between the individual rivers can be drawn, the absence of microplastics found in the fish, does however, not exclude that the area is affected by this form of pollution. The average width of the Flemish rivers studied is estimated to be about ten times lower than that of French rivers in the study of Sanchez et al. (2014). Therefore, besides differences in the geographical region, also the sampling size and the river size could further explain the lower microplastic amounts in this study. Remarkably, the sampling sites with contaminated fish all had a WWTP upstream (VMM, 2018). This further raises

the question if the microplastics have reached the river downstream of the WWTP or if the facility is unable to extract the (micro)plastics from wastewater. Others have also pointed out the likeliness of microplastics to be capable of passing WWTPs (Browne et al., 2007, Gregory, 1996).

CONCLUSION

To date and to our knowledge, this study provides a first observation on microplastic contamination in a wild freshwater fish species in Belgium. While the fish sampling size used in the screening process might be too small to draw any specific conclusions regarding individual river contamination levels, four rivers were identified to have microplastic contaminated fish. Out of the eight microplastics observed in this study, seven different polymers were identified. This shows that the variety of sources contributing to microplastics are diverse and items have most likely fragmented from consumer products (secondary microplastics). Microplastics were found in 9% of the gudgeons across all Flemish rivers, a number relatively low compared to most other marine and freshwater studies. Nonetheless, this might still represent a worryingly large prevalence considering that microplastic research, as an upcoming field of science, is still facing several challenges. The main difficulty is the comparison between studies due to the lack of a standardization in protocols and quality control, making this a clear area to be resolved in upcoming research. Despite the increasing production and usage of plastics, the ecological implications and the impact of microplastics on biota largely remain unanswered. Especially the possibility of microplastics to translocate and bioaccumulate is worrying. The scientific community should further increase their research efforts to include freshwater, estuarine and terrestrial environments if we wish to uncover and tackle the total extent of this increasing global problem.

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FIGURES

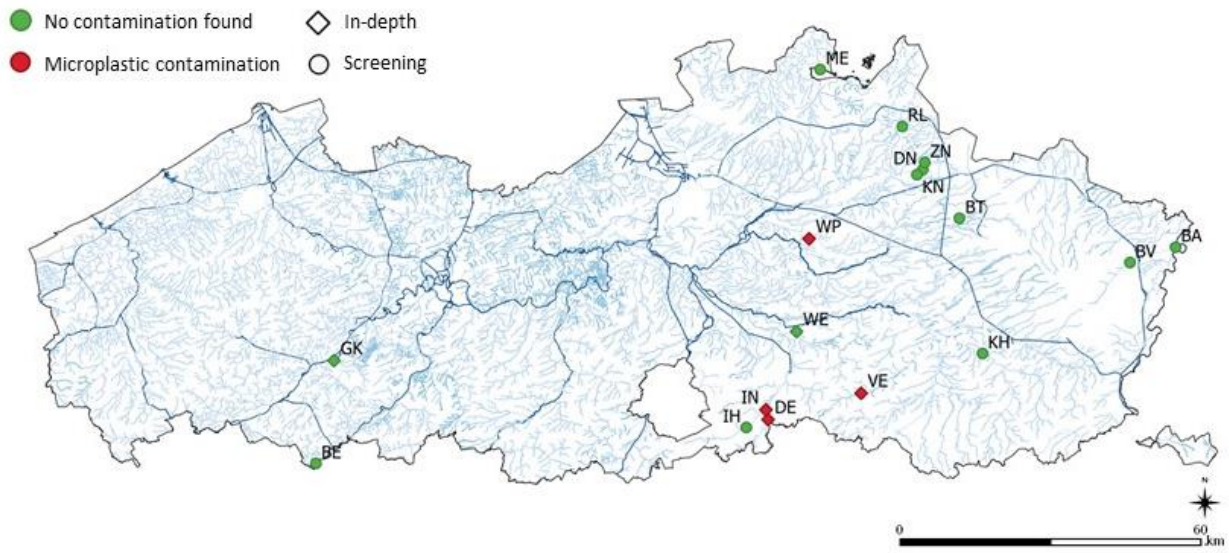


Figure 1. Sampling locations; circles indicate sites only used in the screening process; rhombs have been examined more in-depth. Red labels are locations with identified microplastics, green labels without. Map generated in QGis 3.0. Site abbreviations refer to the code presented in table 1.

