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This research was subsidised by the Dutch Ministry of Agriculture, Nature and Food Quality (project number KB-36-005-003).

Wageningen Environmental Research
Wageningen, April 2023

Reviewed by:
Wouter Jan Strietman, Researcher North Sea and Arctic, Wageningen Economic Research

Approved for publication:
Marion Kluivers-Poodt, team leader of Animal Ecology

Report 3253
ISSN 1566-7197

WAGENINGEN
UNIVERSITY & RESEARCH

Polling, M., G.A. de Groot, I. Laros, M.J. van den Heuvel-Greve, 2023. *Identification of non-indigenous and other fouling species on marine litter using DNA metabarcoding; A comparison of marine taxa found on plastic litter and settlement plates collected from beaches and a harbour in the Netherlands, and a comparison to taxa found on marine litter in Iceland.* Wageningen, Wageningen Environmental Research, Report 3253. 32 pp.; 9 fig.; 4 tab.; 27 ref.

Plastic afval afkomstig van economische activiteiten en consumenten belandt in toenemende hoeveelheden in zee. Niet alleen kan dit drijvende afval sterfte opleveren onder zeevogels en zeezoogdieren, het kan ook dienen als een transportmiddel voor planten en dieren die ze onder invloed van wind en zeestromingen meevoeren naar plekken waar ze van nature niet thuishoren. Dit rapport beschrijft een onderzoek naar de samenstelling van dieren en algen op plastic afval gevonden langs de Nederlandse kust tussen 2019 en 2021. Daarnaast werden 'settlement plates' gebruikt, waardoor een onderscheid kon worden gemaakt tussen al aanwezig zijnde uitheemse soorten in Nederland en nieuw gearriveerde exoten die met plastic afval meekomen. Daarbij werd gebruik gemaakt van DNA metabarcoding voor de identificatie. Het combineren van twee markers (18SV4 en CO1) maakte het mogelijk om organismen veelal tot op soortniveau te identificeren. Op de totale set monsters werden 46 uitheemse algen en dieren aangetroffen. Hieronder zaten voornamelijk exoten waarvan de aanwezigheid in Nederland al langer bekend was, maar ook twaalf mogelijke nieuwkomers voor Nederland en 6 soorten die mogelijk niet eerder in de Noordoost Atlantische regio gevonden zijn. Identificatie van dieren en algen/wieren op plastic afval is van belang voor 1) het bepalen van eventuele risico's van drijvend plastic als vector van de introductie van nieuwe soorten in ecosystemen, en 2) om de minimale leeftijd en hiermee de mogelijke geografische herkomst van plastic te kunnen herleiden. Dit levert informatie voor het onderzoek naar bronnen en potentiële risico's van drijvend plastic afval in zee.

Plastics from consumers and economic activities enter the oceans in increasing numbers. The resulting debris can not only cause mortality for seabirds and marine mammals, but by means of winds and currents can also function as a floating vessel for algae and animals that transport these species to places where they do not naturally occur. Here, we studied fouling animals and algae on marine litter found along the Dutch coast (2019-2021). In addition, 'settlement plates' were used to be able to make a distinction between exotic species already present in the Netherlands and newly arrived exotics that come along with the plastic litter. To identify the animal and algal species, DNA metabarcoding was used. Combined application of two barcoding markers (18SV4 and COI) showed the potential to identify fouling in many cases to species level. In total, 46 non-indigenous algae and animals were detected, including mostly taxa that have already been recorded in the Netherlands before, but also twelve potential newcomers for the Netherlands and 6 not recorded before in the NE Atlantic region. Identification of fouling species can therefore help to 1) assess potential risks of floating marine litter as a vector for the introduction of non-indigenous species, and 2) to establish a minimum age and origin of the plastic that can help in determining and/or modelling where the plastic may have come from. Geographical origin assessment can be used to identify the sources and potential risks to the ecosystem of floating marine litter.

Keywords: marine litter, fouling, DNA metabarcoding, geographical origin, non-indigenous species

Wageningen Environmental Research Report 3253 | ISSN 1566-7197

Photo cover: Martine van den Heuvel-Greve

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Verification

Report: 3253
Project number: 5200047524

Wageningen Environmental Research (WENR) values the quality of our end products greatly. A review of the reports on scientific quality by a reviewer is a standard part of our quality policy.

Approved reviewer who stated the appraisal,

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date: 06/02/2023

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date: 20/04/2023

Summary

Under the influence of wind and currents, marine litter is known to travel long distances. This litter is also known to act as vectors for fouling species, potentially allowing these species to travel from one region to another and becoming invasive at the location of arrival. In this report, fouling species on marine litter found along the Dutch coast (2019-2021) were studied, using DNA metabarcoding for species identification. Additionally, fouling was investigated on settlement plates to identify non-indigenous species already present in the Netherlands. Results from Dutch beaches were further compared with fouling on marine litter from a beach in Iceland (2019) to investigate whether these contain similar non-indigenous species communities.

The use of two barcoding markers in this study (18SV4 and COI) enabled the identification of many of the animals and (macro-)algae growing on marine litter and settlement plates to species level. Different communities of animals and algae were found on the floating litter from Dutch beaches versus the settlement plates and BESE elements. These results therefore further underline that floating marine litter forms a substrate for the transport of both native and non-indigenous species, and can facilitate the introduction and range extension of non-indigenous species.

It was possible to detect a total of 46 non-indigenous species on the beached marine litter. Many observed non-indigenous species were already recorded before in the Netherlands, but twelve potential newcomers for the Netherlands were also observed of which six have, for as far as we could find, not even been reported before in the North East Atlantic region: the annelid worm species *Myrianida convoluta,* two bryozoan species *Conopeum tenuissimum* and *Alcyonidium verrilli,* the cnidarian *Haliclystus inabai,* one species of red algae *Ceramium gardneri,* as well as one species of yellow-green algae *Scytosiphon* (= *Hapterophycus*) *canaliculatus*. We also found that it remains highly important to manually double-check species identifications generated by automated DNA annotation scripts, because of the high number of erroneous records in NCBI and/or BOLD, as well as taking into account potentially missing sequences of closely related species.

A comparison between fouling species on hard and soft marine litter from Dutch beaches showed that different substrate types attract a different community of animals growing on them: while hard plastics were dominated by barnacles (Sessilia) and red algae (Rhodophyta), the soft plastics were dominated by moss animals (Bryozoa) and polyps (Hydrozoa).

While some non-indigenous species identified on settlement plates and BESE elements from the Netherlands were also found on the floating marine litter on Dutch beaches, many were unique to the marine litter. This indicates that marine litter can act as an important vector for non-indigenous species and shows that the analysis of fouling on floating marine litter can form an important early indicator for the risk of introduction of non-indigenous species into the local ecosystem. Along with this, fouling identification can also be used to establish a minimum age of the collected floating marine litter. Datasets need to be constructed for this to enable such estimates of minimum age that a piece of litter has been floating in the sea. Models can consequently use the parameter of ‘age’ to backtrack what the potential geographical origin of the piece of marine litter is. Geographical origin assessment can be used to identify the sources of marine litter. This project is therefore directly connected to the Litter-ID research work carried out by Wageningen University & Research that focuses on identifying the sources, origin and causes of marine litter and assesses the interaction of marine litter with the environment.

1 Introduction

1.1 Background

Plastics from consumers and economic activities enter the oceans in increasing numbers, either directly at the beach or via our rivers. This is an undesirable situation that is receiving more and more attention due to the increasing demand from society for more sustainable food production and circularity. Not only do these plastics cause mortality in seabirds and marine mammals due to entanglement or ingestion, they can also function as a floating vessel for plants and animals that transports them to places where they do not naturally occur. In other words, non-indigenous species can hitch a ride on marine litter and influence biodiversity in other places (Miralles et al., 2018). It has already been shown that species may disperse long distances away from their native ranges. Studies reported that marine litter may have roughly tripled the chances for marine biota to spread at high latitudes (>50°; Barnes, 2002). The introduction of non-indigenous species is seen as the second most important threat to biodiversity next to habitat loss, and can have major consequences for local ecosystems, both in terms of ecology and economy.

Besides potential environmental impact of fouling species that are present on marine litter, fouling can offer information on the minimum amount of time that the litter has been floating in the water, and hereby provide information that can be used to model the potential geographical origin of the plastic.

1.2 Goal

The goal of this report is to provide answers to the following research questions:

1. What fouling species can be found on beached plastics in the Netherlands?
    a. Can different species be found at different locations in the Netherlands?
    b. Do items made of soft plastic contain a different species composition than hard plastics?
    c. Which non-indigenous species can be found on beached plastic items?
2. How does the fouling on beached plastics in the Netherlands compare with:
    a. Settlement plates from the Netherlands?
    b. Fouling on marine litter from a beach from a remote location (Iceland)?
3. Can identification of fouling species help in:
    a. Assessing the potential risk that floating marine litter poses to ecosystems where such litter is transported to?
    b. Providing a minimum amount of time (i.e., age) that the litter has been floating at sea?
    c. Further assessing the geographical origin of the marine litter (where does it come from)?

