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Blue Carbon eDNA

A novel eDNA method to trace macroalgae carbon in marine sediments

Photo: Janne K. Gitmark (NIVA)

Norwegian Institute for Water Research

REPORT

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Summary

Blue forests, such as rockweed beds and seagrass meadows, are considered important natural sinks for carbon and thereby play a vital role in climate regulation, mitigation and adaptation. Kelp forests grow on rocky bed substrates and have therefore no local sedimentation of produced biomass. Kelp has therefore not yet been considered to contribute significantly to carbon sequestration and has been left out from the IPCC methodology for blue carbon. However, there is evidence that kelp biomass is transported to deeper seafloor locations where it is sequestered in sediments.

The advent of eDNA methods for tracking animal or vegetal organisms in water or sediment matrices, has enabled not only geographical detection without collection of the organism but also temporal study of their presence in dated sediment cores. The eDNA methodology presented here enables sediment species-specific identification of biomass demonstrating the presence of kelp species that were transported and stored away from where they were produced. This technique will make it possible to assess the capacity of atmospheric CO2 draw-down in kelp forests and inform about their potential for carbon sequestration and climate mitigation. The method was initially developed as part of the Nordic Blue Carbon Project (2017–2020).

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Blue Carbon eDNA

A novel eDNA method to trace macroalgae carbon in marine sediments

Preface

This report describes in detail the procedure and methodology for tracing and quantifying macroalgae organic carbon in marine sediments applying a novel environmental DNA (eDNA) methodology. The method was initially developed as part of the Nordic Blue Carbon Project (2017–2020) funded by the Nordic Council of Ministers and published in Frigstad et al. (2021). Here it is described in further details making it available to the broader research community. A detailed step-by-step lab protocol is made available online through protocols.io, "MarineSediment-BlueC-eDNA", doi: 10.17504/protocols.io.btk4nkyw.

The Blue Carbon eDNA project was financed by the NEA (contract #17080044), with Snorre Birkelund Wille as the contact person. The writing of this report was led by Marc Anglès d'Auriac, senior researcher at the Norwegian Institute for Water Research (NIVA).

We thank Siri Moy for assistance in the lab with slicing of cores and sample preparations and Paul Ragnar Berg for quality assurance of the report. NIVA appreciates the opportunity to publish this method and acknowledges everyone involved for their dedicated and enthusiastic interdisciplinary cooperation.

Oslo, May 14th, 2021

Marc Anglès d'Auriac
Project manager

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Terms and definitions

Blue carbon – carbon captured by living organisms in coastal vegetated ecosystems (e.g. kelp forests, rockweed beds, seagrass meadows, salt marshes, and mangroves) and stored in biomass and sediments.

Blue carbon budget –an assessment of carbon cycle sources and sinks for blue forests.

Blue forests – coastal vegetated habitats or ecosystems occupied with kelp forests, seagrass meadows, rockweed beds, salt marches, or mangroves (not in Norway).

Carbon pool – the carbon stored in a specific species, ecosystem or geographic location.

Carbon sequestration – long-term removal of carbon dioxide (CO_2) or other forms of carbon from the atmosphere, with secure storage on climatically significant time scales (decadal to century). For particulate organic carbon (POC) this is defined as the carbon that is buried in the shelf or deep-sea sediments. Equivalent to **long-term storage of carbon**.

Carbon uptake – a process by which plants and algae in oceans (or plants on land) absorb carbon from the atmosphere.

ddPCR – droplet digital PCR, separates a sample into tens of thousands of nanolitre size droplet partitions and PCR is carried out in each partition individually. A presence/absence (1/0) result is provided for each partition. Assuming that the molecule population follows the Poisson distribution, the method provides absolute quantification of the total target DNA copy number initially present.

DNA Barcoding – is a method of species identification using short DNA sequences from a specific gene or genes. The premise of DNA barcoding is that, by comparison with a reference library, individual sequences can be used to uniquely identify organisms to species.

eDNA – genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material.

Kelp forest – areas with a high density of kelp, often more than 50% coverage. In Norway the most common species are tangle kelp (*Laminaria hyperborea*), sugar kelp (*Saccharina latissima*), and oarweed (*Laminaria digitata*).

Long-term storage of carbon – see "**carbon sequestration**".

Macroalgae – large algae attached to the bottom of the sea, such as tangle kelp, sugar kelp, and different species of rockweed.

Metabarcoding – is the barcoding of DNA/RNA (or eDNA/eRNA) in a manner that allows for the simultaneous identification of many taxa within the same sample. The main difference between barcoding and metabarcoding is that metabarcoding does not focus on one specific organism, but instead aims to determine species composition within a sample.

Next Generation Sequencing (NGS) – NGS technology is typically characterized by being highly scalable, allowing entire genomes to be sequenced at once. Usually, this is accomplished by fragmenting the genome into small pieces, randomly sampling for fragments, and sequenced using one of a variety of technologies. It is also used for metabarcoding.

Particulate organic carbon (POC) – the organic matter that is present in the form of particles and is operationally defined as the fraction of carbon that is retained in a filter (with pore size ranging from 0.22 to 0.70 micrometers).

qPCR – quantitative polymerase chain reaction also known as real-time polymerase chain reaction, is a laboratory technique of molecular biology based on the polymerase chain reaction (PCR). It monitors the amplification of a targeted DNA molecule during the PCR (i.e., in real time), not at its end, as in conventional PCR.

Sedimentation – the particulate organic carbon (POC) that sinks out of the water column and settles in the coastal shelf or deep-sea sediments.

Sequencing – DNA sequencing is the process of determining the nucleotide order of a given DNA fragment.

Sink – any process, activity or mechanism that removes carbon from the atmosphere.

Summary

This report describes in detail a novel environmental DNA (eDNA) methodology developed at NIVA to identify the presence of organic carbon from specific species of macroalgae (kelp) in marine sediments.

Blue forests, such as rockweed beds and seagrass meadows, are considered important natural sinks for carbon and thereby play a vital role in climate regulation, mitigation and adaptation. Kelp forests grow on rocky bed substrates and have therefore no local sedimentation of produced biomass. Kelp has therefore not yet been considered to contribute significantly to carbon sequestration and has been left out from the IPCC methodology for blue carbon. However, there is evidence that kelp biomass is transported to deeper seafloor locations where it is sequestered in sediments. The eDNA methodology presented here enables sediment species-specific identification of biomass demonstrating the presence of kelp species that were transported and stored away from where they were produced. This technique will make it possible to assess the capacity of atmospheric CO2 draw-down in kelp forests and inform about their potential for carbon sequestration and climate mitigation. The method was initially developed as part of the Nordic Blue Carbon Project (2017–2020).
Established methods, like stabile isotope, pigments and lipid analysis, are known to underperform when it comes to detection and identification of kelp carbon in marine sediments. This has until now hampered robust estimates of the role of kelp forests in long-term carbon storage (termed carbon sequestration) in marine sediments.

The advent of environmental DNA, defined as genetic material obtained directly from environmental samples (soil, sediment, water, etc.) without any obvious signs of biological source material, has provided researchers with new tools for species-specific tracing of organisms in various contexts and through timescales. The methodology is universal in the sense that all DNA traces are collected and purified. The subsequent DNA analysis will then verify the presence or absence of the target group, by for example using specific qPCR protocols.

The methodology described here includes guidelines for sampling of marine sediment cores, their transport and conservation, and how kelp organic matter can be identified in marine sediments using eDNA. Especially, this report explains how novel eDNA methods are applied for tracing species-specific kelp DNA in sediments. We have used a DNA barcode marker [the cytochrome oxidase subunit-1 (COI) gene] and developed species-specific primers used for the identification of the two most common species of kelp in Norway – the tangle kelp (*Laminaria hyperborea*) and the sugar kelp (*Saccharina latissima*).
Special care was given to the selection of DNA extraction and polymerase kits, to reduce false negatives. Adequate negative controls were used to avoid false positives and to validate the obtained positive detections. Further, the first initial steps were taken to develop a quantitative technique to estimate the amount of organic kelp carbon from sediment cores. The latter is not trivial, but in principle applicable through linking the quantity of macroalgae DNA to macroalgae biomass.

This novel method enables identification of species-specific macroalgae contributions to carbon deposition and long-term storage in marine sediments. The method can further be developed to trace carbon sources through marine food webs, such as quantifying the importance of macroalgae as diet for zooplankton or other filter feeders in the water column or at the seafloor.
A detailed laboratory protocol is available online through protocols.io, "MarineSediment-BlueC-eDNA", doi: 10.17504/protocols.io.btk4nkyw.

Sammendrag

Tittel: Blue Carbon eDNA – A novel eDNA method to trace macroalgae carbon in marine sediments
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Denne rapporten beskriver i detalj en ny metode utviklet ved NIVA for å identifisere organisk karbon fra enkelte arter av makroalger i marine sedimenter ved bruk av miljø-DNA (eDNA).

Blå skog, som tang-, tareskog og ålegressenger, regnes som viktige naturlige karbonlagre. Tareskog vokser på steinsubstrater og har derfor ingen lokal sedimentering av produsert biomasse. Tare er derfor ennå ikke ansett å bidra vesentlig til karbonbinding og har blitt utelatt fra IPCC-metoden for blå karbon. Imidlertid er det bevis for at tarebiomasse transporteres til dypere havbunnssteder der den er bundet i sedimenter. Den eDNA-metode som presenteres her muliggjør identifikasjon av sediment art spesifikk biomasse som viser tilstedeværelsen av tarearter som ble transportert og lagret vekk fra hvor de ble produsert. Teknikken som brukes her kan bidra til en vurdering av tareskogens rolle i det marine karbonkretsløpet, gjennom beregninger av dens evne til å ta opp karbon fra atmosfæren. Med dette er vi et skritt videre på veien mot et karbonbudsjett for blå skog. Metoden ble opprinnelig utviklet som en del av det Nordiske blått karbonprosjektet (2017–2020).

Etablerte metoder, som stabile isotoper, pigmenter og lipidanalyse, har vist seg å være utilstrekkelige når det gjelder å påvise karbon fra tare i marine sedimenter. Derfor har man til nå ikke klart å skaffe gode tall på tareskogens rolle i langsiktig karbonlagring (sekvestrering).