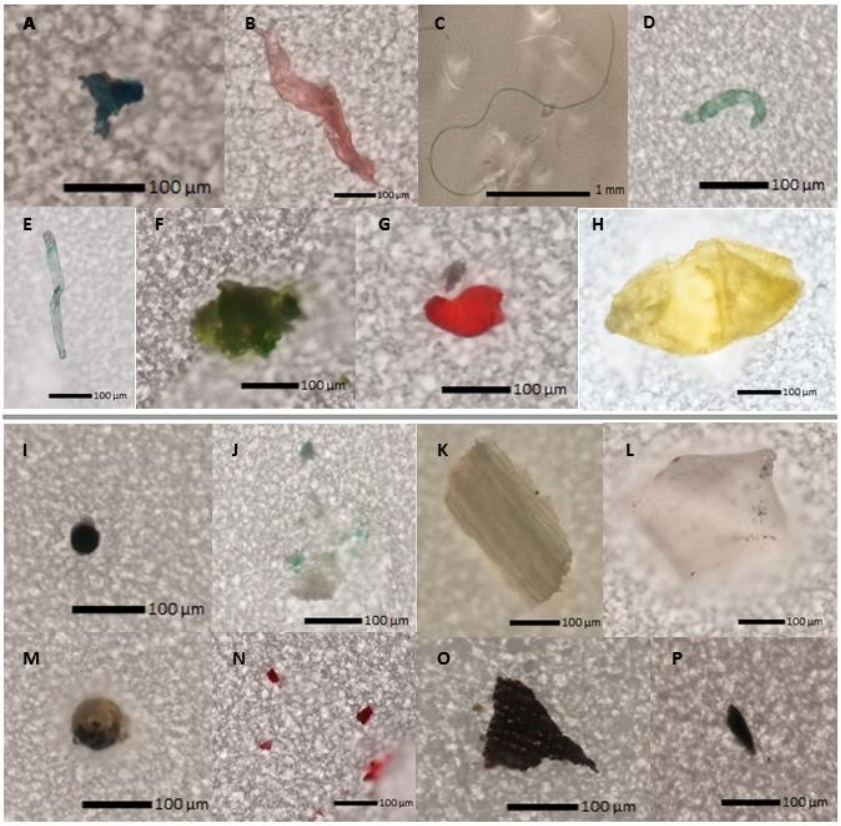


Figure 2. Suspected microplastics; Identified microplastics (A, B, C, D, E, F, G and H) and non-accepted items (I, J K, L, M, N, O and P).

TABLES

Table 1. Sampling sites (coordinates in World Geodetic System 84, date and method), with the number of male and female fish per location, the average biometric values and weight of the digestive system (DS) with the standard deviation displayed.

River (code)	GPS coordinates (WGS 84)	Date catch (dd-mm-yy)	Method	No° Gobio gobio (M/F)	Av. length (cm) ± stdev	Av. weight (g) ± stdev	Av. DS wet weight (g) ± stdev	Av. DS dry weight (g) ± stdev
Balengracht (BT)	51°09'37.0"N 5°11'22.7"E	30/10/15	electrofishing	2 (1/1)	11.2 ± 0.3	12.13 ± 0.93	0.56 ± 0.15	0.11 ± 0.00
Bosbeek (BA)	51°06'09.2"N 5°48'14.7"E	23/07/15	electrofishing	2 (2/0)	13.1 ± 0.4	18.59 ± 2.06	0.61 ± 0.17	0.11 ± 0.00
Bosbeek (BV)	51°04'36.1"N 5°40'24.7"E	04/11/15	electrofishing	2 (0/2)	11.6 ± 0.4	15.52 ± 0.80	0.57 ± 0.07	0.11 ± 0.04
Bovenschelde (BE)	50°43'13.7"N 3°21'57.0"E	29/09/15	fyke	2 (1/1)	13.2 ± 0.5	19.74 ± 0.73	0.60 ± 0.10	0.22 ± 0.06
Desselse Neet (DN)	51°14'51.0"N 5°05'11.2"E	29/10/15	electrofishing	2 (1/1)	12.1 ± 0.0	15.46 ± 0.85	0.54 ± 0.10	0.15 ± 0.00
Dijle (DE)	50°48'10.0"N 4°38'33.2"E	23/09/15	electrofishing	10 (4/6)	12.1 ± 0.9	17.41 ± 3.99	0.70 ± 0.24	0.19 ± 0.08
Gaverbeek (GK)	50°54'17.2"N 3°24'46.8"E	07/05/15	electrofishing	10 (7/3)	11.4 ± 0.9	16.07 ± 4.78	0.71 ± 0.23	0.22 ± 0.07
Ijse (IH)	50°47'20.2"N 4°34'51.4"E	13/11/15	electrofishing	2 (1/1)	12.0 ± 0.6	19.89 ± 5.16	1.07 ± 0.59	0.39 ± 0.19
Ijse (IN)	50°49'13.1"N 4°38'11.2"E	29/04/15	electrofishing	10 (6/4)	13.4 ± 1.2	24.89 ± 7.19	0.92 ± 0.32	0.24 ± 0.08
Kleine Herk (KH)	50°55'04.8"N 5°15'04.5"E	06/11/15	electrofishing	2 (2/0)	11.0 ± 0.3	10.20 ± 1.74	0.34 ± 0.02	0.09 ± 0.00
Kleine Nete (KN)	51°14'20.1"N 5°04'12.1"E	23/10/15	electrofishing	2 (1/1)	12.4 ± 1.1	17.02 ± 4.32	0.66 ± 0.01	0.17 ± 0.00
Merkske (ME)	51°25'42.1"N 4°47'44.3"E	18/11/15	electrofishing	2 (0/2)	11.9 ± 0.3	12.54 ± 1.16	0.32 ± 0.05	0.11 ± 0.00
Rode Loop (RL)	51°19'31.4"N 5°01'46.2"E	18/11/15	electrofishing	2 (1/1)	10.3 ± 0.4	8.07 ± 0.12	0.42 ± 0.08	0.13 ± 0.00
Velpe (VE)	50°50'56.3"N 4°54'22.6"E	06/11/15	electrofishing	10 (3/7)	11.7 ± 0.6	13.98 ± 2.48	0.58 ± 0.16	0.16 ± 0.07
Wimp (WP)	51°07'33.3"N 4°45'40.7"E	08/04/15	electrofishing	10 (7/3)	10.8 ± 0.5	11.64 ± 1.70	0.39 ± 0.13	0.10 ± 0.04
Winge (WE)	50°57'35.7"N 4°43'26.8"E	20/11/15	electrofishing	6 (4/2)	11.8 ± 1.1	14.43 ± 3.59	0.58 ± 0.26	0.17 ± 0.08
Zwarte Neet (ZN)	51°15'38.7"N 5°05'33.6"E	09/10/15	electrofishing	2 (0/2)	10.9 ± 0.7	12.47 ± 2.18	0.82 ± 0.05	0.13 ± 0.05

661 **Table 2. Characterization of the microplastic particles from the different locations: sample name, length, colour, shape, polymer type, density and possible uses or origin.**

