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OPTIMIZATION OF EXTRACTION METHOD FOR MICROPLASTIC FROM INVERTEBRATES USING PANCREATIC ENZYMES

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Abstract

Microplastic is recognized as an emerging contaminant in aquatic environments. To understand the microplastic fate in the system, efficient screening methods applicable to different ecosystem components are needed. Standard methods for the extraction of microplastics from biota are currently lacking, and most methods employing strong acids or bases can also degrade the polymers. Recent study revealed gentle and easy way of extracting microplastic from mussels with pancreatic enzymes. This thesis focuses on optimization of aforementioned enzymatic digestion method from the selection of the invertebrates.

Pancreatic enzymes were used to digest biota samples including small crustaceans, *priapulid worms and jellyfish* from Baltic Sea. Optimal conditions for digestion were chosen based on experiment data with amphipods *Monoporeia affinis* and mysids *Mysis mixta* used as test species. Several concentrations of pancreatic enzymes were tested as well as two methods of digestion in order to determine the highest digestion efficiency and the lowest load of undigested biological matter. Further, the method was applied to a range of pelagic and benthic invertebrates collected in the Baltic Sea. In addition, reference polymers particles were exposed to enzymes to investigate if protocol alters the polymers appearance in any way.

The optimal concentration of enzymes was chosen to be the lowest one out of tested – 0.1 mg/ml. With this method, digestion efficiency up to 87.60 $\pm$ 0.04 % was achieved. Even though enzymatic products were influencing the weight of filter, the results were still acceptable for a successful identification of microplastic with both visual and FT-IR analysis. No changes were found in reference polymer after the exposure, which leads to the conclusion that pancreatic enzymes do not modify plastic particles during the digestion process.

Keywords

Enzymatic digestion, microplastic, microplastic extraction, pancreatic enzymes

CONTENTS

NOMENCLATURE

PA polyamide

PC polycarbonate

PET polyethylene terephthalate

LLDPE linear low-density polyethylene

LDPE low-density polyethylene

HDPE high-density polyethylene

PP polypropylene polymers

PUR polyurethane

PVC polyvinyl chloride

PS polystyrene

PE polyethylene

POPs persistent organic pollutants

PBTs persistent bioaccumulative and toxic substances

HNO_3 nitric acid

H_2O_2 hydrogen peroxide

HCl hydrochloric acid

NaOH sodium hydroxide

KOH potassium

FT-IR Fourier transform infrared spectroscopy

Pyrolysis-GC/MC pyrolysis-gas chromatography/mass chromatography

DE digestion efficiency

LC level of coverage

DW dry weight

GF/F glass microfiber filter

1 INTRODUCTION

Plastic is a synthetic organic polymer with a long chain structure, therefore high molecular weight. It has been widely used in various industries and everyday life since the industrialization period in 1940s - 1950s. The applicability of plastic is extensive for the reasons such as low cost, long durability, light weights and simple manufacturing process. Plastic production has been increasing with every year, reaching more than 348 million tons in 2017 (Plastics Europe, 2018). The properties that make this material so favourable, also make plastic a global environmental threat. Plastic is often used as single-use packaging material, which leads to an increasing amount of plastic litter. Reusing and recycling rate of plastic is much lower comparing to other materials such as paper, metal and glass (European commission, 2018).

Recent studies suggested that 5-13 million metric tons of plastics ends up in the ocean every year (Jambeck *et al.,* 2015). Marine debris is estimated to consist 80 % of plastic litter. Moreover, under various conditions, plastics brake down to smaller particles, called Microplastic which is an emerging environmental problem. The great concern of microplastic is raised because of its small size (1-1000 μm), it is able to enter various food web. Numerous studies have indicated that microplastic is ingested by different marine animals including fish, seabirds, reptiles and mammals and various invertebrates (Dehaut *et al.,* 2016; Gesamp, 2015; Lusher *et al.,* 2013;).

With a growing concern of microplastic, reliable data on occurrence and fate of plastic particles in environment has to be provided. However, investigation of microplastic pollution is currently hindered by analytical challenges. For the successful representation of occurrence of microplastic data, particles must be safely separated form biological sample. It is necessary to ensure, that particles have not been damaged or misplaced during separation and that sample have not been contaminated during the process. Work intensity, time efficiency and cost of the methods are important factors to consider. Currently there is no standardized method for extraction of microplastic from biota. Digestion of tissue and sediment has been commonly used to extract microplastic (Lusher *et al.,*

2017). Applying strong oxidizing acids, base hydrolyzing or oxidizing agents has been done in order to separate microplastic from the biota (Cole *et al.,* 2013, 2015; Claessens *et al.,* 2013; Avio *et al.,* 2015; Catarino *et al.,* 2017). Some of the method showed high digestion efficiency (e.g. >98% with nitric acid treatment (Cole *et al.,* 2015)). However, some of them can potentially cause damage to shape or destroy plastic particle which are sensitive to pH, such as nylone fibers, polyethylene terephthalate and polystyrene polymers (Dehaut *et al.,* 2016; Claessens *et al.,* 2013). Moreover, some of the methods are not suitable for implementation on the large scale due to high price or long exposure time (e.g. 3 weeks of KOH exposure (Dehaut *et al.,* 2016)). Enzymatic digestion showed some promises as it is both time efficient and gentle to polymer (Catarino *et al.,* 2017; Cole *et al.,* 2015). Recently commercially available pancreatic enzymes (PEz) were used for mussel's digestion with high effectiveness (von Friesen *et al.,* 2019).

The aim of this thesis was to investigate the applicability of enzymatic protocol to separate microplastic from biota matrix. Investigation was performed on variety of benthic and pelagic invertebrates such as amphipods, mysids, worms and jellyfish. The following objectives of the thesis were put forward:

- To optimize method for extracting microplastics from small invertebrates with pancreatic enzymes using amphipods *Monoporeia affinis*, mysids *Mysis mixta,* priapulids *Halicryptus spinulosus,* jellyfish *Mertensia ovum, Aurelia aurita* and *Cyanea capillata,* and polychaetes *Marenzelleria* as a test species
- To investigate if enzymatic digestion damages the reference microplastic during the process

2 LITERATURE REVIEW

2.1 Microplastic

Commonly used size categorization by National Oceanic and Atmospheric Administration (NOAA) considers microplastic all polymer particles smaller than 5 mm. With a further research, more detailed nomenclature on size

characterization was developed (Hartmann *et al.,* 2019). In 2015, Gesamp suggested to use following characterization (*Table 1)*:

Table 1: Characterization of plastic by size (Gesamp, 2015)

Nanoplastic	< 1 μm
Microplastic	1 – 1000 μm
Mesoplastic	1 – 25 mm
Macroplastic	2.5 – 100 cm

Another commonly applied characterization is based on the origin of the polymers. Microplastic divided into primary and secondary microplastics. Primary microplastic is intentionally produced in the small sizes, like microbeads for the cosmetic uses or micro pellets for the industrial purposes. It is released into the environment during manufacturing, use or maintenance of plastic products. The examples of primary plastic sources are tyre abrasion during driving, discharge of fibres from synthetic clothing during washing, release of microplastic to waste water from cosmetics use (Boucher and Friot, 2017). Secondary plastics created as a result of degradation xof larger plastic items. Weathering and fragmentation is happening during exposure of plastic to various natural factors. Under solar UV light, chemical reactions in the environment, physical forces such as wind, waves or animal activities, plastic degrades and breaks down to smaller particles. The degradation process is highly influenced by the presence of oxygen, temperature, additives in microplastic which can enhance or reduce photo degradation. All in all, formation of microplastic in the environment depends on environmental factors and properties of the polymer. (Gesamp, 2015).

Occurrence of microplastic in the marine environment was first reported in 1972 (Carpenter *et al*., 1972). Estimated input of plastic litter in ocean considered to be 10% of worldwide production (Thompson, 2006). Plastic was found in meso- macro- and microplastic forms (Shim and Thompson, 2015). Plastic was located floating on the water surface, in the seabed sediment, in the water column and on the coastal lines. Distribution of microplastic in the ocean depends on polymer properties, location of the sources of entry and complex chemical, physical and

biological processes in environment (Kowalski *et al.,* 2016). Primary microplastic enter the ocean from waste water treatment facilities, factories and as a result of accident or loss during transhipment of virgin pellets (Boucher and Friot, 2017). For the secondary microplastic it is particularly difficult to estimate distribution due to unknown and unsystematic degradation and distribution processes. There are many land- and sea-based activities that contribute to plastic pollution, but accurate estimation on local or global scale input is yet to be found (Gesamp, 2015).

