The use of emerging techniques to understand seagrass ecosystems: Case studies using *Posidonia oceanica* in the Eastern Aegean

Sarah Kate Meek

A thesis submitted in partial fulfilment of the requirements of the University of the West of England, Bristol for the degree of Doctor of Philosophy. This research programme was carried out in collaboration with Archipelagos Institute of Marine Conservation, Greece.

Centre for Research in Biosciences, Department of Applied Sciences,
Faculty of Health and Applied Sciences, University of the West of England, Bristol

November 2020

Word count: 39,202

Author's Declaration

As far as possible, where permission is required I confirm that I have gained third party copyright permission for all relevant material and that I have attributed copyright permissions within the thesis where required.

Abstract

Seagrass meadows are vital coastal habitats that support a wide array of species and provide numerous ecosystem services. The area of seagrass meadow has declined significantly, at a rate of about 5% per year, since 1980. Emerging techniques for seagrass research has the potential to provide new insights to fill knowledge gaps and improve our understanding of seagrass ecological function and ecosystem services. This improved understanding will help us to inform policy makers about protection measures. Using *Posidonia oceanica* dominated habitats as a case study, this thesis assesses emerging techniques for mapping seagrass habitats, monitoring biodiversity with seagrass habitats and assessing microplastic pollution loads within seagrass sediments.

Kayak-borne down-scan sonar is shown to provide an accurate and cost-effective method for mapping the distribution of seagrass meadows. Sonar-derived data suggested current estimates of seagrass extent in the Aegean, based on analysis of satellite imagery, may contain considerable inaccuracies particularly in areas of complex bathymetry. It is suggested that kayak-borne sonar mapping can provide accurate reference data for larger scale satellite mapping, delivering benefits in terms of our ability to survey seagrass distribution and monitor temporal changes in extent and health.

Environmental DNA is proven to be an effective tool for the non-invasive detection of, *Pinna nobilis*, a culturally important yet Critically Endangered bivalve species associated with *P. oceanica* habitats. The technique developed in this study is capable of detecting concentrations of DNA as low as 5.50×10^{-10} ng μl^{-1} from sea water samples. This technique can be used at different spatial scales dependent on the

season, allowing eDNA to be a sensitive and precise tool in locating and identifying a key species inhabiting seagrass meadows.

A fine-scale analysis of microplastic distribution within the sediment under a seagrass meadow using recently developed Sediment Microplastic Isolation techniques, indicated that seagrass did not influence the deposition of microplastics to sediment at a semi-isolated bay. Microplastics were recovered at relatively low densities across the entire study area. Analysis of sediment patterns suggested that most sediment input was from terrestrial sources immediately adjacent to the seagrass bed and, therefore, that seagrass beds that are closer to terrestrial sources of microplastic pollution are likely to show much greater microplastic loadings.

It is concluded that, emerging techniques such as down-scan sonar, eDNA and microplastic extraction can provide novel insights into the distribution and ecological functioning of seagrass habitats. These insights provide avenues for the development of existing monitoring methods and for conservation policies.

Acknowledgments

I would like to thank everyone involved for their continued support, encouragement and patience over the course of this PhD, without which, this PhD would not have been possible. Firstly, I would like to say a special thanks to my supervisory team Dr Mark Steer, Dr Stephanie Sargeant, Dr Lyn Newton and Prof. Neil Willey. Particular thanks goes to Mark and Steph who have offered much appreciated support and guidance throughout, especially during the months of field work. You have pushed me to become more comfortable in a laboratory than I ever thought possible.

A special mention must also be given to our collaborators at Archipelagos institute of Marine Conservation. I would like to acknowledge and thank the director, Thodoris Tsimpidis, and the scientific director Anastasia Miliou, for their support and facilitation of field work. I would also like to thank the volunteers from Archipelagos, many of that have helped with the field work and data collection that would not have been possible alone. In particular I would like to thank Emma Ward for her shared love of seagrass, unwavering support, teaching me how to kayak, despite the weather conditions and for keeping me sane when island fever hit. Other volunteers without which this work would not have been possible are, Harry, Amy, Camille, Ben, Abbie, and Clara. There are many others who have helped but not been listed and I would like to thank you all. I would also like to thank the Microplastics research group at Plymouth Marine Laboratory, particularly Dr Matt Cole, for their help and guidance.

