Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

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Investigating the link between microplastics and diet from pellets of European shags (*Gulosus aristotelis*)

Master's thesis in Environmental Toxicology
Supervisor: Veerle L. B. Jaspers
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Abstract

Plastic pollution is an emerging global threat towards seabirds. The quantities and types of plastics seabirds are exposed to depend on their foraging mode, feeding range, life span and excretion ability. Some seabirds eliminate indigestible food items as pellets, where microplastics have been detected. This thesis investigated the presence of microplastics and fish otoliths in European shag (*Gulosus aristotelis*, hereafter shag) pellets from Sklinna (65°12'N, 10°58'E), a remote island in Central Norway where plastics have been detected. The shag is a piscivorous, pursuit-diving seabird, where the exposure to microplastics could be through secondary ingestion from its diet, consisting largely of fish. One of the largest shag populations is found on Sklinna, where Norway holds a responsibility to monitor the population.

To uncover knowledge about microplastic presence and diet, this thesis aimed to determine if microplastics were present in shag pellets. Characterize the shape, colour and size of potential microplastics. Otoliths identified the species and age class of the fish consumed. Connections between fish age and microplastics were investigated.

Two pilot studies were conducted in the lab. Pilot 1 assessed the recovery of spiking pellets with plastics from Sklinna. The second pilot included known and blind spiked samples. The recovery rates approved the method for the main study. The final lab protocol included incubation in ultrapure water, removing otoliths, incubation in KOH (10%) and filtering the pellets through metal filters. The cut-off for microplastics was 100µm. The otoliths were analysed by taking images with a Zeiss-AXIO Zoom microscope and analysed using the ShapeR package. A random forest model was developed to predict the unknown otoliths in the pellets.

The main study analysed 60 samples (48 pellets, 6 field blanks and 6 lab blanks), which contained 4068 otoliths. From visual inspection, 158 potential particles were characterized. Most particles were fibers (n = 134), black (n = 68), blue (n = 25) or white (n= 23) within the length range of 1000-1999µm (n = 56). No microplastics were found in the field or lab blanks. There was a loss of 35 particles from the initial 158 particles during handling. Microplastics were confirmed by processing the remaining 124 potential particles through Fourier Transform Infrared spectrometry. The ten microplastics found in this thesis were on average 2540 x 132 µm. These microplastics were eight fibers, one film and one fragment that were black, blue or white. The polymers were 4 Polypropylene, 2 Polyethylene, 2 Poly(ethylene terephthalate) and 2 Polyamide. It was observed that heavier pellets generally contained fewer otoliths of larger fish (Spearman's rank correlation, p = 0.0064). Otolith analysis investigated a subsample of pellets with and without microplastics with 1309 otoliths. Saithe in age class 0 was the main consumed fish species. No significance was found between fish age classes and species in samples with or without microplastics from a quasibinomial generalized linear model.

The results of this thesis indicate that pellet collection could become a suitable minimally invasive approach to monitor microplastics and diet in seabirds. Pellet analysis is limited to seabirds that produce them. This thesis uncovered the shape, size, colour and polymer of the detected microplastics in the shag pellets. Indicating that the shags on the remote archipelago of Sklinna are also exposed to microplastics. The diet for the shags on Sklinna was for the first time analysed using image processing.

Sammendrag

Plastforurensning er en voksende global trussel mot sjøfugl. Hvilke mengder og typer plast sjøfugler utsettes for avhenger av næringssøkadferd, habitat, levetid og elimineringsevne. Noen sjøfugler eliminerer ufordøyelige harde materialer fra byttet gjennom gulpeboller, der mikroplast er påvist. Denne oppgaven undersøkte tilstedeværelsen av mikroplast og fiskeotolitter i toppskarv (*Gulosus aristotelis*) på Sklinna. Sklinna (65°12'N, 10°58'E), en øy i Midt-Norge hvor plast er påvist. Toppskarv er en fisketende, jaktdykkende sjøfugl, hvor eksponeringen for mikroplast kan være gjennom sekundær inntak fra dietten deres fra fisk. En av de største toppskarvbestandene finnes på Sklinna, der Norge har et ansvar for å overvåke bestanden.

For å avdekke kunnskap om tilstedeværelsen av mikroplast og diett, hadde denne oppgaven som mål å avgjøre om mikroplast var tilstede i gulpeboller. Potensiell mikroplast ble karakterisert etter form, farge og størrelse. Otolitter ble brukt til å identifisere arten og aldersklassen av fisken som ble konsumert. Sammenhenger mellom fiskealder og mikroplast ble undersøkt.

To pilotstudier ble gjennomført på laboratoriet. Pilot 1 vurderte gjenfinningen av spiked gulpeboller med plast fra Sklinna. Den andre piloten inkluderte kjente og blinde spiked prøver. Gjennfinningsgraden av plasten godkjente metoden for hovedstudiet. Den endelige laboratorieprotokollen inkluderte inkubasjon i ultrarent vann, fjerning av otolitter, inkubasjon i KOH (10%) og filtrering av gulpeboller gjennom metallfiltre. Grenseverdien for mikroplast var 100 µm. Otolittene ble analysert ved å ta bilder med et Zeiss-AXIO zoom mikroskop og analysert med ShapeR-pakken. En random forest modell ble utviklet for forutsi den konsumerte fisken fra otolittene.

Hovedstudien analyserte 60 prøver (48 gulpeboller, 6 feltblanks og 6 laboratorieblanks), som inneholdt 4068 otolitter. Fra visuell inspeksjon ble 158 potensielle partikler karakterisert. De fleste partiklene var fibre (n = 134), svart (n = 68), blå (n = 25) eller hvit (n = 23) innenfor lengdeområdet 1000-1999 µm (n = 56). Det ble ikke funnet mikroplast i felt eller laboratorieblanks. Det var et tap på 35 partikler fra de første 158 partiklene fra håndtering. Mikroplast ble bekreftet ved å behandle de resterende 124 potensielle partiklene gjennom Fourier Transform Infrarød spektrometri. De ti mikroplastene som ble funnet i denne oppgaven var i gjennomsnitt 2540 x 132 µm. Disse mikroplastene besto av åtte fibre, en film og ett fragment som var svart, blå eller hvit. Polymerene var 4 polypropylen, 2 polyetylen, 2 poly(etylentereftalat) og 2 polyamid partikkler. Det ble observert at tyngre gulpeboller generelt inneholdt færre otolitter av større fisk (Spearmans rangkorrelasjon, p = 0,0064). Otolittanalyse undersøkte et underutvalg av gulpeboller med og uten mikroplast med 1309 otolitter. Sei i aldersklasse 0 var den mest konsumerte fiskearten. Det ble ikke funnet signifikans mellom fiskealdersklasser og fiskearter i prøver med og uten mikroplast fra en kvasibinomisk generalisert lineær modell.

Resultatene fra denne oppgaven indikerer at innsamling av gulpeboller kan bli et egnet og minimalt invasiv tilnærming for å overvåke mikroplast og diett hos sjøfugl. Analyse av gulpebollene er begrenset til sjøfugler som produserer dem. Denne oppgaven avdekket formen, størrelsen, fargen og polymeren til de påviste mikroplast i gulpebollene fra toppskarv. Noe som indikerer at toppskarvene på den Sklinna også er utsatt for mikroplast. Dietten til toppskarven på Sklinna ble for første gang analysert ved hjelp av bildeanalyse.

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List Of Abbreviations

CAP Canonical Analysis of Principal Coordinates
EtOH Ethanol
FTIR Fourier Transform Infrared spectrometry
GLM Generalized linear model
HDPE High density polyethylene
KOH Potassium hydroxide
LDPE Low density polyethylene
MPs Microplastics
MSFD EU Marine Strategy Framework Directive
NINA Norwegian Institute for Nature Research
NPs Nanoplastics
OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic
PA Polyamide
PE Polyethylene
PET Polyethylene terephalate
POPs Persistent organic pollutants
PP Polypropylene
PS Polystyrene
PVC Polyvinyl chloride
RF Random forest model
rpm Rotation per minute
SEAPOP SEAbird POPulations, Norwegian Seabird Monitoring Program
UP-water Filtered ultrapure water
WW Wet Weight

1 Introduction

1.1 Plastic as a product

The ongoing production and frequent use of plastic products since the 1950s could be explained by its immense and so far, irreplaceable societal and economic value [49]. Plastic is a cheap, lightweight, hydrophobic, and durable material with a long lifetime as it is resistant to degradation [53]. These versatile qualities make plastic an ideal candidate for countless of products with many applications [74].

Plastics are produced from natural or fossil fuel-based products [53]. They form a large, heterogeneous group, where their structure consists of many repeating units called monomers. The monomers are held together by strong carbon double bonds connected into larger polymer chains [12]. Associated chemicals, called additives, are added to the polymers during or after production to yield the desired properties of the plastic products [12], [53]. Frequently used additives include plasticizers for flexibility, fillers for texture, pigments for colouring, flame retardants and antimicrobial chemicals [134].

The most frequently produced and used plastic polymers account for 90% of the global plastic production. These polymers include polypropylene (PP), polyvinyl chloride (PVC), polystyrene (PS), high- and low-density polyethylene (HDPE and LDPE), and polyethylene terephthalate (PET) [14], [77]. Consequently, the high production and inadequate waste management of plastics means plastics are commonly encountered in the environment. Large amounts of polyethylene ((PE) from HDPE or LDPE) followed by PP, PET, PS and polyamide (PA) are readily detected in aquatic environments [89], [65].

Characterizing plastics based on size, they could be distinguished as macroplastics ($>$5 cm), mesoplastics (5 mm – 5 cm), microplastics ((MPs) 0.1 µm – 5 mm), and nanoplastics ((NPs) $<$ 0.1 µm) [121]. Primary MPs include microbeads or pellets, produced on a microscale, found in cosmetics, detergents, textiles and paint [14], [101], [127], [134] [135]. Secondary plastics are larger pieces, films or fibers of plastic broken down or degraded over time into smaller particles by weathering in the environment [14], [101], [127].

1.2 Abundance, distribution and fate of marine plastic pollution

From the plastic waste entering the oceans, 80 % of waste is related to land-based sources and 20 % has a marine-based origin, often traced back to the fishing industry [130]. MPs are the most encountered type of marine plastic debris, where fibers are the most abundant often from the wastewater of washing machines from washing textiles [135], [108], [47], [101].

A 2019 estimate of the global abundance of plastic pollution on the ocean's surface was set to 82 - 358 trillion particles, weighing a staggering 1.1 - 4.9 million tonnes [38]. In 2018, out of the global 358 million tonnes of produced plastic, 14.5 million tonnes were estimated to annually enter the ocean, which equals an input of 4 % of the total global production [70], [130]. The future of plastic production is estimated to triple by 2060, with a shocking 155 - 265 million tonnes of plastic waste per year

[12].

The quantities of plastic regarding the vertical and horizontal distribution profiles in the ocean are unknown [37]. Plastic debris is found to concentrate in surface waters, where the concentration drops when proceeding to lower depths [37], [72]. PP and PE are frequently encountered along shorelines and in surface waters. These polymers are buoyant and could reside in surface waters for years [14]. Denser particles such as cellulose fibers and polyester reside in deep-sea sediments on the ocean floor [14]. MPs are omnipresent and transcend the water column from floating in surface waters to the bottom of the ocean floor, residing in the sediments [29]. Negatively buoyant plastic particles such as PVC fragments, PET fragments and fibers have been associated with kelp forests in temperate regions [124].

The fate of plastic particles once in the marine environment includes fragmentation via mechanical stress, biodegradation and photodegradation. Fragmentation occurs when particles are broken down into smaller pieces from abiotic factors such as pH, temperature, UV-light and salinity [34]. Biodegradation occurs when microorganisms such as bacteria break down plastics into monomers [8]. Photodegradation, which is degradation induced by UV-light has been stated to be one of the most efficient modes of degrading plastic, which applies primarily to plastic in surface waters or on land [76]. Plastics could also be ingested by marine organisms and deposited in sediments on the ocean floor [130]. Another factor, that could influence MPs fate and cause them to sink is biofouling. This occurs when microorganisms or algae accumulate on the surface of the plastics and decrease the buoyancy of the particles, which causes particles to sink [130]. Seagrass meadows in coastal areas have been noted to heighten the sedimentation of smaller plastic particles and may become hot spots with increased MPs levels. This is concerning as many wildlife species, including fish and seabirds, depend on such habitats [125].

1.3 Interaction of plastic pollution with marine organisms

The persistence and abundance of plastics in the marine environment constitute a threat towards marine organisms [46]. When it comes to MPs it is important to consider both the physical particle and the fact that it could contain various chemicals including additives or absorbed contaminants [12], [77].

1.3.1 Physical interactions and effects

The physical impacts, including interactions and effects of plastic towards organisms could be divided into entrapment and ingestion. These have been the focus of the effects of plastics towards marine organisms during the past two decades [2], [98]. As of 2020, 701 species were found to ingest plastic and 354 species were reported to be entangled in marine plastics [67].

The extent of entrapment and ingestion depends on the species, their size and the size of the plastic debris. Ingestion further depends on the organisms' feeding habits, habitat, and susceptibility to be in contact with plastic debris also known as bioavailability [101], [105], [135]. The bioavailability of a particle to a marine organism is determined by particle size and density.

Entanglement occurs when marine organisms get their bodies or parts of their body trapped in marine anthropogenic debris [60], [71]. The type of plastics organisms are found to be trapped is often large plastic items (macro- and mesoplastics) such as nets, ropes or bags, often from discarded fishing equipment [35], [105]. Entanglement could lead to amputations, infections, injuries, and death via starvation or suffocation [51]. It is challenging to account for and quantify all the cases of injuries and mortalities from entanglement in marine debris and an underestimation is often presented [60]. The effect of entanglement has been reported to elicit a threat towards cetaceans, whales, seabirds, and sea turtles [67].

Once ingested, macro- and mesoplastics could cause wounds and lesions in the digestive tract of organisms [101]. Studies have reported the ingestion of plastics by sea turtles, fish, seabirds, marine mammals and even zooplankton which results in physical harm, as their gastrointestinal tract could become blocked or punctured [16], [51], [104]. In cases where large volumes of plastic accumulate in the stomach, the space for food becomes reduced and a fake feeling of satiation could arise. This could reduce food intake leading to starvation as reported in fish, seabirds and marine mammals ultimately leading to death [112].

The ingestion of marine plastics could occur directly (primary ingestion) or indirectly (secondary ingestion) [101]. Primary ingestion could be direct when the plastics are mistaken for food or ingested passively [112]. Secondary ingestion is indirect and occurs via ingesting prey containing plastic [101].

Primary ingestion has been reported for many species mistaking plastic particles as their prey. Sea turtles have confused transparent plastics as cephalopods and jellyfish [101]. The little auk (*Alle alle*), a seabird diving and feeding on zooplankton, mistook light, translucent coloured particles to be zooplankton [4]. Passive primary ingestion is the case for blue mussels (*Mytilus edulis*. This could be explained as they are filter-feeders, unselectively ingesting plastics by filtering the surrounding water [112]. Secondary ingestion from consuming prey that contains MPs has been noted in plankton, from mussels to crabs and from fish to marine top predators such as seabirds and seals, all indicating the potential for MPs to be transferred across one trophic level however the extent of trophic transfer and potential accumulation is not known [40], [97], [117].

Studies have found that the toxicity of MPs and NPs tends to increase with a decrease in particle size [10], [49]. MPs could accumulate in marine organisms, causing physical damage such as internal abrasions and blockages in filter feeders [135]. NPs could translocate to other tissues and bioaccumulate, being reported to pass the blood-brain barrier of fish, altering their behaviour [85].

1.3.2 Chemical interactions and effects

Once plastics are ingested, they could become a potential route and vector for plastic-associated chemical transfer and accumulation in an organism of heavy metals, phthalates or persistent organic pollutants (POPs) [113], [116]. Chemical toxicity could occur from two routes, either from the leaching of additives, added during manufacture or from the adsorbance of extraneous POPs and metals onto the MPs surface which is leached from the particle [89].

Important factors for POPs to adsorb on MPs are the contaminants' hydrophobicity and the increased surface area : volume ratio of the MPs from their small size. MPs could adsorb nonpolar, hydrophobic POPs, concentrating to magnitudes higher than the surrounding environment [48], [110]. Detected levels of POPs depend on the surrounding environmental levels, plastic polymer, the organisms' age and trophic level [128], [110]. [119] highlighted the importance of addressing plastic as a particle with additives as one contaminant and not two distinguish pollutants, as they co-occur.

1.4 Biomonitoring of microplastics in seabirds

Seabirds are one of the largest groups in numbers and diversity of marine megafauna [7]. Pollutants could bioaccumulate in seabirds as they are long-lived, large, top predators [20], [106]. They are, therefore, important sentinel species reflecting the state of the environment and pollution levels [106]. The extent and susceptibility of exposure for seabirds to plastic pollution depends on sex, reproductive status, foraging behaviour, diet and age. In addition, it is important to consider regional and species differences [20], [93].

Plastics are an emerging threat towards seabird populations [33]. It is estimated that 99% of seabird species with 95% of individuals are predicted to ingest plastic by 2050 [133]. One of the highly studied seabird species in the context of marine plastic pollution is the Northern Fulmar (*Fulmarus glacialis*; hereafter fulmar) [15]. The fulmar is a bioindicator for plastic pollution. It is a surface feeder that non-selectively forages on prey in the surface waters, ingesting high amounts of floating plastic debris [15], [45]. The fulmar is part of an international monitoring programme for marine plastics coordinated by the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR). Under OPSAR, the fulmars are being monitored for plastic in the North Sea using beached, dead fulmars for necropsies of their stomachs to uncover plastic loads utilizing opportunistic sampling [44]. The fulmars may be more susceptible to ingesting and encountering plastics when compared to species that for example pursuit dive after prey and are exposed via secondary ingestion [6].

1.5 Do microplastics bioconcentrate, bioaccumulate or biomagnify?

In ecotoxicological studies, the concepts of bio-concentration, accumulation and magnification become important factors when assessing the potential risks of contaminants. The three concepts describe how pollutants enter an organism. This could be either from the surrounding abiotic environment being bioconcentration or from the environment and food, referring to bioaccumulation or as the increase across trophic levels, which is biomagnification. The bioaccumulation of MPs is suggested to occur in respective tropic levels. Biomagnification still needs support in the field and there are currently no clear trends indicating gits occurrence [88]. The question and extent of whether MPs bioconcentrate, accumulate or magnify has been discussed [86], [88].

Bioaccumulation of MPs has been noted to occur for both the physical particle and associated additives within trophic levels, yet no clear trends indicate the occurrence of MPs biomagnification [88].

The vertical transfer of MPs from fish to their predators has previously been reported between fish and seabirds, and fish and seals [59]. Furthermore, zooplanktivorous fish have been reported to ingest MPs. These were either attached to, or by their prey. The fibers commonly ingested by zooplankton-feeding fish, were similar to the size and colour of their prey [100]. Forage fish, occupy a lower trophic level than the fish of interest in this thesis. However, forage fish may transfer MPs in piscivorous food webs [59].

Trophic transfer was further illustrated in [26] in common terns (*Sterna hirundo*), a pursuit-diving, fish-feeding seabird. The MPs levels in the gastrointestinal tract of the terns were higher than its regurgitated prey. The internal levels in the birds were also higher than their prey indicating bioaccumulation [26]. Both the common tern and roseate tern (*Sterna dougallii*) seem to ingest MPs fibers from their prey, where secondary ingestion could be a potential route for MPs exposure [23].

However, if trophic transfer of MPs occurs in the marine foodwebs is still up for debate. It has been argued that the persistence of MPs and trophic transfer depend on MPs concentration in the prey, retention, and elimination rate [115]. The relationship between when the prey was ingested and when the prey ingested MPs is important as excretion could influence levels. MPs shape could also influence the retention time and excretion ability, where retention in the stomach of some seabirds has increased with irregularly shaped particles [115]. When MPs uptake is greater than the rate at which the predator excretes them, the ingested plastic and MPs may bioaccumulate [12], [88].

1.6 Excretion of microplastics

The excretion of MPs could reduce the ingested MPs and reduce potential adverse effects an organism is exposed to [77]. Excretion pathways for MPs in seabirds include excretion via guano, regurgitation of pellets and stomach contents [1], [3], [103]. The excretion of chemical additives from plastics has other excretion pathways in seabirds, for example, preen oil or eggs have been found to contain UV-stabilizers and phthalates [55], [82].

MPs have been detected in the guano of fulmars, King Penguins (*Aptenodytes patagonicus*), Dominican gulls (*Larus dominicanus*), Magellanic penguins (*Spheniscus magellanicus*) [73], [87], [103]. Differences in diet were noted to lead to differences in the number and types of MPs in common tern and roseate tern faeces [23]. The elimination rate of MPs depends on their size, where the gut retention time increases with smaller particles [101].

[17] and [52], have noted that fulmar guano has established local MPs hotspots, and act as vectors for MPs transport and movement into environmental compartments. MPs have been detected in atmospheric deposition, soil and water surrounding seabird colonies [17]. Excretion pathways are crucial for both internal levels of MPs detected in an organism and for the further fate and transport of MPs in the surrounding environment.

Regurgitated pellets are typically produced to excrete indigestible prey items, such as fish bones, otoliths, fish lenses, shells, crustacea remains and stones [9]. Regurgitation of pellets is common in several seabird taxa, including cormorants (*Phalacrocoracidae*), skimmers (*Rynchops*), gulls (*Laridae*), skuas (*Stercorariidae*) and terns

(*Sternidae*) [109]. For these seabirds, indigestible food items from the gizzard are forced back up as a pellet [9]. Seabirds that cannot regurgitate pellets, such as the fulmar, have a lower elimination ability of MPs compared to ones that could [13]. Regurgitating seabirds may be able to eliminate more MPs, as they have an additional elimination route, which could affect levels detected in the stomach and faeces.

Besides producing pellets, several seabird species, including fulmars, feed their young by regurgitating pre-digested prey. This regurgitation of food by adult seabirds to their young during the breeding season could act as an offloading towards their young and be an elimination pathway of MPs for the adult [77]. Significantly higher plastic levels were detected in young, fledgling fulmars that are 50-60 days old when compared to adult fulmars from dissecting their gastrointestinal tract. This indicated that parent fulmars were feeding their chicks large amounts of plastic [123].

1.7 Seabird pellets in diet and microplastics analysis

Pellet analysis is applied to study the diet of pellet-producing seabirds [9]. Identifying diet items in the pellets uncovers the composition of their diet from when the pellet was produced [9].

Otoliths from fish are used in diet studies of seabirds, as seabirds often are piscivores. These calcium carbonate structures are sensory organs that play an important role in balance, sensing gravity, navigation, and the hearing systems of fish [56], [118].

Three different pairs of otoliths are present in the cranium of bony fish, located in individual membranes in the inner ear [56]. In symmetrical fish, the otoliths occur in pairs, and the left and right are mirror images of each other [56]. The three different pairs of otoliths include the sagitta, lapillius and astericus (Figure 1).