River (code)	Sample	Length (µm)	Color	Shape	Polymer type	Density (g/cm³)	Sources and usage
Dijle (DE)	A	80	blue	foam	Ethylene vinyl acetate copolymer	0.93 - 0.94[d,e]	Food packaging, film[d,e]
Ijse (IN)	B	520	red	film	Polypropylene	0.89 - 0.91[c]	Drink caps, rope[a]
Ijse (IN)	C	3400	green	fibre	Polyethylene terephthalate	1.29 - 1.40[c]	Drinking bottles[a]
Ijse (IN)	D	170	green	film	Polyvinylchloride	1.30 - 1.58[c]	Cups, bottles, film
Ijse (IN)	E	300	green	fibre	Cellophane	1.50 - 1.52[b]	Food packaging, film[b]
Velpe (VE)	F	210	green	foam	Polyvinyl acetate	1.17 - 1.20[f]	Adhesive resin, coating[f]
Wimp (WP)	G	120	red	fragment	Polyethylene terephthalate	1.29 - 1.40[c]	Drinking bottles[a]
Wimp (WP)	H	450	yellow	pellet	Polyamide (nylon)	1.07 - 1.10[c]	Netting[a], fishing line

662 [a]Andrady (2011); [b] CJSC«TECHNOCLIP» (2011); [c] Nuelle et al. (2014); [d] TOTAL (2013b); [e] TOTAL (2013a); [f] WACKERPOLYMERS (2013)

663 **Table 3. Prevalence of plastics (micro-, meso- and macroplastics) in wild freshwater fish (excluding estuaries).**

Fish species studied	Prevalence (%)	Area	Extraction method	Spectroscopic confirmation	Author(s)
Lates niloticus; Oreochromis niloticus	20%	Lake Victoria (Tanzania)	NaOH-digestion; Visual	Yes	Biginagwa et al. (2016)
Esox lucius; Catostomus commersoni; Notropis atherinoides; Pimephales promelas; Eucalia inconstans	73.5%	Waskana creek (Canada)	$NaClO/HNO_3$-digestion; Visual	No	Campbell et al. (2017)
Alburnus alburnus; Perca fluviatilis; Rutilus rutilus; Leuciscus leuciscus	7.5%	Lake Geneva (Switzerland)	Visual	Yes	Faure et al. (2015)
Cyprinus carpio; Carassius auratus; Hypophthalmichthys molitrix; Pseudorasbora parva; Megalobrama amblycephala; Hemiculter bleekeri	95.7%	Lake Taihu (China)	H_2O_2-digestion; NaCl separation; Visual	Yes	Jabeen et al. (2017)

Lepomis macrochirus; Lepomis megalotis	45%	Brazos River Basin (USA)	Visual	No	Peters and Bratton (2016)
44 species	8%	Streams in Gulf of Mexico (USA)	Visual	Yes	Phillips and Bonner (2015)
Neogobius melanostomus; Barbus barbus	24%	River Rhine (Germany/France)	$NaOH/HNO_3$-digestion; NaI separation; Visual	No	Roch and Brinker (2017)
Gobio gobio	12%	Rivers and streams (France)	Visual	No	Sanchez et al. (2014)
Hoplosternum littorale	83%	Pajeú river (Brazil)	Visual	No	Silva-Cavalcanti et al. (2017)
Gobio gobio	9%	Rivers and streams (Belgium)	H_2O_2-digestion; NaI separation; Visual	Yes	Present study

665

SUPPLEMENTARY INFORMATION

Supplementary information 1

Nuelle et al. (2014) already showed that a high density NaI solution could be reused, but warned for the reaction with H_2O_2, which is the particular digestive compound proposed by Avio et al. (2015). Following the modification of the protocol (Avio et al., 2015), to include this higher density NaI solution, several trials were made. The trials were necessary to optimize the entire procedure and to avoid or minimize the reaction between the two chemicals. All with the purpose to increase the density separation and consequently the extraction efficiency.

The first trials are all performed using a cellulose nitrate filter of 4.7 cm in diameter (Table 4). While they followed the entire procedure described in the practical methodology section below, their small diameter made it impossible to filter all the decanted material only using one filter as it quickly got clogged with the ground up digestive system. Consequently, up to three filters had to be used during the vacuum filtration for collecting the decanted materials. To collect the decanted digestive system and to get rid of the NaI present in the filter and the filter cake, an extra step had to be performed (not necessary when using larger filters) involving rinsing, flushing and cleaning (Table 4). The rinsing part is used to describe rinsing the filter cake from the "NaI-dirty" filters in a 200 mL MQ filled beaker. The contents of this beaker were then filtered over a "clean" cellulose nitrate filter and the residuals were flushed with MQ-water. Lastly, the cleaning comprised of MQ-water being filtered over the filter cake that came into contact with the NaI solution. After having performed several trials and mixes in the amount of MQ-water used for the "rinse/flush/clean-step", the final results were found to be insufficient to check for microplastics. Brown crystal like structures (become gelatinous after water addition) from the reaction of the H_2O_2 and the reused NaI were found on the entire filter, making it next to impossible to check for any other particles. Although it has to be mentioned that with more MQ-water used to wash the NaI from the organic material, the final results improved. Nevertheless, more MQ-water usage involved a large prolongation of time needed for filtration. In addition to different quantities in the NaI washing step, different methods (data not presented) were performed to collect as much filter cake from the "NaI—dirty" filters as possible. These methods ranged from lightly scratching to gushing MQ-water from a glass syringe. Once more, differences were found in the outcome, but the method using the glass syringe was quickly preferred as it was found to be less time consuming and better at flushing the particles in the 200 mL beaker. In the end, the usage of the smaller cellulose nitrate filters for the samples was discarded, being regarded as too time consuming and insufficient in delivering proper results.

