Abstract

Plastic pollution has emerged as a pressing global issue, with microplastics (MP), plastics smaller than 5mm, being particularly present in marine environments. Reliable methods are paramount to assess and monitor MP contamination in seabirds. Sound monitoring programs and the acquisition of robust data are the basis of science-based decision- and policymaking. Regurgitated pellets from European shags (*Gulosus aristotelis*), a sentinel species, are routinely investigated for diet analyses. This thesis introduces an additional analysis for studying MPs from the same samples that are used for diet analyses. In this study, I developed a tool that can be integrated into existing monitoring initiatives and provides insight into levels of local MP pollution.

Samples were collected at Sklinna, the most important seabird breeding site in central Norway. Several methods inspired by sediment analysis, including both density-dependent and density-independent approaches, were tested with pellets. A protocol was established and optimized for pellet analysis, which allowed for both otolith extraction for diet analysis and MP analysis. The final proposed protocol is non-invasive to the study species, as sampling of pellets leads only to minor disturbance, and the methods chosen in the laboratory are not harmful to the environment. The protocol was tested with artificial MP spikes, yielding a high recovery rate of 83%. This was followed up by a case study with original samples (N=10), of which 40% contained MPs. These were identified as polypropylene (PP) with a Raman spectroscope. 83% of MPs found (N=5) can be classified as fibers of a similar grey color, whereas 17% (N=1) were transparent fragments. The extracted otoliths (N=403) were measured and identified. In total, 94% of otoliths stem from Gadidae, for which the length, mass, and age-class were calculated. The majority of Gadidae were younger than 1 year (N=270) or 1-2 years old (N=124). However, due to the limited sample size, no link between diet and MP contamination could be established.

This study is the first in Norway to report MP pollution in pellets from European shags, thereby providing an insight into the marine MP pollution of Mid-Norway. Through further development of this study, future research will be more adept at comprehending the impacts of MPs on shags and other regurgitating seabird species.

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Preface

The ability to gather and process samples, and extract and analyze data is the basis of biological research. Unfortunately, like many students of my generation, the opportunities during my bachelor's studies were incredibly limited by pandemic restrictions. To catch up on these skills and experiences, I sought a Master's project that would allow me a hands-on approach, both in the field and the lab. My awareness of society's overuse of plastic products was first roused when I encountered the zero-waste movement in Hamburg, Germany. My interest in researching plastic waste, subsequent plastic pollution, and its impact on marine environments was amplified by living in that densely populated harbor metropolis during my time as a bachelor student.

I moved to Norway intending to research the impact of plastic pollution on nature and was delighted to find a project that aligned with all my wishes and interests. I have a background in general biology, but I enjoyed learning about environmental toxicology, interacting with my study species, and developing a methodology for future research.

This thesis was written to attain a Master of Science degree in Natural Resource Management (NARM) at the Institute of Biology (IBI) at the Norwegian University of Science and Technology (NTNU). The NARM program is dedicated to interdisciplinary and solution-oriented knowledge exchange between biologists and geographers. This thesis is part of an ongoing collaboration between the Department of Environmental Toxicology at IBI, NTNU, and the Norwegian Institute for Nature Research (NINA), both located in Trondheim.

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III. List of Abbreviations

$CaCl_2$	calcium chloride
FTIR	Fourier-Transformed Infrared Spectroscopy
h	Hour(s)
H_2O_2	hydrogen peroxide
HCl	hydrogen chloride
HNO_3	nitric acid
IBI	Institute of Biology
KI	potassium iodide
KOH	potassium hydroxide
M	molar
MP(s)	microplastic(s)
MSFD	Marine Strategy Framework Directive
Mt	Million tons
NaCl	sodium chloride
NaI	sodium iodide
NARM	Natural Resource Management
NGO	Non-Government Organization
NINA	Norwegian Institute for Nature Research
NOK	Norwegian crowns
NPI	Norwegian Polar Institute
NTNU	Norwegian University of Science and Technology
OSPAR	The Convention for the Protection of the Marine Environment of the North-East Atlantic
PE	polyethylene
PP	polypropylene
R	residue
rpm	rounds per minute
RT	room temperature
S1	supernatant 1
S2-4	supernatants S2-4
SDG	Sustainable Development Goal
SEAPOP	Seabird Populations
UN	United Nations
WG-GES	Working Group on Good Environmental Status

1 Introduction

1.1 Plastic and Microplastic Pollution

1.1.1 The Global Scale

Plastic pollution is a daunting global problem that poses risks to ecosystems, wildlife, and human health. While the use of plastics has revolutionized some fields, such as the health sector, the downsides of unsustainable plastic use are startling (Joseph et al., 2021; OECD, 2021; Romeo, 2020). Plastic has been found in all major ecosystems, stretching from Antarctica to remote mountain lakes and the deep sea and its trenches (J. Barrett et al., 2020; Courtene-Jones et al., 2019; Free et al., 2014; Peng et al., 2020; Sfriso et al., 2020). For over 50 years, researchers have expressed concerns about plastic pollution. However, it was only in the early 2000s that the issue of microplastic pollution gained traction and received attention from wider scientific communities and coverage in popular media (Bailey, 2022; Carpenter & Smith, 1972; Napper & Thompson, 2020; Thompson et al., 2004)

Despite growing international awareness and efforts to reduce plastic use, the amount of plastic waste is set to triple from 460 Mt (Million tons) in 2019 to 1231 Mt in 2060 (OECD, 2021). It is estimated that 11% or 19 – 23 Mt of plastics generated in 2016 have ended up in aquatic ecosystems (Borrelle et al., 2020). Unfortunately, based on current developments, this trend is expected to double to 53 Mt per year as early as 2030 (Borrelle et al., 2020). Estimates of total plastic waste in the world's oceans amount to more than 170 trillion floating pieces, weighing 1.1 – 4.9 Mt in total (Eriksen et al., 2023).

1.1.2 Plastic Waste Management

Complex issues arise around the proper management of plastic waste. For a long time, developed countries have outsourced the burden of dealing with plastic waste to developing countries, especially in East- and Pacific Asia (Barnes, 2019). However, these countries now buckle under the ever-growing plastic masses, and some countries have introduced import bans to reduce the load of incoming plastic waste (Brooks et al., 2018). The mismanagement of plastic waste, in both developed and developing regions, is a leading cause for evermore plastic waste in the world's oceans (Schmidt et al., 2017). While a considerable amount of plastic originates from marine sources, i.e., fishery activities, many plastics also stem from land-based sources and are often transported through river systems (Rech et al., 2014;

van Emmerik et al., 2022). This plastic waste eventually ends up in the ocean and floats along its' many gyres, often fragments, and distributes in the water column (Choy et al., 2020; Lebreton & Andrady, 2019; Phuong et al., 2018). These findings highlight the need for immediate international policies on reducing plastic production, use, and waste management (Borrelle et al., 2017).

One such initiative is led by the United Nations (UN). The UN expanded its previous efforts (i.e., the Millennium Development Goals), resulting in the Sustainable Development Goals (SDGs) which were adopted in 2015. The SDGs address a wide variety of problems facing the globe and the global population to mitigate them. They are divided into 17 goals, each containing specific targets and indicators, and are to be accomplished by 2030. According to the Plastic Soup Foundation, a Non-Governmental Organization (NGO) campaigning for reducing plastic production (esp. microbeads), seven of the goals are linked to plastic waste. These are SDG 6 – Clean Water and Sanitation, SDG 11 – Sustainable Cities and Communities, SDG 12 – Responsible Consumption and Production, SDG 13 – Climate Action, SDG 14 – Life Below Water, and SDG 15 – Life on Land (Plastic Soup Foundation, 2023).

1.2 The Dangers of Plastics

Plastics are synthetic polymers derived from petroleum oil, which are multifunctional, highly versatile, durable, and inexpensive to produce (Andrady & Neal, 2009). There is a wide variety of additives that can be used to change the nature of the polymer, such as fillers, plasticizers, flame retardants, colorants, stabilizers, and lubricants (Andrady & Rajapakse, 2016). Following the rise in commercial popularity in the late 1950s, plastics soon found application in technology, medicine, science, and day-to-day life. Since then, the production of plastics has increased dramatically, and it is now ubiquitous (Andrady & Neal, 2009). However, the issue of plastic litter and its mismanagement has become startling (Schmidt et al., 2017). Scientists argue that humanity now lives in a plastic age, as synthetic polymers are what we leave behind in the geological sediments of our era (Brandon et al., 2019). One of the prime advantages of plastics has become a major threat to planetary health: plastic fragments into smaller and smaller pieces, i.e., microplastics (MPs), but it barely decomposes (Sivan, 2011). The term "microplastic" describes a heterogeneous mix of different polymers, that come with different chemical properties as well as various shapes and forms, such as granules, flakes, fibers, and beads amongst others (Lambert et al., 2017). Two types of MPs are distinguished based on

their origin: Primary MPs are already small items, e.g., microbeads from cosmetics or fibers from clothing. Secondary MPs originate from large plastics that fragment due to prolonged exposure to sunlight and weathering (European Food Safety Agency, 2016). The composition of microplastics in the ocean is dependent on the sampled region and season, and can therefore vary (Courtene-Jones et al., 2022; Van Franeker et al., 2021). Furthermore, particles are distributed throughout the water column based on their specific gravity (Andrady, 2011).

The discussion about plastic particles is greatly complicated by the fact that there is no scientific consensus on how to define the limitations of different plastic size classes. For example, a definition proposed by Andrady (2011), suggests the distinction between mesoplastics (500 µm – 5 mm), microplastics (50 – 500 µm), and nanoplastics (<50 µm). In 2013, the European Marine Strategy Framework Directive (MSFD) Working Group on Good Environmental Status (WG-GES) published "Monitoring Guidance for Marine Litter in European Seas" and divided MPs into large MPs (5 mm – 1000 µm) and small MPs (1000 µm – 20 µm) (Joint Research Centre Institute for Environment and Sustainability, 2013). While the lower limit remains a topic of debate, the upper limit is most commonly defined as 5 mm. Frias & Nash (2019) highlighted the need for a universal definition, as it would improve comparability between studies and streamline international monitoring efforts across research disciplines.

1.2.1 Dangers to Seabirds

Large plastics, such as ropes and nets, pose threats to larger organisms such as seabirds, i.e., through strangulation, entrapment, and ingestion (Browne et al., 2015; Kühn et al., 2015; Provencher et al., 2017). Ingestion of such plastic pieces can lead to blockages of the digestive tract (Ryan, 1987). Hard plastics were shown to be damaging physically due to sharp edges, which can injure the digestive tract of an individual and lead to internal wounds (Ryan, 1987; Van Franeker & Meijboom, 2002). Soft plastics, such as balloons or latex films, can also have devastating effects (Roman et al., 2019). A study by Roman et al. (2019) found that balloons or balloon fragments cause the highest mortality among petrels (Procellariiformes), although they are consumed comparatively less than other plastic debris. Films such as balloons or condoms can easily block the entrance to the intestines (Roman et al., 2019).

When plastic debris is ingested, it can fragment further and thus release MP into the organism (Ryan, 2015). A recent study of Flesh-footed shearwater (*Ardenna carneipes*) fledglings

revealed a correlation between macroparticles consumed and MP particles embedded in internal organs, where they led to inflammation, fibrosis, loss of organ structure, and possibly affecting nutrient absorption (Rivers-Auty et al., 2023). However, there is no guarantee that the consumed macroplastics are the primary source of the found MPs, which can both be primary or secondary. Nonetheless, very small MPs and nanoplastics are especially worrying, as they can cross from the gastrointestinal tract into the bloodstream and disperse throughout an organism (Smith et al., 2018; Wright & Kelly, 2017). Mattsson et al. (2017) discovered that when ingested by fish, nanoplastic particles can be absorbed into the bloodstream and even cross the blood-brain barrier, causing behavioral changes.

As major consumers of fish, seabirds are likely exposed to microplastics via ingestion, although some species, like the Northern fulmar (*Fulmarus glacialis*), also unselectively ingest plastic that is floating at the surface (Hammer et al., 2016; O'Hanlon et al., 2017; Van Franeker & Law, 2015). Seabirds consume fish in their entirety, stomach, and guts included, and are therefore especially vulnerable to MP exposure. Trophic transfer of plastic particles is a risk to seabirds and other predators, as the particles can rise through trophic levels, e.g., seals (Nelms et al., 2018). This can be a concern for human health, too, as humans occupy a high trophic level. Globally, humans eat on average 20.4 kg of fish per capita and year, however, it is important to note that human fish consumption consists largely of filets and not the entire fish (OECD & FAO, 2023). Nonetheless, the presence and effects of MPs in fish are highly concerning as many effects remain unknown (FAO, 2023).

Another major problem of MP exposure is the near countless (>10 000), and often poorly regulated, chemical additives used in or for plastic production today (Wiesinger et al., 2021). Many studies have assessed the amount of leachate of various additives from MPs and documented their alarming negative effects on marine wildlife (Bridson et al., 2021; Gandara e Silva et al., 2016; Hermabessiere et al., 2017; Koelmans et al., 2014; Luo et al., 2019). MPs can also serve as vectors for potentially hazardous contaminants, e.g., heavy metals, which can leach from the MPs and be highly toxic to organisms (Brennecke et al., 2016).

It seems natural that disturbance of the gut microbes, multi-organ damage, slower development, nutritional stress, and poor body condition due to MP exposure could cause a deterioration of seabird fitness (Fackelmann et al., 2023; Lavers et al., 2014, 2019, 2021; Rivers-Auty et al., 2023). It is furthermore hypothesized that the bacterial biofilm on MPs can carry pathogens

and could play a role in disease emergence, though further investigation of the role of MP on pathogen population transport is needed (Bowley et al., 2021; Frère et al., 2018). Overall, the effects of MP on seabird populations are still unknown (Fackelmann et al., 2023).

1.3 State-of-the-Art Methods to Study Diet and Microplastic Exposure in Seabirds

1.3.1 Microplastic Analysis

In the past, regurgitated pellets from seabirds have been used for either diet analysis or MP analysis (R. T. Barrett et al., 2007) and occasionally for both analyses (Acampora et al., 2017a; Álvarez et al., 2018).

Acampora et al. (2017a), collected pellets from Great cormorants (*Phalacrocorax carbo*) in Ireland and rinsed through a 1 mm sieve. The remaining items (otoliths, lenses, algae, parasites, etc.) and MPs were grouped and quantified. However, no information about field and procedural blanks is provided. Critically, the plastic types were not identified. Notably, no fibers were found, even though they were a very common group of marine MPs. This might be due to the sieve that was used, as even longer fibers can easily bend and washed through.

In a study by Álvarez et al. (2018), who investigated pellets from European shags from a colony off the Spanish coast, fibers were found abundantly. The plastic polymers were, furthermore, analyzed, as were the fish remains. The latter were also divided grouped by habitat, i.e., depths in the water column, to investigate possible links of MP content and depths of the ocean. However, no information about field or laboratory blanks is given and no clear lower limit for MPs is set.

Protocols for diet analysis and MP analysis exist mostly separately, although Acampora et al. (2017a) and Álvarez et al. (2018) provide early combinations of the analyses. Nonetheless, both studies neglect field and laboratory blanks and rely on subjectively locating possible MPs in their samples. Both studies also use different size ranges when defining MPs, especially the lower limits and provide impressions rather than insight about MP pollution in their respective locations. While these studies are important and of interest, the need for a protocol that maximizes the outcome of the combination of analyses becomes clear.

One major challenge when investigating MPs is to isolate them from the unimportant parts of whatever type of sample is being analyzed. In marine sediment samples, this can mean separating MPs from sand and organic matter.

This was the case for Crichton et al. (2017) who were interested in extracting MPs from aquatic soil sediments. The authors explored the oleophilic nature of plastic particles and proposed a cost-effective and density-independent oil-extraction protocol, followed by optical analyses using Fourier-Transform Infrared Spectroscopy (FTIR), a well-established method and popular choice for identifying polymer types. This approach was compared to other established density-dependent extraction methods, namely with sodium iodide (NaI) (Nuelle et al., 2014), and calcium chloride ($CaCl_2$) (Stolte et al., 2015). Crichton et al.'s (2017) comparisons suggest that the oleophilic and density-independent approach was a reliable alternative to density-based approaches.

When investigating MP contamination in fish, Karami et al. (2017) faced the problem of having to isolate MPs from whole organisms. Hence, an appropriately strong digestion step was required before the separation of MPs. The authors tested and evaluated several digestion methods, including the digestion of organic matter, e.g., with potassium hydroxide (KOH), hydrogen peroxide (H_2O_2), various concentrations of hydrogen chloride (HCl), and nitric acid (HNO_3) as well as their effects on MPs. In the first phase of their experiment, KOH was shown to be the most efficient agent to dissolve organic matter, while simultaneously keeping the integrity of MP particles. The second phase of their investigation tackled the separation of MPs from the remaining detritus, i.e., fish bones that were not (entirely) digested. Similarly to Crichton et al. (2017), Karami et al. (2017) adapted the protocol by Nuelle et al. (2014) with some modifications. They expanded their analysis to testing different concentrations of NaI solutions ranging from 3.3 M (molar) to 4.4 M. Karami et al. (2017) therefore proposed a new protocol for digesting fish, using a 10% KOH solution, followed by three rounds of density-dependent separation using 4.4 M NaI and optical analysis using a Raman spectroscope.
A Raman spectroscope excites the molecules of a substance of interest with a focused laser beam and picks up the characteristic vibrations these molecules make in response. The vibrations are registered by the spectroscope and visualized by a software as tables or graphs. Comparing thus generated data makes it possible to identify the substances, e.g., different types of polymers, by comparing them to comprehensive libraries. This method of analysis has been a recommended staple in MP research (Joint Research Centre, Institute for Environment and Sustainability, 2013) and is the method of choice in this study.

1.3.2 Diet Analysis in Seabirds

A vital part of monitoring seabird health is to assess their diet. There are several well-established approaches, mainly necroscopy, regurgitated stomach contents, collection of regurgitated pellets, and feces (R. T. Barrett et al., 2007; Maaseide, 2022; Provencher et al., 2018, 2019). While each of these approaches comes with its benefits and limitations, the least invasive and arguably most ethical method is pellet collection. In this thesis, the terms 'pellet' and 'regurgitate' are used interchangeably and both refer to 'regurgitated pellets', not regurgitated stomach contents. Pellets are regurgitated voluntarily by seabirds and can be picked up throughout the nesting grounds (Provencher et al., 2019). It is however important to note that not all seabirds regurgitate pellets. This approach is therefore only applicable to some seabirds such as cormorants and shags (Phalacrocoracidae), gulls (Laridae), and terns (Sternidae) (Provencher et al., 2019). The collection of pellets is the only entirely non-invasive method, as no handling of the birds is required.

This approach is also very easy to implement into existing seabird monitoring programs, as it is cheap and repeatable throughout seasons and years (Dehnhard et al., 2019). While it is difficult to find pellets from the same individual repeatedly, they can provide an understanding of the diet composition and MP exposure of the colony. Although it is still unclear how much plastic is regurgitated, remains in the digestive tract, and/or is excreted, pellets can provide some insight into the degree of contamination and should therefore not be disregarded. They might not provide a full picture of the total plastic consumption, but they can be used as indicators.