Once in the marine environment, surface of microplastic can be colonized by macrobiotic fouling organisms, developing biofilm which changes polymer's sinking rate as well as decreases photodegradation. Usually biofouling can increase particles density causing it to flow. However, laboratory tests showed that large particles tend to sink with biofouling development (Kowalski *et al.,* 2016). Moreover, role of marine organisms plays huge role in microplastic distribution as animals may transfer microplastic for large distances. For some cases, particles may even be taken back to land carried by sea birds or mammals. Microplastic can also be trapped in sediments for a long period and released due to wave current or bioturbation (Gesamp, 2015).

Marine organisms can be exposed to microplastic through different routes of entry. Some microplastics can enter organism through gills, however it is possible only for particles smaller than 40 μm (Watts *et al.,* 2014). The main way of exposure is ingestion, as both laboratory and field studies demonstrated that microplastic is ingested by large variety of organisms from different trophic levels (Lusher *et al.,* 2013, Foekema *et al.,* 2013, Gesamp, 2015, Cole *et al.,* 2014, Farell and Nelson, 2013). Small size of plastic cause marine animals to confuse it with food and ingest it. These organisms include fish-eating birds, sea mammals, fish and various invertebrates like zooplankton, worms, crustaceans and bivalves. Microplastic has known to be able to pass through the food web. Numerous studies confirmed that microplastic was found in the predator organisms as they consumed plastic contaminated food (Farell and Nelson, 2013). Additional concern is related to occurrence of microplastic in commercially interested

species. It was found in number of organisms consumed by human e.g. blue mussels and oysters (Van Cauwenberghe and Janssen 2014).

After microplastic has entered the organism, it could either accumulate in tissue and/or body fluid, or be excreted depending on size, shape and particle composition. Excretion of microplastic in organism based solely on laboratory studies. (Farrell and Nelson, 2013).

Due to limited information on exposure level and established effects level, risk assessment of microplastic remains to be hazardous. The effect of microplastic ingestion is based either on its chemical or physical properties. The size and shape of the particle is important for physical effect. Physical harm of ingested particles may be resulted from entanglement, blocking of feeding organs, interference with feeding rate/capacity. It is especially dangerous for small animals such as zooplankton, crustaceans and bivalves. Chemical effect depends on two key factors combined together: surface area and reactivity, and toxicity of polymer and absorbed contaminants (Teuten *et al.,* 2009). In theory, smaller particles have larger surface area, therefore likely to absorb more contaminants as well as exhibit more internal toxicity. However, this hypothesis has not been proved by studies (Gesamp, 2015). The risk of exposure to microplastic from ecotoxicological prospective associated with its ability to absorb contaminants from surroundings among with ability to leach out additives/absorbed chemicals. Ingested particles, have potential to cause chemical toxicity in the organisms as well as transfer contaminants to other trophic levels. Hydrophobic contaminants such as POPs, PBTs and some other petroleum hydrocarbons tend to sorb onto plastic surface (Teuten *et al.,* 2009). Moreover, some studies showed that weathered particles can potentially accumulate heavy metals from environment, although in smaller concentrations compared to natural particles. Metals can be leached from the particles, and in the acidic conditions the leaching is enhanced. In general, factors that are influencing sorption/desorption rate are hydrophobicity of the contaminant, plastic size and type (Nakashima *et al.,* 2012). In addition, microplastic can act as a new

vector of invasive species transportation which potentially threats marine communities. (Gesamp, 2015).

2.2 Review on microplastic extraction methods

2.2.1 Oxidizing method

This method is known for efficient removing of biofilm from plastic surfaces. Hydrogen peroxide treatment has been widely implemented for sediment, waste water, filters samples and fish and mussels tissue (Zhao *et al*., 2017). Hydrogen peroxide had been applied to dissolve and/or discolour organic matter, but was noted to damage polyamide (PA), polycarbonate (PC), polyethylene terephthalate (PET), linear low-density polyethylene (LLDPE) and polypropylene polymers (PP) in concentration of 30 % (Nuelle *et al.,* 2014). However, lower concentration (15 % H_2O_2) was found to be efficient in removing organic matter and did not damage polyethylene (PE) and polystyrene (PS) polymers. (Avio *et al.,* 2015). Such method involves treatment with H_2O_2 for 7 days at the room temperature, or incubation at 75 ^{O}C temperature with 15 % H_2O_2 for 24 hours.

2.2.2 Enzymatic digestion

Studies were conducted with enzymes that effectively digest soft biological matter (efficiency up to 97 %) without any damage to polymers (Catarino *et al.,* 2017; Cole *et al.,* 2015). For example, enzymatic treatment with Proteinase-K reported by Cole *et al.* is more efficient in digestion of plankton biota than acid or alkaline methods. Catarino *et al.* reported 100 % digestion efficiency of mussel's tissue with protease. However, some of methods were either too expensive (Proteinase-K, Cole *et al.,* 2015), either required incubation time up to 16 days (Löder *et al.,* 2017). The risk of contamination of some methods (Löder *et al.,* 2017, Cole *et al.,* 2015) is high due to numerous working steps. Even though enzymatic treatment did not show any change in polymers used for spiking, studies were not conducted on weathered particles, which mostly present in the filed samples. Recent study by W. von Friesena *et al.* reported that pancreatic enzymes is both efficient in removing mussel's tissue (efficiency up to 97.7 %) already over a

night, and gentle to weathered LDPE particles collected from sea surface, therefore already subjected to natural degradation.

2.2.3 Acid digestion

Nitric (HNO_3) and hydrochloric acids (HCl) have been used to digest biota. Nitric acid was observed to efficiently digest mussel's tissue. However, fibres were damaged in the process too (Claessens *et al.,* 2013). Concentrated boiling nitric acid not only degrades pH sensitive polymers such as PA, PS and PE (Avio *et al.,* 2015), but poses a health threat, which also should be considered (Budimir *et al.,* 2018). HCl was used to digest zooplankton without damaging the polymers particles, but was less efficient than sodium hydroxide used for the same purpose (Cole *et al.,* 2013). Hydrochloric acid was found to be efficient in removing calcium carbonate which are very beneficial in digestion of shell or exoskeleton parts. Very often combination of the chemicals is implemented to achieve the best results. For example, samples digested in sodium hydroxide often treated with hydrochloric acid afterwards to remove calciferous structures (Budimir *et al.,* 2018).

2.2.4 Alkaline digestion

To digest biological tissue potassium hydroxide (KOH) and sodium hydroxide (NaOH) have been commonly applied. KOH is usually used for digestion of fish tissue with high efficiency over 48 hours at 40 ^{O}C. On other hand, same procedure under room temperature can take up to 3 weeks (Foekema *et al.,* 2013). Sodium hydroxide was used for extract microplastic from biota as well as plankton rich water. However, such method showed damage to plastic particles in the concentration of 10 M and 60 ^{O}C temperature (Dehaut *et al.,* 2016). Reduced concentration of NaOH (1 M) and 10% KOH efficiently removes biota tissue but was noticed to damage cellulose acetate particles as well (Budimir *et al.,* 2018, Dehaut *et al.,* 2016). Such protocol does not remove chitin; thus, addition of hydrogen peroxide or hydrochloric acid is recommended.

2.3 Review on microplastic identification methods

2.3.1 Visual identification

After sample preparation, microplastic can be visually detected under light microscopy, based on morphological properties such as shape, colour and overall structure. Typically, it is easy to distinguish plastic particles (1-5 mm in size) from other organic and inorganic material. Plastic lacks visible structure and their surface is not shiny (Zhao *et al.,* 2017). Microscopic identification is very easy and cheap technique of microplastic identification. However, size limitation hinders this kind of analysis for particles less than 1000 μm. Moreover, besides morphological appearance it is not possible to conclude chemical composition of polymer presented, and more advanced techniques of identification is required.

