Extraction of Microplastics from Fish Tissue: Towards Improved Efficiency using Alkaline Digestion and Detergents with Acid Titration

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Master Thesis

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Content

Abstract

Microplastics (MPs) are ubiquitous in the marine environment. They are ingested by marine organisms and may cause harm for them, or find its way to the top consumer, humans. The resulting effects are not fully known, and more research on the effects of MPs is required, especially with environmentally relevant combinations and concentrations. However, environmentally relevant concentrations are not fully known either, including marine organisms. For these reasons, there is an urgent need to quantify MPs in seafood organisms. Before this can be achieved, quantification methods require further development.

Protocols for the extraction of MPs from biological matrices usually consist of three steps: Sample preparation, sample purification, and analysis. The focus of this study is on the sample purification. For this purpose, acidic-, alkaline-, oxidative-, or enzymatic digestion are used commonly. Alkaline digestion using KOH stands out as the best digestion agent as it is cost-effective, time-efficient, and is better at degrading biological tissue but not the plastic polymers. However, different protocols are used for different matrices, and even within matrices. Currently there are no standardized protocols. For this thesis, several protocols for this purpose were compared on their effectivity on salmon fillet, the best protocol was optimized and tested for robustness with further, different fish fillets, i.e. haddock and mackerel. Important assessment criteria were the digestion efficiency for the tissue and time efficiency in combination with the damage to MPs. The optimization was performed by varying those factors influencing filtration time and digestion efficiency. Damage evaluation of MPs was performed with gravimetric and spectroscopic analysis.

In conclusion, of the tested protocols, a protocol using KOH and detergents with an acidic titration step prior to filtration was the most effective method regarding digestion efficiency. Investigation of factors involved showed that the detergents Tween20® and Triton™ X-100 were important regarding filtration complications. No significant mass loss was recorded for 8 MPs tested in the size range of 1-4 mm. FTIR-analysis indicated no significant changes to the polymers' chemical integrity. Therefore, this protocol was employed for evaluation of the digestion efficiency of the additional matrices salmon-, haddock- and mackerel tissue. Digestion efficiencies were > 99.96 % for all three matrices. Compared to published literature per today, the optimized protocol was more effective for digestion of fish fillet.

List of abbreviations

RT	Room temperature
ABS	Acrylonitrile butadiene styrene
CA	Cellulose acetate
EPS	Expanded polystyrene
EVA	Ethylene-vinyl acetate
HDPE	High-density polyethylene
LDPE	Low-density polyethylene
PA	Polyamide (nylon), also PA-6 and PA-66 (type of polyamides)
PC	Polycarbonate
PES	Polyethersulfone
PET	Polyethylene terephthalate
PMMA	Poly(methyl methacrylate)
PP	Polypropylene
PS	Polystyrene
PSXL	Polystyrene (crosslinked)
PTFE	Polytetrafluoroethylene (teflon)
PUR	Polyurethane
PVC	Polyvinyl chloride
PVC-P	Plasticized polyvinyl chloride
PVC-U	Unplasticized polyvinyl lchloride
RE	Rubber elastomer
GIT	Gastrointestinal tract
PFA	Perfluoralkoxy (tubing)

1. Introduction

Microplastics (MPs) have become ubiquitous in the marine environment (Browne et al., 2011; Derraik, 2002; Eriksen et al., 2014; Hammer et al., 2012; Van Cauwenberghe et al., 2013). The implications of MP being present in the marine environment are not fully understood, although laboratory experiments exposing marine organisms to MPs indicate negative alterations on physiology, metabolism and general behaviour (Jeong et al., 2017, 2016; Mattsson et al., 2017, 2015; Pedà et al., 2016). A multitude of methods have been published, however there are no standard methods used for extraction of MPs from marine organisms (Rochman et al., 2017). The goal for research on this topic should lead to an implementation of routine monitoring of biota, including surveillance of concentrations of MPs in marine organisms, freshwater and oceanic waters, to further assess the impact on biota. This work aims at contributing to MP monitoring effort (regarding e.g. risk assessment for MP intake through seafood consumption), by developing an effective protocol for matrix digestion and MP analysis.

1.1. Plastics

Plastic is well described by its etymology; the word plastic derives from the Greek word *plastikos* (fit for moulding) and *plastos* (moulded), referring to the material's ductility during manufacturing (Lusher et al., 2017). Plastics are polymers that are versatile for many applications. The first fully synthetic plastic – bakelite – was created in the early 1900s ("Bakelite First Synthetic Plastic - National Historic Chemical Landmark," n.d.), exhibiting characteristic properties of plastic polymers, i.e. rigidness and heat resistance ("Characteristics, Applications and Properties of Polymers," 2008). Plastic pollution is well known for entangling marine organisms in the ocean. Additionally, MPs are a potential threat. Although persistent, plastics degrade in marine environments over time through different mechanisms to MPs and nanoplastics (NPs).

1.1.1. Production and use

Common classes of plastics are produced from hydrocarbons that are derived from fossil resources (coal, natural oil, crude oil) or from biomass (grains, corn, potatoes etc; Lusher et al. (2017)). Due to its low cost, the manufacturing of plastics has skyrocketed since the start of mass production in the 1950s. 359 million tonnes of plastics were produced in 2018 ("Publications :: PlasticsEurope," n.d.). Roughly 50 % of produced plastics were PP and PE (*Figure 1*).

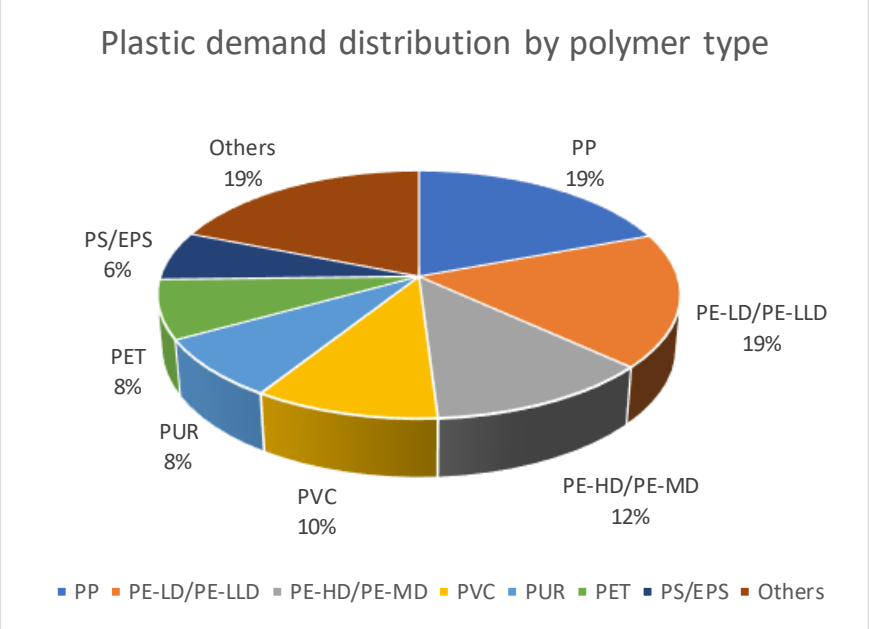


Figure 1: Distribution of polymers produced on world basis ("Publications :: PlasticsEurope," n.d.)

Plastic polymers are formed through a polymerization reaction or a polycondensation reaction (Lusher et al., 2017). As shown in *Figure 2*, plastic polymers may be formed in a polymerization reaction , where monomers starts linking to a chain using a catalyst. The other reaction is called polycondensation, where the elimination of small molecules, such as H_2O, forms the polymer by chemical condensation.

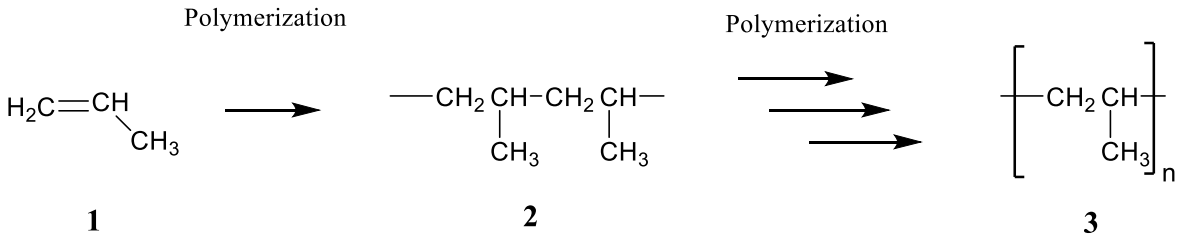


Figure 2: The making of polypropylene: Monomers of propylene (1) get linked after a dimerization reaction occurs to make the dimer of propylene (2) and after n *polymerization reactions makes the polymer polypropylene (3).*

The different characteristics of plastic polymers make them versatile for different applications. Packaging mainly uses three polymer types PE, PP and PET. Building and construction mainly uses PVC, while automotive applications use PP and PUR. The distribution of applications for plastics is packaging (39.9 %), building and construction (19.8 %), automotive industry (9.9 %), electrical and electronic (6.2 %), agriculture (3.4 %), and household, leisure and sports (4.1 %). Other applications include appliances, mechanical engineering, furniture and medical (16.7 %)("Publications :: PlasticsEurope," n.d.).

In Europe, the production of plastics in 2018 was 61.8 million tonnes. Of the 29.1 million tonnes of collected plastic consumer waste, 32 % was recycled and 42.6 % was used for energy recovery. However, 24.9 % ended up at landfills ("Publications :: PlasticsEurope," n.d.). Plastic waste that is not accounted for might potentially end up in the ocean. Eriksen et al. (2014) calculated that there were more than 5 trillion plastic pieces weighing over 250 000 tons afloat at sea at the time of publishing, although this is most likely a substantial underestimation as nets used in this study ranged between 333 µm and 335 µm, which would exclude all smaller sized MPs.

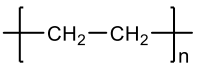
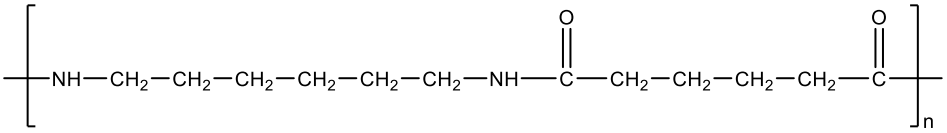
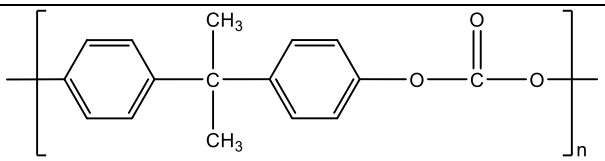
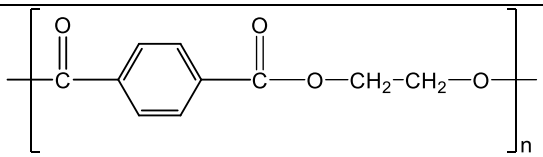
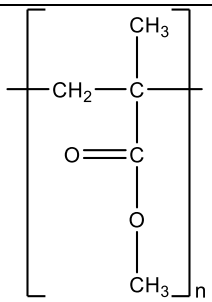
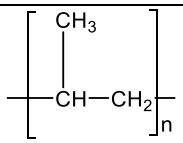
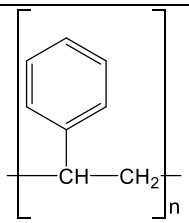
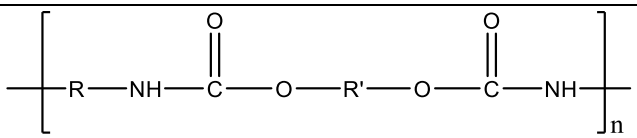
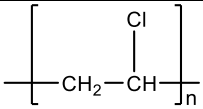
1.1.2. Chemical and physical characteristics

Plastic is a general term used for a wide range of synthetic polymers with different compositions, as exemplified in *Table 1*. Monomers could be as simple as ethylene and more complicated as PA-66. The way the polymers are linked also determine the polymers' characteristics. Polyethylene can be more branched and less dense (low-density PE/LDPE) or more compact with less branching (high-density PE/HDPE). The difference can be seen for the characteristics of PE: LDPE has less tensile strength but greater ductility and is used for i.e. plastic bottles and plastic bags, while HDPE is more rigid with more tensile strength and is used for i.e. plastic containers and toys ("Polyethylene (PE) Plastic," n.d.).

Based on the polymers' ability to be shaped after hardening, plastics can be divided into three categories: Thermoplastics, thermosets and elastomers. Thermoplastics soften on heating and harden on cooling (e.g. PE, PP, PA). Thermosets are moulded during manufacturing and do not soften thereafter (e.g. PUR, Epoxy resins, Bakelite). Elastomers are polymers that can return to its original shape after stretching (e.g. Rubber elastomers and neoprene, Lusher et al. (2017)).

Plastics are versatile due to their useful characteristics, such as high strength to weight ratios, toughness, resistance to corrosion and water, lack of conductivity to both heat and electricity, easy processing and low cost ("Characteristics, Applications and Properties of Polymers," 2008). For additional improved performance, polymers are mixed with chemicals such as phthalates, bisphenol A, flame retardants and nonylphenols. However, these chemicals are not within the scope of the project even though they could potentially contribute to negative impacts of plastic on marine organisms, e.g. as leachate into the marine environments (Browne et al., 2013).

Table 1: Common produced polymers with structural formula

Polymer	Polymer composition
Polyethylene (PE) (HDPE, LDPE)	
Polyamide 6,6 (PA-66)	
Polycarbonate (PC)	
Polyethylene terephthalate (PET)	
Poly(methyl-methacrylate) (PMMA)	
Polypropylene (PP)	
Polystyrene (PS)	
Polyurethane (PUR)	
Polyvinyl chloride (PVC)	

1.1.3. Microplastic and nanoplastic classification

MPs are commonly defined as plastic particles smaller than 5 mm, usually referring to Arthur et. al., 2009, from a workshop meeting regarding the occurrence, effects and fate of microplastic marine debris ("TM_NOS-ORR_30.pdf," n.d.). However, the field has not arrived at a consensus regarding the size intervals for MPs and NPs, as there are different definitions depending on authors. Lusher et al. (2017) defines MPs <0.5 mm and NPs <0.1 for marine plastic litter. Kögel et al., 2020 defines NPs as plastic particles smaller than 1 µm applying the metric scale(*Table 2).*, which will be used for further definition of MPs (1-5000 µm) and NPs (1-999 nm).

Table 2: Classification of size ranges according to its relative size toxicology (From Kögel et al., 2020)

Definition	**Abbreviation**	**Abbreviation used in this thesis**	**Size range**
Nanometer range	NP	NP	1-999 nm
Small micrometer range	SMP	MP	1-9 µm
Medium micrometer range	MMP		10-500 µm
Larger than 500 µm.	LMP		>500 µm

MPs are often subdivided into groups by shape. *Table 3* shows terms used to describe MPs.

Table 3: Categories used when classifying shapes of MPs (From Lusher et al., 2017)

Shape classification	**Other terms used**
Fragments	Irregular shaped particles, crystals, fluff, powder, granules, shavings, flakes, films
Fibres	Filaments, microfibers, strands, threads
Beads	Grains, spherical microbeads, microspheres
Foams	Polystyrene, expanded polystyrene
Pellets	Resin pellets, nurdles, pre-production pellets, nibs

1.1.4. Fate in marine environment

MPs in the marine environment can be classified as primary or secondary MPs (Lusher et al., 2017). Primary MPs are plastics that are already <5 mm in size before entering the environment. Secondary MPs are the product of a degradation mechanism from bigger to smaller plastic polymers through various mechanisms (*Figure 3*).

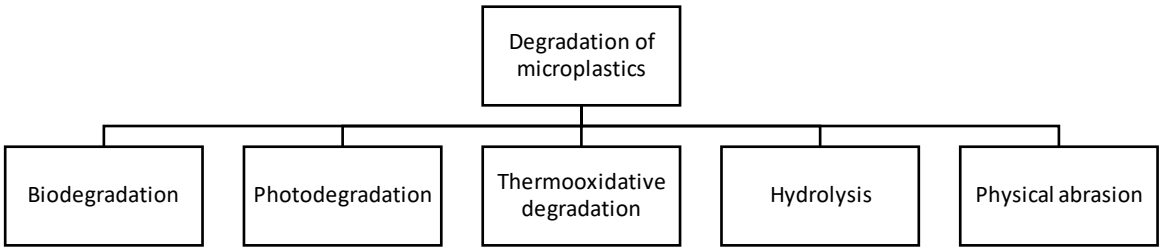


Figure 3: Mechanisms for plastic degradation in marine environment (Andrady, 2011)

Biodegradation occurs through the action of living marine organisms feeding on plastics. These are usually microbes. Photodegradation occurs when plastics are exposed to light. Thermo-oxidative degradation occurs at moderate temperatures while hydrolysis is a reaction through contact with water. Physical abrasion is the erosion of plastics caused by physical impacts as well as wave-driven impact of marine litter on rocks and shores. An additional mechanism not shown in *Figure 3* is thermal degradation, as it is the action of higher temperatures than found in common marine environments.

These mechanisms reduce the mass of the plastic polymers and weaken the polymer integrity. After a while, degraded plastics undergo even further degradation and they start being incorporated into marine biomass through biodegradation. This is referred to as complete mineralisation. At this point the organic carbon in the polymer has been converted. However, plastics degrade slowly, and this process is time consuming (Klein et al., 2018). Anytime during this process, MPs might get ingested by marine organisms, and the potential impact of this is discussed in section 1.2.

The bioavailability of MPs to marine organisms are among other factors based on particle density (Botterell et al., 2019). Polymers such as PP and PE are less dense than seawater (1.02-1.03 g/cm^3), which makes them accessible to pelagic species feeding near the water surface. Polymers such as PVC and PS have a density higher than seawater and will be available to benthic species. Microorganisms attached to MPs, biofouling, can increase density and make the lighter polymers PP and PE sink (Kaiser et al., 2017).

1.2. Impact on biota

To create a better understanding of the impact of MPs on biota, investigating effects on marine organisms exposed to MPs in laboratory environments is important, and quantification of MPs concentrations and sizes in marine organisms.

1.2.1. MPs impact on marine organisms in exposure studies

In the study "Altered behaviour, physiology, and metabolism in fish exposed to polystyrene nanoparticles", crucian carp (*Carassius carassius*) was exposed to 24 nm and 27 nm PS through trophic transfer from Algae (*Scenedesmus sp.*) via zooplankton (*Daphnia magna*) and ultimately to the crucian

carp in a laboratory-controlled environment. Compared to the control group, the fish exposed to NPs through trophic transfer displayed lower activity, increased feeding time, stayed close together and were less explorative. Fish organs were analysed with NMR spectroscopy to evaluate the effects on the metabolite concentrations of the NP diet. Increases in metabolite concentrations were found, i.e. ethanol in the liver and adenosine and lysine in muscles. Morphological changes of brain, such as a more heavy and "more fluffy, whiter and appeared swollen" brain was found in the nanoparticle-fed fish compared to the control fish (Mattsson et al., 2015).

Monogont rotifer (*Brachionus koeranus*) was exposed to three size classes of MPs (and NPs): 0.05 µm, 0.5 µm and 6 µm fluorescent labelled PS beads. Size dependent effects were observed; reduced growth rate, reduced fecundity, decreased lifespan and longer reproduction time. Observation under a fluorescence microscope showed that rotifers exposed to 0.05 µm and 0.5 µm beads displayed fluorescence for 48 hours after ingestion, compared to rotifers exposed to the 6 µm beads which exhibited almost no fluorescence after 24 hours. The difference in persistence indicated that smaller sizes of MPs may be more persistent in marine organisms, as the bigger size class was easier egested (Jeong et al., 2016). In agreement with these results, a review of available literature until 2018 concluded that size is an important factor for MP toxicity (Kögel et al., 2020).

Other studies report brain damage and behavioural disorders in fish induced by nanoparticles (Mattsson et al., 2017), compromised intestinal functions in European sea bass exposed to PVC for 90 days (Pedà et al., 2016) and decreased growth rate and fecundity for the copepod *Paracyclopina nana* exposed to MPs in the sizes 0.05, 0.5 and 6 µm (Jeong et al., 2017) (which was a follow up study from Jeong et al. (2016)).

1.2.2. Occurence of MPs in marine organisms

It is documented that MPs can be found in e.g. the gastrointestinal tract (GIT) of fish, plankton and in mussels (Avio et al., 2015; Beer et al., 2018; Budimir et al., 2018; Catarino et al., 2017; Cole et al., 2014). Due to lack of methodical standardization, results achieved are not fully comparable. Nonetheless, the data shows that MPs are ubiquitous in the environment and are ingested by marine organisms.

Avio et al., (2015) studied the GIT of pelagic, benthonic and bentho-pelagic fish species and reported the presence of MPs, where the predominant polymer was PE. MP sizes between 5 mm and 100 µm were reported to constitute only 20 % of the found MPs, while 80 % were below 100 µm. This signifies the importance of the pore size used for filtration in sample purification, which in this case was an 8 µm pore size filter. More MPs will be discovered the smaller the pore size of the filter is.

Beer et al., (2018) studied the GIT of sprat and herring in addition to plankton that were collected between 1987 and 2015 from the Baltic Sea and filtered with a 100 µm filter. Fibres were the predominant shape of the MPs discovered. It was reported that the MP concentration was 0.21 ± 0.15 per m^{-3} for plankton, for sprat 0.21 ± 0.47 per fish and for herring 0.25 ± 0.52 per fish. However, as already mentioned in the study of Avio et al., (2015), this may be a gross underestimation of plastic concentrations as no MPs under 100 µm could be found due to the methods used. This is an especially important factor to consider, as it is concluded that the MPs concentration in both plankton and in the GIT of herring and sprat have remained constant for three decades. In the light of this data, the conclusion that the MP concentration in both plankton and in the GIT of herring and sprat have remained constant for three decades should be reconsidered.

Budimir et al., (2018) also used a 100 µm mesh filter for the GIT of herring, sprat and three-spined sticklebacks after digestion. It was reported that 1.8 % of herrings, 0.9 % of sprat and 0 % of three-spined sticklebacks contained MPs. The authors mention that MPs under 100 µm were not considered but should be included in future studies. Nonetheless, without reporting the size of the filter pore size, the title "(…) Extraction method shows low number of MPs in offshore planktivorous fish from the northern Baltic sea" can be misleading.

1.2.3. Biota monitoring

GESAMP ("Guidelines for the Monitoring and Assessment of Plastic Litter in the Ocean," n.d.) provides four policy relevant aspects of biota monitoring for plastic particles; impact on biota, impact on human health and well-being by MPs, impact on the ecosystem, and overall indicator of ecosystem contamination.

The quantity of MPs in biota provides information about MP concentration in water, and this will vary for different species. Blue mussel (*Mytilus edulis*) feeds of microorganisms in seawater by filtering large amounts of seawater. Blue mussel was used as an example of a bioindicator in GESAMP, an organism providing information about the environmental conditions.

If MPs are present in marine organisms used for human consumption, they can be transferred to higher trophic levels such as humans. What implications this has for humans is unknown, and there is a need for more data to evaluate potential health risks related to MP ingestion ("Presence of microplastics and nanoplastics in food, with particular focus on seafood," 2016).

The impact on ecosystems is far from clear, however, there is enough data that warrants investigation. For example, the presence of MPs in coral reefs contributes to adverse effects such as necrosis and bleaching (Saliu et al., 2019). In the case of larger sized marine plastic litter there are clear signs of

impact. Marine organisms are getting entangled in plastic litter and ingest larger plastic litter that leads to reduced uptake of nutrients (Werner et al., 2016).

By monitoring biota using bioindicators, e.g. filter feeders, one can assess the environmental contamination of litter. To be able to monitor these bioindicators (or in the case of the current study, a product used for human consumption) a series of steps that are yet to be standardized must be performed.

1.3. Extraction of microplastic from marine organisms

Figure 4: Graphical abstract for extracting microplastics from marine organisms.

There are several steps involved in extraction of MPs. The isolation of MPs is performed differently depending on the matrices.

First step (section 1.3.1) is to prepare the sample. Contamination risk should be minimised by maintaining a clean lab environment to prevent contamination during pre-treatment and storing of sample.

Second step (section 1.3.2.) is to isolate the MPs from biota tissue (sample purification), which in case of biological marine samples means to efficiently digest the organic material without impacting or contaminating MPs during the procedure.

Third step (section 1.3.3.) is to chemically identify and quantify the analytes, which are the MPs. The small size of MPs often makes it hard to quantify them gravimetrically in small sample sizes such as biota, and for this reason, chemical identification is the method of choice for this purpose.

Chemicals used for digestion of biological matrices might degrade MPs, which signifies the choice of chemicals used in a protocol for prevention of potential underestimations of MPs. Impact from different chemicals on MPs is elaborated in section 1.3.4.

The scope of this project was extraction of MPs from fish tissue, and for that reason only methods for extraction from marine organisms will be discussed.

1.3.1. Sample preparation

Following collection of samples, preventing contamination by adhering to measures such as clean storage, lab environment and equipment is important (see section 2.1.).

The sample is prepared, and target organs are selected according to the purpose of the study. The focus of the study can be e.g. environmental monitoring or seafood safety.

The most covered organisms in the literature were mussels (bivalves) and fish (mostly parts of/the gastrointestinal tract (GIT)). Mussels are bivalves that are filter feeders that make them easily exposed to potential MPs in seawater and can work as a bioindicator. GIT of fish may contain ingested MPs and tissue from fish is an important subject for the investigation of food consumed by humans.

1.3.2. Sample purification

This step involves the isolation of MPs from the matrix through digestion or decomposition of the organic material with as little as possible damage to the MPs. The protocols are matrix-dependent, and for an optimal result, the general sample composition should be known in advance. MP isolation is performed with either acidic-, alkaline-, oxidative- or enzymatic digestion of the biological matrix. After the digestion, filtration is performed to isolate the MPs from the biological matrix. The pore sizes from different filtration procedures reported in this chapter varied between 0.8 µm to 300 µm and are essential for the size of MPs expected to be found. To improve filtration, a neutralization step may be performed as seen in a study by Thiele et al. (2019).

Following are different chemicals used in protocols for digesting marine biological matrices for extraction of MPs found in literature.

Acidic digestion

Acidic digestion has been used to digest both fish- and mussel tissue in several studies (Catarino et al., 2017; Claessens et al., 2013; Enders et al., 2017; Karami et al., 2017a). 16 M nitric acid (HNO_3) was previously recommended for digestion of marine biological samples (Matusiewicz, 2003), without regarding potential impact on MPs. It has been reported to efficiently digest tissue in the studies mentioned, although it has been shown to degrade a range of polymers (see chapter 1.3.4.) 12 M hydrochloric acid (HCl) has also been used (Karami et al., 2017a), however it was also found to degrade some polymers.

Alkaline digestion

Alkaline digestion applies bases such as potassium hydroxide (KOH) (Dehaut et al., 2016; Foekema et al., 2013; Karami et al., 2017a, 2017b; Kühn et al., 2017; Phuong et al., 2018; Piarulli et al., 2019; Roch and Brinker, 2017; Rochman et al., 2015; Thiele et al., 2019) and sodium hydroxide (NaOH) (Catarino

et al., 2017; Karami et al., 2017a; Roch and Brinker, 2017). 10 % KOH (≈1.26 M) has been the most frequently used alkaline agent for digesting organic material for extraction of MPs. NaOH has been used to successfully digest mussel tissue (Catarino et al., 2017) and the GIT of fish with an additional neutralization step with HNO_3 (Roch and Brinker, 2017). However, it did not give a satisfactory digestion of fish tissue (Karami et al., 2017a).

Oxidative digestion

Studies with oxidative digestion of fish components may use hydrogen peroxide (H_2O_2) (Karami et al., 2017a; Thiele et al., 2019). H_2O_2 was effective for digestion of mussels at temperatures at 50 °C and 60 °C (Karami et al., 2017a), although it changed the colour of some polymers.

Enzymatic digestion

Enzymatic digestion applies specialized enzymes chosen based on the composition of the sample. It is common to include a detergent, such as SDS, to make the cellular structure more accessible for digestion ("Guidelines for the Monitoring and Assessment of Plastic Litter in the Ocean," n.d.). Although enzymatic digestion often is effective (Löder et al., 2017; Piarulli et al., 2019), it consists of many steps, the procedures require several days and the enzymes are expensive. With the goal of developing methods for monitoring MPs with a high sample throughput, these aspects are unfavourable.

Density separation

Density separation based on buoyancy is used to separate MPs in solutions from denser matter. This method is used when there are particles such as sand, shells, and scales in the sample after digestion (Avio et al., 2015; Karami et al., 2017b; Li et al., 2015; Lusher et al., 2016; Mathalon and Hill, 2014). However, some polymer types could be lost during density separation for more dense polymer types such as tire wear particles (Wagner et al., 2018).