Diet analyses are based on examining the birds' stomach content or pellets for the remains of consumed fish (R. T. Barrett et al., 2007). One type of remains that is commonly found in pellets are otoliths, the fish ear bones. The otoliths of every species have characteristic features and can therefore be used for identification (Härkönen, 1986). Furthermore, the length, mass, and age class of the fish can be determined (Dehnhard et al., 2021; Hillersøy & Lorentsen, 2012).

While Provencher et al. (2019) highlight some valid disadvantages of pellet MP analysis, the value of pellets for diet analysis is undeniable (R. T. Barrett et al., 2007).

One species that has been shown to ingest MPs and whose pellets have already been used in both diet and MP studies is the European shag (*Gulosus aristotelis*, L.), hereafter simply referred to as 'shag' (***Fig. 1.1***) (Álvarez et al., 2018; Hillersøy & Lorentsen, 2012; Howells et al., 2018).

Figure 1.1 – Two European shags with characteristic crests photographed during the summer of 2022 at Sklinna. The crest feathers are lost towards the end of the breeding season. (Photo: N. Dehnhard)

Shags are benthic feeders with a fish-dominated diet. At Sklinna, this study's field site (***Ch. 2.1.1.***), the diet of these seabirds consists mostly of Saithe (*Pollachius virens*), Poor cod (*Trisopterus minutus*), and Cod (*Gadus morhua*) (Hillersøy & Lorentsen, 2012). Shags regurgitate indigestible prey items, like fish bones, in the form of slimy pellets. These can be easily collected in the breeding colonies, without having to handle the birds. As stated before, the fish bones in the pellets, particularly the otoliths, allow us to identify not only the fish species, size, and mass, but also the age class (Dehnhard et al., 2021; Hillersøy & Lorentsen, 2012). This allows, for example, to assess diet differences between years (Lorentsen et al., 2018, 2019). For shags, regurgitates can also be used to assess MP exposure, as they represent the first step on the route of exposure (Álvarez et al., 2018). Shag pellets thus present a medium that allows us to relate the exposure to plastics with the diet of the birds, and thus the main route of intake.

1.3.3 Current Challenges in Microplastic Research

As mentioned in Chapter ***1.2***, there is neither inter- nor intradisciplinary consensus on what "microplastic" is. This overarching deficit affects not only science itself but by extension, policy makers who rely on peer-reviewed knowledge to make informed decisions.

A principal challenge across MP research is the absence of standardized protocols and interpretation guidelines for MP monitoring and investigation (Borrelle et al., 2017; Provencher et al., 2017). Currently, there is only one monitoring program for plastics in seabirds in place (as part of "The Convention for the Protection of the Marine Environment of the North-East Atlantic" or "OSPAR" Convention). OSPAR has several programs for plastic (intake) monitoring, however, the only seabirds included are Northern fulmars in the North Atlantic region (Kühn et al., 2022). While the Northern fulmar has been studied extensively (e.g., Van Franeker, 2004; Van Franeker et al., 2021; Van Franeker & Meijboom, 2002) and the establishment of this program is a step in the right direction, there are still vast knowledge gaps with regards to other species and regions (R. T. Barrett et al., 2007). Data for other seabird species are scarce and mostly opportunistically collected (Acampora et al., 2017b; Baak et al., 2020; Benjaminsen et al., 2022; O'Hanlon et al., 2017). A positive development in MP research is that interdisciplinary knowledge transfer can be observed. For instance, Karami et al. (2017) took inspiration from sediment studies for their fish analysis project.

There are plenty of studies about MPs in (marine) sediments, which are, to a degree, applicable to MP separation in pellets. However, most suggested methods involve chemicals that are hazardous and/or expensive (Cashman et al., 2020). In recent years, the number of suggested methodologies has increased, which has necessitated comparative studies such as Karami et al. (2017), or Tsangaris et al. (2021), who compared the effectiveness between two digestion methods (H_2O_2 and KOH) on fish and mussels, and the chemicals' effects on MPs.

1.4 Aim and Objectives

In this thesis, I aimed to develop a novel and optimized approach that allows for both MP and diet analysis from the same regurgitated pellets. The overall goal was to obtain additional information from wildlife samples that are already being collected for scientific purposes. The inspiration of this thesis is to provide a more sustainable research practice by choosing this non-invasive sample type and by using safer chemicals. In this study, I developed and tested a new protocol that can be used in the monitoring of European shags and possibly other pellet-regurgitating seabirds.

To identify the best methodology, several approaches for separating and isolating MP were tested, i.e., oil separation and density separation, as well as different filtration techniques. The main objective was to develop a protocol for the extraction of MPs from regurgitated pellets from European Shags, which simultaneously allows for MP (300 µm – 5 mm) and diet analysis, and can be used for non-invasive monitoring purposes.

The specific objectives of my thesis were to:

1. Optimize existing methods to find a new and more sustainable protocol that allows pellets to be used for both diet analysis and MP analyses.
2. Validate the new protocol on pellet samples experimentally spiked with MP.
3. Use the developed protocol to quantify MP contamination in original pellet samples from European shags.

2 Method

This chapter is divided into four main sub-chapters. Chapter ***2.1*** *Sampling* describes where and how the samples for this thesis were collected. In Chapter ***2.2*** *Pilot Studies*, several approaches to isolate MPs from the samples were tested. This is followed by Chapter ***2.3*** *Selection of the Best Protocol*, in which the most promising approaches are compared. In Chapter ***2.4*** *The Final Protocol* the best method was performed, which was the basis for my case study. Chapters ***2.5*** *Microplastic Analysis*, ***2.6*** *Diet Identification by Otoliths,* and ***2.7*** *Data Analysis* are not part of the developed method but describe how data was generated and analyzed.

Due to the exploratory nature of this thesis, several results of the methods are presented in this chapter. Whenever a decision is based on an interim result, a cross-reference to the corresponding sub-chapter in Chapter ***3*** *Results* and/or the Appendices are given. In addition, ***Appendix D*** shows the step-by-step processing of one sample as an example.

2.1 Sampling

2.1.1 Site Description

Sklinna (65°12'N 10°59'E) is a small and isolated archipelago approximately 40 km off the coast and belongs to Leka municipality in Northern Trøndelag, Norway. Historically, the islands and their surroundings were used for fishery. Some of the islands were, at least during the summers, inhabited. Today the archipelago is uninhabited by humans, although a harbor for coastal fishing vessels remains. Of all previous buildings, only the lighthouse complex still stands today. Sklinna is the most important seabird breeding site in central Norway, with one of the largest colonies of European shags in Norway and worldwide (SEAPOP - Sklinna, n.d.), as well as substantial breeding populations of Common guillemots (*Uria aalge*), Atlantic puffins (*Fratercula arctica*), Black guillemots (*Cepphus grylle*) and Razorbills (*Alca torda*), and smaller populations of other species, including Great Cormorants (*Phalacrocorax carbo*), Common eiders (*Somateria mollissima*), Herring gulls (*Larus argentatus*), and Great-black-backed gulls (*Larus marinus*) (SEAPOP - Sklinna, n.d.).

In total, over 2,100 pairs of European shags were breeding on Sklinna in 2022, with nests scattered across most of the two main islands, Heimøya and Hansholmen. Shags on Sklinna typically breed under boulders as protection from predation by White-tailed sea eagles (*Haliaeetus albicilla*). The breeding density is highest on two breakwaters, which together hold more than half of the population.

Because of its importance as a seabird breeding site, the archipelago is a Ramsar site (Sklinna - Ramsar Sites Information Service, 2023) During the breeding season, traffic within the archipelago, including the main islands (Heimøya and Hansholmen) is greatly restricted. Landing on the other islands is forbidden, and without having a permit, the outer area cannot be approached, either. While the sizes of seabird populations on Sklinna have been monitored since the 1980s by the Norwegian Institute for Nature Research (NINA), more thorough monitoring, also including the breeding success, survival, diet, and habitat use of seabirds, was established in 2007 through SEAPOP. SEAPOP (SEAbird POPulations) is a collaborative project between NINA and the Norwegian Polar Institute (NPI) founded in 2005, which monitors seabird colonies along the entire Norwegian coastline, including on Svalbard and Jan Mayen (SEAPOP - About, n.d.)

The increasing amount of plastic pollution on Sklinna, a SEAPOP key site since 2007, and other SEAPOP key sites has been observed for many years and it is of scientific interest to quantify the level of plastic contamination in the seabirds (Dehnhard et al., 2019).

2.1.2 Collection Sites

The primary collection site of regurgitated pellets was the outside (i.e., north-east side) of the northern breakwater "gammelmolo" which connects the main island of Heimøya with Hansholmen (***Fig. 2.1***). During the annual seabird monitoring by NINA, 96 European shag nests were counted there in June of 2022 (***Table A.2***).

Rainy weather and high waves are risk factors for the safe collection of regurgitates. When weather conditions were too harsh at gammelmolo, samples were instead collected at the south-western side of the second breakwater "nymolo" in the western part of Heimøya (***Fig. 2.1***). On the south-western side, 674 nests of European shags were counted in June of 2022 (***Table A.2***).

On the 17th of July 2022, plastics that had washed ashore over an unknown period were collected on a beach on Heimøya. Some of the collected plastic items were used for creating MP spikes in a spike experiment (***Ch. 2.2.5***)

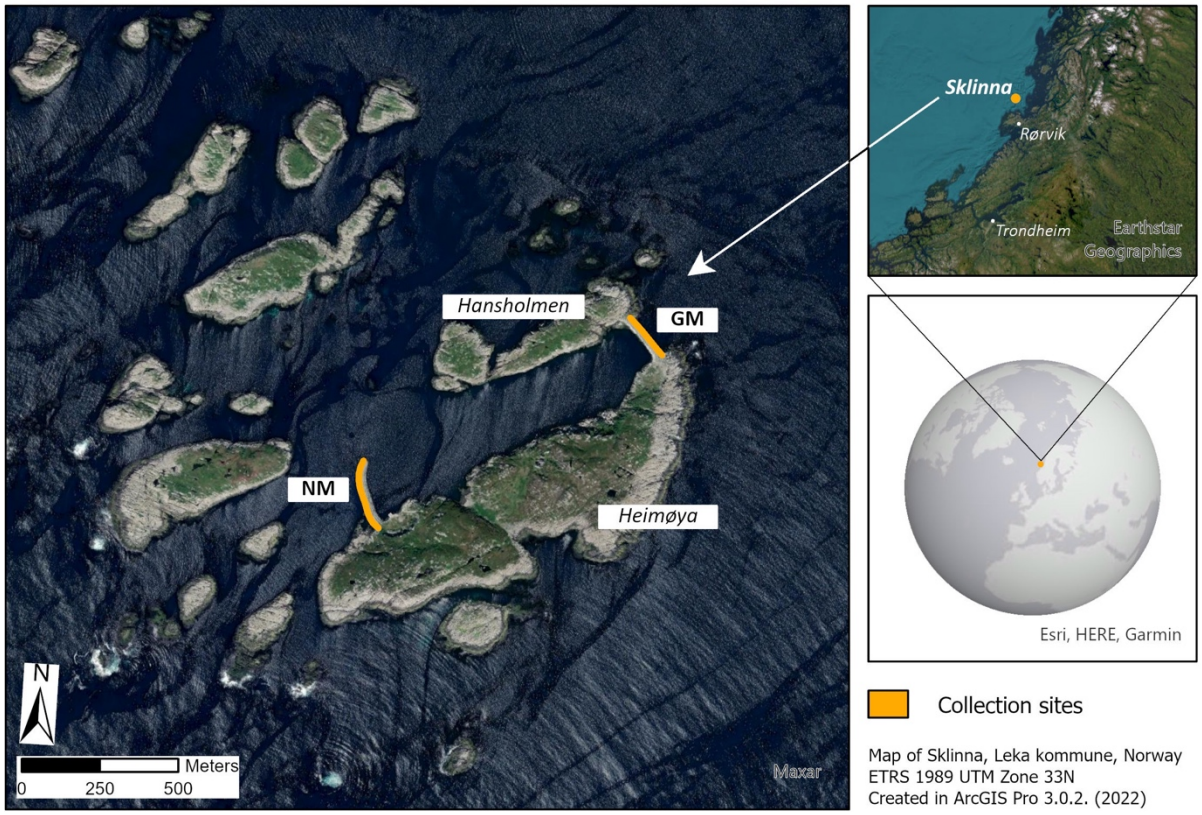

Figure 2.1 – Map of the Sklinna archipelago in Leka municipality, Northern Trøndelag, Norway*. Highlighted in orange are the outsides of the two breakwaters, where collection took place. GM = gammelmolo, NM = nymolo. (Map by K. Buchholz)*

2.1.3 Sample Quality Evaluation Before Collection

Shags regularly regurgitate pellets, which contain mainly indigestible organic matter, such as fish bones wrapped in tough mucus (Johnstone et al., 1990). Pellets can easily be found on the rocks under which the shags are nesting. Regurgitates differ in size, color, and quality. While some fresh pellets are runny, others can be firm or springy to the touch. Fresher pellets are usually of better quality than older pellets. This is because the fresh pellets are less likely to be damaged and are exposed to the environment for only a short time. The color can vary from greyish white to yellow and green. Only intact pellets were collected, to not miss any of the content (***Fig. 2.2*** & ***2.3***).

Figure 2.2 – High-quality sample. Whole, dense, and lying on algae and rock. (Photo: K. Buchholz)

Figure 2.3 – Low-quality sample. Runny, contaminated with feathers and lying in a puddle of dirt. (Photo: K. Buchholz)

2.1.4 Collection and Storage of Samples

Regurgitates were collected over six weeks during June and July of 2022. The collections were made in intervals of five days, resulting in a total of eight intervals (***Table A.1***). On some days, not enough samples could be found, so a new collection round was performed the day after. To store the regurgitates, glass jars (50 mL volume) with a wide screw opening had previously been prepared at NINA's laboratories. They were covered with a double layer of aluminum foil and had been autoclaved at 400°C for 24 hours (h). To reduce the risk of contaminating samples in the field with plastic particles or fibers, only wool or cotton clothing was worn during the collection. Suitable samples were collected by hand and wearing latex or nitrile gloves (i.e., 1 glove per sample). To collect a sample, the aluminum foil was removed, paying careful attention not to touch the inside of it. The regurgitate was then transferred to the glass jars and the foil was immediately placed back, facing the same way it did before. The jar was then sealed with a plastic lid that was screwed in place over the foil. The samples were labeled, frozen, and stored at -20°C. At Sklinna, depending on weather conditions, only 20 – 60 minutes are needed to search the approximately 150 m long breakwaters (***Figure 2.1***) with two people, and most importantly, no handling of the birds is required and the disturbance to the colony is minimal.

Per sampling interval, one field blank was collected. For this, the jar was opened for approximately the same time as for actual samples, and a new glove was worn to imitate the movements and exposure during regular collection.

Additional samples were collected opportunistically for diet analyses, as previously done by Lorentsen et al. (2018) and Hillersøy & Lorentsen (2012). These samples were not initially intended for this study but were collected during everyday fieldwork without wearing gloves, and stored in plastic zip-loc bags at -20° C. The opportunistic samples were used for pilot studies (***Ch. 2.2.***)

2.2 Pilot Studies

The initial laboratory procedure was performed with 25 of the opportunistic samples collected in 2022 (i.e., samples collected in plastic zip-loc bags). While they are neither intended nor suited for MP quantification, they are useful for testing different approaches without losing any actual samples in the process.

The main goal of the tests was to establish a protocol that a) enables the removal of otoliths, b) uses as little equipment as possible (increased risk of contamination), c) separates plastic spiked particles without destroying or losing too many of them, and, most importantly d), has a high recovery rate.

2.2.1 Test 1: Preliminary Digestion

Due to the difficulty of removing otoliths from the original regurgitates (i.e., they are embedded in the mucus (***Fig. 2.2*** & ***D.1***), the first task was to evaluate the best method to dissolve the mucus of the pellets enough to loosen the otoliths. For the pilot study, both the effectiveness of Biotex® solution (as used in previous diet studies; Hillersøy & Lorentsen (2012)) and ultrapure water (Ultrapure type 1 water (ELGA® Veolia Water Solutions & Technology) were tested. Ultrapure water was a viable alternative because it is a common dilutant and was readily available.

Biotex® is a common household laundry soap that utilizes enzymes to break down organic matter. It is strong enough to dissolve the dense mucus of the regurgitates, however, it is not strong enough to break down fish bones. A saturated solution was produced by mixing 10 g Biotex per 1 L ultrapure water and filtering it with a folded filter (Grade 597 ½, Whatman, Germany) and a glass funnel. The solution was stored in a 1 L glass reagent bottle.

Samples with either Biotex® solution or ultrapure water were placed on a heated orbital shaker at 40° C for 24 h. Both resulted in dissolutions (***Ch. 3.1.1*** & ***Fig. D.2***), meaning slimy remnants of mucus mixed with residues (sand, fishbones, algae, etc.), which hereafter are also referred to as 'slurry'.

2.2.2 Test 2: Otolith Removal

The otoliths from the pellets were needed for later diet analyses (***Ch. 2.6***) and therefore had to be removed from the slurries. The jars were opened on a sterile bench and the otoliths were picked out from the bottom with a pair of tweezers. However, this was difficult due to the relatively narrow opening of the glass jars. To ease this, the slurry was poured into a clean glass Petri dish. While this made it easier to remove the otoliths from the slurry, several factors led to this approach being dropped (***Ch. 3.1.2*** & ***4.2.2***). Instead, the best alternative in terms of availability, accessibility, and effective rinsing was clean glass beakers (100 mL) (***Ch. 3.1.2***). An additional LED-lamp was used to see the otoliths, as they shine bright white through the slurry under well-lit conditions.

2.2.3 Test 3: Filtration

As described in Chapters ***2.2.1***, ***3.1.2*** & ***4.2.2***, substantial amounts of detritus remained in the slurries after the pre-digestions. For MP analyses it is important to isolate the particles from their matrix as much as possible. The next step was, therefore, to gain an understanding of how the liquids act under vacuum filtration, and to filter the slurries onto surfaces that were suitable for later analysis (i.e., paper or nitrocellulose filters for Raman spectroscopy, see ***Ch. 2.5.2*** & ***3.3.3***).

While the goal was to find a filtration method that ensured the retention of particles ≥300 µm, no filter with this specific requirement could be sourced. The filtrations were hence tested with the best available alternatives: Two metal meshes, paper filters (quantitative filter paper 454 (VWR), particle retention 12-15 µm), and nitrocellulose membrane (MF-Millipore™) (***Table 1***).

Table 1 – Types of filters tested and their measurements. The pores of the metal meshes are square, hence for the fine metal mesh the diagonal is 141 µm, and for the coarse metal mesh the diagonal is 212 µm (Pythagorean theorem). All listed meshes/filters are fine enough to retain particles ≥300 µm.