When samples loaded with organic matter and it is challenging to find microplastic due to transparent colour or small size, staining is applied. Nile Red had been commonly use to stain polymers such as PE, PP, PS, nylone 6, PC, PET, polyvinyl chloride (PVC), low-density polyethylene (LDPE), high-density polyethylene (HDPE) and polyurethane (PUR), and observe them under fluorescence light (Shim *et al.,* 2016; Erni-Cassola *et al.,* 2017). This lipid fluorescence dye showed most effective adsorbent and fluoresce intensity criteria (Maes *et al.,* 2017). It is dissolvable in organic solvents as acetone, methanol or n-hexane (Shim *et al.,* 2016). From few drops to 100 μL of staining solution is needed to cover a filter. After drying the filters are analysed under either blue (excitation wavelength (ex.): 365 nm; emission wavelength (em.):445 nm), red (ex.:534-558 nm; em.: >590 nm) or green (ex.:450-490 nm; em.:515-565 nm) excitation and emission wavelength (Shim *et al.,* 2016; Erni-Cassola *et al.,* 2017). Synthetic polymers become easily detectable from the background and could be counted and analysed. Interestingly, studies showed (Erni-Cassola *et al.,* 2017) that wood lignin and chitin parts also fluoresces after Nile Red application, even though not as strong as highly hydrophobic plastic material (PE, PS, PP). Nile Red strongly fluorescence in staining of hydrophobic polymers rather than less hydrophobic. Thus, there is a risk of misanalysing organic matter such as lignin

with less hydrophilic particles (PC, PVC, PUR, PET) (Erni-Cassola *et al.,* 2017; Maes *et al.,* 2017; Shim *et al.,* 2016).

2.3.2 Raman Spectroscopy

Raman spectroscopy is highly reliable analytical technique to analyse chemical composition of unknown plastic polymers. When sample is irradiated with monochromatic laser source, it interacts with molecules and atoms. Laser wavelength may vary between 500 and 800 nm. These low frequency interactions change backscattered light, creating the difference between it and original monochromatic laser frequency. This difference called Raman shift, corresponds to substance-specific Raman spectra. Plastic polymers has very distinguishable Raman spectra, which leads to accurate and fast identification of polymer type. One great advantage of this techniques is allowance of identification of small particles size down to 1 μm with micro-Raman spectroscopy (Löder *et al.,* 2015).

2.3.3 Fourier transform infrared spectroscopy

One of the most used techniques – FT-IR spectroscopy – allows to accurately identify plastic polymers based on their infra-red spectra. FT-IR based on the ability of infra-red radiation to excites molecules, which results in molecular vibrations. These vibrations depend on the composition and molecular structure of sample, and have very specific wave length. It is possible to measure absorbed IR radiation for specific wave lengths, therefore obtain characteristic IR spectra. Similar to Raman spectroscopy, plastic polymers have very characteristic IR spectra leading to accurate composition identification. Moreover, FT-IR spectroscopy has been used for analysis of weathered and degraded particles as it can measure intensity of oxidation (Löder *et al.,* 2015). Although, technique enables to efficiently detect small plastics, it is extremely time-consuming. FT-IR has single detector element, allowing it to measure only one particle in a time. Sample should also be dried before analysis as any substances including water influence IR spectra. In addition, unlike Raman spectroscopy, FT-

IR spectroscopy is limited to size detection of 10 μm (Löder *et al.,* 2015; Dis *et al.,* 2018).

2.3.4 Pyrolysis-GC/MS

Another way to detect chemical composition of plastic particles is to subject them to pyrolysis-gaschromatography (GC) in combination with mass chromatography (MC). This technique analyses thermal degradation products of sample, which allows to identify not only polymer type, but plastic additives too. Combustion products of particles are compared with reference virgin-polymer type. Main disadvantages of this techniques are size limitation due to manual input of each particle into the pyrolysis tube, and high time consumption due to the same reason (one particle per run) (Löder *et al.,* 2015).

3 MATERIALS AND METHOD

3.1 Test organisms

Several benthic and pelagic organisms found in Baltic Sea were tested: amphipods *Monoporeia affinis*, mysids *Mysis mixta*, priapulids *Halicryptus spinulosus*, polychaetes Marenzelleria) *and jellyfish (Mertensia ovum, Aurelia aurita,* and Cyanea capillata)*. Amphipods and mysids were* collected near coast of Sweden on 10.09.2008 in the 20-120 m deep levels. Jellyfish samples were collected on 27.07.2009, Marenzelleria on 23.09.2010 and Halicryptus on 26.08.2010. All samples were frozen at -18^{O} C except for jellyfish which were dried already prior to analysis and stored in aluminum foil at room temperature. All samples were provided by Stockholm University.

3.2 Method optimization

3.2.1 Digestion efficiency tests

To determine the efficiency of tissue removal, protocol was tested on amphipods and mysids (*Monoporeia affinis, Mysis mixta).* To conduct the test, frozen

organisms were placed in glass petri dishes separately and dried at the temperature 60^{O} C degrees for 24 hours to achieve the constant dry weight.

After preparation, all samples were subjected to digestion and filtration. First tested on amphipods, digestion protocol was assessed for 24 hours. For each test, 3 replicates and 1 blank solution was used. Pancreatic enzymes tested were commercially available Creon 25000 (Abbott Laboratories GmbH, Germany Mylan). Amphipod tests were conducted with 2 different concentrations of Creon – 30 mg/ml and 20 mg/ml. 300 mg of Creon capsule's content was weighted on analytical scale (Sartorius BP 211D) and places in 20 ml glass bottles. Then, 10 ml of Tris buffer solution (Trizma® hydrochloride solution, 1 M, pH 8.0, 0.2 µm filtered, Sigma-Aldrich, ID T3038, USA) as well as magnetic stirrer were added to bottles. Tris was added to ensure sample pH would maintain at 8 – the optimal performing range of pancreatic enzymes (von Friesen *et al.,* 2019). Bottles were places on magnetic mixer for 10-20 minutes to ensure that all of the Creon came to solution. Finally, 5 individuals of roughly 10-20 mg of dry weight were added to bottles and incubated on magnetic mixer at 37,5 ^{O}C and 200 rmp for 24 hours. The same procedure was repeated with 200 mg of Creon. Blank samples for each concentration tested without biological matter were processed in the same fashion to determine if enzymes influence the final weight of the filter as well as highlight any possible contamination during the procedures. Grounding the dry sample with pestle and mortar before digestion was applied to samples with 30 ml/ml Creon solution to speed up the digestion time.

To test protocol on mysids smaller quantities of reagents were used. To prepare series of standards 100 mg of enzymes were weighted with analytical scale and diluted in 10 ml of Tris. Concentration of 10 mg/ml, 5 mg/ml, 1 mg/ml, 0,5 mg/ml and 0,1 mg/ml were tested in order to determine which one will give the best result. To achieve as precise results as possible, 20.19 $\pm$ 0.21 mg of DW of mysids were used. Reaction volume was set to 5 ml in economic reasons. Creon solutions and uncrushed mysids were placed in 20 ml bottles and put in incubator on magnetic mixer for 24 hours.

As an alternative way of incubation, Eppendorf ThermoMixer® C was tested at the same temperature and 600 rmp to decrease the chance of particle's destruction during mixing with magnetic stirrer. To test this method, samples (n=3 and blank) with 0.1 mg/ml and 1 mg/ml Creon concentrations were used with exposure time of 24 and 48 hours. Results of amphipods and mysids tests were compared.

After digestion, all the samples were filtered by 1,5 μm glass microfibers filters (GF/F) 47 mm in diameter (Whatman, France). Before filtering, filters were dried at 60^{O} C temperature over a night and pre-weighed on the analytical balance. Samples were vacuum filtered and vigorously rinsed with warm Milli Q-water. After that, filters were placed in the oven at 60 ^{O}C temperature until dry weight was stabilized. All the undigested tissues, contamination particles and possible plastics originated from the samples stayed on the filter.

After filtering, filters were visually analysed under microscope (Leica DMRBE, Germany) under 4 X and 10 X power lenses. Level of coverage (LC) (1-4 scale) was assessed in terms of undigested biological matter which can potentially influence microplastic identification on the filter. 4 grades of LC were used: 1 – all/most of the tissue is digested, very easy to analyse; 2 – low load of undigested tissue, easy to analyse; 3 – filter partly covered by undigested tissue, hard but possible to analyse; 4 – filter fully covered with biological tissue, impossible to analyse. LC of level 1, 2, 3 considered to be acceptable since microplastic is visually recognisable.

To calculate the dry weight after digestion, weight of original filter was subtracted from the weight of the filter after digestion. Digestion efficiency was calculated with the *Formula 1*:

$$DE\ (\%) = \frac{DWb - DWa}{DWb} \times 100 \qquad (1)$$

Where, DE (%) is digestion efficiency, DWb is dry weight of the sample before digestion and DWa is dry sample after digestion.