2 Material and methods

2.1 Sample collection

Thirty-seven plastic samples were collected between 2019 and 2021 (Table 1). They consist of various types of plastic litter coming from consumers (e.g., bags, bottles), as well as fisheries or aquaculture (rope, BESE elements, see below). A distinction was made between pieces of soft and hard plastics as these may attract different communities of organisms (Table 1).

Beached plastic items were collected from several locations between 2019 and 2021:

1. Beaches around the island of Griend, Wadden Sea, the Netherlands: 2019 and 2020. This also included four BESE elements (Biodegradable Ecosystem Engineering Elements) that are used for ecosystem restoration. These were found beached along with other marine litter during a beach clean-up and originated from an ecosystem restoration project close to the island of Griend. The pieces collected had originally been part of artificial reef structures made with BESE elements and got separated before they were transported to the beach under the influence of wind and currents.
2. Kwade Hoek/Ouddorp beach, southwest the Netherlands: 2020 and 2021 (see Figure 1A).
3. Oostkapelle beach, southwest the Netherlands: 2021.
4. Vlissingen harbour, Westerschelde, the Netherlands: 2019 (fouling on floating litter as well as on settlement plates placed there in the water near the emergency jetty of Vlissingen harbour, within an area with known presence of non-indigenous species as positive control; see Figure 1B).
5. Húsavík beach, Westfjords, Iceland: 2019 (remote reference location).

Figure 1A *Left: Marine litter on the beach of Kwade Hoek, the Netherlands (2021). Middle: Fouling of barnacles on a plastic cup, Kwade Hoek, the Netherlands (2021). Right: Collected litter from Kwade Hoek, the Netherlands (2021).*

Figure 1B *Left: Set-up of settlement cubes placed in Westerschelde Harbour (Vlissingen) showing extensive algal growth. Middle: Close-up of settlement cube after collection and right: the skeleton shrimp Caprella mutica (non-indigenous species) was commonly encountered on the settlement cubes in Westerschelde Harbour (all images from MvdG-H).*

During beach clean-up activities, plastic items with fouling were separately stored. Either directly on site or back in the laboratory, the fouling species were scraped off the plastic into plastic tubes. For each piece of litter, a new pair of gloves and new stainless steel knife were used to prevent (cross) contamination. The tubes were filled with >97% ethanol to preserve the DNA of the fouling species. As DNA can break down rapidly, only samples were collected of litter items that could be processed directly after collection.

This project is directly connected to the work carried out by Wageningen University & Research using the Litter-ID methodology (Strietman et al., 2020; Strietman et al., 2021). By applying the Litter-ID methodology, local stakeholders and marine litter experts are engaged in identifying the sources, origin, causes, as well as the interaction of beach litter with the local environment (fouling, ingestion, entanglement). The current project provides information on 1) potential risks of floating marine litter as vector for the introduction of non-indigenous species, and 2) establishing a minimum age of the plastic that can help in determining and/or modelling where the plastic may have come from. Both can provide (and have provided) input in the research work carried out using the Litter-ID methodology.

2.2 DNA Extraction and metabarcoding protocol

From each collected piece of marine litter, the attached fouling was removed by scraping it into a clean plastic 50 ml tube containing >97% ethanol using a clean scalpel and pair of tweezers. At the laboratory of Wageningen Environmental Research in Wageningen, the samples were mechanically homogenised (Ultra Turrax T25), and DNA was extracted using the commercially available Powermax Soil Extraction kit (Qiagen) which is designed to deal with complex environmental samples that may include soil. A metabarcoding approach was used to identify the fouling species: this technique allows the large-scale taxonomic identification of complex environmental samples via analysis of DNA sequences for short regions of one or a few genes (called DNA markers). Since many different organisms from various taxonomic groups including both animals and (macro-)algae were known to be present as fouling, two different DNA markers were used. Part of the COI region was amplified with adapted Leray primers from van den Heuvel-Greve et al. (2021) and the 18SV4 region was amplified using primers adapted from Stoeck et al. (2010). Because DNA is present within organisms in concentrations too low to detect directly, PCR reactions were used to multiply the DNA[1]. To generate the DNA data (reads), metabarcoding relies on high-throughput DNA sequencing (HTS) technologies, which yield millions of DNA sequences in parallel and allow large-scale analysis of environmental samples. The samples were therefore sent to Genome Quebec, Canada where all samples were cleaned, received a unique tag and were subsequently sequenced on an Illumina MiSeq PE250bp (base-pair) setup.

The following section describes the technical details of the bioinformatics performed to get from the raw sequencing data (fragments of DNA) to the species assignment. In short, this includes quality check of raw reads, removal of adapter sequences, demultiplexing (assigning reads to particular samples), filtering of erroneous sequences, sequence dereplication, removal of singletons and PCR/sequencing errors, clustering/denoising identical sequences into unique DNA sequences (so-called ASVs: amplicon sequence variants), and taxonomic annotations using reference databases. To those interested in the details of the bioinformatic analysis: raw fastq files were demultiplexed by Genome Quebec, who provided the forward and reverse files for each sample. The bioinformatics for all samples was performed within the QIIME2 platform version 2021.11 (Bolyen et al., 2019). The cutadapt plugin was used to delete forward and reverse primers from both the forward and the reverse sequences, using a minimum sequence length of 200 bp and discarding any reads that were untrimmed, allowing an error-rate of 0.25 (5bp) (Martin, 2011). The sequences were subsequently merged and denoised into ASVs, and chimeras were removed using the DADA2 plugin (Callahan et al., 2016). Forward and reverse reads were truncated at 220bp to get rid of low-quality reads while keeping sufficient room for expected amplicon lengths (313bp for COI and 200-400bp for 18SV4). The 18SV4 ASV's were not clustered into OTUs to retain as much variability as possible, VSEARCH plugin was used to cluster the COI ASVs into 97% de novo OTUs (Rognes et al., 2016).

To assign the ASVs to species, they are compared to reference databases: these contain DNA sequences with known species assignment. This process was performed via the program *blast* for 18SV4, using the public DNA reference database of NCBI (called the nt database), and via the curated COI database BOLD using the program BOLDigger (Buchner and Leese, 2020) for COI. The resulting list of taxa was screened for [1]potentially non-indigenous species, using the following websites and literature to assess the status of the species (non-indigenous or native):

- https://www.nederlandsesoorten.nl
- https://www.gbif.org
- https://www.sealifebase.se/
- Zenetos et al. (2022).

For all ASV's identified as presumed non-indigenous species for which the identification was uncertain using the software, a further manual verification was performed. Per ASV, the top 10 results from the reference database were evaluated to check for ambiguity in species assignment among them. Online DNA reference databases are known to host reference barcodes uploaded under an erroneous name, caused either by misidentification of the specimen or by cross-contamination of the DNA template with DNA from another specimen (e.g., Steinegger and Salzberg, 2020). Therefore, we also checked the genetic distance tree as presented in the program BLAST based on the pairwise alignments of the top hits. This is especially relevant in cases where the top hits showed an incomplete overlap with the query sequence or when along with the top hit very different taxonomic groups were seen. Any potential non-indigenous species for which the assignment was considered uncertain were either collapsed to a higher taxonomic level (e.g., genus or family) or removed from the list of non-indigenous species completely (e.g., in cases where the genus contains both native and non-native taxa). A final source of uncertainty is the incompleteness of the reference databases as well as the marker not being able to distinguish between different species. Fouling on two collected BESE elements (Griend-1 and -2) that were found on the beach of Griend in 2019 have been analysed before, and results were described within the report "*Resultaten bronanalyse zwerfafval Griend*" (ISBN 978-94-6395-492-1). However, since the bioinformatics were performed a few years ago and the methods have been further developed, the bioinformatics for these samples have been redone following the protocol as described above.

To assess to what extent the observed taxonomic compositions observed on plastic items differed among sampling locations, NMDS plots were constructed using *vegan* in R (Jari Oksanen et al., 2018). Goal of an NMDS (Non-Metric Multidimensional Scaling) is to represent the position of data in multidimensional space as accurately as possible using a reduced number of dimensions that can be easily plotted and visualized. In this way, samples that are more similar will cluster closely together while samples with very different species

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[1] PCRs were performed in 25µl, existing of 1U Platinum Taq (Fisher Scientific), 1x PCR buffer, 2.5 mM MgCl2, 5%(m/m) Trehalose, 200ng/µl BSA, 200µM dNTP and 250µM water. The program consisted of 2 min at 94°C, followed by 15 cycli of 30 s at 94°C, 3 min annealing using a touchdown program starting at 56°C and decreasing by 1°C each cycle, 1 min at 72°C, 20 cycli of 30 s at 94°C, 3 min at 42°C and 10 min at 72°C.

composition will plot further away from each other. For the NMDS only samples with at least 500 reads were used.

For this report the following animal/plant groups (phyla) were considered to be of main relevance for this study as they are known to have potential to settle and grow on plastic substrates:

- Segmented worms (Annelida)
- Copepods, barnacles, crabs, lobsters, shrimps and isopods (all Arthropoda)
- Moss animals (Bryozoa)
- Hydra, jellyfish, sea fans etc. (Cnidaria)
- Molluscs (Mollusca)
- Flatworms (Platyhelminthes)
- Sponges (Porifera)
- Green algae (Chlorophyta)
- Yellow-green algae (Ochrophyta)
- Red algae (Rhodophyta)
- Sea stars and the like (Echinodermata)
- Tunicates (Chordata; Tunicata).