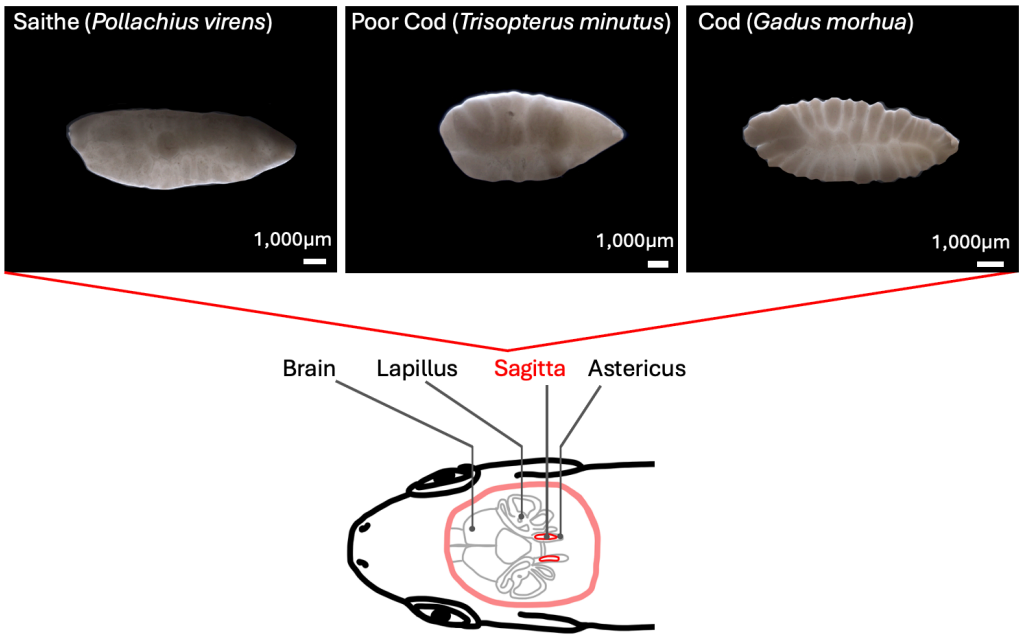

Figure 1: Location of the three pairs of otoliths on the right side of the cranium of a bony fish, with the location and images of three sagitta otoliths (dorsal view). Mirrored otolith pair on the left side modified from [56] and [102]. Otolith images were taken with (*Zeiss-AXIO Zoom Microscope*), magnification x20, 1000µm scale bottom right. Otoliths were from the reference collection at NINA, Trondheim.

The sagitta otoliths are often recovered after seabird digestion being found in regurgitates, stomachs or the faeces of seabirds. These are the largest and most characteristic of the three otolith types [9]. The other two types of otoliths are smaller in size and are not often part of pellets as these may dissolve during the seabirds' digestion [118].

Analysing otoliths allows identification of the species and age-class of the ingested fish by seabirds. The shape and surface of the otoliths, especially the sagitta is species-specific allowing visual identification [22], [118].

Two important characteristics for identifying fish species from otoliths include the rostrum and the sulcus (Figure 2). The rostrum makes a defined edge on the otoliths and influences its contours. The inside contains a groove close to the otolith's centre, which is the sulcus. The size and shape of the sulcus are important for identification. The inside of an otolith could be concave, convex or flat and be hyaline or opaque [19]. The surface could be smooth or with lobes and the shape of an otolith could be oval, lanceolate, or drop-like depending on the species [25], [56].

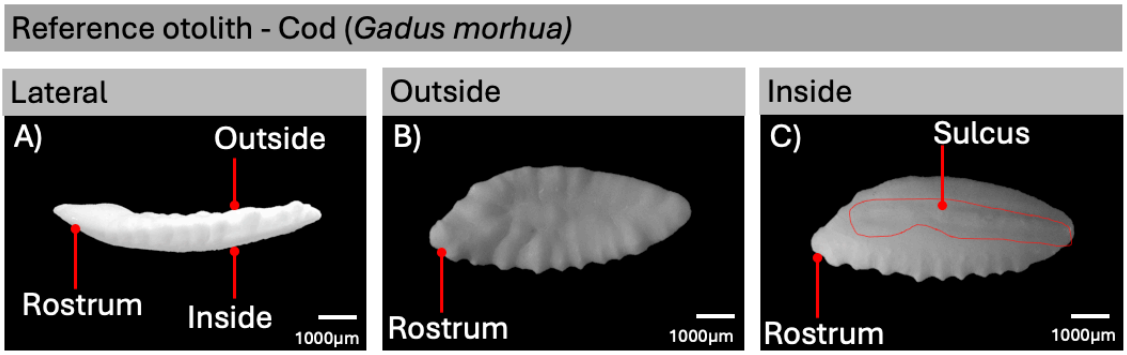

Figure 2: Images of one reference otolith from the cranium of a Cod (*Gadus morhua*), with (A) lateral view, (B) concave outside and (C) convex inside with outlined and labelled sulcus. This lobed, lanceolate otolith is from the reference collection at NINA, Trondheim.

Otolith length is an important measurement as it is correlated to fish length. Linear regressions are applied to back-calculate fish length and identify the age class of the fish [56].

Otoliths from juvenile fish differ from adult fish as they often lack clear species characteristics, and otoliths become more developed with age [56]. It could be challenging to differentiate juvenile fish of related species and identify them to species level as these are similar without distinct characteristics [118].

MPs have been detected in pellets of regurgitating seabirds and constitute a pathway for the seabirds to excrete plastic particles [1], [3]. MPs have been detected in both Atlantic Cod (*Gaudus morhua*, hereafter Cod) and Saithe (*Pollachius virens*) in remote areas [18], [66]. Whether there is a relationship between fish age and the number or size of MPs, with a higher number of MPs in older and larger fish (in this case Cod fish (*Gadidae*) and similar bony fish) is not known. The lack of correlation could be explained by fish being able to excrete particles from their stomachs, reducing the amount of MPs in them. The transfer of MPs from their stomach to tissue is deemed to be minimal [128]. Other studies have noted that Cods with full guts contained more MPs than empty ones, indicating that fish could excrete the MPs from their stomachs and that MPs amounts may relate to gut fullness [18]. The gut fullness and the gut

retention of MPs seem to be important factors for detected MPs levels, MPs ability to accumulate and potential trophic transfer [128], [43].

1.8 Study organism: European shag (*Gulosus aristotelis*)

In most parts of Europe, the European shag (*Gulosus aristotelis*; hereafter: shag) populations are declining [21]. Norway holds approximately 35% of the shag population in the Northeast Atlantic with a stable population [32]. Shags have an all-year coastal distribution and in Norway, they are distributed along the entire coast. As Norway holds a substantial proportion of the breeding population, recording up to 28,000 breeding pairs, Norway has a responsibility to safeguard and monitor its shag population [5], [32], [41], [80]. The largest shag population is on Sklinna an island in Central Norway.

Shags are coastal foragers and pursuit divers that often perform benthic dives, and look for potential prey, which they follow during foraging [27], [90]. They forage at shallow depths ranging from 15 - 30m. In Norway, shags typically forage in areas with kelp forests close to their colonies (within 30 km) [27], [32].

During the breeding season, Gadidae and Sandeels (*Ammodytes marinus*) constitute the majority of the shags' diet in Norway, where demersal Gadidae are found in kelp forests. Benthic sandeels reside in areas with sandy floors [27]. At Sklinna, the shags mainly feed on juvenile Saithe, Poor cod (*Trisopterus minutus*) and Cod during the breeding season [58], [80]. The shags on Sklinna depend especially on juvenile Saithe (age class 0 - 1), which is strongly associated with kelp forests, and constitutes $>$70% of their diet [58]. It has been shown that in years with low Saithe availability on Sklinna there are fewer breeding shags [80]. The breeding success in those years is also lower, thus the importance of fish stocks and food availability in the area are crucial parameters for sustaining the seabird populations and their reproductive success ([58], [80].

Shags are dimorphic, with males weighing 15% more than females which could influence foraging and ingested prey [27]. On Sklinna female shags have been found to go on farther trips than males. Males were found to dive deeper while foraging, which may result in differences in foraging and diet [27].

As with the rest of the world, plastic pollution is present on Sklinna. Entangled adult shags in marine debris have been found in Norway [99]. In Norway 422 nests were examined from 10 shag colonies and 96 of the nests contained plastic. The frequency of occurrence for the different debris types was mainly thread and sheet with some hard plastic [99]. When compared to the other seabirds, shags had a higher tendency to incorporate plastics into their nests [122]. In 2023, 35 of 105 monitored shag nests on Sklinna contained visual meso- and macroplastics [99] (Figure 3).

Figure 3: (A) Adult shag with thread in beak from marine rope, (B) Shag nest with shag chick and orange macroplastics threads in nest and marine ropes surrounding nest on old breakwater where samples were collected. (C) Two shag nests indicated with red arrows, without any visible plastics (left) and visible plastic and marine debris (right) on Sklinna.

2 Aims and hypotheses

This thesis analysed pellets from adult shags during the 2023 breeding season (June to mid-July) on Sklinna. It had the aim to expand knowledge regarding consumed MPs and fish consumed by shags in their pellets. Fish may be an important route for MPs exposure based on the foraging behaviour of the shags, investigating connections between diet and MPs number and size. The use of pellets to monitor and eliminate MPs for shags was also assessed. As such, this thesis seeked to contribute to future developments in monitoring shag populations using pellet analysis for diet and MPs co-occurrence, developing a protocol for analysing them.

The thesis derived the three following hypotheses on MPs occurrence, diet, and connections between MPs and diet in shag pellets:

1. Ingested microplastics were predicted to be detected in shag pellets, being previously found by [3]. Potential particles in the pellets would have their shape, colour, size and polymer characterized.
2. The diet of the shags was expected to consist of different fish species of different age classes. The majority of the shags' diet on Sklinna has previously consisted of Saithe, Poor cod and Cod, hence these were the three main expected fish species to be found in the pellets from the shags [58], [80]. The consumed species were predicted to be of different age classes.
3. Shags that consumed larger and older fish were hypothesized to have a larger size or greater number of MPs in their pellets. Significance between the diet related to the age of the fish species and potential MPs could be tested.

3 Materials and Methods

3.1 Sample site

Fieldwork was conducted on Sklinna (65°12’N, 10°58’E), a remote archipelago and protected nature reserve, 40 km off the coast in Northern Trøndelag, in Central Norway (SEAPOP, 2021). With approximately 2,000 breeding pairs of shags, Sklinna holds one of the largest shag colonies in Norway and worldwide (SEAPOP 2021). The monitoring of seabird populations at Sklinna started during the 1980s. In 2008 the islands became one of the key sites in the Norwegian Seabird Monitoring Programme, SEAbird POPulations, also known as SEAPOP, which is co-run by the Norwegian Institute for Nature Research (NINA) and the Norwegian Polar Institute. Since 2008, breeding success, survival, diet, and since 2010 habitat use of several seabird species, particularly shags, has been studied at Sklinna.

3.1.1 Pellet collection

Sample containers were glass vials (30 mL) covered with a double layer of aluminium foil, that were closed with a metal (n = 68) or plastic (n = 38) screw lid. With the aluminium foil in place (without the screw lid), the vials were heated (400°C, 24 hours) to remove any contamination before the start of fieldwork as in [31]. The vials were transported in cardboard boxes in an upright position to Sklinna.

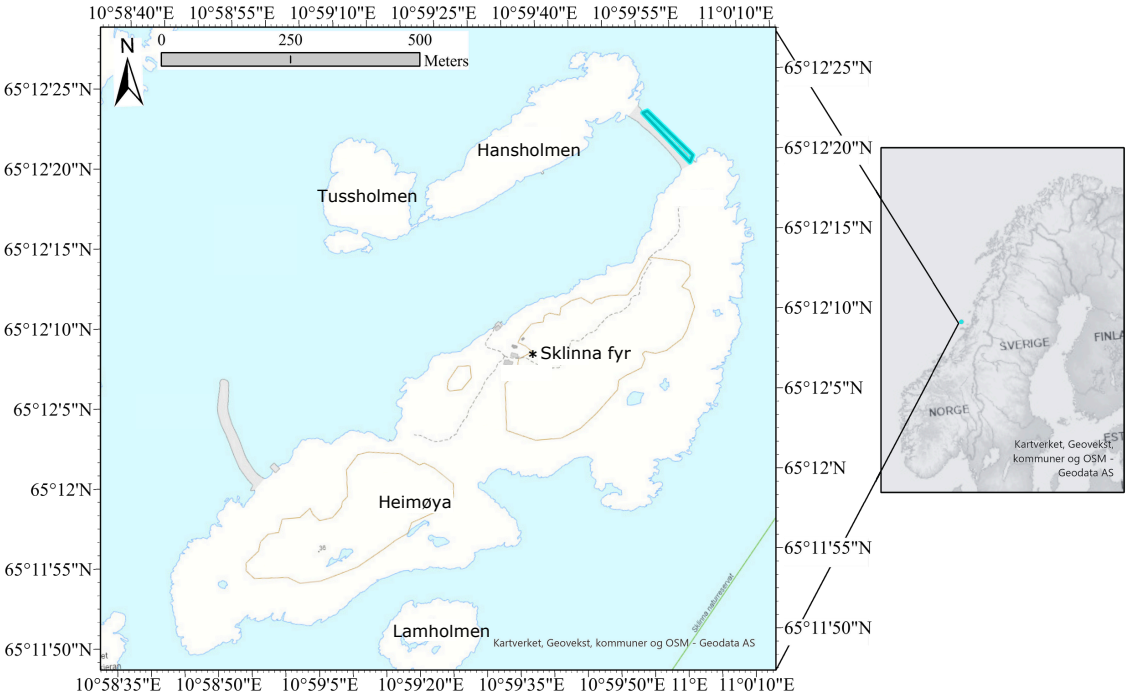

Figure 4: The two main islands of Sklinna (65°12’N 10°59’E), Heimøya and Hansholmen where the collection site at the Northeastern side of the breakwater is highlighted in turquoise. Sklinna´s location in Norway could be seen to the right. Map was made using ArcGIS Pro 2022.

Pellets were collected every 5th day in the morning along the northeastern side of the breakwater (Figure 4) during the 2023 field season. The sex and breeding status of the birds was unknown. For each collection date, 10 pellets and one field blank were collected. If 10 samples were not collected on the initial sampling day, subsequent collections were included on the following day. A total of 106 samples were collected, consisting of 97 pellets and nine field blanks (Table 1).

Table 1: The 10 collection dates for pellet collection and number of pellets collected during 2023 field season on Sklinna. Subsequent collection dates 06.07.2023-07.07.2023 and 11.07.2023-12.07.2023 were pooled together to equal 10 pellets.

Collection date (dd.mm.yy)	**Number of pellets**	**Number of field blanks**
08.06.23	20	1
11.06.23	11	1
16.06.23	11	1
21.06.23	14	1
26.06.23	11	1
02.07.23	10	1
06.07.23	5	-
07.07.23	5	1
11.07.23	9	1
12.07.23	1	1
Total	97	9

Pellet collection was conducted by five people trained by the same professional. Wool or cotton was worn when collecting the pellets. Synthetic clothes and plastic-containing materials were minimized to reduce external contamination. One transparent vinyl glove was worn on one's dominant hand to collect the whole pellet. The double layer of aluminium foil covering the opening of the glass vial was removed with one's non-dominant, glove-free hand. The pellet was placed into the glass, sealed with the aluminium foil, and secured with the screw lid. Field blanks were collected following the same steps as pellet collection but, no pellet was touched or collected, accounting for potential external particles from the field. Collected pellets were intact, without scattered otoliths, feathers, or other visible external contamination (Figure 5). Glass vials with the samples were labelled and stored in a freezer (-20°C) until analysis. Pellet collection occurred on dry days, when there was no rain, as the breakwater would be too slippery for sampling and pellets could be washed away or degraded.

Figure 5: (A) Depicted not collected pellet (B) Collected pellet (C) Collection and (D) Pellet placed in a glass vial.

3.2 Laboratory analyses

Two pilot studies were conducted for this thesis to identify the best digestion and filtration protocol to retrieve otoliths and MPs from the pellets (Figure 6). Pilot 1 (P1) assessed the overall applicability of the chosen method to analyse MPs in pellets from spiking the samples with plastics (Figure 7, steps 2-10). P1 checked the effectiveness and comparability of either the base, potassium hydroxide (KOH, 10%) or the detergent Biotex with spiking plastics and their ability to digest the mucus of the pellets during incubation [75], [57], [58], [120], [101]. Both KOH (10g KOH pellets : 100 mL UP-water for 10%) and Biotex (8.9 g BT / 1L UP-water) were made in the lab. Previously, KOH has been used to degrade organic matter and to start the digestion of the mucus, whereas Biotex has traditionally been used in diet analysis dissolving the pellet for analysing otoliths. P1 also assessed the ability of pellets to be filtered through metal filters (stainless steel, mesh size = 100 x 100 µm, diameter = 4.4 cm).

Pilot 2 (P2) was conducted with one additional step (Figure 7, step 1) to the methodology of P1. It included spiked samples of known and unknown plastic contents to validate the recovery of the MPs. Most importantly, both pilot studies evaluated if MPs used for spiking had a good recovery, and that no MPs were lost at the different steps after conducting the method.

Plastics used for spiking were cut in the lab with metal scissors under a staticmaster (NRD, LLC, Model 2U500). These plastics were collected on Sklinna during a beach-cleanup in the 2022 field season. The collected plastic included remains of hard plastics (parts of plastic boxes, bottle caps and other unidentifiable hard plastic products of nine different colours). The fibers originated from household items such as rugs, blouses, and marine-like ropes. The polymers of the MPs used for spiking were checked with the FTIR. The cut fragments were on average 1030 × 1550 µm and fibers were 1670 × 170 µm. The fibers used for spiking (green, blue, red) were PP. Five of the coloured particles were PP (purple, pink, green, red and grey), three were PE (white, transparent, light blue) and one dark blue particle (polymethylacrylate) (Figure 6).

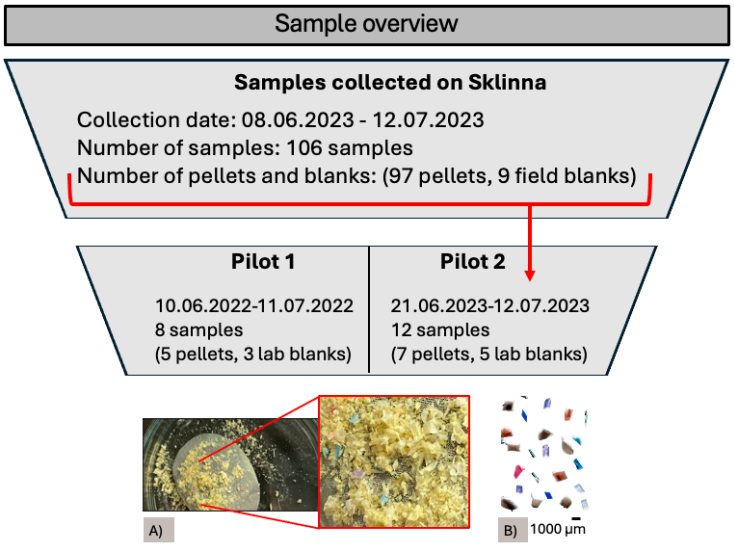

Figure 6: Sample overview of pellets chosen for pilot 1 and pilot 2. (A) Filtered pellet after conducted lab protocol with organic pellet contents and spiked plastics in sample (middle) and (B) Plastics used for spiking with 1000 µm scale bar.

3.2.1 Pilot 1

P1 consisted of eight samples, with five pellets and three lab blanks (Table 2). Four samples were spiked with nine particles and three fibers in triplicates (n = 36). Three samples were not spiked and seven samples were incubated in KOH (10%). The remaining sample was not spiked and was incubated in Biotex (BT).

Table 2: Overview of P1 pellets with their collection date, incubation liquid (KOH (10%) or Biotex (BT)) and whether they were spiked (✓n=36 MPs or not (X).

	Lab ID	Collection date (dd.mm.yy)	Incubation liquid	Spiked samples (✓, n=36 MPs / X)
Pellet	Pellet 1	06.07.22	KOH(10%)	X
	Pellet 2	06.07.22	KOH(10%)	X
	Spiked Pellet 1	11.07.22	KOH(10%)	✓
	Spiked Pellet 2	06.07.22	KOH(10%)	✓
	Pellet Biotex	10.06.22	Biotex	X
Blank	Blank 1	-	KOH(10%)	X
	Spiked Blank 1	-	KOH(10%)	✓
	Spiked Blank 2	-	KOH(10%)	✓

The frozen pellets (collected during 2022 for P1 and 2023 for P2) were taken out of the glass vials from field collection and weighed in an empty Erlenmeyer flask (250 mL, 6 cm wide opening). Glass vials from the field were rinsed with filtered distilled ultrapure water (UP-water, 100 mL) which was poured into the Erlenmeyer flask that contained the pellet. Aluminium foil was placed over the opening of the Erlenmeyer flask to avoid external airborne contamination. Holes were punctured in the foil to allow aeration of the samples during incubation for 24 hours, at 40°C with 80 rotations per minute (rpm) in an IKA-KS 4000 i control-incubator shaker to break up the pellet in solution (Figure 7, step 2).

After incubation, the dissolved pellet in the Erlenmeyer flask was poured into a large glass Petri dish (20 cm diameter). Otoliths were removed from the sample using tweezers in a Holten LaminAir portable laminar flow and were put into individual drams glasses (one glass per pellet) (Figure 7, step 3). The otoliths in the drams glass were rinsed with UP-water which was poured back into the Erlenmeyer flask. This was done to ensure that potential MPs were not lost and continued to be part of the analysis. Otoliths were left to dry before images were taken for species and age class prediction (Figure 7, step 4).

After otolith removal, samples were vacuum filtered through the metal filters set between two rubber rings between the funnel and filtrate flask. The dissolved pellet was poured into the upper glass beaker of the vacuum filtration (Figure 7, step 5).

After filtration, metal filters were carefully placed in the Erlenmeyer flask using tweezers. Samples were incubated for 72 hours at 40 °C at 80 rpm in KOH (10%) (Figure 7, step 6). During P1, the final KOH (10%) was made by first pouring UP-water (50 mL) and then KOH (20%, 50 mL) into the erlenmeyer flask. For the samples incubated in Biotex the same steps were followed, replacing any use of KOH with the Biotex solution.

Proceeding incubation, the samples were filtered again but, this time the dissolved pellet was filtered twice. The sample was first filtered through the metal filter (Figure 7, step 7). Followed by the filtrate being filtered through a paper filter (particle retention = 12-15 µm retention particle size; Figure 7, step 8). This accounted for fibers that were pulled through the metal filter during filtration. The metal filters were rinsed with UP-water and stored in glass Petri dishes (diameter = 9 cm) with their glass lid on top. Once dry, the filters were visually inspected for potential particles that could be MPs using a stereomicroscope (Figure 7, step 9). This was followed by identifying polymers of the particles with an FTIR (Figure 7, step 10).