In total, different concentration of Creon and 2 ways of incubation for 24 and 48 hours were tested. Amphipods and mysids samples were assessed for digestion efficiency percentage and level of coverage *(Table 2).*

All work was done in 100% cotton lab coat and protective gloves. All equipment was rinsed with Milli Q-water. Preferably, manipulations with samples were executed under ventilation hood to minimize the chance of contamination from air borne particles.

3.2.2 Implementation of the method on variety of invertebrates

Experiments on jellyfish and worms was conducted after digestion efficiency tests had been performed. Dry jellyfish samples of 10 individuals in total (5 of *Mertensia ovum,* 2 of *Cyanea capillata* and 3 of *Aurelia aurita*) were subjected to the same protocol of digestion in incubator for 24 hours with 5 mg/ml of Creon in reaction volume of 5 ml (one individual per sample). Then procedure was repeated with 9 individuals of Halicryptus (one individual per sample) in the same concentration of Creon. However, exposure time was assessed for 24 hours and 4 days. Simultaneously, 5 samples of Marenzelleria (0,2 ml volume of wet weight) were subjected to digestion in Thermomixer for 48 hours with same amount of reagents. After digestion, all samples were treated in the same fashion with further air vacuum filtration, drying and visual analysis under microscope with magnification 10 X. For all listed species, level of coverage was assessed in the end of analysis.

Table 2 represents totalization of experiments during the study including methods and assessment criteria used for each species tested.

Table 2: Totalization of experiment: tested species, Creon amount, digestion method, exposure time and assessment criteria

Digestion method	Species	Creon	Exposure time	Coverage Level	Digestion Efficiency
Magnetic mixer in incubator	Monoporeia affinis	30 mg/ml, 20 mg/ml	24 h	+	+
	Mysis mixta	10 mg/ml, 5 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.1 mg/ml	24 h	+	+
	Jellyfish: Mertensia ovum, Cyanea capillata, Aurelia aurita	5 mg/ml	24 h	+	-
	Halicryptus	5 mg/ml	24 h; 4 days	+	-
Thermomixer	Marenzelleria	5 mg/ml	48 h	+	-
	Mysis mixta	1 mg/ml, 0.1 mg/ml	48 h	+	+

3.3 Degradation of the polymer

Second part of the study focused on any possible effect that enzymatic digestion can have on plastic particles of ≥ 300 μm in size. As a reference polymer polystyrene from black computer keyboard was used. Keyboard particles were crushed to random sizes and exposed to UV light for 4 days. Particles were black in color with distinguishable sharp surfaces. Suspension of plastic particles in water was made. To spike solutions (n=1) with Creon and Tris, 20 μL (one drop) of suspension was used. All particles equal or more than 300 μm in size were selected to conduct the analysis, whereas other particles presented in one drop were left out of study. Selected particles were counted and photographed by Leica DMRBE light microscope with Leica DFC280 camera. Bigger size of keyboard particles was used to avoid confusion with possible contamination particles that could appear after manipulations with the samples as well as to conduct visual analysis more accurately and comprehensively. Particles were

exposed to 20 mg/ml and 10 mg/ml Creon solutions (n=1) in reaction volume of 10 ml and 5 ml respectively. Both samples underwent the same procedures of digestion and filtering described in Digestion efficiency chapter except for the addition of biological matter.

First exposure test with 10 mg/ml Creon solution was carried out in Thermomixer whereas second test with 20 mg/ml of Creon solution was carried out in incubator on magnetic mixer. These methods were assessed to see any potential differences between them. Filters then were examined under microscope to detect any changes in amount, size, shape and colour of the particles. Photos of particles were compared before and after.

To detect any degradation of the polymer, keyboard particles were analysed before and after by FT-IR spectrometer (Varian 610-IR FT-IR spectrometer with Varian 610-IR FT-IR microscope). Spectra of original keyboard powder were taken and compared to spectra of selected keyboard particles left after exposure to enzymes. Changes were assessed in terms of correlation with $\geq$ 90 % considered to be satisfactory (von Friesen *et al.,* 2019). Correlation was obtained by subtraction of particles spectra after enzymatic exposure from original keyboard powder spectra in the Resolutions Pro software.

4 RESULTS

4.1 Results on digestion efficiency and method optimization

Solutions with digested amphipods were clear, transparent with slightly yellowish color, no visible biological matter, but highly glutinous (*Figure 1*). Filtration of such solution was not immediate as expected, but at rather slow rate. Nonetheless, weight after digestion of amphipods were much higher than initial weight of the samples. The amount of Creon highly altered the results as it could be seen from the blanks (*Table 3*). Therefore it was not possible to calculate digestion efficiency. No difference between crushed and uncrushed samples were observed.

Table 3: Experiment results on amphipods: dry weight of the samples, residue weight on filters after filtration and level of coverage grades

Creon, mg/ml	DW of amphipods, mg	Residue on filter, mg	Level of coverage
30	Blank	76.65	
30	12.61 ± 1.82	47.82 ± 3.34	2.8 ± 0.3
20	Blank	65.90	
20	18.92 ± 3.15	50.22 ± 14.17	2.7 ± 0.3

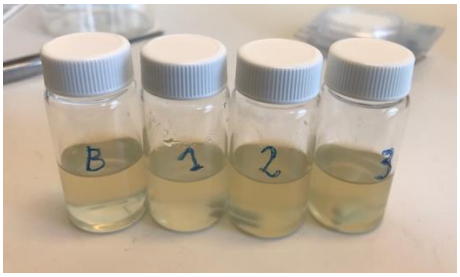

Figure 1: Samples with 20 mg/ml of Creon concentration after digestion in incubator for 24 hours

Unlike amphipods, solutions with digested mysids were a bit cloudy with beige-yellowish color (*Figure 2*), while the filtration of the tested solutions were almost immediate, which allowed quick and convenient analysis of filters (*Figure 3*). As chitin of amphipods and mysids is very soft and not calcinated, it was digested quite well with only some small particles of it remained. Even though the level of coverage was satisfactory, the solutions left small residue on the filters (*Table 4)*.

Table 4: Experiment results on mysids: dry weight of the samples, residue weight after filtration, digestion efficiency percentage and level of coverage grades

Creon, mg/ml	Mysids, mg	Residue, mg	DE, %	Level of coverage
10	Blank	1.95		
	19.98 ± 0.03	5.05 ± 1.63	74.69 ± 0.08	2.0 ± 0.0
5	Blank	6.13		
	20.10 ± 0.29	6.91 ± 0.49	65.62 ± 0.03	1.5 ± 0.0
1	Blank	8.68		
	20.18 ± 0.07	7.56 ± 0.93	62.56 ± 0.04	1.3 ± 0.3
0.5	Blank	6.98		

	20,08 ± 0.03	7.53 ± 1.99	62.47 ± 0.09	1.75 ± 0.35
0.1	Blank	0.12		
	20,27 ± 0.03	2.51 ± 0.87	87.60 ± 0.04	1.3 ± 0.3

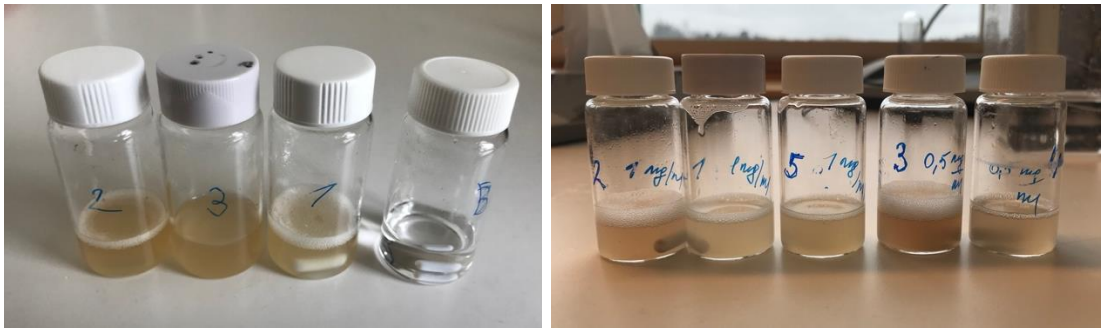

Figure 2: Samples with 1 mg/ml (right), 0.5 mg/ml (left) and 0.1 mg/ml (left) of Creon concentration after digestion in incubator for 24 hours