Many other phyla were found, especially using the 18SV4 marker, including e.g. Ciliophora, Bacillariophyta (diatoms), fungi and protozoa. These all represent microscopic organisms that are invisible to the naked eye, and for that reason they were excluded in this report.

Table 1 *Overview of plastic samples and settlement plates/cubes collected in the Netherlands and Iceland in 2019, 2020 and 2021.*

Year	Location	label	Exact location	Collection date	Extract code	Type of material	Substrate	Identified organisms
2019	Húsavík, Iceland	Iceland-1	65.643N, 21.637W	06-09-19	E190672, E190692	Underside white container	Hard	
	Húsavík, Iceland	Iceland-2	65.643N, 21.637W	06-09-19	E190673, E190693	Grey large tube	Hard	
	Húsavík, Iceland	Iceland-3	65.643N, 21.637W	06-09-19	E190674, E190694	Cream coloured butter package	Hard	
	Húsavík, Iceland	Iceland-4	65.643N, 21.637W	06-09-19	E190675, E190695	White side piece of a bucket	Hard	
	Húsavík, Iceland	Iceland-5	65.643N, 21.637W	06-09-19	E190676, E190696	Black soft piece of a flap (door)	Insulation	
	Húsavík, Iceland	Iceland-6	65.643N, 21.637W	06-09-19	E190677, E190697	White part of a plastic jerrycan	Hard	
	Húsavík, Iceland	Iceland-7	65.643N, 21.637W	06-09-19	E190678, E190698	Black part of plastic	Hard	
	WS harbour, NL*	Settle-1	51.454N, 3.714E	02-07-19	E190681, E190701	Settlement plate - code SP1	Hard	
	WS harbour, NL*	Settle-2	51.454N, 3.714E	02-07-19	E190682, E190702	Settlement cube - code SC1-P1	Hard	
	WS harbour, NL*	Settle-3	51.454N, 3.714E	02-07-19	E190683, E190703	Settlement cube - code SC1-P2	Hard	
	WS harbour, NL*	Settle-4	51.454N, 3.714E	02-07-19	E190684, E190704	Settlement cube - code SC1-R	Hard	
	WS harbour, NL*	Settle-5	51.454N, 3.714E	02-07-19	E190685, E190705	Settlement cube - code SC1-SP	Hard	
	WS harbour, NL*	Settle-6	51.454N, 3.714E	02-07-19	E190686, E190706	Settlement cube - code SC1-L	Hard	
	WS harbour, NL*	Settle-7	51.454N, 3.714E	02-07-19	E190687, E190707	Settlement cube - code SC1-B	Hard	
	WS harbour, NL*	WS-1	51.454N, 3.714E	22-08-19	E190688, E190708	Floating plastic-1 (transparent)	Soft	
	WS harbour, NL*	WS-2	51.454N, 3.714E	22-08-19	E190689, E190709	Floating plastic-3 (transparent)	Soft	
	WS harbour, NL*	WS-3	51.454N, 3.714E	22-08-19	E190690, E190710	Floating insulation material	Insulation	
	WS harbour, NL*	WS-4	51.454N, 3.714E	22-08-19	E190691, E190711	Floating piece of rope	Rope	
	Griend, NL	Griend-1	53.252N, 5.254E	09-09-19	E190679, E190699	BESE-1 (Strietman et al., 2020)	Hard	
	Griend, NL	Griend-2	53.252N, 5.254E	09-09-19	E190680, E190700	BESE-2 (Strietman et al., 2020)	Hard	
	Griend, NL	Griend-3	53.252N, 5.254E	19-11-19	E210810	Grey bucket	Hard	Barnacles, red seaweed, tubeworms
	Griend, NL	Griend-4	53.252N, 5.254E	19-11-19	E210811	Grey bucket	Hard	Barnacles, red seaweed, tubeworms
	Griend, NL	Griend-5	53.252N, 5.254E	19-11-19	E210812	White plastic bottle	Hard	Tubeworms
2020	Griend, NL	Griend-6	53.252N, 5.254E	28-08-20	E210803	White transparent jug	Hard	Tubeworms, barnacles
	Griend, NL	Griend-7	53.252N, 5.254E	28-08-20	E210806	BESE element	Hard	Barnacles
	Griend, NL	Griend-8	53.252N, 5.254E	28-08-20	E210809	BESE element	Hard	Barnacles
	Kwade Hoek/Ouddorp	Kw-Ou-1	51.841N, 4.012E	24-10-20	E210804	Green part of lobster tag ‘DFO LOBST’	Hard	Algae
	Kwade Hoek/Ouddorp	Kw-Ou-2	51.841N, 4.012E	24-10-20	E210805	Soft plastic ribbon/sheet	Soft	Algae
	Kwade Hoek/Ouddorp	Kw-Ou-3	51.841N, 4.012E	24-10-20	E210807	Piece of white hard plastic	Hard	Algae
	Kwade Hoek/Ouddorp	Kw-Ou-4	51.841N, 4.012E	24-10-20	E210808	Piece of black hard plastic	Hard	Barnacles
2021	Kwade Hoek/Ouddorp	Kw-Ou-5	51.841N, 4.012E	21-04-21	E210813	Soft plastic	Soft	Dog whelk eggs
	Kwade Hoek/Ouddorp	Kw-Ou-6	51.841N, 4.012E	18-03-21	E210815	Soft plastic	Soft	Bryozoans
	Kwade Hoek/Ouddorp	Kw-Ou-7	51.841N, 4.012E	18-03-21	E210819	Soft plastic	Soft	Hydroids
	Kwade Hoek/Ouddorp	Kw-Ou-8	51.841N, 4.012E	18-03-21	E210820	Soft plastic	Soft	Bryozoans
	Kwade Hoek/Ouddorp	Kw-Ou-9	51.841N, 4.012E	18-03-21	E210821	Soft plastic	Soft	Hydroids, bryozoans
	Oostkapelle	O-Kap-1	51.586N, 3.545E	04-03-21	E210814	Hard plastic	Hard	Barnacles
	Oostkapelle	O-Kap-2	51.586N, 3.545E	04-03-21	E210816	Hard plastic	Hard	Barnacles
	Oostkapelle	O-Kap-3	51.586N, 3.545E	09-04-21	E210817	Plastic bottle	Hard	Barnacles
	Oostkapelle	O-Kap-4	51.586N, 3.545E	19-03-21	E210818	Hard plastic	Hard	Barnacles

*Calamiteitensteiger, Westerschelde harbour, Vlissingen-Oost, the Netherlands.

3 Results and Discussion

3.1 Composition of animals and plants on marine litter

Fouling was studied on 28 pieces of marine litter collected from beaches and a harbour in the Netherlands, seven from a beach at Húsavík (Iceland), four from BESE elements found on Griend Island and seven from settlement plates (cube form) from the Westerschelde harbour, Vlissingen (**Fout! Verwijzingsbron niet gevonden.**). The plastic debris samples from the Netherlands were dominated by barnacles (Sessilia), molluscs (Mollusca) and hydra and polyps (Cnidaria) (both in COI and 18SV4 results). The marker 18SV4 also picked up many green algae (Chlorophytes) that were mostly missing in COI results. Compared to the fouling on the beached marine litter, the settlement plates from the Westerschelde harbour picked up a distinctly different set of animals with both 18SV4 and COI, showing a dominance of Chordata, Amphipoda, Rhodophyta and Cnidaria. The Icelandic samples, on the other hand, were dominated by yellow-green algae (Ochrophyta), barnacles (Sessilia) and segmented worms (Annelida). The 18SV4 data on the Icelandic samples showed high amounts of ciliates (Ciliophora), but since these are microscopic and there is little to no information on the native ranges of these species, these results were not included here. Similarly to the COI data, the yellow-green algae (Ochrophyta) and barnacles (Sessilia) were abundant on the Icelandic marine litter, but 18SV4 also picked up a lot of green algae (Chlorophyta; 27%).