703 **Table 4. First trials in optimizing the methodology, displaying the sampling information (river, GPS coordinates,**
704 **catch date and municipality) with the biometric parameters (entire fish length and weight), the gender and**
705 **the amount of MQ-water used in the different steps.**

River	GPS coordinates (WGS 84)	Municipality	Date catch (dd/mm/yy)	Individual	Length ± stdev (cm)	Weight ± stdev (g)	F/M	Rinse/flush/clean (mL)
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	1	11.0 ± 1.1	13.2 ± 5.53	F	75/75/200
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	2	11.1 ± 1.1	15.3 ± 5.53	F	75/75/200
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	3	10.2 ± 1.1	10.4 ± 5.53	F	75/75/200
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	4	11.4 ± 1.1	13.0 ± 5.53	F	50/75/100
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	5	10.9 ± 1.1	12.3 ± 5.53	F	120/75/200
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	Blank 1	/	/	/	50/50/150
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	Blank 2	/	/	/	50/50/100
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	Blank 3	/	/	/	75/75/75
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	1	11.1 ± 1.1	15.28 ± 5.53	F	125/50/70
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	2	14.3 ± 1.1	30.34 ± 5.53	M	140/50/0
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	3	14.1 ± 1.1	30.79 ± 5.53	M	125/20/0
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	4	13.0 ± 1.1	20.69 ± 5.53	M	100/20/80
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	5	12.5 ± 1.1	20.79 ± 5.53	M	100/20/400
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	6	11.1 ± 1.1	15.16 ± 5.53	F	25/40/20
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	7	12.3 ± 1.1	19.44 ± 5.53	F	100/40/300
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	8	11.3 ± 1.1	15.97 ± 5.53	F	100/50/500
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	9	11.4 ± 1.1	14.80 ± 5.53	M	100/40/500
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	10	11.9 ± 1.1	19.62 ± 5.53	F	100/50/500
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	11	12.0 ± 1.1	15.67 ± 5.53	F	100/50/500
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	12	11.7 ± 1.1	15.33 ± 5.53	M	50/50/0
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	Blank 4	/	/	/	60/50/300
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	Blank 5	/	/	/	25/50/300
Woluwe	50°52'28.0"N 4°27'30.3"E	Zaventem	03/04/15	Blank 6	/	/	/	100/50/500

706

The second trial performed (Table 5), used larger 10 cm diameter cellulose nitrate filters, which lowered the amount of filters needed to check a singular digestive system to one. This increased the speed of the protocol, but also improved the outcome, providing much cleaner filters. Once more different amounts of MQ-water were used to clean the NaI from both the filter cake and the filter, at least 1.5 L was chosen as it combined both time efficiency and clean filters needed for proper microscopic assessment.

Table 5. Second trials optimizing the methodology, displaying the sampling information (river, GPS coordinates, catch date and municipality) with the biometric parameters (entire fish length and weight), the gender and the amount of MQ water tried out for rinsing.

River	GPS coordinates (WGS 84)	Municipality	Date catch (dd/mm/yy)	Individual	Length ± stdev (cm)	Weight ± stdev (g)	F/M	Rinse (L)
Stenensluisvaart	51°0'4.4"N 2°49'45.6"E	Diksmuide	22/10/15	1	9.4 ± 0.7	7.19 ± 2.33	F	0.5
Stenensluisvaart	51°0'4.4"N 2°49'45.6"E	Diksmuide	22/10/15	2	10.7 ± 0.7	12.60 ± 2.33	M	1
Stenensluisvaart	51°0'4.4"N 2°49'45.6"E	Diksmuide	22/10/15	3	11.0 ± 0.7	13.13 ± 2.33	F	1.5
Stenensluisvaart	51°0'4.4"N 2°49'45.6"E	Diksmuide	22/10/15	4	11.1 ± 0.7	11.42 ± 2.33	F	2
Stenensluisvaart	51°0'4.4"N 2°49'45.6"E	Diksmuide	22/10/15	Blank 1	/	/	/	0.5
Stenensluisvaart	51°0'4.4"N 2°49'45.6"E	Diksmuide	22/10/15	Blank 2	/	/	/	1
Stenensluisvaart	51°0'4.4"N 2°49'45.6"E	Diksmuide	22/10/15	Blank 3	/	/	/	1.5

Practical methodology

This section describes more into detail the exact steps used in the modified protocol from Avio et al. (2015) using the high density separation of Nuelle et al. (2014).

Practical:

- ⇨ Include three procedural blanks per run
- ⇨ Include two petri dishes filled with MQ water for background contamination
- ⇨ Always cover all samples and liquids with aluminium foil, during the entire procedure!
- ⇨ Work under a laminar flow cabinet
- ⇨ Only use MQ-water
- ⇨ Only use metal or glass laboratory equipment
- ⇨ The number of samples = the amount of time in hours needed to complete day 2 of the protocol
- ⇨ Before NaI usage always prefilter over a 0.45 µm cellulose-nitrate filter

Day 1 - Dissection:

- ✓ Rinse, air dry and check the dissection material under stereomicroscope
- ✓ Rinse and air dry all glass petri dishes
- ✓ Weigh petri dishes separately

- ✓ Defrost, weigh and rinse the outside of the gudgeons
- ✓ Dissect gudgeon + determine sex
- ✓ Take out the digestive system (oesophagus – anal sphincter + liver and gallbladder) and cover with aluminium foil
- ✓ Weigh petri dish + wet weight digestive system
- ✓ Lightly cover with aluminium foil and put into dry oven to desiccate overnight 55°- 60°C

Day 2 – Density separation and digestion:

- ✓ Weigh petri dishes + dry weight digestive system
- ✓ Rinse and air dry all laboratory glasswork