To my friends, colleagues and fellow PhD students I would like to thank you for offering invaluable advice, a friendly ear and support when it was needed most. I would like to thank Lizzy, Liana, James and Josh for making our office a fun, supportive and productive environment in which to work.

Lastly to my friends and family outside of UWE, I am eternally grateful for your consistent and steadfast support. To my parents and Grandma for always believing in me and pushing me to always do and achieve my best. To my sister, Jennifer, and cousin, Kathyrn, who have always offered an understanding ear and to Jess who has been there to offer me encouragement when I needed it most.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	Definition
ATR IR	Attenuated total reflectance
CI	Confidence interval
eDNA	Environmental DNA
FT IR	Fourier transform infrared
GLM	General linear model
GLMM	Generalised linear mixed model
GSI	Gonadosomatic index
HSI	Hepatosomatic index
MP/s	Microplastic/s
PC	Principle component
PCA	Principle component analysis
PCR	Polymerase chain reaction
PE	Polyethylene
PEST	Polyester
PP	Polypropylene
PVC	Polyvinyl chloride
qPCR	Quantitative polymerase chain reaction
SE	Standard error
SMI	Sediment microplastics isolation unit
SST	Sea Surface Temperature
NGO	Non - Governmental Organisation

Chapter 1

Introduction and Literature Review

1.1 Thesis Overview

Marine ecosystems provide an estimated 63% of global value of ecosystem services - the supply of benefits from ecosystems to society (Chan *et al.* 2006) - ($20.9 trillion yr^{-1}), with most of marine contribution coming from coastal systems ($10.9 trillion yr^{-1}) (Costanza *et al.* 1997). Many coastal habitats, however, have been lost completely due to human pressures, primarily via direct removal or degradation and eventual loss (Crain *et al.* 2009). Seagrass meadows are among these key coastal habitats, having once supported considerable biomass of megafauna such as large sirenians (e.g. dugongs and manatees) and turtles, as well as a large array of fishes and invertebrates (Jackson *et al.* 2001). Other ecosystem services provided by seagrass meadows include nutrient cycling (Orth *et al.* 2006), sediment stabilisation (Waycott *et al.* 2009), coastal protection (Mtwana Nordlund *et al.* 2016) and fixing of oceanic carbon (Duarte, 2005). Since the middle of the 19th century mass mortality of seagrass due to anthropogenic stressors has become increasingly common and widespread (Kirkman, 1978; Jackson *et al.* 2001; Waycott *et al.* 2009). Difficulties in the collection of marine environmental and biological data has resulted in inadequate knowledge of biological diversity for many species and regions (Ward, *et al.* 1999). It is therefore fundamental to increase the capacity and knowledge of survey methods used in these habitats to improve the knowledge of their importance and to inform policy decisions.

The overall aim of this thesis was to investigate whether a suite of emerging survey techniques can be used to provide increased understanding of the importance of seagrass meadows using case studies from *Posidonia oceanica* (Linnaeus) Delile dominated habitats in the eastern Aegean Sea.

In order to achieve this the specific objectives of the thesis were to:

1. Develop and validate a low cost, and widely accessible, seagrass mapping tool by combining data derived from down-scan sonar imaging with an emerging software analysis package;
2. Evaluate whether environmental DNA can be used to monitor species presence within seagrass beds and assess the spatial specificity of this tool;
3. Evaluate the use of emerging microplastic analysis techniques in the context of seagrass meadows.

This thesis is comprised of five chapters. Chapter 1 provides an overview of the importance of seagrass habitats and the key gaps in understanding their influences on the wider functioning of the coastal environment. Chapter 2 demonstrates use of kayak-borne down-scan sonar in seagrass mapping and provides an in depth comparison with satellite mapping of the same area. Chapter 3 provides an overview of the issues of some marine surveys and species of particular interest, as well an introduction to environmental DNA (eDNA). This chapter then goes on to demonstrate the capabilities of using eDNA in seagrass ecosystems and the factors affecting the detection of species. Chapter 4 evaluates the use of microplastics extraction and analysis techniques in the context of *P. oceanica* meadows. It then compares the microplastics content of seagrass sediments with adjacent bare sediment to explain the factors influencing microplastic deposition. Chapter 5 then synthesises the research

presented in previous chapters to highlight the key findings and identify areas of future work to build on them.