1.3.3. Analysis

The concentrated sample of potential MPs should be prepared for analysis. For more appropriate quantitative or qualitative analysis, the particles need to be transferred to a substrate that is compatible with the specific analytical method. There are several analysis methods applied for analysis of MPs, with different advantages and drawbacks. The correct analysis method should be fitting to the focus of the study and may also include more than one analysis method to determine and quantify MPs. Current methodology limits the size of MPs that can be analysed, mostly due to the filtration process where the pore size of the filter will be the physical limit. Thus, only MPs with sizes over the pore size of the filter may be analysed.

Visual and manual inspection

Renner et al., 2018 has reviewed more than 170 articles using analytical methods for monitoring MPs and found that manual inspection and quantification was used in 79% of the studies. The authors further go on to say that manual identification of MPs is controversial as it has been shown to underestimate number of MPs present in samples. They conclude that visual identification of MPs down to 500 µm is cheap, fast and relatively accurate (if executed in combination with scanning electron microscopy (SEM)), yet it should not be recommended as a standard method.

FTIR- and µFTIR spectroscopy

Fourier Transform Infrared spectroscopy (FTIR) and µFTIR were used in 28% of the studies reviewed by Renner et al. (2018). FTIR spectroscopy is a non-destructive qualitative and semi-quantitative analysis that is suitable for determination of molecular structure. It is also applicable for comparing spectra and evaluating chemical integrity. FTIR has been used in a number of studies examining polymer degradation from chemicals used when extracting MPs from biological matrices (Hurley et al., 2018; Piarulli et al., 2019; Roch and Brinker, 2017) and was therefore chosen as an analytical tool in this project as the MPs used were between 2-4 mm. For identification of MPs below <500 µm, µFTIR may be applied. µFTIR is also a non-destructive analysis, which enables the possibility of coupling this method with py-GC/MS. However, this was not applied in the current study and will not be discussed further.

Infrared spectra derive from the ability of molecules to absorb energy and emit radiation from transitions between vibrational energy states. The most commonly used IR region for this emitted radiation is 4000-670 cm^{-1}. The different modes such as stretching, twisting, scissoring etc. will radiate at different energy levels and hence lead to different peaks in a spectrum.

For larger molecules, using polymers as an example, there are more intricate spectra with several peaks. Different polymers have different spectral fingerprints and comparisons with library spectra may therefore aid in identification of the polymers. In addition to MP identification, FTIR spectroscopy can also be used to compare spectra of MPs before and after being exposed to chemicals involved in digestion of matrix to examine if any changes to the chemical integrity of the polymers has occurred.

Piarulli et al. (2019) compared control spectra of MPs to spectra of MPs after being exposed to different chemicals, as seen in Figure 5. In this spectrum one can see that PC has been affected by 10 M NaOH. Several peaks have disappeared (≈1600 cm^{-1}, 950 cm^{-1}) and new peaks have appeared (≈3600 cm^{-1}, wide peak 3500-2700 cm^{-1}), which most likely will have compromised the chemical integrity of the polymer. Changes to the chemical integrity of a polymer might lead to misidentification or no

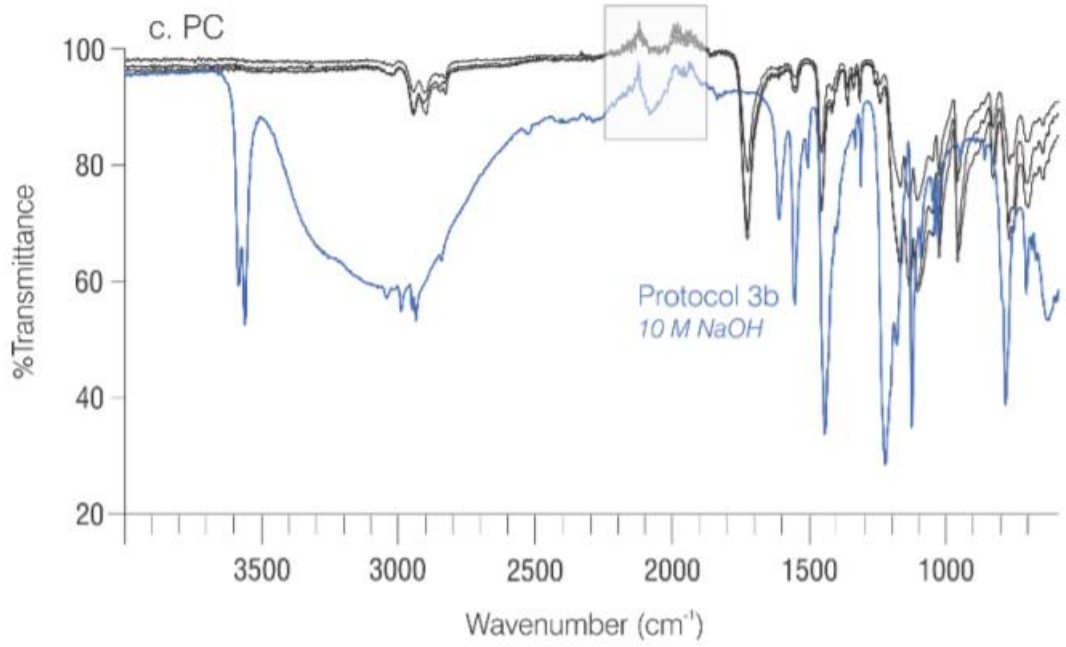


Figure 5: Example of a FTIR spectrum comparing control spectra of PC (depicted in black) to spectrum of PC exposed to 10 M NaOH (depicted in blue). Obtained from supplementary material in Piarulli et al., (2019).

identification at all, which signifies the importance of choosing chemicals for digestion that will not affect the integrity of MPs.

Raman spectroscopy

Raman spectroscopy was used in 14 % of the reviewed studies by Renner et al. (2018). This method is recommended for small fragments below the size of 20 µm. Although it is fit for analysing smaller MPs, problems may arise from fluorescence in spectra deriving from colour pigments, additives or contaminants (Käppler et al., 2016) and the method lacks standardization.

Py-GC/MS and TED-GC/MS

To measure polymers quantitatively, methods using coupled GC/MS in combination with pyrolysis (py) or thermal extraction and desorption (TED) were described in 7 % of reviewed studies. These methods use thermal decomposition of materials at elevated temperatures in a low-oxygen atmosphere. Coupled with GC/MS, fragments can be separated. The disadvantage of py-GC/MS is the small mass input of 0.5 mg, compared to TED-GC/MS being able to have inputs of 100 mg. On the other hand, the detection limit for py-GC/MS is much lower than for TED.

1.3.4. Chemical resistance for MPs

The most critical aspect of digestion methods is to achieve an efficient digestion of the biological matrix while minimizing altering MPs' physical or chemical properties. Hence, data of polymers

directly exposed to chemicals for digesting marine organisms were compiled during a literature search and compared for each chemical. All chemicals/protocols noted achieved satisfactory degree of digestion of organic matter. *Table 4* shows how morphological changes can be described for evaluating damage on MPs.

An essential discovery during the literature search was that elevated temperatures may have an impact on the degradation of polymers, as temperatures above 40 °C (50 °C and 60 °C) led to degradation of PA-66 in one study using KOH (Karami et al., 2017a), and boiling H_2O led to complete recovery loss and severe degradation to polymers (Munno et al., 2018). An important factor to consider about direct exposure to MPs is that tissue may work as a safeguard for degradation of polymers. One of the studies using HNO_3 (Claessens et al., 2013) showed that direct exposure of solution to polystyrene causes them to melt together, but when embedded in tissue it was reported a 93.6 % extraction yield. Nevertheless, it is important to evaluate how direct exposure causes degradation of polymers since smaller MPs (and NPs) are more easily exposed to chemicals due to bigger surface-to-mass ratio.

Table 4: Table from (Enders et al., 2017) describing visually levels of impact on MPs. Additional comments for L2 and L3 was added to easier compare degradation of polymers in Table 11:

Level of impact	**Description**
L1	Initial visually recognizable changes (colour, surface morphology)
L2	Morphological changes and early stages of dissolution/*significant weight change*
L3	Strong morphological disintegration and change of bulk structure/*Yet, still able to weigh or analyse after*
L4	Complete dissolution or disintegration

HNO_3

Karami et al. (2017a) found that 69 % (v/v) HNO_3 at RT (room temperature) for 96 h led to complete loss of two polyamides, PA-6 and PA-66. Furthermore, LDPE, HDPE and PP led to strong morphological disintegration while PET, PVC, PS, HDPE, LDPE and PP had decreased recovery (<95% recovery). Another study (Dehaut et al., 2016) concordantly observed degradation of a polyamide, PA-12, when exposed to 69 % HNO_3. All other polymers tested with HNO_3 (LDPE, HDPE, PP and PS) were observed to change colour.

HNO_3 + $HClO_4$

Enders et al. (2017) observed severe degradation effects on most polymers tested with a mixture of HNO_3 and $HClO_4$ at RT for 30 min, 1 h, 5 h and 10 h, and then at 80 °C for 20 min. PUR, PA "1", PA "2", nitrile, and three rubber elastomers RE "1", RE "2" and RE "3" were completely dissolved during the steps. ABS and PMMA had strong morphological disintegration, two PVC polymers had morphological changes and PS, EPS, PET and PC changed colour or had other visually recognizable changes. After 10 h of exposure, the following heating step at 80 °C was the reason for dissolving two rubber elastomers and degrading and/or visually recognizable changing ABS, the two PVC polymers, PS and EPS.

HCl

When testing HNO_3 (Karami et al., 2017a), it was also tested with 37 % (v/v) HCl at RT for 96 h which led to strong morphological changes to PA-6, PA-66 and PET. There was reduced recovery of PA-6, PA-66, PET and PVC whereas HDPE and LDPE had an increase in recovery.

KOH

There are many polymers tested with KOH at different temperatures. At RT for 96 h, 10 % (w/v) KOH had no visual impact on polymers tested (Karami et al., 2017a), but reduction of mass for PVC was found. An increase in mass was measured for PA-6, HDPE, LDPE and PP. At 40 °C there were no visual impact, but PVC still had a decreased recovery rate. At 50°C and 60 °C PA-66 changed its colour, while PVC and PET for both temperatures had a decreased recovery. Regarding the reduced recovery of PVC, which was <95% for all methods compared (see Table 11 for all chemicals), a search for PVC resistance to KOH was performed, which stated that PVC is not degraded by KOH after 48 h ("PVC (Polyvinyl chloride) Chemical Compatibility Chart," n.d.). Hurley et al., (2018) used 10 % KOH at 60 °C which had no visual impact on tested polymers but increased the mass of PS and decreased the mass of PC. (Enders et al., 2017) used 0.5-1.0 cm MPs for KOH 20 % with the highest temperature used at 80 °C which had no visual impact on any tested polymer in the study. It was not tested for change in mass. (Dehaut et al., 2016) used 10 % KOH (w/v) at 60 °C which had a strong morphological impact on CA and smaller impact on PET, and CA reduced in mass (≈50%). Piarulli et al., (2019) used 1 M KOH at RT which showed signs of discolouration and moderate structural change to one of the PES microfibers after 4 days of exposure. However, no change in the FTIR spectrum was found between control and the PES microfiber.

NaOH

When exposed to 10 M NaOH at 60 °C for 24 h (Hurley et al., 2018), PET and PC was degraded and both had a mass loss of >40 %. It also showed alterations to PC in FTIR. CA, PC and PET was also degraded by 10 M NaOH at 60 °C in another study (Dehaut et al., 2016) which led to mass loss for all three polymers. A rapid protocol (Roch and Brinker, 2017) using NaOH and HNO_3 with 80 °C as highest

temperature used for under an hour degraded a PA and showed visible signs of discolouration or morphological changes for PET, PVC-P and PVC-U.

H_2O_2

30 % (v/v) H_2O_2 at 60 °C for 24 h discoloured PS and discoloured both PS and PP at 70 °C (Hurley et al., 2018). It was also recorded a 27 % decrease of mass for PA-66. H_2O_2 did not have any visual or mass change on PET, but in another study (Karami et al., 2017a) testing 35 % (v/v) H_2O_2 at 60 °C for 96 h, it led to visual recognizable changes to PET and reduced recovery rate for PA-6, PA-66 and PVC. It led to increased recovery rate for PS.

Enzymatic protocol

Piarulli et al. (2019) applied an enzymatic digestion containing SDS detergent, Enzyme F and Enzyme SE at 50 °C for 7 days. The tested MPs PES and PP were not degraded.

1.4. Quality assurance for method validation

The international standard ISO/IEC17025 defines method validation as *"the confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled"* (Daniel C. Harris, 2016). The future aim to routinely monitor MP intake of marine organisms fit for human consumption needs to fulfil certain criteria. The lab needs to have precautions against contamination from both airborne MPs and MPs from chemicals and equipment used. As seen from the literature search, there are several different protocols used with different chemicals. There is an urgent need to standardize research to improve comparison of studies.

1.4.1. Criteria for method validation for extraction of MPs from marine organisms

Criteria listed in "Quality Assurance in Analytical Chemistry" (Elizabeth Prichard and Vicki Barwick, 2007) was rephrased in regard to extraction of MPs from marine organisms.

Specificity, or selectivity, is the method's ability to measure the measurand of interest without interference from the other components in the mixture. During purification, the digestive agent must be able to dissolve all organic material without interfering with MPs in the following analysis.

Precision of a method is defined as the closeness of agreement between independent test results achieved under stated conditions. It is usually calculated using standard deviation (SD), relative standard deviation (RSD) or the standard error of the mean (SEM) of a given number of replicates. Precision measured from short term variations in measurements is called repeatability, while reproducibility means measurements performed in a different environmental condition, other factors have changed, and results are usually obtained in different laboratories (proficiency tests).

Trueness, or bias, is the difference between the mean of a given number of measurements and an accepted reference value. Digestive agents that degrade polymers would lead to a bias towards less MPs, while contamination from equipment, solutions and airborne MPs would lead to a bias towards more MPs. To remove bias, a certified reference material (CRM) in form of MPs would be implemented as a positive control. However, using biological matrices as a negative control introduces a problem; due to MPs being ubiquitous, it is close to impossible to know to what extent there is contamination in a negative control. If it is possible to overcome this issue, the degree of reproducibility can be measured from proficiency tests between accredited laboratories. The European Commission's science and knowledge service encourages expert laboratories to do so, due to the current lack of harmonized sample- and analytical measurement procedures (HANSSENS, 2019).

The sensitivity of a method is the rate of change of the measuring instrument response with change in concentration. This is also known as the slope of the calibration curve, which provides information about its working range, linear range, limit of detection (LOD) and limit of quantitation (LOQ). In relation to extraction of MPs from marine organisms, the analytical tool provided for quantitation will vary for each instrument used. The working range is the range between LOQ and the largest amount that can be quantified.

Ruggedness testing evaluates how small changes of factors in a process affect the measurement result. A ruggedness testing can be performed through an experimental design with many factors involved, without testing each factor independently. Concentrations of chemicals used, incubation time, temperatures and pH are such factors that can be tested with small changes to find the optimal protocol. Such tests will be further elaborated in 1.5.1. Experimental design.

1.4.2. Standardization and reliable research

Published methodical reviews for extracting MPs from marine organisms indicate an urgent need to standardize methods, since the methodical choices affect the generated data.

Due to the differences of biological composition of marine organisms, one universal method is difficult to use. However, comparing methods and evaluating the most efficient methods would be a step in the right direction towards standardization. For the potential future necessity of establishing tolerable intake amount for MP consumption or legal maximal concentrations in commercial products for human ingestion, guidelines for an ISO standard and accreditation need to be followed and these require standardized analysis methods, defined measurement uncertainties and proficiency testing.

Hermsen et al. (2018) has suggested criteria that need to be fulfilled to have a reliable method development. Although the aim of this thesis is to develop an optimized method for sample treatment,

it is essential for new methods being developed to keep in mind quality criteria to achieve reliable results during a method development. Applying criteria for quality assurance for method development will make data obtained more comparable.

1.5. Experimental design

Robustness testing in form of experimental design is used for method development to investigate how small changes in conditions for factors involved affect the outcome of the method. There are many different experimental designs, but 2-level factorial designs and reduced factorial designs dominate in the cases where the purpose is to investigate whether a certain variable has an effect or not.

The design in *Table 5* is a full factorial design with three variables (factors), A, B and C. Each factor is found at two levels, denoted by "+" and "-" and all combinations of high and low values is present in the design.

With 8 experiments, this design allows calculation of a linear model with 8 regression coefficients, which can be a model accounting for the main effects (b_A, b_B, b_c), all possible two-factor interactions (b_{AB}, b_{AC}, b_{BC}), and the three factor interaction b_{ABC} in addition to the constant, b_0 (*Equation 1*)

Equation 1: Linear model for a 2^3 experimental design

$$y = b_0 + b_A + b_B + b_C + b_{AB} + b_{AC} + b_{BC} + b_{ABC}$$

The problem with full factorial designs is that the number of required experiments (*n*) to solve a complete model increase exponentially with the number of studied factors. A full experiment with seven factors will for example require 128 experiments (2^7). Fractional factorial designs are using in cases where the number of experiments in a full factorial design become impractically large.

Table 5: Design matrix for a 2^3 experimental design

Variable	**A**	**B**	**C**
Exp 1	+	+	+
Exp 2	+	+	-
Exp 3	+	-	+
Exp 4	+	-	-
Exp 5	-	+	+
Exp 6	-	+	-
Exp 7	-	-	+
Exp 8	-	-	-

Table 6: Design matrix for a 2^{7-4} experimental design. Also called Plackett-Burman design

Variable	**A**	**B**	**C**	**D**	**E**	**F**	**G**
Generator	A	B	C	AB	AC	BC	ABC
Alias	CE	CF	BF	AB	AC	BC	CD
Alias	BD	AD	AE	CG	BG	AG	BE
Alias	DG	EG	FG	DE	DF	FG	AD
Exp 1	+	+	+	+	+	+	+
Exp 2	+	+	-	+	-	-	-
Exp 3	+	-	+	-	+	-	-
Exp 4	+	-	-	-	-	+	+
Exp 5	-	+	+	-	-	+	-
Exp 6	-	+	-	-	+	-	+
Exp 7	-	-	+	+	-	-	+
Exp 8	-	-	-	+	+	+	-

Adding more factors without increasing the number of experiments will inevitably give less information about each factor. This is explained by the aliases. As shown in the table, each main factor has three aliases that are two-factor interactions. One of these is the generator if this is a two-factor interaction. In the model (*Equation 1*) all aliased factors are explained by a single regression coefficient, and the effect of the main factors cannot be separated from the effects of their aliases without doing further experiments. Reduced factorial designs are therefore most useful in cases where one can assume the interactions are insignificant compared to the main factors. The columns in

Table 6 are also aliased with three-factor interactions that are not shown in the table. In addition, the model constant (b_0) is aliased with three-factor interactions.

1.6. Objectives

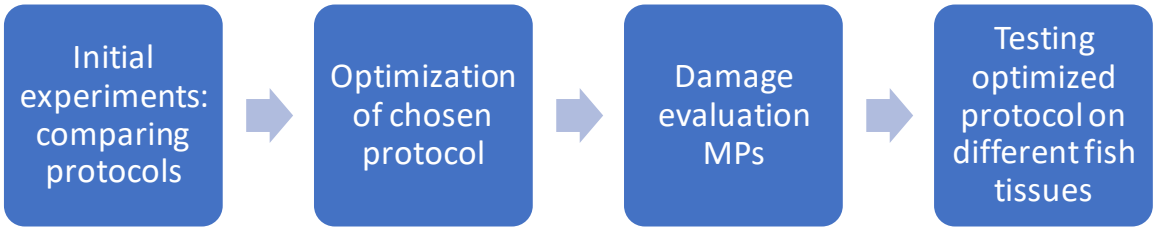


Figure 6: Graphical abstract of objective for thesis

The aim of this project is to improve a time- and cost-efficient and practicable method for extraction of MPs from fish tissue without degrading the MPs.

1. In the initial experiments, different proposed protocols will be compared and evaluated for the ability to digest the tissues efficiently.
2. The protocol most fit for purpose will be optimized and investigated for factors involved for digestion efficiency and filtration time.
3. Damage evaluation of MPs will be performed using eight different MPs (HDPE, LDPE, PA-66, PC, PMMA, PP, PS) and comparing them before and after exposure to the protocol regarding weight change and FTIR-spectra. The use of FTIR will be evaluated according to its ability to differentiate spectra of different MPs and MPs with and without exposure from a performed protocol. Results will be compared to literature.
4. Lastly, the chosen protocol will be tested for robustness with three different fish tissues, salmon, haddock and mackerel, to find potential MPs above the detection limit (10-16 µm (filter pore size)). Methods and results will be discussed and reviewed according to criteria for method development and compared to previously used methods in literature to consider if the optimized protocol is a suitable method for extraction of MPs from fish tissue. Results will be compared to literature.

2. Materials and method

2.1. Materials and chemicals

2.1.1. Table 7: Solutions

Ultrapure MilliQ-water	Used throughout all performed experiments and is just referred to as water throughout all experiments and procedures.
4.2 M KOH-solution	200 g potassium hydroxide (EMSURE® for analysis, Supelco®) was dissolved in water. Diluted *ad* 1000 g solution.
4 M HCl-solution	166 ml 37 % (w/w) hydrochloric acid (EMSURE® for analysis, Supelco®) was added to water. Diluted *ad* 500 ml solution.
PBSTnT-solution (saline aqueous phosphate buffer with detergents)	8 g sodiumchloride (EMSURE® for analysis, Supelco®), 0.2 g potassiumchloride (EMSURE® for analysis, Supelco®), 1.42 g disodium phosphate (EMSURE® for analysis, Supelco®), 0.24 g potassium dihydrogen phosphate (EMSURE® for analysis, Supelco®), 20 g Tween®20 (VWR Chemicals), 14 g Triton™ X100 (Millipore®). Diluted *ad* 1000 g.
1M Tris-solution	60.57 g Tris-(hydroxymethyl)aminomethane (Tris) (VWR Chemicals) was dissolved in water and adjusted by HCl to pH 9.3. Diluted *ad* 500 ml solution.
Protease/Tris solution	1 M Tris-solution and protease (Sigma P3111) 5:1.
Lipase/Tris solution	1 M Tris-solution and lipase (Sigma L0777) 100:1.
H_2O_2 (30 %)	Is a ready-to-use working solution and requires no further preparation.

2.1.2. Materials

8 different polymers were used in experiments: HDPE, LDPE, PA66, PC, PET, PMMA, PP and PS. All MPs were in the size range of 1-4 mm (in their shortest and longest dimension). These MPs were classified as nurdles (see *Table 3*). Additionally, LDPE from cling foil, used in the pH investigation experiment, was cut into pieces < 1 cm. These were classified as flakes (see *Table 3*). Identification of MPs smaller

than 10 µm is restricted by the filtration step. Thus, "MPs" mentioned further would refer to the size range 10-5000 µm.

Atlantic salmon (*Salmo salar*) is a pelagic fish species ("Laks," n.d.), Haddock (*Melanogrammus aeglefinus*) is a benthic fish species ("Hyse," n.d.), and Atlantic mackerel (*Scomber scombrus*) is a pelagic fish species ("Makrell," n.d.). The three fish species were chosen based on different fat compositions, and the order of fat percentage is mackerel > salmon > haddock ("Total fat content (ethyl acetate) | Substance | hi.no," n.d.).

Atlantic salmon from the FHF project "SalmoDetect" was used as matrix for all initial experiments (section 3.1.1-3.1.5), and the first experimental design (section 3.2.2.). Atlantic salmon used in all other experiments was acquired from local stores (Bunnpris and Lærøy). Atlantic haddock and Atlantic mackerel (section 3.4) were acquired from surveillance projects by order of the ministry for Trade, Industry and Fisheries at the IMR.

2.1.3. Equipment and instruments

A muffle furnace (LE 14/11, Nabertherm) was applied at 500 °C for 5 h for all equipment that could tolerate the treatment. This was to remove traces of plastics.

Samples were incubated in a New Brunswick™ Innova® 42 incubator shaker (Eppendorf). Standard 50- or 125- ml filter crucibles pore size 1-16 µm (ISO 4793-0, Por. 4) and 4-5.5 µm (ASTM E128-99, Fine) from ROBU® Glasfilter-Geräte GmbH were used for filtration of digested samples. Standard vacuum filtration setup (VWR International) was used during filtration. pH was measured with a LAQUAtwin pH-11 pocket pH meter (HORIBA), pre-calibrated at pH 4.0, 7.0 and 10.0 using certified Certipur® buffer solutions (Merck KGaA). A tabletop FTIR spectrometer was used for chemical analysis (Nicolet iS50R FT-IR with a monolithic diamond crystal).

Solutions for analysis were pre-filtered through fiberglass filters. During the sample preparation phases, dust trap collectors represented by glass jars filled with 100 ml filtered MilliQ water were used to evaluate possible sample contamination from airborne plastic. Additionally, a procedural control is run together with the processed samples, following the same treatment steps to estimate contamination through the reagents.

2.2. Methods

All protocols were performed at the microplastic lab at the IMR. Complete protocols are found in *Appendix A*: Protocols performed. FTIR analysis and an additional cleansing step for MPs were performed at room 3070/3E5d, Department of Chemistry, University of Bergen.

2.2.1. Sample preparation

Whole fish sample was defrosted overnight was rinsed with water before gut was cut. Intestines and organs were removed. The upper side of fillet was extracted (*Figure 7*) before grinding the fish fillet in a meat mincer for homogenization. 100 g minced fish fillet was weighted in containers and put in freezer. Samples were defrosted overnight before use.

Salmon used in section 3.2.1., 3.2.3., 3.2.4. was removed fish bones before grinding the fish fillet. Both salmon and mackerel in section 3.4. were removed fish bones. Removal of fish bones was performed after fish bones presented a problem when calculating digestion efficiencies in optimization experiments.

Figure 7: Only upper side of fish fillet was used for experiments. Here, the upper side of a salmon fillet is cut from the rest of the fish.

2.2.2. Protocols

Identical samples of minced salmon fillets (each 100 g of salmon except for protocol 3 which contained 20 g per parallel) were exposed to the different protocols in order to study the protocol extraction efficiency. All filter crucibles were weighed with a 4-decimal weight, except for filter crucibles used for **protocol 3** which used a 3-decimal weight.

Protocol 1: Combined KOH and PBSTnT-solution (see 2.1.1) were used for digesting the minced salmon fillet. Samples were incubated at 40 °C for 24 hours. After incubation, samples were titrated with HCl to pH ~ 7 before filtration. Filter crucibles were dried at 40 °C for 48 h after filtration (see A-1). During initial experiments and optimization, some conditions were changed to make an **optimized protocol 1**. Concentrations of chemicals were the same as for **protocol 1**.

Protocol 1b: Applied the same conditions as protocol 1, however citric acid was used for titration.

Protocol 1c: Applied the same conditions as protocol 1, however the titration step was not performed. Instead, PFA-tubing was used with vacuum to transfer matter from solution after incubation. When

only viscous solution remained, the solution was diluted to 1 l and transferred to crucibles without PFA-tubing.

Protocol 2: Separate KOH and diluted solution of PBSTnT was used for digesting the minced salmon fillet. Samples were first incubated at 56 °C for 16 h with PBSTnT-solution, then with added KOH and incubated for 3h at 56 °C. After filtration, filter crucibles were added Protease and Lipase in two steps before a final filtration step. After filtration, protease and lipase were added in two steps before a final filtration step.

Protocol 3: Detergents and enzymes were used as the digestive agents for digestion the minced salmon fillet. Steps included addition of Tween20®, protease, lipase and H_2O_2, ultrasonic bath and several incubation steps.

For all protocols, the digestion efficiency was calculated for samples containing matrices according to *Equation 2*:

Equation 2: Digestion efficiency for fish tissue

$$DE = (1 - (\frac{C_{AP} - C_{BP}}{M})$$

where C_{AP} and C_{BP} is weight of crucible after protocol and crucible before protocol, respectively, and M is weight of matrix (fish tissue).

2.2.3. Optimization of protocols

All crucibles were weighed with a 4-decimal weight. All optimization experiments were performed with 1/5 of the original volume for both solutions and matrices.

pH-Investigation

Part A: **Protocol 1** and **protocol 1b** were performed with 10-16 µm filter crucibles without matrices. Both protocols were performed with one sample titrated to pH ~ 10, one sample titrated to pH ~ 7 and one sample titrated to pH ~ 4. Filtration time was measured.

Part B: **Protocol 1** and **protocol 1b** were performed with 4-6 µm filter crucibles without matrices. Both protocols were performed with one sample titrated to pH ~ 10, one sample titrated to pH ~ 7 and one sample titrated to pH ~ 4. Filtration time was measured. Additionally, HDPE flakes were added to see if there were any alterations to the surface after being titrated with HCl or citric acid.

Part C: **Protocol 1** and **protocol 1b** were performed with 10-16 µm filter crucibles with minced salmon fillet with removed fish bones. Both protocols were performed with 7 samples each titrated to different pH (10, 9, 8, 7, 6, 5, 4). Filtration times were measured, and digestion efficiencies were calculated.