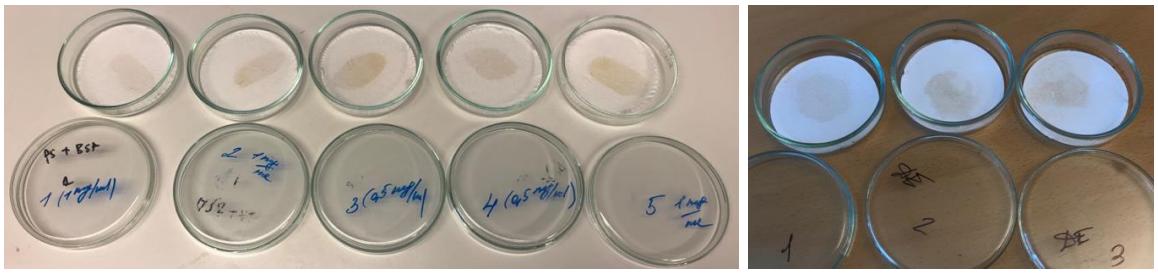

Figure 3: Filters with 1 mg/ml and 0.5 mg/ml (left) and 0.1 mg/ml (right) Creon solutions after drying

In all tests, enzymes did alter the final weight of blanks (*Table 3; Table 4*). *Figure 4* illustrates the relation between Creon concentration tested and weight of the residue on the blank filters.

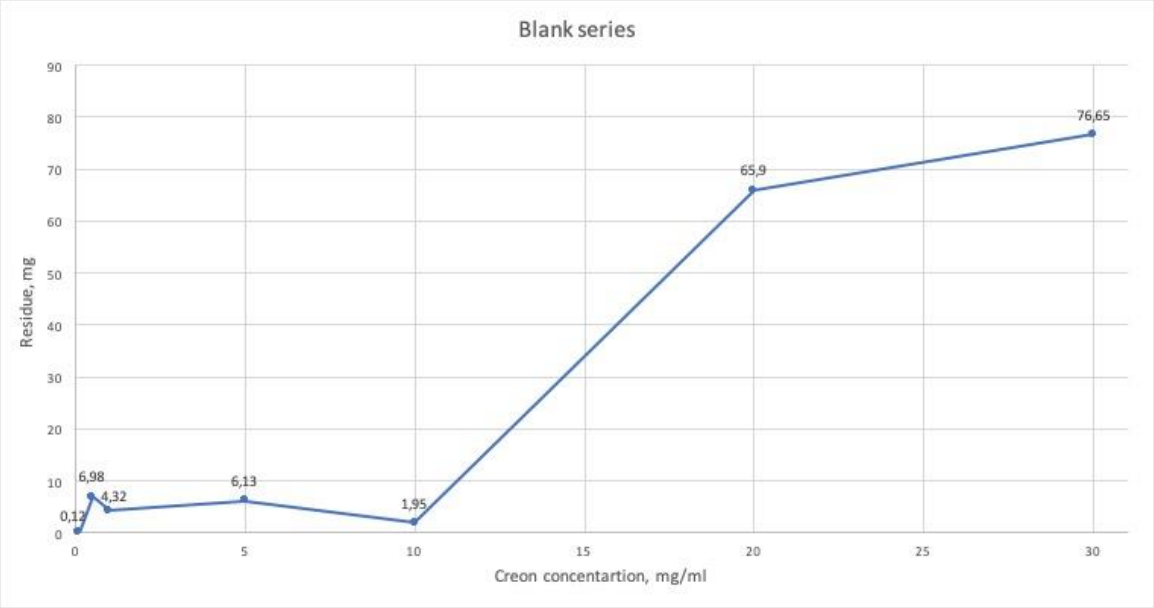

Figure 4: Blank series (n=1): residue weight left by different concentrations of Creon

Results showed that 300 mg and 200 mg of enzymes strongly increased the weight of the filter by 76.65 mg and 65.90 mg respectively, whereas concentration of 10 mg/ml and below increased the weight of filter just slightly (*Figure 4*).

Out of all concentrations tested, the calculated digestion efficiency was the highest for the 0,1 mg/ml of PEz samples with 87.60 $\pm$ 0.04 %. *Figure 5* demonstrates the digestion efficiency percentage for each concentration tested.

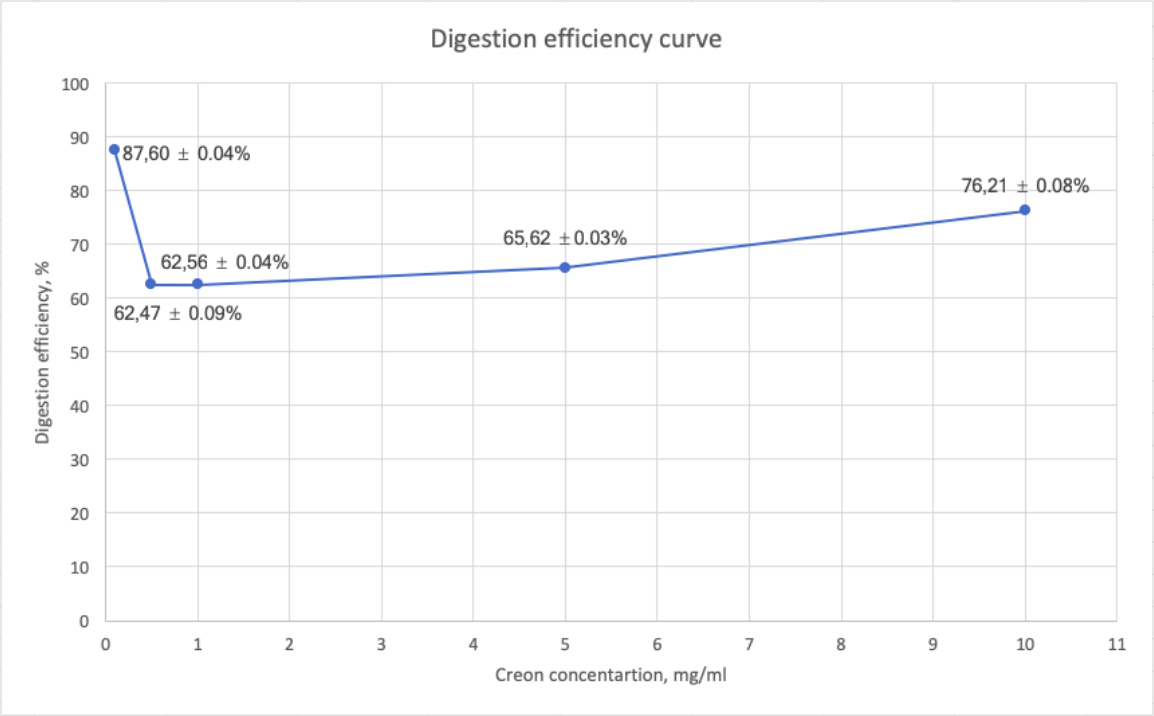

Figure 5: Digestion efficiencies calculated from samples with 10 mg/ml, 5 mg/ml, 1 mg/ml, 0.5 mg/ml and 0.1 mg/ml Creon solution

While conduction experiment in Thermomixer, mysids were digesting at very slow rate. Additional duplicates with 0.1 mg/ml and 1 mg/ml of Creon solution were placed in Thermomixer for 24 hours, but most of the body tissue was still whole. Time was prolonged for another 24 hours and samples were digested a bit more, but still some parts remained visible. Particularly the eyes balls were not affected by enzymes at all. Filters were covered with undigested body parts (*Figure 6*), but it was still possible to recognize the plastics in it. Even though these samples had the worst results (mysids tissue were not fully digested), the final weight of the filters were lighter than in other samples, therefore digestion efficiency was higher (0.1 mg/ml of Creon – 78.73 ± 4.17 %; 1 mg/ml of Creon – 84.77 ± 2.65 %) (*Table 5*).