Miljø-DNA defineres som genetisk materiale hentet direkte fra miljøprøver (jord, sediment, vann, osv.) uten noen åpenbare tegn på biologisk kildemateriale. Utviklingen av denne metoden har gitt forskere nye verktøy for artsspesifikk sporing av organismer i ulike miljø og over tid. Metoden er universell i den forstand at alle DNA-spor samles og renses. Den påfølgende DNA-analysen vil da fastslå om målgruppen er til stede eller ikke, for eksempel ved bruke av spesifikke qPCR-protokoller.

Metodikken som er beskrevet her inkluderer retningslinjer for prøvetaking av marine sedimentkjerner, samt transport og oppbevaring i tilknytning til dette. Videre gis en beskrivelse av hvordan organisk taremateriale kan identifiseres i marine sedimenter ved hjelp av eDNA. Spesielt viser rapporten hvordan nye eDNA-metoder brukes for artsspesifikk sporing av tare-DNA i sedimenter. I denne studien har vi brukt en DNA-strekkodemarkør [cytokrom oksidase underenhet-1 (COI)-gen] og utviklet artsspesifikke primere som brukes til å identifisere de to vanligste artene av tare i Norge, som er stortare (*Laminaria hyperborea*) og sukkertare (*Saccharina latissima*). «Kit» for DNA-ekstraksjon og polymerase ble omhyggelig valgt for å redusere falske negativer. Det ble også benyttet negative kontroller, for å unngå falske positive svar og for å validere positive deteksjoner. Videre ble de første skritt tatt i retning av å utvikle en kvantitativ teknikk for kvantifisering av organisk tarekarbon fra sedimentkjerner. Sistnevnte er langt fra trivielt, men i prinsippet mulig gjennom å koble mengden av makroalge-DNA til -biomasse.

Denne nye metoden muliggjør identifisering av karbonavsetning og langsiktig lagring fra artsspesifikke makroalger i marine sedimenter. Metoden kan videreutvikles for å spore karbonkilder gjennom marine næringsnett, for eksempel for å kvantifisere viktigheten av makroalger som diett for dyreplankton eller andre filtermatere i vannsøylen eller på havbunnen.

En detaljert laboratorieprotokoll gjøres også tilgjengelig på protocols.io, "MarineSediment-BlueC-eDNA", doi: 10.17504/protocols.io.btk4nkyw.

1 Introduction

Current needs for blue carbon accounting in the marine realm
Over the last few years, considerable global research efforts have focused on understanding the flux and transport of carbon in vegetated coastal ecosystems, and quantifying the capacity of these systems to act as sinks for atmospheric carbon (Nellemann et al. 2009; Mcleod et al. 2011). Often, vegetated coastal ecosystems are referred to as **blue forests** or **blue carbon ecosystems** (Moritsch et al. 2021). While regrowth or restoration of blue forest habitats could increase the natural sink capacity regionally and globally, a similar reduction in the distribution could negatively impact the natural sink capacity and potentially reduce the carbon draw-down from the atmosphere (Pörtner et al. 2019). Recognition of this ability has led to the development of strategies for climate mitigation through the conservation and restoration of blue forests (Hoegh-Guldberg 2019), termed blue carbon strategies, and to the construction of blue carbon budgets for these important vegetated coastal habitats (Frigstad et al. 2021).

The role of blue carbon in climate regulation and the link to mitigation actions
Inorganic carbon that is taken up in coastal vegetated systems and transformed into organic carbon pools in kelp forests, rockweed beds, seagrass meadows, salt marshes, and mangroves is referred to as **blue carbon** (Figure 1). The residence time of blue carbon in the ocean interior and sediments determines its role in the regulation of the climate system, and if retained on longer time scales (centuries) the **carbon is defined as sequestered** (equivalent term: **long-term carbon storage**). An important carbon sequestration process is the sedimentation and long-term burial of particulate organic matter from blue forests, including organic biomass from kelp forests (Krause-Jensen and Duarte 2016). In a recent blue forest carbon budget for the Nordic region, the sequestration of kelp particulate organic matter is estimated to ~500.000 tonnes CO_2 per year (Frigstad et al. 2020). On global scales, macroalgae sequestration has been estimated to account for >600 Million tonnes CO_2 per year (Krause-Jensen and Duarte 2016). Kelp forests can export more than 80% of its biomass per year, as for instance documented for Norway (Pedersen et al. 2020). Of this export the major part is either grazed by fauna and utilized as a food source in the food web or remineralized by bacteria. However, a smaller fraction escapes this fate and is eventually buried in softbottom seafloor sediments and sequestered. Thus, marine sediments represent a natural sink of carbon, however how large the sequestration is per year and the seasonal and yearly dynamics remains largely unknown (Gundersen et al. 2021; Frigstad et al. 2021).

Figure 1 Forests of tangle kelp (*Laminaria hyperborea*, left) and sugar kelp (*Saccharina latissima*, right) at the west coast of Norway (Photo: Hartvig Christie, NIVA)

Environmental DNA as a solution to identify and trace blue carbon in marine sediments

Traditional techniques, such as stable isotopes, pigments and lipid tracers, have all been documented to underperform when it comes to the detection and identification of kelp carbon in marine sediments. They are also lacking the necessary precision, robustness and reproducibility (Ortega, Geraldi, and Duarte 2020). The assessment of carbon sequestration associated with blue forests is based on the quantification of the pool of organic carbon stored in sediments under – as in the case of seagrass meadows – or in the vicinity to the blue forests – such as kelp forests and rockweed beds, since they grow on hard substrate. With concurrent dating of sediment cores using ^{210}Pb (lead) and ^{137}Cs (caesium) for short time scales, and/or ^{14}C (carbon) for longer timescales, it is possible to estimate the burial and sequestration rates of organic carbon. In seagrass meadow, the accumulating total organic carbon pool in the sediment below the vegetation has been assumed equal to the seagrass carbon sequestration rate. However, for kelp and rockweeds the organic carbon is exported outside the habitat in which they grow, and the macroalgae carbon is thus mixed up with organic carbon from other sources (e.g. terrestrial, phytoplankton and other marine organisms). Thus, a need to identify the fraction of the total carbon that originates from kelp is critical for accurate carbon accountings and sequestration estimates.

Environmental DNA (eDNA) methods (Thomsen and Willerslev 2015), which emerged more than 20 years ago (Taberlet, Waits, and Luikart 1999), were initially applied for the detection of aquatic species, both in freshwater and marine environments, including detection of mammalians (Foote et al. 2012), fish (Jerde et al. 2011; Thomsen et al. 2012; Engesmo et al. 2019), molluscs (Goldberg et al. 2013), crustaceans (Mauvisseau et al. 2018; Strand et al. 2019), amphibians (Pilliod et al. 2013), reptiles (Piaggio et al. 2013), insects (Valentin et al. 2018) and plants (Fujiwara et al. 2016; Anglès d'Auriac, Strand, et al. 2019). The versatility of the method has led to a steady increase of applications, also bridging with another scientific field known as ancient DNA (Pedersen et al. 2015), for which the samples are historical, dating sometimes many centuries back, rather than fresh and contemporary. This approach has been used for various types of sediments and in particular marine sediments (Armbrecht et al. 2019).

A few recent studies have used eDNA tools for tracing marine macrophyte species in sediments. For instance a method for estimating seagrass carbon sequestration has been developed using specific probe real-time polymerase chain reaction (qPCR) and droplet digital PCR (ddPCR) assays (Hamaguchi et al. 2018). Another approach has used Next Generation Sequencing (NGS) metabarcoding for evaluating general macrophyte contribution to blue carbon in the surface sediments (Ortega et al. 2020; Ortega, Geraldi, and Duarte 2020). In this work, we have applied eDNA tools to alleviate previous limitations associated to specific kelp identification and its contribution to carbon sequestration in marine sediments (Frigstad et al. 2021). This includes using sediment samples dated as long back as several hundred years.

Aim of the report

The aim of this report is to deliver a hands-on protocol for collecting marine sediment core samples, extract eDNA material and identify kelp organic carbon in marine sediments using species-specific DNA barcode primers – here specifically for identification of tangle kelp (*Laminaria hyperborea*) and sugar kelp (*Saccharina latissima*). The report describes in detail the applied process and methodology, from core sediment sampling, transport and storage of samples, preparation of the subsamples for the laboratory work, extraction of DNA, running qPCR, and interpretation of data.

The purpose is to communicate this novel technique to relevant stakeholders and ensure successful application and reproducibility of the method. Strengths and weaknesses of the method are discussed, and future improvements and applications are recommended.

2 Methods

2.1 Core sampling

Sediment cores analyzed in the Nordic Blue Carbon Project were collected from the seafloor in Frohavet outside Trøndelag, mid-Norway, which is a sea area between the Fosen Peninsula and the island of Froan (63–64°N). It is a highly dynamic coastal environment with high primary productivity coming from both benthic macroalgae (kelp and rockweed) and phytoplankton in the water column. The main basin covers around 1300 km^2, with water depths ranging from 100 to 500 m. In total 13 sediment cores were sampled in October 2018 from the NTNU research vessel R/V Gunnerus. Of these 13 cores, four were used to develop and test the novel eDNA method for tracing and identifying kelp with the purpose of estimating long-term sequestration of kelp organic carbon in the sediments. The cores were sampled across a transect with water depths between 242 and 531 m. The sediment type was silt and very fine sand. The length of the four cores that were sampled ranged from 61 to 121 cm, depending on the penetrability of the seafloor.

2.1.1 Equipment used for core sampling

Sediment cores were sampled using a KC Denmark Gravity Corer (Figure 2a). A gravity corer is basically an open tube fitted with a weight so that gravity can force it sufficiently deep into the seafloor in order to retrieve a column of undisturbed sediment. The diameter of the KC Denmark Gravity Corer is 88.9 mm, and is designed with several detachable core tubes each with a length of 150 cm.
The corer is made from stainless steel and consists of a corer body with 6 steering fins and a weight platform, steel corer, internal PVC tube (preferably transparent), lead weights, a core catcher (or so called “orange peel closing system”) and a carver with cutting edge (nose piece) (Figure 2b & c). For a detailed description to how to assemble the KC Denmark Gravity Corer see Table 1 or online manual.

