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Original Scientific Article

ISOLATION OF MICROPLASTICS FROM FRESHWATER MACROINVERTEBRATES IN THE DANUBE RIVER

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Abstract. *The study was conducted on the Danube River, within the project Joint Danube Survey 3 (JDS3). The main aim was to estimate the quantity of microplastics in aquatic ecosystems through passive biological monitoring. Three freshwater species were used for microplastic (MP) isolation from different taxonomic groups of organisms: Mollusca, Oligochaeta, and Chironomidae (Diptera), with the following species: Lithoglyphus naticoides (C. Pfeiffer, 1828), Limnodrilus hoffmeisteri (Claparede, 1862), and Chironomus acutiventris (Wülker, Ryser & Scholl, 1983), respectively. The samples were collected from 6 sites along the Danube River where 540 specimens were examined. The samples were digested by alkaline method (incubation in 10% KOH solution at 60 ºC for 24 h) and filtered through a mill silk, 10 µm mesh size. Collected particles were categorized as: fibre, hard plastic, nylon, rubber, or miscellaneous. Categories were divided into subcategories based on the coloration of the particles. Particles ingested by organisms were represented mostly by fibres and fragmented hard plastics, within the size range were from 0.03 to 4.87 mm. A total of 678 MP particles were collected with an average of 4.64 ± 1.59; 1.64 ± 0.46 and 1.24 ± 0.34 items/organism isolated from L. hoffmeisteri, L. naticoides and C. acutiventris, respectively. According to results, L. hoffmeisteri, L. naticoides and C. acutiventris could be used as proper bioaccumulators of MP pollution in the Danube River.*

Key words: *microplasic, plastic litter, freshwater, alkaline method, macrozoobenthos, the Danube River.*

Introduction

The presence of plastic nowadays has been estimated as ubiquitous. Annual production of plastic has rapidly increased since the 1950s, with constantly increasing rates ever since. Estimated at 1.5 million metric tons (MT) in 1950, the world's production of plastic has reached 368 million metric tons (MT) in 2019 [1]. Constantly increasing world's production over time, durability and low recycling rates of plastic resulted in their high presence and accumulation in the environment [2]. MP is an organic polymer derived from fossil feedstocks within the size 1 µm [3] up to 5 mm [2]. Primary MPs are manufactured in microscopic size as industrial pellets, exfoliating microbeads in personal care products [4], abrasives in blasting, or as a component of paints [5–6]. Secondary MP has primarily been macroscopic size, manufactured as plastic demands of the buyers, with the dominance of packaging items (plastic bags, containers, bottles) and building and construction [1]. Any physical, chemical, or biological process causes degradation of macroplastics into microplastics [7]. The plastic polymers have residual monomers and chemical additives, capable of absorbing toxins from the environment such as persistent organic pollutants (POPs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, PAHs, and dioxins [8–10]. In addition, additives such as phthalate-based plasticizers and bisphenol A (BPA) amplify the toxicity of the plastic particles [11].

MP became a great concern due to potential availability to a wide range of organisms because of similar size fractions as sediment and food particles [12]. Studies on plastic contamination in natural ecosystems have reported MPs within freshwater ecosystems such as rivers [13–16] lakes [17–22] and shoreline sediments [23–24], estuarine areas [25], indicating rivers as pathways for marine plastic debris [26–28], but with incomparably fewer data, and conducted researches. In addition to its presence in the aquatic environment, MPs can cause a mechanical hazard [29] to organisms or be a vector for opportunistic pathogens [30–31], persistent organic pollutants (POPs) [8–10] and heavy metals [32] or invasive species [33], which may result in harmful algal bloom (HAB) [34].

Annex VIII of the Water Framework Directive (WFD) is focusing on the identification of 'Specific Pollutants', such as MPs, to derive Environmental Quality Standards (EQSs) for targeted chemicals to achieve Good Surface Water Status [35].

As MP is a global problem, the Directives on packaging waste ([94/62/EC), waste (2008/98/EC), landfills

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(1999/31/EC), and sewage sludge (86/278/EEC) [36–37] established monitoring of plastic sources of freshwater ecosystems in Europe. In 2013, the European Commission developed 'Green paper on a European strategy on plastic waste in the environment' [37], European strategy on plastic waste in the environment, especially on MP waste, as a wider review of waste legislation. In 2015, the Plastic Bags Directive was adopted with the aim of reducing its consumption through pricing, taxes, and levies [38]. Since the concern for this synthetic pollution is rising, the Union's chemicals legislation (1907/2006/EC) applied relevant production volumes of plastic monomers and additives used in manufacturing processes [39]. The European Commission adopted a Circular Economy Package in 2015 for using the resources in a more sustainable way which refers to plastic, among five priority sectors [38]. As it can be concluded from the enclosed data, MP is a serious issue nowadays.