- Make NaI solution (60% w/w, 1.8 g/cm³) (500 g of solution)
  - ✓ Weigh 300 g of NaI
  - ✓ Weigh 200 g of MQ-water
  - ✓ Stir until everything is dissolved
  - ✓ Filter it over a 0.45 µm cellulose-nitrate filter using a vacuum pump
- Density separation
  - ✓ Ground dried gastrointestinal tract in mortar and pestle (grind well!)
  - ✓ Put it with at least 100 mL of NaI solution in a high 200 mL beaker
  - ✓ Stir and decant for 10 min (while decanting do not use all NaI, do it in stops, organic matter sticks to beaker)
  - ✓ Filter using the vacuum pump, over an 8 µm cellulose-nitrate filter
  - ✓ Collect the NaI and add it to the decanting beaker again
  - ✓ Filter everything twice
  - ✓ The sedimented material (sand,…) gets collected in another 200 mL beaker, to extract the remaining NaI for reuse
- Rinse all NaI from filter
  - ✓ Rinse the vacuum glasswork thoroughly!!!
  - ✓ Place the filter with filter cake in the vacuum glasswork
  - ✓ Rinse the filter cake + filter with at least 1.5 L MQ-water
  - ✓ Cover every spot on the filter and filter cake to rinse all NaI
  - ✓ Put each of the filters in a clean glass petri dish and cover with aluminium foil
- Digestion with H_2O_2
  - ✓ Prepare 15% H_2O_2 solution (for ten samples)
    - Put 75 mL filtered MQ-water into a clean, air dried glass measuring cylinder
    - Add 75 mL 30% H_2O_2
  - ✓ Add 15 to 20 mL of the solution to each petri dish containing the sample
  - ✓ Cover with aluminium foil and put in dry oven overnight at 55°-60°C
  - ✓ Loosely cover the samples to allow the filters to dry after digestion

Day 3 – Visual observation:

- Check for microplastics on the filter under a dissecting microscope (32x)
- Describe the item + background contamination

- Wet a needle tip and pick up the suspected microplastic and/or contaminating particle
- Transfer the item stuck to the needle to a clean piece of cellulose nitrate paper or alternatively if the initial filter was clean enough wet it directly
- Place the wet filter in between two glass microscopic slides and tape up the sides to keep it firmly in place for later observation
- Check the suspected particles again under a higher magnification microscope (up to 400x)
- Take pictures under macroscope
- Use micro-FTIR spectroscopy to analyse a synthetic origin

Supplementary information 2

Table 6. Data on the sampling location, displaying the river, GPS coordinates, the municipality, sampling date and province of the sampling site, including the local human pressure from the municipality displayed as human population, surface area and population density.

River	Code	GPS coordinates (WGS 84)	Municipality	Date catch (dd-mm-yy)	Province	Population[a]	Surface area[a] (km²)	Population density[a] (#/km²)
Balengracht	BT	51°09'37.0"N 5°11'22.7"E	Balen	30/10/15	Antwerp	21570	72.88	296
Bosbeek	BA	51°06'09.2"N 5°48'14.7"E	Maaseik	23/07/15	Limburg	24828	76.91	323
Bosbeek	BV	51°04'36.1"N 5°40'24.7"E	Maaseik	04/11/15	Limburg	24828	76.91	323
Bovenschelde	BE	50°43'13.7"N 3°21'57.0"E	Spiere-Helkijn	29/09/15	West Flanders	2110	10.78	196
Desselse Neet	DN	51°14'51.0"N 5°05'11.2"E	Dessel	29/10/15	Antwerp	9231	27.03	342
Dijle	DE	50°48'10.0"N 4°38'33.2"E	Oud-Heverlee	23/09/15	Flemish Brabant	11046	31.14	355
Gaverbeek	GK	50°54'17.2"N 3°24'46.8"E	Waregem	07/05/15	West Flanders	36751	44.34	829
Ijse	IH	50°47'20.2"N 4°34'51.4"E	Huldenberg	13/11/15	Flemish Brabant	9528	39.64	240
Ijse	IN	50°49'13.1"N 4°38'11.2"E	Huldenberg	29/04/15	Flemish Brabant	9528	39.64	240
Kleine Herk	KH	50°55'04.8"N 5°15'04.5"E	Hasselt	06/11/15	Limburg	74588	102.24	730
Kleine Nete	KN	51°14'20.1"N 5°04'12.1"E	Dessel	23/10/15	Antwerp	9231	27.03	342
Merkske	ME	51°25'42.1"N 4°47'44.3"E	Hoogstraten	18/11/15	Antwerp	20386	105.32	194
Rode Loop	RL	51°19'31.4"N 5°01'46.2"E	Arendonk	18/11/15	Antwerp	12894	55.38	233
Velpe	VE	50°50'56.3"N 4°54'22.6"E	Tienen	06/11/15	Flemish Brabant	32987	71.77	460
Wimp	WP	51°07'33.3"N 4°45'40.7"E	Heist-op-den-berg	08/04/15	Antwerp	40512	86.46	469
Winge	WE	50°57'35.7"N 4°43'26.8"E	Rotselaar	20/11/15	Flemish Brabant	15963	37.57	425
Zwarte Neet	ZN	51°15'38.7"N 5°05'33.6"E	Retie	09/10/15	Antwerp	10799	48.39	223

[a]: source: ADSEI (2013)