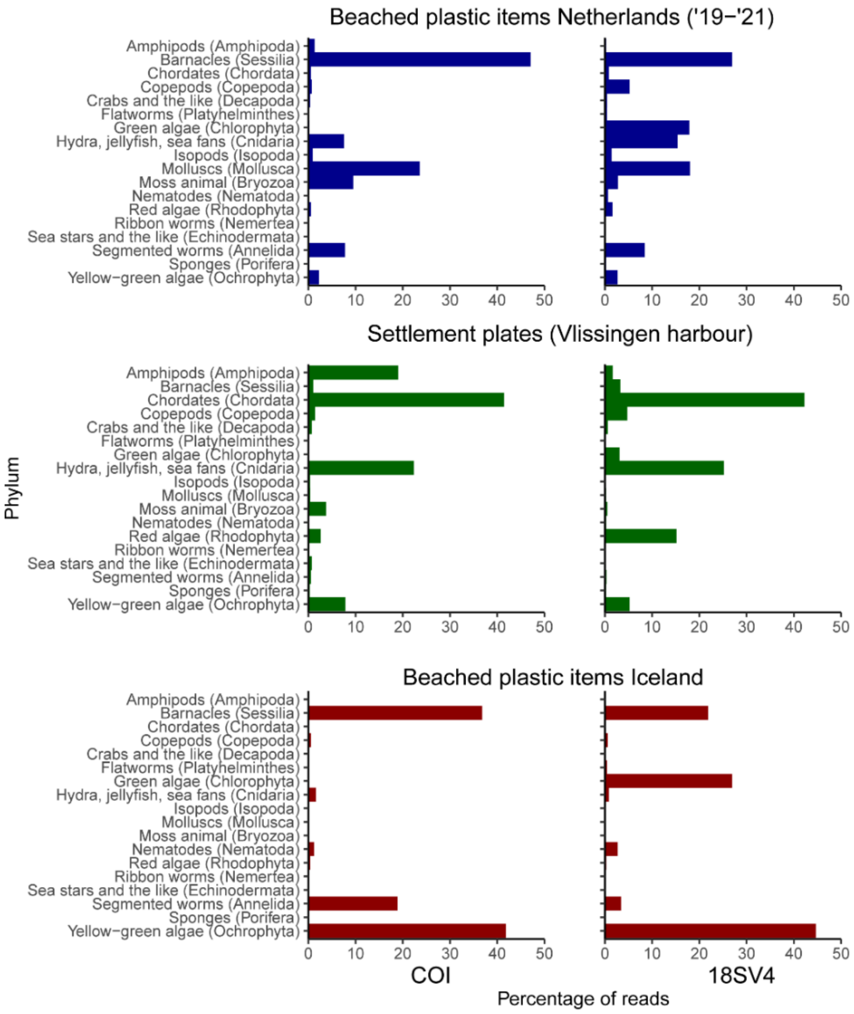

Figure 2 Overview of phyla identified using COI in the marine litter and BESE samples from the Netherlands (n = 32), settlement plates in Westerschelde Harbour, the Netherlands (n = 7) and Icelandic marine litter samples (n = 7).

3.2 Species composition and geographical origin

This section shows the native versus non-indigenous species composition for the samples from the various locations and the geographical origins of the potential non-indigenous species. In the following images, only those taxa that could be identified to the species level were used. Neither marker is able to differentiate all taxa to species level, partly because many taxa are missing reference in the barcode databases, and partly because species may not be distinguishable from very similar species using either marker.**Fout! Verwijzingsbron niet gevonden.**

3.2.1 Marine litter and BESE elements from Griend island, the Netherlands

The samples from the island of Griend, the Netherlands, generally showed 5-10 species identifications using 18SV4. Report "*Resultaten bronanalyse zwerfafval Griend*" (Strietman et al., 2020) showed the identification of several non-indigenous species on the BESE elements (Griend-1 and Griend-2; **Fout! Verwijzingsbron niet gevonden.**): the barnacles *Amphibalanus improvisus* and *Austrominius modestus*, and the shellfish parasite *Mytilicola orientalis* (Table 2), also found in this new analysis.

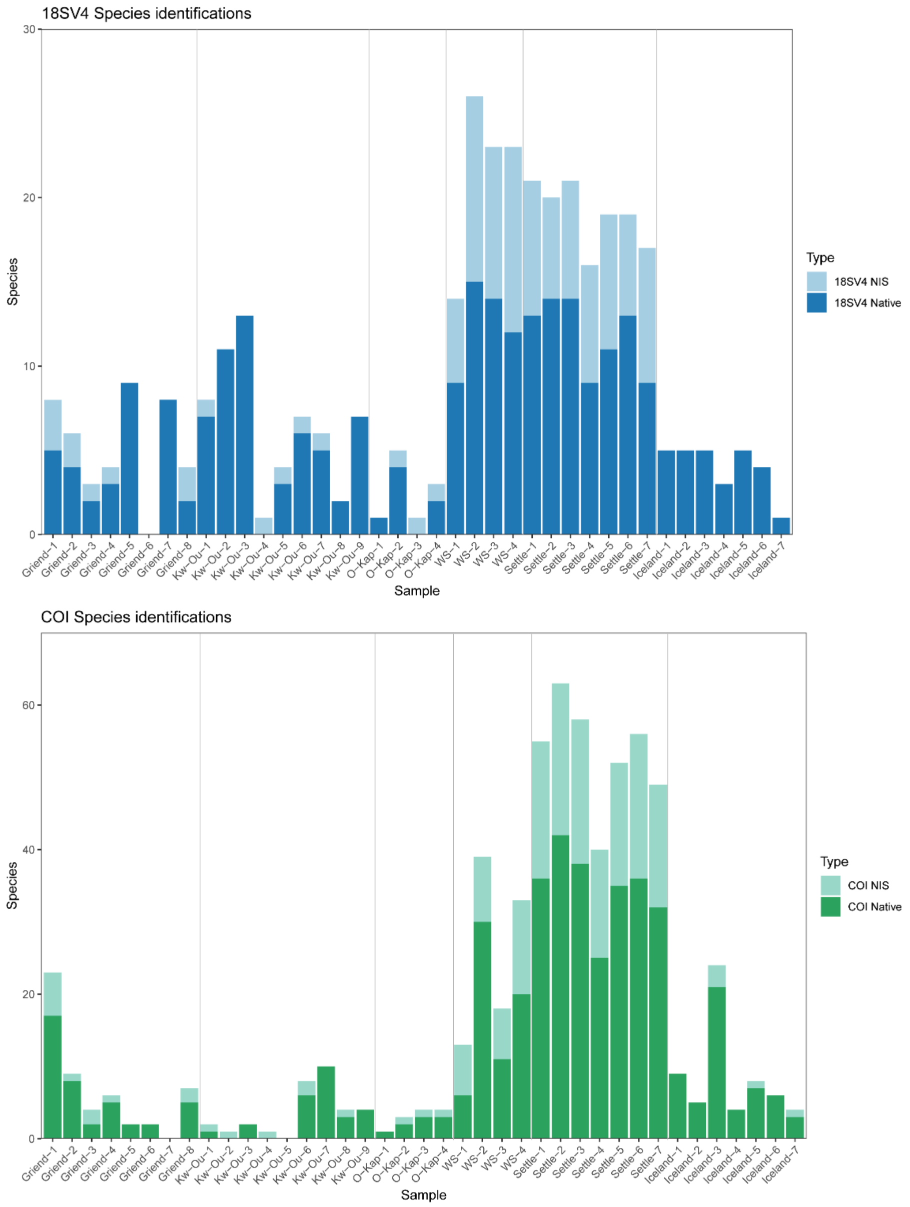

Figure 3 *Total number of species identified on the marine litter and settlement plates from the Netherlands and Iceland (see sample identification code on the X-axis). Please note scale change for the number of identified species using the COI marker.*

Additionally within the BESE elements, COI detected the following established non-indigenous species in the Netherlands: *Smittoidea prolifica* (bryozoan) and red algae *Melanothamnus harveyi*, both being of North Pacific origin (Table 2). For the two newly analysed BESE elements (Griend-7 and 8), one did not show any identified species (Griend-7), while Griend-8 showed the well-established barnacle *Austrominius modestus* and *Molgula manhattensis* (a tunicate native to the North Atlantic; Table 2). *Mytilus galloprovincialis* on Griend-8 is a potential non-indigenous species, but since it covers two distinct lineages, one present in the Mediterranean Sea, and the other in the Atlantic, it is uncertain whether it is a non-indigenous species or not (Zenetos et al., 2022).

When comparing all species found on the BESE elements (of local origin) to those found on floating plastic items (potentially from further away), it is clear that most species were found on the BESE elements for both DNA markers (Figure 4). Only four species were found both on the BESE elements and on the litter, including one non-indigenous species, *Amphibalanus improvisus*. Several non-indigenous species were only found on the BESE elements (*Austrominius modestus, Molgula manhattensis, Mytilicola orientalis, Alcyonidium verrilli, Ectocarpus crouaniorum, Melanothamnus harveyi*). The Griend marine litter samples showed some additional non-indigenous species, including a red algae (*Antithamnionella spirographidis* from South Pacific waters) not known to occur in NE Atlantic. An oyster-shell-boring annelid from Asia, *Polydora websteri*, was also identified, which has not been recorded before in the Netherlands, but has been found in other parts of the European Wadden Sea (Germany and Denmark; Waser et al., 2020). Furthermore, *Ficopomatus enigmaticus* was identified with high read numbers on one plastic sample (Griend-4). This tubeworm is native to Australia and has been found throughout Europe including the Netherlands, at least since 1921 (Zenetos et al., 2022), presumably entering Europe by means of hull fouling and ballast water (Dittmann et al., 2009).