Experimental design 2 factors (Incubation time and KOH)

A 2^2 factorial design was used (*Table 8*). Triplicates of each experiment was performed. Digestion efficiency was calculated. Minced salmon fillet was used as matrix.

X1 = incubation time (24 h (+) and 48 h (-)).

X2 = concentration of KOH (4.2 M (+) and 2.1 M (-)).

Table 9: A factorial 2^2 design

Exp. no.	X1	X2
1	+	+
2	+	-
3	-	+
4	-	-

Table 8: A fractional factorial 2^{5-1} design

Exp. no.	X1	X2	X3	X4	X5(X1X2X3X4X5)
1	+	+	+	+	+
2	+	+	+	-	-
3	+	+	-	+	-
4	+	+	-	-	+
5	+	-	+	+	-
6	+	-	+	-	+
7	+	-	-	+	+
8	+	-	-	-	-
9	-	+	+	+	-
10	-	+	+	-	+
11	-	+	-	+	+
12	-	+	-	-	-
13	-	-	+	+	+
14	-	-	+	-	-
15	-	-	-	+	-
16	-	-	-	-	+

Experimental design 5 factors (Incubation time, PBSTnT-solution, Triton™ X100, Tween20® and KOH)

A 2^5 fractional factorial design was used (*Table 9).* Digestion efficiency was calculated and filtration time measured. Minced salmon fillet was used as matrix.

X1 = Incubation time (48 h (+) and 24 h (-)).

X2 = PBSTnT-solution (100 % of solution (+) and 25 % of solution diluted with water (-)).

X3 = Triton™ X100 (Presence (+) and absence (-)).

X4 = Tween20® (Presence (+) and absence (-)).

X5 = Concentration of KOH (4.2 M (+) and 1.05 M).

Experimental design 2 factors (Incubation time and Tween20):

A 2^2 factorial design was used (*Table 8*). Triplicates of each experiment was performed. Digestion

efficiency was calculated, and filtration time was measured. Minced salmon fillet was used.

X1 = Incubation time (48 h (+) and 24 h (-)).

X2 = Tween20® (Presence (+) and absence (-)).

2.2.4. Damage evaluation of MPs

Optimized protocol 1 was carried out without matrices with reduced volume of the recipe (1/5 of the original solution). Triplicates of 0.1 g of each MP type were added to solutions. MPs were weighed before and after the protocol was applied, with a 5-decimal weight. Visual examination and pictures of MPs before protocol and after protocol were performed. MPs were analysed with FTIR before and after the protocol. An additional cleaning step was carried out for MPs exposed to the **optimized protocol 1**: MPs were washed with a mixture of 7:3 ethanol/water before a final FTIR analysis**.** Spectra for MPs before and after exposure to **optimized protocol 1**, and after exposure with an additional cleaning step were compared using the software OMNIC and Sirius version 10.0. Spectra were converted by calculating Log 1/R, normalizing scale, then converted the intervals 2600-1900 cm^{-1} and 400-505 cm^{-1} to a blank line in OMNIC. Principle component analysis (PCA) used treated spectra in addition to differentiation to smooth relative differences in intensities (1^{st} degree, width 7, order 3) in Sirius.

2.2.5. Extraction of MPs from salmon, haddock and mackerel

Optimized protocol 1 was carried out with triplicates of minced salmon, haddock and mackerel fillets. Controls were performed for each triplicate. Matter left in crucibles was prepared for analyses with µFTIR and py-GC/MS, although this was not performed due to instrumental errors. Crucibles were weighed with a 4-decimal weight.

2.3. Statistics

Standard deviation was calculated to evaluate the variance between samples, according to *Equation 3*:

Equation 3: Standard deviation

$$SD = \sqrt{\frac{\sum|x - \bar{x}|}{n}}$$

Relative standard deviation was calculated to evaluate relative differences between parallels according to *Equation 4:*

Equation 4: Relative standard deviation

$$RSD = \frac{SD * 100}{\bar{x}}$$

Spearman rank correlation coefficient for calculating correlation between groups with ranked responses was calculated to investigate correlation between digestion efficiency and filtration time, according to *Equation 5*:

Equation 5: Spearman rank correlation coefficient

$$\rho = 1 - \frac{6\sum d_i^2}{n(n^2 - 1)}$$

2.4. Quality assurance

Fish fillets were minced for homogenization. Minced fish fillet samples were prepared with parallel procedural controls, i.e. at least duplicates of open glass jars of filtered water placed in the working area in the laboratory and in the laminar flow bench each working day. However, due to limited working space, working hours, available space in incubator and waiting for equipment in muffle furnace, this was not always performed. All solutions were filtered through a 0.7 µm filter to prevent contamination in samples. The Microplastic laboratory at the IMR is equipped with high efficiency ultra-low penetration HEPA filtration with an efficiency of 99.995 % for the most penetrating particle size. The laboratory has overpressure and an antechamber with airlock and a sticky floor mat to avoid dust entry. The laboratory is entered with dedicated low abrasion shoes and a cotton laboratory coat. Clothing with loosely weaved artificial polymer fibres are avoided. Either no gloves or nitrile gloves are worn. Wherever possible, non-plastic equipment is employed. Samples are handled under a laminar flow bench (Class II biological safety, Thermo Scientific SAFE 2020). All laboratory equipment used for sample treatment was rinsed with ethanol and water between samples.

3. Results

All results for experiments are compiled in *Appendix B*: Results for experiments at the IMR and referred to with their respective experiment number. *Appendix C*: Pictures of crucibles and Erlenmeyer flasks contains photos of crucibles after drying for 48 h. Digestion efficiency was calculated using *Equation 2*. Standard deviation was calculated using *Equation 3.* Filtration time was defined as both the filtration step and the rinsing step for 3.1. Initial experiments, whereas for all other experiments it was defined as filtration time without the rinsing step.

3.1. Initial experiments

Initials experiments were performed to familiarize with equipment and methods used for digestion of organic matrices. The protocols were compared in terms of their digestion efficiency, filtration time and examination of the filter crucibles after filtration.

3.1.1. Protocol 1 (KOH + HCl neutralization)

The minced salmon fillet was completely dissolved for all parallels after 24 h incubation time. However, undigested matter was observed in flask (*Figure 8*) and thus, the flask was thoroughly washed with water when transferring solution to filter crucible. It was decided to implement this for all further experiments to retrieve all remaining matter in flask, using a 7:3 ethanol/water mixture. There were complications during filtration for sample **1.1a**; thus, sample **1.1b** and **1.1c** were incubated for an additional 24 h due to limited lab hours.

Figure 8: Residues presumed to be fish bones not digested.

A titration graph (*Figure 9*) was made for sample **1.1a**. This was used for the other parallels to reduce titration time. During titration with 4 M HCl a white "opalescent" colour appeared as a layer in the solution for each addition of 4 M HCl (*Figure 10*), also for the control sample **1.2**.. This layer vanished when shaking the flask. The whole solution remained opalescent after shaking when titrated to pH ≈7.

The opalescence reaction occurring worked as an indicator for pH which was used for further experiments, although pH was checked before filtration (acceptable final pH was decided to be pH 7 ± 0.4).

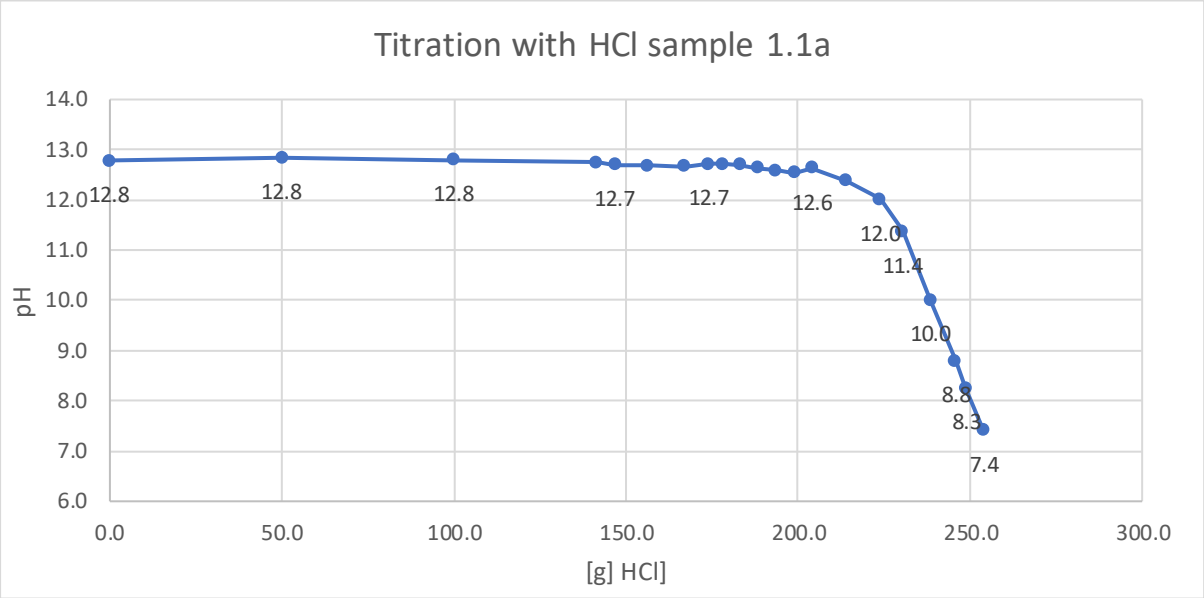


Figure 9: Titration graph for sample 1.1a

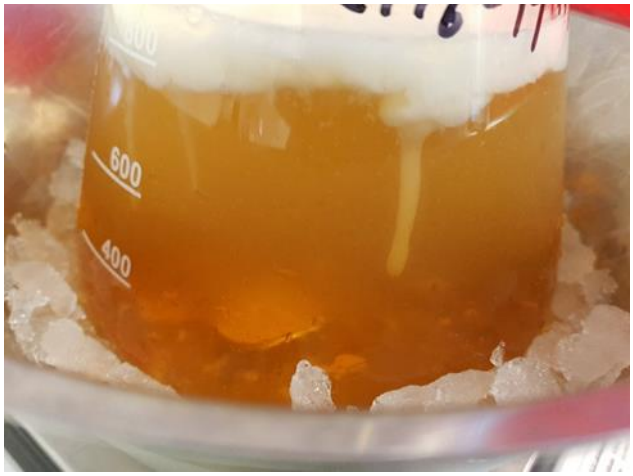


*Figure 10: Sample **1.1a** during titration with 4 M HCl. The white substance (opalescence) is the reaction occurring when HCl is added.*

Filter crucibles (Por-D 10-16 µm) were quickly clogged for sample **1.1a** and **1.2** (control) during filtration due to foam. A possible cause was the filter crucible running dry, permitting an entry of air flow through the filter crucible. 4 M HCl was added to crucible without any effect whereas concentrated HCl dissolved the clogging matter. However, filtration for sample **1.1b** and **1.1c** was performed quicker, possibly due to air being kept out. Thus, no foam was generated when adding solution to crucibles. Hereafter, all solutions were added continuously to crucibles to prevent clogging deriving from foam.

Crucibles contained fragments of what was presumably fish bones from tissue. The digestion efficiencies were 98.95 % for sample **1.1a** incubated for 24 hours and 99.90 % and 99.90 % for samples **1.1b** and **1.1c** incubated for 48 hours.

Protocol 1 was repeated with one parallel (sample **1.3**.) for 24 h. The minced salmon fillet was completely dissolved. Filtration took no more than 12 minutes, which was shorter than for samples **1.1a** (≈ 2 hours) and control sample **1.2.** (≈ 1 hour). However, this time a 7:3 ethanol-water mixture and a diluted KOH-solution (2.1 M) was applied when the filter crucible was clogged in addition to continuously adding digested solution to the filter crucible.

The ice bath during titration was discarded for further experiments as there was no noticeable increase in temperature. Using 2.4 M HCl during titration was used for further experiments to prevent over-titration.

3.1.2. Protocol 1b (KOH + CA neutralization)

The minced salmon fillet was dissolved after 24 h incubation. The solution was titrated with 101.7 g 2 M citric acid to reach pH ≈ 7. The filtration was executed without complications. The filtration step lasted for 12 minutes. The filter crucible showed no signs of fish bones. Calculated digestion efficiency for sample **2.1** was 99.95 %.

3.1.3. Protocol 1c (KOH without neutralization)

The minced salmon fillet was dissolved after 24 h incubation. The solution was transferred to crucible with PFA tubing for filtration. However, the filter became completely clogged long before the dilution step, hence 4 M HCl was added which sped up the filtration (105 minutes). However, this broke with protocol, which was to not add HCl, although it was not possible to clear the filter without it. The filter contained undigested matter after filtration. The digestion efficiency of sample **3.1.** was 99.75 %.

3.1.4. Protocol 2 (KOH + enzymatic digestion)

After the 16 h incubation with PBSTnT-solution, residues from the minced salmon fillet were stuck to Erlenmeyer flasks. This could not be removed after shaking, addition of KOH or after incubation. The minced salmon fillet seemed to be dissolved after 3h incubation with KOH. The filtration of sample **4.1** lasted 4 hours, which led to an additional 24 h incubation for sample **4.2a**, **4.2b** and **4.3** (control) due to limited working hours. The foam generated during filtration was hypothesized to be the reason for the extensive filtration. Adding water did not clear the filter, however, adding diluted KOH (2.1 M) and ethanol/water mixture (7:3) cleared the filter.

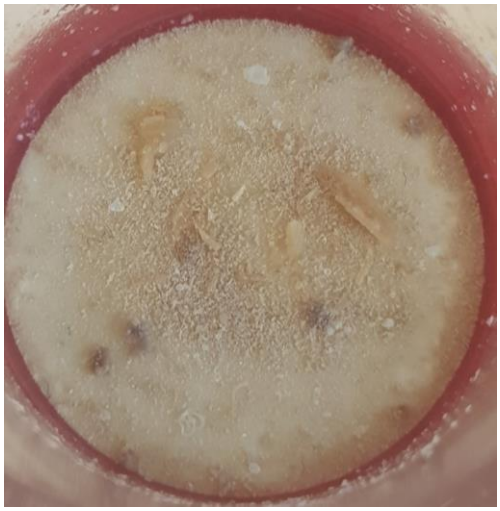


Figure 11: Undigested matter of salmon and possibly fish bones after the first filtration step

There were undigested matter left in crucible prior to the enzyme step for sample **4.2a** (*Figure 11*). Filtration of the three other samples went quicker, by using diluted KOH and ethanol/water mixture. The ultrasonic bath did not have any effect. This may be caused by the lack of direct contact between water in the ultrasonic bath and the filter crucibles (Crucibles were always stored in a glass beaker). The digestion efficiency for sample **4.1**, **4.2a** and **4.2b** were 99.94 %, 100.28 % and 99.96 %, respectively. Crucibles were weighed with a 4-decimal weight with uncertainty of ± 0.0001 g. This meant that the digestion efficiencies calculated were not reliable as there were undigested matter in filter crucibles when weighing. The crucibles used had not been incubated in the muffle furnace at 500 °C after being washed, which could explain the deviating calculations. This signifies the importance of following rinsing procedures of filter crucibles.

3.1.5. Protocol 3 (Enzymatic digestion)

Sample **5.1a**, **5.1b** and **5.1c** with 20 g minced salmon fillet each could not be stirred during incubation due to the impractical form of the filter crucibles. The H_2O_2-step for 36 hours was only performed once for the triplicates. After all filtration steps, undissolved tissue was stuck to the filter crucibles (Figure 12). Additionally, there was matter left in all crucibles after all steps were performed (Figure 13). The same observations were seen for samples **5.3a**, **5.3b**, **5.3c**, **5.3d** and **5.3e**. For the five replicates performed, it was estimated that each filtration step was 40, 30, 45 and 30 minutes each spanning over a total of 8 days, where the H_2O_2-step was performed for 72 hours. In addition to undigested matter stuck to filter crucibles, matter was left in each crucible for all experiments using **protocol 3** (excluding control sample, **5.2**.). The digestion efficiencies were 93.48 %, 92.89 % and 93.86 % for sample **5.1a**, **5.1b** and **5.1c**. For sample **5.3a**, **5.3b**, **5.3c**, **5.3d** and **5.3e**, their respective digestion efficiencies were 94.41 %, 94.78 %, 94.28 %, 92.21 % and 91.32 %.

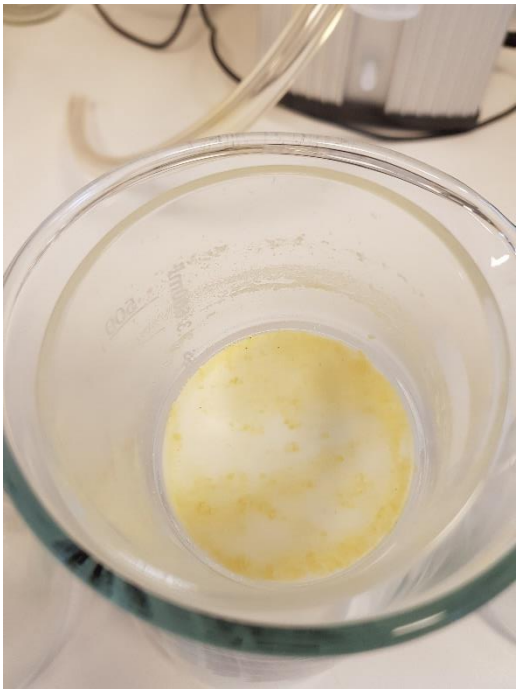


Figure 12: Crucibles after H_2O_2-step for 36 hours

Figure 13: Undigested matter after digestion stuck to crucible

3.1.6. Comparison of protocols

Factors involved in protocols were compared in *Table 10*. An acceptable protocol should be as time-efficient as possible, have a satisfactory digestion efficiency and not use incubation temperatures over 40 °C, as higher temperatures could potentially degrade polymers (Karami et al., 2017a; Munno et al., 2018). Thus, protocol 2 and 3 was discarded. All protocols excluding protocol 3 achieved a digestion efficiency > 99 %. Visual inspection of the filter crucible, however, showed less undigested matter present when a titration step was implemented. Foam during filtration most likely deriving from the PBSTnT solution was found to be prevented by adding 7:3 ethanol/water solution to crucibles, which solved the complication of long filtration time. This method was applied for all further experiments to prevent foam. Additionally, absence of hazards like concentrated HCl and protease is preferred.

In conclusion, **Protocol 1** and **protocol 1b** was applied for further experiments as they were the most satisfactory protocols tested regarding digestion efficiency, undigested matter in filter crucible, lab hours, and (few) complications.

Table 10: Comparison of initial experiments. Good digestion efficiency was defined as > 99% while poor efficiency was defined as < 99%. Bad visual inspection of filter was defined as undigested matter left while poor was defined as small quantities of undigested matter left.

Protocol	Digestion efficiency	Visual inspection of filter	Total lab days	Incubation temperatures [°C]	Hazards involved	Complications	Accepted protocols
Protocol 1 (KOH + HCl titration)	Good	Good	2	40	4.2 M KOH 4 M HCl	Foam during filtration	✓
Protocol 1b (KOH + citric acid titration)	Good	Good	2	40	4.2 M KOH	Foam during filtration	✓
Protocol 1c (KOH without titration)	Good	Poor	2	40	4.2 M KOH Conc. HCl	Foam during filtration. Long filtration time	✗
Protocol 2 (KOH + enzymes)	Good	Poor	4	56, 50, 40	4.2 M KOH Protease	Long filtration time	✗
Protocol 3 (Enzymes + H_2O_2)	Poor	Bad	8	50, 30	Protease H_2O_2	Undigested matter stuck to crucibles	✗

3.2. Optimization

3.2.1. pH Investigation protocol 1 and 1b

The pH investigation was carried out before the optimization experiment with 5 factors as pH was not expected to have a significant impact for digestion efficiencies or filtration time for the protocols. Due to differences between parallels (undigested matter present in some, others not), titrating samples with different pH with both HCl and citric acid was carried out to investigate how pH and the type of titration agent affected the digestion efficiency and filtration time.

Part A.

No significant differences in filtration time was found between HCl and citric acid at all applied pHes (pH 4, 7 and 10) with negative controls (without matrix) using Por-D (10-16 µm pore size), although the filtration times for samples titrated with citric acid was filtrated more rapidly. Negative control samples **10.1.** (pH 3.16), **10.2.** (pH 7.40) and **10.3.** (pH 10.64) titrated with HCl were filtrated in 26, 27 and 32 seconds, respectively. Negative control samples **10.4.** (pH 4.80), **10.5.** (pH 7.31) and **10.6.** (pH 10.69) titrated with citric acid were filtrated in 16, 16 and 29 seconds, respectively.

Part B.

The same experiment was repeated with same conditions but with smaller pore size of the crucibles and LDPE flakes spiked to the control.

It was not found any significant differences in filtration time between HCl and citric acid at pH 4, 7 and 10 with negative controls using Por-F (4-5 µm pore size). Negative control sample **10.7.** (pH 1.69) and **10.8.** (pH 6.90) titrated with HCl were filtrated in 344 and 394 seconds, respectively. Due to complications with setup of filtration, the filtration time was not measured exactly for sample **10.9**. (pH 11.95). Negative control sample **10.10**. (pH 4.52), **10.11**. (pH 6.50) and **10.12**. (pH 11.35) titrated with citric acid were filtrated in 343, 274 and 418 seconds, respectively.

Positive control with spiked LDPE flakes did not lose any significant mass after exposure for titration with HCl nor citric acid at pH ≈4, pH ≈7 or pH ≈10**.** The changes of mass registered can be explained by the uncertainty of the weight, which is accurate to the last decimal (4-decimal weight).

µFTIR results on LDPE flakes were not interfered by citric acid or HCl.

Part C.

For these experiments, minced salmon fillet with removed fish bones was used. No fish bones were found in crucibles after filtration.

When titrating with HCl aggregates in solution were observed for sample **11.7.** (pH 4.16), as seen in *Figure 14* (top). When titrating with citric acid, aggregates in solutions were observed for sample **11.13**. (pH 5.14) and **11.14**. (pH 4.12), also seen in *Figure 14* (bottom). Same figure also demonstrate difference in opalescence that can be used as an indicator for pH.

All samples were filtrated except for sample **11.13.** and **11.14**. (citric acid titration) that clogged the filter crucibles completely.

Filtration times for samples titrated with HCl and citric acid were compared in *Figure 15*. From this graph one can observe the trend where the filtration time increases when samples are below pH 6 (filtration time for sample **11.13** and **11.14** were not measured as the filter crucibles got clogged). Acceptable filtration time for samples titrated with both HCl and citric acid was at pH 6 and above.

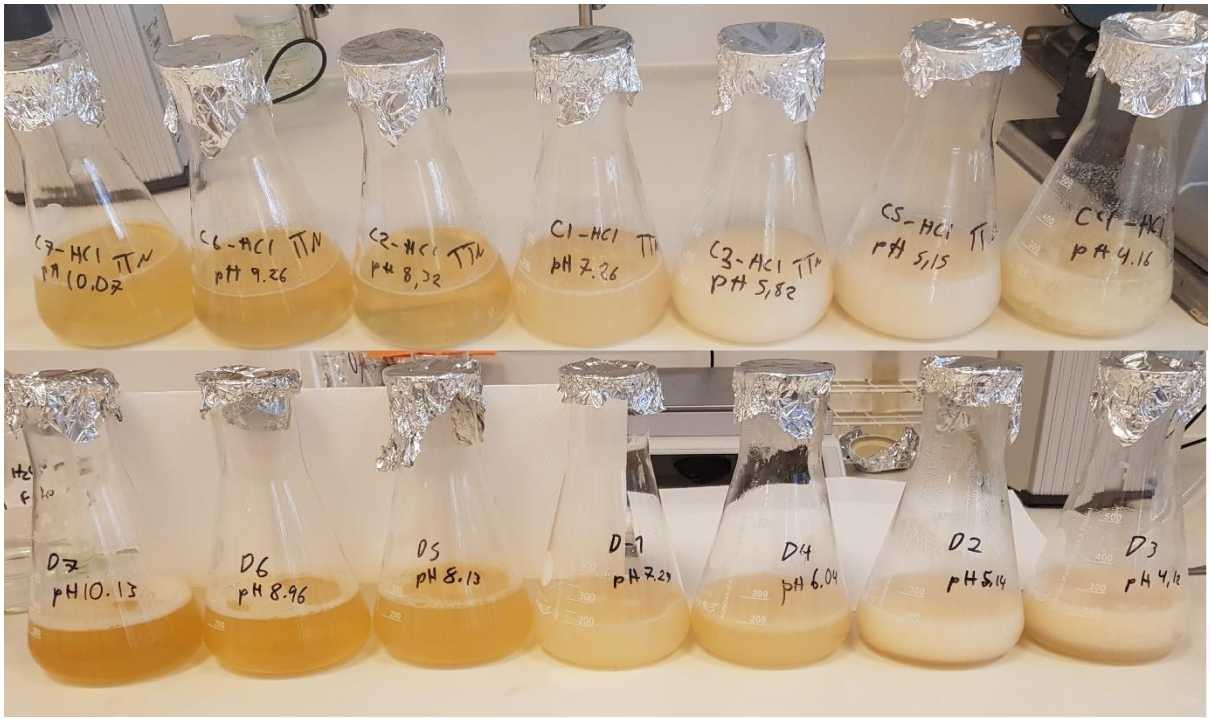


Figure 14: Erlenmeyer flasks sorted from basic to acidic (pH 10 → 4). Picture on top shows solutions titrated with HCl, while picture on bottom shows solutions titrated with citric acid.

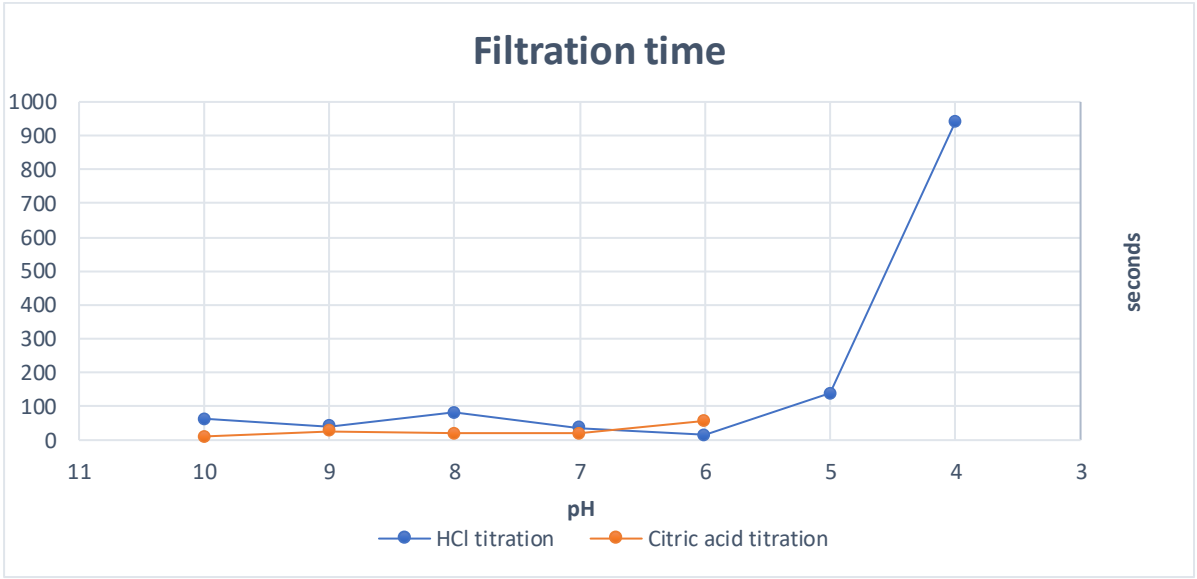


Figure 15: Filtration time for samples 11.1-11.7 titrated with HCl (blue), and samples 11.8-11.12 titrated with citric acid (orange). Sample 11.13 and 11.14 clogged the filter crucibles completely during filtration, and thus, no filtration time was measured.

Digestion efficiencies for samples titrated with HCl and citric acid are shown in *Figure 16*. Interestingly, samples titrated to pH between 7 and 6 for both acids achieved the best digestion efficiencies. Below pH 6, aggregates created from the acidic environment clogged the filter that led to longer filtration time for titration with HCl. Samples titrated to pH 5 and 4 with citric acid clogged the filter crucible completely.