1.2 Seagrass Meadows

Seagrass meadows are widespread coastal habitats with global distribution (Duffy, 2006, Orth *et al.* 2006). Seagrasses are a group of marine flowering plants (figure 1.1) that inhabit coastal waters in all but the most polar seas (figure 1.2; Short *et al.* 2007). Despite this worldwide distribution, seagrasses exhibit low taxonomic diversity, comprising approximately just sixty species that all evolved from a single lineage of monocotyledonous flowering plants (Orth *et al.* 2006; Badalamenti *et al.* 2015).

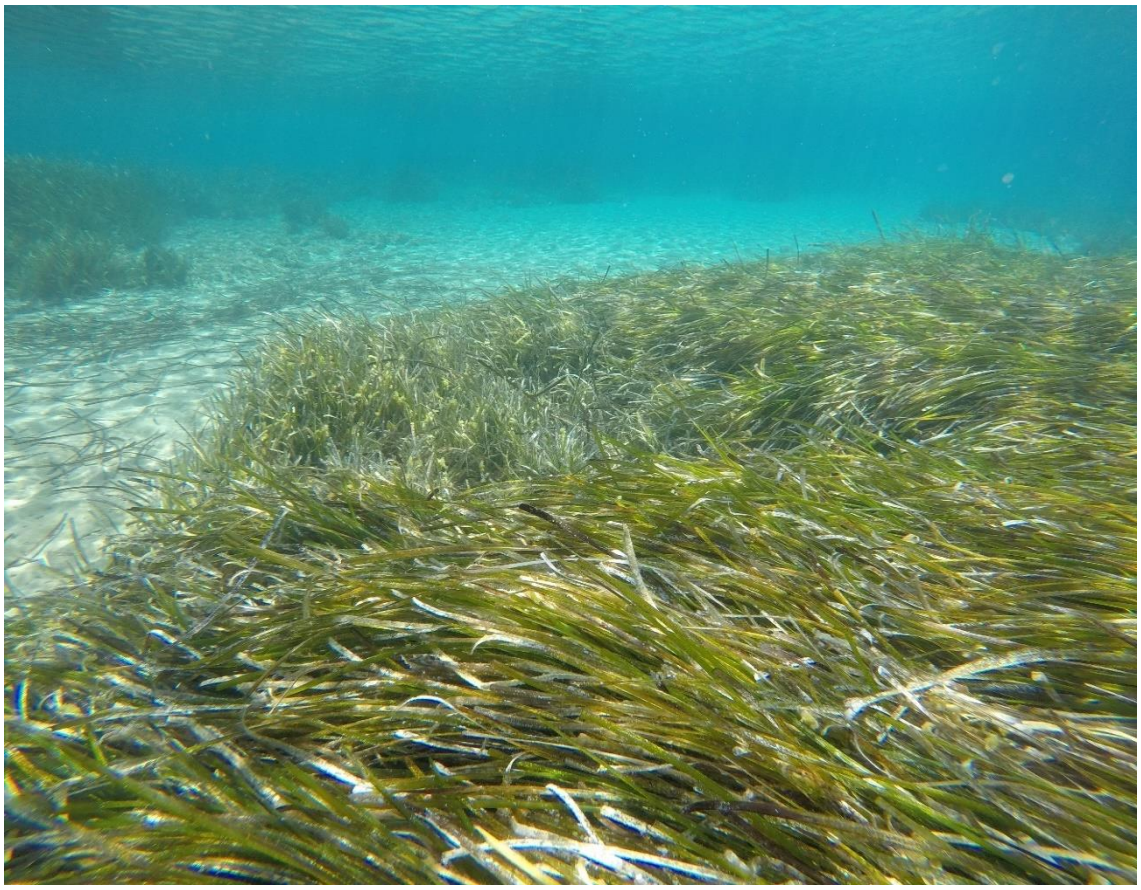


Figure 1.1 Example of a shallow seagrass meadow in Greece, species: *Posidonia oceanica*. (Author's own)

Seagrass species can be either fully submerged or intertidal and have developed many unique ecological, physiological and morphological adaptations to survive in these environments (Orth *et al.* 2006). These adaptations include internal gas transport,

epidermal chloroplasts, submarine pollination and marine seed dispersal (Orth *et al.* 2006).