Table 1 Assembling the KC Denmark Gravity Corer (link to manual)

1. Add the necessary number of lead weights at the main rack.
2. Mount the supporting frame and secure it by fastening the bolt.
3. Mount the unit with the top lid. Open the fastening device and push the tube with the mechanical stop into the bottom. Lock the handles and secure with a bolt.
4. Attach the carver and fasten with two nails.

Other required equipment includes nails, hammer, cutting tools, ruler, PVC/gaff tape, waterproof writing utensils and safety equipment. In order to handle the gravity corer in a safe way, the vessel must be equipped with hydraulic cranes and winches capable of lifting the corer, that weights up to 290kg, out of the sediments. Also, the rope or wire used for lifting must be strong enough to support the heavy equipment. Preferably, the vessel may also be equipped with a dynamic positioning system or at least have sufficiently maneuverable abilities, so that the vessel can stay in the same position during sampling.

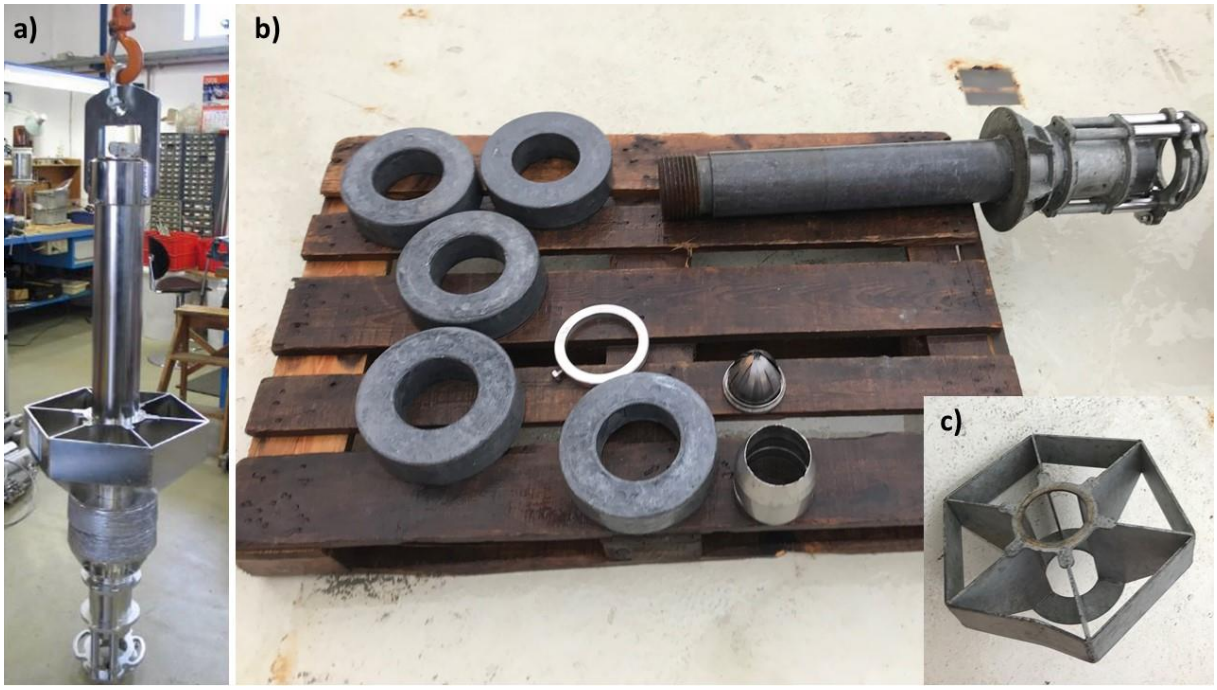


Figure 2 a) Fully assembled KC Denmark Gravity Corer (Photo: www.kc-denmark.dk) b) Corer body, five lead weights, core catcher ("orange peel closing system") and cutting edge (nose piece) (Photo: NIVA) c) Steering fins (Photo: NIVA).

2.1.2 Core sampling and processing on vessel

The gravity corer was lowered into the sea and towards the sea floor at a controlled speed until it reached 10m above the sea floor at which point it was left to freefall (Figure 3a). The corer penetrates the sediments by gravity, and compact, fine-grained sediment will be retained in the corer. The core catcher was not used in this project, as it entails a risk of physically disturb the sediment surface. A top lid is released when the corer hits the sea floor in high speed and close off the top of the corer. Upon retrieval of the corer, a vacuum is then created that retain the sediment core inside the corer during retrieval, given that the corer is handled with caution when exiting the sea water and entering the vessel. This is crucial to avoid the sediment from falling out, especially with coarser sediment types such as sand.

Once the corer entered the vessel, the carver was removed (Figure 3 Figure 3b and c) and the bottom of the internal PVC tube was sealed with a tight-fitting plastic cap and secured with waterproof tape. The internal tube was removed carefully from the corer in an upright position in order to keep the sediment-water interface intact. The tube, with the sediment core inside, was capped at both ends and secured with waterproof tape. The cores were stored and transported with sea water from the sampling at the top, and care was taken to avoid air being trapped between the sea water and the cap to ensure an undisturbed sediment surface during transport.

The internal PVC tube, that collects the core, was new for every cast. The corer, nose piece and other equipment was washed with running sea water from a medium pressure hose on deck between every core cast. The travel through 500 meters of water column between each cast must also be regarded as part of the cleaning process.

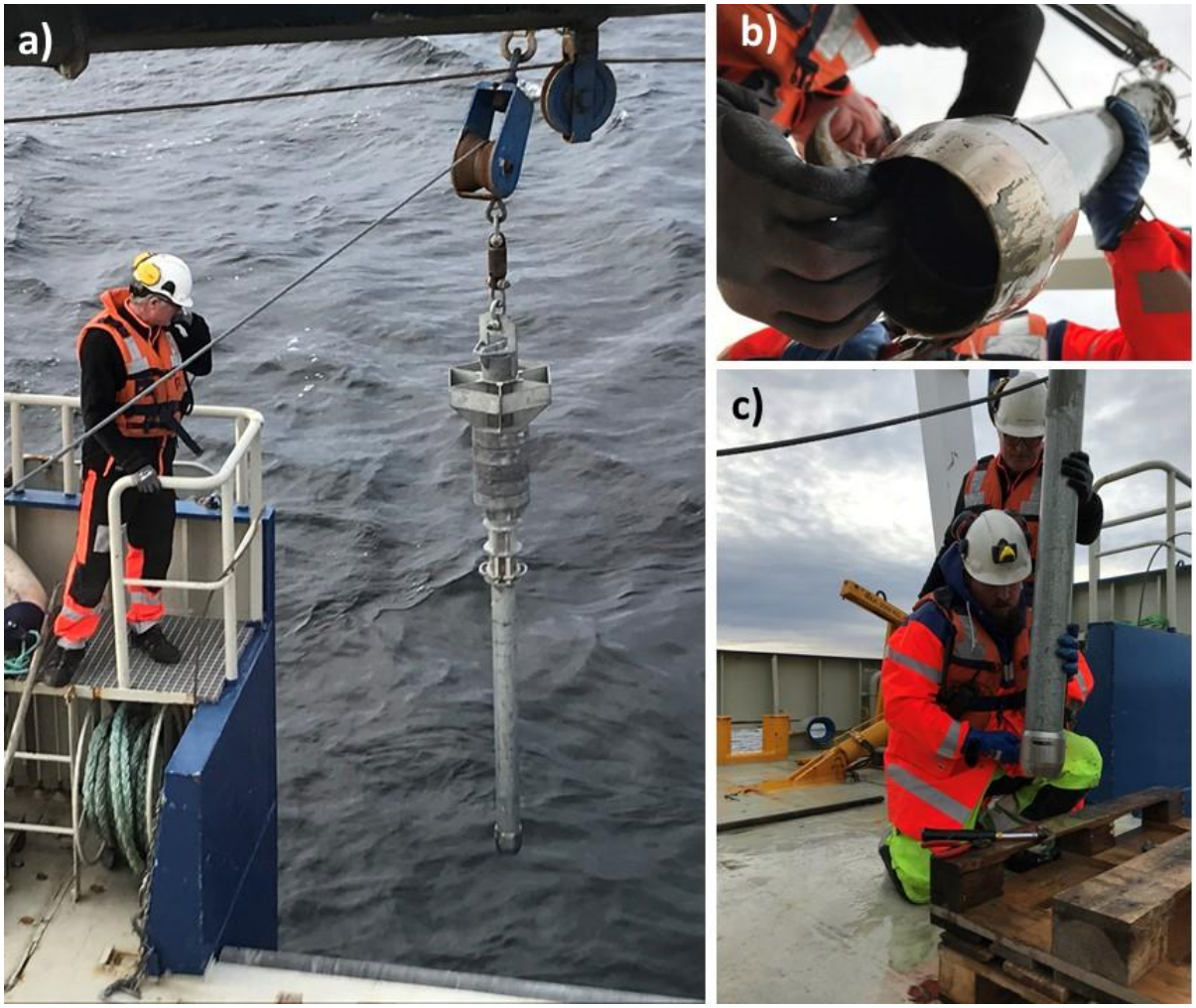


Figure 3 Launching of the gravity corer (a) and details showing the carver be taken off by removing the two nails with a hammer (b and c). Photo: K. Hancke & G. Borgersen (NIVA).

2.1.3 Core sample transportation and storage

Each core was photographed in order to record basic characteristics of sedimentary layers while the sediment was fresh, and to be able to identify any major disturbances during transport (Figure 4a). Cores with a disturbed sediment surface, or non-intact for any other reason, were discarded immediately after sampling. Intact sealed-cores were transported and stored standing up in a predesigned box (Figure 4c) and were maintained in their PVC tubes until they were opened in the laboratory for further analysis. They were stored undisturbed in the dark at 4 °C until processed 11,5 weeks later.