According to Framework of the European Water Framework Directive [35], macroinvertebrates are used in monitoring studies to assess the ecological water quality as a group of different bioindicators of organic pollution. Despite the huge problem posed by MPs in the environment, there are no indicators developed for passive monitoring of MPs in aquatic ecosystems. In Annex VIII there is a list of the main pollutants, among others, 'persistent and bioaccumulative organic toxic substances' and 'persistent hydrocarbons' which may include synthetic polymers [35].

MP has reached a significantly high level of abundance during the past few decades. Since the first report on MP debris from 1972 [40] in the aquatic ecosystem (marine ecosystem) popularization has been rising constantly. In this article, we present results from the survey on plastic debris in the Danube River in Serbia. The main aim of the study was to estimate the amount of microplastics in aquatic ecosystems through passive biological monitoring. To that end, we set the following tasks: (1) to quantify the number of MP particles per organism and per biomass and (2) to estimate the distribution of particles per categories and subcategories, based on their shape and color.

Materials and Methods

Sampling Site and Procedure

The study was conducted on the Danube River, which flows into the Black Sea and is the second largest river in Europe [41]. This international river basin occupies the territory of 19 countries, with an area of 817.000 km^2 and gathering 83 million inhabitants near it. The Danube River System is situated in nine ecoregions and classified as a special case study in terms of conservation and management issues [42]. In this study three species from different groups of organisms (Mollusca, Oligochaeta, and Chironomidae (Diptera)) were used for MPs isolation: *Lithoglyphus naticoides* (C. Pfeiffer, 1828), *Limnodrilus hoffmeisteri* (Claparede, 1862) and *Chironomus acutiventris* (Wuelker, Ryser & Scholl, 1983), respectively. Within the JDS3 project, the most diverse components of the total community were Chironomidae (Diptera), Oligochaeta, and Mollusca [43]. *L. hoffmeisteri* (Naididae:Tubificidae) represents one of the most dominant species along the whole stretch of the Danube River, tolerant to organic pollution [44]. *L. naticoides* has Ponto-Caspian origin and it is considered as cryptogenic for the upper and middle stretch of the Danube, while it is native to the Lower Danube [45]. *C. acutiventris* one representative of the chironomids, which are considered as useful bioindicators suitable for determining the biological effects of different pollutants in the aquatic environment [46]. Since chironomids are non-specific feeders, ingestion of MPs instead of food particles is very common [47].

Samples were collected between August and September 2013, at six sites along the Danube. Following the multi-habitat procedure [48], macrozoobenthos was sampled using a hand net (ap. 25 cm× 25 cm, mesh size 500 μm) by the kick & sweep (K&S) sampling technique (EN 27828:1994). For the deep water area, a triangle shaped dredge (ap. 25 x 25 cm, mesh size 500 μm) was pulled five times per sampling site in a length of 80 cm. Each transect was considered as a separate sample. A detailed description of the sampling methodology is presented in Liška et al. [49]. The samples of macrozoobenthos were sorted in the laboratory and the specimens of Oligochaeta, Mollusca, and the larvae of Chironomidae were counted, separated, and identified to the lowest possible taxonomic level, by the use of the following identification keys: Moller Pillot [50–51], Schmid [52], Vallenduuk and Moller Pillot [53], Pfleger, [54], Timm [55].

Methodology of Isolation MPs from Macroinvertebrate

Although numerous approaches have been developed for the extraction of MPs, all are classified into six protocol groups within the following methods: acidic [56–57], alkaline [58-59], oxidizing [60–61], and enzymatic methods [58]. Dehaut et al. [62] have tested all of the protocols and found out that five out of six have shown significant degradation of plastic particles or insufficient tissue digestion. The alkaline method appeared to be the best protocol for isolating MPs from biological samples and for later identification.

The protocol is based on using 10% potassium hydroxide (KOH) solution as a medium for the samples and incubation at 60 ºC during a 24 h period. This leads to an efficient decomposition of biological tissues with no significant degradation on all tested polymers, except for cellulose acetate [62]. They suggested it for the implementation in further monitoring studies on MPs.

For each species, 180 specimens were randomly selected from 6 sites, 30 specimens per site. One sample contained 10 specimens, meaning three replicates per site. In total, 540 specimens were measured on an analytic scale in order to estimate potential MP litter per biomass (Table 1). In the experiment setting, the control did not contain any single entity.