KOH (10%) proved to be a better incubation liquid than Biotex. KOH was more efficient at digesting the mucus. The Biotex being a detergent left residues on the glassware. As a result, it was difficult to get the glassware clean and to make sure the entirety of the sample was rinsed and included on the filter after filtration. The method using KOH as the incubation liquid was accepted for further analysis of the pellets.

3.2.2 Pilot 2

P2 was conducted with seven pellet samples and five blanks (Table 3).

Table 3: Overview of P2 pellets with their collection date, incubation liquid (KOH (10%) and whether they were spiked (✓n=5-39 MPs) or not (X).

	Lab ID	Collection Date (dd.mm.yy)	Incubation liquid	Spiked samples (✓, nMPs/X)
Pellet	Known Spiked Pellet 1	12.07.23	KOH (10%)	✓, 39 MPs
	Known Spiked Pellet 2	06.07.23	KOH (10%)	✓, 39 MPs
	Known Spiked Pellet 3	26.06.23	KOH (10%)	✓, 39 MPs
	Blind Spiked Pellet 1	21.06.23	KOH (10%)	✓, 5 MPs
	Blind Spiked Pellet	07.07.23	KOH (10%)	✓, 13 MPs
	Blind Spiked Pellet	06.07.23	KOH (10%)	✓, 15 MPs
	Not Spiked Pellet	21.06.23	KOH (10%)	X
Blank	Known Spiked Blank 1	-	KOH (10%)	✓, 39 MPs
	Known Spiked Blank 2	-	KOH (10%)	✓, 39 MPs
	Blind Spiked Blank 1	-	KOH (10%)	✓, 15 MPs
	Blind Spiked Blank 2	-	KOH (10%)	✓, 10 MPs
	Not Spiked Blank	-	KOH (10%)	X

Known spiked samples as in P1 had triplicates of the different coloured plastics. The grey, white, transparent fragments and additional white fiber (likely cotton, added for P2) with five particles each instead of three as these were more challenging to find (n = 44 MPs). However, as the white fibers frayed they were excluded from analysis (n = 39). The blind spiked samples contained 5-15 MPs and were spiked by a lab technician, remaining unknown to the investigator (Table 4). In addition to the 10 spiked samples, one pellet and one blank were not spiked.

Table 4: Overview of content in blind spiked pellets and blanks from Pilot 2. Particles marked with (-) indicate no spiking of the given plastic

Pilot 2 contents of Blind Spiked Samples						
Particle type	**Colour**	Pellets			Blanks	
		Blind Spiked Pellet 1 (n=5)	Blind Spiked Pellet 2 (n=13)	Blind Spiked Pellet 3 (n=15)	Blind Spiked Blank 1 (n=15)	Blind Spiked Blank 2 (n=10)
MPs	Red	-	-	2	-	1
	Dark Blue	-	2	3	-	1
	Light Blue	1	-	-	3	-
	Pink	1	3	-	2	-
	Purple	1	-	-	2	-
	Grey	1	3	5	3	-
	Transparent	-	-	2	-	2
	White	1	-	-	4	3
Fibers	Blue	-	3	-	-	1
	Green	-	-	1	-	1
	Red	-	1	1	-	1
	White	-	1	2	1	-

One additional step was added to P2: Before the first incubation with UP-water 0.02 g of the pellet's mucus was weighed and extracted to identify the sex of the shags that produced the pellet (Figure 7, step 1). The extracted mucus was placed in a 1.8 mL cryotube with ethanol (70%, EtOH). The mucus was cut using a clean and new scalpel blade for each sample to avoid cross-contamination. During this step, care was taken to not include other organic contents from the pellet. After the mucus removal, all steps from P1 (Figure 7, steps 2-10) were conducted for P2.

The metal filters were inspected under a stereomicroscope for any remaining particles after rinsing the filter before they were changed between incubation and filtering (Figure 7, step 7). Samples with large amounts of mucus or organic matter were divided into smaller batches using multiple filters during all filtration steps (Figure 7, steps 5, 7 and 8). This avoided blocking the filter, aiding the filtration. In cases where the samples were not evenly spread out on the metal filters during the last filtration step, tweezers or UP-water would be used during filtration to spread them out. The visual inspection of the filter became less demanding once dry, as otherwise, fish bones could be lying on top of each other.

To obtain the correct MPs dimensions, a stereomicroscope with ocular was applied to measure all potential particles, before using the FTIR. When looking for potential MPs in P2 (Figure 7, step 9), suspicious particles were removed from the filtered and dried sample and stored in individual wells in a 96-well plate.

3.2.3 Main study

Good recoveries from P1 and P2 (see Results 4.1 and 4.2 respectively), accepted the method. The method with a streamlined workflow from P2 was applied to the actual samples in the main study (Figure 7).

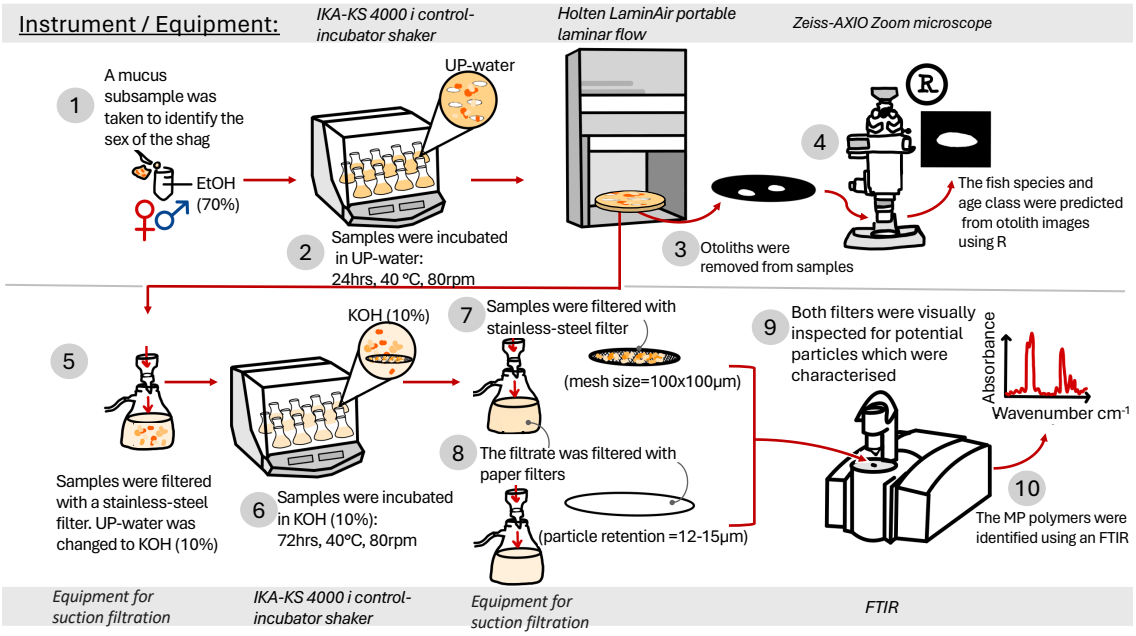

Figure 7: Schematic outline of the final ten lab steps applied in the main study. The main instruments/equipment required is written in the grey fields at the top and bottom of the figure. The steps included: (1) Removing 0.02g of mucus from the pellets to identify the sex of the shag that produced it, stored in 70% EtOH. (2) Incubation of pellet in UP-water in IKA-KS 4000 i control-incubator shaker (24 hours, 40°C, 80rpm). (3) Removal of otoliths in Holten LaminAir portable laminar flow cabinet, followed by (4) identification of fish species and age class from otolith images taken with a Zeiss-AXIO zoom microscope (x17.6 or x25 magnification) analysed using R. (5) Filtration of the sample without otoliths. (6) The second incubation, in KOH (10%) to dissolve organic matter in Holten LaminAir portable laminar flow cabinet (72hrs, 40°C, 80rpm). (7) Suction filtration of samples, rinsed with UP-water, obtaining the processed pellet on the metal filter. (8) The filtrate underwent suction filtration with a paper filter. (9) Metal and paper filters were visually inspected for potential particles once dry. Characteristics of particles were noted. (10) All particles were checked with FTIR to identify their polymer, and to confirm that they were a MPs.

A total of 60 samples were analysed (48 pellets, 12 blanks consisting of six lab and six field blanks) in the main study (Figure 8). Data for the processed samples in the main study can be found in Appendix A.

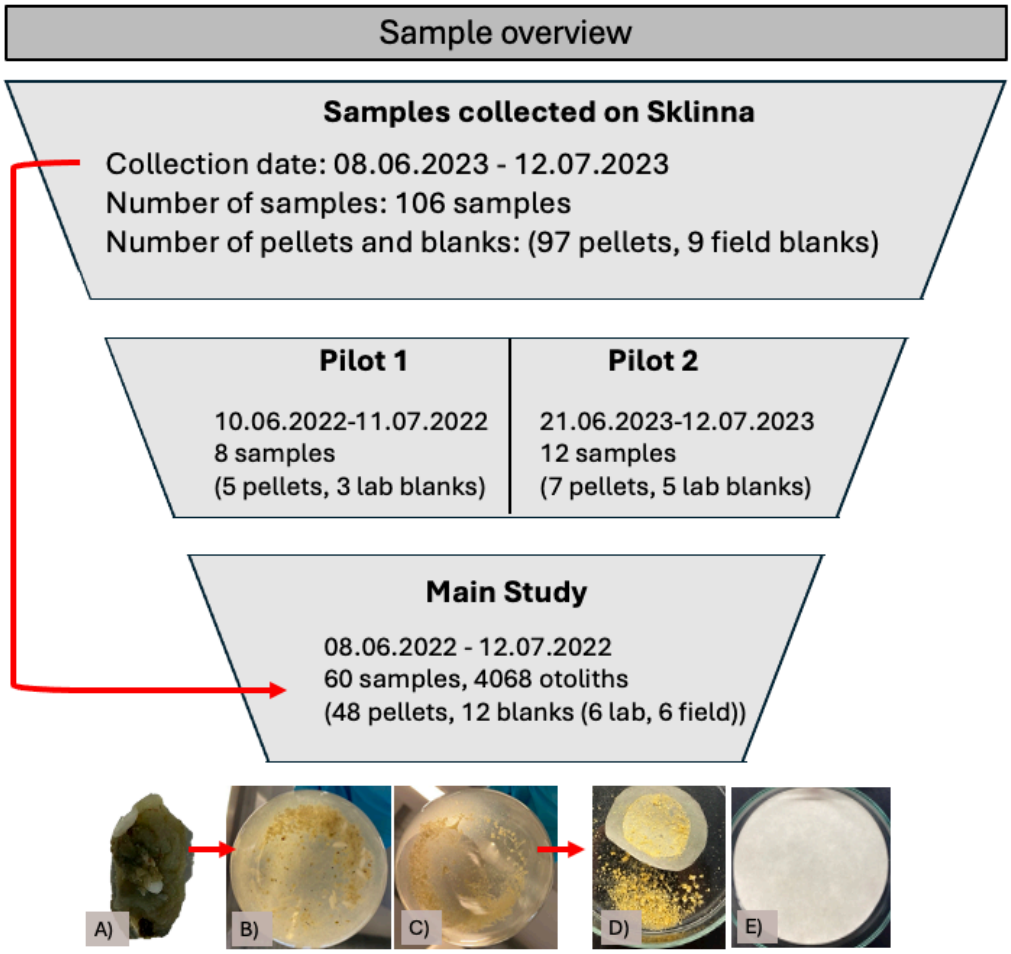

Figure 8: Sample overview of samples from the main study. Pellet processing included (A) Pellet, (B) Dissolved pellet in UP-water after incubation, otoliths can be seen before removal (C) Pellet after incubation in KOH (D) Resulting filtered on metal filter and (E) Paper filter.

The processed samples (pellets and field blanks) were spread evenly across the breeding period, with six samples for each collection date seen in Table 5.

Table 5: Selected pellets and blanks for the main study. Collection dates 06.07 - 07.07.2023 and 11 - 12.07.2023 were pooled. (-) indicates no sample was selected from the given date.

Collection date (dd.mm.yyyy)	**Number of analysed pellets**	**Number of analysed field blanks**
08.06.2023	6	1
11.06.2023	6	-
16.06.2023	6	1
21.06.2023	6	1
26.06.2023	6	1
02.07.2023	6	-
06.07.2023	3	-
07.07.2023	3	1
11.07.2023	6	-
12.07.2023	0	1
Total	**48**	**6**

The mucus subsamples extracted for sexing were analysed at NINAGEN, the Centre for biodiversity genetics. The sex of the shags that produced the pellets were identified based on genetical analyses.

Diet items such as fish lenses, squid beaks and crustacea remains were quantified in the pellets when the otoliths were removed (Figure 7, step 4). Diet items have previously been identified along with MPs in pellet studies [1]. Additional pellet content, classified as external contaminants that were not significant in the shags diet including mites, stones, seaweed and feathers were also quantified (all quantified items are found in Appendix B).

3.3 Microplastic analyses

3.3.1 Visual inspection of filters

Potential particles were found via systematic visual inspection of the 48 filtered pellets distributed on 130 filters (metal filters n =60, paper filters n = 70). The limit of detection (LOD) was at 100 µm, based on the dimensions of the metal filter, visual inspection limits and handling in addition to it being a commonly used LOD [128]. Both filter types had at least one filter per sample and were scanned under a stereomicroscope following a zigzag pattern from left to right to cover the entire surface of the filter. The metal filters were checked for MPs using both a black and white piece of paper under the samples to give different contrasts to the samples. The contrast of white particles from fish bones on a black background and darker fibers on a white background made identification easier.

Potential particles were pinched with tweezers or a dissection needle to determine their texture if they broke under pressure being brittle organic matter or not. Particles that did not break under pressure were processed with the FTIR (MSFD, 2013). Fibers, fragments and films were distinguished based on dimensions between length and width in addition to their hardness and shape.

All potential particles were measured with the ocular (scaled to 100 units on the ocular = 1 cm), noting the particle shape, size (length x width) and colour [39]. Tweezers were carefully used to remove the particles from the glass Petri dish or filter and translocated to a 96-well plate with one particle per well. The wells were closed with a lid and sealed with parafilm until further polymer identification with the FTIR. Initially, 80 MPs were transferred to the 96-well plate, 35 particles were analysed with the FTIR from the well plate. The remaining 89 particles analysed by the FTIR were directly transferred from their sample to the FTIR. A higher transfer of potential particles was obtained when the MPs were directly transferred from the sample to the FTIR and was the preferred method.

3.3.2 Polymer identification using FTIR

FTIR analysis in the context of MPs analysis allows the identification of polymers based on vibrations of the atoms from the energy of infrared light. The resulting spectrum therefore becomes a fingerprint for the MPs based on the bonds and molecular structures present, where different bonds generate different peaks of absorbance or transmission [95].

A total of 124 particles were analysed using the OPUS 7.5 BRUKER ALPHA FTIR sample compartment RT-DLaTGS at the Faculty of Natural Sciences at the Norwegian University of Science and Technology in Trondheim. The polymer of the particles were identified through the spectrum search with the FTIRs´ three reference libraries. The reference libraries included a general IR library Demolib.s01 (350 entries), Bruker Raman Demolibrary RAMDEMO.S01 (246 entries), and a demo database SR.IDX (200 entries).

Background air measurements were taken before measuring the samples, and the particle was placed in the optical path of the sample cell, followed by a single measurement of its absorbance. A ZnSe attenuated total reflection (ATR)-crystal analysed

the particles using eco-ATR to identify the polymer types. The wavenumber range of the FTIR absorbance was 4,000-400 cm^{-1} and the number of scans = 4. The spectrum was baseline corrected, peaks were labelled with their absorbance, and the polymer was identified through spectrum search.

The spectra of the particles that did not match the spectrum search in the FTIR OPUS 7.5 (BRUKER), were manually matched using two reference libraries one was from polymers from environmental plastic samples (Références "Environnementales" version 08.04.2020) and another reference library (Références bibliographiques version 31.03.2020) [75].

3.4 Quality assurance and quality control

The use of plastic-containing materials (in the field and lab) was minimized during processing, using glass equipment and non-synthetic materials when possible.

All lab equipment was machine washed and the openings were immediately covered with aluminium foil to avoid contamination from external plastic particles. After use, equipment was rinsed three times with filtered UP-water between each sample and machine washed between lab rounds (each batch of samples) to avoid cross-contamination between samples.

The final 60 samples were processed in five rounds with 12 samples, where each round took one week. One lab and field blank were processed with each round of samples, to account for potential contamination of particles from the lab and field. All lab steps including filtration and otolith removal were conducted in a Holten LaminAir portable laminar flow cabinet, in a closed lab with little activity. Plastic gloves made of bright blue or green nitrile material were worn in the lab. Cotton clothing and a white cotton lab coat were worn when processing samples during filtration and MPs analysis. The response of the metal filter and the rubber rings to KOH was checked by placing them in a Petri dish with KOH (20 %) for one hour, where there was no noted effect of KOH. All UP-water was filtered through a Whatman 10311843 Grade 597 1/2 paper folded filter as MPs could be present in the water [94]. The same filtered UP-water was used to make the stock solution of KOH.

To avoid bias when looking for MPs after the samples were filtrated in P1, P2 and the main study the order of analysis was randomized to reduce the effects from improving at identifying the MPs from experience. Recurring light purple PP MPs were excluded from the results. These were external contamination from the rim of a glass bottle used to pour water in the lab and one small fragment was observed in one of the laboratory blanks with 12 MPs divided on the 130 filters, both on metal and paper filters [52]. Other encountered materials such as nitrile gloves used in the lab, paper filter for the filtrate and blue rims and lids of lab equipment were checked with the FTIR and their spectras were crosschecked with the other spectras of the potential particles.

Using metal filters as in [75], obtains the filtered regurgitate on a metal filter and allows steps to be streamlined. It reduces the number of filters required and allows incubation of the filter and sample together. Allowing the same filter to be used for incubation and filtration, minimising loss and handling of the sample.

Cleaning the metal filters included rinsing them with UP-water, followed by sonication in an ultrasonic bath for approximately five minutes. Filters were placed in a beaker filled with UP-water, these were vertically suspended from leaning on a clean pair of metal tweezers. Once sonicated, the filters were rinsed with EtOH (96%) and placed in one large glass Petri dish covered with aluminium foil and left to air dry.

3.5 Identification of fish species and age class from otoliths in pellets

3.5.1 Otolith imaging

The 48 analysed pellets contained 4068 otoliths, of which 10 pellets contained MPs (see Results 4.3.1b). To have a balanced sample size for the otolith analysis, only otoliths from the samples with MPs (n=10) and samples without MPs (n=10) collected on the same dates and additional dates to include all collection dates, were further analysed. As a result, 20 pellets with 1309 whole and intact otoliths were analysed to identify fish species and their age class.

Otolith images were acquired using a Zeiss-AXIO Zoom light microscope (model Axio Zoom.V16) with a digital camera (Axiocam 506 color) and the ZEISS ZEN microscope software (ZEN 2012, ZEN pro for taking images). Otoliths were positioned in the middle of the frame with their sulcus side up, and rostrum to the left [92]. Both right and left otoliths were analysed, and otoliths were not paired. As each fish contains one pair of otoliths, in most cases (unless single otoliths were retained in the stomach by the bird, lost or broken during processing), one fish was thus represented by two otoliths. In this thesis approximately 66 otoliths were broken and not included in the analysis (Appendix B).

Before capturing images, the exposure and aperture were manually adjusted in small increments for clarity and consistent lighting across samples. One could ensure that all images were consistent and reproducibly processed by standardising otolith orientation and microscope settings. Each otolith image was linked to a scale bar calibration and exported as a .jpg for further processing. The raw images were edited using Preview Version 11.0 (1044.2) on a Mac, using the smart lasso tool to select the otolith and apply a black background. The exposure and colour of the images were adjusted so that the image was black on white, without texture (Figure 7).

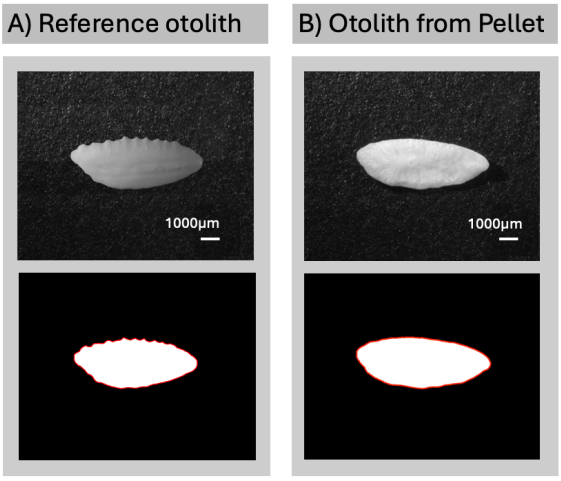

Figure 9: (A) Cod reference otolith and (B) a predicted Cod otolith by R from the pellets (right). Images are taken with a Zeiss-AXIO Zoom microscope (x25 magnification). Otoliths were orientated with the sulcus up and rostrum towards the left. All images were edited (bottom) and processed in R to obtain red outline which forms the basis of the analysis with Shape R.

3.5.2 Otolith shape analyses

To quantify the differences in the otolith shape the ShapeR package was used in R version 4.2.3 (Libungan and Pálsson 2015). outlines were extracted using the "detect.outline" function with a threshold of 0.2. The resulting outlines were manually checked and re-analysed if the initial shape extraction had failed to capture the outline of the otolith. Outlines were smoothed using the "smoothout" function with 100 iterations. Wavelet coefficients were extracted using the "GenerateShapeCoefficients" in the ShapeR package and used to draw the reconstructed otolith outlines. Wavelets provide an alternative to the more commonly used Fourier transform shape analysis as it provides a better approximation for sharp edges in otolith outlines. The quality of the Wavelet reconstructions was assessed by measuring how it deviated from the original otolith outline using the available functions in the ShapeR package. For each sample, four otolith size parameters and standardized wavelet coefficients were extracted, and then six additional otolith shape indices were calculated (Table 6).

Otolith size parameters and shape indices are important for morphological analysis of otoliths and species identification. The otolith size parameters could easily be measured. OL (mm) is derived from the longest measured dimension, whereas OW (mm) is the shortest and is perpendicular to the length. The OA refers to the surface area, and OP encompasses the measured length around its outline. The shape indices are calculated, AR is the ratio between length and width, indicative of the otoliths elongation. C refers to the closeness in shape the otolith has to a circle, in turn E is how off from an ellipse the otoliths shape is. As C, FF measures shape, accounting for OA and OP. Re measures how rectangular an otolith is and Ro relates the similarity of the otoliths shape to a circle [91], [92].