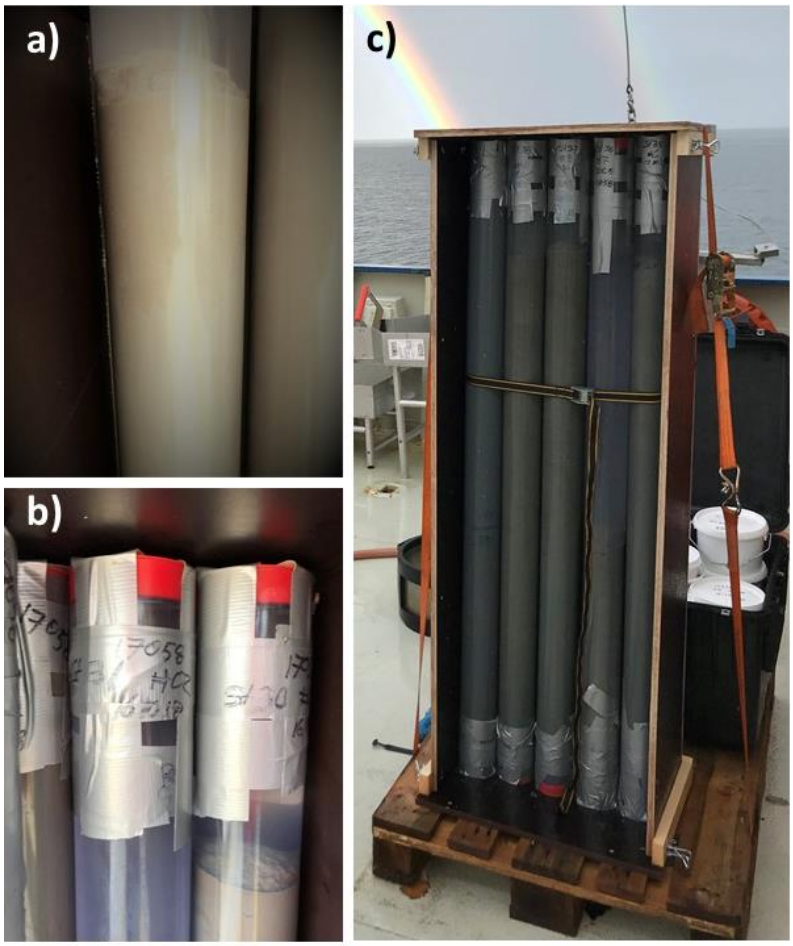
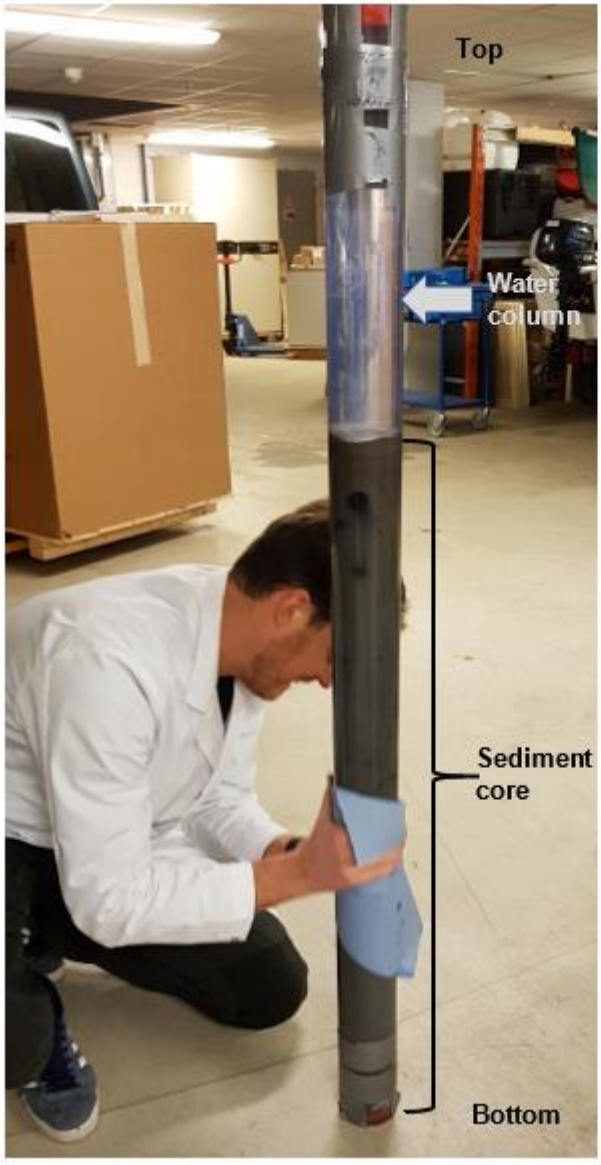


Figure 4 Each sediment core was photographed (a). Thereafter the internal PVC tube was sealed at both ends with a tight-fitting plastic cap and secured with waterproof tape (b) and placed vertically in a box predesigned for transport and storage (c).

2.2 Core processing

The sediment cores were processed in NIVAs premises (Figure 5). The PVC tube was opened at both ends and placed on top of a plunger fitting the inner diameter of the tube. The tube was pushed slowly and carefully downward until all the overlying seawater had spilled over the top and the sediment surface was even with the top of the tube. The tube was then pushed further down so that a 1 cm thick section of the sediment core could be sliced off with a clean steel plate (slicer) (Figure 6). In this way, cores can be sliced at desired thickness (representing a range of year of sediment accumulation) and further subsampled for targeted analysis, see below and Figure 6. Each core was sliced into 1 cm thick sections, until we reached down to 50 cm depth to make sure to cover at least that past 150 years of sediment accumulations. From each core, 12 sections were chosen for further analysis: 0–1, 2–3, 4–5, 6–7, 8–9, 10–11, 13–14, 17–18, 22–23, 28–29, 35–36, 43–44 cm depth layers, with fewer sampling on the oldest (deepest) layers, as we expected a consistent reduction of total eDNA towards the bottom of the core.



Upper section of the sediment core

Figure 5 Example of an intact sediment core >100 cm long when back in the lab and ready to be subsampled in 1 cm thick slices. The sediment surface and core were examined prior to subsampling to ensure that it was intact. The sediment cores were sealed at both ends, with overlying seawater filling up the top part of the tube (left).

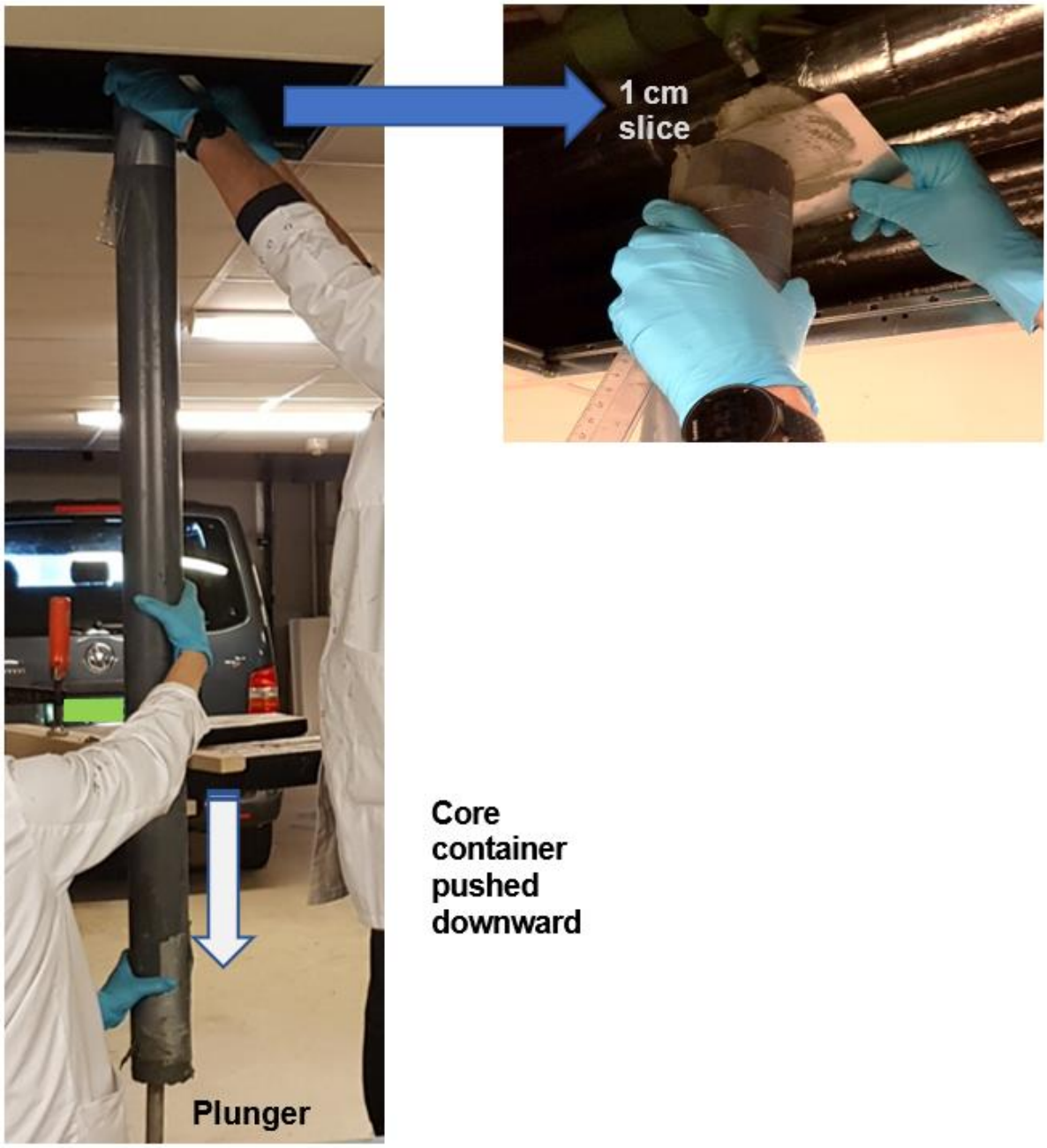


Figure 6 The PVC tubes were placed on top of a plunger and pushed slowly and carefully downward until all the overlying seawater had spilled over the top and the sediment surface was even with the top of the tube. The tube was then pushed further down so that a 1 cm thick section of the sediment core could be sliced off with a steel plate (slicer). If not taking into consideration the height of the plunger and the tube, it may be necessary to remove parts of the garage ceiling to get the job done.

Each 1 cm slice was portioned into equal parts (i.e. aliquoted) for different analyses (Table 2 and Figure 7). The equipment was rinsed with ethanol (95 - 100%) between each core slice and the inner part of each slice section was selected for sampling in order to further reduce the risk of cross contamination.