The samples were treated by the suggested alkaline method, using a 10% solution of KOH and incubation at 60 ºC for a 24 h in a water bath. The samples of *C. acutiventris* remained undigested after the suggested incubation time, due to the presence of the chitin. For its degradation, samples were additionally treated with the double volume of nitrate acid (HNO_3) in controlled conditions - vials with the samples were placed in the digester in cold water with ice and 3 ml of HNO_3 was added carefully by micropipette. This reaction formed potassium nitrate (KNO_3), which started an instant reaction with the chitin and dissolved the tissue of *C. acutiventris*. The samples were filtered through a milling silk, as filter, with a 10 µm mesh size. The filtered material was treated with 30% hydrogen peroxide to remove the remaining organic matter if needed. The particles were carefully collected, photographed and categorized, based on their shape (Fig. 1). Subcategories were defined according to the colorization of the particle.

Table 1 Total and average weight of species. Total weights are in grams.

	L. naticoides	*L. hoffmeisteri*	*C. acutiventris*
Total number of individuals	180	180	180
Total weights	1.74	0.07	0.26
Average	0.097	0.004	0.015
SD*	0.004	0.07	0.006

* Standard deviation

Particles were counted manually with a Leica MZ16A stereomicroscope (10 X/21 B ocular; from 20 X to 50 X objective magnification), photographed with a Leica DFC320 Digital Camera system, and measured in program ImageJ [63]. Fibres from the air were excluded.

Results

The collected particles were assigned to one of 5 major categories: fibre, hard plastic, nylon, rubber, or miscellaneous (Fig. 2). In the present study, fibres were the dominant group of MPs with 49.48 % of the total count and the second major category was hard plastic with the percentage share of 43.21 % of collected particles in total (Fig. 3). Measurement of particles by the longest length has shown that fibres had a length from 0.19 to 4.87 mm and hard particles from 0.046 to 0.23 mm (Table 2).

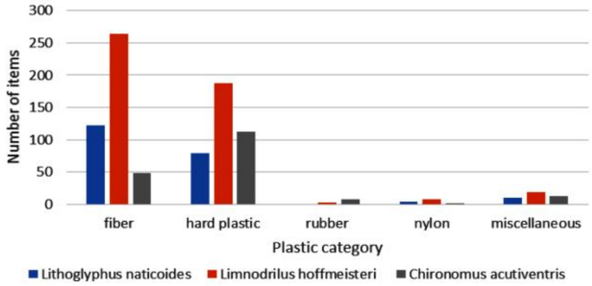

Fig. 2 Types of MPs collected form *Litogliphus naticoides, Limnodrilus hoffmeisteri* and *Chironomus acutiventris.*