Type	Pore size	Filter Diameter	Reusable
Metal mesh, fine	100 µm * 100 µm (141 µm diagonal)	ca. 50 mm	Yes
Metal mesh, coarse	150 µm * 150 µm (212 µm diagonal)	ca. 50 mm	Yes
Paper	12 – 15 µm	90 mm	No
Nitrocellulose membrane	0.8 µm	47 mm	No

The metal meshes were placed in between two flat rubber rings to ensure the vacuum (***Fig. 2.4***). After filtrating the slurry, the funnel was rinsed thoroughly with ultrapure water. The metal meshes were then carefully removed from the setup and stored in individual glass beakers filled with ultrapure water. After sonicating and rinsing of the meshes, the residues were filtered onto paper filters using the Büchner funnel setup shown in Chapter ***2.4.4***.

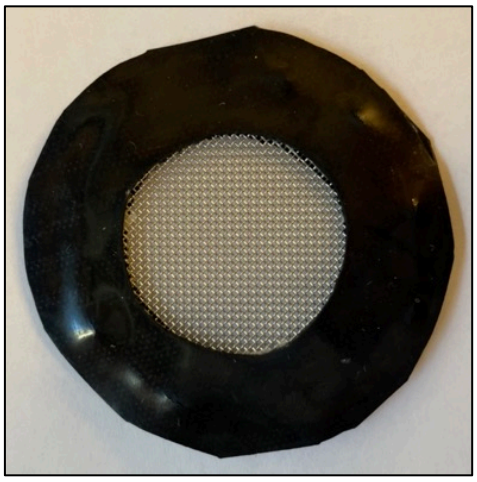

Figure 2.4 – Fine metal mesh in between two rubber rings. The rubber rings ensure the vacuum during filtration. (Photo: K. Buchholz)

The transfer to paper filter was done for practical reasons, namely improved visibility of particles on white background, and better handling under the Raman. The paper filters were placed in upside-down plastic Petri dishes. Lastly, the lids were put on the dishes tilted to avoid condensation and the entire drying area was loosely covered with aluminum foil to reduce the risk of contamination.

The nitrocellulose filters had the same diameter as the metal meshes and were tested accordingly, but without the rubber rings which were obsolete for this type of filter. This filter

was tested with one sample, and, more specifically, with only the filtrate that was collected after a paper filtration instead of a regular slurry. The two filters were placed in a single plastic Petri dish and left to dry under an aluminum foil cover.

2.2.4 Test 4: Potassium Hydroxide Digestion and Subsequent Filtration

Since all types of filters clogged (***Ch. 3.1.3*** & ***4.2.3)***, a second step was required to dissolve the detritus. This method had to be strong enough to digest the organic matter, but not so strong that it damaged or even digested MP particles. All pre-digestions were done for 24 h with either saturated and filtered Biotex® solution or ultrapure water, as described in Chapter ***2.2.1***. Following the procedure established by Kühn et al. (2017), 12 of the 25 additionally collected test samples were then treated with potassium hydroxide (KOH) to digest the organic matter. To learn how much KOH was necessary to digest pellets, different concentrations and incubation times were tested. To balance out the dilution, a 5 M KOH solution was used, which led to 1 – 2 M in the samples. All samples were placed on an orbital shaker for 24 – 72 h at room temperature (RT). After this step, the samples were filtered onto paper, as described and shown in Chapters ***2.2.1*** & ***2.4.4***.

The resulting filters (***Ch. 3.1.4***) were, to varying degrees, still filled with fishbones and detritus. None of the 12 samples could be used directly for MP analysis with a Raman spectroscope. Another step was needed to separate possible MPs from the leftovers.

2.2.5 Test 5: Oil Separation with Spikes

When investigating MP contamination in aquatic sediments, Crichton et al. (2017) faced similar problems of finding MP in a mass of detritus and sand. They proposed the separation of plastics from detritus, in this case sand and other sediments, with an oil separation protocol utilizing the oleophilic properties of MP. This became the inspiration for this second approach.

While peanut oil (Sigma®Life Sciences) was available in the laboratory, commercial canola oil and an olive-sunflower oil mix were sampled from the Institutes' lunchrooms. The goal of this method was to learn how the plastic particles behaved when exposed to oil and to check whether this technique was feasible for the proposed scale of this study. For this, plastic spikes were created from plastics gathered from a beach on Heimøya (***Ch. 2.1.2***).

A thin strip of each plastic listed in ***Table 2*** was carefully shaved from its source with a scalpel. The strips were then cut into fine particles (approx. 0.3 – 5 mm) using scissors. Particles were kept in separate clean beakers and covered with aluminum foil.

Table 2 - Overview of the plastics that were cut to be used as spikes. The suspected source is based on subjective impressions of the items from which parts were cut. The parts can be viewed in Appendix B.

Color	(Suspected) source	Consistency
Grey	Riffled tube	Hard
Purple	Bottle cap	Somewhat flexible
Dark blue	Seal or tape	Flexible
Light blue	Unknown	Hard
White	Cosmetics bottle	Somewhat flexible
Green	Lid	Somewhat flexible
Red	Double layered sheet	Flexible
Transparent	Jug	Somewhat flexible
Pink	Unknown	Hard

For the spiking of samples, particles were counted and assembled in small plastic dishes (***Fig. 2.5***). The handling of particles was complicated by static electricity, as they would adhere to the metal tools used to transfer them. Hence, a Staticmaster® (NRD, LLC, Model 2U500) emitting α-waves, was placed aiming at the cut plastic particles, which greatly improved their handling. The particles (***Fig. 2.5***) were later identified with a Raman spectrometer (WITec confocal Raman imaging system alpha300 R, WITec GmbH, Germany), where 56% of the particles were found to be polypropylene (PP) and polyethylene (PE), the other 44% were inconclusive. However, most known sources (***Table 2***) were very likely plastics.

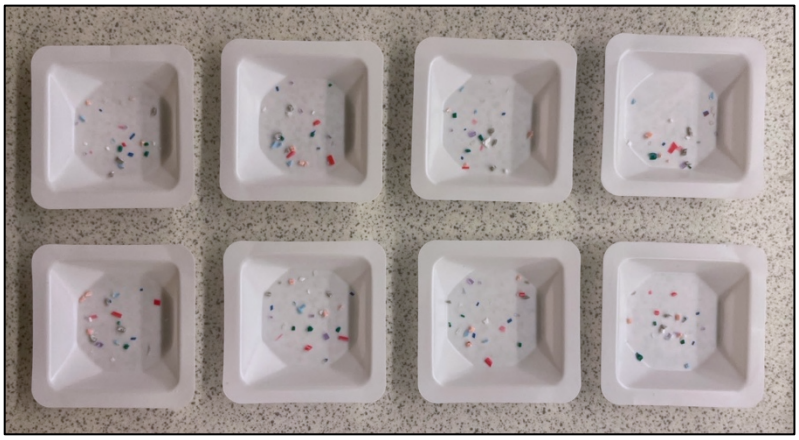

Figure 2.5 – Artificially created microplastic particles. These particles were cut from plastic collected on Sklinna during the field season of 2022. Each dish contains one batch of spikes. (Photo: K. Buchholz)

In glass beakers, approximately 30 mL of ultrapure water or KOH solution were combined (but not emulsified) with approximately 10 mL of one of the oils and a spike batch each. In addition, the samples were treated with 4 M HCl to test its' effect on the buoyancy and fish bones.
While Test 5 was effective (***Ch. 3.1.5***), the associated downsides, e.g., time expenditure, were deciding factors in not proceeding with this method and another approach had to be tested.

2.2.6 Test 6: Density Separation with Spikes

Since neither KOH digestion nor oil separation on their own resulted in filters that could be analyzed further (***Ch. 3.1.4*** & ***3.1.5***), yet another approach had to be found. Karami et al. (2017) found sodium iodide (NaI) to be highly effective when extracting MPs from whole fish. NaI was not readily available at the institute and had to be ordered.
In the meantime, the concept of density separation was tested using saturated sodium chloride (NaCl) solution as well as potassium iodide (KI) solution. In imitation of a sample, the same ratio of empty pre-digestions (Biotex® solution or ultrapure water) was mixed with 5 M KOH solution (resulting in 1 – 2 M KOH), spiked with MPs (***Ch. 2.2.5***) and mixed with saturated NaCl solution.
Based on the positive indications from the NaCl test, as detailed in Chapter ***3.1.6.***, the KI test was applied to three regurgitates and spiked with a batch of MPs each (***Ch. 2.2.5***). A solution of similar density to the proposed NaI solution density was easy to set up, as the salt was available in the laboratory. After the filtration of these samples onto paper (***Ch. 2.2.3***), the recovered MPs were counted.
Karami et al. (2017) further proposed the use of a plastic membrane after the KOH digestion of fish. This step was irrelevant for regurgitates because the samples were already digested once by the shags and were mostly free of large bones (compared to those of the whole fish used in the Karami et al. (2017) study).
Based on initial testing, KI appeared to be a reasonable alternative to NaI (***Ch. 3.1.6***). A 5.5 M KI solution has a density of 1.5g/mL at RT, and it is substantially cheaper to make than NaI solution. It thus seemed useful to compare if KI, as the cheaper and less dangerous alternative, would perform equally good or better than NaI.
KI separation outperformed both NaCl separation and oil separation in efficacy, efficiency, and practicality (***Ch. 3.1.5*** & ***3.1.6***), but the NaI approach recommended by Karami et al. (2017) remained of interest (***Ch. 1.3***).

2.3 Selection of the Best Protocol

Based on initial testing, KI appeared to be a reasonable alternative to NaI (***Ch. 3.1.6***). This finding caused the focus of this study to slightly shift from only testing the Karami et al. (2017) method. It seemed useful to comparing if KI as the it this a cheaper and less dangerous alternative would perform equally good or better than NaI. To compare the effectiveness's of NaI and KI, 3 replicates were processed for both zebrafish and regurgitated pellets (***Fig. 2.6***).

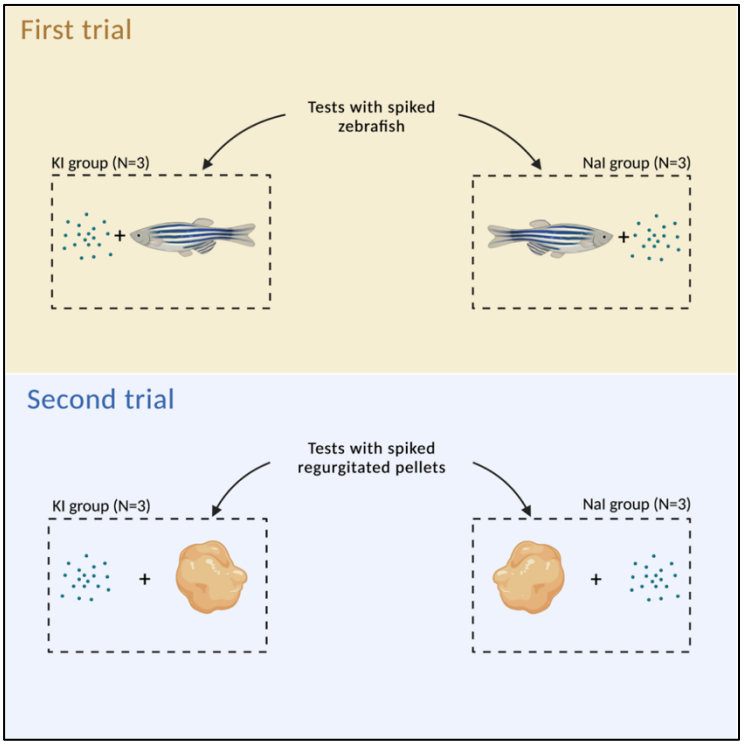

Figure 2.6 – The trial setup to identify the best protocol. All samples were spiked with one batch of MPs each. In both trials, two groups were formed, one for KI treatment and one for NaI treatment respectively. (Created with BioRender.com)

Firstly, zebrafish from the Animal Facilities at the Department of Biology, NTNU, were used, as they lived their entire lives in controlled surroundings, hence contamination is minimal. The fish were not euthanized for this study, but for other studies or died of natural causes. All zebrafish were stored frozen. Although they were frozen in plastic bags, tests could be performed with them because the spikes used are distinctly different from the thin transparent sheet of the bags. The average weight of the collected regurgitated pellets was around 4g. Therefore, the zebrafish were thawed, manually homogenized, distributed into samples of approximately 4g, and processed in falcon tubes.

The zebrafish samples were then each spiked with one spike batch of the artificial MP described in Chapter ***2.5.5*** and divided into two groups. A batch of spikes contained 3 particles of each of the 9 colors, totaling 27 particles per batch (***Ch. 2.2.5*** & ***Table 2***). One group was treated

with KI while the other was treated with NaI. For both groups, laboratory blanks were processed accordingly.

The second trial used the properly collected pellets stored in glass vials (***Ch. 2.1.4***) (with otoliths removed after pre-digestion), which were spiked with one spike batch of MPs each and processed in two groups for KI and NaI (with laboratory blanks respectively). Both trials followed the modified Karami et al. (2017) protocol as detailed in Chapters ***1.3, 2.2.6*** & ***2.4***.

It is important to note that the NaI and KI react differently when added to ultrapure water. The dissolution of NaI in ultrapure water is exothermic, hence, the Florence flask was placed on ice to cool the solution to RT. Contrary, the dissolution of KI in ultrapure water is endothermic, hence, the solution needed to be warmed up to RT by leaving it standing safely in the laboratory for some time.

2.3.1 Extraction Efficiency Validation

For the selection of the best method, 3 filters per sample were created: The initial pour-off from each sample, supernatant 1, was filtered onto one filter (S1). Repeating the density separations three times created three additional supernatants, numbers 2 – 4, which were combined on one filter (S2-4). And lastly, the residue (R) was rinsed out and filtered. Across all filters, the recovered particles were counted, and the recovery rates were determined. This was done by comparing the number of plastic particles added and retrieved after the two methods were completed for both zebrafish and pellets (***Ch. 3.2***). For the colored particles this was easily possible without magnification, however, to find white and transparent particles in the filtered residue, a stereo microscope was used.

2.4 The Final Protocol

Based on the best results from the pilot studies (***Ch. 3.1***) and the comparison study (***Ch. 3.2***), the following protocol was developed. It consists of the dissolution of the samples with ultrapure water, followed by a KOH digestion, a density separation protocol using KI, and a filtration protocol (***Fig. 2.7***). This protocol was applied to a total of 20 regurgitates, collected across the 2022 field season on Sklinna, Norway. Of these 20 samples, 10 were further analyzed with a stereo microscope, see Chapter ***2.5.1***. Moreover, three field blanks and three laboratory blanks were processed.

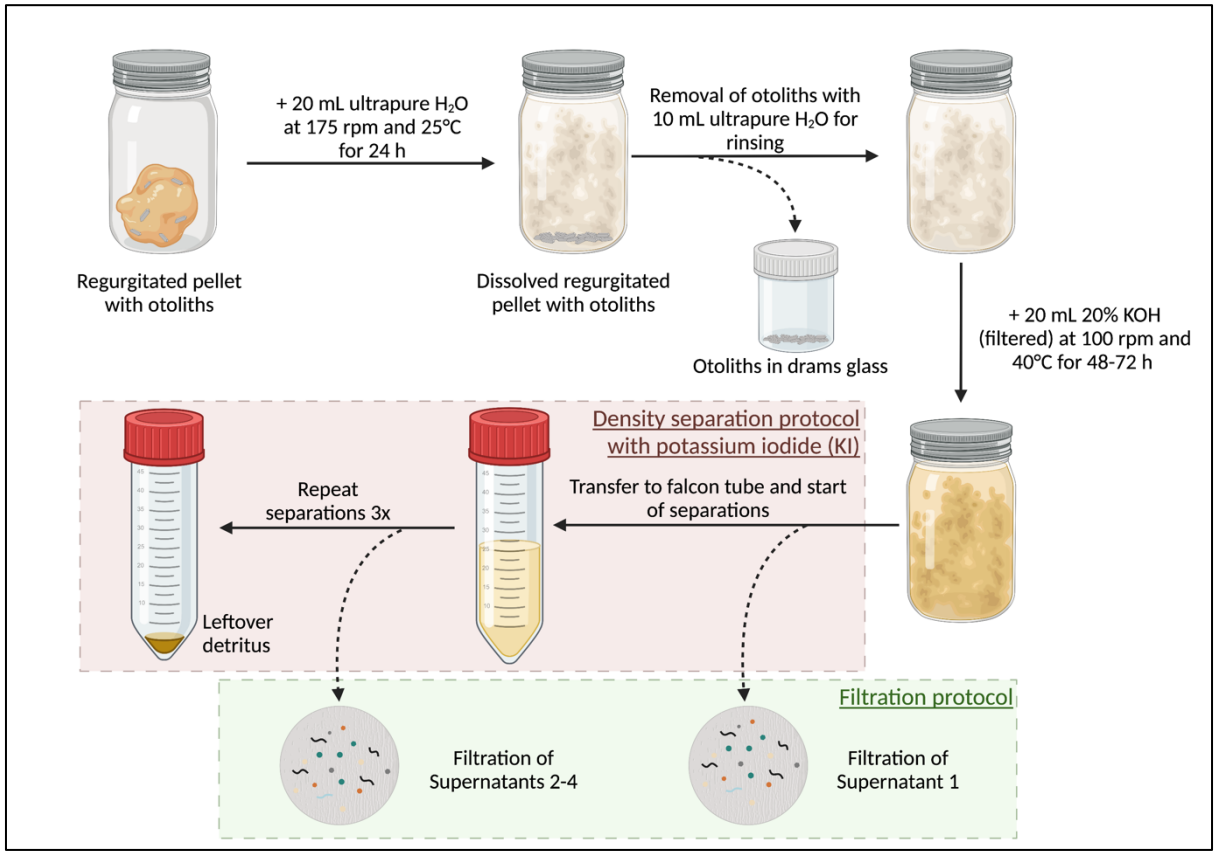

Figure 2.7 – Illustration of the entire final protocol created for this study. The protocol can be divided into three distinct phases: 1. the preparation of the sample to remove otoliths, 2. the density separations, and 3. the filtrations. (Partially adapted and modified from Karami et al. (2017); created with BioRender.com)

2.4.1 Step 1: Dissolving of Regurgitates and Removal of Otoliths

After thawing the samples to room temperature in their original glass jars, mucus subsamples for further studies were taken under sterile conditions. The regurgitates were then carefully pulled apart and 20 mL ultrapure water was added to each jar. The aluminum foil was removed, the original lid secured with parafilm and the jars were placed on a horizontal shaker table at 25° C and 175 rpm for approximately 24 h.

After this interval, it was possible to pick out the otoliths. To ease access, on a safety bench (Holten Lamin Air), the diluted pellet was poured off into a 100 mL beaker and covered with aluminum foil immediately. The otoliths were removed with tweezers and stored in labeled drams glasses. Once all otoliths were removed, the liquids were poured back into the original jars. The beaker, tweezers, and otoliths in the drams glass were rinsed twice with glass pipettes and a maximum of 10 mL ultrapure water, which were then also added to the original glass jar. Following this, the open drams glasses were placed in a heated cabinet to dry for 3 – 4 h at 40° C, after which they were sealed with plastic lids.