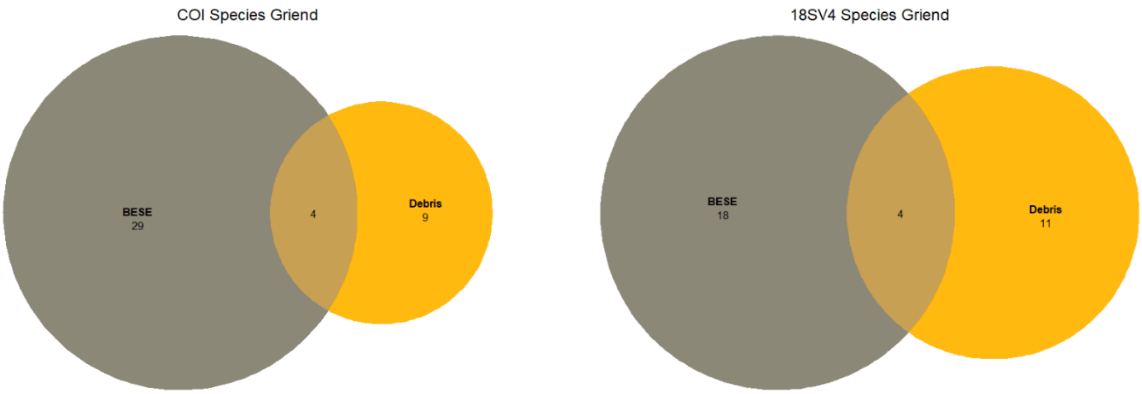

Figure 4 *Venn diagrams of all species found on BESE elements (n = 4) on the island of Griend versus those found on plastic debris on Griend (n = 4) for COI and 18SV4.*

3.2.2 Marine litter from Kwade Hoek/Ouddorp and Oostkapelle, the Netherlands

Marine litter samples from Kwade Hoek/Ouddorp showed high numbers of species of microscopic chlorophytes based on the 18SV4 marker that have unknown native ranges and will therefore not be discussed here. Several non-indigenous species were also identified (Table 2). The non-indigenous, but well-established barnacle *Austrominius modestus* was identified on several pieces of plastic (Figure 5). Furthermore, the red algae *Caulacanthus okamurae* was found on a piece of soft plastic. This algae is native to Japan and Korea and is increasing in abundance along the Dutch coast, especially in the Oosterschelde (Stegenga and Karremans, 2015). The North-Pacific *Sargassum muticum* was also identified, a well-established species within Europe and the Netherlands since at least 1972 (Zenetos et al., 2022).

Some species were not found on the websites and the literature that was used for checking the status of the identified species in the Netherlands. These may potentially form new records for the Netherlands. It includes the marine bryozoans *Conopeum tenuissimum* native to the American coasts (both NE Pacific and NW Atlantic) and *Alcyonidium verrilli* (NW Atlantic) (Table 2).

The annelids *Myrianida langerhansi* and *Myrianida edwarsi* have also not been recorded in the Netherlands before, but the first species has a native range along the UK coastline, while the second has an 'expected' status on www.nederlandsesoorten.nl. A check with taxonomic experts (Lodewijk van Walraven, WUR) revealed that species that were found on floating litter in earlier studies may not have been qualified as 'present in the Netherlands' and may have therefore not yet been included in Dutch databases. The only certain non-indigenous species detected on samples from Oostkapelle was the barnacle *Austrominius modestus* (Table 2).

Table 2 *Identified (potential) non-indigenous species (based on DNA) on beached marine litter collected in the Netherlands, their known native ranges, and the current status of invasion at the country where the non-indigenous species was observed. Established 2a: present since at least 100 years in the Netherlands, 2b: established for at least 10-100 years, 2c: established <10 years in NL and 2: unknown time of establishment.*

English name	Phylum	Non-indigenous species	Sample	Marker	Origin	Status in NL
Australian tubeworm	Annelida	*Ficopomatus enigmaticus*	Griend-4	18SV4, COI	Australia, S Pacific	Established, 2b

-	Annelida	*Myrianida langerhansi*	Kw-Ou-7	18SV4, COI	British Isles	Unknown
-	Annelida	*Myrianida edwarsi*	Kw-Ou-7	COI	NE Atlantic and the Mediterranean Sea. Also Denmark	Expected
Oyster mudworm	Annelida	*Polydora websteri*	Griend-3	COI	Asian coasts	Unknown, recorded in eastern Wadden Sea
-	Bryozoa	*Alcyonidium verrilli*	Kw-Ou-8	COI	NW Atlantic (Canadian coast)	Unknown
Lacy crust bryozoan	Bryozoa	*Conopeum tenuissimum*	Kw-Ou-6	COI	NE Pacific, NW Atlantic	Unknown
-	Bryozoa	*Smittoidea prolifica*	Griend-1	COI	N Pacific	Established, 2b
Oyster redworm	Copepoda	*Mytilicola orientalis*	Griend-1	18SV4, COI	Japan	Locally common, 2b
Mediterranean mussel	Mollusca	*Mytilus galloprovincialis*	Griend-8	COI	Mediterranean, Black Sea, NE Atlantic	Uncertain, see (Zenetos et al., 2022)
-	Ochrophyta	*Ectocarpus crouaniorum*	Griend-1,2	COI	Unknown	Unknown
Jap weed	Ochrophyta	*Sargassum muticum*	Kw-Ou-2	COI	E Pacific	Established
-	Rhodophyta	*Caulacanthus okamurae*	Kw-Ou-5	18SV4	Indian Ocean (Japan, Korea)	Established
Harvey's siphon weed	Rhodophyta	*Melanothamnus harveyi*	Griend-1	COI	NW Pacific	Established
-	Rhodophyta	*Antithamnionella spirographidis*	Griend-3	18SV4	S Pacific	Established
bay barnacle	Sessilia	*Amphibalanus improvisus*	Griend-1	18SV4	Atlantic Ocean	Established, 2a
New Zealand barnacle	Sessilia	*Austrominius modestus*	Many	18SV4, COI	S Pacific (New Zealand)	Established, 2b
Common sea grape	Tunicata	*Molgula manhattensis*	Griend-8	18SV4	N Atlantic	Established, 2a

Figure 5 *Examples of non-indigenous species on marine litter from the Netherlands. A. Austrominius modestus B. Molgula manhattensis and C. Melanothamnus harveyi (source for all images: Flickr).*

3.2.3 Floating marine litter and settlement plates in Westerschelde harbour

For the Westerschelde harbour location in Vlissingen, a comparison was made between species identified on floating marine litter in the harbour and fouling on the settlement plates that were deployed in the harbour at the same location (**Fout! Verwijzingsbron niet gevonden.**6). Of the 94 species found on settlement plates using COI, 31 were also found on the floating marine litter, including 11 non-indigenous species (examples include well known established non-indigenous species such as *Caprella mutica, Botrylloides violaceus, Austrominius modestus* and *Tricellaria inopinata*; see Table 3). However, some species were also found on the settlement plates and/or marine litter that we could not find in the Dutch databases mentioned in section 2.2. These include the Bryozoans *Alcyonidium verrilli* from the NW Atlantic coasts (Canada) and *Schizoporella japonica* (W Pacific, Japan) first observed in Western Europe in 2014 (Ryland et al., 2014) but in the NE Atlantic already back in 1976 (Zenetos et al., 2022). The stalked jellyfish (Cnidaria) *Haliclystus inabai* and *H. tenuis* (both from W Pacific, Japan) were also found of which only *H. tenuis* is known to occur in the NE Atlantic, at least since 2010 (Zenetos et al., 2022). Furthermore, the yellow-green algae (Ochrophyta) *Ectocarpus crouaniorum* of unknown origin*, Myrionema balticum* (N America?) and *Scytosiphon (Hapterophycus) canaliculatus* (N Pacific) have not been described before in the NE Atlantic. Additionally, the red algae *Ceramium gardneri* from the NE Pacific (American West Coast) was found.

Some non-indigenous species were found on the floating marine litter that were not found on the settlement plates (**Table 3**). This included the copepod *Pseudodiaptomus marinus*, an established non-indigenous species in the North Sea (Deschutter et al., 2018), the established spionid worm *Streblospio benedicti* and the yellow-green algae *Fibrocapsa japonica*. Furthermore, two Asian crab species (most likely represented by their larvae) were identified: *Hemigrapsus sanguineus* on two of the floating marine litter samples and *H. takanoi* on all four of these samples. Both species are well established in both the Ooster- and Westerschelde (Faasse, 2004; Van den Brink et al., 2012). Using 18SV4, some additional non-indigenous species were identified on the floating marine litter, including the known established non-indigenous species of segmented worm (Annelida), *Boccardiella hamata* and *Pseudopolydora paucibranchiata*, the copepod *Acartia tonsa* and the tunicate *Molgula manhattensis*. Additionally, we observed the annelid worm *Myrianida convoluta,* a species with uncertain status in the Netherlands and thus seen as a putative non-indigenous species.