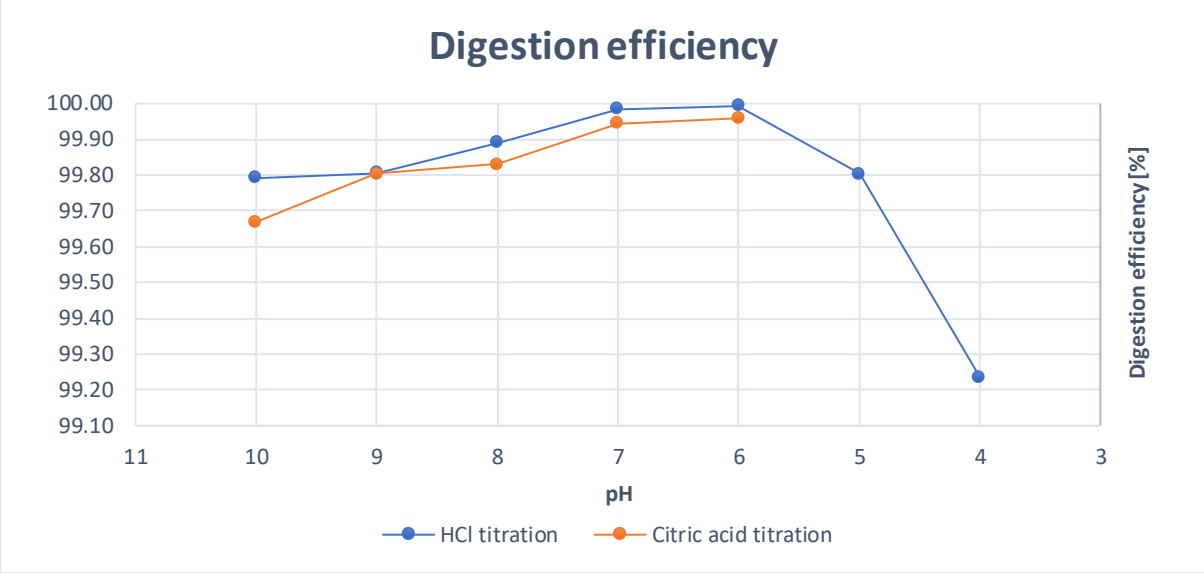


*Figure 16: Digestion efficiency for samples **11.1-11.7** titrated with HCl (blue), and samples **11.8-11.12** titrated with citric acid (orange). Sample **11.13** and **11.14** clogged the filter crucibles completely during filtration, and thus, no digestion efficiencies were calculated.*

Undigested matter in filter crucibles from titration with HCl and citric acid (*Figure 17*) indicated that the optimal pH for digestion efficiency was between pH 6 and 5 for samples titrated with HCl, and between pH 7 and 6 for samples titrated with citric acid. It was decided that pH should be more closely monitored when executing **protocol 1** and avoid over-titration as this causes aggregates in solution that increases filtration time and leaves residue in the filter crucibles.

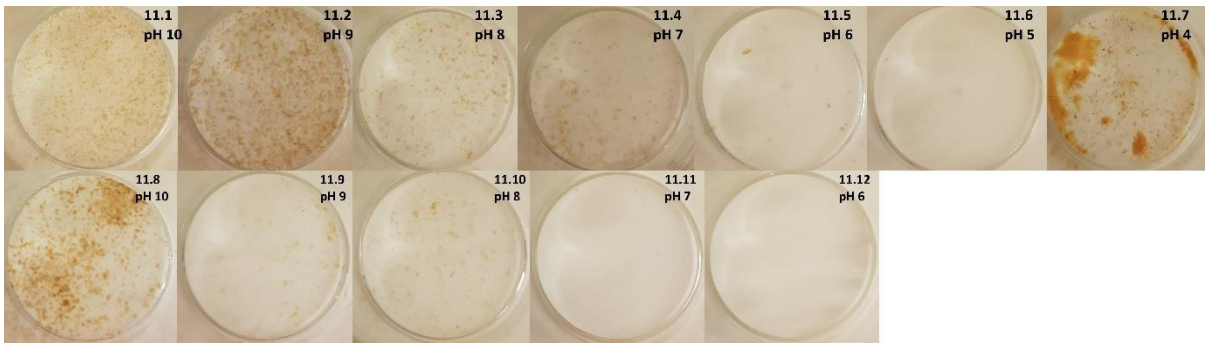


*Figure 17: On top: crucibles for samples **11.1-11.7** (HCl titration). On bottom: crucibles for samples **11.8-11.12** (Citric acid titration).*

In conclusion, based on the trends shown in *Figure 15*, *Figure 16* and *Figure 17*, **Protocol 1** with HCl titration was decided to be applied for further experiments. Titration to pH interval 7.0-5.5 was decided to be implemented in the protocol.

3.2.2. Experimental design two factors (KOH and incubation time)

Protocol 1 was used with varied factors incubation time (24 h/48 h) and concentration of KOH (4.2 M/2.1 M) following a 2^2 factorial design (*Table 8*). During filtration, adding 7:3 ethanol/water mixture

was found to clear clogged filters immediately (clogged filters due to foam) and this was implemented in **protocol 1** from here on. A possible reason for the clearing of clogged filters by using the ethanol/water solution could be that the surface tension for the generated form is decreased when adding the ethanol/water solution to the filter crucible.

Filter crucibles contained uneven amounts of fish bones (*C-4, C-5* and *C-6*). Sample **6.1b** contained the most fish bones which lead to a calculated digestion efficiency of 94.28 %. All other samples achieved a digestion efficiency of > 99.3 %. Due to the presence of fish bones which complicated the calculation of factors, minced salmon tissue where bones were removed prior to grinding (during sample preparation) were decided to be used for further experiments. The calculated effect of factors incubation time and concentration of KOH regarding digestion efficiency was negligible and was decided not worth to take into consideration.

Matter present in filter crucibles was also different between parallels (*C-4, C-5* and *C-6*). This was not investigated at the time, although it was later presumed to be caused by differences in pH after titration. The big changes during inspection of filter crucibles between parallels suggests that the factors calculated in chapter 3.2.3. and chapter 3.2.4. are not completely reliable.

3.2.3. Experimental design five factors (KOH, incubation time, Triton X-100, Tween20® and PBSTnT)

Minced salmon tissue with removed fish bones were used for optimization with protocol 1. After incubation, samples were titrated within a 7.0-5.5 pH interval as this was regarded as the optimal pH for digestion efficiency and filtration time (pH Investigation **protocol 1** and **1b**).

The experimental setup performed was a 2^{5-1} fractional factorial design with a total of 16 unique experiments (*Table 9*). Sample **7.12**. and **7.14.** got completely clogged during filtration. Therefore, no reliable numerical values for these two experiments were achieved and they were just regarded as failed experiments with regards to filtration time and digestion efficiency, and values both for filtration time and digestion efficiency were far from normally distributed. A reliable model of the factors requires reliable numerical values in all experiments. To get around the problem with the failed experiments it was decided to rank the results which a method commonly used in robust statistics. An inverse rank was used for filtration time (The shortest filtration time was the value 16 and the two failed experiments were given value of 1.5) and digestion efficiency (The highest digestion efficiency was the value 16 and the two failed experiments were given value of 1.5). Positive regression coefficients in the models (*Figure 18* and *Figure 19*) therefore indicate a wanted effect (high digestion efficiency and short filtration time). The ranks for the experiments are listed in *B-2*.

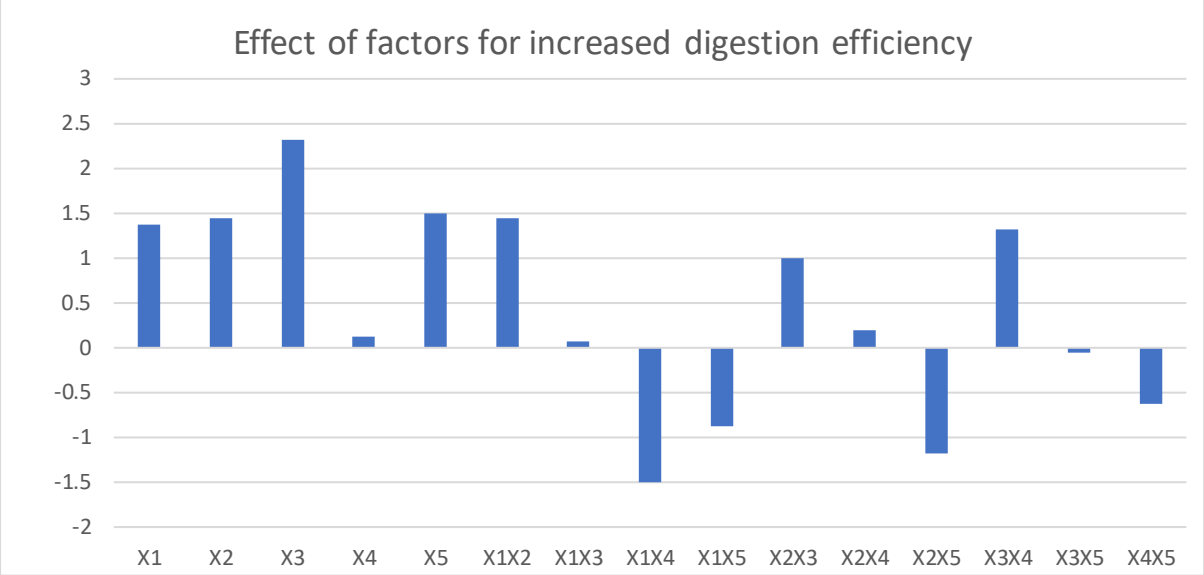


Figure 18: Diagram showing effect of factors with increased digestion efficiency.

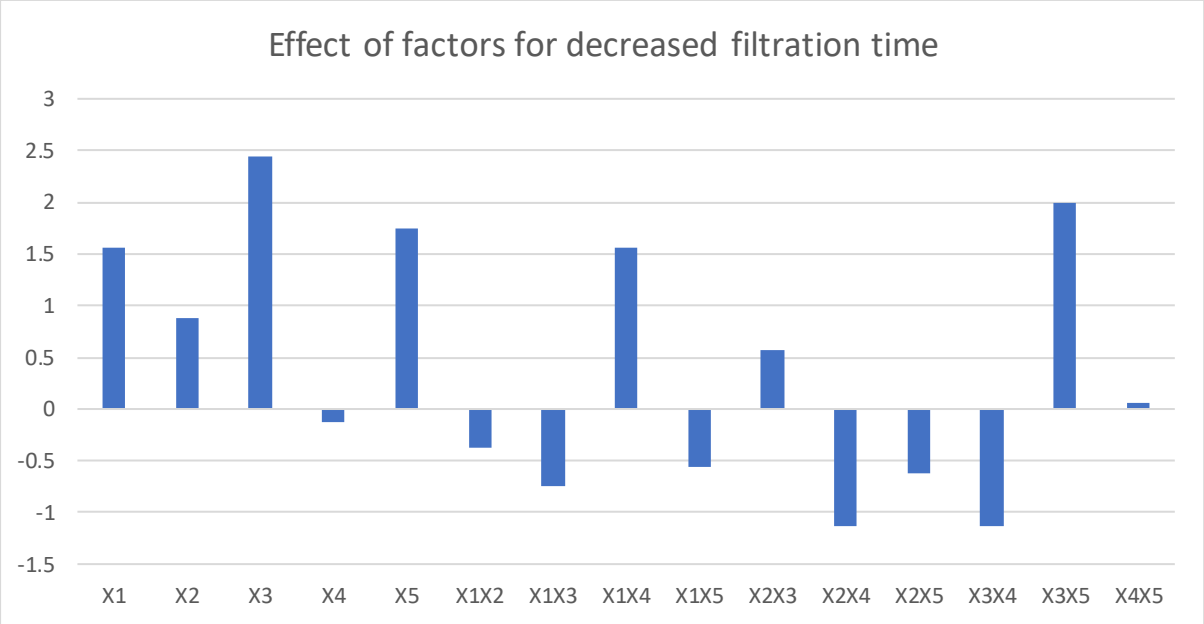


Figure 19: Diagram showing effect of factors with decreased filtration time.

After the ranking there is a clear correlation between the two responses (decreased) filtration time and (increased) digestion efficiency, as shown in *Figure 20*. The correlation coefficient, r, between the two ranked variables is 0.86, which is equal to the Spearman's rank correlation coefficient between the raw data.

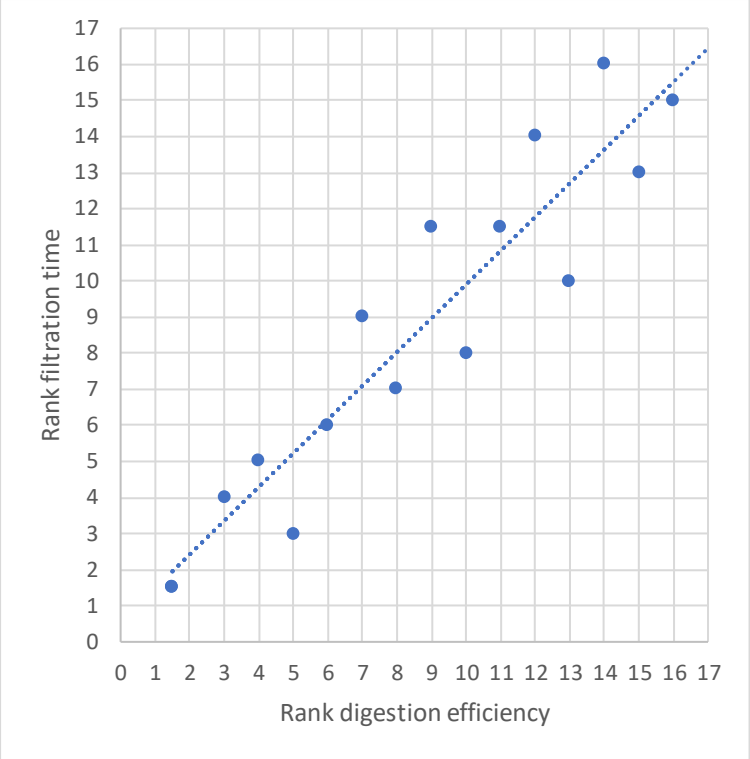


Figure 20: Plot of trend line between rank for experiments 7.1-7.16 for digestion efficiency and filtration time

The general picture is that the regression coefficients for the main factors in *Figure 18* are higher than the coefficients for the interactions. They are also positive, except for factor X4 (Tween20®) that is around zero, indicating that Tween20® alone does not contribute to the digestion. It has interactions with other variables, but these have to some degree opposite signs (X1X4 and X3X4), depending on the response variable chosen. This indicates that Tween20® may not contribute to the digestion efficiency. Discussion of other interactions is of limited relevance as the main effects show that all other factors seem to have a clear positive contribution. Thus, they should be included in the protocol irrespective of what the interaction effects show.

Replicates of experiments **7.2.**, **7.3.**, **7.5.** and **7.9.** were carried out (two new replicates for **7.2.**) to examine deviations between replicates. Responses for sample **7.2b**, **7.3b**, **7.5b** and **7.9b** replaced responses for sample **7.2a**, **7.3a**, **7.5a** and **7.9a**, respectively, and new values for effect for factors were calculated. These did not change the overall picture (*Figure 21*).

Relative standard deviation was calculated for digestion efficiency (matter left) using formula *Equation 4*: 0.0082 g (±112%) for experiment **7.2**, 0.0332 g (±54.4%) for experiment **7.3**, 0.0515 g (±14.4%) for experiment **7.5** and 0.0239 g (±59.8%) for experiment **7.9.**

In conclusion, Tween20® needed to be investigated whether it has any impact on the digestion efficiency. A new optimization design was made for incubation time and Tween20® in chapter 3.2.4.

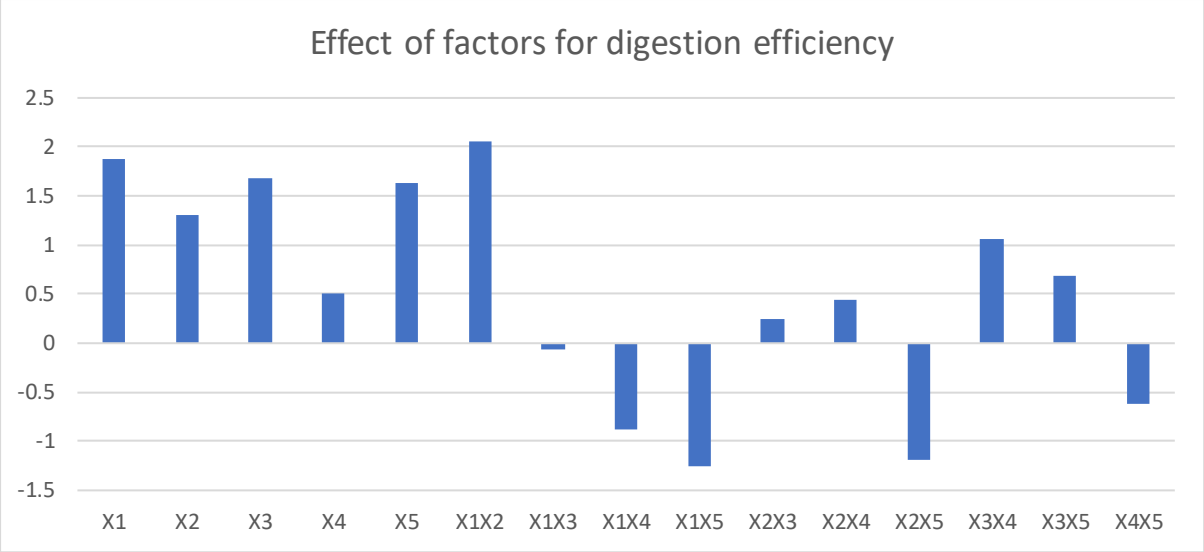


Figure 21: Diagram showing effects of factors for experiments 7.1-7.16 with replicates used in calculation instead.

3.2.4. Experimental design two factors (Incubation time and Tween20®)

Protocol 1 was performed with a 2^2 experimental design (*Table 8*). X1 was incubation time (48 h (+) and 24 h (-)) and X2 was Tween20® (Presence (+) and absence (-)). Triplicates of all 4 experiments were carried out. Minced salmon fillet was used with removed fish bones. Only factors regarding digestion efficiency was planned to be investigated.

Sample **8.2c** (48 h incubation and absence of Tween20®) was titrated to a lower pH (6.3) than its other parallels **8.2a** and **8.2b** (pH 6.6 and 6.9, respectively). This resulted in creation of aggregates in solution. The aggregates in solution was present in crucible after filtration and it was decided to remove this sample from calculations of factors.

Sample **8.4b** (pH 6.2) and **8.4c** (pH 5.6) were also titrated with more HCl which resulted in aggregates. However, there were no aggregates present in crucibles after filtration. This was likely due to the shaking of solutions for ~ 30 seconds prior to filtration.

The mean digestion efficiencies with associated standard deviation was calculated; 99.88 % ± 0.08 % for sample **8.1a-c**, 99.85 % ± 0.01 % for sample **8.2a-b**, 99.74 % ± 0.27% for sample **8.3a-c** and 99.86 % ± 0.10 % for sample **8.4a-c**.

Due to the insignificant changes in digestion efficiencies combined with (relatively) large relative standard deviations, it was decided to rather interpret the results for each sample.

In conclusion, the three samples that experienced aggregates in solution all had absence of Tween20®. Thus, it was decided to continue having Tween20® implemented in the protocol.

3.3. Damage evaluation of MPs

Optimized protocol 1 was performed with triplicates for each polymer tested. No visual changes were observed for MPs before exposure or after exposure to **optimized protocol 1** (*D-1, D-2, D-3, D-4, D-5, D-6, D-7, D-8).*

The gravimetric analysis was carried out with a 5-decimal weight. Sorted from smallest to largest loss in percent with associated standard deviations; PA66 (0.01% ± 0.02%), PS (0.06 % ± 0.14 %), LD (0.10 % ± 0.11), HD (0.15 % ± 0.31 %), PP (0.15 % ± 0.16), PET (0.27 % ± 0.21 %), PMMA (0.39 % ± 0.17) and PC (0.48 % ± 0.29 %). Mass loss with relative standard deviations is shown in *Figure 22*.

To investigate if FTIR spectra of MPs were able to differentiate between different plastic polymers, a PCA plot was made for MPs not exposed to **optimized protocol 1** (*Figure 23* with associated loadings *D-17*). All spectra were transformed using differentiation (1st degree, width 7 and order 3) due to differences in relative intensities in spectra. The PCA plot shows a clear differentiation between the 8 polymers. One MP of LDPE can be observed alongside the cluster with PC, which is most likely due to mistakenly analysing and giving the name PC to a misplaced LDPE particle.

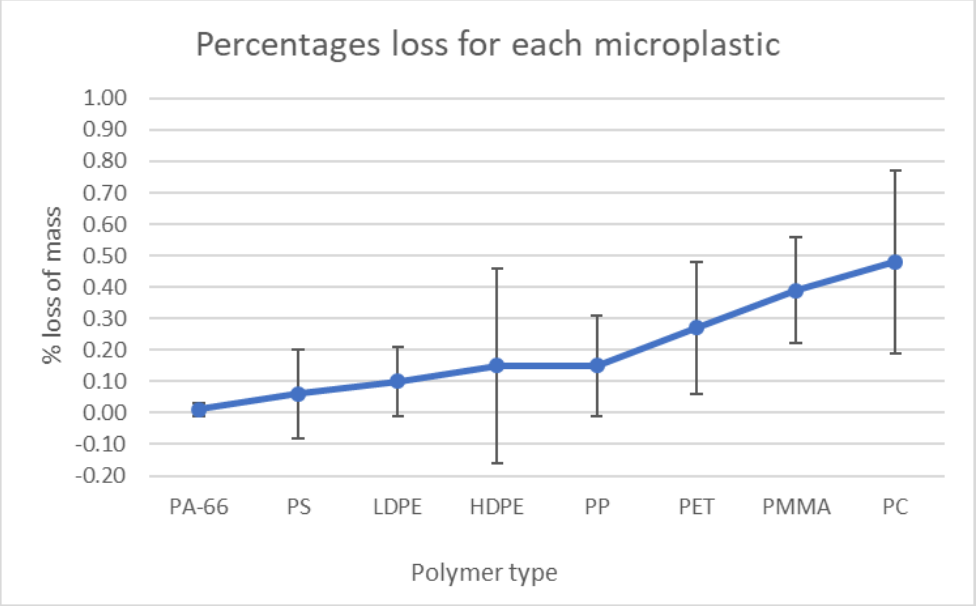


*Figure 22: Mass loss after exposure to **optimized protocol 1** for different polymers with relative standard deviation*

Another PCA plot (*Figure 24* with associated loadings *D-18*) was made for 3 parallels of HDPE before and after exposure using same data-treatment as for *Figure 23*. *Figure 24* shows that parallels of HDPE MPs before and after exposure can be differentiated using principal component 1 (Comp. 1) which explains 77.7% of the total variance; thus, it was decided that spectra could be compared by using one representative parallel of each spectra for before exposure, after exposure, and after exposure with an additional cleaning step.

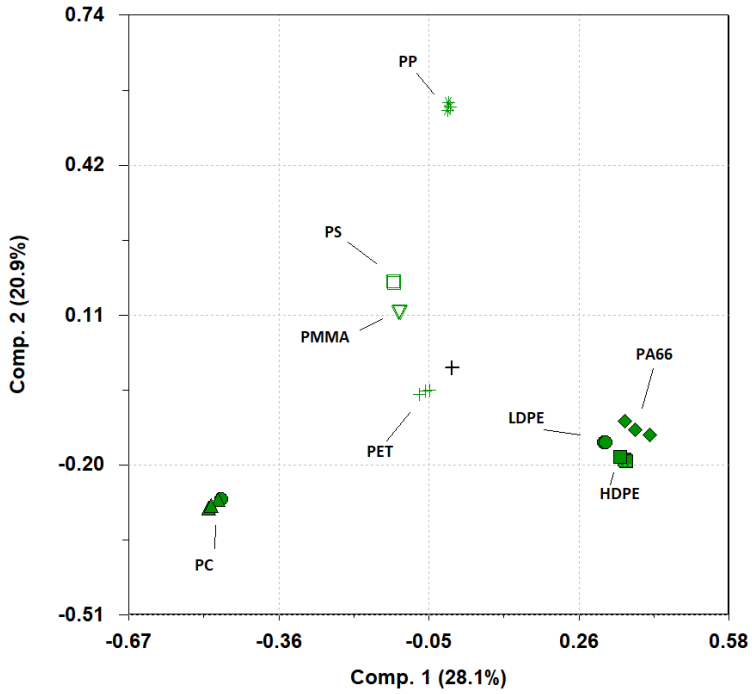


Figure 23: PCA Scoreplot of 3 parallels of 8 polymers before protocol. Comp. 1 explains 28.1 % of the variance whereas Comp. 2 explains 20.9 % of the variance.

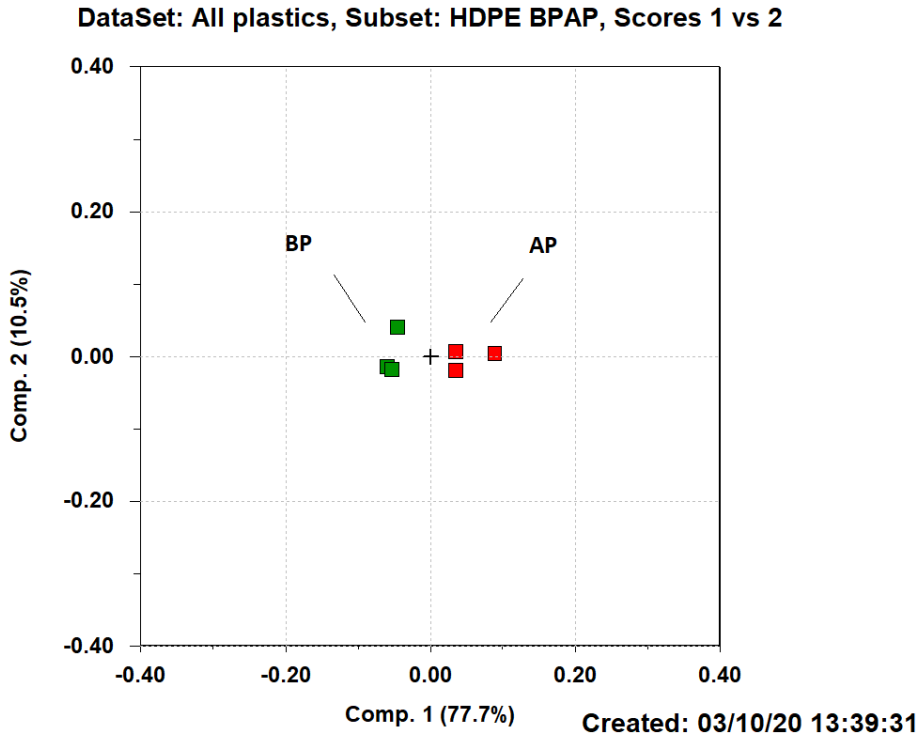


Figure 24: PCA score plot of 3 parallels of BP and AP for HDPE. Comp. 1 explains 77.7 % of the variance whereas Comp. 2 explains 10.5 % of the variance.

Spectra for MPs before exposure, after exposure, and after exposure with an additional cleaning step were compared (*D-9, D-10, D-11, D-12, D-13, D-14, D-15, D-16*). No major changes to the fingerprint regions were observed which is further elaborated below.

No significant changes in spectra for PET or PMMA were found after exposure. New peaks were found in spectra for MPs after exposure for HDPE (1110 cm^{-1}, 1150 cm^{-1}, 1750 cm^{-1}), LDPE (1110 cm^{-1}, 1150 cm^{-1}, 1750 cm^{-1}), PP (1150 cm^{-1}, 1750 cm^{-1}) and PS (1150 cm^{-1}, 1750 cm^{-1}). Spectrum for PA66 also indicated higher intensities at 1110 cm^{-1}, 1150 cm^{-1} and 1750 cm^{-1}, however the change of intensity of the peaks was small and did not compromise the spectrum. The same spectrum for PA66, however, has decreased intensities in spectra after exposure at 2850 cm^{-1} and 2920 cm^{-1}, and increased intensity for after exposure at 3290 cm^{-1}. Spectrum for PC has indications of a peak at 1750 cm^{-1}, and two small peaks with higher intensity at 2870 cm^{-1} and 2930 cm^{-1}.

There was a suspicion regarding potential contamination from the solution during **optimized protocol 1** with the new peaks found and hence, library spectra of Tween20®, Triton™ X-100 and KOH were found (*D-19*, *D-20*, and *D-21*, respectively). Triton™ has major peaks at 2950 cm^{-1}, 2870 cm^{-1}, 1510 cm^{-1}, 1250 cm^{-1} and 1110 cm^{-1}. Tween20® has major peaks at 2920 cm^{-1}, 2870 cm^{-1} and 1110 cm^{-1}. KOH has major peaks at 2920 cm^{-1} and 2850 cm^{-1}. Peaks for the three suspected contaminants do not correlate with all the new peaks found in spectra for MPs after exposure, although it was decided to see if an additional cleaning step after exposure would remove the new peaks found potentially deriving from chemicals present in solution in **optimized protocol 1**. Spectrum for HDPE indicated a lowered intensity of the new peaks found, although this was not observed for any of the other spectra of the other MPs. The additional cleaning step was decided to be redundant.