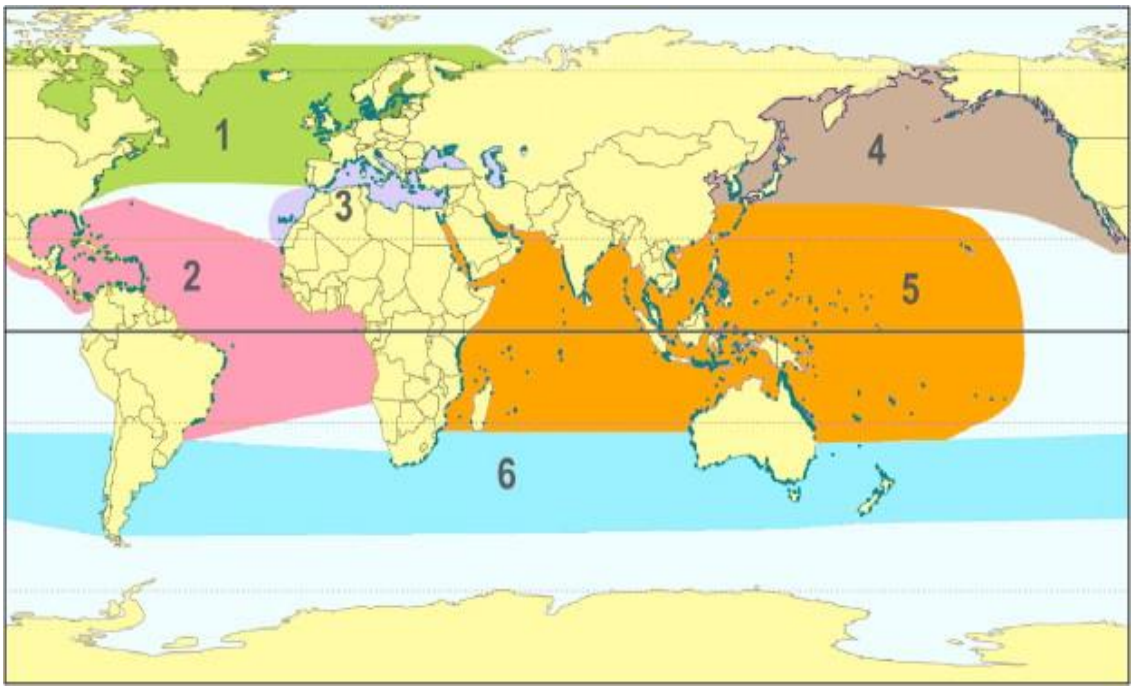


Figure 1.2 Global seagrass distribution shown as blue points and polygons (data from 2005 UNEP-WCMC) and geographic bioregions: 1. Temperate North Atlantic, 2. Tropical Atlantic, 3. Mediterranean, 4. Temperate North Pacific, 5. Tropical Indo-Pacific, 6. Temperate Southern Oceans (from Short *et al.* 2007).

Seagrass meadows are highly productive habitats, providing high value ecosystem services at multiple spatial scales (Dewsbury *et al.* 2016). In some coastal communities, quality of life depends on the state of seagrass meadows and the services they provide (Dewsbury *et al.* 2016). Ecosystem services provided by seagrass meadows include the support of commercial fisheries (e.g. Heck *et al.* 2003; Dorenbosch *et al.* 2006; Grol *et al.* 2008), nutrient cycling (Orth *et al.* 2006), sediment stabilisation (Waycott *et al.* 2009), and coastal protection (Mtwana Nordlund *et al.* 2016). It has also been suggested that seagrass meadows are effective bioindicators of water quality and light intensity (Orth *et al.* 2006; Boudouresque *et al.* 2009). They also play an important role in fixing oceanic carbon, by sequestering it within their root systems, thereby acting as an important global carbon sink (Duarte, 2005; Duffy, 2006; Orth *et al.* 2006).