Table 6: Otolith measurements (parameters and indices) with units and formulas for calculated otolith morphological indices following common definitions from otolith literature [91]

Otolith size parameters	**Unit**
Otolith length (O_L)	mm
Otolith width (O_W)	mm
Otolith area (O_A)	mm^2
Otolith perimeter (O_P)	mm
Otolith shape indices	**Formula**
Aspect ratio (AR)	O_L/O_W
Circularity (C)	O_P^2/O_A
Ellipticity (E)	$(O_L - O_W)/(O_L + O_W)$
Form-factor (FF)	$(4\pi O_A)/O_P^2$
Rectangularity (Re)	$O_A/(OL \times OW)$
Roundness (Ro)	$(4O_A)/(\pi O_L^2)$

3.5.3 Fish species classification

A random forest model was developed to classify fish species based on otolith shape [91]. The model was based on a reference otolith collection from NINA, Trondheim. The dataset consisted of n $>$ 10 otoliths for Saithe, Poor cod, Cod, Haddock (*Melanogrammus aeglefinus*), and Capelin (*Mallotus villosus*). No correction was applied to the otolith shape indices for fish size as this was unknown. First, variations in otolith shape among the different species using Canonical Analysis of Principal Coordinates (CAP) was analysed using the vegan package in R and contrasted the ranges of the different otolith indices using boxplots. This was followed by building a random forest (RF) model to classify individual fish to their corresponding species using the ranger and caret packages in R. The reference data was down-sampled to 10 otoliths for each species and then split into a training (70%) and test (30%) dataset, as in [91]. The RF model was tuned using cross-validation with cv = 10 and trees = 500, and variable importance was assessed. The RF model was evaluated on the test data using a confusion matrix. RF should be robust for highly correlated variables, for example, otolith length and otolith widths. Other variables might not always provide accurate results and should be treated carefully.

3.5.4 Fish size and age class reconstructions

Otolith length was used to reconstruct individual fish size using established relationships between otolith lengths and fish length. The formulas given in [56] were used for all fish except Cod. To calculate the fish length of Cod, the formula published in [62] was used. This followed the same methodology applied earlier to calculate fish length in the diet of shags on Sklinna [58], (equations in Appendix C). The age class of Saithe and Cod were determined using fish size ranges based on the calculated fish length (FL, mm) based on formulas in [81] and [58], while for Poor cod [131] was used (Table 7).

Table 7: Main fish species consumed by the shag, with their derived fish age classes related to fish length (mm).

Fish Species	Age class	Fish Length (mm)	Reference
Saithe	0	$<$ 120	[58]
	1	$\geq$120 < 250	[58]
	2+	$\geq$250	[58]
Poor cod	0	$<$ 80	[131]
	1+	$\geq$80	[131]
Cod	0	150	[81]
	1	$\geq$150 < 250	[81]
	2	$\geq$ 250 < 300	[81]
	3+	$>$300	[81]

3.6 Statistical analyses

For each sample, the numerical proportion of otoliths belonging to a specific fish species and age class was calculated (assuming a 100% fish-based diet). This was to test if dietary composition differed between samples with and without MPs, hence General Linear Models (GLMs) with a quasi-binomial distribution were used and ran in R. The dependent variable was the numerical proportion of fish of a given species/age class in the diet, and the variable of interest was whether the sample contained plastics (1) or not (0). GLMs were run separately for each fish species and for each class, also including one model each for Cod, Saithe and Poor cod where fish of all age classes were pooled. Model assumptions were validated using the protocols described in [136].

A Shapiro test was conducted to determine if pellet mass (ww) and the number of otoliths were normally distributed. A Spearman's rank correlation was performed to investigate if there was a correlation between pellet mass and the number of otoliths present (Linear regression can be seen in Appendix D).

4 Results

4.1 Pilot 1

P1 consisted of eight samples, including five pellets and three blanks. The mass of the pellets used in P1 ranged from 1.60 - 3.11 g (wet weight, ww) and contained 56-182 otoliths. Four samples were spiked, and the recovery rates of the two spiked pellets and two blanks were calculated (Table 8).

Table 8: Overview of number of otoliths and mass of pellets from Pilot 1 (n=8). Indicating if samples were spiked (n=36 MPs, ✓) or not (X) and their calculated recovery.

	Lab ID	Number of otoliths	Pellet mass (g, ww)	Spiked samples (✓, n=36 MPs/X)	Recovery of spiked plastics (%)
Pellet	Pellet 1	182	2.23	X	-
	Pellet 2	82	1.60	X	-
	Spiked Pellet 1	92	1.84	✓	100
	Spiked Pellet 2	56	2.49	✓	94
	Pellet Biotex	59	3.11	X	-
Blank	Blank 1	-	-	X	-
	Spiked Blank 1	-	-	✓	100
	Spiked Blank 2	-	-	✓	100

The recovery rates were 100% except for one spiked pellet where two particles were missed. The resulting recovery was 94%, which was the sample with the lowest recovery rate. Particles were found on both the metal and paper filters. For both the spiked pellets, two fibers were found on the paper filters.

For samples with high loads of organic matter such as fish bones in the spiked samples, it became challenging to distinguish and find the white, transparent, and grey particles as these were similar to the organic matter. It was also challenging to find the red fiber, being the thinnest and smallest of the three types of fibers used for spiking (green, blue and red).

4.2 Pilot 2

P2 had 12 samples and contained 96-280 otoliths. The mass of the pellets ranged from 1.93- 3.78 g (ww). The pilot had both known and blind spiked samples, and their recovery rates were calculated (Table 9). The white fibers frayed and were not possible to analyse, hence the plastics used for spiking were 5 - 39 MPs.

Table 9: Overview of number of otoliths and mass of pellets from Pilot 2 (n=12). Indicating if samples were spiked (n = 5-39 MPs ✓) or not (X) and their calculated recovery.

	Lab ID	Number of otoliths	Pellet mass (g, ww)	Spiked? (✓, nMP/X)	Recovery of spiked plastics (%)
Pellet	Known Spiked Pellet 1	150	2.88	✓, 39 MP	90
	Known Spiked Pellet 2	208	3.78	✓, 39 MP	74
	Known Spiked Pellet 3	280	2.53	✓, 39 MP	82
	Blind Spiked Pellet 1	158	2.04	✓, 5 MP	80
	Blind Spiked Pellet 2	159	1.93	✓, 13 MP	92
	Blind Spiked Pellet 3	222	3.04	✓, 15 MP	71
	Not Spiked Pellet	96	3.03	X	-
Blank	Known Spiked Blank 1	-		✓, 39 MP	92
	Known Spiked Blank 2	-		✓, 39 MP	92
	Blind Spiked Blank 1	-		✓, 15 MP	93
	Blind Spiked Blank 2	-		✓, 10 MP	100
	Not Spiked Blank	-		X	-

Both known spiked and blind spiked samples obtained similar recovery rates (74-90% and 71-92% respectively). It was difficult to count the transparent particles, as some samples had a lot of organic matter. Transparent particles were in some cases defined as grey instead of transparent for blind spiked blank, but all particles were recovered.

4.3 Main study

The following results are from analysing the pellets from the main study for microplastics (4.3.1) and otoliths (4.3.2).

4.3.1 Microplastics

4.3.1.1 Characterizing potential particles

Number and shape

The original 60 samples (48 pellets, 12 blanks), were filtered on 60 metal filters and 83 paper filters. From these, 158 particles were found from visual inspection. The particles consisted of 134 fibers, 16 fragments and eigth film-like fragments.

For the field blanks (n = 6), no particles were found on the metal filters or paper filters. As for the lab blanks (n = 6) two paper filters from different samples had potential particles. One of the filters had three white fibers in a bundle (1000 x 100 µm) which was recognized to originate from the filter itself. One thin black fiber (1500 x 100 µm) was found on a paper filter and had no match when checked with both the FTIR and manually looking over the absorbance spectra from the FTIR.

Colour

The main colours found for the 158 potential particles were black, blue, white, transparent, and red. The remaining six colours were detected at a lower frequency ($<$ 5 particles) (Figure 10).

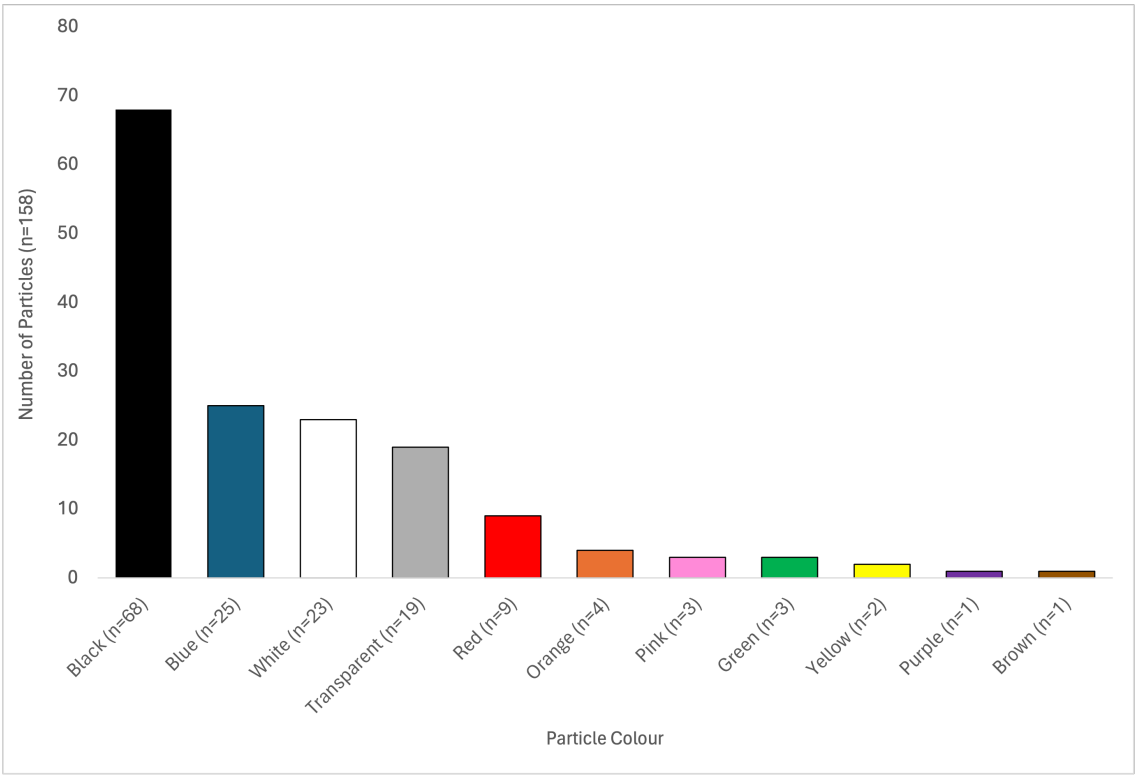

Figure 10: Colour frequency distribution for the potential particles.

Size

The average dimensions (length x width) for the measured potential particles (n = 158) was 1790 x 323 µm, with a lower threshold of 100 µm for all (Figure 11). The average dimensions for the fibers (n = 134) were 2244 x 100 µm, and 766.7 x 586.7 µm for fragments (n = 16), and 1375 x 600 µm for film (n = 8). The longest recorded fiber had a length of 10500 µm, the largest fragment was 2500 x 2000 µm, and the film-like fragment was 2500 x 1400 µm. The smallest recorded dimension, with an LOD of 100 µm for the smallest fiber was 200 x 100 µm, the fragment was 100 x 100 µm and film was 500 x 100 µm. All measured fibers can be found in Appendix E. The size distribution of the potential particles, based on their longest dimension (length), had the highest frequency of occurrence in the size range of 1000 - 1999 µm, with 56 particles and lowest at 800 - 899 µm with no particles recorded (Figure 11).

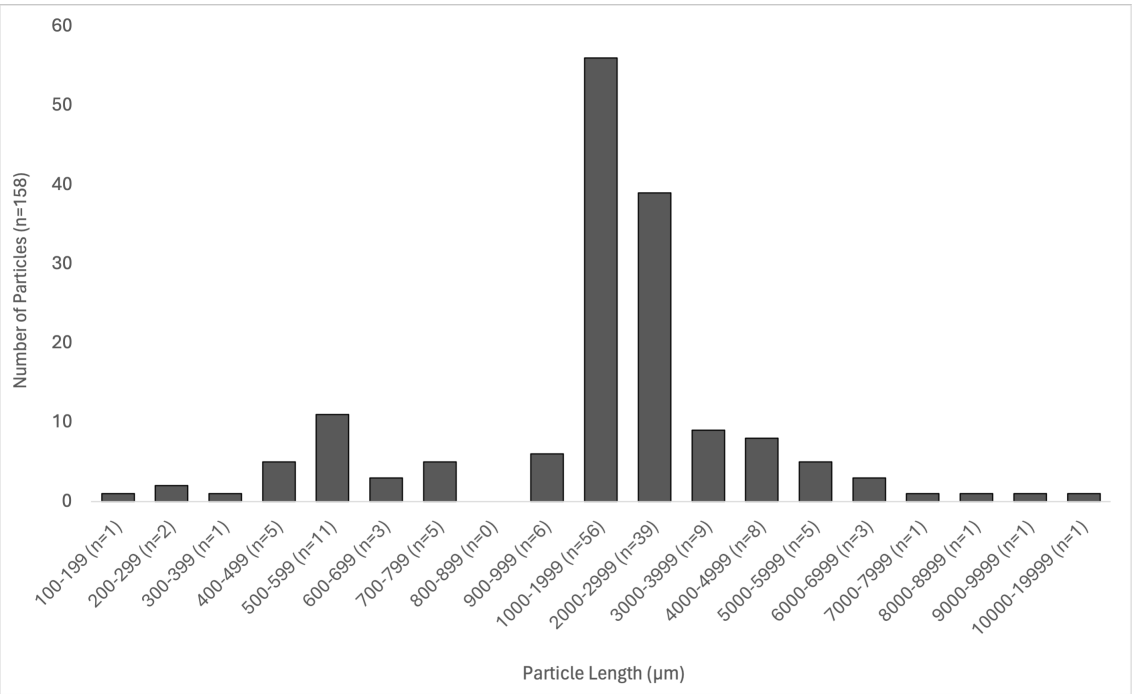

Figure 11: The length distribution of the potential particles.

4.3.1.2 Identification of polymers with FTIR

A total of 124 particles were analysed with FTIR, as 34 potential particles were lost during handling from the original 158 potential particles. Of the potential particles, six MPs were identified as a plastic polymers with the reference libraries in the FTIR (Figure 12 A-F).

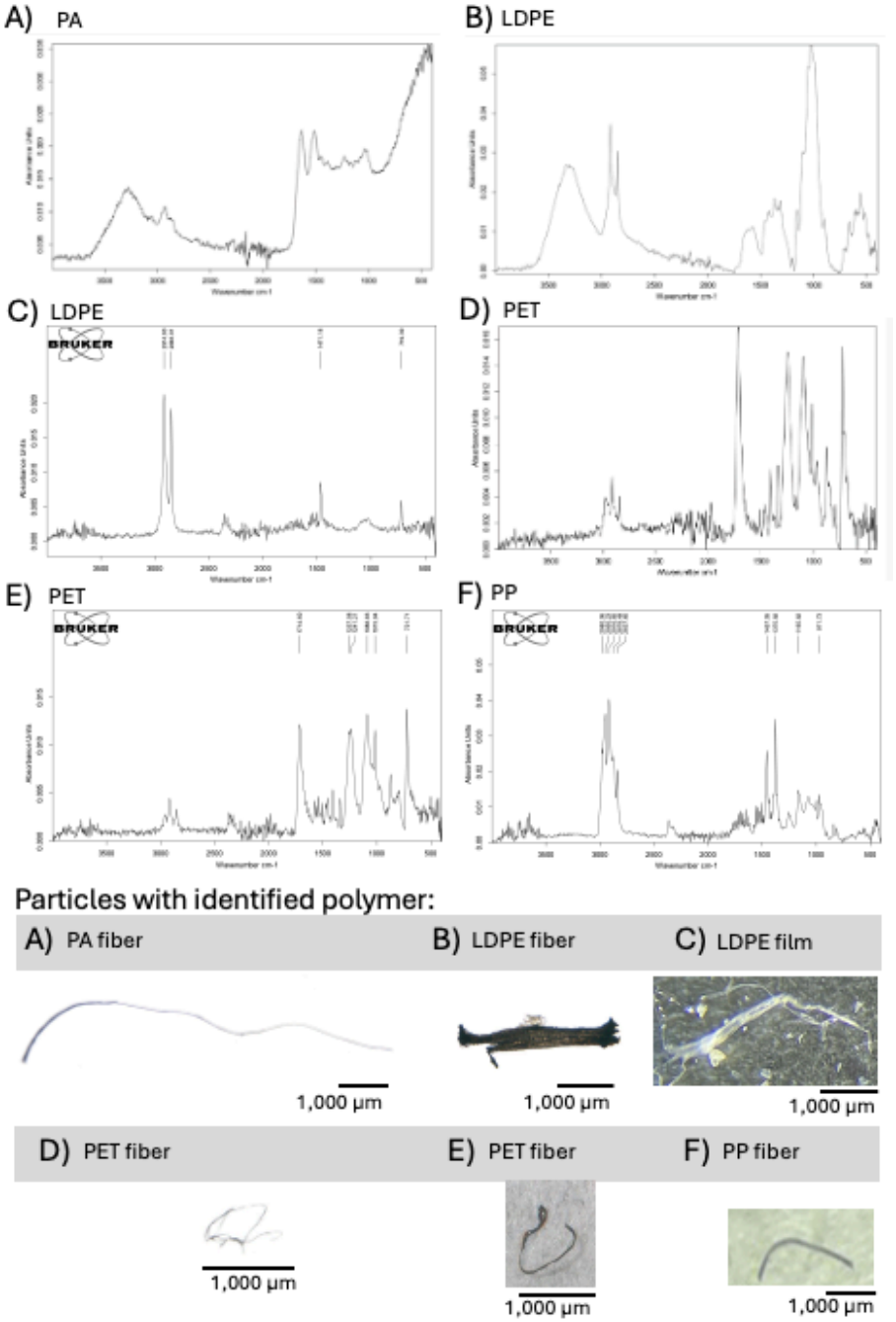

Figure 12: BRUKER ALPHA FTIR absorbance spectra of MPs matched with the three reference libraries in the FTIR. The identified polymers included (A) PA fiber, (B) LDPE fiber, (C) LDPE film, (D) PET fiber, (E) PET fiber and (F) PP fiber. Image of the particles with an identified polymer is taken with a Zeiss-AXIO Zoom Microscope (x50 magnification). All images have a 1000 µm scale bar.

In total 118 spectra had no match with the FTIRs reference industrial libraries. The 54 spectra that had a high degree of noise were excluded from manual spectra identification. As a result, 60 potential particles underwent manual spectrum matching. Four spectra were manually matched to plastic polymers (Figure 13). In the unmatched spectra, 12 cellulose fibers were identified from manual identification. These were excluded from the analysis since they were not plastic polymers.

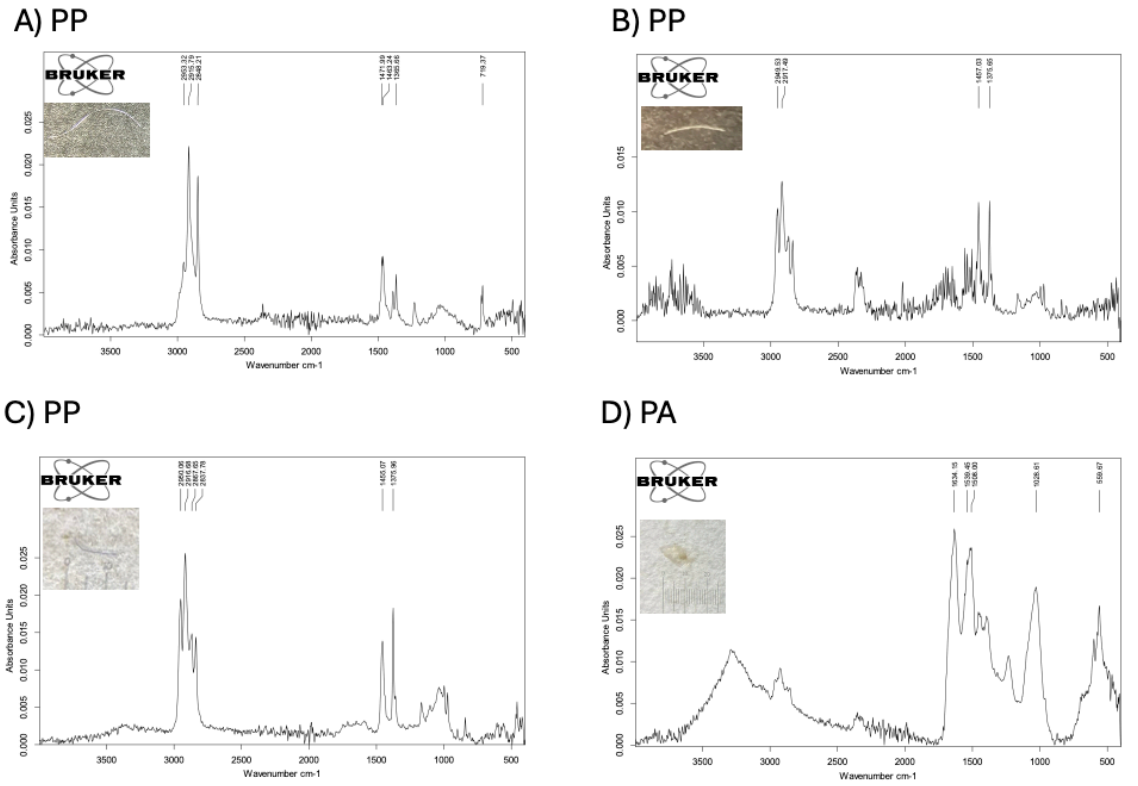

Figure 13: Manually identified absorbance spectra from the FTIR using reference libraries, [75]. Identified polymers include (A) PP fiber, (B) PP fiber, (C) PP fiber and (D) a potential polyamide particle. Images were taken through the eyepiece of the stereomicroscope (1 cm on the ocular = 100 ticks) with either black or white paper underneath.

In total 10 MPs were identified as plastic from their polymer's characterization after FTIR. Hence, 20.8 % of the pellets contained plastic. The different MPs in the pellets consisted of 40 % PP particles, 20 % PE, 20 % PET and 20 % PA (Figure 12 and 13).

4.3.1.3 Microplastic presence in relation to the sex of the shag

Of the pellets in the main study, 44 were produced by males and four by females. Nine of the pellets that contained MPs were produced by males, and one by a female (Figure 14).