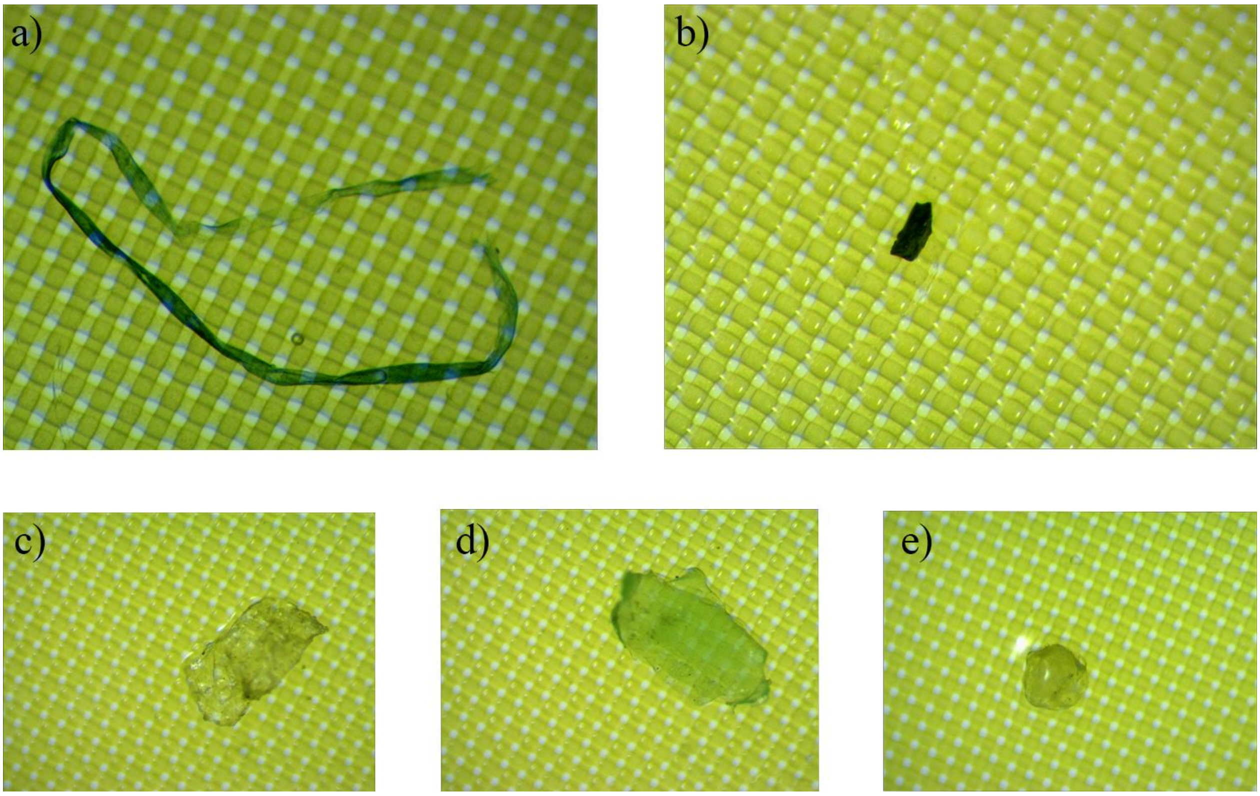

Fig. 1 Photographs of particles from 5 major categories: a) fibre; b) hard plastic; c) nylon; d) rubber; e) miscellaneous.

Table 2 Percentage share and length of particles divided in subcategories. Lengths are in millimeters.

Subcategory	No. of particles	Percentage share [%]	Minimum length [mm]	Maximum length [mm]	Average length [mm] ± SD*
blue fibre	349	39.79	1.97	4.8	2.62 ± 0.55
red fibre	85	9.69	0.19	4.87	1.35 ± 0.65
red rubber	5	0.57	0.096	0.026	0.066 ± 0.025
green rubber	3	0.34	0.085	0.11	0.097 ± 0.012
white rubber	2	0.23	0.058	0.14	0.099 ± 0.041
black hard plastic	79	9.00	0.05	0.16	0.083 ± 0.03
blue hard plastic	64	7.29	0.07	0.199	0.119 ± 0.085
white hard plastic	54	6.16	0.064	0.23	0.152 ± 0.122
grey hard plastic	12	1.37	0.052	0.077	0.065 ± 0.008
brown hard plastic	39	4.45	0.1	0.21	0.144 ± 0.037
crystal hard plastic	33	3.76	0.046	0.11	0.084 ± 0.019
transparent hard plastic	98	11.18	0.046	0.086	0.067 ± 0.013
white nylon	3	0.34	0.11	0.15	0.127 ± 0.017
transparent nylon	10	1.15	0.055	0.89	0.225 ± 0.239
miscellaneous	41	4.68	0.059	0.14	0.106 ± 0.019

* SD – standard deviation.

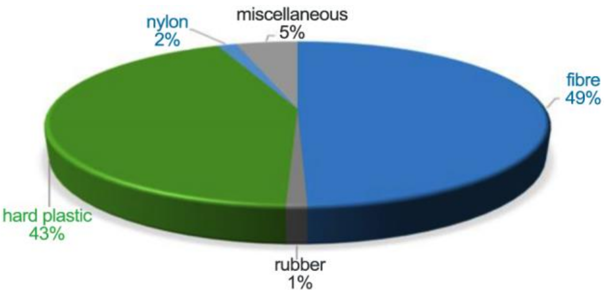

Fig. 3 Main categories with precentage share in total number of collected particles.

A total of 678 MPs were collected, whereas majority of the particles were isolated from *L. hoffmeisteri* (61.6 %), followed by *L. naticoides* (21.8 %) and *C. acutiventris* (16.6 %). An average, 4.64 ± 1.59; 1.64 ± 0.46 and 1.24 ± 0.34 items/organism or 0.000421 ± 0.000409; 0.009661 ± 0.005247 and 0.001465 ± 0.000598 items/g wet weight were isolated from *L. hoffmeisteri*, *L. naticoides* and *C. acutiventris*, respectively.

Discussion

There is still scarce information assessing microplastic pollution in the freshwater environments due to a lack of data on the presence and quantities of MPs within the bodies of freshwater biota. In this study, MPs were recorded in the tissue of *L. naticoides*, *L. hoffmeisteri* and *C. acutiventris* in the Danube River, thus supporting earlier reports on the worldwide presence of MPs [64-66].