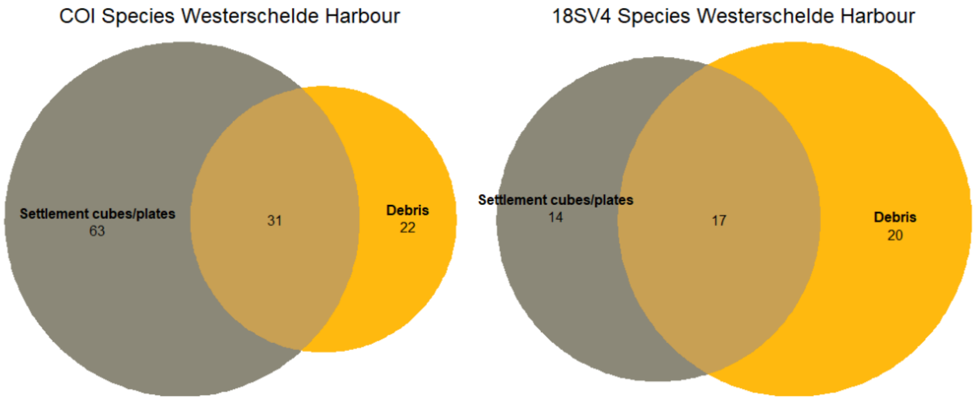

Figure 6 *Venn diagrams of all species found on settlement plates and cubes (n = 7) in Westerschelde harbour versus those found on plastic debris (n = 4) for COI and 18SV4.*

Table 3 *Identified non-indigenous species (based on DNA) on floating marine litter collected in Westerschelde harbour, Vlissingen, as well as on the settlement plates including their known native ranges, and the current status of invasion in the Netherlands.*

English name	**Phylum**	**Non-indigenous species**	**Sample**	**Marker**	**Origin**	**Status in NL**
-	Annelida	*Boccardiella hamata*	WS-3 (insulation)	18SV4	NW Pacific	Established, 2c
Australian tubeworm	Annelida	*Ficopomatus enigmaticus*	Settle	18SV4, COI	Australia, S Pacific	Established, 2b
-	Annelida	*Myrianida convoluta*	WS-1,3, Settle	18SV4, COI	Western Indian Ocean, W Atlantic, Mediterranean	Unknown
Elkhorn slough spionid	Annelida	*Pseudopolydora paucibranchiata*	WS2,3	18SV4, COI	Indo-Pacific and NE Atlantic	Established, 2c
Bar-gilled mudworm	Annelida	*Streblospio benedicti*	WS-1,2,4	COI	Atlantic and E Pacific	Established, 2b

English name	Phylum	Non-indigenous species	Sample	Marker	Origin	Status in NL
-	Amphipoda	*Monocorophium acherusicum*	Settle	COI	N Atlantic?	Established, 2
Japanese skeleton shrimp	Amphipoda	*Caprella mutica*	Settle, WS-1-4	18SV4*, COI	Asia	Established, 2b
-	Amphipoda	*Jassa marmorata*	Settle	COI	N Atlantic	Established, 2
-	Bryozoa	*Alcyonidium verrilli*	WS-2	COI	NW Atlantic (Canadian coast)	Unknown
-	Bryozoa	*Amathia gracilis*	All	18SV4, COI	Unknown	Established, 2a
-	Bryozoa	*Bugulina stolonifera*	All	COI	Unknown	Established, 2b
-	Bryozoa	*Tricellaria inopinata*	All	COI	N Pacific	Established, 2b
-	Bryozoa	*Schizoporella japonica*	Settle	COI	W Pacific	Unknown
Chain tunicate	Chordata	*Botrylloides violaceus*	All	COI	N Pacific	Established, 2b
Star tunicate	Chordata	*Botryllus schlosseri*	Settle	18SV4, COI	N Atlantic	Established, 2b
-	Cnidaria	*Haliclystus inabai*	Settle	COI	W Pacific (Japan)?	Unknown
-	Cnidaria	*Haliclystus tenuis*	Settle	COI	W Pacific (Japan)?	Unknown
-	Copepoda	*Acartia tonsa*	WS-2-4	18SV4	Indo-Pacific, Atlantic and Mediterranean	Locally present, 2a
-	Copepoda	*Pseudodiaptomus marinus*	WS-4	18SV4, COI	Indo-West Pacific: Sea of Japan	Established, 2c
Asian shore crab	Decapoda	*Hemigrapsus sanguineus*	WS-2,4	18SV4, COI	W Pacific, E Atlantic	Established, 2b
brush-clawed shore crab	Decapoda	*Hemigrapsus takanoi*	WS-1-4	18SV4, COI	W Pacific, E Atlantic	Established, 2b
False angelwing	Mollusca	*Petricolaria pholadiformis*	Settle	COI	N Atlantic, N America	Established, 2a
-	Nemertea	*Cephalothrix simula*	Settle	COI	N Pacific	Established, 2c
-	Ochrophyta	*Ectocarpus crouaniorum*	Settle	COI	Unknown	Unknown
-	Ochrophyta	*Fibrocapsa japonica*	WS-2,4	COI	Northeast Atlantic	Established, 2b
-	Ochrophyta	*Scytosiphon (Hapterophycus) canaliculatus*	Settle-5	COI	N Pacific (American West coast to Japan)	Unknown
-	Ochrophyta	*Myrionema balticum*	Settle-1	COI	N America?	Unknown
Crumb-of-bread sponge	Porifera	*Hymeniacidon perlevis*	Settle	COI	N Atlantic	Established, 2b
-	Porifera	*Haliclona xena*	Settle	COI	Unknown	Established, 2b
-	Rhodophyta	*Dasysiphonia japonica*	All	18SV4, COI	N Pacific (Japan)	Established, 2b
-	Rhodophyta	*Ceramium gardneri*	All	COI	NE Pacific (American West Coast)	Unknown
Harvey’s siphon weed	Rhodophyta	*Melanothamnus harveyi*	All	COI	NW Pacific	Established, 2b
bay barnacle	Sessilia	*Amphibalanus improvisus*	All	18SV4, COI	Atlantic Ocean	Established, 2a
New Zealand barnacle	Sessilia	*Austrominius modestus*	All	18SV4, COI	S Pacific (New Zealand)	Established, 2b
Common sea grape	Tunicata	*Molgula manhattensis*	WS-2,4	18SV4	N Atlantic	Established, 2a
stalked sea squirt	Tunicata	*Styela clava*	All	18SV4	N Pacific	Established, 2b
	**Caprella* sp.					

In total, 46 (potential) non-indigenous species were identified on the marine litter collected from the Dutch beaches and in the harbour (including the settlement plates). A comparison of the observed non-indigenous species on the plastic that was collected from beaches in the Netherlands (Table 2) with those observed on floating plastic and settlement plates from Vlissingen harbour (Table 3), showed that 16 non-indigenous species were observed on the pieces of plastic collected from the beach and 36 non-indigenous species on the pieces of plastic and settlement plates of Vlissingen harbour. Of these, nine non-indigenous species found on plastic from Dutch beaches and 27 non-indigenous species from Vlissingen harbour were well known and established in Dutch waters. The following six species showed an overlap in presence on both plastic items from beaches in the Netherlands and plastic items or settlement plates from Vlissingen harbour: the worm *Ficopomatus enigmaticus* (found both on one item on Griend and the settlement plates, both markers), the bryozoan *Alcyonidium verrilli* (found on a piece of plastic in Kwade Hoek and one in Vlissingen harbour, COI), the red algae *Melanothamnus harveyi* (on a plastic item on Griend and all samples in Vlissingen harbour, COI), the barnacles *Amphibalanus improvisus* (plastic item on Griend and all samples in Vlissingen harbour, both markers), *Austrominius modestus* (on many of the beached plastics and all Vlissingen harbour samples, both markers) and the tunicate *Molgula manhattensis* (on a plastic item on Griend and a piece of plastic in Vlissingen harbour, 18SV4).

3.2.4 Marine litter from Iceland

The Icelandic plastic debris samples showed a low number of identified species using 18SV4 (1 to 5). These plastic items were stored in bags with all collected beach litter for at least a few days before the items with fouling were processed. This supports our approach that storage diminishes the potential for identification of fouling species based on DNA. With COI a similar picture arose, although one sample (Iceland-3; a butter package) showed a relatively high number of identified species (24) including three non-indigenous species for Iceland. The dominant species in this sample was the yellow-green algae *Hecatonema terminale* (syn. *H. maculans*), a species with a near cosmopolitan distribution (Table 4). Furthermore the yellow-green algae *Scytosiphon (Hapterophycus) canaliculatus* and *Coilodesme californica* (an epiphyte of *Cystoseira osmundacea,* which itself was not found here) were found on this sample, two species that are native to the Northern Pacific.

Table 4 *Identified (potential) non-indigenous species (based on DNA) on beached marine litter collected in Iceland, their known native ranges, and the current status of invasion, if known.*

English name	Phylum	Non-indigenous species	Sample	Marker	Origin	Status in Iceland
-	Ochrophyta	*Coilodesme californica*	Iceland-3	COI	NE Pacific (American West coast)	Unknown
-	Ochrophyta	*Hecatonema terminale*	Iceland-3	COI	NE, W Atlantic Ocean, Pacific Ocean, Australia and Mediterranean Sea	Unknown
-	Ochrophyta	*Scytosiphon (Hapterophycus) canaliculatus*	Iceland-3	COI	N Pacific (American West coast to Japan)	Unknown

3.3 Differences in taxonomic composition among sampling locations

To visualize whether the beached plastic samples as well as the settlement plates and BESE elements contained a composition of species unique to the sampling locations, the COI results were plotted in a non-metric multidimensional space (NMDS).