2.4.2 Step 2: Digestion of Organic Matter

To each jar, 20 mL of filtered 20% KOH solution (≈3.8 M) was added. Because of the danger of developing gases, the lid was neither tightly shut nor sealed, but placed loosely on the glass jars. Depending on the volume of the regurgitate, the total volume in each jar was around 50 mL with an approximate concentration of 10% KOH (≈1.9 M) remaining.

The jars were then placed on the horizontal shaker table at 40° C and 90 rpm for 48 – 72 h. To disturb the settled pellet, the jars were screwed shut, shaken manually, and replaced with loosened lids every 24 h.

All following steps in which samples are opened occurred on a safety bench (Holten Lamin Air), that pushed filtered air down and outwards. This was to minimize any potential contamination from flying MP particles in the laboratory.

2.4.3 Step 3: Density Separation

This part of the protocol, with some small modifications, was adapted from Karami et al. (2017). After 48 – 72 h, the jars were taken from the heated horizontal shaker table and left to cool down so that the diluted slurry can settle. After 2 – 3 h, the supernatant was poured off into a 100 mL glass beaker and covered with aluminum foil immediately. Approximately 20 – 30 mL of ultrapure water was used to rinse the slurry into falcon tubes (Sarstedt, 50 mL) and to rinse the original glass jar twice. The tubes were then left to settle once more. After 1 – 2 h, the supernatant was poured and pipetted off into the corresponding glass beakers, until only 5 mL of volume remained (***Fig. 2.8***). The liquid in the beaker constituted supernatant 1 (S1) (***Fig. D.10***). To prevent further digestion and damage to possible plastic particles, S1 should be filtered immediately.

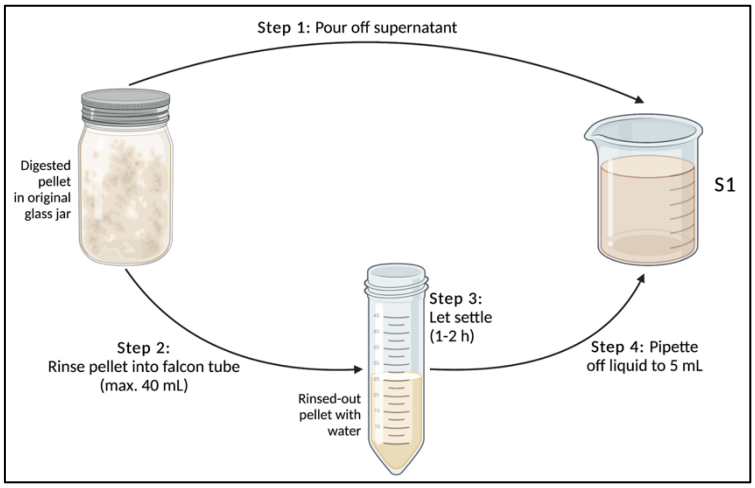

Figure 2.8 – Handling of the sample after KOH digestion and creation of supernatant 1. (Created with BioRender.com)

To each tube, 20 mL of filtered 5.5 M KI solution was added. The tubes were then placed in a rack and submerged in water in a hypersonic bath at RT for 5 minutes. After that, the tubes were transferred to a shaker table. There, the liquid was briefly agitated at 400 rpm and then left to shake for another 5 minutes at 300 rpm. In the last step, the tubes were centrifuged at 500 rpm. The samples were then carefully removed from the centrifuge and the liquid was pipetted off into new glass beakers until the 5 mL marks were reached again. Another 20 mL of KI was added to each tube and the density separation process was repeated two more times, meaning three times in total. The supernatants of these three repetitions were collected in the same glass beaker for each sample. This was supernatant 2 – 4 (S2-4).

Lastly, and only if a filtration of the pellet was needed, 50 mL ultrapure water could be added to the tube. This constituted the residue (R).

2.4.4 Step 4: Filtration

For the filtration of supernatants, a glass flask was attached to a water-driven vacuum pump. A Büchner funnel with rubber seals was placed on the flask. This setup was simple and efficient but poses a safety risk: The flask can implode and shatter under the pressure of the vacuum. It was therefore wrapped in bubble wrap and a thick plastic bag to protect against possible shards (***Fig. 2.9***). Additionally, personal safety gear (laboratory coats, safety glasses, etc.) was used.

Figure 2.9 – Filtration setup for paper filters. It is important to pay close attention to the vacuum intensity, as the flask can implode. It is also advisable to prepare filtration as depicted: The labelled Petri dish, sufficient water for rinsing and all other necessary tools lie ready. (Photo: K. Buchholz)

After starting the vacuum pump, the funnel was rinsed twice with ultrapure water, paying careful attention to the inside, to rinse off any possible contaminants. With clean tweezers, a round filter paper (VWR, Quantitative filter paper, 454) is placed in the funnel. The filter was also rinsed twice with ultrapure water to moisten it and ensure the vacuum drew through the funnel pores and filter paper. The supernatant was then be poured onto the filter in a slow and controlled manner, as the liquid should not rise above the edges of the filter paper. The glass beaker was rinsed twice with ultrapure water. Using a glass pipette the inside of the funnel was carefully rinsed with ultrapure water. With a second pair of clean tweezers, the filter can then be lifted into an upside-down plastic Petri dish, which was closed immediately. After this, the funnel was rinsed thoroughly at least two more times with ultrapure water. The filters within the Petri dishes were be left to dry by lifting the lid slightly, replacing it on an angle, and covering them with a layer of aluminum foil.

2.5 Microplastic Analysis

2.5.1 Stereo Microscope Analyses

The corresponding pairs of filters (S1 and S2-4) of the samples are viewed under a stereo microscope. Including this step helped to find suspected MP items before using the Raman spectrometer as well as reducing the exposure to air and lowering the costs. The filter within the petri dish was divided into four quarters, much like a compass, and marked accordingly on

the edges of both the lid and bottom of the petri dish (***Fig. 2.10***) This allowed the two dishes to be readjusted in case of misalignment. Suspicious items, including particles and fibers, were marked on the lid and pictures of each marked-up Petri dish were taken (***Fig. D.14***).
Without removing the lid, the dish was placed under the stereomicroscope. To ensure every part of the filter was examined, the search for possible MPs followed a pre-determined zigzag pattern (***Fig. 2.10***). This pattern was repeated at least two times per filter.

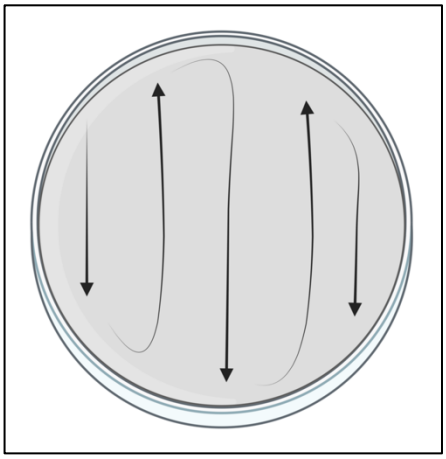

Figure 2.10 – Petri dish search pattern for stereo microscope. Each dish was carefully and systematically searched (Created with BioRender.com)

2.5.2 Raman Spectrometer Analyses

With possible MPs marked on the Petri dishes, the samples were then analyzed with a Raman spectrometer (WITec confocal Raman imaging system alpha300 R, WITec GmbH, Germany), which has a laser intensity range from 0.1 – 66 mW. The software (Control Five 5.2 Plus Version, WITec GmbH, Germany) operating the spectrometer was linked to an analysis program (WITec TrueMatch GmbH, Germany) which included a library of known chemicals and polymers (ST Japan-Europe GmbH, Germany). The spectra of potential MPs were compared to this library, for which the software provides a likelihood percentage. Moreover, the spectrometer was connected to a camera, so that images of the particles/fibers could be taken before and after the laser analyses. This allowed for precise calibration of laser intensity, time, and reiterations. Correct calibrations were critical: A too-intense laser or prolonged exposure can melt and burn MPs, especially fibers, and the resulting spectra cannot be used. For plastic analysis, it was therefore best to start with a low laser intensity setting, e.g., 0.5 mW, and carefully increase from there. A good starting point were the settings listed in ***Table 3***; however, adjustments had to be made according to each particle (fiber, particle, general size).

Table 3 – Settings and values used as a baseline for analysis with the Raman spectrometer.

Setting name	Value
Laser intensity	0.5 mW
Single spectrum s	1
Accumulations	20

2.6 Diet Identification by Otoliths

Otoliths extracted in Step 1 (***Ch. 2.4.1***) were examined under a photomicroscope (Zeiss Axio Zoom V16, Carl Zeiss Microscopy GmbH, Germany) using the accompanying microscopy software ZEN5 (Blue Edition, Carl Zeiss Microscopy GmbH, Germany). Out of the 20 original samples, 10 samples were analyzed in the following manner.

The content of the Drams glasses was transferred into a glass Petri dish each and placed under the microscope. With tweezers and a needle, the otoliths were carefully turned so that the inside was facing up (***Fig. 2.11***). The ZEN5 software was equipped with a live mode with rulers, in which the otoliths can be measured without the need to take photographs. The width and length of each otolith were measured to the first decimal. Three guides were recommended to identify the family level (for gadoids) and/or species: Camphuysen & Henderson, 2017; Härkönen, 1986; Jobling & Breiby, 1986. For the case study, 403 otoliths were thus measured and identified.

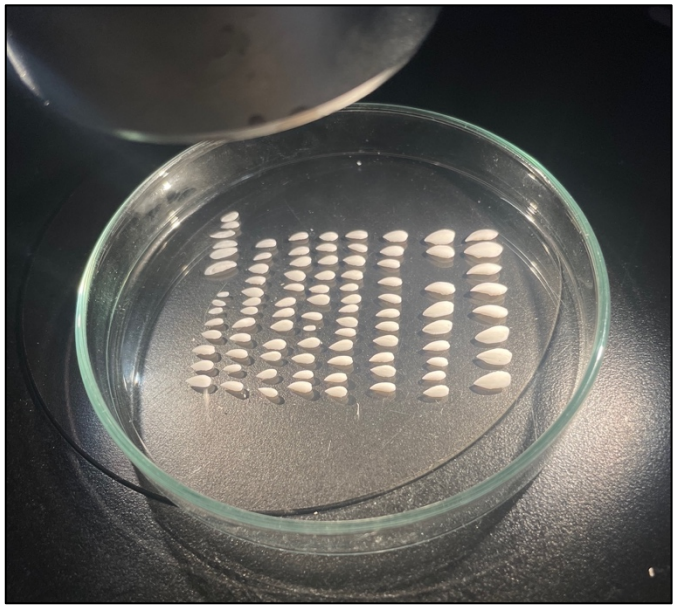

Figure 2.11 – Photograph of the otoliths found in a single pellet. They are loosely sorted by species/family and size so that the same frame can be used for measurement without readjusting the scale between otoliths. (Photo: K. Buchholz)

2.7 Data Analysis

Data was stored in and visualized with Excel (Microsoft Cooperation, 2023). Visualization of diet analysis was performed with RStudio (R Core Team, 2022) and the ggplot2 package (Wickham, 2016). Whenever identification of the fish was possible to the species level and fish-specific formulas were available, the calculations for fish mass and length followed the protocols described in Dehnhard et al. (2021), namely Härkönen (1986) and Jobling & Breiby (1986; exclusively for cod fish length). Likewise, when identification was only possible to the family level, the frequency distribution among species was applied when calculating fish length and mass, as previously done by Dehnhard et al. (2021). The frequency distribution among species was provided from diet samples collected in the period 2007 – 2018 (Lorentsen, unpublished data), and analyzed in the same way as described by Hillersøy & Lorentsen (2012). In this dataset from 2007 – 2018, 82% of otoliths belonging to the family Gadidae were identified to be from Saithe, 11% were from Poor cod, 6% were Cod, and the remaining 1% were Rockling sp. (*Ciliata mustela* or *Rhinonemus cimbrius*), Pollack (*Pollachius pollachius*), and Tadpole fish (*Raniceps raninus*).

As Saithe was the predominant diet species in the family Gadidae for shags at Sklinna, the cut-off values for age classes for Saithe were used for all fish belonging to the family Gadidae (Hillersøy & Lorentsen, 2012). Thus, fish of the family Gadidae that were < 120 mm length were defined as 0-group, 120 – 250 mm as 1-group, and those >250 mm as 2-group or older.

3 Results

3.1 Pilot Studies

3.1.1 Test 1: Preliminary Digestion Tests

The dissolutions with Biotex® solution and ultrapure water resulted in states of varying homogeneity (ranging from liquid to thin mucus strips) and coloring (ranging from milky-grey to yellow and orange). However, Biotex® resulted in a more homogenous slurry than the dissolution in ultrapure water. In both 'slurries' (***Fig. D.2***), the loosened otoliths and residue were visible at the bottom of the glass jar.

3.1.2 Test 2: Otolith Removal

Due to the narrow opening of the original glass jars, otolith removal was difficult. Transferring the slurry to a Petri dish made it easier to remove the otoliths. However, this method was discarded due to reasons discussed in Chapter ***4.2.2***, i.e., practicality, volume limitation, and risk of contamination. Instead, 100 mL glass beakers were used. The wider opening was better suited for using multiple tools, the surface exposed to air was smaller, and they were easier to flush.

3.1.3 Test 3: Filtration

The vacuum pump in combination with the coarse metal mesh (150 – 212 µm) effectively pulled the slurry through the meshes, thus removing any particles smaller than approximately 212 µm. Even pellets containing a lot of debris could easily pass through this mesh without too much difficulty. The finer metal mesh (100 – 141 µm) retained particles larger than approximately 141 µm. However, the finer mesh clogged more easily than the coarse mesh (***Fig. 3.1***). The metal filters were sonicated and filtered onto paper filters (see the setup in ***Fig. 2.9***). The amount of residue remaining on the paper filters was observed to be moderate. Especially the sand and fine detritus previously observed in the glass jars was absent after these steps.

To test nitrocellulose filters, which have the smallest particle retention (0.8 µm), only one sample was used. Here the filtrate from a previous paper filtration was filtered, which nonetheless clogged the nitrocellulose filter almost immediately. Two nitrocellulose filters were required to filter the entire filtrate.

Figure 3.1 – A sample clogging the fine metal mesh filter (Photo: K. Buchholz)

Irrespective of treatment, the paper filters clogged fast, too, and 2 – 8 filters were needed per sample. Even the more homogenous slurries from the Biotex® pre-digestions clogged the paper filters easily. All filters held large quantities of fish bones and other organic matter, such as seashells and algae.

The filters were completely covered in layers of fish bone, shell fragments, algae, sand, and other residue, making it almost impossible to detect smaller MPs, as these could be underneath, in between, or stuck to the detritus. No obvious MP >5 mm could be observed.

3.1.4 Test 4: Potassium Hydroxide Digestion and Subsequent Filtration

The filtrations after ultrapure water/Biotex® treatment both revealed large amounts of detritus, which hindered further analysis (***Fig. 3.2***). All concentrations of KOH (1 – 2 M) (***Table C.1***) improved the state of the slurry. However, the higher the concentration, the better dissolved the mucus was. Hence, the 2 M KOH solution was the most efficient.

Compared to the initial tests, fewer fishbones and organic matter were left on the filters, however, their number was still profound.

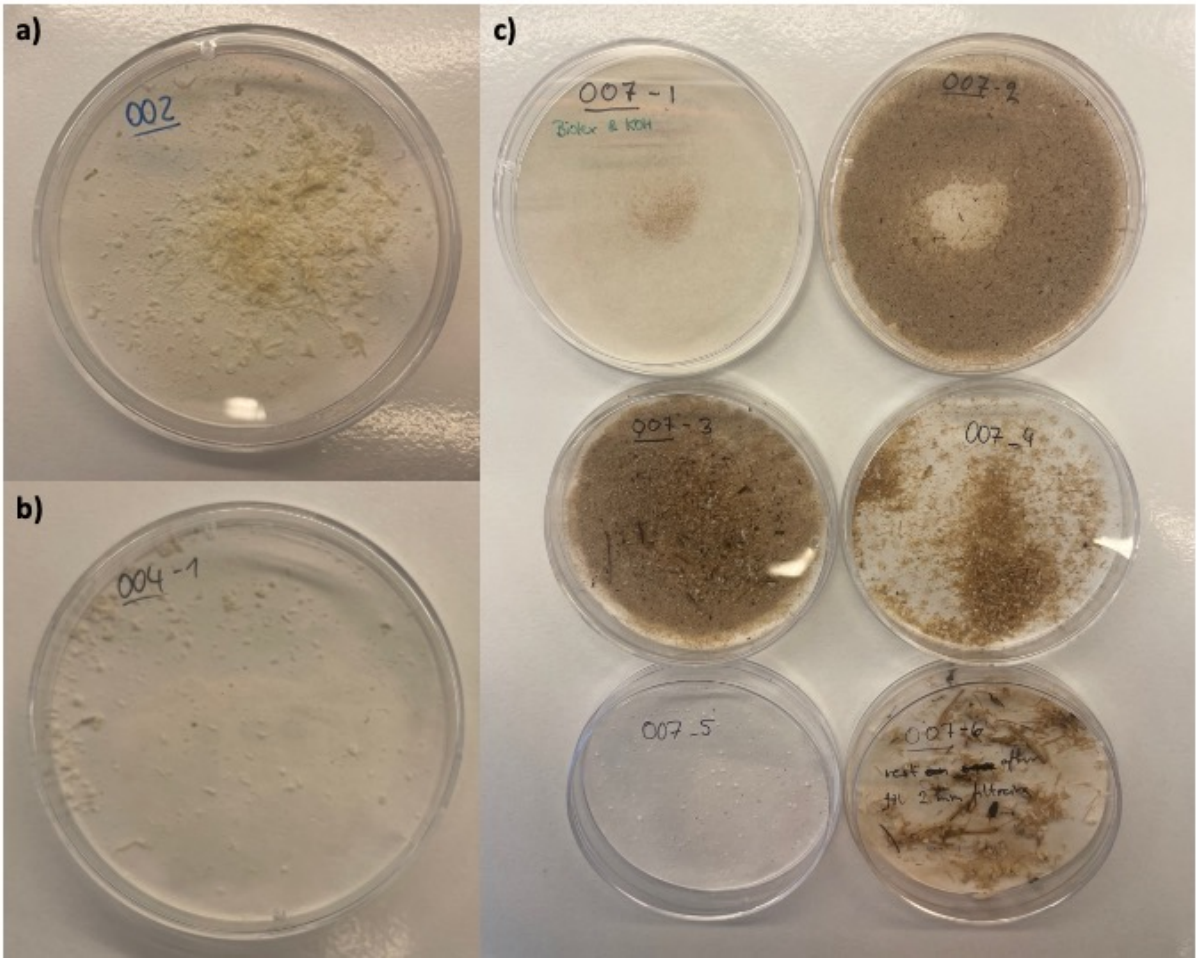

Figure 3.2 – Samples with different pre-digestions and KOH digestions filtered on paper. The final concentration of KOH in these three examples was approximately 1 M. **a)** Result of filtering sample 002 (ultrapure water + 5 M KOH) on paper. A decent number of fishbones remained. **b)** Filtration of sample 004 (ultrapure water + 5 M KOH) resulted in a few fishbones on the paper. **c)** Worst-case example. Six filtrations were needed to filter the entirety of sample 007 (Biotex + 5 M KOH). The number of small to large fishbone and organic detritus is high and much sand made this sample hard to filtrate. (Photos: K. Buchholz)

3.1.5 Test 5: Oil Separation

The separation worked for all spikes except the dark blue particles, which proved very resistant to adhere to any oil layer. Contrary to all other particles used for spiking (***Ch. 2.2.5***), the dark blue MPs only rose to the surface when the pH of the liquid was lowered by adding several drops of 4 M HCl.