Table 2 Sediment slices were prepared for the following analyses

1. Sediment dating (^{210}Pb)
2. Total organic carbon and total carbon content
3. Chlorophyll-a content
4. eDNA (quantitative PCR)
5. 13C and 15N stable isotopes

Figure 7 Each 1cm thick sediment slice was aliquoted for different analyses

Sediment dating (^{210}Pb)

The 210Pb dating technique (Arias-Ortiz et al. 2018) was used to determine the chronology of each core and to estimate the sediment age of the different sediment layers. The method is accurate for sediment age up to approximately 120 years and will also provide sedimentation rates for each layer. The Department of Geosciences and Natural Resource Management at the University of Copenhagen

carried out the 210Pb analysis and sediment dating. Sediment age for layers older than 120 years was extrapolated based on a function derived from a polynomial curve with R2 > 0.99.

Total organic carbon and total carbon content
Total organic carbon (TOC) and total carbon (TC) content was analysed using a CHN (i.e. carbon, hydrogen and nitrogen) elemental analyser after removal of inorganic carbon by adding hydrochloric acid (HCl). Analysis was carried out in NIVA's laboratory following standard procedures for TOC/TC analysis. See Frigstad et al. 2020 for further details and results.

Chlorophyll-a content
Chlorophyll-a pigments were extracted from the sediments by adding a defined volume of 96% ethanol. Concentrations were determined spectrophotometrically (Parsons and Strickland 1963) using extinction coefficient as suggested by (Wintermans and De Mots 1965). Chlorophyll-a samples were analysed at the Department of Biology at the Norwegian University of Science and Technology (NTNU) following standard procedures for chlorophyll-a analysis. See Frigstad et al. 2021 for further details and results.

eDNA (quantitative PCR)
Samples for eDNA analysis (Figure 7, lower right corner) were put into 2.0 or 1.5mL tubes, that were completely filled and immediately frozen at -20^{o}C for preservation until DNA extraction was performed. Analysis was carried out in NIVA's laboratory, see the section below for details.

^{13}C and ^{15}N stable isotopes
Prior to the stable isotope analyses, the sediment samples were treated with acid to remove carbonates before they were washed in distilled water to remove acid residues. Samples were loaded into tin cups and analysed using Isotope Ratio Mass Spectrometry (IRMS). See Frigstad et al. 2021 for further details and results.

2.3 DNA extraction

Pure reference material was collected from living tangle and sugar kelp biomass near the city of Ålesund at the following geographic positions 62.709959 N / 6.342591 E (62°42'35.8524" N / 6°20'33.3276" E) and 62.778985 N / 6.476473 E (62°46'44.346" N / 6°28'35.3028"E), respectively. Collected material was preserved dry in silica beads (Figure 8). DNA extraction from the core samples as well as from the kelp reference material was performed using DNeasy PowerSoil Pro Kit (#47014 © QIAGEN), with about 250 mg of material (wet weight for the sediment) per sample following the user manual instructions. A final elution volume of 50µL was used. This kit helped mitigate the challenges posed by PCR inhibiting compounds found in environmental samples as well as in Laminariales macrophytes, which both tangle and sugar kelp belongs to (McDevit and Saunders 2009). After comparing with DNeasy PowerMax Soil kit (#12888-50 © QIAGEN) in this study, the DNeasy PowerSoil Pro Kit was found to provide the best results, in particular regarding sensitivity, i.e. improving the Limit Of Detection (LOD).

Figure 8 A few grams of *L. hyperborea* tissue material preserved in a 15mL Falcon tube filled with Silica beads

2.4 qPCR protocol

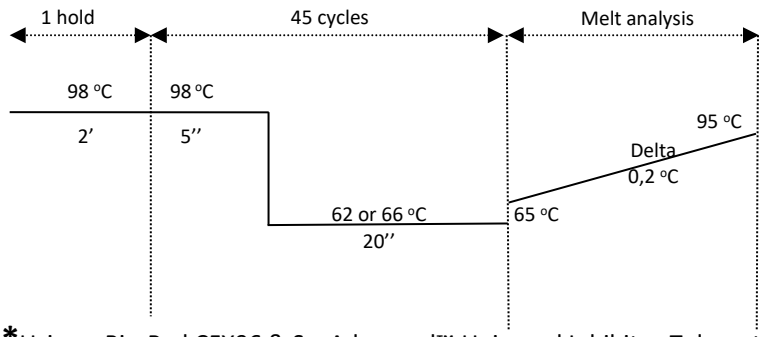
The qPCR protocol uses a kit with SYBR® staining of the amplified DNA, providing product specificity information by melt curve analysis. The Bio-Rad SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix (hereafter named "Supermix") kit was chosen for qPCR amplification as it is developed to tolerate inhibitors which are often present in environmental samples and provided best LOD and best specificity when tested in this study. As non-specific double stranded DNA dye is used in the assay for monitoring the product amplification in the qPCR assay, the specificity of the product(s) is assessed by using the melt curve analysis to ensure the right melt profile is generated, corresponding to the target amplicon.

A Bio-Rad CFX96 instrument was used to perform all qPCR assay optimization and sample testing. The assays annealing temperature were determined by running gradient qPCR on both extracted species reference material and sediment cores. For assay design it is important to take into account that DNA fragments show a pattern of degradation from approximately 350 base pair (bp) in the first 10 cm layer and approximately 100 bp in lower layers (Lejzerowicz et al. 2013). Therefore, assays producing the smallest amplicons, ideally less than 100 bp, were favoured in order to improve target detection from eDNA sample. Hence, smaller amplicon increases chances for amplification, and therefore detection, versus larger ones. For tangle kelp, a primer pair producing an 88 bp amplicon was successfully developed (Table 1). However, sugar kelp COI sequences were more challenging for designing specific oligos resulting in this work in a primer pair producing a 312 bp amplicon (Table 3). Specificity of the selected oligos was challenged in-silico and in the laboratory although only with the two studied related species *L. hyperborea* and *S. latissima*.

Table 3 Primer information

Target	Species specificity	Primer name	Primers (Forward & Reverse)	Ta. °C	Product (bp)	Reference
cytochrome oxidase subunit-1 (COI)	*Laminaria hyperborea* (tangle kelp)	L_hyper_coi471F20 L_hyper_coi538R21	CTCCCGGTATGACAATGGAT AAAACAGGAAGCGATAACAGT	62	88	This protocol
cytochrome oxidase subunit-1 (COI)	*Saccharina latissima* (sugar kelp)	S_lat_coi56F19 S_lat_coi350R18	ATTCGTTTGCAATTGGCTA GTGTGCCTGAATACCACT	66	312	This protocol

A two-step amplification protocol was used for both assays (Figure 9) with an initial denaturation step at 98 °C for 2 minutes followed with 45 cycles, 5 seconds 98 °C denaturation and 20 seconds elongation at 62 °C for tangle kelp and 66 °C for sugar kelp. A total mastermix reaction volume of 15 µL was used with 7.5 µL Supermix, with 0.75 µL for each of the two primers (final concentration 0.5 µM), a 1.5 µL sample, and completed with 4.5 µL sterilized pure water.



*Using a Bio-Rad CFX96 & SsoAdvanced™ Universal Inhibitor-Tolerant SYBR® Green Supermix

Figure 9 qPCR protocol

2.5 eDNA practices

Proper eDNA practices are essential for ensuring the validity of the results. Strict measures and controls need to be set in place to mitigate cross contamination risks. These precautions are implemented at all stages of the work, starting with the sampling in the field, transportation, sample processing, sample DNA extraction and finally qPCR analysis. All laboratory surfaces were wiped with hypochlorite 2% solution to neutralise DNA (Kampmann, Borsting, and Morling 2017), followed with a wipe using deionised water to neutralise hypochlorite residues. Cap-strips, rather than films, were used to enable progressive protection of the various plate positions. Reagents and mastermix (see 2.4) were prepared and dispatched in the PCR plate in a Laminar flow (LAF) bench. Mastermix negative controls, using deionized water, were dispensed and caped in the LAF-bench. The plate was thereafter transported to the sample laboratory. All field samples were then first dispensed and caped followed by at least 3 sample plate negative controls with deionized water. Finally, the standard samples, also used as positive controls, were dispensed and caped. White, rather than see-through PCR plates were used to provide increased sensitivity.

2.6 Quantification estimation rationale

Extracted DNA target kelp reference dry weight material was used for quantification estimation of the eDNA samples. The extracted DNA was eluted in 50µL volume, and DNA concentration was measured in ng/µL. This is the reference material used for calibrating the qPCR results by using serial dilutions using DNA concentration as quantification units. Kelp DNA concentration in the core eDNA samples can therefore directly be measured in the extracted samples using the standard curve. As the initial dry weight (of reference kelp material) used for the extraction is known, it can potentially be used as calibration units. For example, 10 mg dry weight for a final 50 µL eluate will correspond to 0.2 mg dry weight/µL eluate used in the calibration curves. As the core samples were "wet" when weighted and extracted, a correction factor of x2 was used as the water content of these field sediment core samples in this study was 50%.
This is an approximation as DNA extraction from dry tissue and from sediment will most likely not have the same extraction efficiency, the latter assumed to be less efficient. A correction factor of 10 was used to account for this difference. Another limitation is the weathering of DNA which increases with age while organic carbon remains stable. This is exemplified in Figure 13 in (Frigstad et al. 2021), which shows relatively little variation between different core depths for TOC quantities, while total DNA consistently diminishes with increased core depths. A correction factor of 20 was assumed to account for DNA degradation.

3 Results & discussion

The outcome of using this protocol and thus the results of the Nordic Blue Carbon project has been published already in Frigstad et al. (2021). In this section we have included the most central results from Frigstad et al. (2020) but complemented with more of the intermediate results from the development and testing of the eDNA tracing method, to document the development and performance of the method.

3.1 Species-specific kelp qPCR assays

We developed and tested several specific qPCR assays for *L. hyperborea* and *S. latissima* for demonstrating and tracing their presence in sediment cores, and by doing so assess their contribution to carbon sequestration. Standard curves using calibrated reference material were used to roughly estimate kelp mass and hence relate the amount of eDNA to the kelp carbon biomass. To our knowledge, no species-specific qPCR assay has previously been developed for tangle kelp and sugar kelp.