The resulting plot shows that samples from the different locations are significantly different (p = 0.001). The Dutch beach samples (Griend, Kwade Hoek/Ouddorp and Oostkapelle) clustered relatively close to each other (Figure 7). The settlement plates from Vlissingen harbour were clearly distinct from all other samples and showed a very high overlap with the floating plastic items of Vlissingen harbour termed ‘Westerschelde’ in Figure 7 (see section 3.2.3). All settlement plate samples clustered closely together, indicating they had highly similar communities growing on them (*Caprella mutica, Botrylloides leachi, Asterina* sp., *Botryllus schlosseri* and many more).

One of the BESE elements from Griend island (Griend-1) clustered relatively close to all samples from Westerschelde (Vlissingen), showing that the composition of species on this sample is likely of local origin while the BESE samples Griend-2 and Griend-8 clustered more closely with floating plastic litter, based on the abundant growth of barnacles (*Amphibalanus improvisus* and New Zealand barnacle *Austrominius modestus*), both non-indigenous species. The two plastic litter samples found on Griend island show species compositions more similar to samples from Kwade Hoek/Ouddorp and Oostkapelle.

The Icelandic samples could also clearly be distinguished from the other locations based on the unique presence of species like *Fucus vesiculosus*, *Dictyosiphon foeniculaceus*, *Coilodesme californica* and the high amounts of *Semibalanus balanoides* (although in low number of reads). This means that clustering based on fouling composition can be used to separate locations.

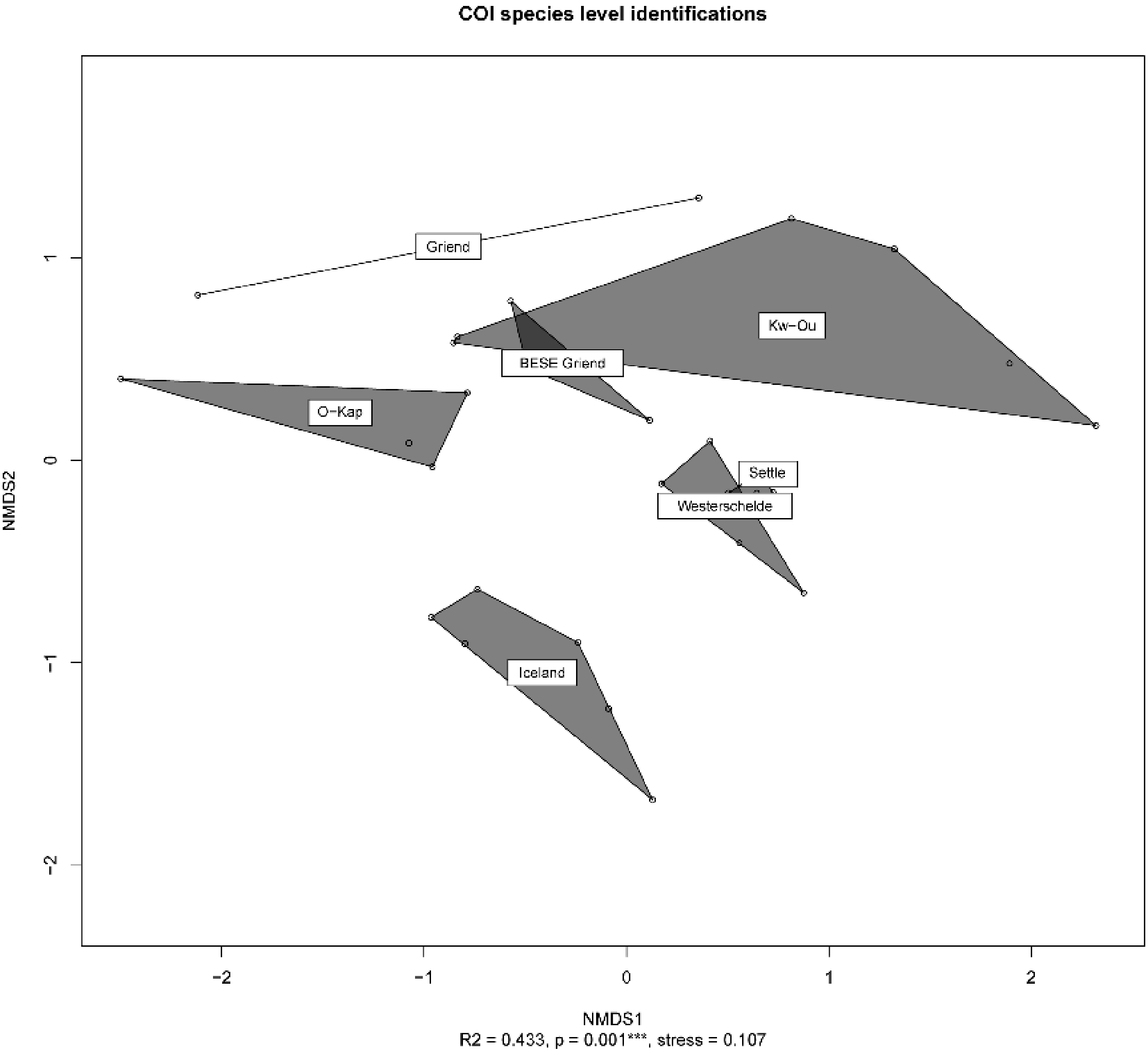

Figure 7 *Two-dimensional NMDS plots on relative abundance based Bray-Curtis dissimilarities of species level identifications using the COI marker, grouping the samples based on their sampling location.*

3.4 Differences in taxonomic composition among substrate types

A comparison was made between the type of substrate the fouling was growing on for the Dutch samples: hard or soft marine plastic or litter. Only six samples consisted of a soft plastic (four from Kwade Hoek/Ouddorp and two from Westerschelde harbour), while the remaining 18 samples consisted of hard plastic (including the settlement plates), isolation material (one piece) and rope (one piece).

A significant (p=0.009) separation was visible between the soft and hard plastic samples on the NMDS plot (Figure 8). Species occurring more on the soft plastics include the bryozoans *Electra pilosa, Membranipora membranacea* and the hydrozoa *Tubularia indivisa, Eucheilota maculata* and *Obelia bidentata*. Of these, only *M. membranacea* was also found on one of the pieces of hard plastic, in low numbers of reads. The settlement plates showed some other bryozoans, including *Obelia longissima* and *Gonothyraea loveni*, while *Cryptosula pallasiana* was found on Griend-4.

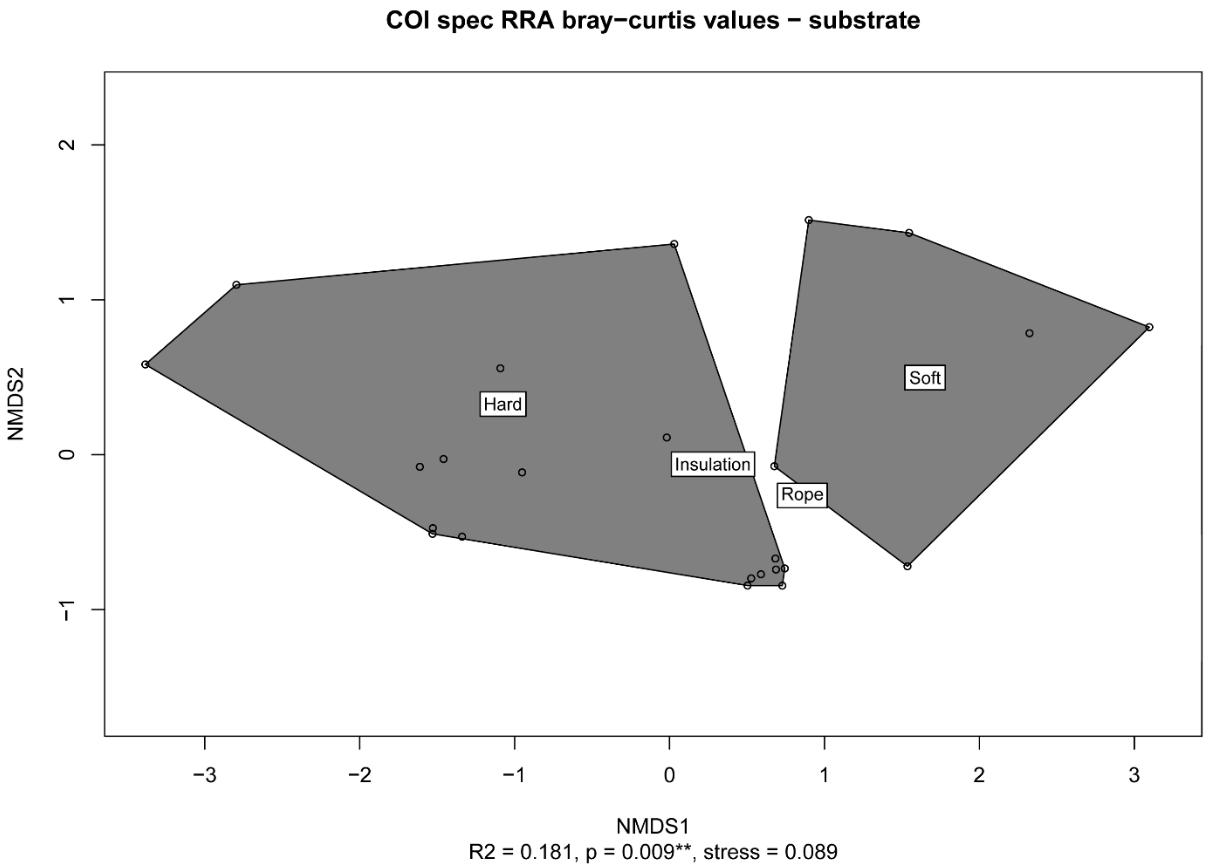

Figure 8 *Two-dimensional NMDS plots on RRA-based Bray-Curtis dissimilarities of species level identifications using COI, grouping the samples based on their substrate (hard versus soft plastics).*

Species only found on hard substrate included all barnacles and most red algae (Rhodophyta) and seven out of the total eight species of amphipods found on the marine litter. Only the amphipod *Caprella mutica* was also found on soft plastics, although only on the marine litter samples from Westerschelde harbour. The (larvae of) *Hemigrapsus* crabs were also only found on soft plastics (in Vlissingen harbour), but as these are freely floating in the water column, they may not be representative for the marine litter substrate.