To test how fishbones from pellets would interact with the now acidic solution, some fishbones were added and dissolved within minutes. As it is known that acids also damage and/or dissolve plastics, this approach was deemed unsuitable.

3.1.6 Test 6: Density Separation Tests with NaCl and KI

Tests containing Biotex® solution did not work well and most MP particles did not rise. Adding NaCl solution to ultrapure water and KOH, however, worked well. Most particles rose to the top. Density separation with NaCl in ultrapure water and KOH worked far better than NaCl solution with a Biotex® and KOH.

The KI density separation tests were performed with 3 samples and one batch of MPs each. The samples underwent one round of KI density separation and were filtered onto paper, which resulted in an overall MP recovery rate of 83%. As the KI approach looked highly promising, the protocol was developed further from this.

3.2 Comparison of Two Density Separation Methods with Spiked Samples

3.2.1 Recovery Rates

To compare the effectiveness of KI and NaI density separation, all zebrafish samples and pellets from shags were spiked with a known number of MPs in different colors (***Ch. 2.2.5***). The first supernatant (S1) was created by pouring off the liquids after KOH digestion and rinsing the pellet with ultrapure water. While neither KI nor NaI was used in this step, it was important to note that the MP particles already reacted to the ultrapure water and KOH. By inspecting the S1 filters, it was determined that pouring off and rinsing the pellet led to an average MP recovery rate of 62-80%. This affected the impact of the density separation, as some samples contained more MPs than others.

For comparing the performance of sodium iodide and potassium iodide as density separation methods, only supernatants 2-4 (S2-4) were relevant (***Fig. 3.3***). Counting the MPs on the S2-4 filters of zebrafish, showed that KI was more effective than NaI, with an average recovery rate of 30% compared to NaI's 16%. Contrary to this, in pellets, NaI was slightly more effective with a 14% recovery rate compared to KI's 12%.

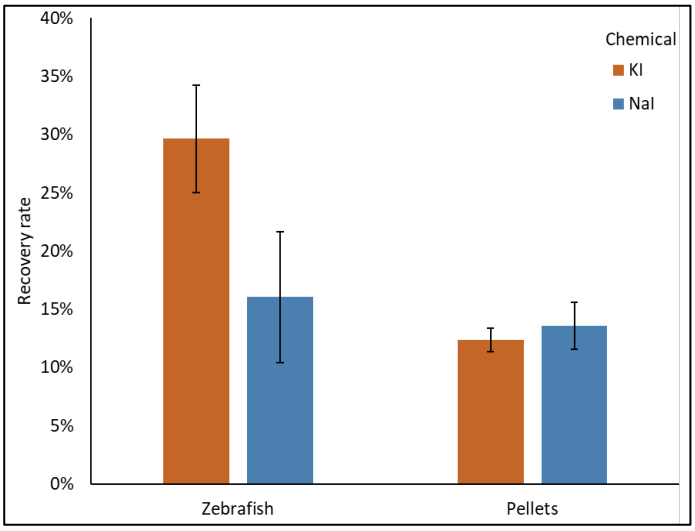

Figure 3.3 – Recovery rates of KI and NaI. This figure depicts the recovery rates of KI and NaI density separation, based on three replicates each. The whiskers represent the standard errors.

The overall average recovery rate was 91% for KI and 92% for NaI, respectively. Disregarding the colors of recovered particles, both methods were thus equally effective overall (***Fig. E.1*** & ***E.2***). It is important to note that the KI and NaI tests in both zebrafish and pellets have only three replicates each.

Analyzing the residues allowed to determine how much of the lost MPs could be accounted for and how many were lost during the procedure. For KI, 2% were recovered from the residue, meaning approximately 7% were lost and unaccounted for. Across all NaI-treated pellets, 6% of the MPs were lost, while 2% were recovered.

3.2.2 Impact of Color

As stated in Chapter ***2.3.1***, the colorful spiked particles stood out immediately and could therefore be counted without the need for magnification. Transparent and white particles, however, were more challenging to detect or were not detectable at all.

Table 4 shows that transparent particles were lost most frequently with 28% of all transparent particles used to spike. This is particularly the case for KI, where 33% of transparent particles were lost. For KI, this is closely followed by 22% loss of white particles. For NaI, the largest loss was also in transparent particles, with 22%. The other critical group for NaI is the purple MPs, of which 17% were lost during the process. No particles were found in the blanks.

Table 4 – Overview of the MPs recovered during KI and NaI testing with both zebrafish and regurgitated pellets as samples. N = 3 for both zebrafish- and pellet samples per KI and NaI treatment. This results in a total of 12 samples. Every sample was spiked with 3 MPs of each color, resulting in 27 particles per sample.

	Percentage of particles recovered in						
Color (N=3 per color)	**Zebrafish KI**	**Pellets KI**	**∑ KI**	**Zebrafish NaI**	**Pellets NaI**	**∑ NaI**	**∑ Total**
Purple	89%	89%	89%	78%	89%	83%	86%
Green	89%	100%	94%	100%	100%	100%	97%
Light blue	100%	100%	100%	89%	89%	89%	94%
Dark blue	100%	100%	100%	89%	100%	94%	97%
White	78%	78%	78%	100%	89%	94%	86%
Transparent	56%	78%	67%	78%	78%	78%	72%
Red	100%	100%	100%	89%	89%	89%	94%
Pink	100%	100%	100%	100%	100%	100%	100%
Grey	100%	89%	94%	100%	100%	100%	97%

3.3 Case Study on European shag Pellets

The final protocol was applied to 20 original pellets from the start, middle, and end of the 2022 field season (***Table A.1***). Of these, 10 samples were analyzed further (for otoliths, light microscopy, and Raman spectroscopy). For quality control purposes, 3 laboratory blanks and 3 field blanks were processed alongside the original samples.

3.3.1 Diet Analysis

In total, 403 otoliths from 10 regurgitated pellets from European shags were measured and analyzed. The 10 pellets contained vastly varying numbers of otoliths, ranging from 3 to 91 otoliths (***Table 5***). Although some fish species could be identified, for this summary, only the families were counted, as they are the common denominator.

Altogether, 4 fish families were present. Of the 403 recovered otoliths, 380 otoliths, or 94% were identified as Gadidae. The other identified families were Ammodytidae (3%), Labridae (2%), and Pholidae (0,2%).

The average estimated fish length per sample varied from 87 mm to 258 mm (***Table 5***), however within the samples, the variance was even higher. For example, the shortest fish of this study was found in sample M1-01 with an estimated length of only 17.36 mm, while the largest fish in the same sample had an estimated length of 218.62 mm; both were Gadidae not identified further to species level.

Table 5 – Summary of the results from the diet analyses of 10 regurgitated pellets collected during the start, middle, and end of the Sklinna field season 2022. Ø = Average. Age classes were only assessed for fish of the family Gadidae, based on cut-offs for their most frequent prey species, Saithe.

Sample ID	No. otoliths	No. fish families	Ø length [mm]	Ø mass [g]	Ø age class	MPs	MP type
S2-01	21	2	133.07	61.16	0.63	-	-
S2-03	54	2	119.39	19.13	0.29	1	fiber
S2-05	91	2	115.27	13.35	0.21	-	-
M1-01	24	2	138.37	19.34	0.50	-	-
M1-02	3	1	257.53	180.18	1.67	2	fibers
M1-03	25	3	111.56	16.95	0.18	-	-
M1-05	15	1	107.97	23.46	0.27	-	-
E1-01	37	2	94.08	15.29	0.23	1	fiber
E1-02	80	2	87.30	9.25	0.28	-	-
E1-03	53	1	129.36	20.90	0.43	2	1 fiber, 1 fragment

The average estimated fish mass per sample varied from 9 g to 180 g (***Table 5***), but here too, the variance within the samples was much higher. Predictably, the shortest fish in this study was also the lightest with an estimated weight of only 0.02 g. Within the same sample (M1-01), the heaviest fish was estimated to weigh 93.9 g; again, both were Gadidae not further identified to species level.

Fish age classes were estimated only for fish of the family Gadidae, and the average fish age class per sample varied from 0.18 to 1.67, however, in 7 out of 10 samples, the predominant age class was the 0-group (N = 270 otoliths). Fewer fish fell into the 1-group (N= 124 otoliths) and only 9 otoliths belonged to fish that were 2 years old or older.

The obvious outlier in this table is sample M1-02, which contained the largest, heaviest, and oldest fish of this study, but totaled only 3 otoliths. All other samples contained fish that were considerably shorter, lighter, and younger.

3.3.2 Stereo Microscope Analysis

Using a stereo microscope, across all 10 samples (N=20 filters) (***Fig. 2.10***) as well as the 3 field blanks (N=6 filters) and 3 laboratory blanks (N=6 filters), a total of 100 items were found that could potentially be MPs. These included both fragments and fibers.

Across 10 sample filters, 50 items of interest were found, with an average of 4.9 items per sample. These were mostly dark items or spots and fibers. On the field blank filters, 20 items of interests were found (on average 6.6 items/sample). The laboratory blanks contained 30

items of interest (10 items/sample), which were visually similar those found in the field blanks. The items found on the field and laboratory filters were alike, and only partially obvious on the sample filters.

One of the primary contaminants after the separation protocol was arachnids, most likely mites (Acari) (***Fig. 3.4***) and their partial remains. Numerous mite legs were observed on the final filters from pellets.

Figure 3.4 – One of many mites (Acari) found *on the filter paper after the last filtration step.*
At first glance, broken off legs were easy to confuse with fibers. (Photo: K. Buchholz)

3.3.3 Raman Analysis

Out of the 100 items deemed suspicious under the stereo microscope, seven items were lost, meaning 93 items were found again under the Raman spectroscope. Of these, 51 items were not identified, because they were irrelevant items after all (i.e., mite parts, discoloration, shadows in the filter paper). Further 36 items were analyzed but yielded highly inconclusive results (Hit Quality Index (HQI) 12 – 88%). While the spectra matched chemicals from the ST Japan database, the majority were heavily distorted by fluorescence and/or were identified as non-plastics (with low to moderate certainty).

The only clear and identifiable spectra were the six shown in Figure ***3.5***. All six items were identified as polypropylene (PP) with HQI's ranging from 85% – 95%. Of the six items found, five can be classified as fibers (***Fig. 3.5.a – e***). These five fibers, i.e., Figure ***3.5.a – e***, were of similar dark grey color. In length, the fibers varied from approximately 500 – 3,900 µm.

The item depicted in Figure ***3.5.f*** was a transparent particle, also identified as PP (HQI = 93.25%) and approximately 200 – 300 µm in diameter. For all spectra except for Figure ***3.5.a*** some level of fluorescence can be observed.

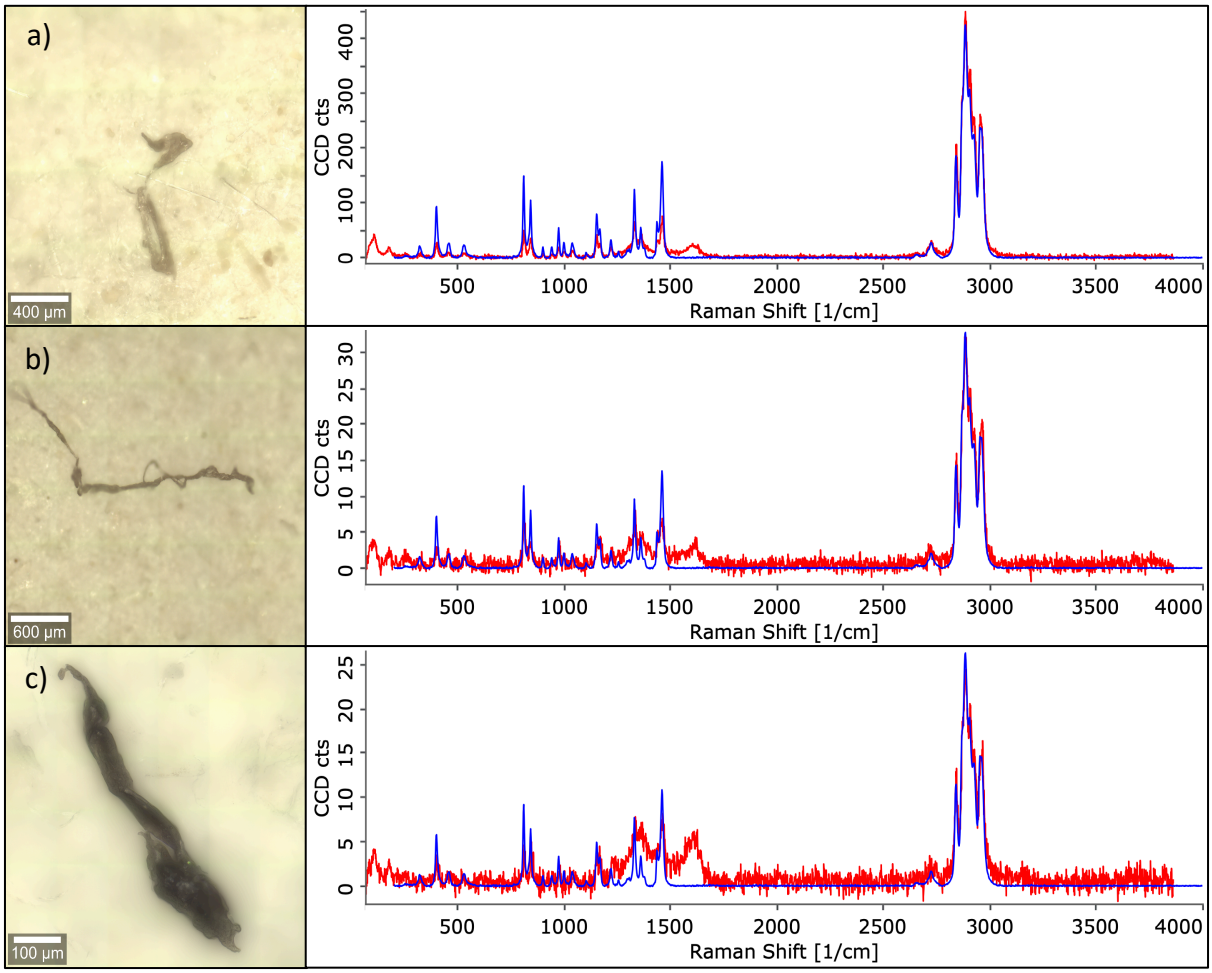

Figure 3.5 **– Items found in regurgitated pellets and their respective Raman spectra.** Images were taken using the "image stitch" function in the Raman software, which automatically inserts the matching scale. The corresponding spectra to the right side of the images are the results of comparing the spectra to the library of "ST Japan". HQI = Correlation Coefficient. ***Figure 3.5.a*** „POLYPROPYLENE, ISOTACTIC ", HQI: 95.26; ***Figure 3.5.b*** „POLYPROPYLENE, ISOTACTIC ", HQI: 93.92; ***Figure 3.5.c*** „POLYPROPYLENE, ISOTACTIC ", HQI: 85.09

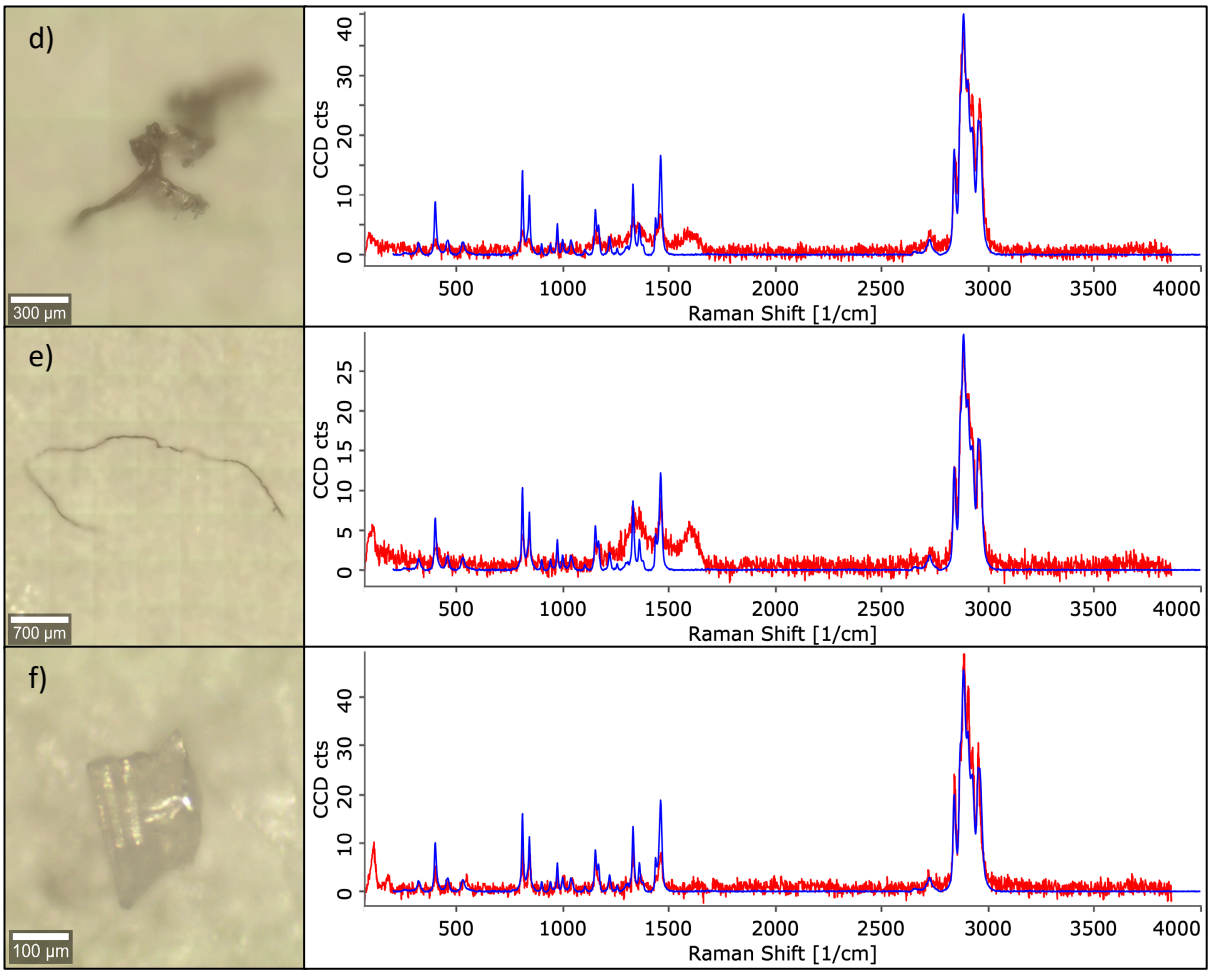

Figure 3.5* – Items found in regurgitated pellets and their respective Raman spectra (continuation).** Images were taken using the "image stitch" function in the Raman software, which automatically inserts the matching scale. The corresponding spectra to the right side of the images are the results of comparing to the library of "ST Japan". HQI = Correlation Coefficient. ***Figure 3.5.d „POLYPROPYLENE, ISOTACTIC ", HQI: 91.97; ***Figure 3.5.e*** „POLYPROPYLENE, ISOTACTIC ", HQI: 87.97; ***Figure 3.5.f*** „POLYPROPYLENE, ISOTACTIC ", HQI: 93.25

All attempts to identify the suspicious items from lab and field blanks described in Chapter ***3.2.2*** with the Raman spectrometer yielded inconclusive results, as the spectra depicted mostly fluorescence, and the ST Japan library did not suggest matches with satisfactory correlation coefficients (i.e., all HQI <85%).