3.4. Extraction of microplastics from salmon, haddock and mackerel

Optimized protocol 1 was performed on triplicates of minced salmon, haddock and mackerel fillets. Prior to the experiments, fish bones were removed from salmon and mackerel. One negative control was performed for each triplicate of fish tissue.

Sample **9.1a-c** containing minced salmon tissue were incubated for 48 h due to undigested tissue in solution. This was unexpected, as the minced salmon fillet in all previous experiments using **protocol 1**was dissolved after 24 h. One possible explanation could be human error, e.g. forgetting to but on the shaking function during incubation, or instrumental errors. Both samples containing haddock and mackerel were dissolved after 24 h and filtrated swiftly after. **Sample 9.1c** was over-titrated to pH 4.98, which led to complete clogging of the filter crucible, and thus no digestion efficiency could be calculated. It was decided to keep the pH between 7 and 6 for all samples, however it was decided to

not add base to return to the desired pH. This decision was made to investigate if the solution could be filtrated in case of over-titration.

The 1 l Erlenmeyer flasks were full after addition of HCl for all samples. This could potentially lead to complications when transferring solution to filter crucibles by spillage.

The mean digestion efficiency with standard deviation (*Equation 3*) calculated for salmon, haddock and mackerel were 99.97 % ± 0.01, 99.98 % ± 0.01 and 99.96 % ± 0.01, respectively. What was presumed to be fish bones were present in crucibles for both haddock- and mackerel samples.

The planned analysis of potential MPs present in filter crucibles was not executed due to technical problems.

4. Discussion

4.1. Initial experiments

Protocol 1, with 4.2 M KOH (Diluted to 1.05 M/5 % (w/w) in solution) and PBSTnT-solution for 24 h at 40 °C with a titration step with HCl to pH ≈ 7 prior to filtration, was regarded the most suitable method for initial experiments regarding the criteria in *Table 10*, with digestion efficiencies for minced salmon tissue of 99.00 % and 99.85 %. KOH has been used in previous studies for digesting fish tissue, although for 48 h at 40 °C with a 10 % w/w KOH solution (Karami et al., 2017a), and for 24 h at 60 °C with a 20 % w/v KOH solution (Dehaut et al., 2016). **Protocol 1** demonstrates that it can digest tissue for shorter time, lower temperature and lower concentration than other protocols using KOH. This is likely due to the presence of the PBSTnT-solution, containing detergents that makes the tissue more available for digestion. The PBSTnT-solution also works as an indicator during titration, as the solution turns opalescent when pH is close to 7. **Protocol 1** using citric acid for titration (**protocol 1b**) was also accepted for further experiments (digestion efficiency of 99.95 %), although discarded in section 3.2.1. (further elaborated in section 4.2.).

The titration with HCl facilitated the filtration. The filtration was a major issue for **protocol 1c** and **protocol** 2 during the initial experiments, due to clogging of filter crucible caused by undigested minced salmon tissue in solution. Thiele et al. (2019) used a neutralization step with citric acid prior to filtration which reportedly resulted in successful filtration of mussels digested in KOH solution with filter crucibles with pore size 1.2 µm. The authors' results correlate with our observations and results; the filtration step is more practicable executed when a titration step is present. **Protocol 2** also applied an incubation temperature of 56 °C, which is a temperature with KOH present that may degrade PA-66 (Karami et al., 2017a). Due to the complications during filtration for both protocols and the undesired temperature of **protocol 2**, **protocol 1c** and **protocol 2** were discarded.

Protocol 3 was not effective in digesting fish tissue (< 99 %). In addition of the disadvantage of being a time-extensive protocol, using temperatures over 40 °C during incubation might result in degradation of MPs. Piarulli et al. (2019) used enzymes to achieve a successful digestion of the GIT of fish. However, the authors concluded that the KOH digestion tested was a more cost-effective method. Thus, due to low digestion efficiency and the extended time needed, **protocol 3** was discarded. One possible reason for not achieving a successful digestion efficiency for **protocol 3** could be that a chitinase-step was not performed, which was a part of the original **protocol 3**. However, it was excluded when performing this protocol.

4.2. Optimization

The pH-investigation in section 3.2.1 indicated that the pH in solutions that were titrated prior to filtration affected the digestion efficiency, filtration time and visual inspection of filter crucible. It was shown that HCl was better suited for titration, as samples titrated with citric acid below pH 6 could not be filtrated due to clogging. The trend shown in *Figure 17* signifies the importance of careful titration, as over-titration of acid will cause aggregates to be formed in solution which will clog the filter. Under-titration leads to more undigested matter being present in filter crucibles after filtration, also shown in *Figure 17*. The optimal pH for titration with HCl, which visual inspection of the filter crucibles, filtration time and digestion efficiencies indicated, was between pH 7 and 6. As there were no parallels performed, this is not a reliable conclusion. More detailed investigation regarding pH should be executed in future experiments. Because of the failed experiments for citric acid titration of samples with pH below 6, **protocol 1** with HCl titration was preferred. This decision was also made because of the suspicion that citric acid would create complexes with potential MPs that could complicate the endpoint chemical analysis. No complexes were found, however if citric acid would have been the preferred for titration, more MPs should have been investigated for potential complexes made on the MPs' surface.

In hindsight, the pH of solution prior to filtration should have been a factor implemented in the experimental designs. A new uniform design investigating pH intervals as low as 0.2 between each pH would provide more information about the optimal pH achieved with titration. The pH was difficult to keep constant for all experiments, and small changes would cause differences between parallels. However, all factors tested in experimental design in section 3.2.3. (Incubation time, PBSTnT concentration, Triton™ X-100 and KOH concentration) except for Tween20® were shown to have positive correlation with increased digestion efficiency.

The presence of fish bones in the first experimental design from section 3.2.2. complicated the calculations of factors. The removal of fish bones in further experiments was proven effective, as there were not observed any fish bones in the other two optimization experiments.

The relative standard deviation for the parallels measured in section 3.2.3. with 5 factors suggests that reproducibility still is difficult at the current stage of the protocol development. Additionally, the issue with reproducibility suggests that the effect of factors is not completely reliable and should be interpreted carefully. However, calculation performed in section 3.2.3. of getting regression coefficients above 1 suggested that the effects of the factors incubation time, PBSTnT concentration, Triton™ X100, and KOH concentration, were not likely to occur by chance. Thus, the mentioned factors are most likely positively correlated with increased digestion efficiency. Because a protocol for

extraction of MPs from biological matrices is preferred to be time-efficient, the incubation time should not be extended to 48 h. This means that all factors should have the conditions that was already proposed in **protocol 1**; thus, the **optimized protocol 1** have the same conditions as **protocol 1**.

There was a significant positive correlation between increased digestion efficiency and decreased filtration time in the experimental design with 5 factors in section 3.2.3. This is a reasonable correlation, as undigested matter will prevent the solution to be filtrated by clogging the pores in the filter crucibles. Incubation time for 48 h was more effective for digestion efficiency as compared to 24 h, although it was preferred to keep the incubation time as short as possible. The solution recipe for PBSTnT was preferred compared to the diluted PBSTnT solution (25 %). Positive correlation for digestion efficiency suggested that the presence of Triton™ X-100, however Tween20® only had a small positive correlation with increased digestion efficiency. Tween20® was shown to have an effect for digestion (section 3.2.4); it prevented the solution from creating aggregates when titrated to pH < 6.3. Increased KOH concentration also led to increased digestion efficiency, although this was expected. The robustness test with experimental design could not be used for a predictive model for digestion efficiency due to the failed experiments. However, it demonstrated the importance of the presence of PBSTnT-solution with both Triton™ X-100 and Tween20®.

The discussed results led to new implementations in the protocol which formulated the **optimized protocol 1**. Following this protocol, the problem deriving from clogging due to foam formation was solved. Additionally, the new steps implemented involving complete transfer of matter to solution with ethanol/water-mixtures and from flask to filter crucible is now executed more thoroughly (see *A-4*).

4.3. Damage evaluation of MPs

The low mass loss of < 0.5 % for all MPs tested suggests that **optimized protocol 1** is a protocol fit for purpose for degrading fish tissue without harming MPs, regarding mass loss. This is, however, only for the size range of MPs used for this experiment (1-4 mm). The FTIR-spectra comparison of MPs before and after exposure shows no significant alterations to the chemical integrity. Surprisingly, the spectrum of PA-66 after exposure (*D-11*) showed moderate changes in intensity at wavelength 2850 cm^{-1} and 2920 cm^{-1}, despite being the least degraded MP regarding mass loss (0.01 % ± 0.02 %, *Figure 22*). The new peaks found in spectra after treatment with **optimized protocol 1** for HDPE, LDPE, PP and PS (*D-9*, *D-10*, *D-15*, *D-16*) correlate with some of the peaks found in library spectra of Triton™ X-100 and Tween20®. The peaks are however not completely explained as other peaks at 1510 cm^{-1} and 1250 cm^{-1} for Triton X-100 was not observed. Additionally, the peak at 1750 cm^{-1} could not be

explained. Nevertheless, using py-GC/MS for chemical analysis would allow to separate contamination of Triton™ X-100 and Tween20® from MPs present if these were adsorbed to the MPs' surface.

The additional cleaning for MPs after exposure did not have any effect for the new peaks in spectra, although a more thorough rinsing step after filtration could be implemented to prevent contaminants adhered to the surface of potential MPs in filter crucibles.

MPs were directly exposed to the KOH- and PBSTnT solution in **optimized protocol 1**, which is a worst-case scenario for MPs in solution. One study (Claessens et al., 2013) demonstrated that MPs embedded in tissue was more protected from degradation. This observation makes it reasonable to suggest that MPs found in fish matrices might be more protected from degradation and chemical integrity changes than for MPs exposed directly to the solution in **optimized protocol 1.** This hypothesis could be investigated by exposing cellulose acetate (which is reported to be degraded by KOH (Dehaut et al., 2016)) to **optimized protocol 1** embedded in matrix and exposed to the solution without matrix.

Since no major changes were found in spectra before or after exposure and that all spectra were able to be matched with library spectra of respective plastic polymers suggests that the chemical integrities were not compromised.

One major flaw in this thesis was performing the damage evaluation of MPs after choosing one protocol for optimization. The most common order is to check for damage evaluation of protocol for MPs before optimization, as the protocol should be rejected if there are signs of degradation on MPs. However, Karami et al. (2017a) reported that KOH does not degrade plastic polymers at 40 °C (except for cellulose acetate). This led to the assumption that our KOH-solution that contained lower concentration of KOH solution (1.05 M/ 5 % (w/w) would not degrade the MPs tested here.

Another flaw with the damage evaluation was that smaller sizes of MPs were not tested. Comparing spectra of smaller MPs before and after **optimized protocol 1** would be interesting to investigate, as the surface to mass ratio increases with decreasing size, which will lead to increased damage to the chemical integrity. Performing a damage evaluation of smaller sizes of MPs would, however, be hard to perform without using µFTIR. One possibility to alleviate this problem would be to extrapolate from the results of mass loss of MPs. This way one could calculate how much mass would be lost for MPs with smaller sizes.

The results for damage evaluation was compared to other studies compiled during literature search as shown in *Table 11*.

Table 11: Procedures from 6 studies exposing MPs directly to chemicals for damage evaluation + 1 study (Karami et al., 2017a) that embedded MPs to the biological material. FTIR was included as criteria as this was used in the current study. Only FTIR spectra of MPs with major changes after protocol was mentioned.

Reagent			Damage to polymers				Study
Chemical	Conc.	Highest temperature used [°C]	Polymers tested in study	Visual impact	Change of weight	FTIR	
KOH + PBSTnT-solution	**1.05 M / 5 % (w/w)**	**40**	**(HDPE, LDPE, PA66, PC, PET, PMMA, PP, PS)[4]**	**No impact**	**< 0.5 %**	**No change**	**Current study**
H_2O_2	30 % (v/v)	60	(PP, LDPE, HDPE, PS, PET, PA66, PC, PMMA)[4]	PS[L1]	-	No change	Hurley et al., (2018)
H_2O_2	30 % (v/v)	70		PP[L1], PS[L1]	PA-66	No change	
NaOH	10 M	60		PET[L3], PC[L3]	PET, PC	PC	
KOH	10 % (w/v)	60		No impact	PS, PC	No change	
4:1 HNO_3 + HClO	69 % (v/v) + 70 % (v/v)	80	(PP, LDPE, HDPE, PS, EPS, ABS, PU, PA“1”, PA“2”, EVA, PET, PC, Nitrile, PVC“1”, PVC“2”, PVC“3”,	PS[L3], EPS[L1], ABS[L3], PU[L4], PA"1"[L4], PA"2"[L3], PET[L1], PC[L1], Nitrile[L4], PVC"1"[L2], PVC"2"[L2], PMMA[L3], "RE1"[L4], "RE2"[L4], "RE3"[L4]	-	-	Enders et al., (2017)
KOH	1120 g/l	80	PMMA, PTFE, “RE 1”, “RE 2”, “RE 3”)[4]	No impact	-	-	
KOH	10 % (w/v)	60	(CA, EPS, HDPE, LDPE, PA-12, PA-6, PC, PET, PMMA, PP, PS, PTFE, PUR, PSXL, PVC-U)[4]	CA[L3], PET[L2]	CA	-	Dehaut et al., (2016)
NaOH	10 M	60		CA[L3], PC[L3], PET[L3]	CA, PC, PET	-	
NaOH + HNO_3	1 M + 69 % (v/v)	80	(HDPE, LDPE, PP, EPS, PET, PA, PVC-P, PVC-U)[4]	PA[L4], PET[L2], PVC-P[L1], PVC-U[L1]	PET	No change	Roch and Brinker, (2017)
KOH	10 % (w/v)	RT	(LDPE, HDPE, PP, PS, PET, PVC, PA-6, PA-66)[3]	No impact	PA-6, PVC, HDPE, LDPE, PP	-	Karami et al., (2017a)
KOH	10 % (w/v)	40		No impact	PVC	-	
KOH	10 % (w/v)	50		PA-66[L2]	PVC, PET	-	
KOH	10 % (w/v)	60		PA-66[L2]	PVC, PET	-	
H_2O_2	35 % (v/v)	60		PET[L1]	PA-6, PA-66, PVC, PS	-	
HNO_3	5 % (v/v)	RT		PA-6[L4], PA-66[L4], LDPE[L3], HDPE[L3], PP[L3]	PET, PVC, PS, HDPE, LDPE, PP.	-	
HCl	37 % (v/v)	RT		PA-6[L3], PA-66[L3], PET[L3]	PA-6, PA-66, PET,	-	

					PVC, HDPE, LDPE.		
KOH	1 M	RT	(PES, PP)[4]	PES[L3]	-	No change	Piarulli et al., (2019)
Enzymatic digestion	10 ml SDS + 5 ml Enzyme F + 5 ml Enzyme SE	50		No impact	-	No change	

[3] Size range 10-500 µm (using size scale from *Table 2*)

[4] Size range >500 µm (using size scale from *Table 2*)

[L1,L2,L3,L4] See Table 4 for degradation impact on MPs

4.4. Extraction of MPs from salmon, haddock and mackerel

It is unknown why salmon was not degraded during the first 24 h of incubation with **optimized protocol 1**, which was the first time this occurred. Sample **9.1c** contained aggregates due to over-titration. Adding diluted KOH-solution to reach pH between 6 and 7 could have been performed and should be done in the future in cases where aggregates are present due to over-titration.

80 % of the original solution should be applied for future experiments, as the 1 l Erlenmeyer flasks were full after titration with HCl.

Both mackerel and haddock were dissolved after 24 h and achieved high digestion efficiencies, compared with other studies in *Table 12*. The current study using **optimized protocol 1** demonstrates the best achieved digestion efficiencies for fish fillets, as compared to the studies found in the literature search (Dehaut et al., 2016; Karami et al., 2017a).

Undigested matter that were presumed to be fish bones were found in filter crucibles for both haddock and mackerel, despite that fish bones were removed from the mackerel fillet prior to grinding. This raises the question whether fish bones should be removed or not. For experimental design experiments, removal of fish bones was necessary as an eventual density separation following the filtration step would interfere with calculation of the digestion efficiency. If fish bones are removed from tissue, yet still found in filter crucibles, a density separation may be performed. NaI with density 1.5 g/cm has been used for this purpose in a study by Karami et al., (2017b) and this could be implemented in case there are fish bones present. Density separation could, however, lead to an underestimation of more dense particles such as tire wear polymers (Wagner et al., 2018). Another suggestion to remove potential undigested matter before an eventual density separation (if required) is to add diluted KOH to filter crucible until all matter is digested.

The quality criteria to standardise methods for extracting MPs from matrices mentioned in section 1.4.2. by Hermsen et al. (2018) are important to implement for **optimized protocol 1**. By following these criteria, this method can be validated and used for routine monitoring of MPs in fish tissue.

Table 12: Selection of chemicals with reported efficient digestion of marine organisms' tissue or intestinal organs.

Chemical[a]	Highest temperature used [°C]	Incubation time	Filter pore size [µm]	Matrix	Digestion efficiency [%][b]	Reference
HNO_3 (22.5 M)	100	14 h + 2 h	5	Mussel tissue	99.85	(Claessens et al., 2013)
HNO_3 (35 % v/v)	60	1 h	0.8-1.6	Mussel tissue	n.n.	(Catarino et al., 2017)
HNO_3 (69%)	RT	96 h	8	Fish tissue	99.9 (0.14)	(Karami et al., 2017a)
HCl (37%)	25-60	96 h	8	Fish tissue	98.8-100	(Karami et al., 2017a)
KOH (5%) + PBSTnT-solution	**40**	**24 h**	**10-16**	**Salmon tissue Haddock tissue Mackerel tissue**	**99.97 (0.01) 99.98 (0.01) 99.96 (0.01)**	**Current study**
KOH (20%)	RT	2-3 weeks	200	Fish stomach	n.n.	(Foekema et al., 2013)
KOH (20%)	60	12 h	-	Fish GIT	n.n.	(Rochman et al., 2015)
KOH (20% w/w)	60	24 h	1.6	Tissue from fish, mussels and crab	99.6-99.8	(Dehaut et al., 2016)
KOH (20% w/v)	40	48 h	8	Fish tissue	98.6 (0.05)	(Karami et al., 2017a)
KOH (20% w/v)	80	24 h	5	Mussel tissue	99.775 (0.009)	(Phuong et al., 2018)
KOH (20% w/v)	40 60	48 h	1.2	Mussel tissue	98.0 (0.5) 96.8 (0.7)	(Thiele et al., 2019)
KOH (1M)	RT	48 h	300	Fish stomach and intestines	n.n.	(Kühn et al., 2017)
KOH (1M)	RT	48 h	20	Crab GIT	n.n.	(Piarulli et al., 2019)
NaOH (1M)	60	12 h	0.8-1.6	Mussel tissue	n.n.	(Catarino et al., 2017)
NaOH (1M)	80	15 m + 30 m	8	Fish GIT	n.n.	(Roch and Brinker, 2017)
H_2O_2 (35 %)	50	96 h	8	Fish tissue	n.n.	(Karami et al., 2017a)
Corolase 7089	60	1 h	0.8-1.6	Mussel tissue	n.n.	(Catarino et al., 2017)
Biozym F & Biozym SE	50	24 h + 48 h	20	Crab GIT	n.n.	(Piarulli et al., 2019)
Trypsin	60	4 h	63	Mussel tissue	95.8 % (2.4)	(Thiele et al., 2019)

[a] Protocols which chemicals are mentioned may contain other components or is a simplified protocol

[b] n.n. when adequate digestion efficiency rate was reported but not stated in numbers.

Standardising protocols for extracting MPs from matrices should be of high importance, to achieve comparable reliable data. Further experiments should investigate how **optimized protocol 1** works on other fish tissues as well, formulated in section 6 Further work.

Filter crucibles applied in experiments (pore size 10-16 µm) should have smaller pore size to find smaller sizes of MPs. However, long filtration time would be a complication. Numbers from the pH investigation in section 3.2.1. (with negative controls containing 1/5 of the volume of the recipe) showed ≈ 20 times longer filtration times when filtrating with a 4-5 µm filter crucible compared to a 10-16 µm filter crucible (*B-1*). This indicates that extended filtration time will be expected for even smaller pore sizes of filter crucibles.

5. Conclusions

KOH has been widely reported in studies as a suitable chemical for digestion of biological matrices for the extraction of MPs. Here, such a protocol was selected out of five tested protocols as the most suitable method regarding digestion efficiency, short filtration time, and an acceptable incubation temperature. This protocol was optimized, and for the resulting protocol, KOH was combined with the detergents Tween20® and Triton™ X-100 and HCl titration to pH 6-7 prior to filtration. The protocol was demonstrated to be time-efficient for extracting MPs from minced salmon, haddock and mackerel fillets. The chemical integrity of HDPE, LDPE, PA66, PC, PET, PMMA, PP or PS was not compromised according to FTIR spectra. Multivariate analysis could differentiate between FTIR spectra for different plastic polymers, and for spectra of MPs before and after treatment with the optimized protocol.

6. Future work

The optimized protocol has the potential to be developed towards a standard protocol for fish fillet, possibly also more matrices. However several aspects remains to be tested first: 1) Investigating the robustness of the protocol using other fish fillet matrices and organs e.g. the GIT, liver, kidney and brain, 2) Optimizing pH-interval for titration prior to filtration to achieve optimal digestion efficiency without undigested matter in solution 3) Determining retrieval rates of spiked samples with known amounts of smaller MPs than used in this experiment 4) Damage evaluation of cellulose acetate and PVC. These investigations will contribute to the aim of routinely using a standardized protocol and aid in producing more comparable and reliable data.

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Supplementary material

Appendix A: Protocols performed

A-1: ***Protocol 1***

Alkaline digestion	- Add 100 g minced salmon fillet into a 1000 ml Erlenmeyer flask - Add 600 g PBSTnT - Add 200 g 4.2 M KOH - Shake at 40 °C, 125 rpm, 24 h
Titration	- Put flasks in ice water - Add 4 M HCl (*aq*) until solution turns opalescent, around 250-260 g results in pH 7. Add in 50 g intervals until 200 g, then add in smaller intervals until 250 g. - Shake for every addition of HCl - Control with pH-meter after shaking
Filtration	- Filter with Por-D crucible (10-16 µm). - If clogged, rinse with 1 M HCl. - If still clogged, rinse with conc. HCl.
Rinsing	- Rinse with 2.1 M KOH (aq) - Rinse with 1 M HCl (aq)
Drying	- Incubate filter crucibles at 40 °C for 48 h

A-2: ***Protocol 2***

Alkaline digestion	- Add 100 g minced salmon fillet into a 1000 ml Erlenmeyer flask - Add 200 g PBSTnT - Shake at 56 °C, 130 rpm, 16 h - Add 200 g 4.2 M KOH slowly - Shake at 56 °C, 130 rpm, 3 h - Add 400 g water and shake flask - Settle for 1 h at 56 °C
Filtration	- The supernatant is transferred to perfluoralkoxy (PFA) tubing aided by vacuum to the Por-D (1 filter crucible. Dilute remaining viscous solution to approximately 1L and transfer without PFA tubing. - Matter and remaining supernatant are transferred from Erlenmeyer flask to filter

	- Use spatula and wash out of flask onto crucible using 7:3 water/ethanol mixture - Wash through crucible filter (vacuum) with 7:3 water/ethanol mixture
Enzyme step	- Add 40 ml Protease (Sigma P3111)) /Tris solution (1:5) onto each crucible - Shake and put on 50 °C, 100 rpm, 20 h - Filter and wash through (vacuum) with 1M Tris-solution - Add 40ml Lipase (Sigma L0777)/Tris solution (1:10) onto each crucible - Shake and put on 40 °C, 100 rpm, 20 h
Second filtration	- Wash through (vacuum) with water then ethanol
Drying	- Incubate filter crucibles at 40 °C for 48 h

A-3: ***Protocol 3***

Detergents and enzymes	- Add 20 g minced salmon fillet into pre-cleaned sintered glass Por-D crucible (10-16 µm) - Add 70 ml Tween20® (5%, v/v) - Sonicate in ultrasonic bath for 1 min - Incubate at 50 °C for 3 h - Filtrate and rinse with water - Add 90 ml Protease (Sigma P3111) and glycine buffer (0.1 M, pH 9) mixture (1:20) - Incubate at 50 °C for 48 h - Filtrate and rinse with water - Add 50 ml Lipase (Sigma L0777) and PBS-buffer (0.1 M, pH 7.4) mixture (1:10) - Incubate at 30 °C for 48 h. - Filtrate and rinse with water
Filtration and H_2O_2-treatment	- Add 50 ml 30 % H_2O_2 - Incubate at 50 °C for 36 h - Filtrate and rinse with water. If necessary, the H_2O_2-treatment is repeated
Drying	- Incubate filter crucibles at 40 °C for 48 h

A-4: ***Optimized protocol 1***

Alkaline digestion	- Add 100 g minced salmon fillet into 1000 ml Erlenmeyer flask - Add 600 g PBSTnT - Add 200 g of 4.2 M KOH - Check for remnants of fish remaining inside flask, flush down with water dispenser and/or with the help of a spatula - Shake at 40 °C, 125 rpm, 24 h
Titration	- Add 2.4 M HCl (aq) until solution turns opalescent, use magnetic rod at 500 rpm - Add 0.8 M HCl (aq) until pH is between 7.0-6.0 - Control with pH-meter when pH is between 7.0-6.0.
Filtration	- Filter with 10-16 µm crucible (Por. 4). - If clogged due to foam, rinse with ethanol-water (7:3). - If clogged due to layer of fat/precipitation, add 2.1 M KOH, then ethanol-water (7:3). - Remove all remnants from flask first with 50 ml ethanol-water (3:7), then with water dispenser if there are still remnants.
Rinsing	- Rinse with 20 ml 2.1 M KOH (aq) - Rinse with 50 ml ethanol-water (7:3) then 50 ml water. If there are signs of foam after addition of water, repeat with ethanol-water then water.
Drying	- Incubate filter crucibles at 40 °C for 48 h

Appendix B: Results for experiments at the IMR

B-1: Results for all experiments excluding experiments with MPs

Exp. No.	Matrix [g]	pH	Crucible BP [g]	Crucible AP [g]	Mass difference crucibles [g]	Digestion efficieny	Filtration time	Protocol description
1.1a	99.07	7.40	49.618 + 49.095	50.050 + 49.641	0.978	99.01	≈ 2 hours	
1.1b	99.45	7.54	48.500	48.595	0.095	99.90	-	
1.1c	99.22	6.93	48.809	48.913	0.104	99.90	-	**Protocol 1.**
1.2.	Control	≈ 7	49.902 + 48.922	49.954 + 48.926	0.056	-	≈ 1 hour	Exp. no. 1.1b and 1.1c were incubated for 48 h.
1.3.	99.52	≈ 7	49.3505	49.4931	0.1426	99.86	15 min	
2.1.	99.32	≈ 7	49.4971	49.5491	0.0520	99.95	12 min	**Protocol 1b**
3.1.	99.62	-	48.7178	49.9710	0.2532	99.75	105 minutes	**Protocol 1c**
4.1	99.42	-	49.7225	49.7788	0.0563	99.94	> 4 h	
4.2a	99.48	-	49.9923	49.7091	-0.2832	100.28	-	**Protocol 2.**
4.2b	99.91	-	48.8086	48.8456	0.0370	99.96	-	
4.3	Control	-	48.4830	48.4665	-0.0165	-	-	Exp. no. 4.1b and 4.1c were incubated for 48 h
5.1a	19.99	-	105.573	106.876	1.303	93.48	-	
5.1b	20.09	-	103.445	104.874	1.429	92.89	-	
5.1c	20.36	-	105.510	106.761	1.251	93.86	-	
5.2.	Control	-	48.907	48.899	-0.008	-	-	
5.3a	19.97	-	103.778	104.894	1.116	94.41	40 + 30 + 45 + 30 minutes	
5.3b	19.82	-	105.546	106.580	1.034	94.78	-	**Protocol 3.**
5.3c	19.97	-	101.745	102.888	1.143	94.28	-	
5.3d	19.73	-	104.371	105.907	1.536	92.21	-	Duration of exp. no. 5.3a included all parallels 5.3a-e
5.3e	19.16	-	103.414	105.077	1.663	91.32	-	
6.1a	19.86	≈ 7	48.7226	48.7526	0.0300	99.85	-	
6.1b	19.84	≈ 7	48.8665	50.0023	1.1358	94.28	-	
6.1c	19.97	≈ 7	48.8360	48.8720	0.0360	99.82	-	
6.2a	19.87	≈ 7	49.0351	49.1088	0.0737	99.63	-	
6.2b	18.49	≈ 7	50.5573	50.5991	0.0418	99.77	-	
6.2c	19.80	≈ 7	-	-	-	-	-	
6.3a	19.83	≈ 7	50.1022	50.1860	0.0838	99.58	-	
6.3b	19.86	≈ 7	48.9160	48.9861	0.0701	99.65	-	
6.3c	19.93	≈ 7	48.4208	48.4683	0.0475	99.76	-	
6.4a	19.95	≈ 7	48.8964	48.9088	0.0124	99.94	-	
6.4b	18.83	≈ 7	49.1603	49.2807	0.1204	99.36	-	
6.4c	18.91	≈ 7	48.7590	48.7909	0.0319	99.83	-	**Optimization 2 factors:** Incubation time and KOH.
7.1.	20.12	≈ 7	48.9226	48.9264	0.0038	99.98	23 s	
7.2a	19.37	≈ 7	48.3602	48.3587	- 0.0015	100.01	14 s	
7.2b	19.93	≈ 7	48.9216	48.9309	0.0093	99.95	64 s	**Optimization 5 factors**: Incubation time, PBSTnT, Triton, Tween20, KOH. Sample **7.12** and **7.14** were not filtrated.
7.2c	20.42	≈ 7	49.7808	49.7976	0.0168	99.92	60 s	
7.3a	19.43	≈ 7	49.4929	49.5388	0.0459	99.76	226 s	