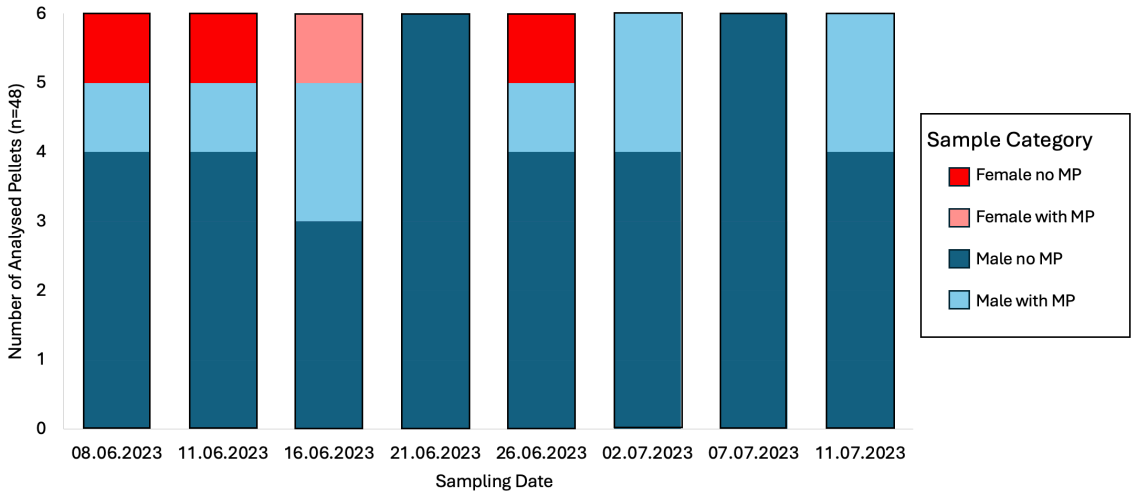

Figure 14: All analysed 48 pellets across the sampling dates (08.06.2024 - 11.07.2024, 06 - 07.07 pooled and 11 - 12.07 pooled into one collection date each) with identified sex of the shag that produced the pellet

Due to the sex bias in the samples, no clear trends could be deciphered between MPs in the pellets and the sex of the shag producing the pellet during the shags' breeding season (Figure 14).

For the 10 MPs the average dimension (length x width) was 2540 x 132 µm. The distribution of the MPs particle size, colour, polymer and sex of the shag for the eight fibers, one film and one fragment, is displayed in Figure 15.

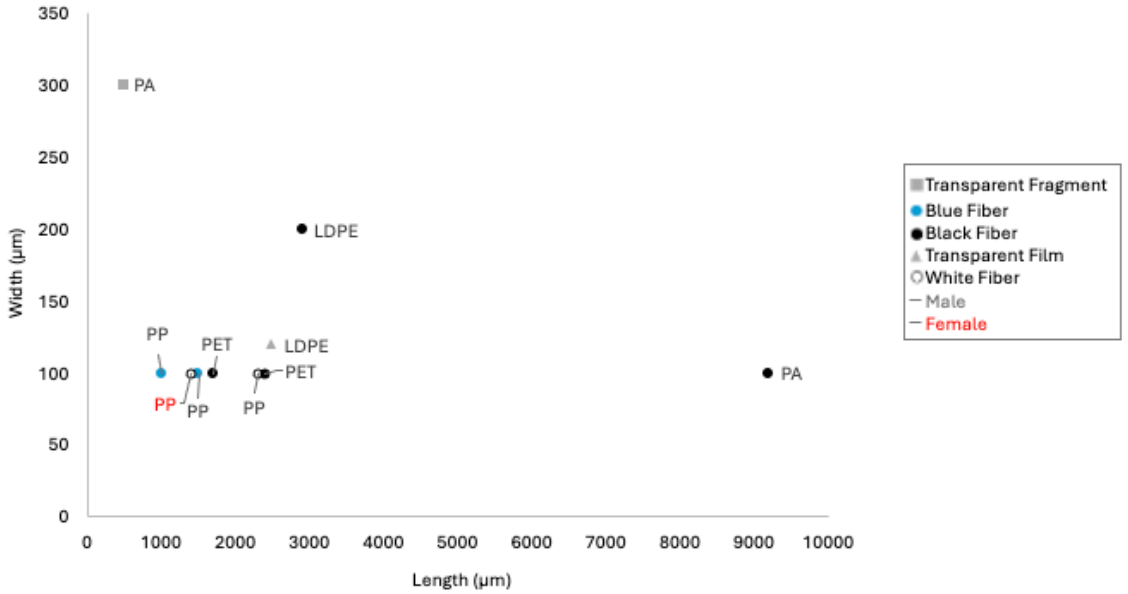

Figure 15: Distribution of MPs based on size (length (µm) x width (µm)), with their colour, shape, polymer and sex of the shag that produced the pellet.

Except for the PA fibre and fragment, all particles were within the length of 1400 - 2900 µm. It should be noted that the width of the fibers was cut off at 100 µm. These may be much thinner, however they were measured to the LOD and the smallest increment of the ocular tickmark. Both male and female pellets had PP fibers with a length of around 1500µm. Overall, the female fiber had a similar colour, size and shape as the other male MPs (Figure 15).

4.3.2 Otoliths

A total of 4068 otoliths were found in the 48 pellets from the main study, ranging between 0 - 312 per sample (raw data for all samples in Appendix A).

A subsample of pellets was analysed in detail for otolith analysis (10 with MPs and 10 without). From these 20 pellets, 1309 otoliths were present and photographed (Table 10). One sample from a female was analysed and thus assessments of potential changes in diet concerning sex were not possible. For samples with MPs, the number of otoliths ranged from 4-189 and samples without MPs had 10-240 otoliths.

Table 10: Samples containing MPs and no MPs, with their identified sex, collection date covering the entire breeding season, number of otoliths and identified polymer from FTIR.

Sample ID	Sex	Collection Date	Number of otoliths	Polymer (no MPs (-))
F2R1	Male	08.06.2023	14	PP
F3R8	Male	11.06.2023	18	PET
F4R3	Male	16.06.2023	189	PP
F5R9	Male	16.06.2023	31	LDPE
F3R6	Female	17.06.2023	36	PP
F1R5	Male	26.06.2023	67	PA
F4R8	Male	02.07.2023	4	LDPE
F5R6	Male	02.07.2023	65	PA
F4R10	Male	11.07.2023	76	PP
F5R8	Male	11.07.2023	71	PET
F1R1	Male	08.06.2023	22	-
F4R1	Male	08.06.2023	10	-
F5R7	Male	11.06.2023	193	-
F2R3	Male	16.06.2023	26	-
F4R5	Male	21.06.2023	240	-
F4R7	Male	26.06.2023	27	-
F3R4	Male	02.07.2023	22	-
F3R3	Male	07.07.2023	87	-
F1R9	Male	11.07.2023	68	-
F3R1	Male	11.07.2023	43	-
Total			1309	

A Spearman’s rank correlation indicated a significant negative correlation between pellet mass and the number of otoliths ($p= 0.0064$, $r= - 0.16$). Hence, heavier pellets could contain fewer otoliths indicating that larger fish consumed (Appendix D).

4.3.2.1 Random forest model performance

Important features for predicting the correct species based on an unknown otolith for the RF are found in (Appendix F). The averaged smoothed outlines based on wavelet reconstructions for the seven extracted reference otoliths could be seen in Figure 16.

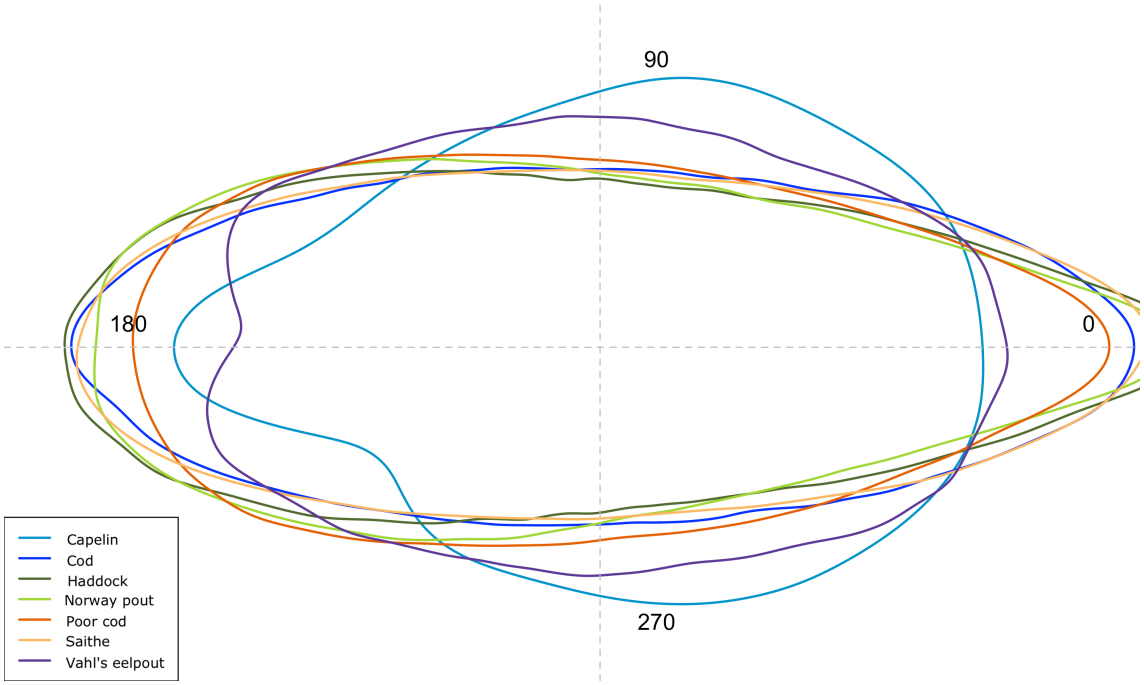

Figure 16: Otolith outline derived from image processing in R. Degrees 0-270 are the polar coordinates relating to the otolith orientation, with their centre located in the dashed cross.

Most of the otoliths had similar outlines that overlapped. Many of the reference otoliths had an elongated otolith outline. Capelin and Vahl's eelpout (*Lycodes vahlii*) seemed to have smaller and rounder outline (Figure 16). These observations coincided with the observed characteristics when images were taken of the reference otoliths. Capelin and Vahl's eelpout also seem on average to be smaller than the rest. The otolith size parameters and shape indices from Table 6, were derived from the image analysis and the otolith outlines, with boxplots including all of the calculated ranges of the parameters and indices (Figure 17).

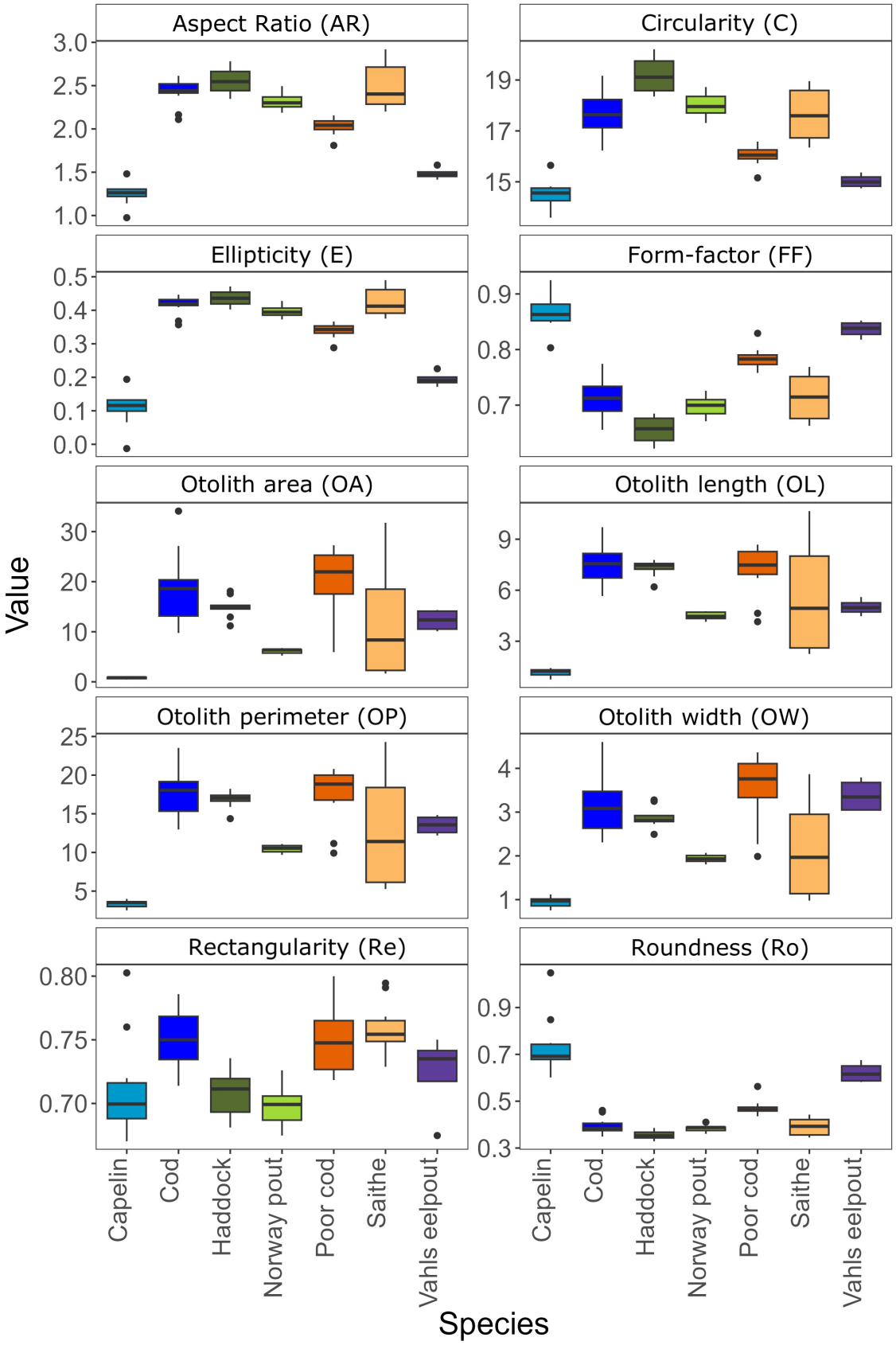

Figure 17: Box plot including morphometric outputs from R analysis, with variation within and between different species. The morphometric indices include AR (OL/OW), E $((O_L - O_W)/(O_L + O_W)), OA(mm^2), OP(mm), Re(O_A/(OL \times OW), C(O_P{}^2/O_A), FF(4\pi O_A)/O_P^2, OL(mm), OW(mm) and Ro((4O_A)/(\pi\ O_L^2))$.

For the majority of the box plots, Capelin and Vahl's eelpout obtains different values than the other fish species otoliths. The difference in outline for the two (Figure 16), could influence the parameters and indices (Figure 17). Generally, Capelin and Vahl's eelpout are less elongated, with a lower AR. Similar trends were seen for E, where Cod, Haddock, Norway pout (*Trisopterus esmarkii*), Capelin and Vahl's eelpout were less elliptical than the other two, also seen from their outlines. For OA, Cod, Poor cod and Saithe consist of a greater area and range of sizes when compared to the other otoliths. In addition, looking at OP, the same trend could be seen, indicating that the three aforementioned reference species have a larger range in otolith size for the reference otoliths used. As for Re, few of the otoliths were round, yet there was some variety between species. As for C, as values are high, deviated from circularity close to 1, suggests that the otoliths have more complex shapes as FF also suggests. OL and OW highlight that the reference otoliths used for Saithe, with the greatest range consisted of a range of otolith sizes. Ro as seen from the outlines of the otoliths indicates again that Capelin and Vahl's eelpout are more round than the other otoliths [91], [92].

A CAP (Figure 18) was also conducted illustrating the variation in the reference otoliths and patterns in their morphology based on all of the morphometric indices from Figure 17. All otoliths except for Capelin and Vahl's eelpout are towards the right side of the plot. Hence, as seen in Figure 16 and 17, then Capelin and Vahl's eelpout have more similar outlines and morphometric indices. Saithe and Cod had similar otolith shapes and morphometric outputs seen in the CAP (Figure 18). Vahl's eelpout consisted of the four otoliths in the reference collection hence the variation is the greatest as this species group had the smallest sample size (Figure 18).

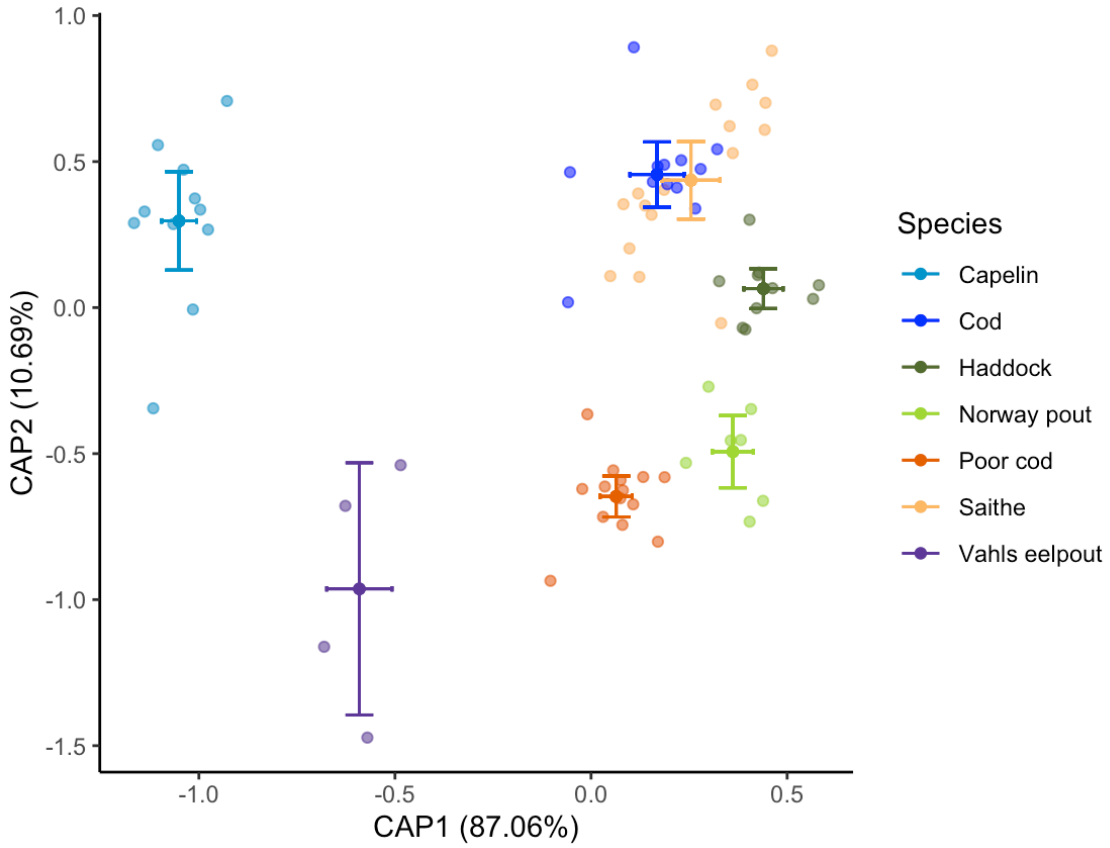

Figure 18: Variation in otolith shape and all morphometrics for the different reference species. Individual otoliths could be seen as dots, with average filled dot in the middle and two standard error values for each species is shown as the lines in the plot.

The fish species distributed in the CAP plot (Figure 18) relates to otolith shape, morphometric outputs and their variance. CAP1 explains the majority of the variance (87.06%) indicative of the most important shape features among the otoliths. CAP2 could account for more subtle characteristics as less variation (10.69%) is explained here. The underlying features were not further investigated as the plot was mainly used to identify species and patterns in otolith shape between the reference species for developing the model. The general appearance of the plot and distribution of the species seems to agree with the previous trends in Figure 16 and 17 for the reference otoliths [91].

Reference otoliths which had 10 otoliths were selected for further analysis (Vahl's eelpout was not included). The model was trained to identify unknown otoliths. The model was first balanced with the training data (70% of the 10 reference otoliths). This was followed by training the model with the remaining 30% of the reference otoliths (3 otoliths per species) to determine if it could predict the correct fish species. The models' accuracy obtained a kappa value of 0.92 (Figure 19).

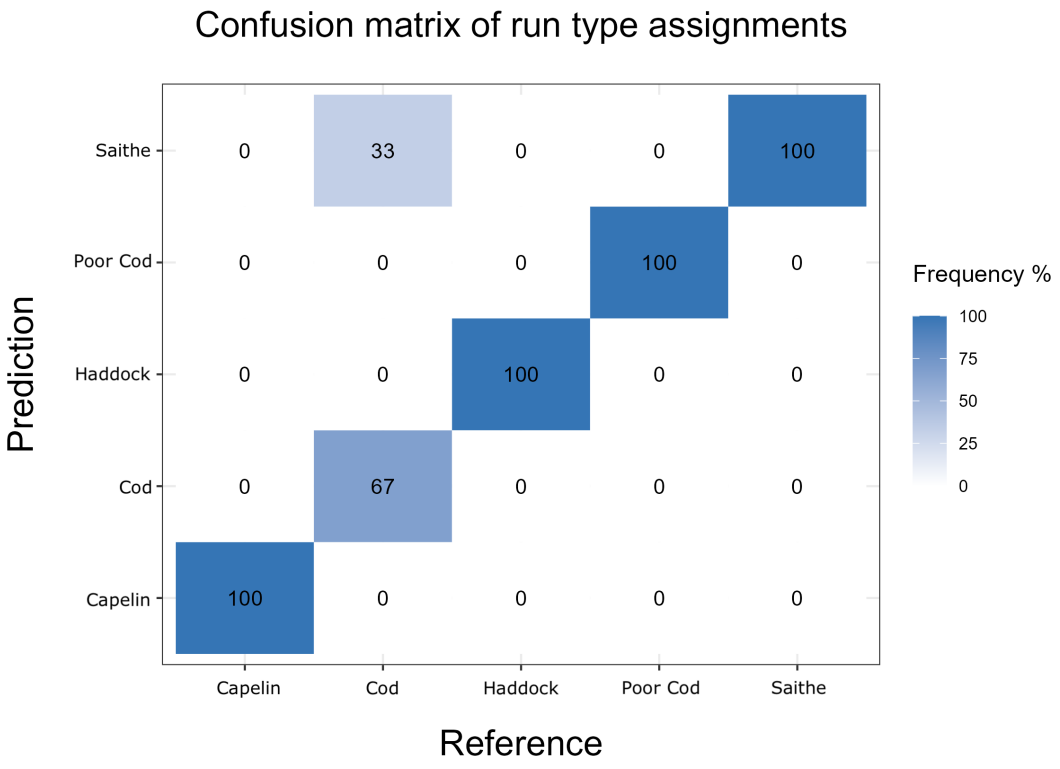

Figure 19: Confusion matrix of species assignments from otoliths evaluated on the test data (n=15) with three otoliths per species the for predictions. For the predictions of Cod then 1/3 otoliths (33%) were misidentified as Saithe. The intensity of blue with the respective value in the plot indicates the frequency (%) of the predicted otoliths.

All otoliths except for Cod were predicted to be the correct species obtaining accurate predictions (Prediction Frequency of 100%). The predictions for Cod otoliths resulted in 1 out of 3 otoliths in the prediction could be misclassified as Saithe (Figure 19).