3.3.4 Link between microplastic and diet

For illustrative purposes, the presence (=1) or absence (=0) of MP was plotted in a logistic regression against the mean age class, the number of otoliths, the number of species, the mean length, and the mean mass (***Fig. E.3***). The confidence intervals for the age class, length, and mass plots are mainly driven by a single outlier (M1-02) described in Chapter ***3.3.1*** (***Table 5***) and shown in Figure ***E.3***. However, no meaningful statistical tests can be performed with only the 10 samples and 6 detected MPs.

4 Discussion

4.1 Key Findings

Plastic and microplastic pollution is a serious environmental threat, due to its ubiquity in ecosystems, especially in freshwater and marine environments. We are still only beginning to grasp the many problems linked to this anthropogenic pollutant. Therefore, the development of monitoring tools that allow for quantification, assessment of contamination levels, and identification of trends and sources is crucial to further our understanding of the issues. A solid knowledge base is the starting point for informed decision and policy making, which are desperately needed to protect the environment and thereby human health.

At the start of this thesis, there were no established protocols that combined both diet analysis and microplastic analysis, that included MPs <1 mm, in the regurgitated pellets from seabirds. With my project I contribute to the growing pool of methods that are shared across research disciplines. I have reviewed methods from seabirds, fish, and soil sediment research to develop a single protocol, that is not harmful to the study species, the European shag, or the environment. Moreover, it can easily be implemented into already existing shag monitoring efforts and provides insight into local MP contamination levels. When applied to original samples, the KI-based approach I propose successfully separated six MPs from their matrices, i.e., regurgitated pellets. The MPs were all identified as PP. All plastics observed in this study were smaller than 5 mm and larger than approximately 200 µm.

4.2 Pilot Studies

4.2.1 Test 1: Preliminary Digestion Tests

Biotex® is a commercial enzymatic household laundry soap which was re-purposed due to its useful characteristics. There is, however, no guarantee that the Biotex® powder or its packaging process is plastic free, hence, the solution was filtered to minimize this risk.

For loosening the otoliths, both dissolutions (ultrapure water and Biotex® solution) worked sufficiently well (***Ch. 3.1.1***). In both approaches the dissolution was improved by pulling the pellets apart before placing on the heated shaker. This is most likely because Biotex® solution/ultrapure water had more sample surface to interact with. However, Biotex® was disregarded later due to its interference in the density separation tests, as described in Chapter ***4.2.6***.

The reason for using the original vials and their lids during this and the following steps was to expose the samples to potential contamination as little as possible. All exposure to air, tools and other containers poses contamination risks. The original lids were also used on the field blanks, which were processed alongside the samples, and no contamination was found on their respective filters later.

4.2.2 Test 2: Otolith Removal

It was easier to remove otoliths from Petri dishes, because it allows for the use of more than one tool at a time. However, this method was abandoned due to the limited number of glass Petri dishes available and the need for constant rinsing and cleaning. Moreover, the rinsing of the dish required a lot of ultrapure water. This had to be returned to the glass jar in addition to the slurry, thereby increasing the volume of the samples, which was limited by the 50 mL volume of the original jars. Using a large open surface like a Petri dish could also potentially increase the risk of contamination. Therefore, glass beakers were used, as they exposed only a small portion of the sample to the air and could be rinsed easily with only a small amount of ultrapure water.

4.2.3 Test 3: Filtration Tests

In this series of filtration tests, metal meshes, nitrocellulose filters and paper filters were tested (***Ch. 2.2.3, Table 1***). A great benefit of using the metal meshes lies in the fact that they are reusable and removed finer unwanted contents (sand, fishbone fragments, etc.). However, the meshes had to be sonicated and rinsed with ultrapure water before the residue could be filtered for a second time, but onto a paper filter (better suited for Raman spectroscopy). These additional steps lengthened the process significantly and required a lot of additional equipment. An important issue to note is that flexible fibers can bend and pass through the mesh, especially the coarser mesh, and thus be lost.

A major issue with paper and nitrocellulose filtrations was clogging. Especially the fine detritus (mucus residue, fine bones, other organic matter, sand etc.) led to slow filtrations or stopping them entirely. When a filter was blocked, the remaining slurry had to be pipetted off, which was particularly difficult since the slurry contained debris that barely fit in the 5 mL pipette tips.

Of the three approaches, paper filtrations worked best. However, the overwhelming amount of fish bone made it impossible to check for MPs, as they could potentially stick to the debris or

be covered by it. Any MPs, but especially those of similar color to the debris, i.e., white, transparent, and yellow, would be very difficult to spot. Hence, the decision was made to find an additional digestion step to decrease the amount of fish bone and detritus on the paper filters.

4.2.4 Test 4: Potassium Hydroxide Digestion Tests

The implementation of a digestion step with KOH reduced the amount of fish bones on the paper filters (***Fig. 3.2***), and the highest concentration, 2 M KOH, worked best. This matches the protocol from Karami et al. (2017), who suggested a 10% KOH solution, which equivalates circa 1.9 M. Despite this, a considerable amount of fishbone and detritus remained even after the KOH digestion. Some variation in outcome might also be due to the quality of the samples, which varied greatly. The KOH digestions were thus useful, but further steps to separate potential MPs from fishbones and other detritus were necessary.

4.2.5 Test 5: Oil Separation

Combinations of oils, ultrapure water and KOH solution were spiked with artificially created MP batches to observe the behavior of MPs when exposed to oil. The oil separation test confirmed the oleophilic nature of the MPs used for spiking, as 8 out of 9 MPs separated from the fish bones. HCl had to be added to make the dark blue particles separate, most likely by affecting the buoyancy between the pre-digestions and oil layer. However, the HCl also caused fishbones to dissolve entirely, and the lowered pH could potentially damage the MPs.

Overall, the downsides of the oil separation method outweighed the methods' benefits, and ultimately, this density-independent approach was regarded as unsuitable for this thesis. The original protocol by Crichton et al. (2017) was designed for MP extraction from marine sediments and was optimized for FTIR. Unfortunately, the Institutes' FTIR was unavailable for this study. However, having to focus the Raman spectroscope's laser under the oil layer, while possible, would be difficult and time-consuming. To ease these difficulties, all filters and tools would have to be rinsed thoroughly with a detergent and ethanol to remove all oil residues, which likewise is a time-intensive and resource intensive step.

4.2.6 Test 6: Density Separation with Spikes

Density-dependent approaches, as described in Chapters ***1.3.1*** and ***2.2.6***, like the NaI protocol suggested by Karami et al. (2017) for MP separation from whole fish, are common practice in MP research.

To gain an idea of how MPs behave when exposed to NaCl solution, an approach often used in MP/sediment research (e.g., Fries et al., 2013; Le Guen et al., 2020; Nuelle et al., 2014), preliminary density separation tests were performed and the MPs reactions to combinations of either Biotex® and KOH or ultrapure water and KOH were assessed. As detailed in Chapter ***3.1.6***, mixtures that contained Biotex® did not work well. This is likely due to the nature of Biotex®, which is a common laundry detergent, and as such acts like an amphiphilic surfactant. This means that microplastics, which are "naturally" hydrophobic, become less so when they are binding to the surfactant molecules, thereby improving their suspendability and ability to migrate within a solution (Jiang et al., 2021). This appeared to be corroborated by the tests conducted without Biotex® (i.e., NaCl, water, and KOH), most particles easily rose to the surface. Studies show that low-density salt solutions like NaCl can be insufficient in separating denser MPs (Cashman et al., 2020; Coppock et al., 2017; Hidalgo-Ruz et al., 2012).

In addition, KI was tested with MP spikes, which yielded surprisingly good results, i.e., a recovery rate of 83%. KI is effective in MP separation in marine sediment research before (Phuong et al., 2018). Seeing that KI poses no serious health and safety issues, whereas NaI does, and KI being cheaper than NaI, density separation with KI seemed a good alternative to continue with for this master thesis. This was further investigated in a comparative study.

4.3 Comparison of Two Density Separation Methods with Spiked Samples

The pilot studies were crucial to understand how the samples and MPs behave during various treatments and the lessons learned were applied to one more set of samples and one last test. This was the comparison of the density separation with NaI as detailed by Karami et al. (2017) and KI as alternative.

Regarding the results of the comparison (***Ch. 3.2***), it is firstly important to note that the pour-off after KOH digestion and the subsequent rinsing of the leftovers removed a large quantity of the MPs (62 – 80% found on filter S1) before density separation was even started. The number of MPs possible to extract after this step is therefore limited by the unknown number remaining in the leftovers. This became evident in the zebrafish tests, where for unclear

reasons, the pour-off removed a much larger number of MPs in samples designated for NaI density separation, than in those designated for KI density separation (***Fig. 3.3***). Hence, the recovery rates might differ. However, for the three pellets analyzed per treatment, on average the same number of MPs was poured-off, and both density separation treatments yielded similar recovery rates.

The recovery rates, including the pour-offs, from both sample types, were calculated for KI and NaI (***Ch. 3.2.1*** & ***Appendix E***). The overall effectiveness of KI separation (91% MP recovery) and NaI separation (92% MP recovery) are almost identical. Also analyzed were the loss of MPs and how much could be accounted for. These values, too, implicate similar effectiveness, i.e., in KI samples 2% of lost MPs were found in the pellet, meaning that 7% were lost during the procedure, and in NaI samples 2% of lost MPs were found in the pellet, meaning 6% were lost in the procedure (***Ch. 3.2.1*** & ***Appendix E***). Visual bias for certain colors is a likely reason for this, since the most frequently lost colors were transparent, white, and purple (***Ch.* 3.2.2**, ***Table 4***). Firstly, white, and transparent particles are very difficult to spot on the white filter paper and thus easy to overlook when scanning the filters for MPs. Secondly, transparent particles might have also been overlooked while pipetting and rinsing. Thirdly and contrary to all other colors, the purple particles were cut from a plastic piece that did not have a solid color but was transparent with a purple tint. Therefore, it is likely, that these MPs, too, were overlooked during the procedure. Lastly, the residue (R) was analyzed to account for MPs that were not caught by the methods. There, independent from which chemical was used, the problem of fish bones on the filters naturally persisted, making it difficult to spot all MPs. It is therefore possible that the "lost but accounted for" portion was actually larger for both treatments (***Ch. 3.2.1*** & ***Appendix E***).

Based on these results, both KI and NaI are equally good approaches for density-dependent separation. Thus, other criteria became deciding factors. NaI costs approximately 1300 NOK/kg (Norwegian crowns per kilogram) while KI only costs 560 NOK/kg, which is less than half the price of NaI.
Another important criterion is the safety and health risk associated with the use of NaI and KI. According to Norwegian regulations ("CLP-forskriften"), NaI is a moderately strong skin and eye irritant and has weak acute toxicity for aquatic life. KI is not listed as a risk for any of these. Both chemicals are rated as equally dangerous (low risk for organ damage) through prolonged and repeated exposure. KI was therefore overall the better choice.

4.4 The Final Protocol – A Case Study and its' Limitations

4.4.1 Otoliths and Diet Analysis

The true number of otoliths per sample, and thereby fish consumed, is likely higher than reported in this thesis. Some initial filters revealed miniscule otoliths and/or otolith fragments (<2 mm), which indicate that the transfer to the glass beakers (***Ch. 2.4.1***), did improve but not guarantee full visibility of otoliths. With regards to identification, otoliths should ideally be identified to species level and paired, whenever possible (Hillersøy & Lorentsen, 2012). However, this requires specific expertise and is a very time-consuming process and thus it was omitted from this thesis. The identification can be further convoluted by the fact that otoliths, although very resistant, dissolve when exposed to seabird stomach acids for a prolonged time. The acid attacks the surface, shrinking the otoliths and blurring their characteristic traits, a process known as "wear and tear" (R. T. Barrett et al., 2007; Dehnhard et al., 2021). This means that the true otolith measurements are probably larger, and, in the worst case, it is possible to assign them to the wrong age-class. However, sensitivity analyses, assuming that corrosion shortened otoliths by 10% in a large-scale diet analysis of great cormorants, found otolith corrosion to be overall negligible for their results (Dehnhard et al., 2021).

In the 10 analyzed samples, fish from the gadoids dominated the diet of European shags on Sklinna in 2022 with them constituting 94% of the total biomass consumed. This is consistent with previous studies by Hillersøy & Lorentsen (2012) and Lorentsen et al. (2004), that found gadoids to make up 92 – 98% of the shags' diet at Sklinna.

Also matching these previous studies at Sklinna (Hillersøy & Lorentsen, 2012; Lorentsen et al., 2018, 2019) most fish were belonged to the 0-group and 1-group. Young gadoids of these age classes, and especially Saithe, inhabit kelp forests in the coastal zones (Christensen-Dalsgaard et al., 2017). Kelp forests are also the preferred foraging habitats of shags from Sklinna (Christensen-Dalsgaard et al., 2017; Dehnhard et al., 2022).

4.4.2 Stereo Microscope Analysis

Across the 10 samples, three field blanks and three laboratory blanks, 100 potential MP items were found, but only six were later confirmed to be plastic (***Ch. 3.3.3***). Marking items of interests on the lids was crucial to reduce the working time with the Raman spectrometer and allowed for a pre-selection of certain items. Indeed, other particles, including mites (***Fig. 3.4***) were observed during stereomicroscopy. The mites were also observed on the breakwaters of Sklinna, and it is not surprising that many of them got stuck to the pellets. However, only the chitinous exoskeletons remained, as the KOH digestion likely dissolved all organic matter on the inside. Due to their size and shape, mite legs can easily be confused with fibers when viewed with the bare eye, emphasizing the need for stereo microscope analysis.

4.4.3 Raman Analysis

Six items were identified as PP. Of these, 5 were fibers of a similar dark grey color, while one item was a transparent fragment. PP is one of the most common plastic contaminants in the oceans (Erni-Cassola et al., 2019), however, other types are also highly prevalent, and the absence of any other polymers is thus somewhat surprising.

Under the assumption that the 91% MP recovery rate for KI, shown in Chapter ***3.2.1.***, also applies to the 10 original samples, then 0.6 (≈1) item(s) were lost during the separation process. However, there is no way to prove this, as the actual number of MPs in the original samples remains unknown.

After unsuccessfully trying to identify suspected MP fibers (***Ch. 3.3.3***; ***Fig. 4.1.a*** & ***Fig. 4.1.b***) on filters from both laboratory and field blanks, the filters with original samples were searched for similar fibers. These filters also contained the suspicious fibers. It can therefore be either assumed that the contamination stems from the procedure, e.g., the air or non-plastic clothing, or that the fibers stem from the filters themselves. This is a possibility, because the filters come in a box made of recycled carton and transfer from the packaging might occur. Several other possibilities for the ambiguity of the items shown in Figure ***4.1*** exist: The fibers could be a blend of natural materials (e.g., cotton, wool) and synthetic fibers, the fibers could be coated or covered in some organic compounds that are not registered in the database, or dyes or other additives was interfering with the Raman spectroscopy. This makes identification of the suspicious items difficult, as there is no guarantee that they are not partially made from plastic.

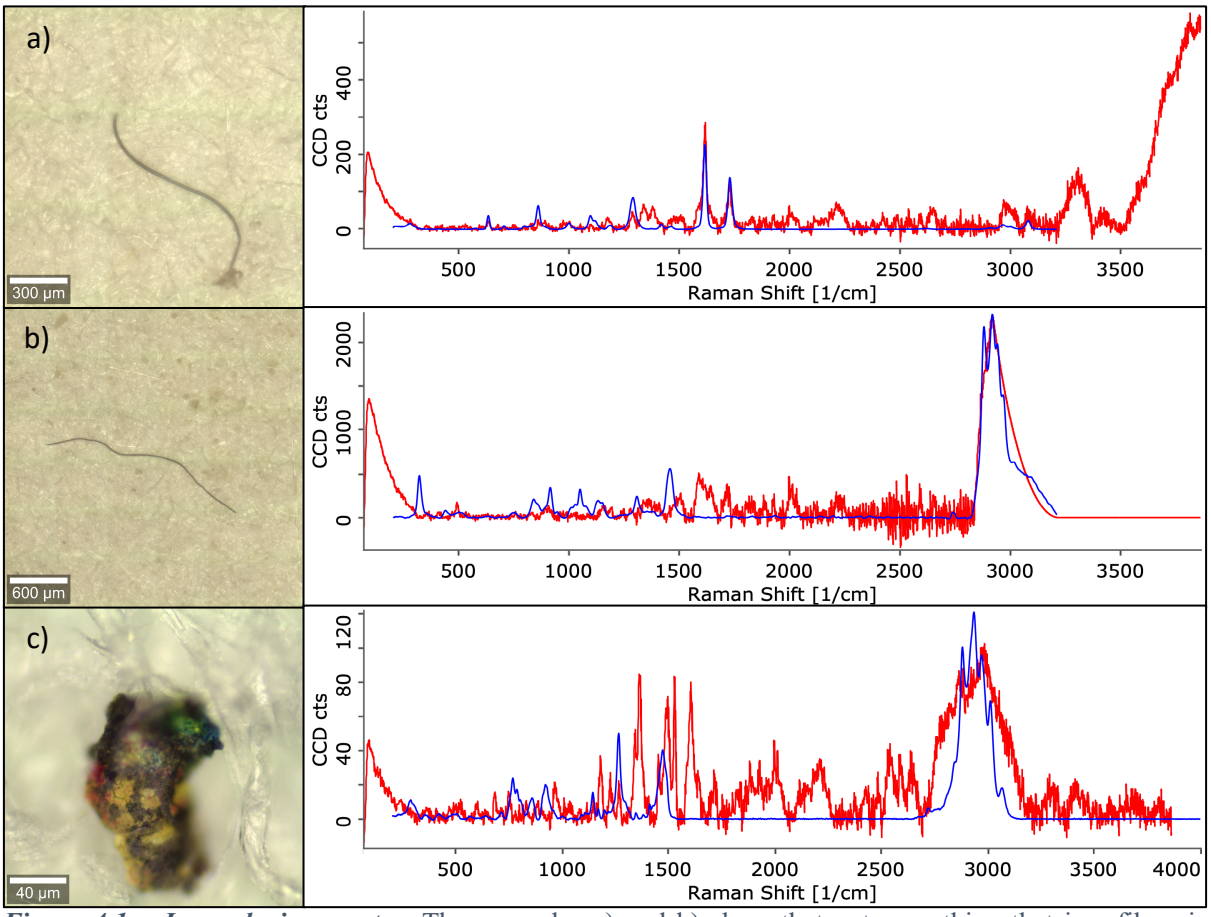

Figure 4.1 – Inconclusive spectra. The examples a) and b) show that not everything that is a fiber, is automatically a MP. The particle shown in c) also suspicious due to its colors is also not a MP. ***Figure 4.1.a*** "TAIKO-FE", HQI: 46.57; ***Figure 4.1.b*** "4-HEPTANOL", HQI: 85.63; ***Figure 4.1.c*** "POLYPOX R14", HQI: 48.39

4.4.4 Shortcomings of the Method

It is necessary to highlight that the samples used in the case study were carefully selected from a large pool of samples, which were collected for this very purpose. This made it possible to choose only the visually cleanest samples, i.e., those that did visibly contained little or no contaminants, such as pebbles and sand. Some contaminants such as algae are resistant to KOH digestion and will float in the density separation.