The qPCR assay for tangle kelp showed excellent performance with an efficiency comprised between 100.2% and 101.1%, and a correlation coefficient R^2 of 0.999 of the calibration curves (example given in Figure 10). An optimal annealing temperature of 62 ^{o}C was chosen based on the results from the gradient qPCR experiment which did not compromise specificity when tested against sugar kelp. The sugar kelp assay was less performant with an efficiency around 77% and an R^2 of 0.993 of the calibration curves (example given in Figure 11). As the assay showed some cross amplification when tested against tangle kelp, the chosen annealing temperature was set to 66 ^{o}C to improve specificity, albeit reducing the sensitivity of the assay.

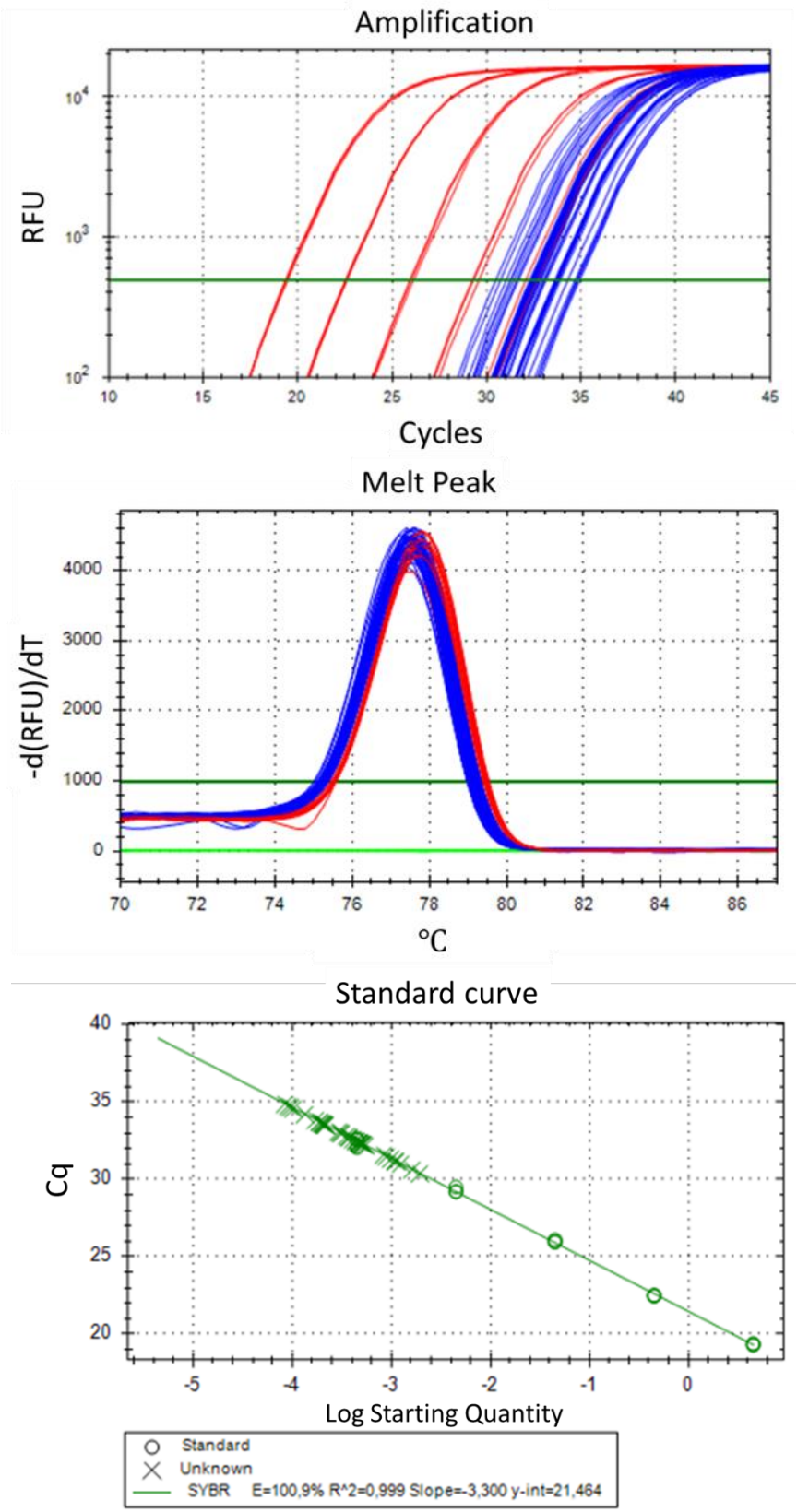


Figure 10 qPCR results example for *L. hyperborea*, standard curve and extracted sediment eDNA samples. Red = standard *L. hyperborea* serial dilutions starting at 4,5 ng/µL; Blue = sediment core samples; Green = Mastermix & sample plate negative controls.

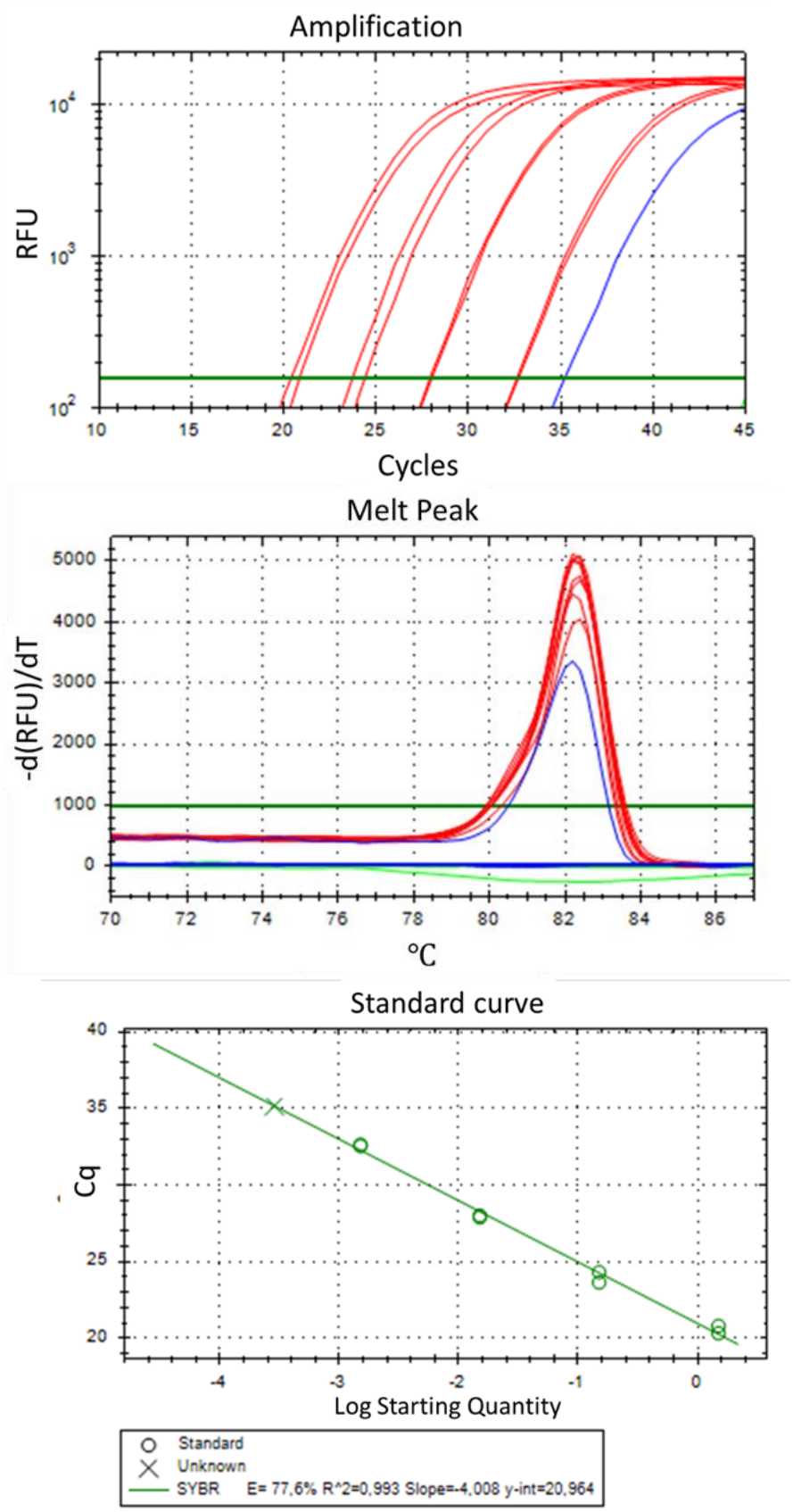


Figure 11 qPCR results example for *S. latissima*, standard curve and extracted sediment eDNA samples. Red = standard *S. latissima* serial dilutions starting at 1,5 ng/µL. Blue = sediment core samples. Green = Mastermix & sample plate negative controls.

3.2 eDNA sediment analysis

Overall results for both species' detection and relative amount of kelp quantified in species-specific DNA ng/g wet sediment samples are shown in Figure 12. Conversion to DNA ng/g dry sediment was performed by accounting for an average water content of 50%. All sampled cores are presented as a function of age (year of sedimentation).