Supplementary information 3

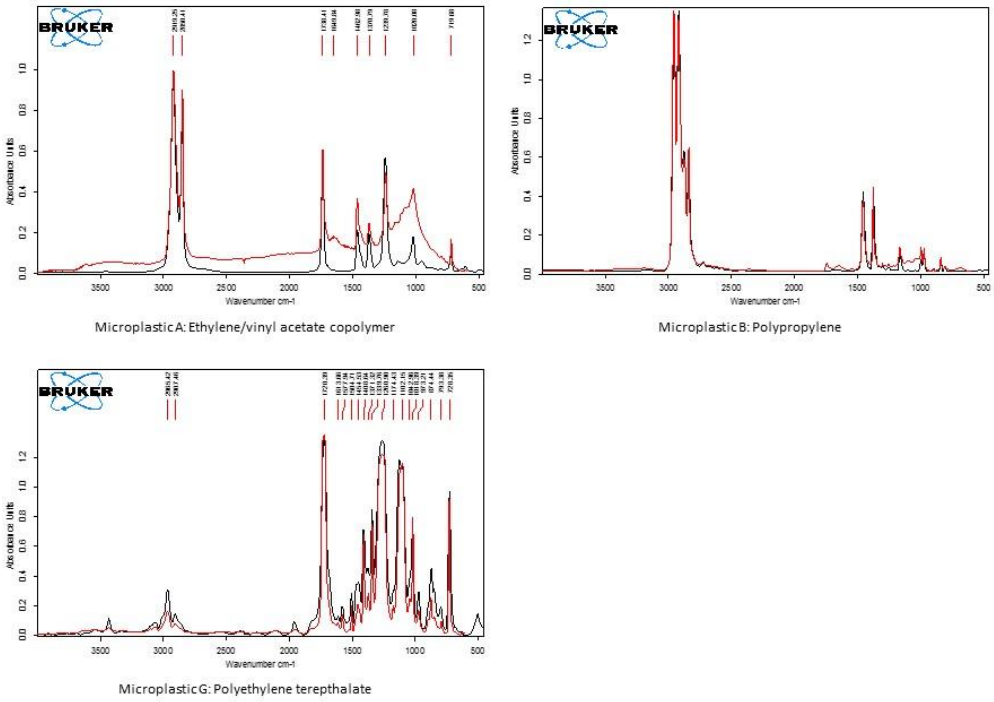
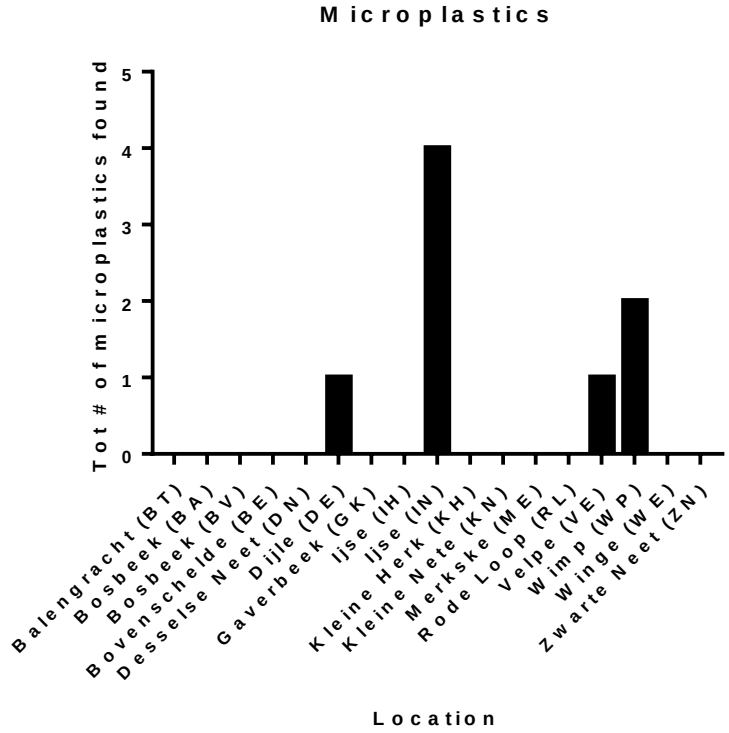


Figure 3. Examples of microplastic A, B and G portraying the spectroscopic analysis using micro-FTIR.

807 Supplementary information 4



808

809 **Figure 4. Microplastic amounts in fish from the different sampling sites.**

810 Supplementary information 5

811 **Table 7. The biometric parameters, the gender, the condition index (K) and the average weight of the digestive**
812 **system (DS) with the standard deviation displayed from all 78 gudgeons sampled at the different rivers,**
813 **including the occurrence of suspected microplastic samples.**

River (Code)	Length ± stdev (cm)	Weight ± stdev (g)	Condition index ± stdev	DS wet weight ± stdev (g)	DS dry weight ± stdev (g)	F/M	MP Sample
Balengracht (BT)[a]	10.9 ± 0.3	11.20 ± 0.93	0.86 ± 0.00	0.42 ± 0.15	0.11 ± 0.01	M	/
Balengracht (BT)[a]	11.5 ± 0.3	13.06 ± 0.93	0.86 ± 0.00	0.71 ± 0.15	0.12 ± 0.01	F	/
Bosbeek (BA)[a]	11.2 ± 0.4	16.32 ± 0.80	1.16 ± 0.14	0.64 ± 0.08	0.14 ± 0.04	M	/
Bosbeek (BA)[a]	11.9 ± 0.4	14.72 ± 0.80	0.87 ± 0.14	0.49 ± 0.08	0.07 ± 0.04	M	/
Bosbeek (BV)[a]	12.7 ± 0.4	16.53 ± 2.06	0.81 ± 0.03	0.44 ± 0.17	0.12 ± 0.01	F	/
Bosbeek (BV)[a]	13.4 ± 0.4	20.65 ± 2.06	0.86 ± 0.03	0.78 ± 0.17	0.11 ± 0.01	F	/
Bovenschelde (BE)[a]	12.7 ± 0.5	19.01 ± 0.73	0.93 ± 0.06	0.70 ± 0.10	0.27 ± 0.06	M	/
Bovenschelde (BE)[a]	13.6 ± 0.5	20.46 ± 0.73	0.81 ± 0.06	0.50 ± 0.10	0.16 ± 0.06	F	/
Desselse Neet (DN)[a]	12.1 ± 0.0	14.61 ± 0.85	0.82 ± 0.05	0.64 ± 0.10	0.14 ± 0.01	F	/
Desselse Neet (DN)[a]	12.1 ± 0.0	16.31 ± 0.85	0.92 ± 0.05	0.44 ± 0.10	0.15 ± 0.01	M	/
Dijle (DE)[a]	13.1 ± 0.9	20.61 ± 3.99	0.92 ± 0.07	0.90 ± 0.24	0.24 ± 0.08	F	/
Dijle (DE)[a]	14.0 ± 0.9	27.31 ± 3.99	1.00 ± 0.07	1.18 ± 0.24	0.27 ± 0.08	F	/
Dijle (DE)	11.6 ± 0.9	16.66 ± 3.99	1.07 ± 0.07	0.98 ± 0.24	0.35 ± 0.08	M	/