7.3b	20.08	≈ 7	48.9721	48.9925	0.0204	99.90	152 s	
7.4.	19.76	≈ 7	49.0221	49.0474	0.0253	99.87	21 s	
7.5a	19.70	≈ 7	50.0654	50.1221	0.0567	99.71	57 s	
7.5b	20.32	≈ 7	48.9962	49.0424	0.0462	99.77	111 s	
7.6.	21.48	≈ 7	49.1943	49.2229	0.0286	99.87	28 s	
7.7.	19.45	≈ 7	49.5763	49.6926	0.1163	99.40	480 s	
7.8.	19.70	≈ 7	50.0091	50.0888	0.0797	99.60	342 s	
7.9a	19.72	≈ 7	49.4756	49.4894	0.0138	99.93	30 s	
7.9b	18.78	≈ 7	48.6637	48.6977	0.0340	99.82	48 s	
7.10.	20.15	≈ 7	49.1779	49.2138	0.0359	99.82	28 s	
7.11.	25.53	≈ 7	48.6751	48.7549	0.0798	99.69	1500 s	
7.12.	19.88	≈ 7	50.0541	51.3993	1.3452	93.23	6000 s	
7.13.	19.61	≈ 7	49.0114	49.0213	0.0099	99.95	12 s	
7.14.	19.22	≈ 7	49.5671	49.8586	0.2915	98.48	6000 s	
7.15.	19.98	≈ 7	49.8019	49.9410	0.1391	99.30	1050 s	
7.16.	20.33	≈ 7	48.8764	48.9099	0.0335	99.84	170 s	
8.1a	19.19	6.5	48.6330	48.6394	0.0064	99.97	30 s	
8.1b	19.45	6.9	48.9137	48.9467	0.0330	99.83	42 s	
8.1c	20.08	6.6	48.3473	48.3771	0.0298	99.85	14 s	
8.2a	19.77	6.6	50.0394	50.0673	0.0279	99.86	38 s	
8.2b	20.44	6.9	49.7409	49.7740	0.0331	99.84	36 s	
8.2c	21.04	6.3	49.9994	50.2016	0.2022	99.04	593 s	
8.3a	19.65	6.8	48.7986	48.8221	0.0235	99.88	19 s	
8.3b	19.85	6.8	48.6734	48.6925	0.0191	99.90	11 s	
8.3c	19.65	6.8	49.4080	49.5197	0.1117	99.43	20 s	
8.4a	20.71	6.2	48.8374	48.8900	0.0526	99.75	40 s	
8.4b	20.07	5.6	48.7487	48.7604	0.0117	99.94	18 s	**Optimization 2 factors:** Incubation time and Tween20
8.4c	19.72	6.7	49.2382	49.2580	0.0198	99.90	38 s	
9.1a	100.29	6.93	49.4150	49.4543	0.0393	99.96	-	
9.1b	100.07	6.93	48.5282	48.5578	0.0296	99.97	-	**Robustness test for digestion of fish tissue:** Salmon. Sample **9.1c** was not filtrated.
9.1c	100.20	4.98	48.8051	49.0569	0.2518	-	-	
9.2.	Control	-	48.9083	48.9094	0.0011	-	70 s	
9.3a	100.53	6.36	48.5264	48.5435	0.0171	99.98	102 s	
9.3b	100.67	6.53	49.2825	49.3161	0.0336	99.97	106 s	
9.3c	100.63	6.36	49.1325	49.1400	0.0075	99.99	159 s	**Robustness test for digestion of fish tissue:** Haddock
9.4.	Control	-	49.9626	49.9606	-0.0020	-	200 s	
9.5a	100.21	6.07	48.5768	48.6202	0.0434	99.96	180 s	
9.5b	99.38	6.85	48.5362	48.5645	0.0283	99.97	82 s	
9.5c	99.32	6.81	48.8649	48.9060	0.0411	99.96	146 s	**Robustness test for digestion of fish tissue:** Mackerel
9.6.	Control	-	50.3335	50.3319	-0.0016	-	113 s	
10.1.	-	3.16	-	-	-	-	26 s	

10.2.	-	7.40	-	-	-	-	27 s	**pH investigation:** Titration with HCl. Filtration of negative control using 10-16 µm pore size crucibles
10.3.	-	10.64	-	-	-	-	32 s	
10.4.	-	4.80	-	-	-	-	16 s	**pH investigation**: Titration with citric acid. Filtration of negative control using 10-16 µm pore size crucibles
10.5.	-	7.31	-	-	-	-	16 s	
10.6.	-	10.69	-	-	-	-	29 s	
10.7.	-	1.69	-	-	-	-	344 s	**pH investigation:** Titration with HCl. Filtration of negative control using 4-5 µm pore size crucibles
10.8.	-	6.90	-	-	-	-	394 s	
10.9.	-	11.95	-	-	-	-	360-480 s	
10.10.	-	4.52	-	-	-	-	343 s	**pH investigation:** Titration with citric acid. Filtration of negative control using 4-5 µm pore size crucibles
10.11.	-	6.50	-	-	-	-	274 s	
10.12.	-	11.35	-	-	-	-	418 s	
11.1.	20.77	10.07	48.9332	48.9765	0.0433	99.79	63 s	**pH investigation:** Titration with HCl. Salmon matrix
11.2.	19.80	9.26	50.0211	50.0596	0.0385	99.81	42 s	
11.3.	18.30	8.32	49.2098	49.2298	0.0200	99.89	82 s	
11.4.	20.65	7.26	49.5071	49.5102	0.0031	99.98	36 s	
11.5.	19.84	5.82	48.3774	48.3788	0.0014	99.99	16 s	
11.6.	19.73	5.15	49.8219	49.8605	0.0386	99.80	139 s	
11.7.	19.67	4.16	50.0792	50.2293	0.1501	99.24	941 s	
11.8.	20.56	10.13	48.7026	48.7706	0.0680	99.67	11 s	**pH investigation:** Titration citric acid. Salmon matrix
11.9.	19.54	8.96	49.3189	49.3573	0.0384	99.80	27 s	
11.10.	19.83	8.13	48.8891	48.9228	0.0337	99.83	20 s	
11.11.	19.43	7.27	48.6879	48.6986	0.0107	99.94	20 s	
11.12.	20.26	6.04	49.5879	49.5962	0.0083	99.96	57 s	
11.13.	-	5.14	-	-	-	-	-	
11.14.	-	4.12	-	-	-	-	-	

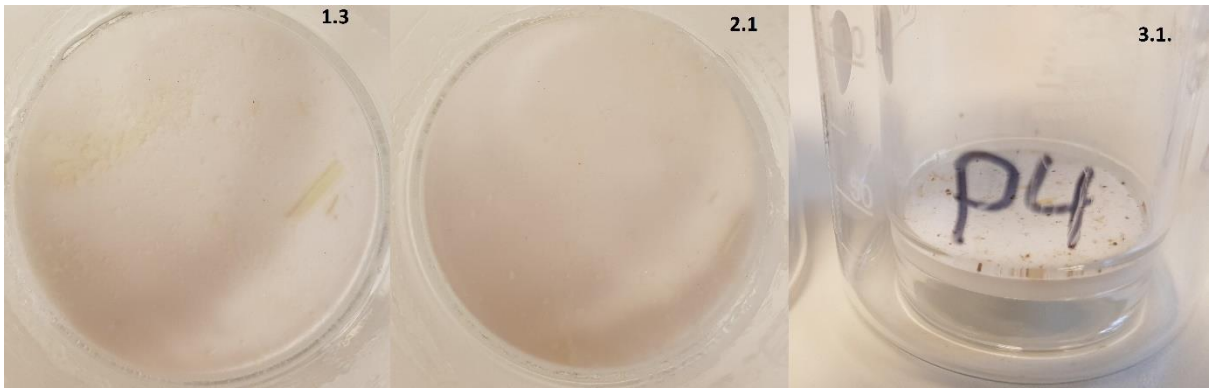
B-2: Rank for experiments carried out in optimization with 5 factors

Experiment	Rank digestion efficiency	Rank filtration time	Experiment	Rank DE with new parallels	Rank FT with new parallels	Protocol description
7.1.	15	13	**7.1.**	16	13	**Optimization 5 factors:** Rank for experiments 7.1-7.16. Effect of factors were calculated for the first 16 experiments and then with 7.2b, 7.3b, 7.5b and 7.9b instead of 7.2a, 7.3a, 7.5a and 7.9a, respectively.
7.2a	16	15	**7.2b**	14	15	
7.3a	8	7	**7.3b**	13	8	
7.4.	12	14	**7.4.**	12	14	
7.5a	7	9	**7.5b**	7	9	
7.6.	11	11.5	**7.6.**	11	11.5	
7.7.	4	5	**7.7.**	4	5	
7.8.	6	6	**7.8.**	6	6	
7.9a	13	10	**7.9b**	9	10	
7.10.	9	11.5	**7.10.**	8	11.5	
7.11.	5	3	**7.11.**	5	3	
7.12.	1.5	1.5	**7.12.**	1.5	1.5	
7.13.	14	16	**7.13.**	15	16	
7.14.	1.5	1.5	**7.14.**	1.5	1.5	
7.15.	3	4	**7.15.**	3	4	
7.16.	10	8	**7.16.**	10	7	

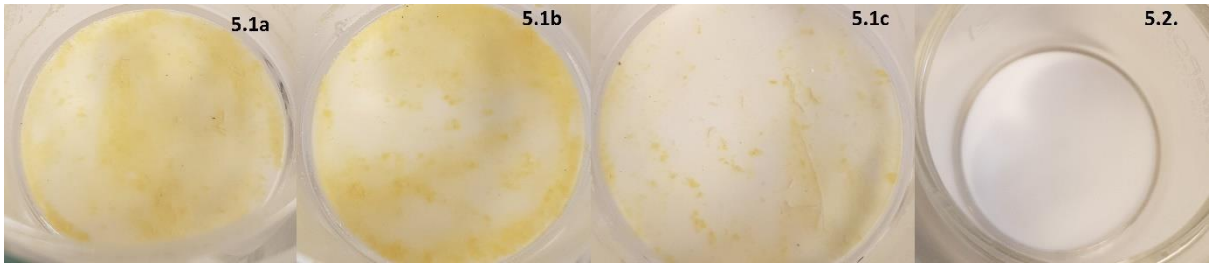
B-3: Results for experiments involving spiked MPs

Exp. No.	pH	*n* BP	*n* AP	MPs BP [g]	MPs AP [g]	Mass difference [g]	Mass loss [%]	Mean and SD	Protocol description
12.1.	<4	3	3	0.0070	0.0070	0.0000	0.00	-	**pH investigation:** Titration with HCl. LDPE spiked controls
12.2.	7	3	3	0.0074	0.0074	0.0000	0.00	-	
12.3.	>10	3	3	0.0065	0.0064	-0.0001	-1.56	-	
12.4.	<4	3	3	0.0078	0.0078	0.0000	0.00	-	**pH investigation**: Titration with citric acid. LDPE spiked controls
12.5.	7	3	3	0.0119	0.0120	0.0001	0.83	-	
12.6.	>10	3	3	0.00640	0.00640	0.0000	0	-	
13.1a	-	4	4	0.10053	0.10071	-0.00018	-0.18	0.15 (0.31)	**Damage evaluation MPs**: HDPE
13.1b	-	4	4	0.09205	0.09165	0.00040	0.43		
13.1c	-	4	4	0.09897	0.09877	0.00020	0.2		
13.2a	-	5	5	0.09354	0.09356	-0.00002	-0.02	0.10 (0.11)	**Damage evaluation MPs:** LDPE
13.2b	-	5	5	0.09155	0.09143	0.00012	0.13		
13.2c	-	6	6	0.10645	0.10625	0.00020	0.19		
13.3a	-	7	6	0.09395	0.08078	0.01317	14.02	0.01 (0.02)	**Damage evaluation MPs:** PA-66
13.3b	-	7	7	0.09237	0.09235	0.00002	0.02		
13.3c	-	7	7	0.10176	0.10177	-0.00001	-0.01		
13.4a	-	4	4	0.09145	0.09125	0.00020	0.22	0.48 (0.29)	**Damage evaluation MPs:** PC
13.4b	-	4	4	0.09203	0.09130	0.00073	0.79		
13.4c	-	4	4	0.09290	0.09251	0.00039	0.42		
13.5a	-	6	6	0.10441	0.10416	0.00025	0.24	0.27 (0.21)	**Damage evaluation MPs:** PET
13.5b	-	6	6	0.09614	0.09566	0.00048	0.5		
13.5c	-	6	6	0.10077	0.10069	0.00008	0.08		
13.6a	-	5	5	0.09546	0.09494	0.00052	0.54	0.40 (0.17)	**Damage evaluation MPs:** PMMA
13.6b	-	5	5	0.09651	0.09631	0.00020	0.21		
13.6c	-	6	6	0.11387	0.11341	0.00046	0.4		
13.7a	-	7	7	0.09486	0.09468	0.00018	0.19	0.15 (0.16)	**Damage evaluation MPs:** PP
13.7b	-	5	5	0.09351	0.09353	-0.00002	-0.02		
13.7c	-	7	7	0.09588	0.09560	0.00028	0.29		
13.8a	-	5	5	0.09473	0.09455	0.00018	0.19	0.01 (0.14)	**Damage evaluation MPs:** PS
13.8b	-	5	5	0.09226	0.09234	-0.00008	-0.09		
13.8c	-	5	5	0.09797	0.09790	0.00007	0.07		

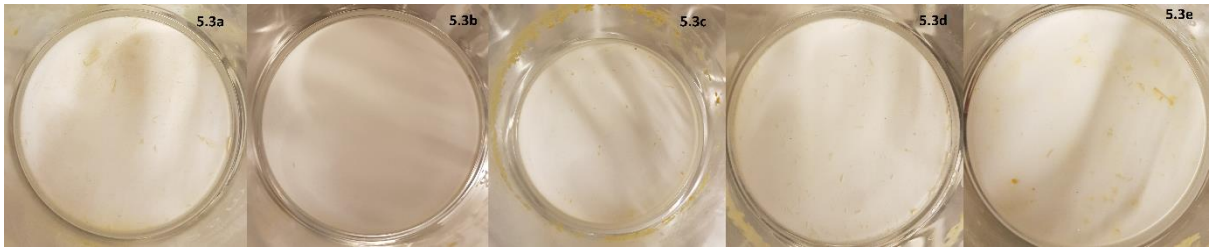
Appendix C: Pictures of crucibles and Erlenmeyer flasks



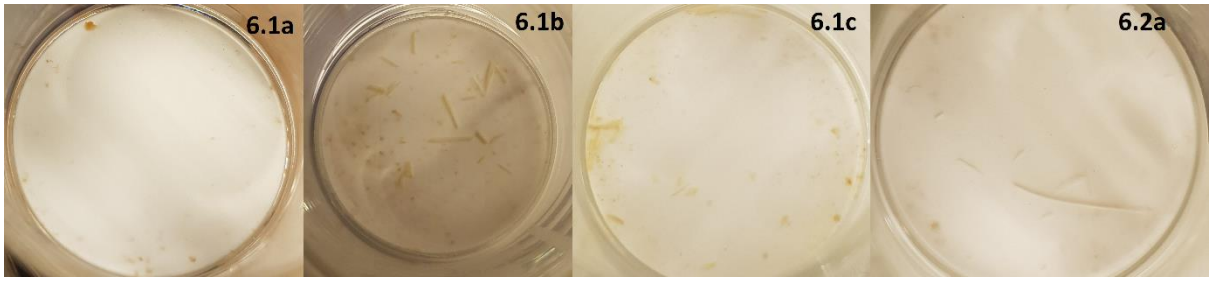
*C-1: Crucibles for samples **1.3, 2.1** and **3.1**.*



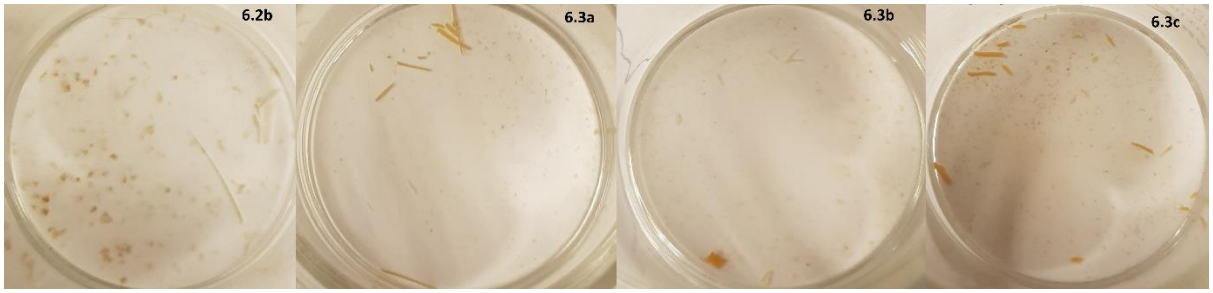
*C-2: Crucibles for samples **5.1a-c** and **5.2***



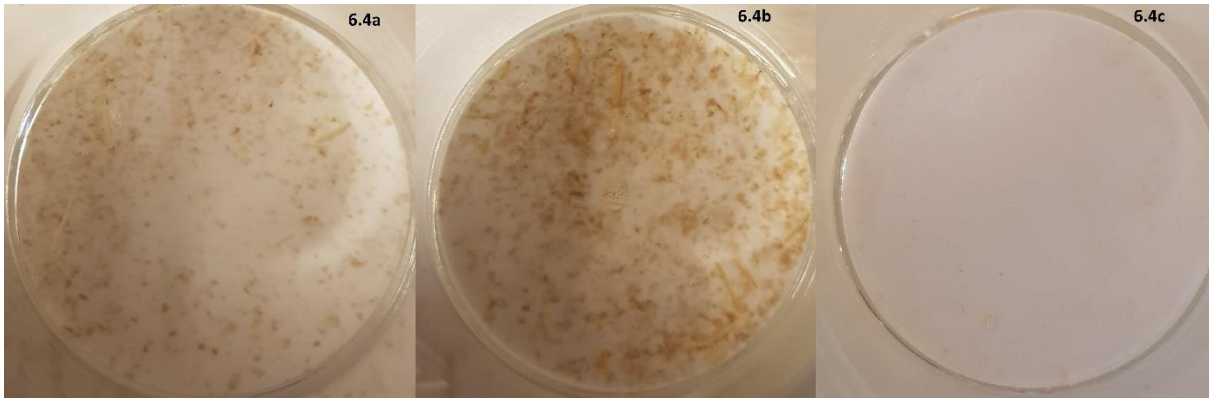
*C-3: Crucibles for samples **5.3a-e***



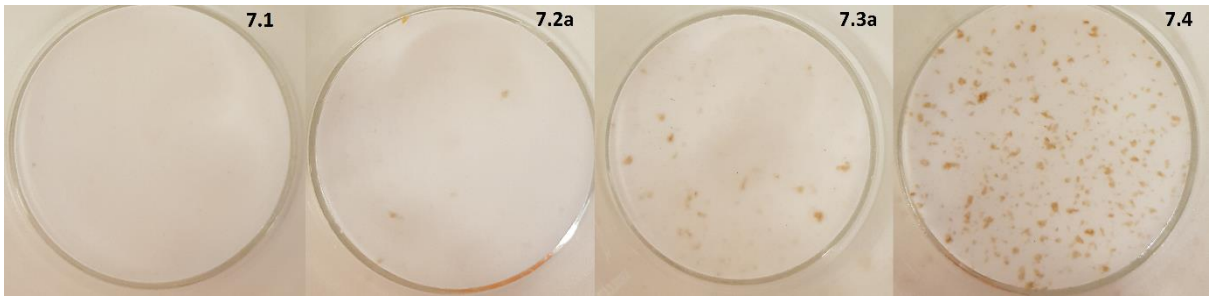
*C-4: Crucibles for samples **6.1a-c** and **6.2a***



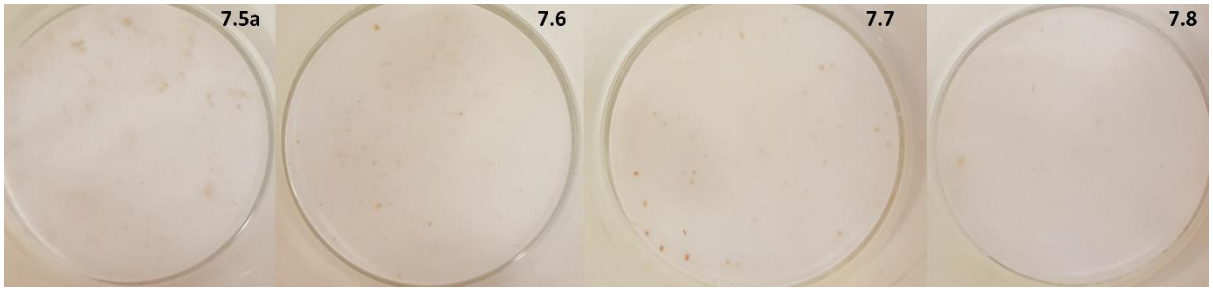
*C-5: Crucibles for samples **6.2b** and **6.3a-c***



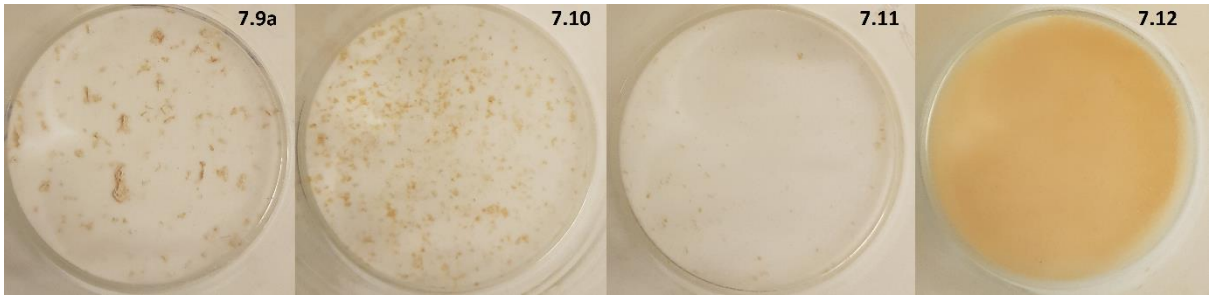
*C-6: Crucibles for samples **6.4a-c**.*



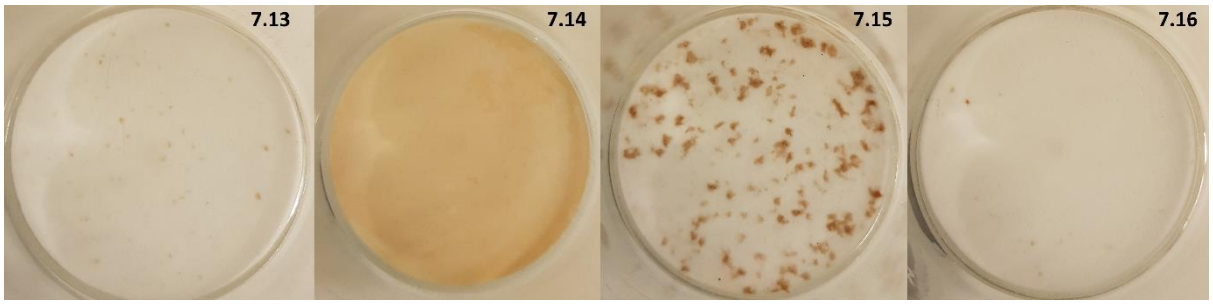
*C-7: Crucibles for samples **7.1**, **7.2a**, **7.3a** and **7.4**.*



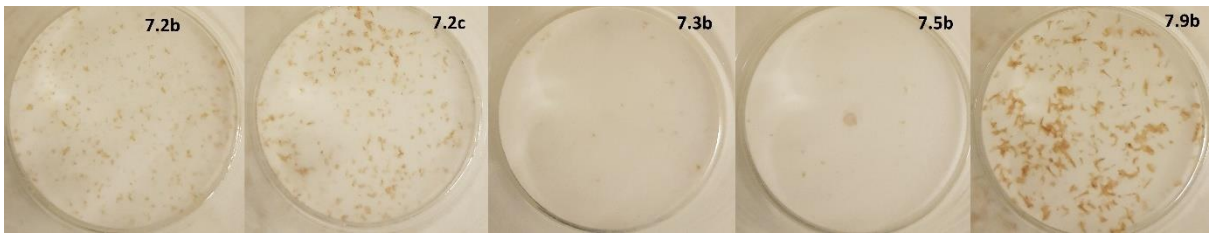
*C-8: Crucibles for samples **7.5a**, **7.6**, **7.7**. and **7.8***



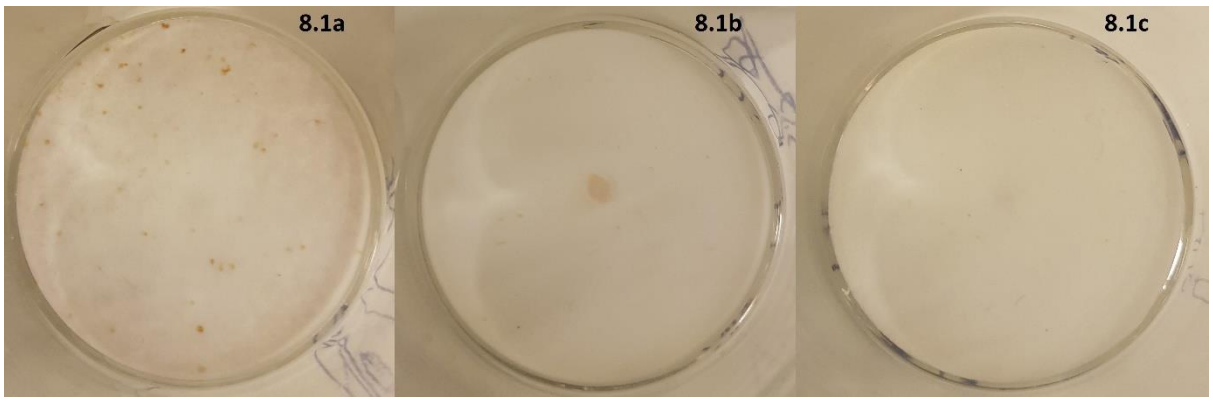
*C-9: Crucibles for samples **7.9a**, **7.10**, **7.11** and **7.12**.*



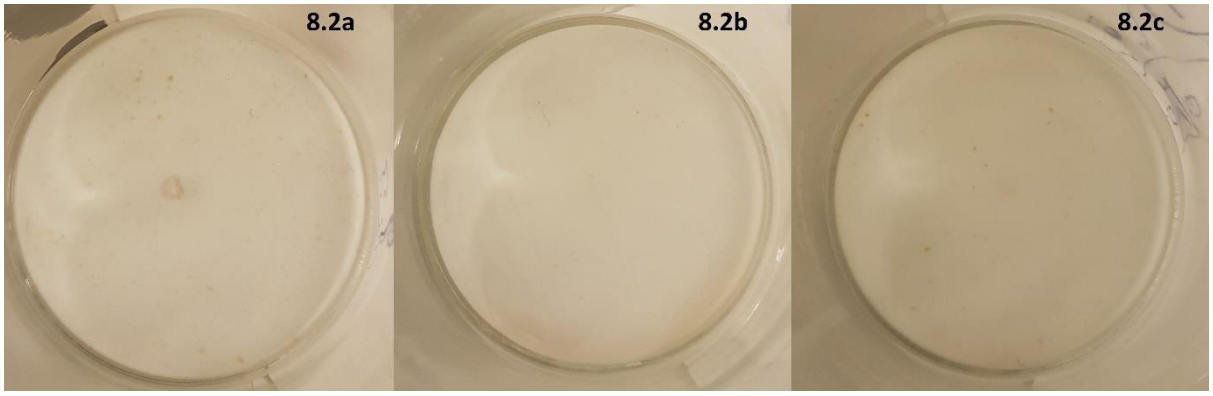
*C-10: Crucibles for samples **7.13**, **7.14**, **7.15** and **7.16**.*