Furthermore, this protocol, while effective, is time-consuming. Considering all incubation times, it takes a sample approximately five days from thawing to filtration onto paper. The duration of KOH digestion plays a pivotal role, carrying both positive and negative implications. On the one hand, the suggested timeframe facilitates the optimal dissolution of organic matter as the base gradually breaks it down. On the other hand, it introduces the potential risk of damaging MPs, a concern substantiated in the study conducted by Karami et al. (2017).

Similar concerns about time arise from the filters used for the Raman analysis. Each paper filter has a surface area of approximately 64cm^2 and locating tiny items on them proved challenging. While marking suspicious items on the lids before Raman spectroscopy reduced the time spent searching, the items remained tricky to find.

Once the items of interests were found, operating the Raman spectroscope itself was also challenging, as the settings had to be adjusted to each item. This resulted in several scans per sample, usually five or more, until the ideal setting was found. However, despite multiple attempts the ideal settings could in many cases not be found, as fluorescence distorted the readings (***Fig. 4.1***), or the laser was too difficult to focus on the uneven surfaces of the items.

4.4.5 Microplastic Exposure and Diet

Due to the small sample size, it was not attempted to link the amount of MP to diet or age class composition. Notably, however, the outlier sample M1-02 (***Table 5***) contained only 3 otoliths, but had the longest, heaviest, and oldest fish in this study and contained two MPs (the highest amount found). One could thus speculate that MP content increases in older fish, thus pellets that contain otoliths from larger fish should be more likely to contain MPs. However, sample M1-05 had the second highest average age class and contained otoliths only from gadoids, but no MP was found. Based on the average length, the sample M1-01, would be of interest. However, this sample, too, contains no MP. For average length, the next highest value belongs to sample S2-01, but here too, no MP was found. Sample S2-01 also has the highest average age class after M1-01, but again, no MP was found (***Table 5***).

In contrast to this, the only other sample containing 2 MPs is E1-02, which, contrary to outlier M1-02, comprised 80 otoliths of very young Gadidae, predominantly of age class 0 (***Table 5***). Another sample was S2-05, which contained 91 otoliths, also predominantly of age class 0, but contrary to E1-02, did not contain any MPs. To conclusively draw a link between diet and MP contamination, a larger number of samples needs to be analyzed.

4.5 Significance of Reported Findings

This thesis can be viewed as a complementary study to Maaseide (2022), who proved MP exposure in shags in the Sklinna population by analyzing their feces (Maaseide, 2022). The approach proposed in my thesis investigated regurgitated pellets as a monitoring matrix and supports Maaseide's (2022) findings that European shags at Sklinna are consuming MPs. It is

the first study to do so for the Norwegian shag population and adds to the pool of information and methods available for monitoring plastic exposure and contamination in seabirds.

Overall, plastic was found in 40% of the samples in this study. This value is lower than the 63% that Álvarez et al. (2018) reported from European shags pellet they collected on the North-Western coast of Spain, but not as low as other records of plastic occurrence in regurgitated pellets from seabirds, which range from 3 – 7% (e.g., Acampora et al., 2017a; Acampora et al., 2017b; Hammer et al., 2016). In a recent study, Benjaminsen et al. (2022) investigated whole stomach contents of shags and other seabirds. In shags, no plastic items were found. The sampled shags were sourced from the North Cape of Norway, which might explain the discrepancy to the results found in my study, since Sklinna is in Mid-Norway and much closer to densely populated areas. Moreover, Van Franeker et al. (2021) described a latitudinal gradient in plastic loads in Northern fulmars, with lower loads further North than further South in the North Sea. The authors also strongly suggest a seasonal variation with higher loads in early summer (May – June) compared to late summer (July – August).

4.5.1 Regurgitated Pellets as a Monitoring Matrix for Microplastic in Shags

The fact that MPs were isolated from regurgitated pellets is contrary to Provencher et al. (2019) who stated that pellets could not be used to assess microplastic. The same authors also stated that the investigation of pellets is typically only useful during the breeding season, as shags then occupy nesting sites on land (Provencher et al., 2019). However, Sklinna shags inhabit the island year-round, and samples could be collected year-round. The value of pellets for MP analysis is further substantiated by Acampora et al. (2017a), Acampora et al. (2017b), and Álvarez et al. (2018), who have successfully investigated seabird pellets for plastic contamination, also outside the breeding season. Pellets are commonly collected for diet monitoring purposes, and integrating the protocol developed here, would allow to draw even more information from the samples.

4.5.2 Implications for Shags at Sklinna

The European shags at Sklinna are exposed to MPs and regurgitate (this study) and excrete them (Maaseide, 2022). It is not possible to say how much plastics are consumed by shags based on these two studies, and therefore, no clear implications can be drawn. While the consumption of MPs has been shown to carry possible health risks, the amount proven so far

is low. Burthe et al. (2014) assessed the vulnerability of Northern Sea marine birds for vulnerability to climate change and other anthropogenic impacts, and classified the risk of plastics to shags as “low”. The negative impacts of entangling and entrapment are likely higher than the risks associated with MP intake (Browne et al., 2015; Kühn et al., 2015; Provencher et al., 2017). Nonetheless, these finding underline the need for monitoring, habitat protection and global anti-plastic policies.

4.5.3 Recommendations for Future Research

The protocol developed in this thesis should be applied to a larger sample size, which would increase the amount of datapoints and allow for meaningful statistical analyses. This could also be expanded to samples from multiple years or investigations on whether the MP content fluctuates throughout the breeding season. This could provide insight into seasonal trends. Future projects should also include an alternative step for the removal of the pellet and sticky residue in the original vial. Changing the containers, e.g., to beakers (>100 mL), would streamline the protocol and make steps such as shown in Figure ***2.8*** obsolete.

The options of staining either the plastic particles or the paper filters could be explored. While staining may affect color analysis, the accuracy of MP quantities would be improved. Since one major hinderance for identification were the issues related to fluorescence (***Ch. 4.4.3***), other tools, such as FTIR, or a combination of both could be tested. In this study, only one database was utilized for identifying the Raman spectra. However, several public databases could be used, especially for spectra to which the ST-Japan library could not provide matches.

4.6 Conclusion

In this study, the primary objective was to develop a practical method for extracting both MPs and otoliths from regurgitated pellets of European Shags. Extensive testing led to the successful development and verification of a protocol that not only enabled both diet and MP analysis, but also met the criteria of being non-invasive to the study species, environmentally friendly, and easily integrates into existing monitoring programs. This research also proves the presence of MPs in regurgitated pellets from Sklinna's shags and is the first study in Norway to do so. The protocol developed here provides a tool, which can be included into existing monitoring efforts to gain insight into the level of local MP contamination in shags.

However, due to low sample size, it was not possible to link diet and MP content. To advance this work further, future studies should use a larger sample size and consider alternative identification methods, such as FTIR, to generate results more efficiently. By building on this study, future research will be better equipped to understand the effects of MPs on shag and other regurgitating seabird species.

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Appendix A Sampling

Table – A.1 Detailed sample collection information from the Sklinna 2022 field season. GM = gammelmolo, NM = nymolo; KB = Katharina Buchholz, ND = Nina Dehnhard, SS = Saria Sato, HBK = Hanne Bjørnås Krogstie, RTJ = Robert Torsvik Jacobsen; fb = field blank

Interval	Date	Site	Collector(s)	N
1 (05. – 09.06.2022)	08.06.2022	GM	KB, ND, SS	9 + fb
	09.06.2022	NM	KB, SS	11 + fb
2 (10. – 14.06.2022)	12.06.2022	GM	KB, SS	20 + fb
3 (15. – 19.06.2022)	17.06.2022	GM	KB, SS	21 + fb
4 (20. – 24.06.2022)	22.06.2022	GM	KB, SS	8 + fb
	24.06.2022	NM	KB, SS	12 + fb
5 (25. – 29.06.2022)	27.06.2022	GM	KB, SS	20 + fb
6 (30.06. – 04.07.2022)	02.07.2022	GM	KB, SS	20 + fb
7 (05. – 09.07.2022)	06.07.2022	GM	KB, HBK	20 + fb
8 (10. – 14.07.2022)	11.07.2022	GM	KB, SS, HBK, RTJ	13 + fb
	12.07.2022	GM	KB	8

Table A.2 – European shag nests counted during the field season of 2022 on Sklinna. Each nests equals two adults. "Inside" refers to the harbor-facing side and "outside" to the sea-facing side.

Location	Inside	Outside
Gammelmolo	241 nests	96 nests
Nymolo	196 nests	674 nests

Appendix B Collected Plastics

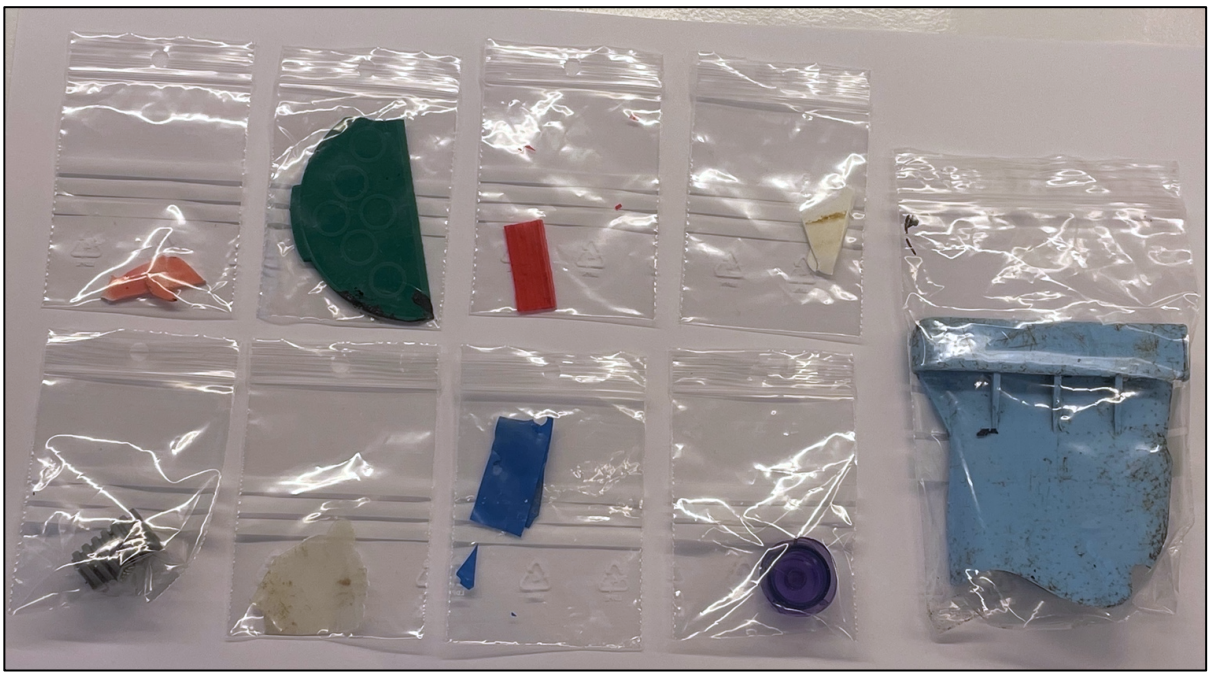

Figure B.1 – Plastic collected on Sklinna on 17. July 2022 and manually cut into MP sized particles to be used as spikes.

Appendix C Digestion Test

Table C.1 – Overview of the 10 samples on which further digestion tests were performed. All samples were pre-digested for 48 h and the otoliths removed. Between steps 1 and 2, approximately 48 h passed.

Sample	Pre-digestion	Step 1	Step 2	Final KOH concentration	Filter
001	Biotex solution 100 mL	10mL 10M KOH	-	1M	Paper
002	50mL ultrapure H_2O	20mL 5M KOH + 30 mL ultrapure H_2O	-	1M	Paper + nitro-cellulose
003	Biotex solution 100 mL	-	-	-	Paper
004	50 mL ultrapure H_2O	20 mL 5M KOH + 30 mL ultrapure H_2O	-	1M	Paper
005	Biotex solution 100 mL	10 mL 10 KOH	-	1M	Paper
006	50 mL ultrapure H_2O	20 mL 5M KOH +30 mL ultrapure H_2O	-	1M	Paper
007	Biotex solution 100 mL	10 mL 10 M KOH	-	1M	Paper
008	50 mL ultrapure H_2O	20 mL 5M KOH + 30 mL ultrapure H_2O	10mL 10M KOH	Approx. 2M	Paper
009	Biotex solution 100 mL	10mL 10M KOH	-	1M	Paper
010	Water	20mL 5M KOH	-	1M	Paper

Appendix D Photographic Documentation of the Final Protocol

This step-by-step series of images and captions are illustrations of what the stages of the protocol look like with an ideal sample (as described in ***Ch. 4.1.4.***).

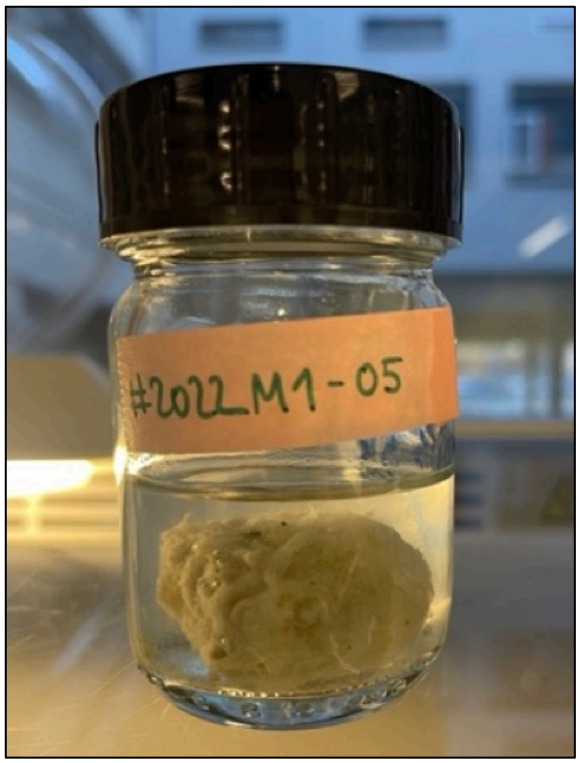

Figure D.1 – Step 1: Dissolving of pellet (a). A thawed pellet in original glass jar to which 20mL ultrapure water were just added.

Figure D.2 – Step 1: Dissolving of pellet (b). Sample after 24h in ultrapure water on shaker table (175 rpm, 25°C). Slurry with loose otoliths.

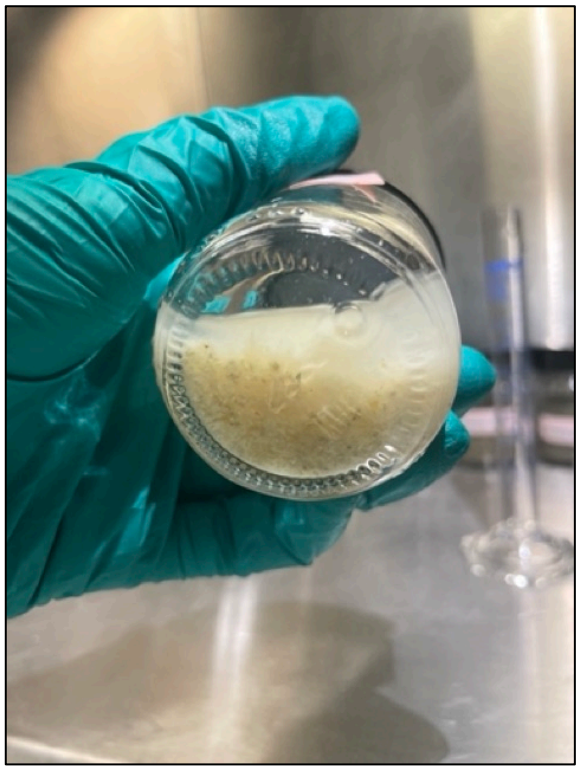

Figure D.3 – Step 1: Dissolving of pellet (c). Slurry after otolith removal. A significant amount of detritus remains at the bottom of the jar.

Figure D.4 – Step 1: Dissolving of pellet (d). Removed otoliths (thrice rinsed and then dried) in drams glass.

Figure D.5 – Step 2: Digestion of organic matter (a). Sample immediately after adding 20 mL of filtered 20% KOH solution. The detritus is still very visible.

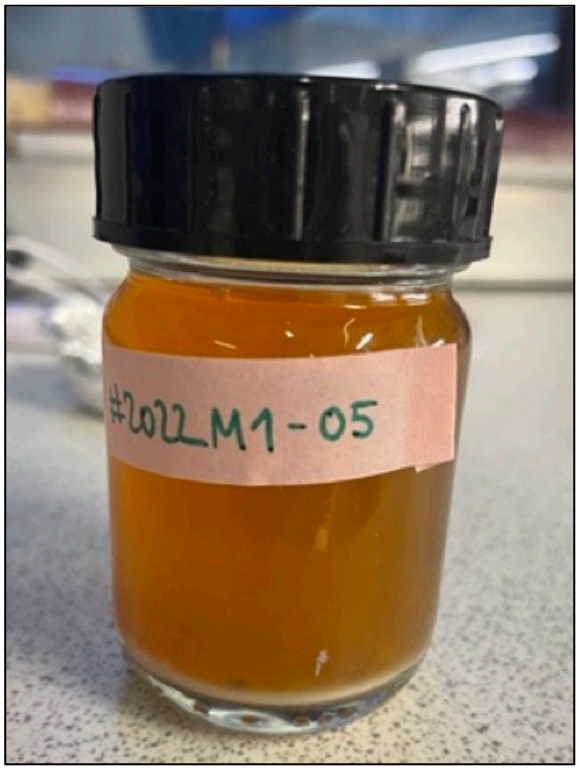

Figure D.6 – Step 2: Digestion of organic matter (b). Sample after 24h on shaker table (90 rpm, 40°C) in KOH solution. The color has changed, and the detritus is more homogenous.