Table 5: Experiment results on mysids in Thermomixer: dry weight of the samples (n=3), residue weight after filtration, digestion efficiency percentage and level of coverage grades

Creon, mg/ml	**Mysids, mg**	**Residue, mg**	**DE, %**	**Level of coverage**
1 mg/ml	20.24 ± 0.08	3.08 ± 0.52	84.77 ± 2.65	3 ± 0.0
0.1 mg/ml	20.62 ± 0.19	4.39 ± 0.69	78.73 ± 3.16	3 ± 0.0

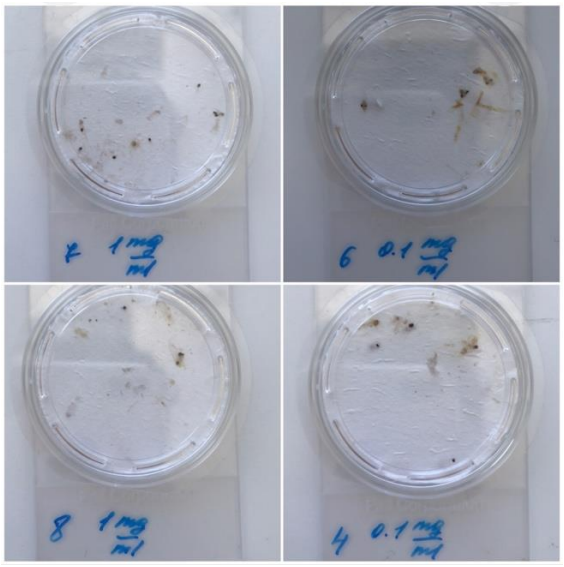

Figure 6: Thermomixer digestion of mysids: filters with undigested tissues

All filters were at the acceptable LC level. While amphipods tests were with the least success (2.7 ± 0.3 for the 20 mg/ml of PEz and 2.8 ± 0.3 for the 30 mg/ml of PEz), mysids tests with low concentration of PEz as well as jellyfish analyses were all within 1-2 LC grades. For the concentration 10 mg/ml all filters were within the 2.0 ± 0.0 LC grade. Thin yellowish layer of glutinous liquid remained on top of the filter through which any particles like microplastic, chitin parts and other remaining body parts were visible. With further lowering the dosage yellowish layer became more transparent and light in color. Filters with 5 mg/ml, 1 mg/ml and 0.1 mg/ml of Creon showed the best results with 1.5 ± 0.0, 1.3 ± 0.3 and 1.3 ± 0.3 LC grade respectively. However, samples with concentration of 0.5 mg/ml of Creon stood out from the series and showed increase in level of coverage with 1.75 ± 0.35 (*Table 4*). Samples that underwent digestion in Thermomixer had the highest level of coverage with 3.0 ± 0.0 for both 1 mg/ml and 0.1 mg/ml concentrations tested (*Table 5*).

10 samples of jellyfish were digested in 5 mg/ml Creon solution. LC coverage level was 1.9 ± 0.5 with thin transparent yellowish layer of undigested biological matter. No correlation between level of coverage and species of jellyfish were observed. Digestion of Marenzelleria in Thermomixer for 48 hours showed LC level of 2.3 ± 0.45. Some of the outer skin was visible, however it did not hinder

the microplastic identification significantly. Halicryptus individuals showed LC level of 2.6 $\pm$ 0.52 after digestion in incubator for 24 hours, while LC level after 4 days in incubator decreased to 2.2 $\pm$ 0.45. Filters were covered by thin brownish layer with a lot of teeth left undigested (*Appendix 2*). Overall results on LC grades of worms were worse than on small crustaceans and jellyfish.

Finally, it was found that digestion in Thermomixer required more time and was less efficient than digestion in incubator with magnetic mixer. Tests with mysids confirmed that under same circumstances the samples in Thermomixer were only partially digested, while digestion on magnetic mixer was completed already over the night. The test was repeated once, to see if the results were the same. Second time mysid's tissue were completely not affected by enzymes. Moreover, LC grade of Marenzelleria was high with 2.3 $\pm$ 0.45 after 48 hours of exposure, even though the tissue of this species is very soft and pulpy, which should be effectively digested by pancreatic enzymes.

4.2 Results on degradation of the polymer

In the first exposure test with 10 mg/ml Creon concentration, 2 particles were selected 300 μm in size (*Figure 7*). Similarly, in the second exposure test with 20 mg/ml, 3 particles were of required size (*Figure 10*).

Digestion with 10 mg/ml solution of Creon in Thermomixer C on the speed of 600 rmp for 24 hours doesn't seemed to have any effect on polymer degradation. The photos taken before and after digestion were almost identical. 2 out of 2 particles of 300 μm were recovered with no visible damage to surface and unchanged color. Moreover, spectra taken from these particles before and after digestion revels correlation of 95 % in FT-IR spectrum (*Figure 9*). The photos with magnification 10x 0.03 are listed below (*Figure 7*):

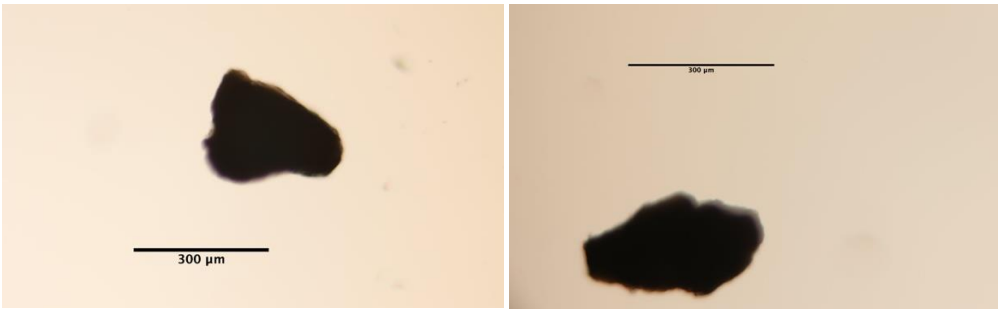

Figure 7: Particles before digestion in Thermomixer

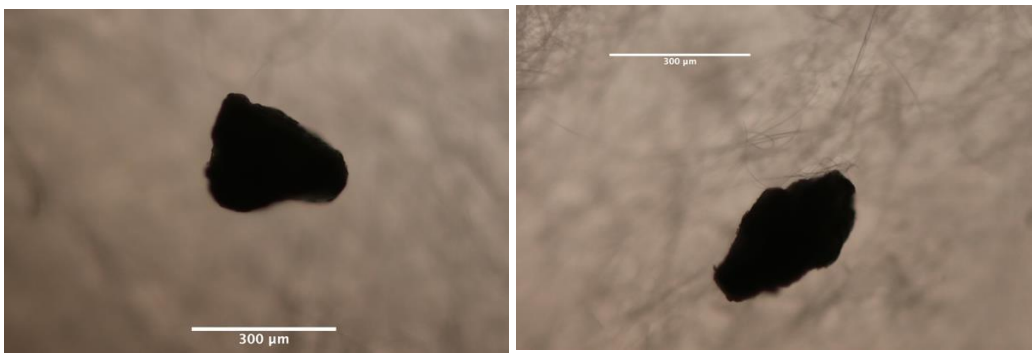

Figure 8: Particles after digestion in Thermomixer

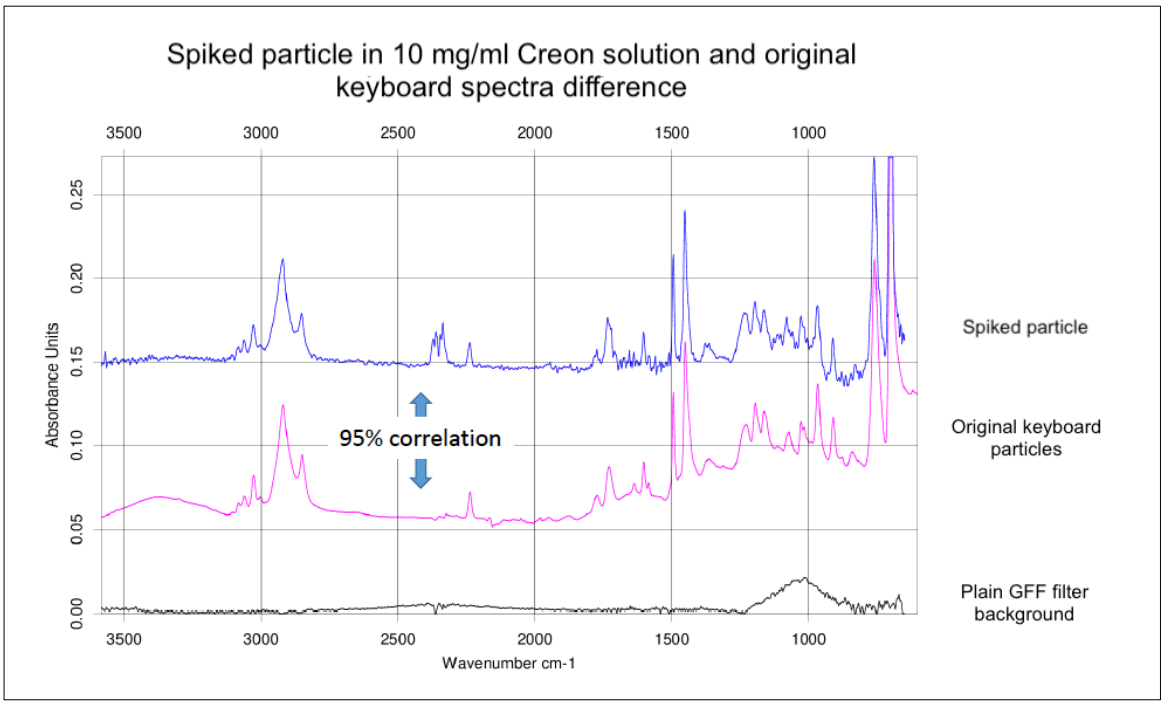

Figure 9: FT-IR spectra differences between original keyboard particle and particle after digestion in ThermoMixer in 10 mg/ml Creon solution