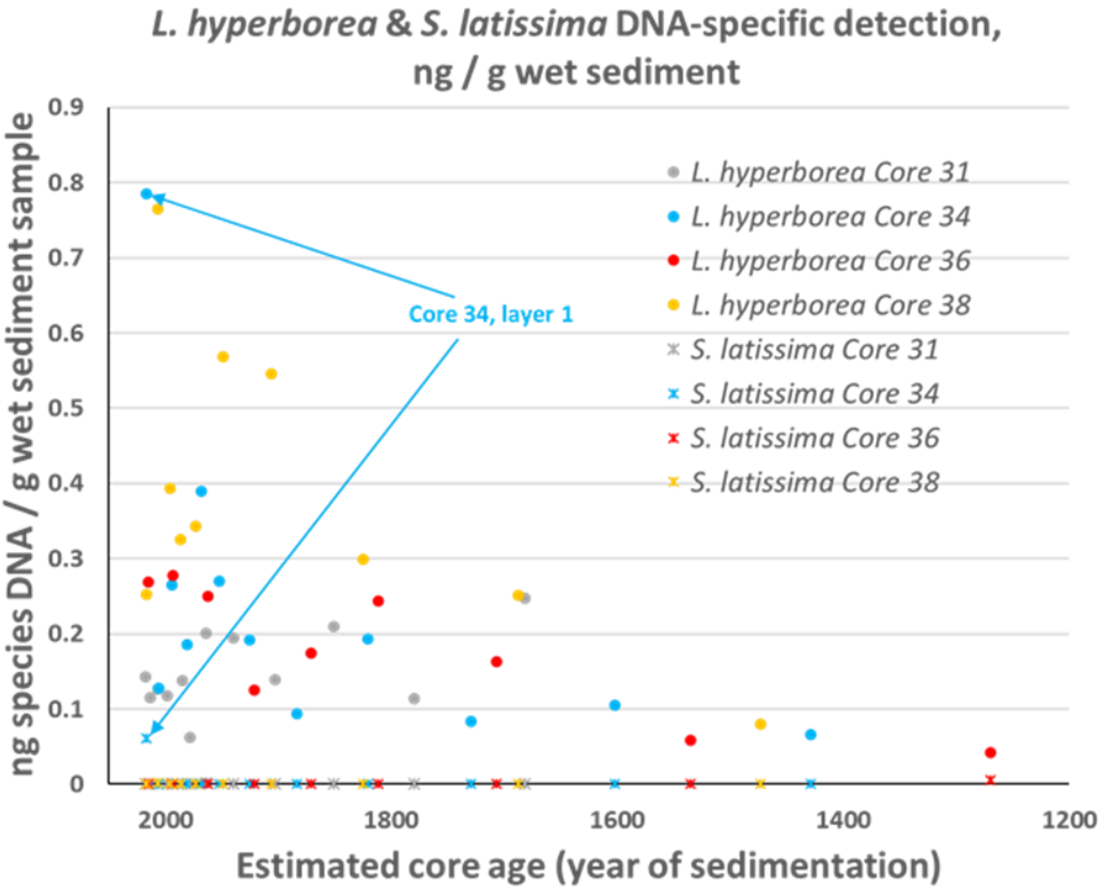


Figure 12 The DNA-specific detection, measured as the proportion of species-specific DNA (ng DNA per g dry sediment) for tangle kelp (*Laminaria hyperborea*) and sugar kelp (*Saccharina latissima*) against the estimated age of the sediments in the cores from the four studied stations in Frohavet, Trøndelag (NW coast of Norway, 63-64°N), described in more detail in Figure 13 and Figure 14. Highest DNA concentration for both kelp species is found in the same sample (Core 34, layer 0-1 cm).

The DNA analysis showed presence of tangle kelp in 47 out of 48 eDNA samples among the four collected cores. A general pattern of the relative quantity of tangle kelp eDNA was a decrease with sediment depth and thus with age of the sediment layer (Figure 12 & Figure 13). In contrast to tangle kelp, sugar kelp was only detected in 6 out of 48 eDNA samples among the four collected cores samples (Figure 12 & Figure 14). The results could indicate a much lesser presence of sugar kelp in these coastal sediments. However, the lower qPCR efficiency as well as amplification of a larger amplicon for the sugar kelp qPCR assay (312 bp compared to the tangle kelp assay of 88 bp), may likely have biased the results towards less signal from sugar kelp relative to tangle kelp. Indeed, it has

also been previously reported that most DNA fragments found in marine ancient sediments are less than 100 bp (Lejzerowicz et al. 2013).

Two stations, #34 and #38, showed higher relative quantities of sugar kelp and in particular the surface layer (0-1 cm) at station 34 showed high relative quantities for both targeted species. As DNA will be more fragmented with time, and therefore in older sediment layers, higher kelp DNA quantities are expected in the top layers, although historical kelp coverage may change this trend. As DNA will be more fragmented with time down the core, it is expected to find higher quantities on the top layer, as long as historical kelp coverage has been stable. These trends were also observed with relative quantities of unspecific total DNA, decreasing with age of the core, indicating a general breakdown of DNA with sediment age (Figure 15). The highest total DNA quantities, reaching up to 12.8 µg DNA per g dry sediment from the upper sediment layers (see Figure 15), are in the range of reported measures (Corinaldesi, Danovaro, and Dell'Anno 2005; Dell'Anno et al. 1999).

For kelp carbon quantification, the qPCR standard curves, built using serial dilution of standardized reference kelp DNA concentrations, are converted to mg organic carbon using the dry weight of DNA-extracted reference material. By using a correction factor of 10 for reduced extraction efficiency and 20 for DNA degradation (see 2.6), the qPCR calculation for organic carbon quantities from tangle kelp varies from 10% to 32% of the measured TOC, as shown in Figure 16. Further research is warranted to confirm and adjust the assumptions made for these calculations.

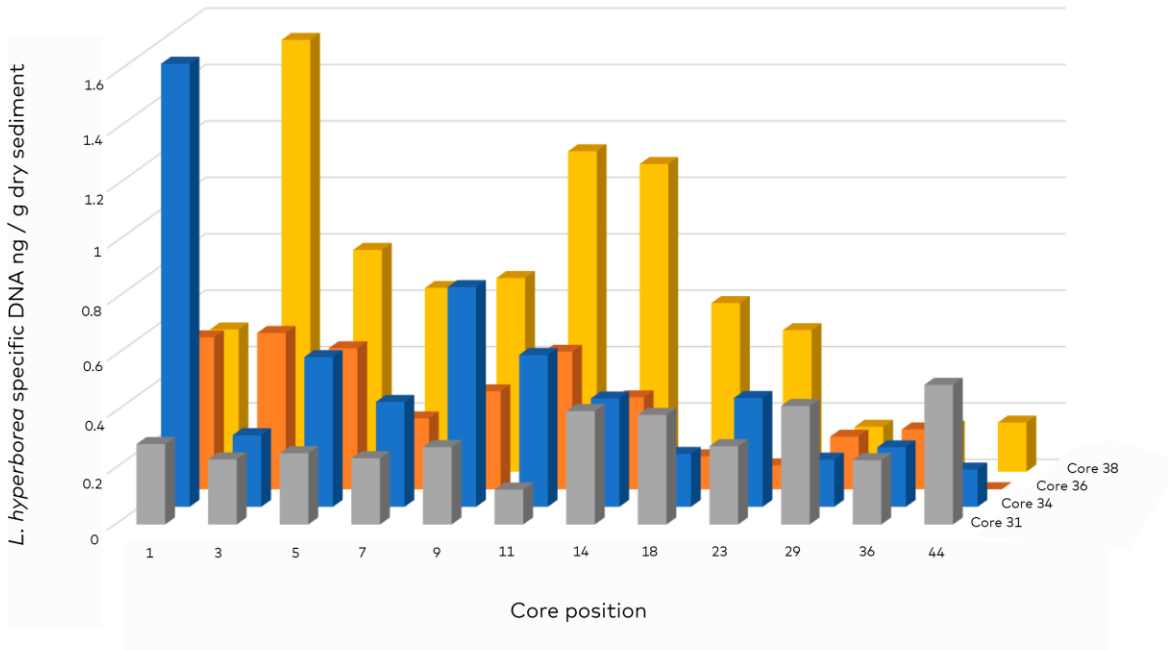


Figure 13 An estimate of the proportion of tangle kelp (*Laminaria hyperborea*) specific DNA (ng DNA per g dry sediment) for each core sample (i.e. the depth in the sediment core, in cm). The different colors represent the cores from the four stations sampled.

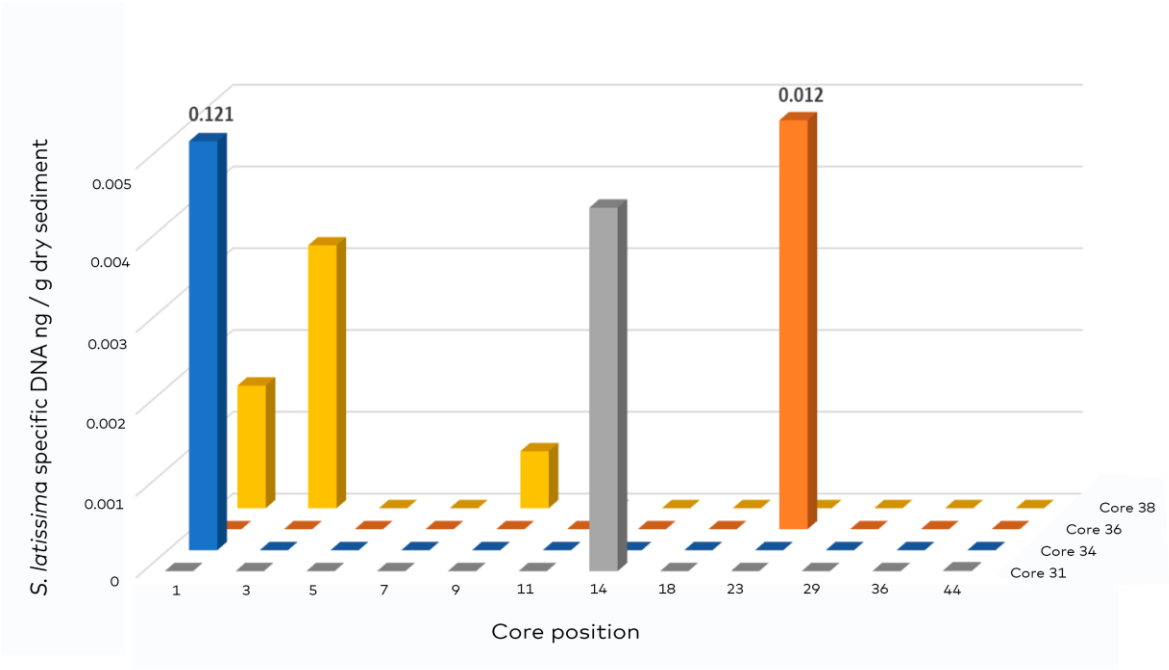


Figure 14 An estimate of the proportion of sugar kelp (*Saccharina latissima*) specific DNA (ng DNA per g dry sediment) for each core sample (i.e. the depth in the sediment core, in cm). The different colors represent the cores from the four stations sampled.