Current estimated valuations of global seagrass ecosystem services vary greatly from \$2,287 ha^{-1} yr^{-1} to \$10.3 million ha^{-1} yr^{-1} depending in part on the valuation method used and services evaluated in the study (Dewsbury *et al.* 2016). There is, however, also significant variation between global bioregions and the ecosystem services provided by the seagrasses (Mtwana Nordlund *et al.* 2016). Mtwana Nordlund *et al.* (2016), showed seagrasses in the tropical Indo-Pacific and temperate Southern oceans, (i.e. those with more seagrass genera), exhibited higher levels of ecosystem services. The authors also showed that the number of services provided varied between genera, with larger seagrasses such as *Posidonia* and *Enhalus* associated with the majority of ecosystem services whereas smaller genera such as *Lepilaena* provided a low frequency of services.

One of the most valuable and common services provided by seagrass is the support of commercial fisheries. Seagrass meadows are widely recognised as crucial nursery grounds for many fish species, including those of commercial importance (Heck *et al.* 2003; Dorenbosch *et al.* 2006; Grol *et al.* 2008). Use of seagrass meadows is dependent on both the fishery species and seagrass species. Meadows provide permanent habitat, feeding grounds, and temporary nursery habitat for juvenile development or protection from predation (Tuya *et al.* 2014; Jackson *et al.* 2015). It has been estimated that 35% (\$762.5 million yr^{-1}) of the total Mediterranean commercial fishery landings were associated with seagrasses at some stage in their life history (Jackson *et al.* 2015). This took into consideration all seagrass species in the region (i.e *Posidonia oceanica, Cymodocea nodosoa, Zostera marina, Zostera noltii, Halophila stipulacea*). Blandon and Zu Ermgassen (2014) estimated the economic value enhancement that seagrass adds to commercial fisheries in southern Australia. According to their study seagrass adds \$160,263 ha^{-1} yr^{-1} to the commercial fisheries in the area, although this only considers

twelve fish species and may be a conservative estimate. In the Caribbean, the most abundant species landed in commercial fin fisheries use seagrass meadows at various life stages (Baker *et al.* 2015). The most abundant species were shown to use exclusively seagrass meadows as a juvenile.

Nutrient cycling and sediment retention are also important services provided by seagrass meadows (Orth *et al.* 2006 & Waycott *et al.* 2009). The complex structure of seagrass leaves, rhizomes (example shown in figure 1.3) and roots modify the hydrodynamics of surrounding water, trapping and storing sediments and nutrients, while also filtering nutrient inputs into the coastal ocean (Orth *et al.* 2006). Seagrass blades in the water column obstruct water flow (figure 1.4), resulting in reduced waves and currents within the seagrass canopies causing particles to be deposited (Koch *et al.* 2006). Densely covered meadows at sandy sites displayed a significant increase in fine sediment fractions in vegetated parts compared to unvegetated areas (van Katwijk *et al.* 2010).

Figure 1.3 Example of seagrass rhizome (outlined in black), from the species *Posidonia oceanica*. (Image: © de Moraes)