However, similar spiking in incubator with 20 mg/ml of Creon on magnetic mixer showed minor destruction to particles. The surface was slightly damaged on one particle. Two out of three particles were recovered. FT-IR spectra before and

after analysis showed correlation of 83 % (Figure 12). Figures 10 and 11 shows the photos taken before and after analysis.

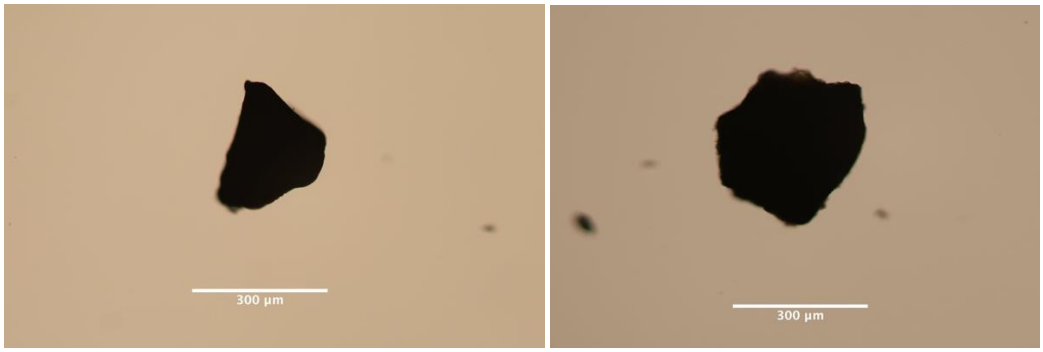

Figure 10: Particles before digestion in incubator

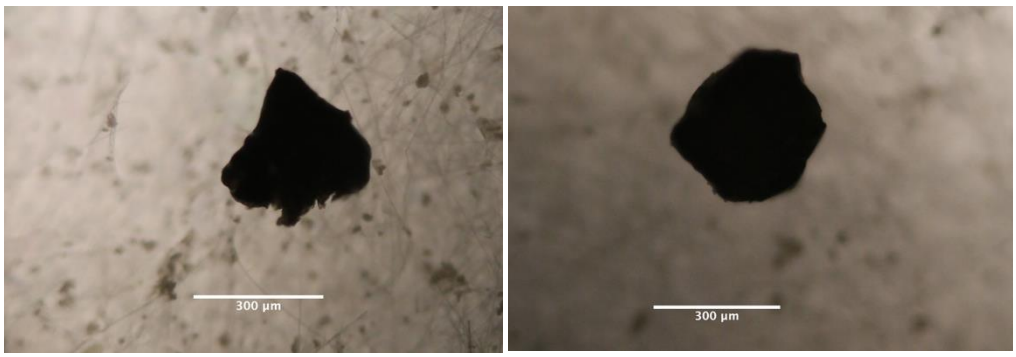

Figure 11: Particles after digestion in incubator

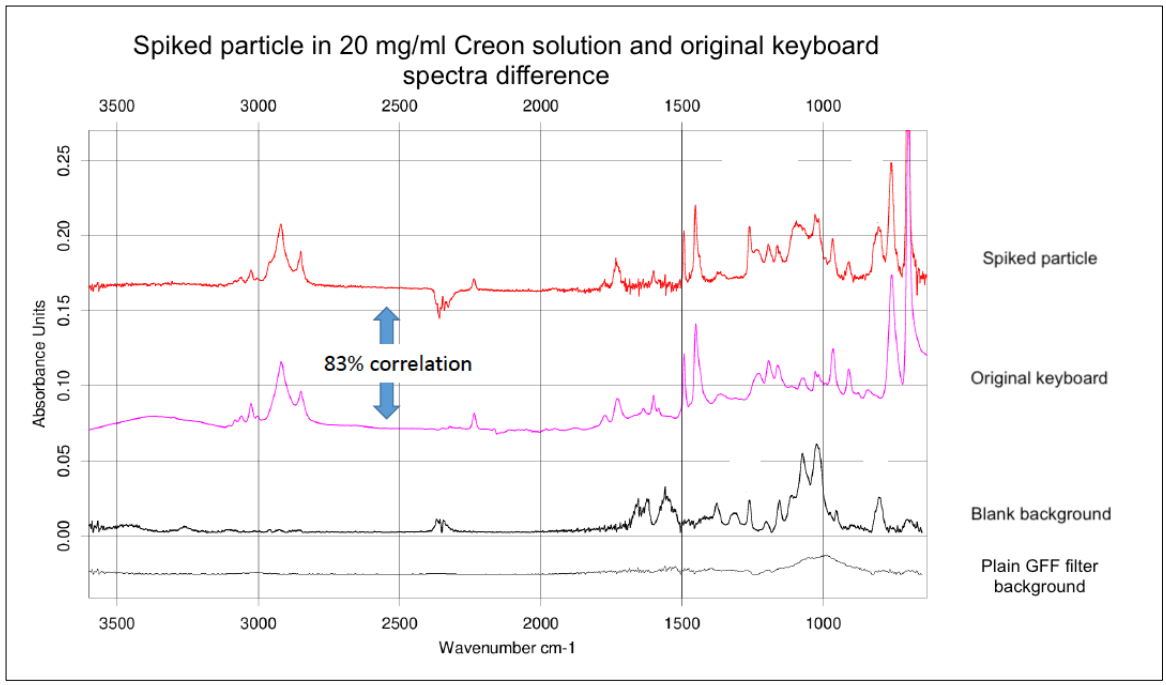

Figure 12: FT-IR spectra difference between original keyboard particle and particle after digestion in incubator in 20 mg/ml Creon solution

5 DISCUSSION

5.1 Digestion efficiency and method optimization

Experiments conducted on amphipods showed that amount of Creon was clearly too high for such small quantities of sample and mostly enzymatic products were left on filters. Thus, 1 pill (300 mg) of Creon highly influenced the final weight of the filter, leaving 76.65 mg of additional weight on the blank filter. With 200 mg of Creon, residue on the blank filter was a bit smaller (65.90 mg). As most of the residue originated from the Creon itself, it was not possible to calculate digestion efficiency for treatment with high concentration of enzymes. Samples with 30 mg/ml and 20 mg/ml of Creon had both unsatisfactory digestion efficiency results and level of coverage grades. Grounding of the sample posed higher risks of contamination as well as loss of sample during manipulation. Moreover, it was not noticed that grounding improved digestion efficiency during 24 hours, therefore it is recommended to exclude this step.

Series of digestion efficiency tests with mysids showed much better results than with amphipods. Further lowering the dosage of Creon showed some significant improvements in both digestion efficiency and level of coverage criteria. Nonetheless, graph of blank series highlighted that some amount of enzyme's products was still left on the filters. This issue could be resolved by using filters with bigger retention size. Von Friesen *et al.* reported no alteration of the final filter weight by whole pill of Creon 40000. Moreover, digestion efficiency up to 97.7 $\pm$ 0.02 % was achieved with her enzymatic protocol on mussels. However, in her study nylon filters with greater retention sizes were used (300 and 20 μm) (Von Friesen *et al*., 2019*),* thus the difference in experiments' results could be relate to divers materials used.