Dijle (DE)	11.6 ± 0.9	15.68 ± 3.99	1.00 ± 0.07	0.71 ± 0.24	0.17 ± 0.08	M	/
Dijle (DE)	12.4 ± 0.9	18.35 ± 3.99	0.96 ± 0.07	0.69 ± 0.24	0.18 ± 0.08	F	/
Dijle (DE)	12.1 ± 0.9	14.42 ± 3.99	0.81 ± 0.07	0.41 ± 0.24	0.09 ± 0.08	F	/
Dijle (DE)	12.5 ± 0.9	18.77 ± 3.99	0.96 ± 0.07	0.7 ± 0.24	0.19 ± 0.08	F	/
Dijle (DE)	11.2 ± 0.9	12.86 ± 3.99	0.92 ± 0.07	0.47 ± 0.24	0.09 ± 0.08	F	/
Dijle (DE)	11.5 ± 0.9	15.09 ± 3.99	0.99 ± 0.07	0.50 ± 0.24	0.14 ± 0.08	M	**A***
Dijle (DE)	11.2 ± 0.9	14.33 ± 3.99	1.02 ± 0.07	0.49 ± 0.24	0.22 ± 0.08	M	/
Gaverbeek (GK)[a]	12.7 ± 0.9	28.18 ± 4.78	1.38 ± 0.17	1.23 ± 0.23	0.27 ± 0.07	F	/
Gaverbeek (GK)[a]	13.2 ± 0.9	21.10 ± 4.78	0.92 ± 0.17	0.87 ± 0.23	0.35 ± 0.07	M	**J**
Gaverbeek (GK)	11.2 ± 0.9	13.63 ± 4.78	0.97 ± 0.17	0.51 ± 0.23	0.13 ± 0.07	M	**L**
Gaverbeek (GK)	10.2 ± 0.9	14.69 ± 4.78	1.38 ± 0.17	0.75 ± 0.23	0.27 ± 0.07	F	/
Gaverbeek (GK)	11.8 ± 0.9	15.86 ± 4.78	0.97 ± 0.17	0.61 ± 0.23	0.21 ± 0.07	M	/
Gaverbeek (GK)	10.7 ± 0.9	11.80 ± 4.78	0.96 ± 0.17	0.57 ± 0.23	0.16 ± 0.07	M	/
Gaverbeek (GK)	11.2 ± 0.9	13.54 ± 4.78	0.96 ± 0.17	0.44 ± 0.23	0.15 ± 0.07	M	/
Gaverbeek (GK)	11.0 ± 0.9	12.90 ± 4.78	0.97 ± 0.17	0.63 ± 0.23	0.18 ± 0.07	M	/
Gaverbeek (GK)	10.9 ± 0.9	12.33 ± 4.78	0.95 ± 0.17	0.58 ± 0.23	0.20 ± 0.07	M	/
Gaverbeek (GK)	11.5 ± 0.9	16.64 ± 4.78	1.09 ± 0.17	0.94 ± 0.23	0.27 ± 0.07	F	/
Ijse (IH)[a]	11.4 ± 0.6	14.73 ± 5.16	0.99 ± 0.13	0.47 ± 0.60	0.20 ± 0.19	M	/
Ijse (IH)[a]	12.6 ± 0.6	25.04 ± 5.16	1.25 ± 0.13	1.66 ± 0.60	0.58 ± 0.19	F	/
Ijse (IN)[a]	13.3 ± 1.2	22.60 ± 7.19	0.96 ± 0.06	0.68 ± 0.32	0.20 ± 0.08	M	**B***
Ijse (IN)[a]	13.2 ± 1.2	22.46 ± 7.19	0.98 ± 0.06	0.92 ± 0.32	0.11 ± 0.08	M	/
Ijse (IN)	13.6 ± 1.2	26.93 ± 7.19	1.07 ± 0.06	1.03 ± 0.32	0.36 ± 0.08	F	**C***
Ijse (IN)	12.4 ± 1.2	18.90 ± 7.19	0.99 ± 0.06	0.62 ± 0.32	0.30 ± 0.08	M	/
Ijse (IN)	12.9 ± 1.2	24.53 ± 7.19	1.14 ± 0.06	0.92 ± 0.32	0.28 ± 0.08	F	/
Ijse (IN)	14.4 ± 1.2	29.59 ± 7.19	0.99 ± 0.06	0.98 ± 0.32	0.18 ± 0.08	M	/
Ijse (IN)	13.3 ± 1.2	24.05 ± 7.19	1.02 ± 0.06	0.89 ± 0.32	0.20 ± 0.08	M	**I + M**
Ijse (IN)	12.0 ± 1.2	16.31 ± 7.19	0.94 ± 0.06	0.58 ± 0.32	0.15 ± 0.08	M	/
Ijse (IN)	12.2 ± 1.2	20.04 ± 7.19	1.10 ± 0.06	0.82 ± 0.32	0.22 ± 0.08	F	**D* + E***
Ijse (IN)	16.3 ± 1.2	43.47 ± 7.19	1.00 ± 0.06	1.78 ± 0.32	0.37 ± 0.08	F	/
Kleine Herk (KH)[a]	11.3 ± 0.3	11.93 ± 1.74	0.83 ± 0.07	0.36 ± 0.02	0.09 ± 3.55	M	/
Kleine Herk (KH)[a]	10.7 ± 0.3	8.46 ± 1.74	0.69 ± 0.07	0.32 ± 0.02	0.09 ± 3.55	M	/
Kleine Nete (KN)[a]	13.4 ± 1.1	21.34 ± 4.32	0.89 ± 0.00	0.65 ± 0.01	0.17 ± 0.01	F	/
Kleine Nete (KN)[a]	11.3 ± 1.1	12.70 ± 4.32	0.88 ± 0.00	0.67 ± 0.01	0.16 ± 0.01	M	/
Merkske (ME)[a]	11.6 ± 0.3	11.38 ± 1.16	0.73 ± 0.02	0.27 ± 0.05	0.10 ± 0.01	F	/
Merkske (ME)[a]	12.1 ± 0.3	13.70 ± 1.16	0.77 ± 0.02	0.37 ± 0.05	0.11 ± 0.01	F	**P**
Rode Loop (RL)[a]	10.7 ± 0.4	8.19 ± 0.12	0.67 ± 0.08	0.50 ± 0.09	0.13 ± 0.01	M	/
Rode Loop (RL)[a]	9.9 ± 0.4	7.95 ± 0.12	0.82 ± 0.08	0.33 ± 0.09	0.12 ± 0.01	F	/
Velpe (VE)[a]	12.1 ± 0.6	15.07 ± 2.48	0.85 ± 0.05	0.48 ± 0.16	0.13 ± 0.07	M	/
Velpe (VE)[a]	13.0 ± 0.6	20.24 ± 2.48	0.92 ± 0.05	0.95 ± 0.16	0.33 ± 0.07	F	**O**
Velpe (VE)	11.0 ± 0.6	11.74 ± 2.48	0.88 ± 0.05	0.40 ± 0.16	0.11 ± 0.07	M	/
Velpe (VE)	11.7 ± 0.6	14.30 ± 2.48	0.89 ± 0.05	0.64 ± 0.16	0.19 ± 0.07	F	/
Velpe (VE)	11.8 ± 0.6	13.73 ± 2.48	0.84 ± 0.05	0.53 ± 0.16	0.13 ± 0.07	F	/
Velpe (VE)	11.1 ± 0.6	12.63 ± 2.48	0.92 ± 0.05	0.70 ± 0.16	0.22 ± 0.07	F	**F* + N**
Velpe (VE)	12.2 ± 0.6	15.74 ± 2.48	0.87 ± 0.05	0.53 ± 0.16	0.10 ± 0.07	M	/
Velpe (VE)	11.5 ± 0.6	11.82 ± 2.48	0.78 ± 0.05	0.38 ± 0.16	0.08 ± 0.07	F	/