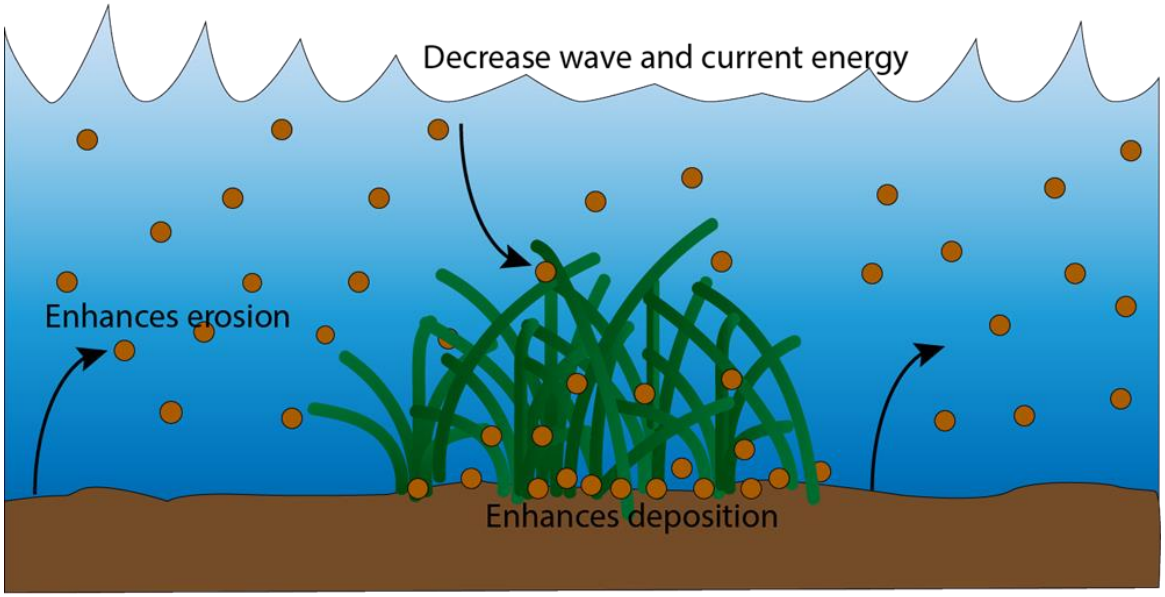


Figure 1.4 Schematic of the mechanics of seagrass enhancing sedimentation due as a result of water column obstruction. (Authors own)

Wave attenuation by seagrass meadows reduces the energy of waves reaching the adjacent shoreline, protecting the coast and controlling erosion (Barbier *et al.* 2011). A 20% reduction in wave height at sites with high *Zostera noltii* seagrass cover has been demonstrated, that was four times the effect shoaling has alone (the effect of surface waves entering shallower water and changing wave height) (Paul & Amos 2011). The authors concluded that a minimum shoot density of between 2,000 and 4,000 shoots m^{-2} was required for this species of seagrass to function in wave attenuation. Even low canopy height can still provide coastal protection with significantly reduced erosion by waves in *Halodule uninervis* meadows in Indonesia (Christianen *et al.* 2013). Infantes *et al.* (2012), tested the wave attenuation of *Posidonia oceanica*, finding that during storms wave height is reduced by up to 50% when passing over a 1 km meadow with an average shoot densisty of 600 shoots m^{-2}.

Losses of seagrasses signal important declines in the ecosystem services they provide and their widespread distribution allows for better large scale assessment than other

comparable coastal habitats that occupy just one broad geographical region (Orth *et al.* 2006). Seagrasses are considered biological sentinels or "coastal canaries", due to their sensitivity to changes in light availability and water quality, making them excellent for biological monitoring of coastal and estuarine water quality (Carlson *et al.* 2003, Orth *et al.* 2006, Montefalcone, 2009).

Carlson *et al.* (2003) studied the response of three seagrass genera, *Thalassia*, *Halodule* and *Syringodium*, in response to light stress caused by phytoplankton blooms during the 1998 El Niño event. Authors found that moderate light stress levels caused a shift in abundance from climax species *Thalassia*, towards the pioneer species *Halodule* or *Syringodium*. Under extreme light stress conditions all three species of seagrass declined. *Thalassia*-associated rhizome sugar, starch and total non-structural carbohydrate showed significant declines during the El Niño event, demonstrating the temporal responsiveness necessary for a good indicator.

Cabaço & Santos (2012) showed reproductive effort of seagrasses increased in response to both natural and anthropogenic stresses in 75% of cases. Overall, the reproductive effort increased 4.1 fold in response to disturbance, however, the reaction to anthropogenic stressors alone resulted in a response three times higher than when solely environmental stressors (i.e. not of direct human origin) were studied. The magnitude of response was shown to be significantly related to rhizome diameter of seagrass species in question. Zhang *et al.* (2014), tested the response of *Thalassia hemprichii* from nutrient loading as a result of aquaculture activities. Phosphorus content was significantly higher in all tissues closer to the nutrient source and showed distribution trends consistent with that of the bay. Nitrogen, however, was only found to correlate with nutrient loading within leaf tissues, while rhizome and root tissues showed no correlation of nitrogen content with that of the water column.