While it was expected that with a decrease of PEz the residue on the filter would decrease as well, the residue on the filter was nearly the same for all listed concentration below 10 mg/ml. Thus, 10.0 mg/ml solution left less weight than 5.0 mg/ml and 1.0 mg/ml solution (1.95 mg compare to 6.13 and 4.32 mg), while it would have been expected to be vise verse. Similarly, the sample with 0.5 mg/ml concentration of Creon had the heaviest weight of 6.98 mg, even though it is the

second lowest concentration tested. The lowest, therefore the best, residue weight was observed for 0.1 mg/ml of PEz, leaving only 0.12 mg on the filter. Samples with this concentration showed good level of coverage grades as well as highest digestion efficiency percentage. Notably, concentrations of 5 mg/ml, 1 mg/ml and 0.1 mg/ml of Creon also showed low level of coverage grades, even though their digestion efficiency values were lower than expected, while Creon concentration of 10 mg/ml had higher DE with higher LC grades.

Tests conducted in Themomixer were lower both in digestion time and digestion efficiency. No possible explanation were found for this issue, as the Thermomixer itself functioned properly and the conditions of the experiment fully complied with the method. Further research on digestion in Thermomixer as well as any other possible equipment to conduct digestion is recommended.

Jellyfish samples showed good LC results after 24 hours of enzymatic exposure with average grade of 1.9 ± 0.5. Most probably, further decreasing of Creon dosage to 1 mg/ml or 0.1 mg/ml could improve the results. However, for both visual and FT-IR analysis these filters considered to be satisfactory.

While testing the method on Halicryptus worms, it was noted that the results were better with longer time period, as less weight was left on the filter after 4 days of exposure to enzymes than after 24 hours. The load of undigested biological matter on the filter was also smaller after 4 days of exposure (LC 2.2 ± 0.45 compared to LC 2.6 ± 0.5). It states that for some species rich in protein such as Halicryptus, 24 hours is not enough for complete digestion and further research is recommended.

5.2 Degradation of the polymer

Results on keyboard degradation tests showed that overall, polymers did not damage. The size remained the same as well as the color and the shape. One particle that was treated in incubator had a rough surface after the treatment with enzymes while the other was in exact same shape and condition as it was before the treatment. Particles that underwent the procedure in Thermomixer seemed to

be exactly the same in shape and color as before the analysis. Correlation of 95 % in FT-IR spectra indicated that chemical composition of particles remained the same after the procedure (Figure 9). Test with 20 mg/ml solution showed lower results with 83 % of correlation, but this could be connected with higher concentration of enzymes. A thicker layer of Creon on the particle surface might have contributed to the spectral differences between the treated and untreated polymer.

As in 20 mg/ml Creon solution 1 of 2 particles were damaged, it could be suggested that physical effect of stirrer is the reason of polymer damage, while enzymes did not have any effect on it. However, visual analysis of particles before and after enzymatic exposure could be hindered by diverse position of particles on the filter since it was not the same. In the second test in incubator 2 out 3 particles were recovered. The third particles most probably was lost during manipulation with sample. The possibility that this particle was destructed is very low as no visible damage was observed for other particles in the same sample. However, considering, that only 5 particles in total were assessed, further research is needed with more spiking replicates to obtain more reliable results.

Overall, results were more promising with lower concentration of Creon. Physical movement of magnetic stirrer could be the reason of polymers damage. However, at the speed of 200 rmp, only the surface of particles was slightly damaged, which did not interfere with microplastic detection in the sample in terms of occurrence. In addition, if the particle's condition and weathering level is important to the study, lower speed might be applied to reduce the risk of destruction. Moreover, this study focused only on particles of 300 μm in size. Smaller particles as well as highly degraded might have higher risk of destruction.

5.3 Limitations of the study

Despite of the outcome, this study considered to be unreliable as number of limitations were discovered during the process. While conducting the polymer exposure assessment, it was found that contamination particles were highly similar with keyboard particles on the filter. Therefore, it was not possible to

indicate which particles were originated from studied keyboard. Only relatively large particles (≥300 μm) were suitable for visual analysis under such circumstances. Unfortunately, there were only small amount of keyboard particles of needed size (5 particles in total) and overall, number of replicates were not sufficient to achieve reliable and accurate results. Moreover, indication of enzymatic residue on the blank filters leads to the suggestion of using bigger filter's retention size in the further research. Because of this, accurate digestion efficiency was not possible to calculate due to fact that some of the enzyme's products were left on the filter. All in all, further research is suggested with more replicates in both digestion efficiency and polymer exposure test.

6 CONCLUSION

To conclude, the optimized method was found to be applicable to a wide range of invertebrates and non-damaging for the synthetic polymers. Sample digestion using low Creon concentrations yielded higher digestion efficiency and low filter coverage, thus making sample examination more feasible. Therefore, for successful analysis of small crustaceans, such as mysids and amphipods (≈ 20 mg), concentrations of <1 mg/ml are recommended. Extraction volume is less important and can be reduced from 10 to 5 ml to save reagents and time for filtration. For digesting small samples, 24 hours is sufficient; however, for more proteinaceous samples (Halicryptus, Marenzelleria) more time is required. Further optimization of the digestion conditions for worms is recommended because samples of Marenzellaria and Halicryptus after filtration had relatively high level of coverage compared to other species tested.

At 10 mg/ml of Creon, the digestion process had no effect on the polymer chemical structure, with high (95 %) correlation for the FT-IR spectra between the treated and untreated reference polymer. Reference particles were found in the same shape and color as before the treatment. However, when conducting digestion in the incubator, high speed of the magnetic stirrer might have mechanically damaged the particle surface. It could be concluded that enzymes did not alter plastic particles in any way and this method could be used for safe and reliable extraction of microplastic particles from the soft tissues.

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APPENDICES

Appendix 1

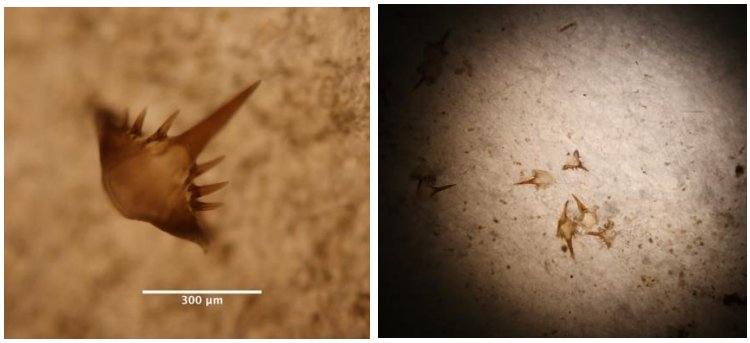
FORMULAS

$$DE\ (\%) = \frac{DWb - DWa}{DWb} \times 100 \qquad (1)$$

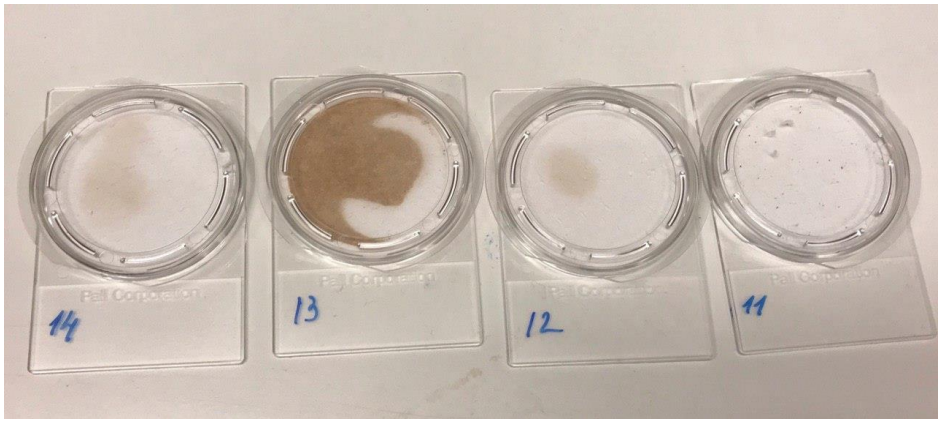
Where,	DE	digestion efficiency	[%]
	DWb	Dry weight before analysis	[mg]
	DWa	Dry weight after analysis	[mg]

Appendix 2

PHOTOS TAKEN DURING THE EXPERIMENT



Filters with digested Halicryptus after 4 days of exposure to 5 mg/ml of Creon solution - Tooth left and brownish background. Photos taken with magnification lenses 10 X (left) and 4 X (right)



Filters with Mertensia (11,14), Aurelia (13), Cyanea capillata (12) after digestion in 5 mg/ml of Creon