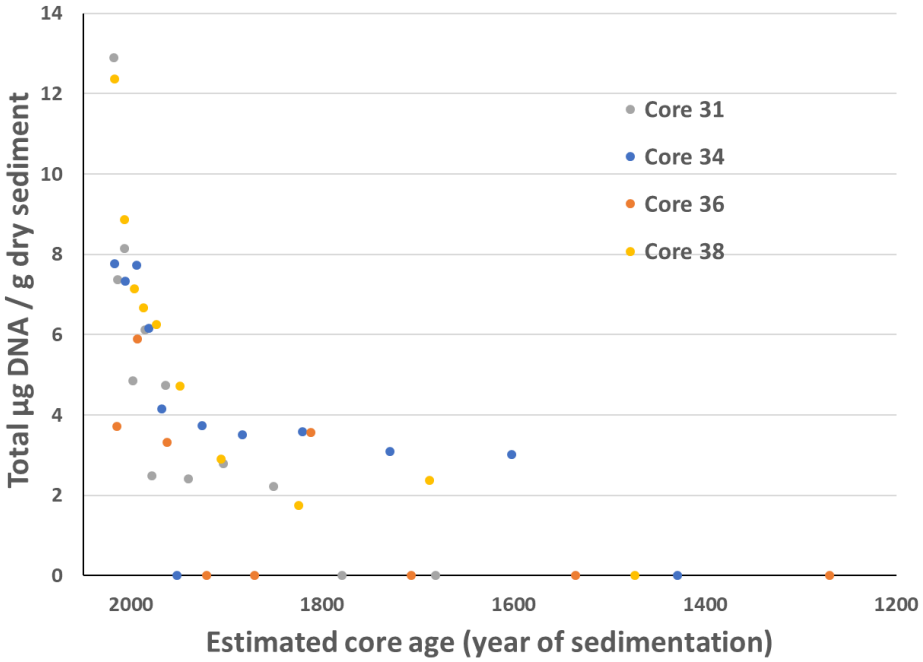


Figure 15 Total unspecific DNA concentrations, measured in µg/g dry sediment, against the estimated age of the sediments in the cores from the four studied stations in Frohavet, Trøndelag (NW coast of Norway, 63-64°N). A cut-off value of 4 ng/µL was used for the measurements of the eluates performed using NanoDrop™. Measurements under the cut-off (4 ng/µL) are considered unreliable and therefore plotted as "0".

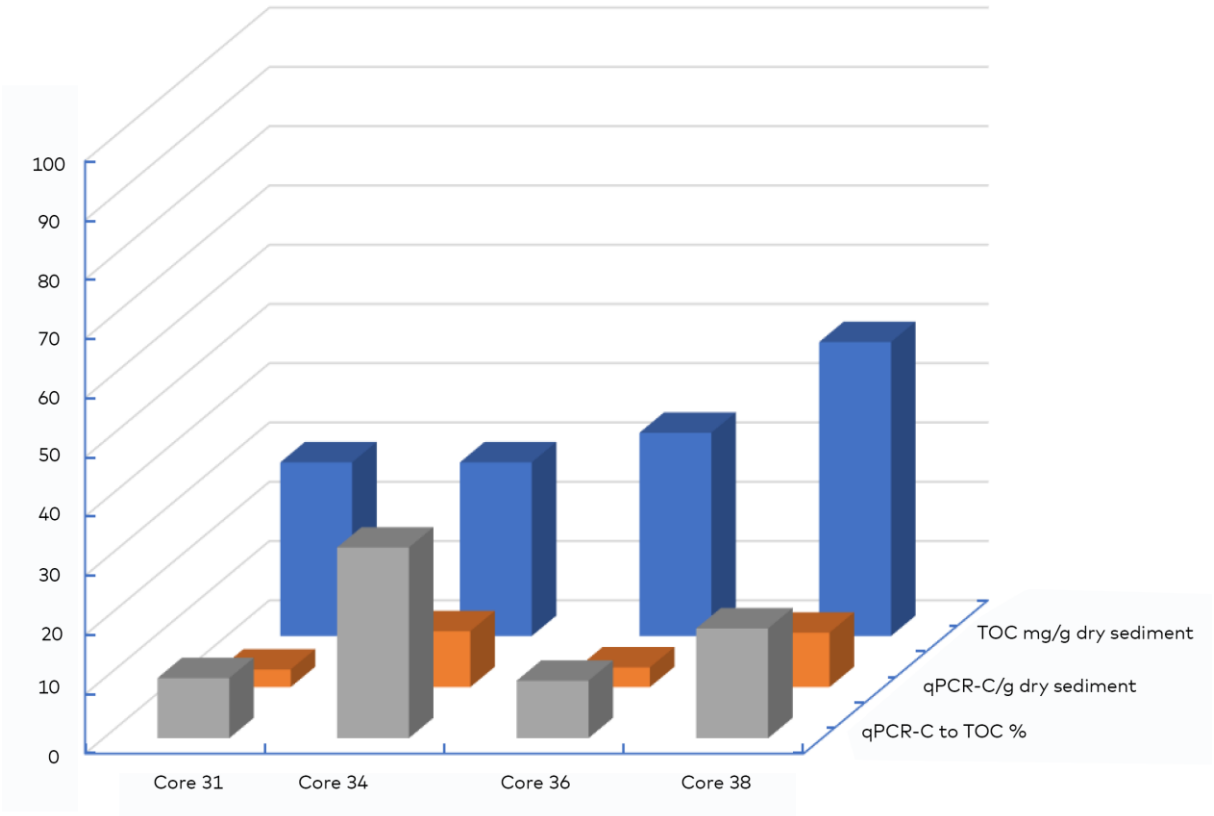


Figure 16 The blue bars show the total organic carbon (TOC) content (mg/g) measured in the sediment cores from the four stations in Frohavet, Trøndelag (NW coast of Norway, 63–64°N). The orange bars show an estimation of organic carbon from tangle kelp measured by species-specific qPCR. A correction factor was used for calculating this estimation (see text). The core position giving the highest qPCR measurement was used. Grey bars indicate the fraction (%) of the total organic carbon (TOC) that originates from tangle kelp.

3.3 Limitations on the current method design

Extracting core samples and transporting them from the ship to the laboratory is both expensive and labor intensive. Solutions may be developed for preparation of the sub-samples core layers on board of the ship, when possible, to avoid the transportation of the whole cores back to land laboratory. This would require appropriate facility on board to ensure adequate procedures are followed to avoid cross contamination. This would also require freezing of the sub-samples on board the ship and during transportation. Possible improvements to avoid the requirement of freezing might be devised (see 4.1). Further, historical analysis of kelp blue carbon storage through sediment layers may not necessarily be sought when only assessing if blue forests carbon is present in sediment and thus used to indicate the involvement of blue forests in marine carbon burial and sequestration in coastal sediments. Surface grabs may be sufficient for that purpose. Currently NIVA is leading an initiative to test this eDNA approach and are for the field season 2021 conducting a survey along the Norwegian coast to assess the geographical distribution of kelp carbon contributions to the marine sediment carbon pool (the project is named KELPCOAST).
Regarding the molecular assays, experimental data has shown that the *S. latissima* qPCR assay is not optimal, lacking both efficiency, specificity as well as sensitivity in connection to the larger amplicon it produces, 312 bp, when less than 100 bp is recommended when working with eDNA samples. These assay limitations, as discussed, may account for the weak detection of *S. latissima* compared with *L. hyperborea* rather than representing a biological fact. We therefore only included the *L. hyperborea* assay in protocols.io and would recommend developing a new *S. latissima* specific qPCR assay.

4 Perspectives

4.1 Alternative solutions for sample storage and transport

As mentioned in 3.3, grab samples, instead of cores, may be satisfactory for assessing the presence of blue carbon in marine sediments and thus offer a simple sampling approach to map the spatial distribution of blue carbon in costal and offshore marine sediments. Transportation, according to the current protocol, requires freezing on board the ship and during the transportation which may be operationally challenging with the risk of sample integrity disruption upon failure of the cooling chain. Alternative solutions may be devised to enable room temperature transportation by using preservation buffers to protect DNA from degradation. Such approaches have been used for eDNA related work, although for water filtrated filter samples (Engesmo et al. 2019; Spens et al. 2016). Sediment samples are considerably more complex to handle and using preservation buffers will require careful design and testing but holds good potential to simplify and boost research in this domain.

4.2 Future application of the technique

Single species detection was developed in this work which is demonstrated to be a powerful technique to trace and target the contribution of specific species to the total carbon pool in marine sediments. Such analysis requires up to 5 µL eluate, run in triplicates. In this study a total amount of 50 µL was used to concentrate eDNA as only two species were targeted. When several species are required for the analysis, a higher eluate volume may be used, as for example 150 µL, to enable the detection of additional species, up to 10 using this scenario. When a wider array of species is studied, including “blind” search for of species with unknown presence knowledge, metabarcoding using NGS technology may be useful. However, traditional metabarcoding typically targets amplicon larger than 600 bp, which is a drawback when analysing environmental samples or museum specimens with degraded DNA. Reducing the size of the targeted amplicon by developing mini barcodes has been studied to increases amplification rates. Initial studies have shown that enough information is retained for taxonomic identification of moth and wasps (Hajibabaei et al. 2006). Similar approach was successfully used for identification of coralline algae individuals (Anglès d’Auriac, Le Gall, et al. 2019). Finally, 18S mini barcode was developed for distinguishing major lineages of marine macrophytes from marine sediments using metabarcoding (Ortega et al. 2020), although the authors indicate that taxonomic resolution remains unsatisfactory. Metabarcoding using mini barcodes holds great potential for wide taxonomic screening from marine sediments. However, quantification using metabarcoding remains challenging related to primer and qPCR bias (Ortega, Geraldi, and Duarte 2020). Single species qPCR will probably remain useful for developing quantitative estimation of carbon pool contribution from defined species. For that purpose, ddPCR technology enables absolute quantification of the amplified target, and was applied by Hamaguchi et al. (2018) for the detection and quantification of seagrass eDNA from coastal sediments. Although the technology also holds limitations, as indicated by these authors, it is a promising approach that merits to be pursued. However, reliable eDNA quantification remain to be correlated to the carbon biomass it is associated with, in order to estimate the carbon pool contribution of the targeted species. In this study we have established standard curves calibrated against the dry weight material used for DNA extraction, and further applied it for analysis of the eDNA samples, with additional approximative correction factors. This is a first step taken for estimating sequestered kelp biomass based on eDNA results. As discussed

in eDNA sediment analysis 3.2, this preliminary approach requires further research to confirm and adjust the assumptions made for these calculations or modify the quantification method.

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