4.3.2.2 Fish species composition

The predicted otoliths from three pellets from the main study (F2R1, F4R10, F5R6 (pellet details in Appendix A) consisting of 155 otoliths) were manually classified and checked by Magdalene Langset, a Senior Technician at NINA, Trondheim. The proposed species from the manual classification and the predicted species from the model applied in this thesis agreed on the fish species consumed.

Of the 20 analysed pellets with 1309 otoliths, a total of 1052 otoliths were from Saithe, 141 from Poor cod, 42 from Cod, 60 from Capelin and 14 from Haddock. Saithe was the dominating species in all pellets, whether they contained plastics or not.

The proportion of otoliths for the predicted fish species was the highest for Saithe (79% for MPs, 81% no MPs), then Poor cod (9% MPs, 12% no MPs). Capelin had the third highest proportions (6% in MPs, 4% in no MPs) followed by Cod (4% MPs, 2% no MPs). Both categories had the same proportion of Haddock otoliths (both 1%) (Figure 20). The otolith proportions for each individual processed pellet can be viewed in Appendix G.

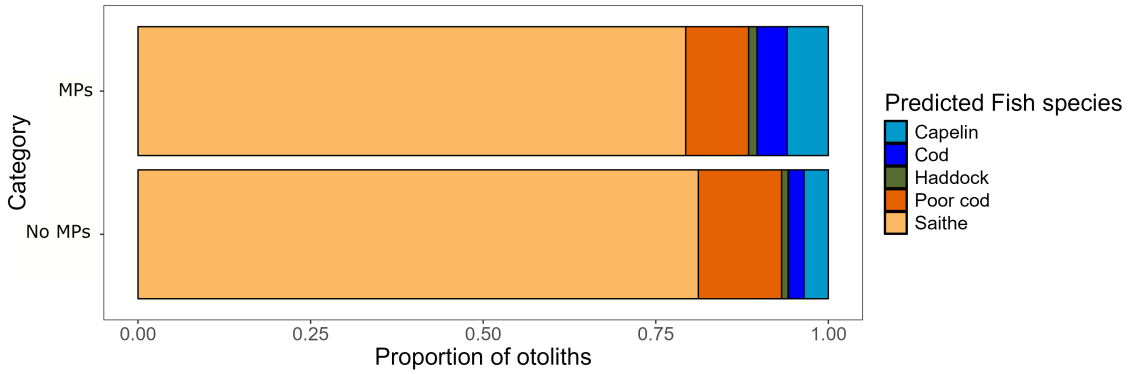

Figure 20: Predicted fish species for the proportion of otoliths. The predicted fish species was from otolith image analysis using ShapeR in R.

The fish count was based on the individual otoliths as they were not paired, hence the number of actual fish consumed could be expected to be half yet the proportion would remain the same. Fork lengths were calculated as in [32].

Of the consumed Saithe, 992 otoliths in age class 0 and 59 were age 1 (Figure 21). For age class 0 some fork lengths resulted in a fish count greater than 200 otoliths, with an average fork length of 45.5 mm for the fish (Figure 21). Otoliths in Saithe age class 1 had an average fork length of 161.3 mm based on the otoliths.

Poor cod was the second most consumed fish, with similar proportions of age class 0 and 1, with 63 and 78 otoliths respectively in each fish age class. Age class 0 did not seem to follow a normal distribution but rather a bimodal distribution, the average fork length was 16.8 mm. Age class 1 seemed to be normally distributed from the 75th percentile, and had an average fork length of 111.1 mm. For the Poor Cods both 0 and 1 age class seemed to reach around 15 otoliths for the fish count at different size intervals.

For Cod, 37 of the Cods were age class 0, reaching fish counts >7.5 for the most frequent fork length, with an average fork length of 109.3 mm. Five otoliths corresponded to age 1 with an average fork length of 168.2 mm.

Capelin and Haddock were identified to be part of the shags diet. Their age class was not determined as they made up 6% of the shags diet (Figure 21).

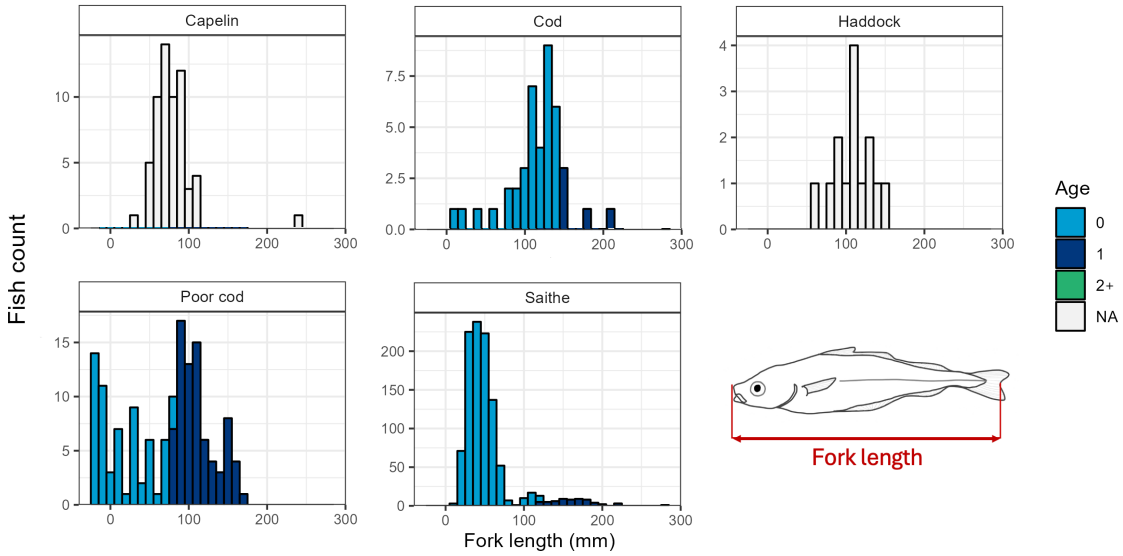

Figure 21: Fish count for each age class (0-1 in graph) of the five species, based on the size of the fish (fork length (mm)). Fork length (mm) in fish is measured from the snout to the middle of the tails fork (right).

Dividing the fish species consumed by the shags into their respective age class, more age 0 Saithe and Cod seemed to be present than age 1 (Figure 22). The opposite seemed to be the case for Poor cod, with a higher proportion of age class 1 than age class 0. There was only one 2 age class Saithe in one pellet, which did not contain MPs.

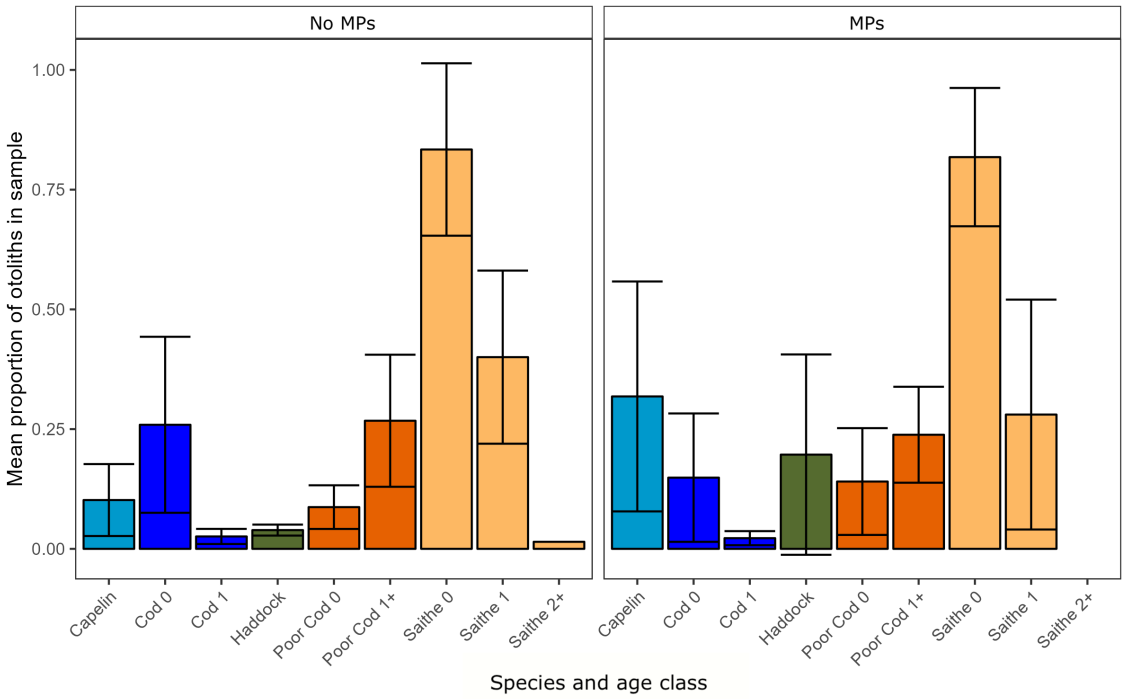

Figure 22: Mean proportion (± SD) of fish species and age classes, separately for samples that contained MPs (left) and those that did not contain MPs (right). Sample sizes: n=1309 otoliths in n = 20 samples.

GLMs revealed no significant difference in the proportions of fish-age groups in samples that contained MPs versus those that contained no MPs (all t-values < 0.54, p > 0.312; Table 11).

Table 11: Results from the GLMs to compare the numerical proportion of fish species and age classes between samples containing MPs and those without MPs. Each row refers to the results from one model. For Poor cod, Saithe and Cod models were run on the individual age classes and on the total proportion of fish for a given species (all age classes pooled).

Fish Species-age class	t-value	p-value
Capelin	-0.546	0.592
Poor cod - all age classes pooled	-0.157	0.877
Poor cod - Age 0	0.323	0.750
Poor cod - Age 1	-0.423	0.677
Saithe - all age classes pooled	-0.539	0.597
Saithe - Age 0	-0.295	0.771
Saithe - Age 1	-0.004	0.997
Saithe - Age 2	-0.005	0.996
Cod - all age classes pooled	-0.281	0.781
Cod - Age 0	-0.256	0.801
Cod - Age 1	-0.319	0.753
Haddock	1.040	0.312

5 Discussion

This thesis investigated MPs presence in shag pellets, the shags diet and potential connections between MPs and diet. The following sections will discuss the results of this thesis and provide context for the detected MPs, consumed fish and potential connections. Considerations and recommendations were also made with details regarding the validation of the protocol and assessing the use and collection of pellets for MPs studies. The present sex bias in the pellets was discussed and the applicability of this thesis method to other shag colonies was evaluated.

5.1 Microplastics detected in shag pellets

The first hypothesis for this thesis was that MPs could be detected in the pellets of the shags. MPs were confirmed in the shag pellets from Sklinna with one MPs per pellet, giving a total of 20.8% of the pellets containing MPs. Based on the detected shape, size and colour, similarities could be drawn to previously detected MPs in the available literature. Recurring MPs detected along the Norwegian coast have been reported to be black and blue fibers [18]. Based on the detected MPs in this thesis, fibers and PET are negatively buoyant and have previously been detected in benthic coastal environments [75]. PP and PE are buoyant particles, and the shags may also be exposed to these MPs on Sklinna. Exposure may be through respiration, drinking water or collecting plastic debris from their nests. It is not possible to exclude this factor from the analysis of MPs exposure. As a result, the MPs detected in this study given their shape, colour and polymer could be found in the coastal habitats that the shags on Sklinna forage in.

The difference in the number of potential particles and MPs is important to note and could be explained by many factors. Weathering and biofouling are reported issues for polymer identification [42]. The degree to which MPs were biofouled in this thesis was not possible to assess as the digestion with KOH would have removed any traces of this during incubation [128]. If particles were not sufficiently cleaned, then residues from organic matter in the Petri dish holding the filtered sample, or solvents used could add noise to the spectra [132]. The particles could also contain additives and external contamination which could interfere with the spectra and MPs match. As reported in previous studies, KOH digestion could have degraded cellulose polymers, and could be a contributing factor to why many of the potential particles were not identified [30]. Cellulose fibers could be included in future studies and incorporated into the FTIR reference libraries as anthropogenic particles as they often occur in the marine environment [75]. As many potential particles in this study were small fibers, then the thin width could make it challenging for the FTIR to identify the polymer [132]. In addition, some crustacea or mite remains were not brittle and could have been mistaken as potential particles. When it comes to the number of MPs per pellet, this thesis found one MPs in each of the 10 pellets. This plastic count per pellet will be compared to other detected MPs and plastics in pellet-producing seabirds.

5.1.1 Studies reporting microplastics and plastics in seabird pellets

Five studies conducted between 1988 and 2018 reported plastics in the pellets of seabirds (Table 12). Including this thesis, one other study analysed MPs in pellets from shags [3]. The remaining four have investigated plastics in pellets from different seabird species including cormorants, gulls and skuas [1], [78], [54], [111].

Table 12: Studies reporting plastic and MPs in pellets from regurgitating seabirds. Grey fields are not filled in as information was not reported in the study.

Species	Location	Pellet collection (mm/yyyy)	Number of pellets	Frequency of occurence of plastic	Mean plastic particles in pellet ± SE	Plastic shape	Colour	Polymers	Average length µm ± SE (n=)	Size cut off (µm)
European shag (*Phalacrocorax aristotelis*) **This thesis**	Skinna, **Central Norway**	06-07 / 2023	48	0.21	0.208 ± 0.059	Fibers	Black (40%), blue (20%), white (20%)	PP	1550 ± 272 (n = 4)	100 µm
								PET	2050 ± 495 (n = 2)	
								PE	2900 (n = 1)	
								Nylon	9200 (n = 1)	
								PA	500 (n = 1)	
						Film	Transparent (10%)	LDPE	2500 (n = 1)	
						Fragment	Transparent (10%)	PA	500 (n = 1)	
European shag (*Phalacrocorax aristotelis*) **Álvarez et al. 2018**	Island of Ons, **NW Spain**	04 / 2017	41	0.63	1.68 ± 0.42	Fibers		PA (Nylon)	7580 ± 820 (n = 32)	
								Polyester	13490 ± 5500 (n = 3)	
								Rayon	4070 ± 1070 (n = 2)	
								Ethylene vinyl acetate	49500 ± 5500 (n = 2)	
Great Cormorant (*Phalacrocorax carbo*) **Acampora et al. 2017**	Money Point and Great Saltee Island, **Ireland**	2011, 2014-2015	92	0.032	0.043 ± 0.026	Sheets				1000 µm
						Foams				
						Fragments				
Glaucous-winged Gull (*Larus glaucescens*) **Lindborg et al. 2012**	Protection Island, **USA**	06-07 / 2007-2010	589	0.12		Films				
						Fragments				
						Foams				
						Filaments				
						Pellets				
Great skua (Stercorarius skua) **Hammer et al. 2016**	Skúvoy, the **Faroes**	04-08 / 2013	1034	0.006		Hard plastics	White/yellow (68%), red (10%), pink (5%), orange (4%), black (3%), green (2%), blue (2%) other (6%)			
						Threads				
						Foams				
						Sheets				
Great skua (*Stercorarius skua*) **Ryan and Fraser 1988**	Island, **South Atlantic Ocean**		591	0.22						

Comparing this thesis and the study conducted by [3] on MPs in shags in Spain both studies analysed a similar number of pellets. However, the frequency of occurrence of MPs was about three times higher in the Spanish colony than in the colony on Sklinna (Table 12). Both studies found fibers, whereas this thesis found one additional film and fragment. The MPs colours found in Alvarez's study were not reported. There was one polymer that was similar between the studies and this was a PA fiber, specifically nylon. The MPs in the pellets in Alvarez's study were also much longer (originally reported in mm) than MPs from Sklinna. The difference in polymers and size of the MPs could indicate potential regional differences in MPs occurrence [6]. The higher occurrence of MPs at the Spanish colony could be due to the Island of Ons being closer to human activities, with a mussel fishery in the area. The Island of Ons is also closer to the mainland than Sklinna, where the proximity to human activities could influence MPs levels. The recorded fishery-related activities close to Sklinna during 2023 included kelp harvesting (*Laminaria hyperborea*) and some fishing activities of Saithe and Angler (*Lophius piscatorius*) using fishing rods and nets [64]. The occurrence of fibers could be from the nets. However, the extent of fishery activities is regulated as Sklinna is a nature reserve.

Differences in the amounts of MPs could also be due to regional differences in MPs distribution. Flumars have previously illustrated a Northern-to-Southern gradient in plastic pollution, where the ones closer to southern latitudes contain more plastic [44]. Fish have also been reported to follow a latitudinal gradient, with higher MPs levels detected at southern latitudes. No trends were found for coastal proximity and MPs amounts for these fish [69]. Sklinna is a remote island situated away from the coast with a northern latitude, which could be a possible spatial reason for the number of detected MPs compared to the more Southern latitudes of Spain.

Differences between the processing of the pellets in this thesis and [3] makes comparability difficult. The MPs in [3] were picked out from the pellets with tweezers without any digestion or filtration, where the lower size cut-off is not known. The smallest length of the fibers found in [3] was on average 4070 µm, whereas the average for the 10 MPs in this thesis was 2540 µm which could explain differences in findings between the two. Fibers and potential particles in the smaller size ranges may have gone undetected in [3]. Another important factor to consider is that [3] exclusively analysed fibers. The inclusion of fibers in MPs studies is debated, as it is difficult to account of external contamination. It is also not known if [3] included blanks when processing their samples. This thesis included adequate lab and field blanks accounting for external MPs contamination and potential airborne fiber contamination.

The remaining studies focused on plastic particles in the pellets rather than MPs. A study focused on plastics in Great Cormorant pellets from Ireland [1]. The noted plastics in these pellets differed from the first two studies on shags, these were sheet-like, foam and fragments. Such differences may account for differences between the species, their preferred prey and the local occurrence of plastics. The colour, polymer and length of these plastics were not reported, so they cannot be compared to the findings of this thesis. [1] may have underestimated the present plastic as the presence of fibers in Acampora's study was not known, as they were excluded from the analysis.

The Glaucous-winged Gull study [78] was conducted using citizen science, where citizens looked for plastic after being trained by professionals. In [78], similar plastic as the study by [1] were found including films, fragments, foams, filaments and plastic

pellets. The frequency of occurrence of plastic for the gulls was higher than [1] cormorants but lower than the shags in this thesis. However, [78] study had a much larger sample size with 589 pellets collected over three years. It should also be noted that many different people conducted the visual inspection of the pellets for plastic hence, this could introduce bias and misrepresentation for the plastic detection.

In the two studies investigating great skua pellets [54], [111] it is important to note that skuas are predatory foragers unlike the shags and cormorants that are pursuit divers. The great skuas select other species such as seabirds as their prey, where high amounts of plastic were found in cases where the skua foraged on fulmars. This may also explain why the colours and shapes were different from the MPs reported in this thesis. The difference could be explained by their foraging behaviour, as the fulmar and the shag forage at different depths in the water column, with different MPs occurrences. The frequency of occurrence was low for the study on great skuas by [54]. However, many of the collected pellets were from prey species, which were not seabirds.

Comparing this thesis to the findings of the studies mentioned above, the frequency of occurrence of plastics in the shag pellets on Sklinna was moderate at 20.8%, obtaining the 3rd highest detection of MPs or plastics in Table 12. The mentioned factors such as location, MPs concentration in the area, proximity to human activities, prey and how the species forage may be external factors that influence the MPs detected in the pellets. Internal factors impacting MPs detection could be associated with metabolism, individual differences, and differences in elimination rates between regurgitation, digestion and defecation.

The mentioned seabird species in Table 12 all have different pellet regurgitation frequencies. This is important to consider as it could influence the retention of MPs and the rate MPs are eliminated. Cormorants are thought to regurgitate daily, while gulls and skuas produce pellets after every meal and shags produce pellets from every 1-4 days [1]. However, a common denominator for pellet analysis is that it allows a short-term scale for investigating diet. Pellets illustrate the immediate food supply and could be more beneficial for MPs analysis as MPs are detected in pellets [6], [78].

MPs in studies could often be underestimated by 20% [61] when particles are smaller than 1mm. The actual MPs load in shag pellets may be higher as there are biases in visual inspection, and difficulties in removing small MPs by hand, where MPs often go undetected. The visual inspection of the pellets in this thesis was challenging when high degrees of organic matter was present as potential particles were challenging to find [18]. Hence, the real quantities of MPs in the pellets from Sklinna are not known [24].

The lack of standardization when studying MPs is important to discuss. In Table 12, it becomes clear that there is missing information regarding the reported characteristics of the plastics found in studies investigating plastics and MPs in pellets from seabirds. Different methodologies were applied in all studies so no uniform treatment for the pellets or plastics was used. Overall, such discrepancies with a lack of standardization and reporting of findings in MPs research makes it challenging to compare findings for different species, conduct spatiotemporal comparisons and relate findings to literature.

5.2 Fish species composition

The second hypothesis for this thesis, was intended to explore the diet of the shags and it was expected to consist of different fish species and age classes. Saithe consumption made up 80% of the diet from the 20 pellets analysed in this thesis. Saithe in age class 0 was the main consumed group. Poor cod and Cod were also consumed but at lower levels. The shags mainly consumed age 0 and 1 Gadidae, which shows similar trends as previous work in the shag colony on Sklinna [58]. Since the start of the monitoring of the shag diet in 2007 and until 2016, Saithe has been the main fish species in the diet of shags for all years (2016 last year with published data) [80].

The proportion of age 0 and age 1 Saithe consumed depends on the spawning success and survival of the fish larvae (Lorentsen et al. 2018). Saithe spawn during late winter in Eastern pelagic regions of the Norwegian Sea. During their first summer, young fish move into kelp forests (*Laminaria spp.*) and Saithe in age class 0 increase in number during the breeding season as the Saithe relocate into the foraging area of the shags around Sklinna [79]. The prevalence of Saithe age class-0 around Sklinna could be illustrated by their high fish count and consumption in this thesis. The fact that the shags do not selectively forage on one size of fish within the different age classes, as the majority were normally distributed could highlight that they forage on healthy fish stock. Poor cod was the only species not normally distributed, this could be due to challenges in distinguishing between age 0 and 1 Poor cod [131].

In this thesis, during the 2023 breeding season, the consumption of age 1 Saithe was low with a greater proportion of Saithe of age class 0. Looking to [80], age 1 Saithe has been an important age class for the consumption and breeding success of the shags. However, the proportion of Saithe in age class 0, 1 and 2 in the shags diet was noted to have a great annual and seasonal variation from 2011-2016. In years with low availability of age 1 Saithe, then the consumption of Saithe in age class 0 increased. The shags were also noted to feed on Cod and Poor cod to make up for the lack of age 1 Saithe [80]. In previous years, the shags have still consumed Gadidae and there were no significant changes in the total biomass of Gadidae consumed across years from 2007-2011 noted in [58]. This could be indicative of Gadidae being the main fish consumed by the shags on Sklinna. Where the shags may not tend to forage on other species of prey outside of Gadidae. Saithe of age class 2 in this thesis and previous studies has not been commonly consumed by the shags or encountered in pellet analysis.