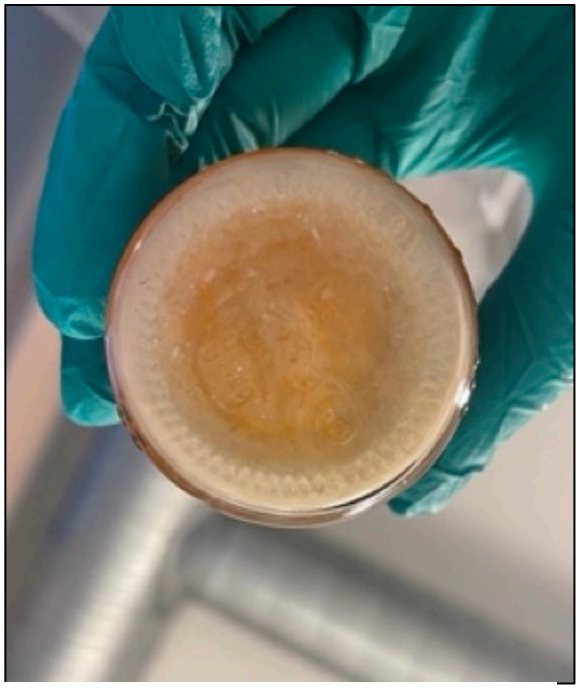

Figure D.7 – Step 2: Digestion of organic matter (c). Bottom view of sample after 24h in KOH solution (same stage as *Fig. 5.4.6*).

Figure D.8 – Step 2: Digestion of organic matter (d). Sample after 48h on shaker table (90 rpm, 40°C) in KOH solution. The detritus is more homogenous than at the previous stage.

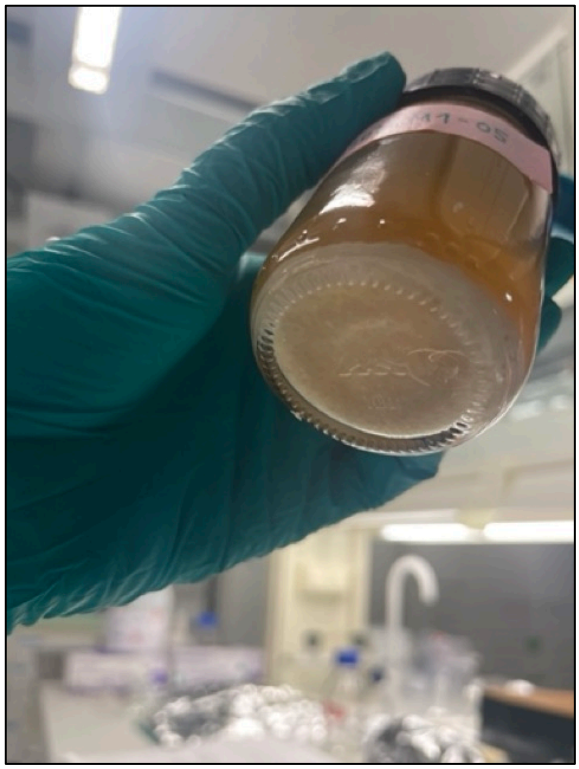

Figure D.9 – Step 2: Digestion of organic matter (e). Bottom view of sample after 48h in KOH solution (same stage as *Fig. D.8*).

Figure D.10 – Step 3: Density separation (a). Sample after transfer to falcon tube and settling (1-2h, RT). The liquid removed to beaker ≙ supernatant 1 (S1).

Figure D.11 – Step 3: Density separation (b). Sample after transfer to falcon tube and settling (1-2h, RT). The liquid removed to beaker ≙ supernatant 1 (S1).

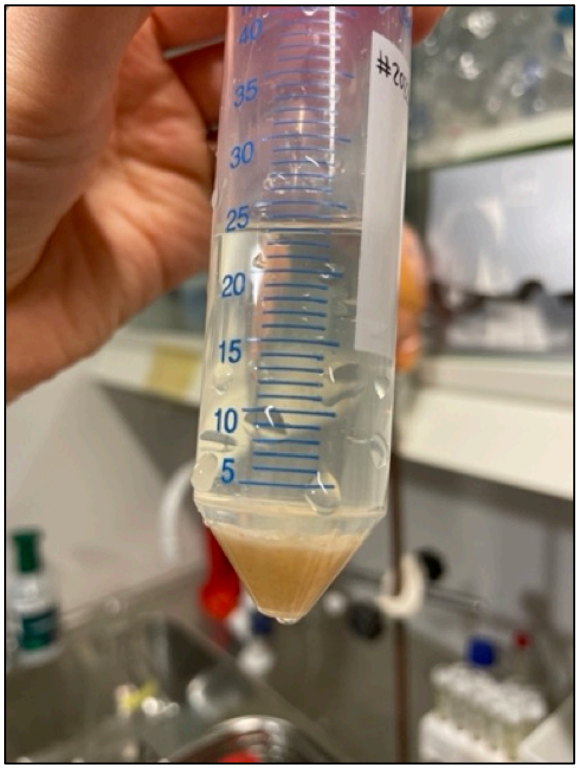

Figure D.12 – Step 3: Density separation (c). Sample after hypersonic bath (RT, 5 min.)

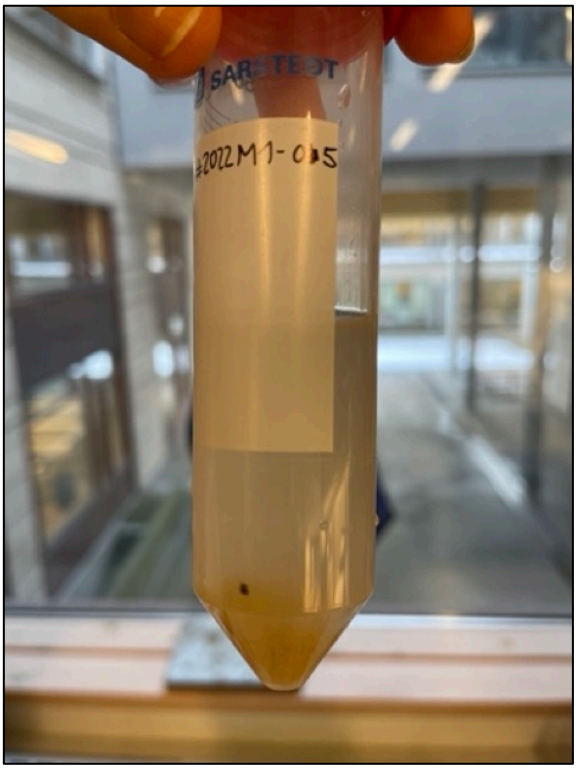

Figure D.13 – Step 3: Density separation (d). Sample after agitation on horizontal shaker (briefly 400 rpm; then 5 mins, 300 rpm)

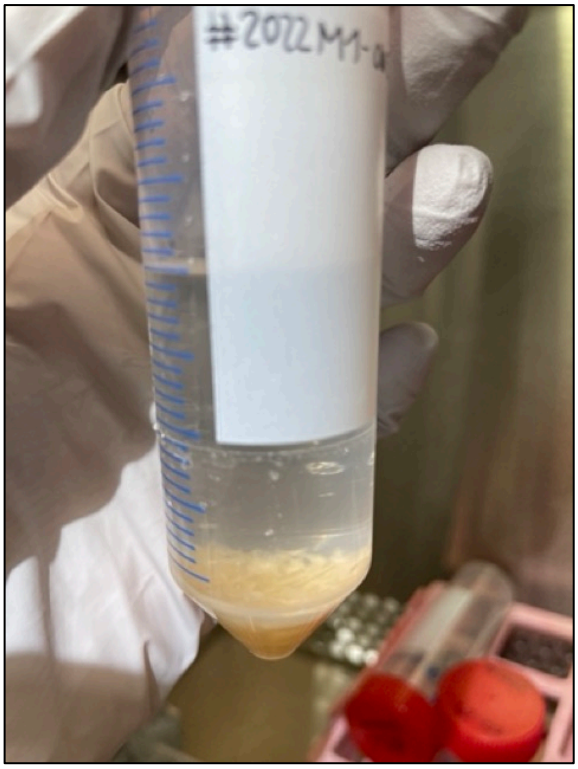

Figure D.14 – Step 3: Density separation (e). Sample after centrifugation (5 mins, 500 rpm)

The illustration of "Step 4: Filtration" is included in the corresponding Chapter ***2.4.4*** (***Fig. 2.9***).

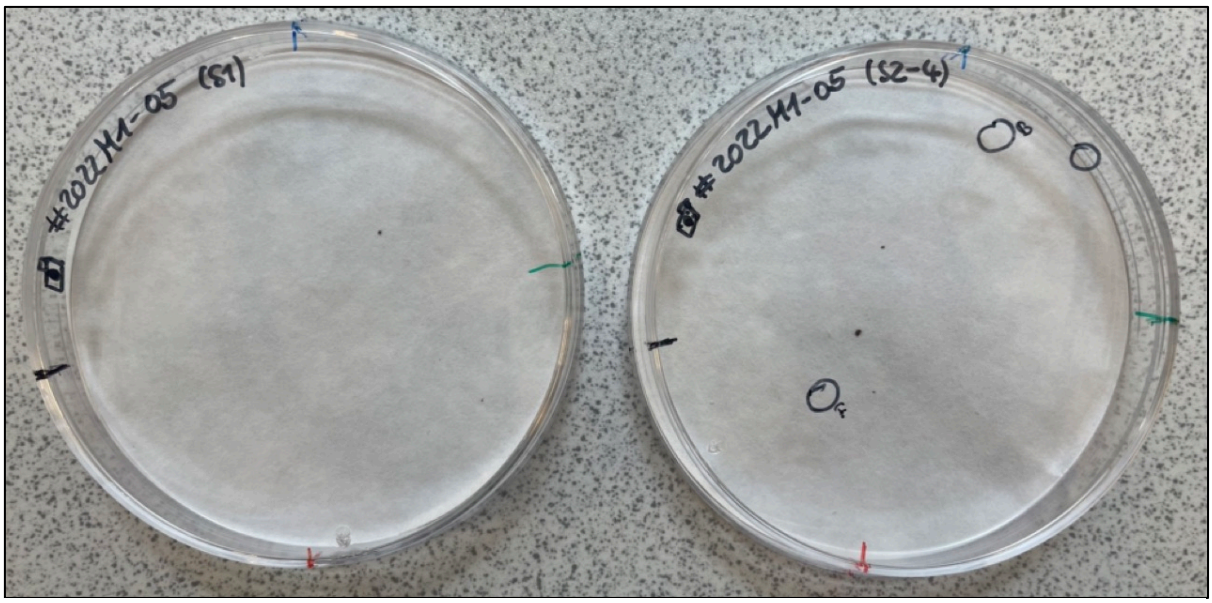

Figure D.15 – Step 5: Stereomicroscope. Filter of S1 (left) and, after three rounds of density separation, filter of S2-4 (right). Petri dishes with differently colored markings for orientation.

Appendix E Recovery Pie Charts and MP/Diet Link Logistic Regressions

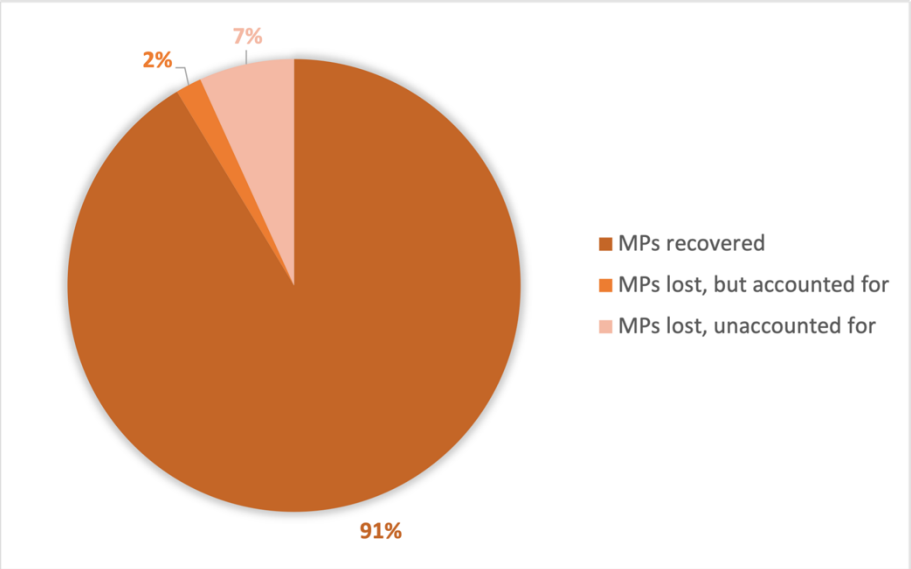

Figure E.1 – Percentages [%] of recovery/loss with KI. A visual representation of the MP spikes recovered and lost including both zebrafish and pellets, and whether the losses could be accounted for by examining the leftovers.

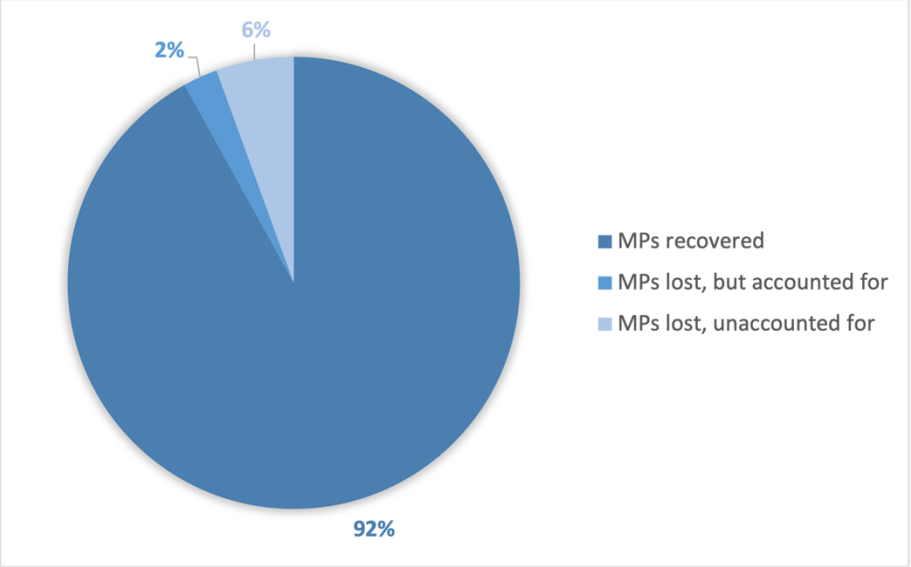

Figure E.2– Percentages [%] of recovery/loss with NaI. A visual representation of the MP spikes recovered and lost including both zebrafish and pellets, and whether the losses could be accounted for by examining the leftovers.

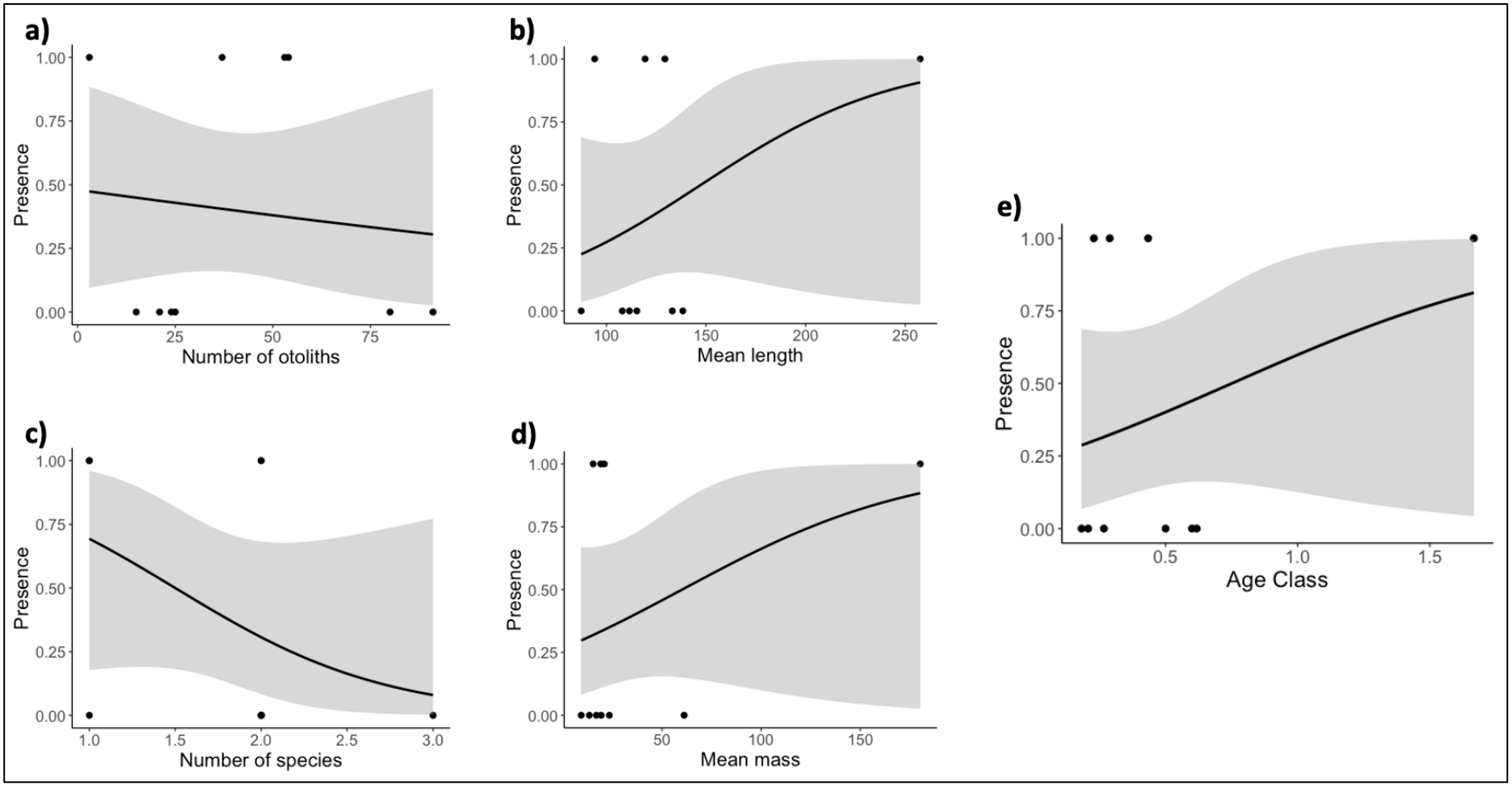

Figure E.3 – Logistic regressions (based on a sample size of 10 regurgitated pellets from European shags, processed according to the protocol developed in Chapter ***2*** plotting the presence or absence of MPs against a) number of otoliths per pellet, b) mean length [mm] of fish per pellet, c) number of species, d) mean mass [g] of fish per pellet, and e) mean age class of gadoids per pellet. The regression is mainly driven by one outlier.