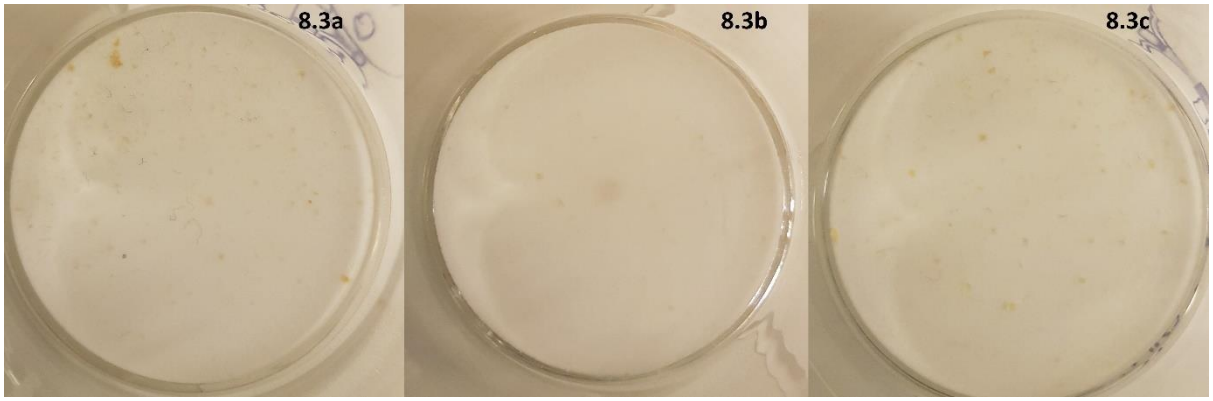
*C-11: Crucibles for samples **7.2b**, **7.2c**, **7.3b**, **7.5b** and **7.9b**.*



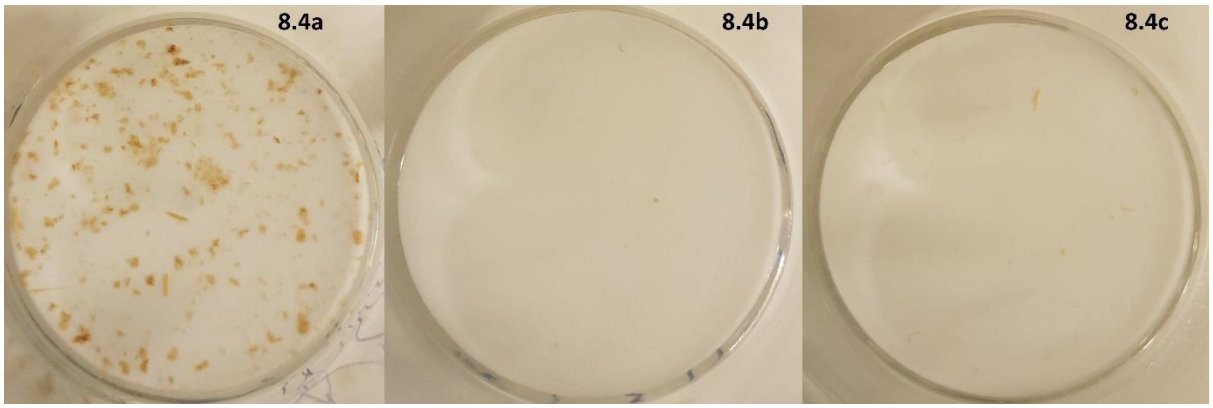
*C-12: Crucibles for samples **8.1a-c***



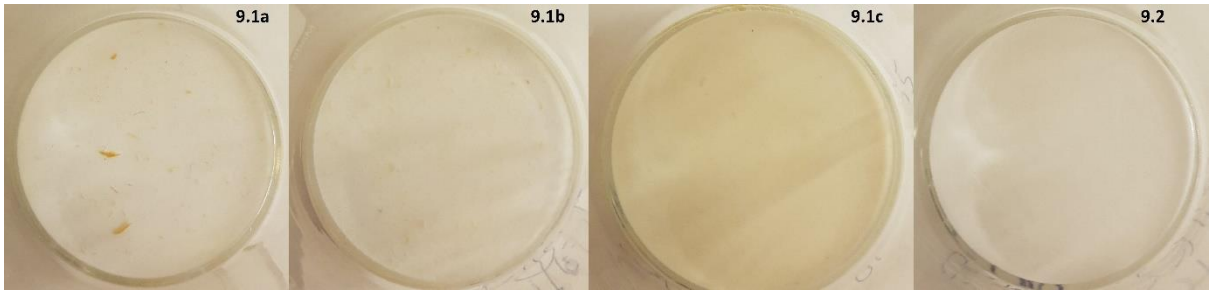
*C-13: Crucibles for samples **8.2a-c***



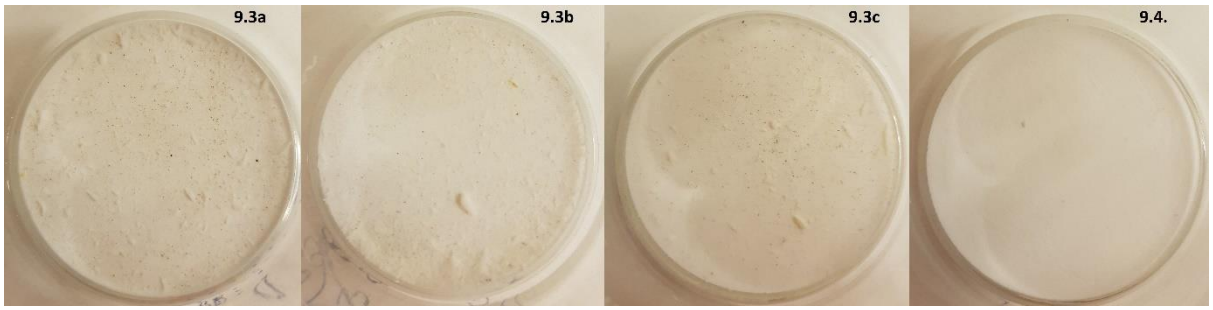
*C-14: Crucibles for samples **8.3a-c***



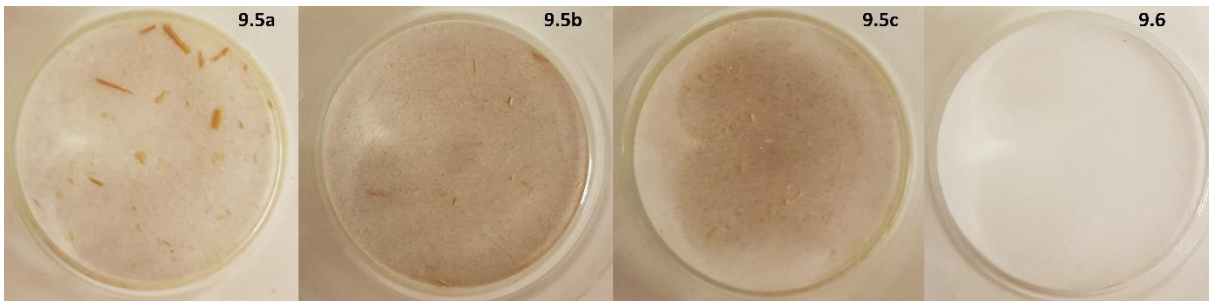
*C-15: Crucibles for samples **8.4a-c***



*C-16: Crucibles for samples **9.1a-c** (Salmon) and **9.2** (control)*

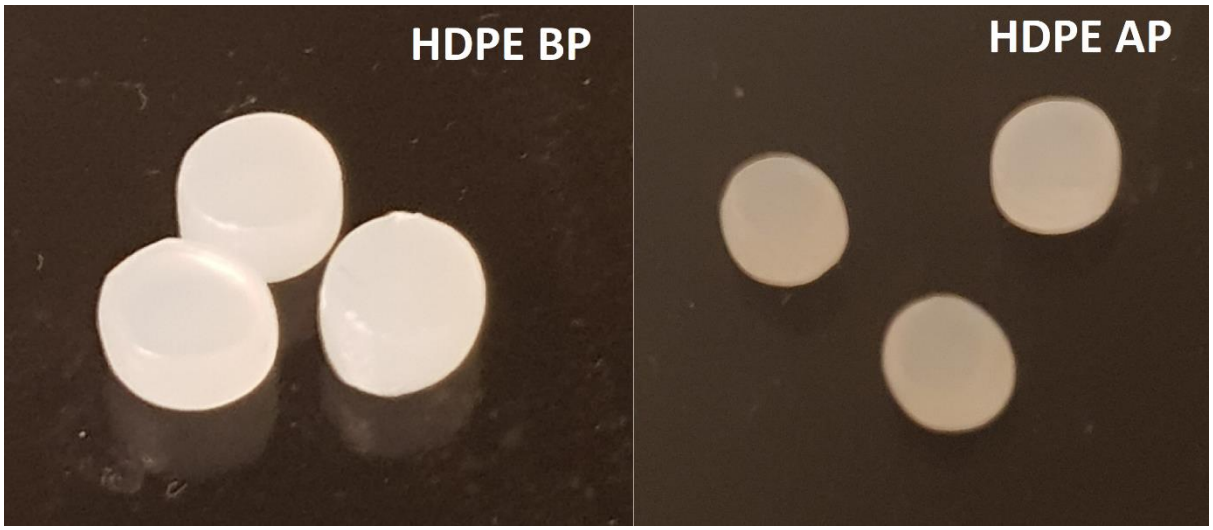


*C-17: Crucibles for samples **9.3a-c** (Haddock) and **9.4** (control)*

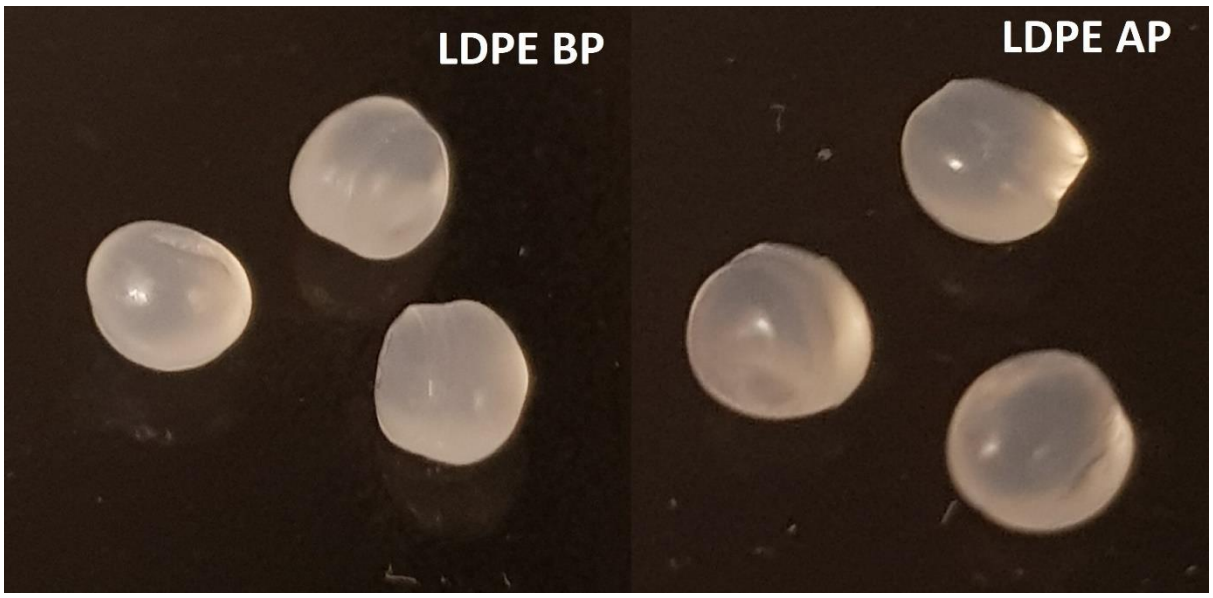


*C-18: Crucibles for samples **9.5a-c** (Mackerel) and **9.6** (control)*

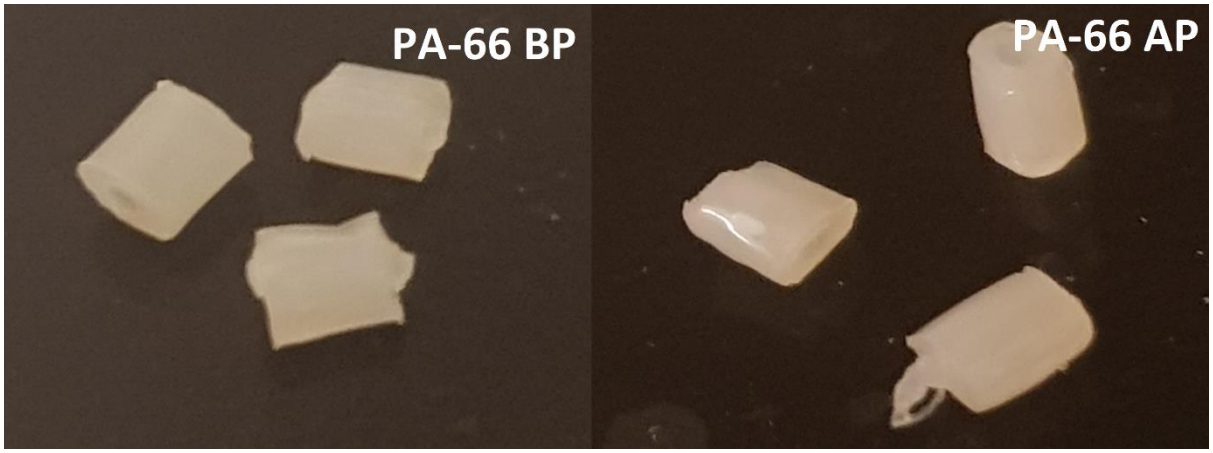
Appendix D: Damage evaluation of MPs



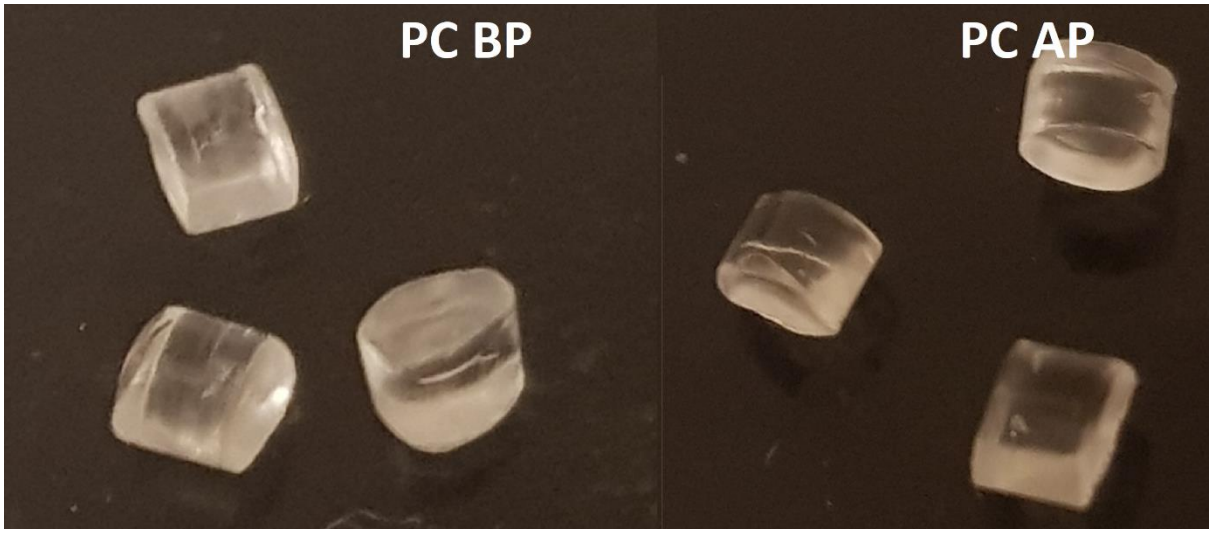
D-1: HDPE BP and AP



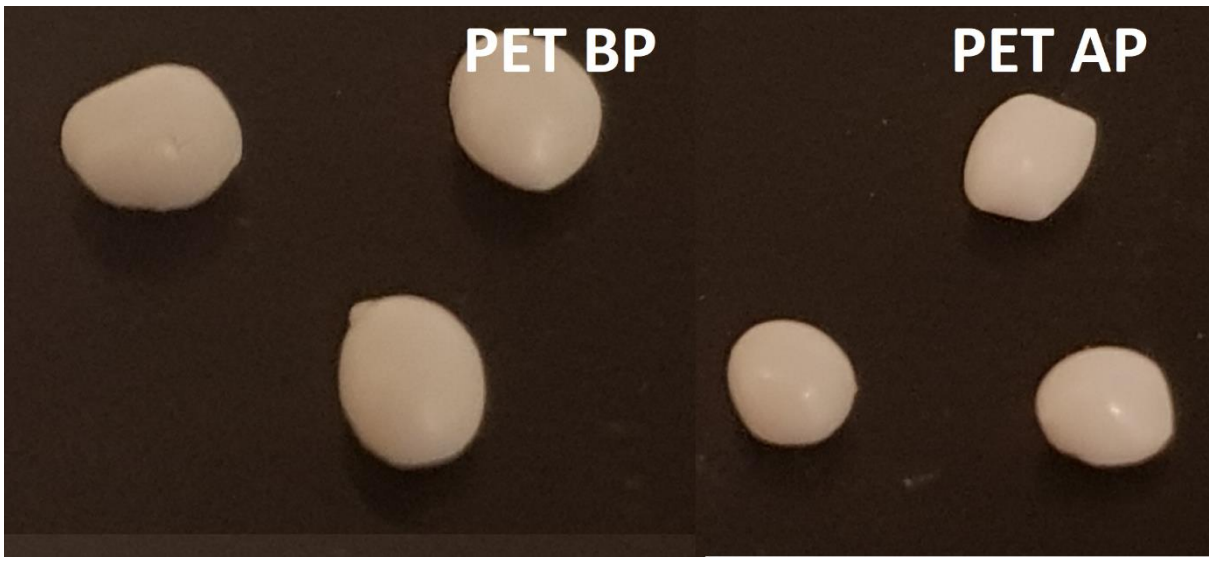
D-2: LDPE BP and AP



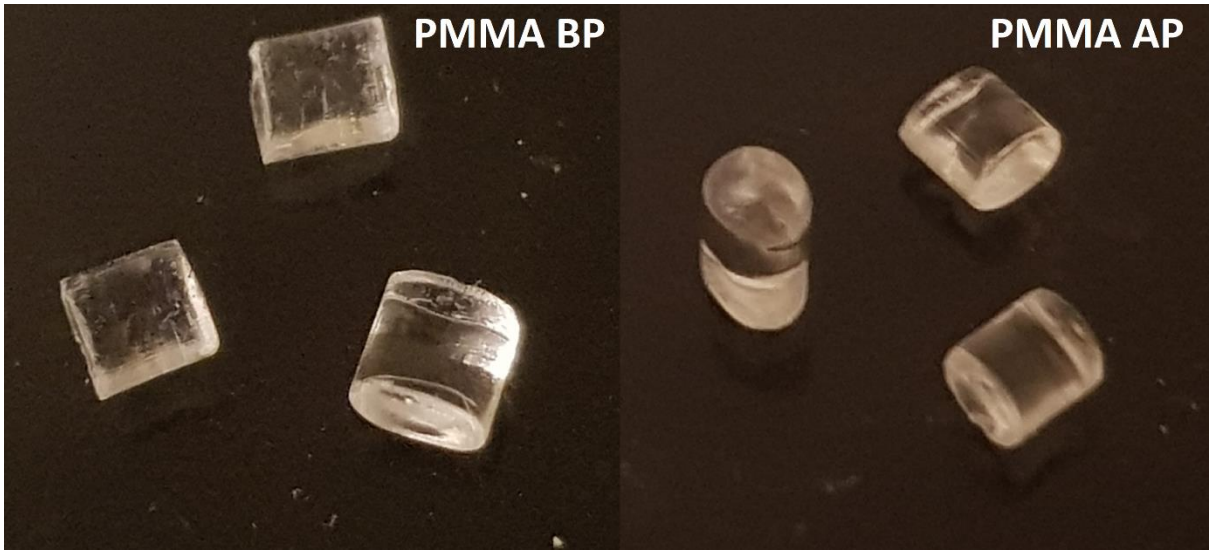
D-3: PA-66 BP and AP



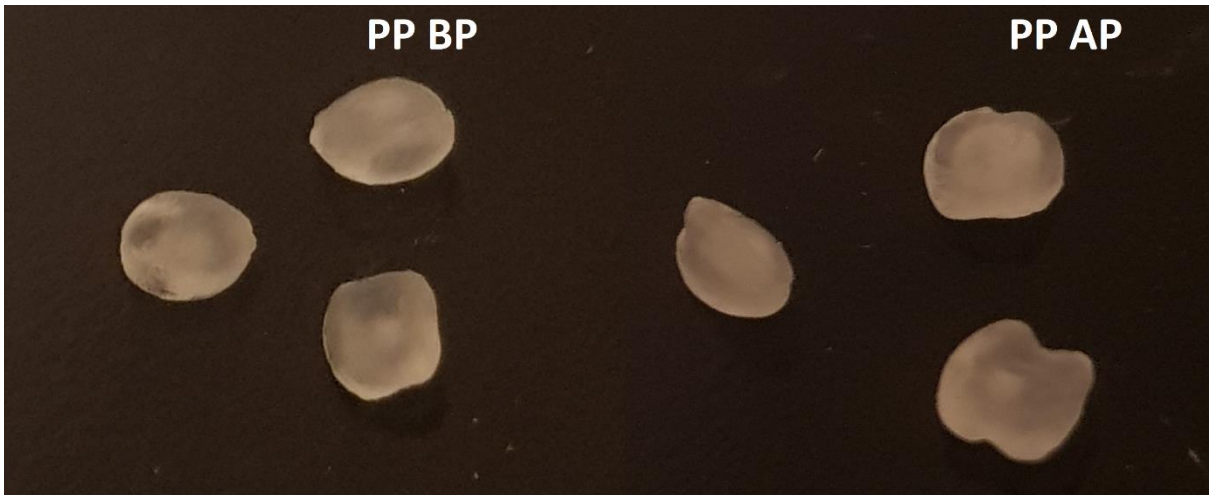
D-4: PC BP and AP



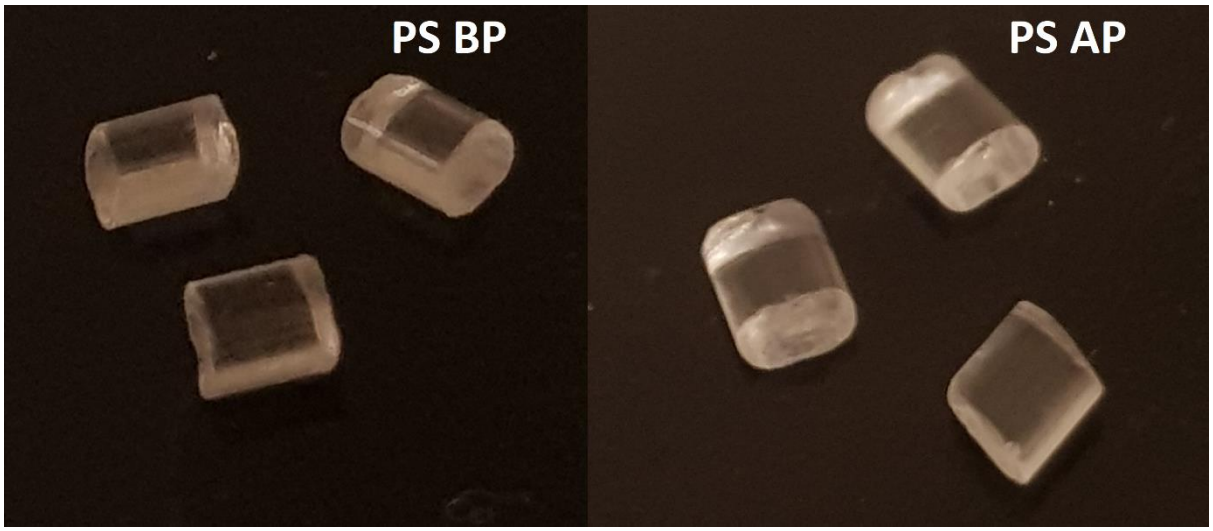
D-5: PET BP and AP



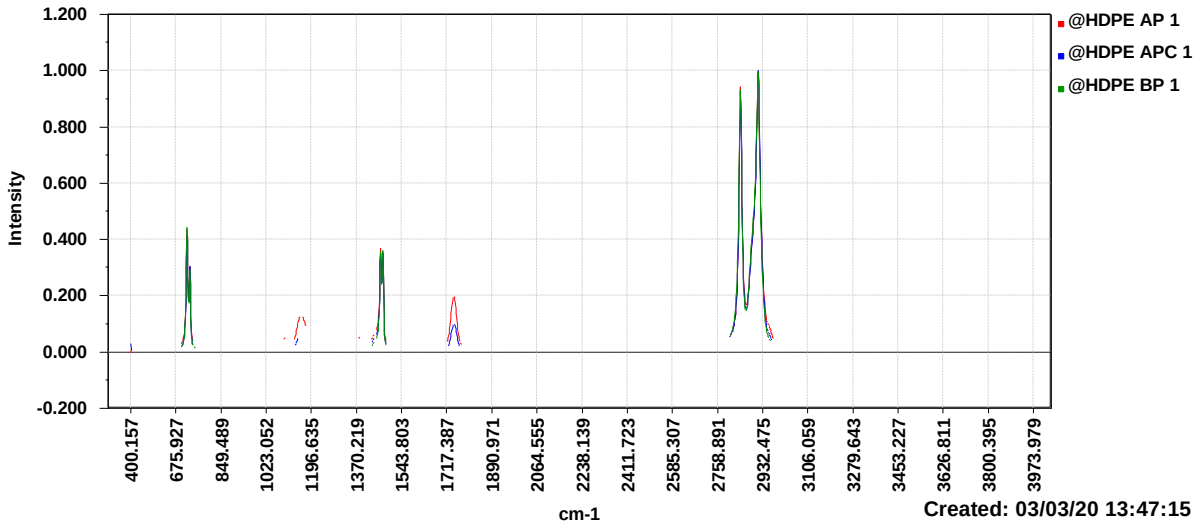
D-6: PMMA BP and AP



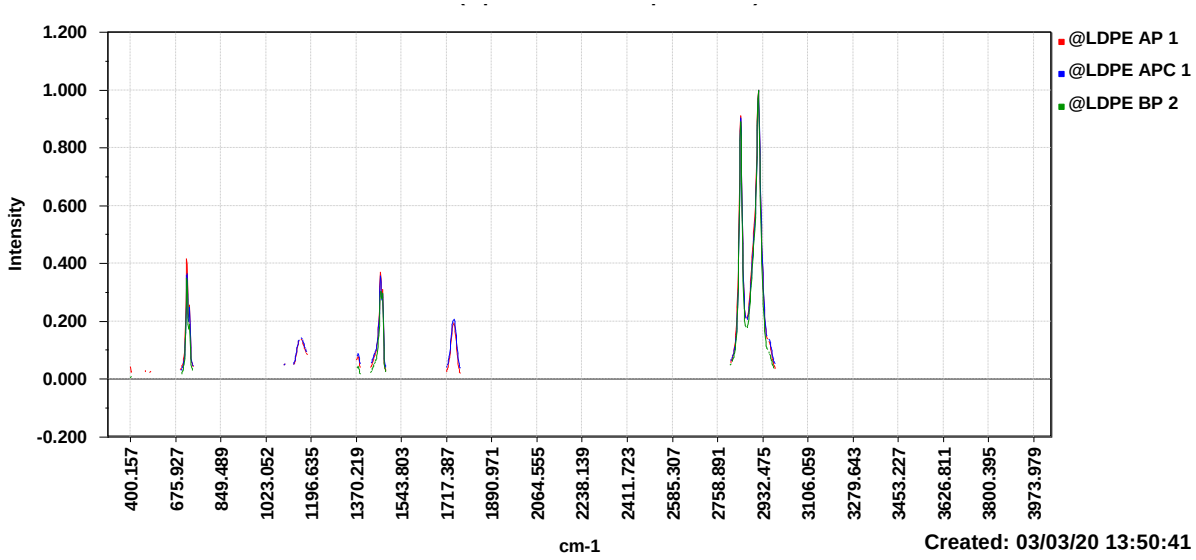
D-7: PP BP and AP



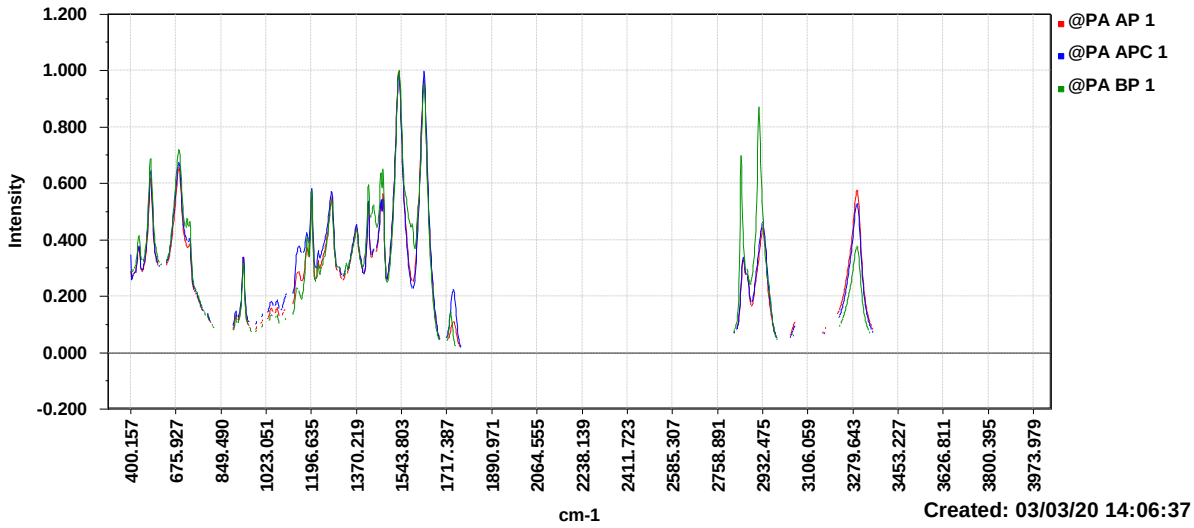
D-8: PS BP and AP



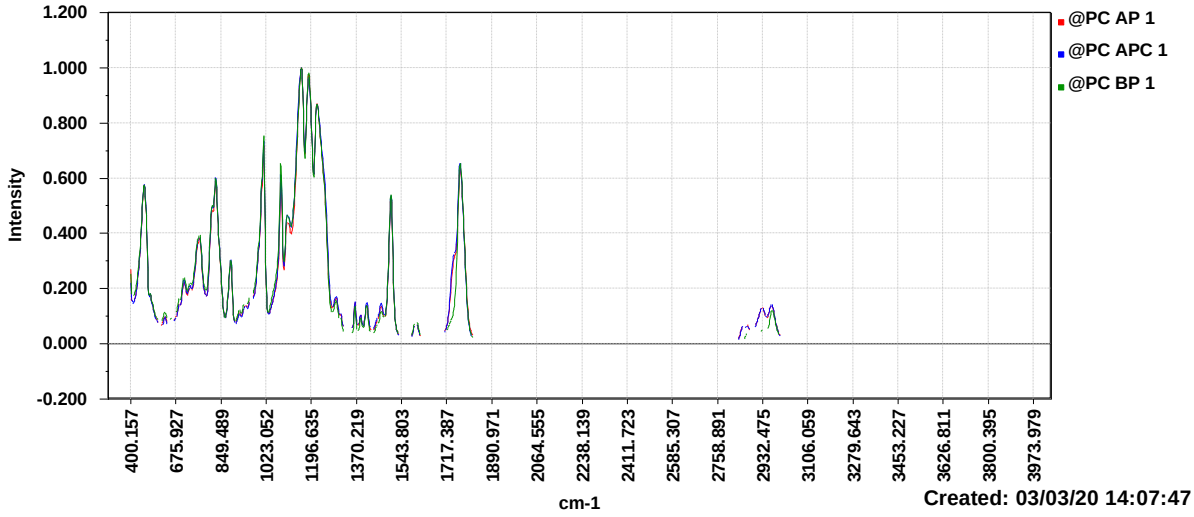
D-9: FTIR spectra of HDPE BP (green), AP (red) and APC (blue)