5.2.1 Otolith identification

Otoliths from the pellets contained a variety of fish species and age classes. Studies have found that there are normally no statistically significant differences between the left and right otoliths therefore, both otoliths were included in the analysis and these were not paired [107]. Manual identification of species and taking measurements of their length and width is labour and time-intensive. The identification requires time to learn, often requiring a professional to teach and check the work [118].

Image processing to identify the otoliths is therefore beneficial as it is much more time-efficient once the images are acquired. The random forest model provided a robust approach to classify fish species based on otolith shape from wavelet coefficients and otolith morphometric indices. One important limitation of the approach

used in this thesis is that the model could only predict fish to the species provided in the reference data, so one fundamental assumption is that the reference data is comprehensive enough to cover the diet range of the studied bird species.

Due to very similar otolith shapes, Saithe could be misidentified as Cod. This could be expected as the species are closely related and the otoliths are very similar in shape and size as seen from the overlapping outlines (Figure 17). These otoliths could be difficult to distinguish even when they become manually identified to species. However, the fact that Cod was misinterpreted to be Saithe in some cases could be argued not to be too detrimental as Saithe was the main species consumed by the shags on Sklinna. Saithe may have been overestimated, and Cod underestimated in this thesis. However, the fish lengths, age classes and proportions coincide with otolith analysis from shag pellets on Sklinna from 2007-2016 [58], [80]. The fish compositions in the pellets according to age, size and species were confirmed in this thesis with similar findings as previous years on Sklinna.

The low sample size, with 10 otoliths per fish species, using seven for reference training and three otoliths to predict their species caused a slight variation in the model performance. In cases where one otolith was misclassified, it could result in high variation as the training data was based on three otoliths, hence one misclassified otolith would account for 33% (Figure 19). The misclassification of some Cod species and low number of otoliths the model was based on could be reflected in the confidence interval (0.68%-99%).

To improve the model, more images should be added including additional species, as shags have been reported to feed on 17 different species of fish, not only five as used in this thesis [58]. The types of species should also be expanded to strengthen the model. This could further increase its applicability to other shag colonies or otolith studies. As otoliths are also found in the faeces and stomachs of seabirds the use of ShapeR could also be applied to analyse otoliths in other matrices. Including an "other" category in future models could account for additional uncertainties or unknown species in the analysis. Previous studies have grouped challenging Saithe, Cod and unidentifiable Gadidae into a Gadidae sp. category [58]. The model could also be applied to new or archived samples. Using ShapeR to analyse otoliths could become efficient over time, as the reference library could be expanded and applied to process new samples. Both digested and reference otoliths could also be included in future models. This could reduce uncertainty when determining the species and account for otolith differences from degradation and digestion. When calculating the fork lengths, one must also consider that different equations should be used for fish species from different regions [58]. Obtaining a sufficient dataset of reference material could establish a good model and reduce the need to identify otoliths at the species level by professionals [118].

5.3 Investigating the link between diet and microplastics in shag pellets

The last hypothesis for this thesis aimed to explore if there was a link between a diet consisting of larger and older fish would result in a larger or greater number of MPs when compared to younger and smaller fish. The oldest fish consumed was one age class 2 Saithe and it was in the pellet containing no MPs. There were also high numbers of young fish consumed, and there were no significant differences between fish species, age class and composition between samples with and without MPs.

Pursuit-diving seabirds have previously been reported to have a low tendency to ingest plastics, often explained by how and where they forage [93]. As the particles are smaller than the fish that the shags prey upon, then one could assume that the shags have not actively selected the MPs, and rather that the fish may have ingested them [128]. However, as buoyant MPs were also found, the MPs could also be from the passive ingestion by the shags as such MPs are detected in the water column.

A meta-analysis found globally that fish ingested MPs PE and PP fibers that were blue or black (Lim et al. 2022). PE and cellulose fibers have also been reported in mesopelagic fish [132]. A study by [59] highlighted the trophic transfer of MPs from fish to seabird. The fibers found in the fish were less than 0.9 mm and were blue, black and transparent coinciding with the findings of this thesis. However, it should also be noted that blue, and black fibers are frequently detected MPs in the marine environment [28]. It can therefore be challenging to uncover discrete trends in MPs consumption of prey and not from passive ingestion from the environment.

The research on MPs in juvenile fish is limited [128]. MPs have been detected in juvenile Polar Cod (*Boreogadus saida*), where fibers were excluded from the analysis. The Polar cod were found to contain particles of epoxy resin and Kaolin with polymethylmethacrylate [69]. For Atlantic Cods found in the Norwegian Sea, along the Norwegian coast, 3% had plastics in their stomachs, with polyester being the most frequent MPs [18]. [83] findings indicated the prevalence of cellulose fibers in the gastrointestinal tracts of 10 different fish. However, for both the pellets containing MPs and without MPs in this study contained similar number of otoliths. Juvenile Saithe feed on plankton, krill, and copepods, where what they prey upon changes with time [96]. It is unknown if the juvenile fish in this thesis mistake or selectively intake MPs as prey.

[3] study investigated diet items in the pellets and found that MPs were significant and frequently encountered in samples with benthic fish. The shags in Spain fed on sandeels that were 5-15 cm long [3], [126]. The shags on Sklinna consumed fish of similar lengths but different species than the shags in Spain. This could be indicative of regional MPs differences, MPs differences in prey or habitat differences for the fish.

As the shags feed on juvenile fish mainly from age class 0 and 1 then the extent of MPs exposure and ingestion may be limited to the age of the fish. As the shags mainly consumed Saithe of age class 0 (75.6% of all fish), then there was little variation in age and size of fish in the samples limiting the assessment to determine the effects of age on MPs.

The frequency of regurgitation of pellets should also be considered, as they give a snapshot of the shags' diet, starting from the last time they were produced [63]. It is not known when the shag last foraged, produced its last pellet, or how much of

the diet is represented in a pellet. In terms of MPs, [105] stated that it is unlikely that one regurgitated pellet could eliminate all MPs from the seabird's stomach. It is therefore important to uncover the retention times of MPs in the gastrointestinal tract of the shags.

The retention and elimination of MPs depend on the physicochemical characteristics of the MPs and the anatomy of the seabirds. Anatomical differences in the gastrointestinal tract could also influence the retention of MPs and excretion in the pellets. The great skua could regurgitate the contents of both its proventriculus and gizzard. The great skua has been noted to retain plastics in their stomachs where MPs may be regurgitated with the remains of later meals. Procellariiformes (petrels and fulmars) are unable to regurgitate, plastics pass the proventriculus into the gizzard which is separated by a sphincter and plastics accumulate in their gizzard [54]. As for cormorants (including shags), the proventriculus and gizzard are slightly visibly separated [36]. The extent and if the anatomy of the shags stomach influences MPs retention and further addition of MPs in pellets is not known.

5.4 Considerations and recommendations

5.4.1 Validation of protocol

There is no established and standardized protocol for processing seabird pellets in the lab. [106], claimed that minimal laboratory steps were required to process pellets from seabirds. It would be challenging to apply the proposed protocol by [106] in this thesis, as the pellets from the shags had a lot of mucus, which had to be removed during KOH digestion for further analysis. Incubation in KOH was applied after otoliths were removed, even though, most otoliths such as Gadidae are not readily impacted by KOH digestion. However, fragile diet items have been noted to disappear after KOH digestion, which could also be the case for more fragile hyaline otoliths [68].

MPs studies rarely conduct recovery experiments [129]. Studies that do conduct recoveries tend to validate the chosen method from spiking samples with virgin industrial plastics. Such plastics will behave differently when processed in the lab or compared with the weathered particles often found in the environmental samples [129]. [114] stated that it is important to conduct laboratory studies with relevant shapes, sizes and polymers of MPs representative of what is found in the field. This thesis gave insight into the type of MPs found in the pellets and conducted spiking experiments using relevant plastic materials from the field.

Previously, the influence of KOH (10%) digestion on plastic particles has not influenced the chemical and structural integrity of MPs [50]. However, [30] found that following KOH (10%) digestion, cellulose acetate the became degraded. Other polymers such as the ones reported in this thesis (PA, PET, PP and PE) remained unchanged. It has also been found that digested MPs in the stomachs of seabirds were also unchanged by KOH [68].

The reference libraries in the FTIR were based on industrial plastics. Studies have previously suggested expanding or creating reference libraries with frequently encountered organic matter and weathered plastics. For instance, fish bones, and crustacea remains could be included so that potential particles of organic origin could be matched and confirmed [3].

5.4.2 Pellets as a matrix for microplastic and diet analysis

Pellets as a sample for MPs detection has been discussed in various studies. [3], [23] proved pellets to be an effective means for monitoring MPs and plastics in pellets. [106] does not regard pellets as a matrix for MPs studies and that they cannot be used to assess the presence of MPs as external contamination is too high. The same study states that it is unlikely that plastics $<$1mm accumulate in seabirds, and that seabirds may not be applicable to indicate the distribution or occurrence of such environmental MPs pollution. However, MPs $\leq$ 1 mm were detected in the pellets in this thesis, hence pellets could be beneficial for assessing this type of MPs as they are excreted.

Pellets could be a beneficial matrix as they could be collected in a minimally invasive way, without having to handle the birds. The collection allows a repetitive and regular sampling for multiple seasons and years. On the other hand, it could be difficult to link the pellet to an individual shag, as samples may not always be whole, and collection often occurs during the breeding seasons while the birds are on land [106].

From pellet analysis, the MPs remaining internally in seabirds are unknown [106]. Lavage, emitics and dissection of the gastrointestinal tract uncovers MPs in the digestion system of the birds, where individual and species differences are important to consider. Even though lavage gives a picture of the contents of the proventriculus, it is a very invasive and labour-intensive method [109]. Beached birds may have died from disease or starvation. This could influence normal feeding and give an incorrect image of their diet and plastic levels as more plastic may be in birds which die from starvation, dependent on the body condition of the seabird. In addition, it is often difficult to know the origin of beached birds, especially in migratory species, where exposure to MPs may differ between breeding and non-breeding grounds [98]. It could be beneficial to harmonize sampling methods, and pair dissections with pellets gaining an overview of MPs in each species in addition to ingestion and elimination [105].

The fullness of the stomach and rate of regurgitation and defecation may influence the otoliths and MPs found in the pellets. Indeed, one sample analysed in this thesis had very little organic matter with no otoliths. The maximum number of otoliths recorded in a pellet was 312, so individual variations in diet should also be considered. The MPs prevalence in other organs, the stomach and faeces of the adult shags producing the pellet is not known. Previously no MPs were detected from the dissection of healthy, bycaught shags in Northern Norway [11]. Pellet analysis could in the future be coupled with other monitoring strategies such as dissection if dead birds are found. Using multiple methods increases the understanding of MPs ingestion and excretion in the shags. Elimination rates of MPs should be considered, as the frequency between regurgitating pellets and defecation, two elimination routes are not established in the shags. This could influence MPs levels in the gastrointestinal tract and further the pellets and faeces.

Fragments were the most frequent particle detected in the faeces of shag chicks [84]. The MPs detected in the chicks are likely from regurgitated food items from their parents [6]. Out of the MPs found in this thesis, fibers were the most found in adult shag pellets, which was also the case in [3]. These differences could be due to elimination routes. Smaller particles that have passed through the proventriculus may become mechanically ground in the gizzard until they pass through into the faeces. Fibers may become attached and tangled with organic matter, be lodged in their gut tissue and more readily expelled in the pellets [24].

5.4.3 Pellet collection

When collecting the pellets in the field it is important to consider collection bias towards fresh, "clean", and whole pellets. As a result, the potential MPs and organic content of "dirty" pellets with a lot of earth or algae attached is unknown. However, analysing such pellets could encompass analytical difficulties as the metal filter could become clogged at a higher rate. The sample would also have to be processed in multiple subsamples. It could also be more difficult to find MPs during visual identification if there was a high degree of organic matter and external dirt, mites or algae. The efficiency of the method may be reduced and as more steps are required to process the pellets and loss of potential MPs might increase.

Diet studies have often opted for collecting pellets at the end of the breeding season after the chicks have fledged as this is less invasive [109]. A downside to collecting pellets at the end of the breeding season is that the dates for when the pellets were produced are unknown and the pellets may be subjected to more external contamination before collection. The physical characteristics of the breakwater at Sklinna allowed a minimally invasive collection, disturbing a limited number of nests during the breeding period. The collection in this thesis allowed a better coverage of pellets and the breeding period coverage of the season compared to the suggestion by [109]. However, the ease of collection and degree of disturbance must be considered individually at each site.

5.4.4 Sex bias in the analysed pellets from the shags

It was expected to obtain a sex ratio closer to 50/50 for the main study as shags of both sexes feed their chicks and attend their nest. The sex of the shags that produced pellets in the second pilot were identified, and here there were four females and three males with a ratio closer to 50/50. Female shags on Sklinna are known to go on longer foraging trips during the breeding period [80], whereas males often occupy the nesting area. All pellets were collected in the morning, as pellets often are produced then, which allows fresh pellets to be collected [63]. If the females were foraging when the pellets were collected, then more males may have been present at the colony a potential effect of the behaviour patterns of the adult shags. Therefore, more pellets from males were collected within this time frame.

The sex bias in the processed samples could be reduced if the sex was known before the samples were processed and the pellets could be selected based on sex before analysis. In this thesis, the results from the sex analysis were ready after all the samples had been analysed. In future studies, a larger proportion of the pellets should be analysed and their sex should be identified.

5.4.5 Applicability of the method to other shag colonies

Assessing the applicability of this method to other shag colonies should be considered. It may ease pellet collection and analysis and give insight into the diet in other colonies and regions. Background levels of MPs and foraging activities will influence the shape, polymer and frequency of occurrence of the MPs found. Shags on Hornøya, a colony in Northern Norway feed predominately on sandeels, and here the topography with sand beds, unlike Sklinnas rocky bottoms, dictates and prey ability influences what they foraged on [27].

Sandeel otoliths are small, fragile and tricky to include in diet analysis as these are often underestimated [58]. Hence there might be differences in MPs between species as plastic ingestion may differ and be related to the specific diet of a species [6]. Regional differences in plastic concentration and foraging behaviour may alter the ingested MPs. Hence, differences between sites, diets of the shags and the consumed prey should be considered when applying and adapting the method.

5.5 Significance of findings

In this thesis, MPs were detected in the pellets from largest shag colony in Norway. The fact that MPs have been found is important, as it highlights that MPs are present in the environment on Sklinna and has been ingested by the shags. As [3] sought to contribute to a baseline for MPs in shag pellets. This thesis has the same ability, as a similar number of pellets were analysed and reporting the MPs characteristics. This thesis has also developed this further as it has reported MPs colour, LOD, included blanks, recovery studies and a suitable laboratory protocol for analysing the pellets in the lab.

The detected MPs in the pellets on Sklinna, indicate that the shags can also eliminate MPs. Even though, pellet analysis proved to be a suitable matrix for MPs and diet analysis, the levels of MPs that remain internally in the shags are not known.

Based on the previous studies reporting MPs in pellets from seabirds, the shags in this thesis had the 3rd highest levels, at 20%. The numbers of MPs was lower than detected at the Spanish colony, the Island of Ons [3].

Even though, there was no significant relationship between diet and MPs in this thesis, [3] found a significant relationship between fish consumed by the shags and MPs, related to the habitat of the fish. Plastic pollution and the threat it elicits towards seabirds is expected to increase [1]. The knowledge that MPs can be detected in pellets and that southern latitudes such as Spain contain MPs levels in shag pellets that are three times higher than Sklinna pushes for suitable and less inflicting, monitoring efforts.

As in [6], this thesis highlights the importance of developing protocols to monitor MPs in regurgitated pellets in live birds. Pellets could therefore serve as a suitable matrix for MPs analysis compared to other more invasive methods [6]. As pellets are already used in diet studies then monitoring of MPs could be coupled with this analysis.

6 Conclusion

The detected colour, shape and polymers of the MPs coincide with what has previously been detected along the Norwegian coast and ingested by fish. The three main consumed fish species by the shags were Saithe, Poor cod and Cod, as in previous studies on Sklinna. The main consumed fish was Saithe in age class 0. The only age class 2 Saithe consumed was in a pellet that did not contain MPs. Shags did not seem to selectively forage on one size of fish within the different age groups. As a result, there was no significance between fish age, species and MPs presence concerning size and number. This may be due to the large consumption of age 0 Saithe, the foraging mode of the shags or regional concentrations of MPs.

The shag population on Sklinna is the largest European shag colony and it is important to monitor them. Due to their foraging mode, shags are likely to be exposed to MPs via secondary ingestion from their prey. Pellet collection and analysis is a suitable method for future MPs and diet studies. This thesis conducted spiking studies calculating recoveries, often not included in existing MPs studies. Furthermore, including a defined cut-off and reporting MPs characteristics increases MPs understanding and comparability and is important to consider and include in future studies. Analysing otoliths using image processing was an efficient approach as the model could easily be expanded and applied in future studies. Adapting image analysis for analysing otoliths would be beneficial as it saves time and does not require expertise and knowledge. Future microplastic studies should push for protocol standardization and reporting details about MPs characteristics to allow comparisons between studies.

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Appendix

A Analysed samples from main study

Overview of analysed pellets from the main study. F1-5 indicates processing batch and R1-10 is pellet number processed. FB are field blanks and LB are lab blanks.

Sample	Lab ID	Collection Date (dd.mm.yyyy)	Pellet mass (g, ww)	Number of otoliths
Field blank	F3-FB	08.06.2023	-	-
Field blank	F1-FB-1	16.06.2023	-	-
Field blank	F5-FB	21.06.2023	-	-
Field blank	F2-FB	26.06.2023	-	-
Field blank	F1-FB-2	07.07.2023	-	-
Field blank	F4-FB	12.07.2023	-	-
Lab blank	F1-LB	LB	-	-
Lab blank	F2-LB	LB	-	-
Lab blank	F3-LB	LB	-	-
Lab blank	F4-LB	LB	-	-
Lab blank	F5-LB1	LB-1	-	-
Lab blank	F5-LB2	LB-2	-	-
Pellet	F2R1	08.06.2023	3.64	13
Pellet	F3R9	08.06.2023	3.76	27
Pellet	F1R1	08.06.2023	4.01	23
Pellet	F3R10	08.06.2023	5.62	13
Pellet	F4R2	08.06.2023	6.63	6
Pellet	F4R1	08.06.2023	7.58	24
Pellet	F2R2	11.06.2023	1.91	179
Pellet	F3R8	11.06.2023	2.75	15
Pellet	F1R2	11.06.2023	4.25	36
Pellet	F5R10	11.06.2023	4.32	188
Pellet	F5R7	11.06.2023	4.71	149
Pellet	F3R7	11.06.2023	5.47	26
Pellet	F1R3	16.06.2023	1.74	0
Pellet	F2R4	16.06.2023	3.75	38
Pellet	F5R9	16.06.2023	3.98	24
Pellet	F2R3	16.06.2023	4.02	29
Pellet	F4R3	16.06.2023	4.63	107
Pellet	F3R6	17.06.2023	2.45	31
Pellet	F1R4	21.06.2023	1.64	183
Pellet	F5R3	21.06.2023	2.1	7
Pellet	F4R5	21.06.2023	2.67	31
Pellet	F4R4	21.06.2023	2.7	160
Pellet	F2R5	21.06.2023	3.55	312
Pellet	F5R4	21.06.2023	5.47	231
Pellet	F2R6	26.06.2023	2.02	274
Pellet	F1R5	26.06.2023	2.05	NA
Pellet	F5R1	26.06.2023	2.25	203
Pellet	F5R2	26.06.2023	2.96	176
Pellet	F4R6	26.06.2023	3.05	124
Pellet	F4R7	26.06.2023	4.89	11
Pellet	F4R8	02.07.2023	1.35	255

Pellet	F1R6	02.07.2023	1.87	208
Pellet	F2R7	02.07.2023	2.64	34
Pellet	F5R6	02.07.2023	5.08	55
Pellet	F3R4	02.07.2023	5.11	22
Pellet	F3R5	02.07.2023	6.28	25
Pellet	F1R7	06.07.2023	2.49	108
Pellet	F2R9	06.07.2023	2.51	112
Pellet	F2R8	06.07.2023	3.35	97
Pellet	F1R8	07.07.2023	1.85	111
Pellet	F4R9	07.07.2023	3.76	107
Pellet	F3R3	07.07.2023	3.77	160
Pellet	F2R10	11.07.2023	3.01	23
Pellet	F3R2	11.07.2023	3.34	92
Pellet	F5R8	11.07.2023	3.34	82
Pellet	F4R10	11.07.2023	4.33	NA
Pellet	F3R1	11.07.2023	5.04	44
Pellet	F1R9	11.07.2023	6.03	66

B Quantification of diet items in pellets

Quantified diet and other items counted in the analysed pellets for the main study. A total of 66 otoliths were broken and not included in the main study. (-) indicates that otolith samples that should have been recounted as the number was not recorded.