Although there are no data on microplastic ingestion of *L. naticoides*, *L. hoffmeisteri* and *C. acutiventris*, other representatives of the groups of Mollusca, Oligochaeta, and Chironomidae (Diptera) have been used in studies of MP ingestion. The categorization of the particles differs in different studies due to the lack of standardization of categories of MP particles. In the tissue of freshwater snail *Sinotaia aeruginosa* (Reeve, 1863) from Taihu Lake, fibres and fragments were the most common categories. Transparent, red and blue subcategories were common within the fibres, while transparent subcategories were dominant within the fragments [67]. Akindele et al. [68] detected only fibres in the tissue of freshwater gastropods *Melanoides tuberculata* (Müller, 1774) and *Theodoxus fluviatilis* (Linnaeus, 1758), and fibres and films in the tissue of *Lanistes varicus* (Müller, 1774). The majority of isolated particles from the tissue of *Tubifex tubifex* (Müller, 1774) in the Salford Quays basin (Manchester City, England) were represented as fibres (87 %), while fragments represented the rest of the particles [69]. Lin et al. [70] detected microgranules (0-28 %), microfilms (0-16 %), microfragments (3-47 %), and microfibres (40-64 %) within the midge larvae (Diptera: Chironomidae) among 5 sampling sites in the Wu river basin, Taiwan. Su et al. [17] detected four categories of MP particles in the tissue of freshwater Asian clam *Corbicula fluminea* (O. F. Müller, 1774): fibre, pellet, film, and fragment, with the dominance of fibres (48-84% in the samples). Within the subcategories, blue items were dominant on the water surface (50-63 %), while transparent and white items were more common in organisms and sediments (29–44 %). Hohenblum et al. [71] conducted preliminary research in Austria on the Danube River and over 50 % of the extracted plastic particles consisted of fragments, 4–10 % were pellets and 2.1–2.8 % were green lenticular flakes. MPs isolated from *C. fluminea*, collected along 2040 km of the Danube, were represented by fibres - dominance of blue subcategory and fragments - dominance of transparent subcategory [72].

According to previous studies, fibres were the most dominant category in the soft tissue of *B. aeruginosa* [67], *M. tuberculata, T. fluviatilis, L. varicus*. [68], *C.*

fluminea [17, 72], *Thienemannimyia* spp., *Chironomus* spp. and *Orthocladius* spp. [68], which is in concordance with this study (49.48 %). Ingestion rates of *B. aeruginosa* [67], *L. varicus*, *T. fluviatilis* [68], *C. fluminea* [72] were higher than ingestion rates of *L. hoffmeisteri*, *L. naticoides* and *C. acutiventris*. Xu et al. [67] detected 96.67% to 100% of one or more types of microplastics within freshwater snail *B. aeruginosa* collected from Taihu Lake, China, with the dominance of polyvinyl acetate fibres (88.0 ± 12.1%), polystyrene fibres (66.3 ± 17.5%), polyamides (49.7 ± 22.4%), and polyethylene terephthalate (30.0 ± 7.4%). Also, previous research of the Taihu Lake [17] hasdetected cellophane, PET, Polyester, and Polypropylene in Asian clam *C. fluminea*. Scherer et al. [46] demonstrated the uptake of polystyrene among different freshwater invertebrate groups, including freshwater snail *Physella acuta* (Draparnaud, 1805) (Mollusca), the blackworm *Lumbriculus variegatus* (Müller, 1774) (Oligochaeta), and *Chironomus riparius* (Meigen, 1804) (Chironomidae, Diptera).

Conclusion

MPs in the environment have been characterized as a global problem nowadays. Due to their bioavailability to a wide range of organisms and ubiquitous presence and distribution, there is a need to determine its amount in the natural environment by the use of bioindicators. Lack of data on the presence of MPs in freshwater biota is one of the reasons for the lack of a solution for plastic pollution. Choice of good indicators for estimation of the MPs in the aquatic environment is necessary. According to the amount and diversity of MPs isolated from three benthic species (*L. hoffmeisteri, L. naticoides* and *C. acutiventris)* it seems that these species could be used as proper bioaccumulators of MP pollution in the Danube River in further studies. The impact of MPs has been documented in recent years for various freshwater species, but it is certain that a lot of data is still missing to form a wider insight on this major synthetic pollution. Therefore, new field data are needed in order to estimate more precise quantities we are dealing with in the environments. In order to accomplish this task, including MPs in the standard analysis procedures could be necessary to gather more data. Further research and continued monitoring on the Danube is a request for a good evaluation of the presence and effects of MPs on aquatic organisms and the environment.

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