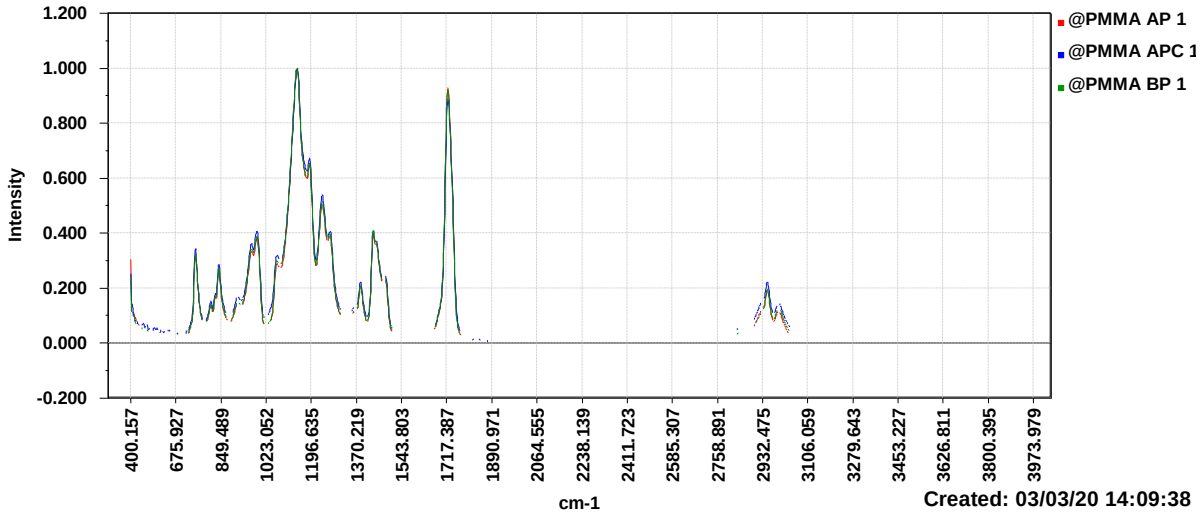
D-10: FTIR spectra of LDPE BP (green), AP (red) and APC (blue)



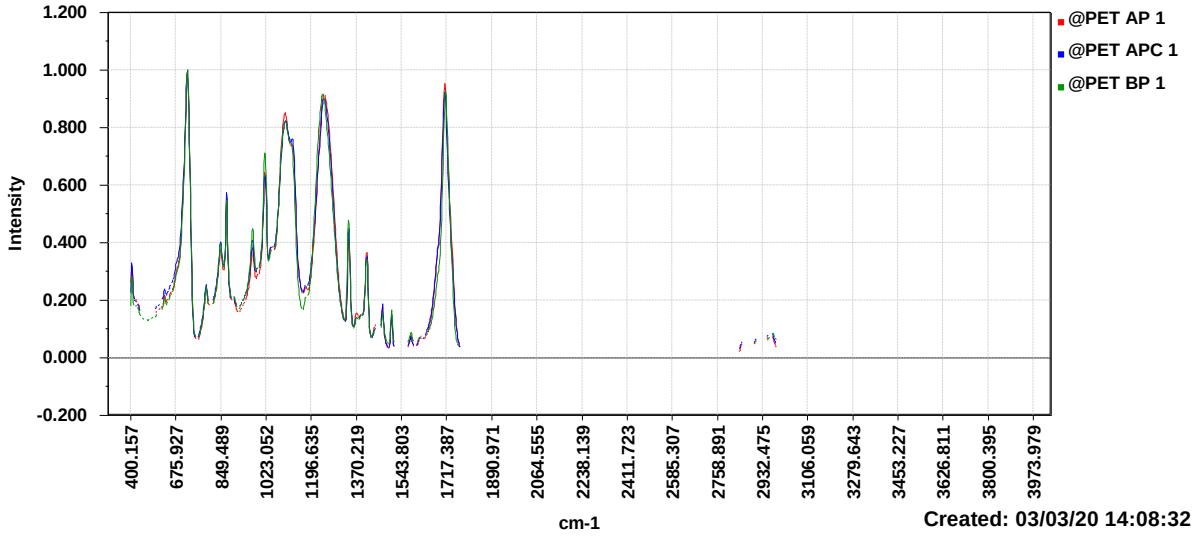
D-11: FTIR spectra of PA-66 BP (green), AP (red) and APC (blue)



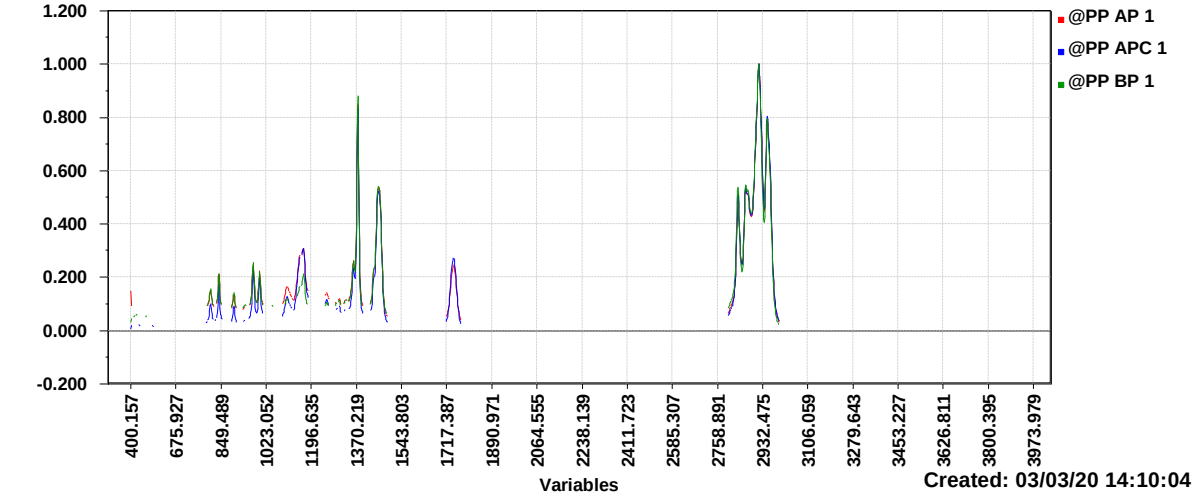
D-12: FTIR spectra of PC BP (green), AP (red) and APC (blue)



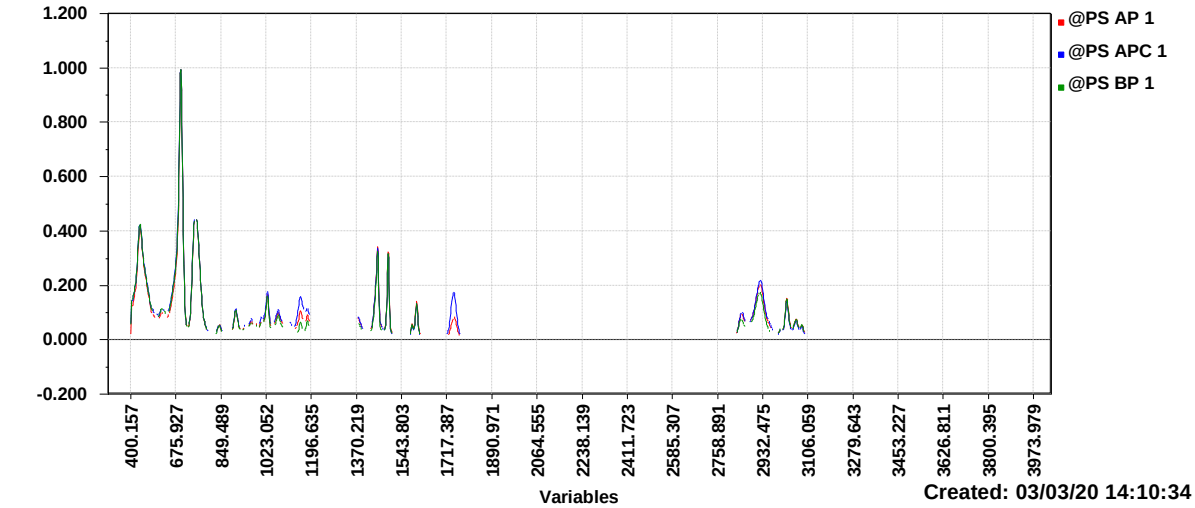
D-13: FTIR spectra of PMMA BP (green), AP (red) and APC (blue)



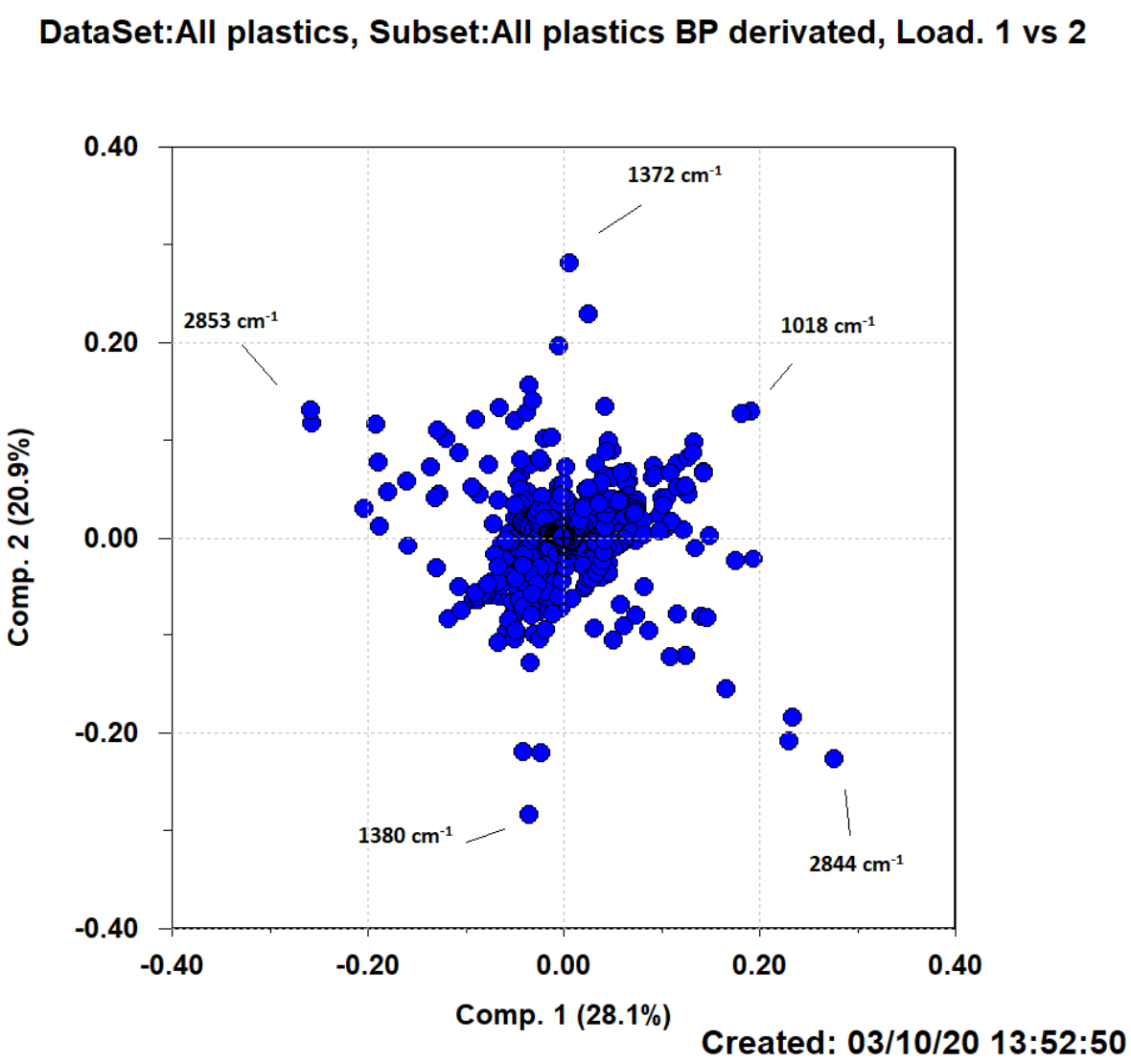
D-14: FTIR spectra of PET BP (green), AP (red) and APC (blue)



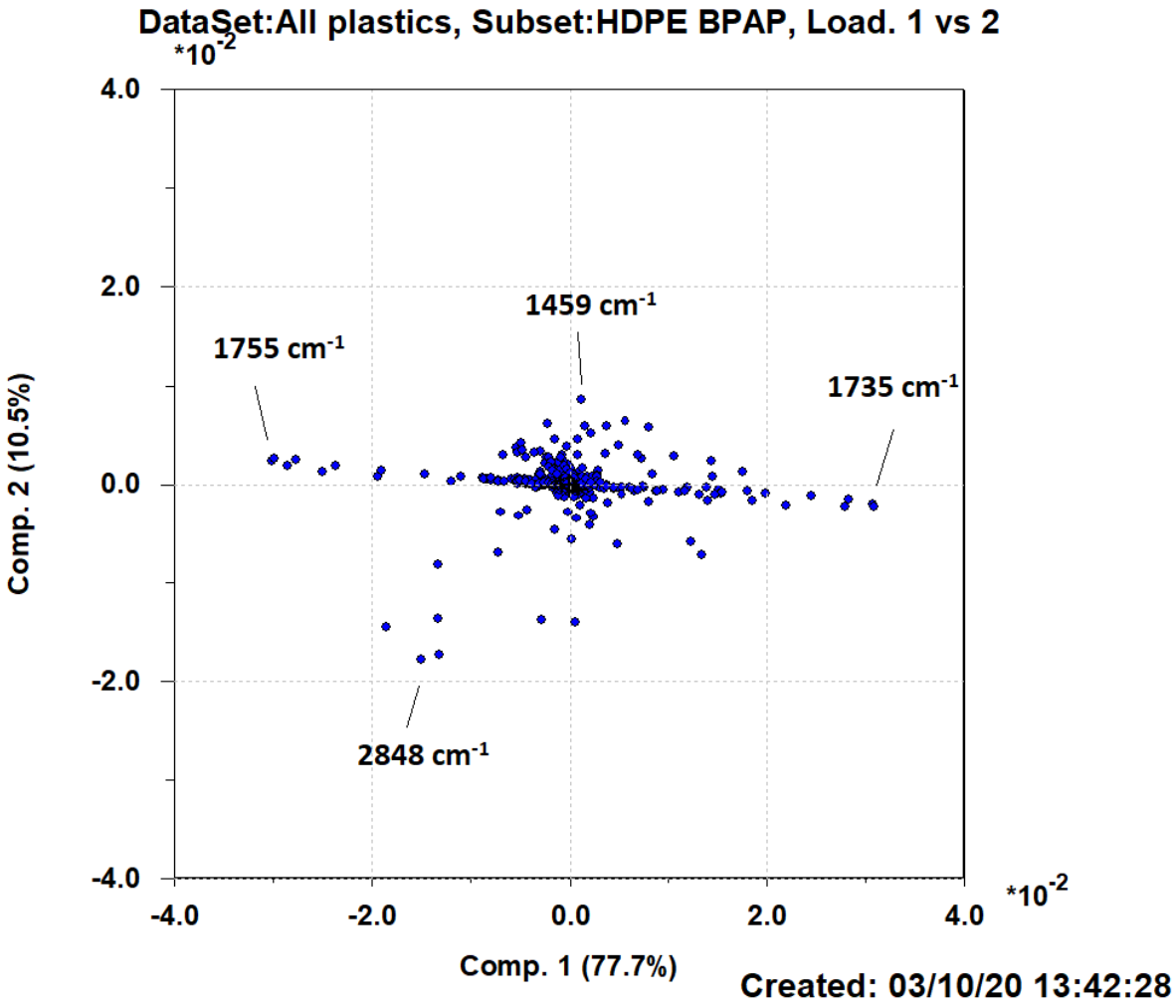
D-15: FTIR spectra of PP BP (green), AP (red) and APC (blue)



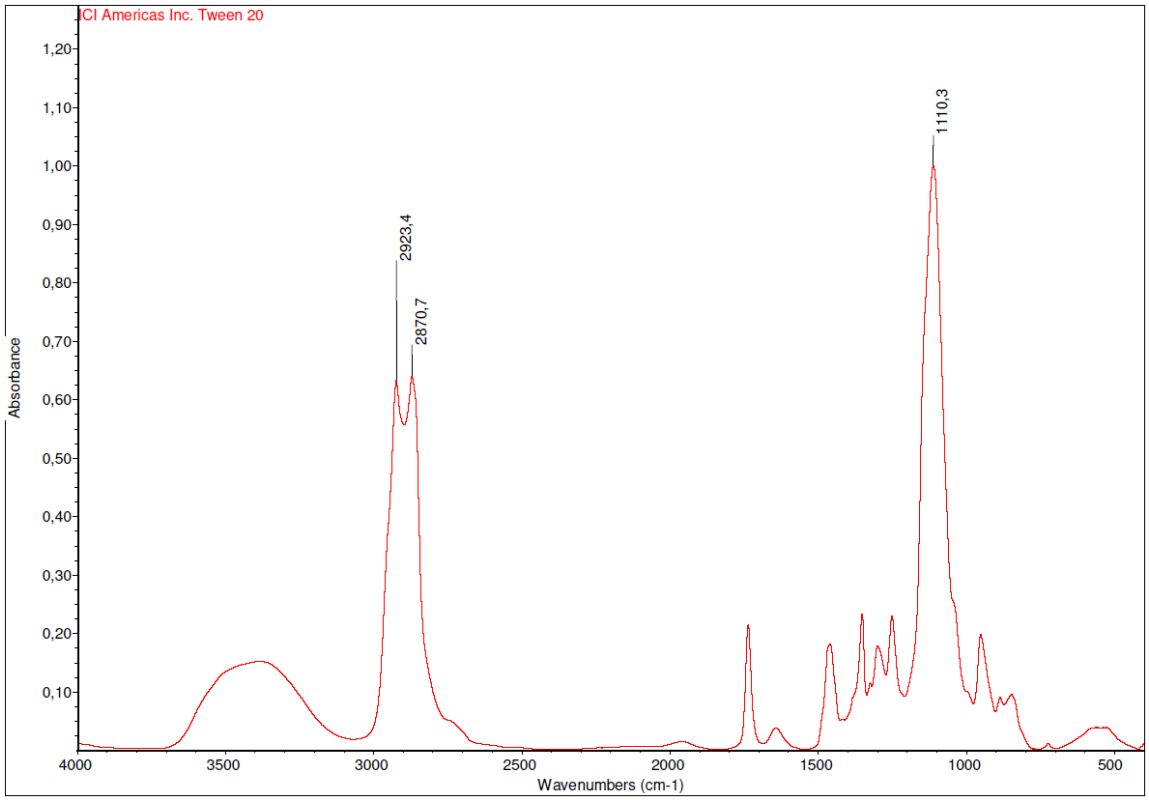
D-16: FTIR spectra of PS BP (green), AP (red) and APC (blue)



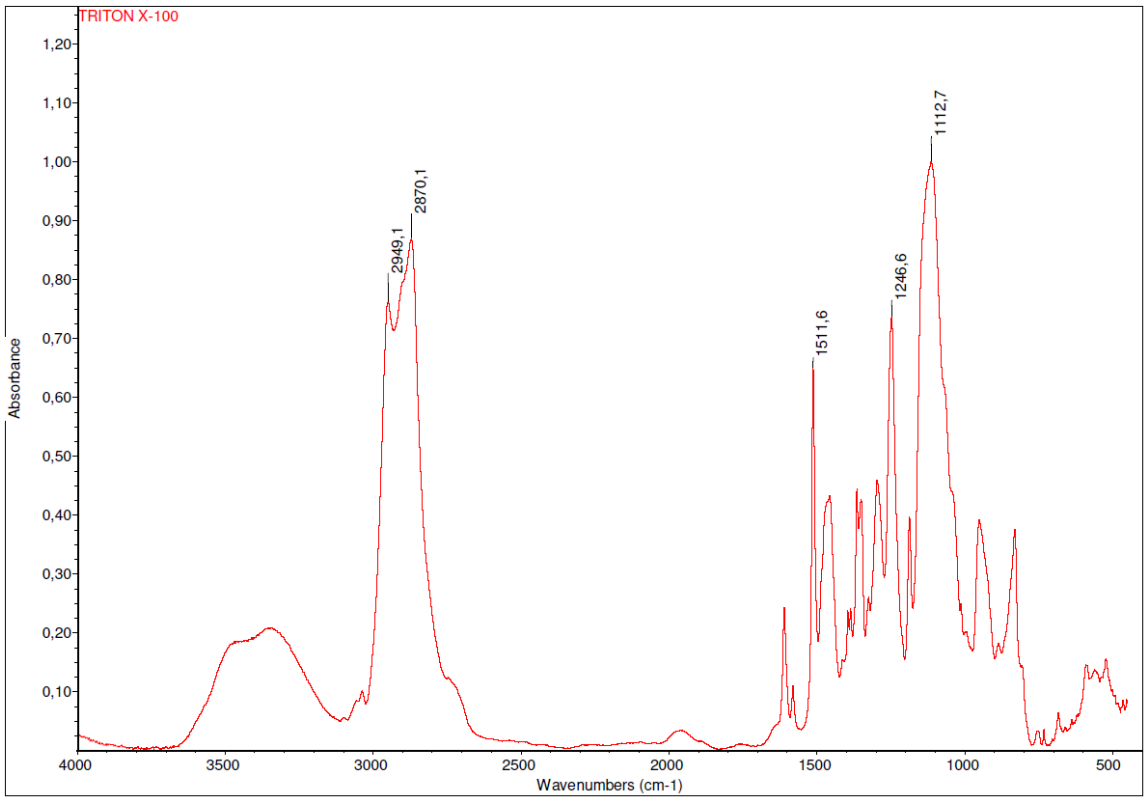
D-17: PCA Loadings plot for the PCA score plot shown in Figure 23.



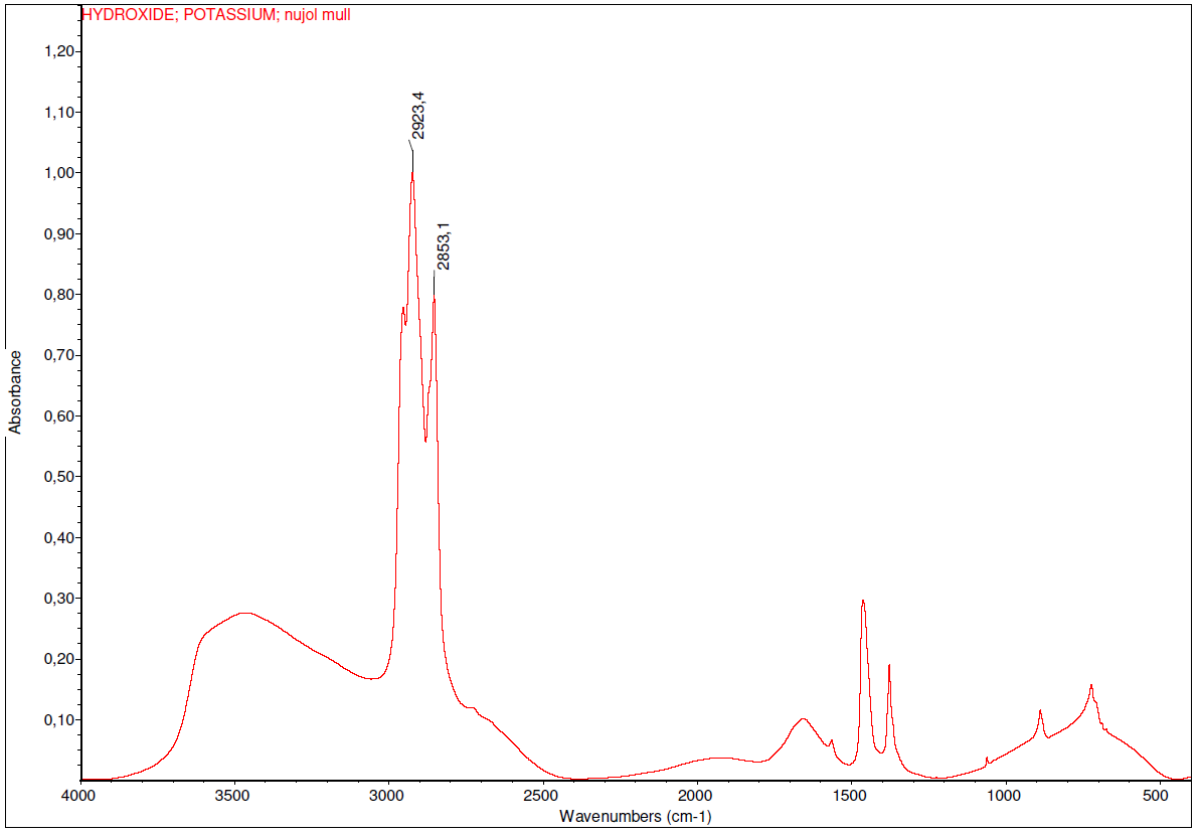
D-18: PCA Loadings plot from the PCA score plot in Figure 24.



D-19: FTIR spectrum of Tween20®



D-20: FTIR spectrum of Triton™ X-100



D-21: FTIR spectrum of KOH