				Diet Items					Other					
Collection Date (dd.mm.yyyy)	Lab ID	Pellet mass (ww, g)	Number of otoliths	Fish lens	Jaws	Vertebrae	Crustacean	Squid beaks	Shells	Stones	Nematodes	Mites	Feathers	Plants
08.06.2023	F2R1	3.64	13											
08.06.2023	F1R1	4.01	23					4						
08.06.2023	F3R9	3.76	27			122								6
08.06.2023	F3R10	5.62	13	7		3	23		2					
08.06.2023	F4R1	7.58	24	10						3				
08.06.2023	F4R2	6.63	6	40					2		4			
11.06.2023	F1R2	4.25	36								3			12
11.06.2023	F2R2	1.91	179	4										6
11.06.2023	F3R7	5.47	26	7								32		
11.06.2023	F3R8	2.75	15	4		32								2
11.06.2023	F5R7	4.71	149	51	6		1				1	2		3
11.06.2023	F5R10	4.32	188	5			1				3			
16.06.2023	F1R3	1.74	0									16		
16.06.2023	F2R3	4.02	29	13	9							10		
16.06.2023	F2R4	3.75	38	19										3
16.06.2023	F4R3	4.63	107	14							3			
16.06.2023	F5R9	3.98	24	5							1		2	2
17.06.2023	F3R6	2.45	31	4	9				1					
21.06.2023	F1R4	1.64	183											
21.06.2023	F2R5	3.55	312	3	8									
21.06.2023	F4R4	2.70	160	2		14					9			
21.06.2023	F4R5	2.67	31											
21.06.2023	F5R3	2.10	7									2		
21.06.2023	F5R4	5.47	231	5				1						
26.06.2023	F1R5	2.05	-			12								
26.06.2023	F2R6	2.02	274											2
26/06/2023	F4R6	3.05	-	7							3			
26/06/2023	F4R7	4.89	11	20							22			
26/06/2023	F5R1	2.25	203	2	10				3			11	1	11

26/06/2023	F5R2	2.96	176	2							3			2
02/07/2023	F1R6	1.87	208		5									
02/07/2023	F2R7	2.64	34	5	7								2	
02/07/2023	F3R4	5.11	22	23								290		
02/07/2023	F3R5	6.28	25	10		71							14	1
02/07/2023	F4R8	1.35	255	6						3	1		2	34
02/07/2023	F5R6	5.08	55	3										
06/07/2023	F1R7	2.49	108											
06/07/2023	F2R8	3.35	97	2										27
06/07/2023	F2R9	2.51	112											
07/07/2023	F1R8	1.85	111			1		1				116		
07/07/2023	F3R3	3.77	160	2							42	20	1	
07/07/2023	F4R9	3.76	124	6		27					3	111		
11/07/2023	F1R9	6.03	66					1			2			1
11/07/2023	F2R10	3.01	23											
11/07/2023	F3R1	5.04	44	26					1				1	
11/07/2023	F3R2	3.34	92	2		30								
11/07/2023	F4R10	4.33	-	24							5			
11/07/2023	F5R8	3.34	82	3	12				2		3			4
	Average mass (g, ww)		**Total number of items**											
		3.66	4134	336	66	312	25	7	11	6	108	610	23	115

C Otolith equations for calculating fork length

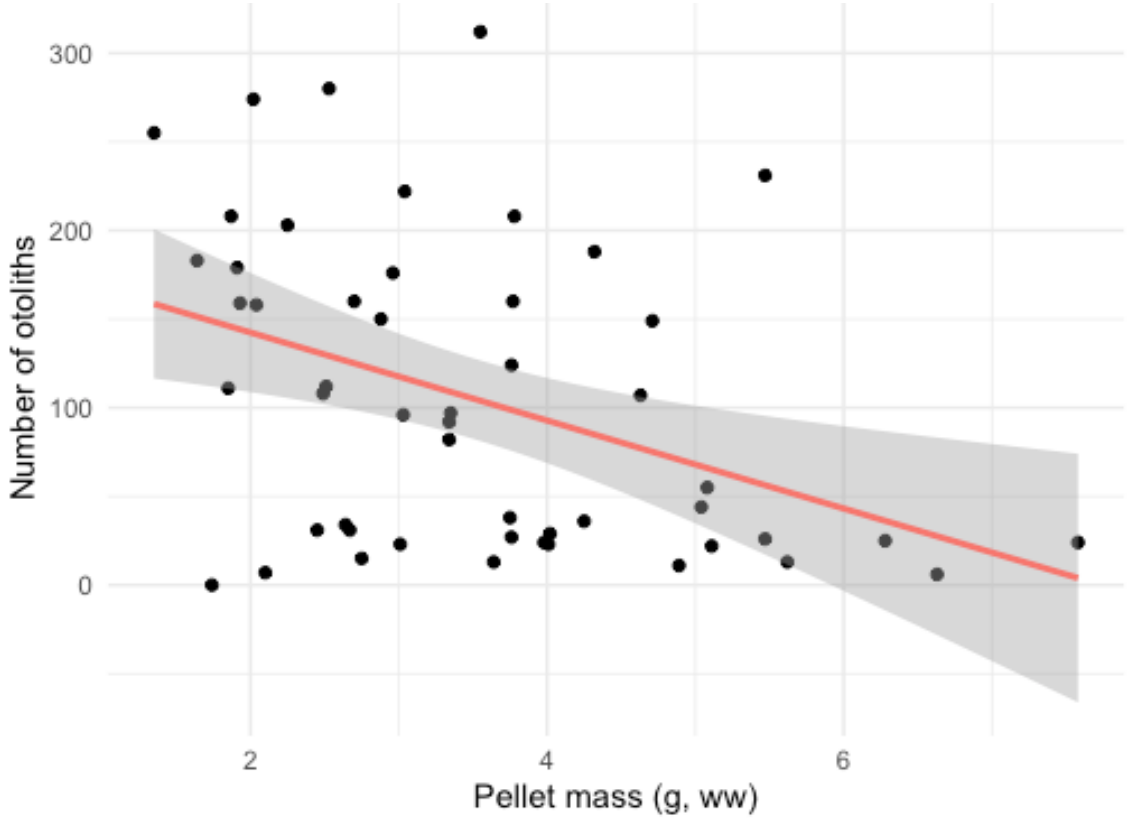
Equations applied to calculate fork length (mm) of fish from otoliths.

Species	Equation of fork length (mm)
Cod	-202.13 + 48.37*OL
Poor cod; OL> 0.5	-49.9 + OL*28.091
Haddock	$8.785*OL^{1.38}$
Norway pout	-42.6 +OL*29.522
Saithe; OL < 5.5	-4.24 + 23.5*OL
Saithe; OL ≥ 5.5	$8.97297*OL^{1.53}$
Vahl 's eelpout	21.19 + 37.74*OL
Capelin	14.83 + 45.58*OL

OL= length of the otolith, species gives species name
Sources for formulas are mentioned in Dehnhard et al. 2022

D Linear regression between pellet mass and number of otoliths

Scatter plot for number of otoliths and pellet mass (g, ww) with linear regression line for number of otoliths and pellet mass. y = -24.262x + 190.54, R^2=0.16



E Overview of potential particles

Measured potential particles from main study, with reported MPs characteristics. Particles were measured with a scaled ocular (1 cm = 100 ticks). Images of the polymers refers to results 4.3.1.2

Lab ID	Metal or Paper Filter	Shape	Colour	Length (µm)	Width (µm)	Polymer	Image of MP
F5R9	Metal	Film	Transparent	2500	100	LDPE	(Figure 10, C)
F4R8	Metal	Fiber	Black	2900	200	LDPE	(Figure 10, B)
F5R6	Metal	Fiber	Black	9200	100	Nylon	(Figure 10, A)
F3R8	Metal	Fiber	Black	1700	10	PET	(Figure 10, D)
F5R8	Paper	Fiber	Black	2400	100	PET	(Figure 10, F)
F1R5	Metal	Fragment	Transparent	500	300	PA	(Figure 10, D)
F2R1	Paper	Fiber	Blue	1500	300	PP	(Figure 10, E)
F4R3	Paper	Fiber	Blue	1400	100	PP	(Figure 11, C)
F3R6	Metal	Fiber	White	1000	100	PP	(Figure 11, B)
F4R10	Metal	Fiber	White	2300	100	PP	(Figure 11, A)
F5(LB1)	Paper	Fiber	White	1000	10		
F5(LB1)	Paper	Fiber	White	500	10		
F2(LB)	Paper	Fiber	Black	1500	10		
F5R3	Paper	Fiber	Black	4000	5		
F5R3	Paper	Fiber	Black	500	100		
F3R6	Paper	Fiber	White	5000	10		
F3R2	Metal	Fiber	White	1700	10		
F3R4	Metal	Fragment	Transparent	600	200		
F3R4	Metal	Fiber	Blue	4000	100		
F3R4	Metal	Fiber	White	100	100		
F3R4	Metal	Fiber	Pink	1600	10		
F3R5	Paper	Fiber	Black	5000	100		
F5R9	Paper	Fiber	Black	1100	100		
F5R9	Paper	Fiber	Green	2500	100		
F5R1	Paper	Fiber	White	3000	100		
F5R1	Paper	Fiber	White	6000	100		
F5R1	Paper	Fiber	White	2400	700		
F5R1	Paper	Fiber	Black	2000	100		
F5R1	Paper	Fiber	Transparent	2500	100		
F5R1	Paper	Fragment	Yellow	600	100		
F5R1	Paper	Fiber	Blue	1500	100		
F5R1	Paper	Fiber	Black	1300	100		
F5R6	Paper	Fiber	Black	1000	100		
F5R8	Paper	Fiber	Blue	2000	200		
F5R8	Paper	Fiber	Blue	1000	100		
F5R8	Paper	Fiber	Black	2500	100		
F4R4	Metal	Fiber	Blue	4000	100		
F4R4	Paper	Fiber	Red	1000	100		
F4R4	Paper	Fiber	White	1700	700		
F4R4	Paper	Fiber	Black	1700	100		
F4R3	Paper	Film	Transparent	700	500		
F4R3	Paper	Film	Transparent	1400	100		
F4R3	Paper	Fragment	Transparent	1000	1500		
F4R3	Paper	Fiber	White	3300	2300		
F4R2	Paper	Fiber	Black	2000	100		
F4R10	Paper	Fiber	White	2300	2000		
F4R10	Paper	Film	Transparent	1000	500		

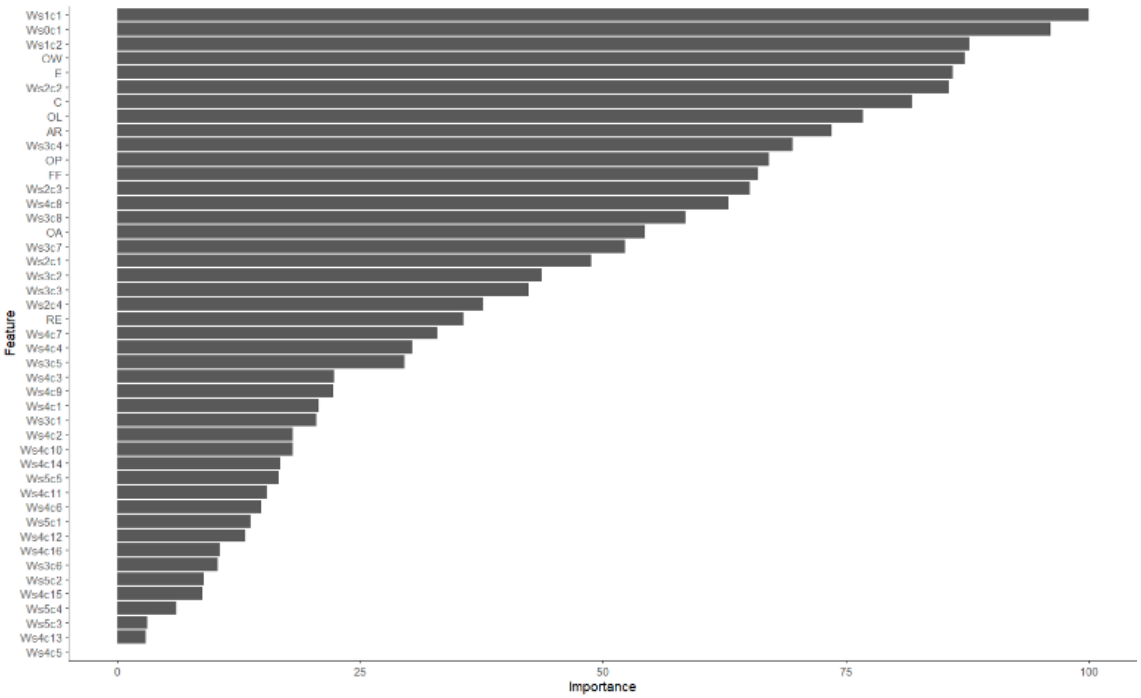
F4R10	Paper	Fiber	Black	2000	100
F4R10	Paper	Fiber	White	8500	100
F4R10	Paper	Fiber	White	2400	100
F4R10	Paper	Fiber	Black	2000	100
F4R1	Paper	Fiber	Black	6800	100
F4R8	Metal	Fiber	Black	2900	200
F1R2-1	Paper	Fragment	Orange	500	100
F1R2-1	Paper	Fragment	Blue	300	400
F1R2-1	Paper	Fiber	Blue	2200	100
F1R2-1	Paper	Fiber	Transparent	3400	100
F1R2-1	Paper	Fiber	Black	1000	100
F1R2-1	Paper	Fiber	Black	700	100
F1R3-2	Paper	Fiber	Green	500	100
F1R2-5	Paper	Fiber	Black	7000	100
F1R2-5	Paper	Film	Transparent	900	1400
F1R2-5	Paper	Fragment	Green	900	200
F1R2-5	Paper	Fragment	Pink	900	500
F1R2-4	Paper	Fiber	Blue	1000	100
F1R2-4	Paper	Fiber	Black	1000	100
F1R2-3	Paper	Fiber	Black	3000	100
F1R2-3	Paper	Fragment	Yellow	1700	1300
F1R8	Paper	Fiber	Black	1400	100
F1R8	Paper	Fiber	Blue	900	100
F1R8	Paper	Fragment	Blue	100	100
F1R8	Metal	Fiber	Blue	900	100
F1R6	Paper	Fiber	Black	1200	100
F1R6	Paper	Fiber	Black	2200	100
F1R6	Paper	Fiber	Red	900	100
F1R6	Paper	Fiber	Black	500	100
F1R4	Paper	Fiber	Blue	1000	100
F1R4	Paper	Fiber	Black	200	100
F1R9	Paper	Fiber	Black	2300	100
F1R9	Paper	Fiber	Black	5300	100
F1R5	Paper	Fiber	Black	700	100
F1R5	Paper	Fiber	Black	400	100
F1R7-1	Paper	Fiber	Black	400	100
F1R7-1	Paper	Fiber	Black	500	100
F1R7-1	Paper	Fiber	Pink	700	100
F1R5	Paper	Fiber	Black	400	100
F1R5	Paper	Fiber	Blue	1400	100
F1R5	Paper	Fiber	Blue	3700	100
R1F5	Metal	Fiber	Black	2100	100
R3F5	Metal	Fiber	Red	1300	100
R3F3	Metal	Fragment	Red	200	100
R3F3	Metal	Fiber	Transparent	1500	100
R3F2	Metal	Fiber	Black	1400	100
R10F5	Metal	Fiber	Transparent	1400	100
R10F5	Metal	Fragment	Red	600	800
R8F4	Metal	Fiber	White	2600	100
F1R8	Paper	Fiber	Blue	1000	100
F1R8	Paper	Fiber	Black	1500	100
R4F4	Metal	Fiber	Blue	2300	100
R5F4	Metal	Fragment	Black	400	100
R1F2	Metal	Fiber	Transparent	1000	100

R5F2	Metal	Fiber	Black	1200	100
R5F2	Metal	Fiber	Blue	1200	100
R5F2	Metal	Fiber	Blue	600	100
R5F2	Metal	Fragment	White	2500	2000
R5F2	Metal	Fiber	Orange	2000	100
R5F2	Metal	Fiber	White	2200	100
F2R7	Metal	Fiber	Red	1500	100
F2R7	Metal	Fiber	White	3000	600
R8F3	Metal	Fiber	White	2200	500
R8F3	Metal	Fiber	Purple	2500	100
F1R4-4	Paper	Fiber	Blue	500	100
R9F4	Paper	Fiber	Red	3000	100
R2F3	Metal	Fiber	Transparent	4500	1200
R1F5	Metal	Fiber	Black	2500	100
R1F5	Metal	Fiber	Blue	1200	100
R1F5	Metal	Fiber	Black	2000	100
R6F2	Metal	Fiber	Black	2500	100
R6F2	Metal	Fiber	White	4900	100
R6F2	Metal	Fiber	White	1500	100
R4F2	Metal	Fiber	Black	700	100
R4F2	Metal	Fiber	Blue	1500	100
R6F4	Metal	Fiber	Black	1600	100
R1F1	Metal	Fiber	Black	5500	100
F1R4	Paper	Fiber	Black	1600	100
F4R3	Metal	Fragment	Orange	1000	1000
F4R3	Metal	Fiber	Black	2500	100
R7F1	Metal	Fiber	Black	1000	100
R7F1	Metal	Fiber	Black	1000	100
R7F1	Metal	Fiber	Red	5000	100
R2F1	Metal	Fiber	Black	1800	100
R2F1	Metal	Fragment	Brown	400	200
F1R7-1	Paper	Fiber	Black	500	100
R4F1	Metal	Fiber	Black	1000	100
R4F1	Metal	Fiber	Black	2000	100
R6F1	Metal	Fiber	White	2700	100
R6F1	Metal	Fiber	Transparent	6000	100
R6F1	Metal	Fiber	Transparent	2200	100
F1R2-5	Paper	Fiber	Black	3500	100
R6F3	Metal	Fiber	Transparent	2500	100
R6F3	Metal	Fragment	Orange	500	300
R8F5	Metal	Fiber	Black	1000	100
R8F2	Metal	Fiber	Black	1500	100
F1R9	Metal	Fiber	Transparent	4700	100
F1R9	Metal	Fiber	Black	10500	100
F1R9	Metal	Fiber	Blue	2000	100
R6F5	Metal	Fiber	Black	1200	100
F3R9	Metal	Fiber	Black	4500	100
R7F4	Metal	Film	Transparent	2500	1000
R8F1	Metal	Fiber	Blue	1100	100
R8F1	Metal	Fiber	Black	3100	100
R8F1	Metal	Fiber	White	5100	100
R5F1	Metal	Fiber	Black	3200	100
R6F1	Paper	Fiber	Black	2000	100
R6F1	Paper	Fiber	Black	1200	100

F1R2-2	Paper	Fiber	Black	1200	100
F1R2-2	Paper	Film	Red	500	200
R9F1	Paper	Fiber	Black	2000	100
R9F1	Paper	Fiber	Black	1000	100

F Random forest model variables for prediction

Variables of importance for predicting reference otoliths in the random forest model based on Wavelet descriptors, otolith size parameters and shape indices.



G Number and species of otoliths per pellet for pellets with and without microplastics

Number of otoliths and predicted fish species per pellet in the otolith analysis, with the sub-sample of 20 pellets from main study.

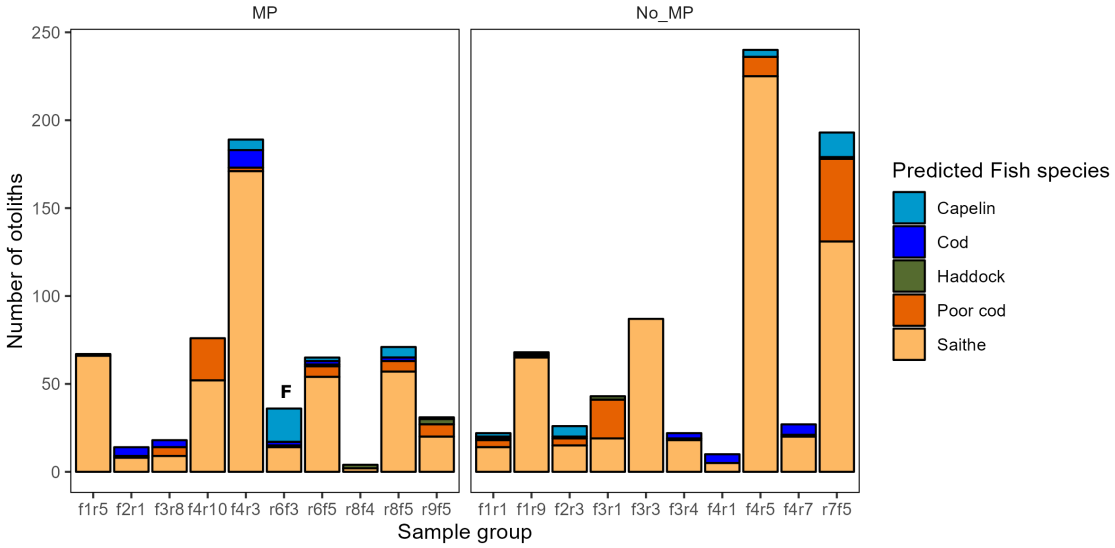
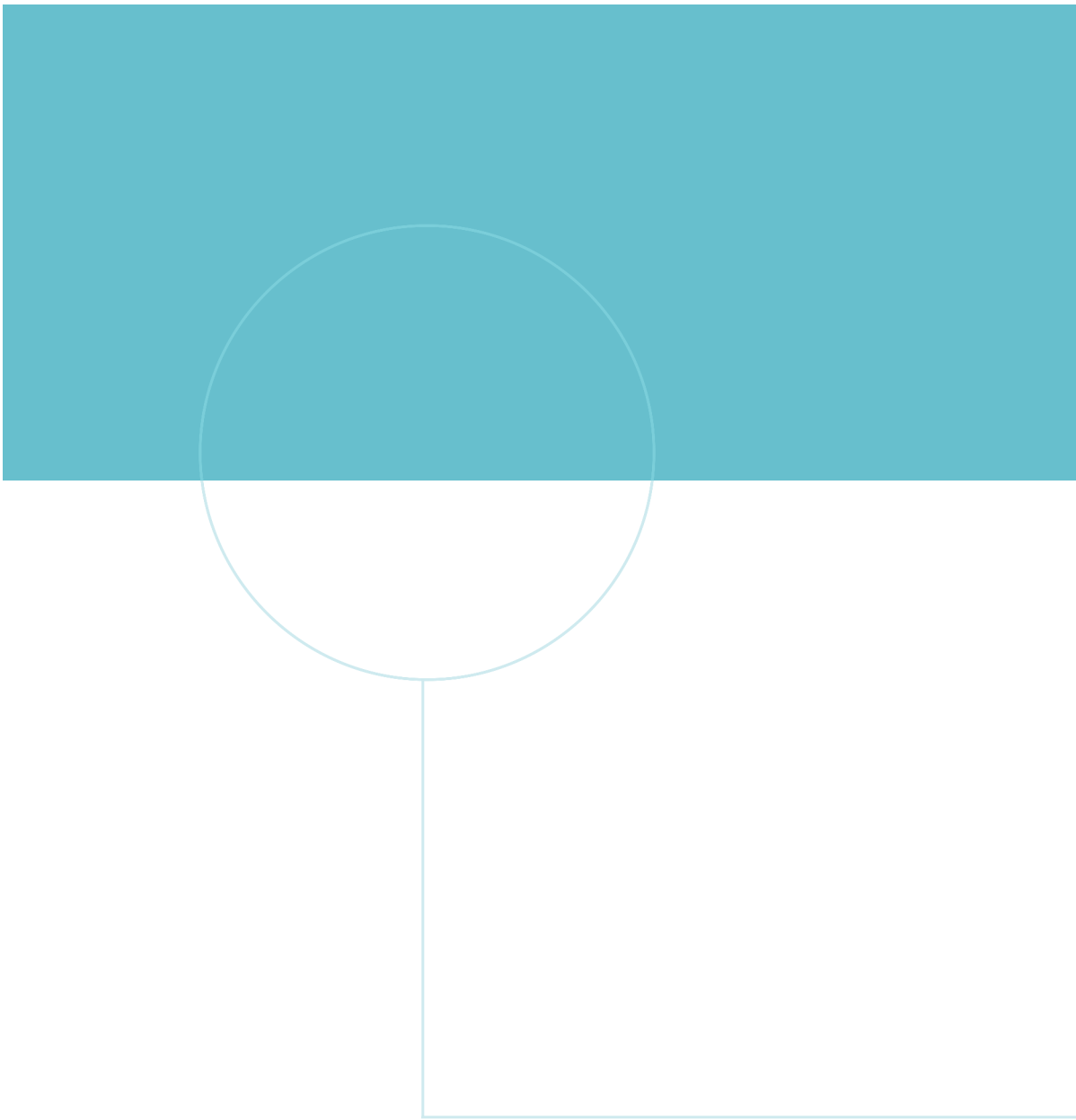


Figure 23: Number of otoliths (n=1309) with predicted fish species per pellet in MP (n=10 pellets) or no MP samples (n=10 pellets). The only pellet from a female is marked with an "F".



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