Velpe (VE)	11.6 ± 0.6	12.75 ± 2.48	0.82 ± 0.05	0.56 ± 0.16	0.12 ± 0.07	F	/
Velpe (VE)	11.3 ± 0.6	11.76 ± 2.48	0.82 ± 0.05	0.61 ± 0.16	0.18 ± 0.07	F	/
Wimp (WP)[a]	11.6 ± 0.5	14.28 ± 1.70	0.91 ± 0.03	0.61 ± 0.13	0.15 ± 0.04	M	**K + G***
Wimp (WP)[a]	11.2 ± 0.5	12.33 ± 1.70	0.88 ± 0.03	0.29 ± 0.13	0.06 ± 0.04	M	/
Wimp (WP)	11.3 ± 0.5	13.80 ± 1.70	0.96 ± 0.03	0.63 ± 0.13	0.19 ± 0.04	M	/
Wimp (WP)	10.8 ± 0.5	11.04 ± 1.70	0.88 ± 0.03	0.41 ± 0.13	0.13 ± 0.04	M	**H***
Wimp (WP)	10.5 ± 0.5	10.84 ± 1.70	0.94 ± 0.03	0.32 ± 0.13	0.08 ± 0.04	F	/
Wimp (WP)	10.8 ± 0.5	11.51 ± 1.70	0.91 ± 0.03	0.30 ± 0.13	0.06 ± 0.04	M	/
Wimp (WP)	9.8 ± 0.5	8.22 ± 1.70	0.87 ± 0.03	0.20 ± 0.13	0.05 ± 0.04	M	/
Wimp (WP)	11.0 ± 0.5	12.74 ± 1.70	0.96 ± 0.03	0.40 ± 0.13	0.10 ± 0.04	M	/
Wimp (WP)	10.1 ± 0.5	9.91 ± 1.70	0.96 ± 0.03	0.34 ± 0.13	0.09 ± 0.04	F	/
Wimp (WP)	10.8 ± 0.5	11.75 ± 1.70	0.93 ± 0.03	0.44 ± 0.13	0.11 ± 0.04	F	/
Winge (WE)[a]	12.2 ± 1.1	15.02 ± 3.59	0.83 ± 0.07	0.64 ± 0.26	0.22 ± 0.08	M	/
Winge (WE)[a]	12.0 ± 1.1	15.67 ± 3.59	0.91 ± 0.07	1.06 ± 0.26	0.27 ± 0.08	M	/
Winge (WE)	12.1 ± 1.1	15.54 ± 3.59	0.88 ± 0.07	0.52 ± 0.26	0.16 ± 0.08	M	/
Winge (WE)	11.1 ± 1.1	13.08 ± 3.59	0.96 ± 0.07	0.50 ± 0.26	0.21 ± 0.08	F	/
Winge (WE)	13.5 ± 1.1	19.60 ± 3.59	0.80 ± 0.07	0.53 ± 0.26	0.11 ± 0.08	F	/
Winge (WE)	10.0 ± 1.1	7.66 ± 3.59	0.77 ± 0.07	0.20 ± 0.26	0.04 ± 0.08	M	/
Zwarte Neet (ZN)[a]	11.5 ± 0.7	14.65 ± 2.19	0.96 ± 0.00	0.77 ± 0.06	0.08 ± 0.05	F	/
Zwarte Neet (ZN)[a]	10.2 ± 0.7	10.28 ± 2.19	0.97 ± 0.00	0.88 ± 0.06	0.17 ± 0.05	F	/

814 [a] Fish investigated during the initial screening; * Particles accepted as microplastics