For the polychaete worms, some species were found to be present on many of the hard substrate samples while missing on the soft plastics (e.g. *Ficopomatus enigmaticus, Streblospio benedicti*), while species of the genus *Myrianida* were only found on soft plastics. Similarly, most of the yellow-green algae were only found on hard substrates (e.g. *Pylaiella littoralis*, *Ectocarpus fasciculatus, Hincksia sandriana*), while *Sargassum muticum* was only found on the soft plastic substrate.

The piece of rope showed a community that was more similar to what was found on the soft plastics, while the insulation material was more similar to the hard substrate samples.

3.5 Age and source identification of marine litter

The origin and range extension of especially newcomers (non-indigenous species) identified on the marine litter, can be further used to assess the source of the collected marine litter by studying the original distribution of the species.

The age of marine litter, or time the litter has been floating at sea, can be used to assess the possible sources of marine litter (van Duinen et al., 2022). Fouling is a useful indication for (minimal) age (Mesaglio et al., 2021). To assess this, information on the settling time, metamorphosis and growth of the identified fouling species is required. For example *Austrominius modestus* has a larval stage of 2-3 weeks, a metamorphosis of 1-3 days and a growth rate of 0.5 - 1.0 mm/month, depending on the water temperature and food availability (https://www.cabidigitallibrary.org/doi/10.1079/cabicompendium.109096). This means that a piece of litter with adult *A. modestus* attached to it, has been in the water for multiple months. Also the spawning season is of relevance. *A. modestus* is able to spawn throughout the year, though with a peak during summer (Knight-Jones and Stevenson, 1950; Knight-Jones and Waugh, 1949). This means that for all relevant fouling species a database needs to be constructed to provide a good overview of the reproduction cycle of the species, needed to develop an estimate of the minimum time the plastic has been floating in the sea.

With targeted modelling that include information on ocean currents, winds and time (age of the litter), potential source locations can be identified, as has been recently done for the southwest of the Netherlands (van Duinen et al., 2022). This study showed that the main sources for litter found in this area were the east coast of the UK, the Dutch coast, the English Channel (fisheries) and several rivers in the Netherlands, the UK and France. Age appears to be an important parameter for distinguishing litter coming from rivers versus litter coming from fisheries (Van Duinen et al., 2022).

In other studies applying the Litter-ID methodology, determination of geographical origin was done using language detection on labels on the collected litter (Strietman et al, 2023). It would be useful to make such a comparison with our samples, but unfortunately, no labels were present on the marine litter used here for fouling identification.

The determination of geographical origin is useful additional information in combination with an assessment of external characteristics, text and logos on marine litter (assessed as part of the Litter-ID methodology). Such a combined analysis helps in identifying relevant mitigation measures to diminish the amount of litter that enters the world’s oceans.

Finally, even differences within species were found using the DNA markers. This so-called haplotype variability was observed between different locations within the COI region (e.g., *Petalonia fusca* had a different haplotype in Iceland than in the Netherlands). This information could potentially be used to further help in determining source populations of non-indigenous species in future studies. A pilot is currently being run (as of 12/2022) to see whether haplotype variability within the barnacle *Austrominius modestus* can be used to identify different populations.

4 Conclusions

Under the influence of wind and currents, marine litter is known to travel long distances in the oceans. Marine litter is also known to act as vectors for fouling species, potentially acting as a stepping stone for plants and animals to travel from one region to another and potentially becoming invasive at the location of arrival. Along with an assessment of external characteristics and language detection on labels characteristics, text and logos, analysing the species composition, specifically whether such species are native or non-indigenous, of fouling on marine litter can help in identifying the origin of litter. In addition, such an assessment helps in determining the potential risk such fouling species pose to the local ecosystem.

In this study we collected the fouling on pieces of marine litter that were found on several beaches along the Dutch North Sea coastline (Griend, Kwade Hoek, Westerschelde and Oostkapelle) and studied using DNA metabarcoding. For comparison, settlement plates were placed in Vlissingen harbour and the fouling species growing on them were collected to identify the locally occurring non-indigenous species. These were also sampled using BESE elements found on the island of Griend which were sourced from a nearby ecosystem restoration project. Furthermore, fouling on plastic litter from on a beach in Iceland was studied to identify if differences in fouling species between locations could be identified.

Dominant biological groups that were identified as fouling on marine litter collected from beaches in the Netherlands consisted of barnacles (Sessilia), hydra and polyps (Cnidaria), segmented worms (Annelida) and molluscs (Mollusca). Significant differences were observed between study locations in the Netherlands, allowing separation between marine litter from Westerschelde harbour (which showed high overlap with the settlement plates placed there) from the other locations. Marine litter samples collected from beaches on Griend and Kwade Hoek were more similar, while the location Oostkapelle showed a more distinct community. Different communities of animals and (macro-)algae were found on the floating litter from Dutch beaches versus the settlement plates and BESE elements. This further underlines the importance of floating marine litter as a vector for native and non-native species facilitating the introduction and range expansion of non-indigenous species (Audrézet et al., 2021). Analysis of fouling on floating marine litter can therefore form an important early indicator for the risk of introduction of non-indigenous species into the local ecosystem.

Marine litter collected in Iceland was dominated by yellow-green algae (Ochrophyta), barnacles (Sessilia) and segmented worms (Annelida). The species composition differed significantly from that found on marine litter from the Netherlands.

A comparison between fouling species on hard and soft marine litter showed that different substrate types attract a different community of animals and/or algae growing on them: while hard plastics were dominated by barnacles (Sessilia) and red algae (Rhodophyta), the soft plastics were dominated by moss animals (Bryozoa) and hydra and polyps (Hydrozoa).

This study shows that integrating multiple DNA markers (18SV4 and COI) allows the identification of non-indigenous species on beached marine litter and settlement plates. Both markers identified species that were missed by the other, showing the importance of a multi-marker approach. It remains, however, important to manually double-check species identifications generated by automated annotation scripts, because of the high number of erroneous records in NCBI and/or BOLD, as well as taking into account potentially missing sequences of closely related species. We advise to take high-resolution pictures of observed fouling before processing the material for DNA-analysis. Furthermore, additional future efforts to improve the completeness of online barcode reference databases will be pivotal to improve identification success.

A total of 46 (potential) non-indigenous species were identified on marine litter collected from Dutch beaches and Vlissingen harbour, based on DNA. These include the potential identification of the following non-indigenous species of which – to our best knowledge - no earlier records in the Netherlands exist:

Alcyonidium verrilli, Ceramium gardneri, Conopeum tenuissimum, Ectocarpus crouaniorum, Haliclystus inabai and *H. tenuis, Myrianida convoluta*, *Myrianida langerhansi, Myrionema balticum, Polydora websteri, Schizoporella japonica* and *Scytosiphon* (= *Hapterophycus*) *canaliculatus.* Six of these species were also not found before in the NE Atlantic, as far as we could find: *Myrianida convoluta, Conopeum tenuissimum, Alcyonidium verrilli, Haliclystus inabai, Ceramium gardneri* and *Scytosiphon* (= *Hapterophycus*) *canaliculatus*. Final conclusions on the arrival of these species lack the support of a taxonomic identification based on visual characteristics.

This study also shows that, in combination with an assessment of external characteristics, text and logos, analysing the species composition of fouling on marine litter can help in identifying the origin of litter in the following manner. The identification of fouling species can help in establishing a minimum age and origin of the collected floating marine litter. Datasets need to be constructed for this to enable estimates of minimum age that a piece of litter has been floating in the sea. Models can use the parameter ‘age’ to backtrack what the potential sources of the marine litter are. Models can use this information to backtrack what the geographical origin is of the marine litter. The determination of geographical origin is useful additional information next to an assessment of external characteristics, text and logos on marine litter (assessed as part of the Litter-ID methodology). Such a combined analysis helps in identifying relevant mitigation measures to diminish the amount of litter that enters the world’s oceans.

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Report 3253
ISSN 1566-7197

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Report 3254
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