This suggests that the phosphorus content of tissues is a better bioindicator of nutrient loading than nitrogen. There was also found to be a higher epiphyte load on the leaves at sites closest to the nutrient sources and a significant correlation was found between the amount of epiphytic algae and nutrient loading.

Seagrass meadows account for 10 -15% of the yearly estimated carbon sequestration in the oceans, despite occupying less than 0.2% of the world's marine surfaces (Duarte *et al.* 2005; Duffy, 2006; Fourqurean *et al.* 2012). Unlike terrestrial soils, the sediments in which healthy coastal vegetation, such as seagrass, grow do not become saturated with carbon because they accrete vertically in response to rising sea levels, therefore seagrasses can sequester organic carbon at a rate thirty five times faster than tropical rainforests (Mcleod *et al.* 2011, Macreadie *et al.* 2015). Seagrass meadows are estimated to store between 48 and 112 gigatons of carbon per year (Mcleod *et al.* 2011). Fourqurean *et al.* (2012) suggested that the total global organic carbon stock of seagrass meadows is even higher. They estimated 9.8 - 19.8 Pg of carbon is stored within the seagrass meadows across the globe, based off cores from at least a metre deep to estimate the soil content and estimates of above ground biomass. Results also suggested a large underestimate of the yearly carbon sequestration as the actual organic content of seagrass soils being conservatively estimated by Fourqurean *et al.* (2012) was double previous estimates. Mateo *et al.* (1997) estimated that the accumulation of carbon deposits in a *Posidonia oceanica* matte were at least 6,000-7,000 years old, similar to some terrestrial peat deposits. This has important implications for long-term storage as seagrass has the capability to store carbon for millennia compared to that of rain forests that sequester carbon for decades.

Concurrent with an improved understanding of the role seagrass plays in the marine environment, there has been an increase in the reports of seagrass losses globally, with

increased monitoring highlighting a declining trend observed since the 1970s (Orth *et al.* 2006; Collins *et al.* 2010). Waycott *et al.* (2009) estimated a total area of 3370 km^2 of seagrass was lost globally between 1879 and 2006 that equated to a loss of 27 km^2 yr^{-1}. The authors also showed that seagrass loss has been accelerating over the past eight decades, from <1% yr^{-1} before 1940 to 5% yr^{-1} after 1980, mainly from anthropogenic induced stress such as coastal development. They also estimate that since 1980 a 35% loss of seagrass area has occurred. Whilst they also demonstrated that seagrass increase has accelerated over the same time period, this has not been enough to compensate the losses that have occurred. Between 1990 and 2000 there was a still a net decrease in seagrass area of 319,670 ha.

Many of the threats to seagrass meadows are anthropogenic (Duarte, 2002; Pergent *et al.* 2014). Human activities can directly cause the decline of meadows though coastal development, destructive fishing practises (e.g. trawling), boat propellers and moorings / anchoring (figure 1.5; Duarte, 2002; Boudouresque *et al.* 2009; Waycott *et*

Figure 1.5 Example of anchor scar in *P. oceanica* meadow. (Author's own)

al. 2009. These physically damage the meadows by uprooting or burying shoots and rhizomes, leaving the meadows scarred and fragmented (Milazzo *et al.* 2004; Orth *et*

al. 2006; Boudouresque *et al.* 2009; Collins *et al.* 2010). Indirect damage from human activities also occurs via the alteration of the environment (e.g. increased turbidity), ecosystem structure and processes (e.g. overgrazing) (Duarte, 2002; Boudouresque *et al.* 2009; Waycott *et al.* 2009). Almost 15% of all seagrass species (8 of 60 species) are currently listed as threatened in some portion of their range, including the Mediterranean species, *Posidonia oceanica* (Hughes *et al.* 2009).

1.3 POSIDONIA OCEANICA

Posidonia oceanica is a seagrass endemic to the Mediterranean (Milazzo *et al.* 2004; Vassallo *et al.* 2013). It grows in coastal waters, and is generally considered to have a depth limit of 40-45 m (Pasqualini *et al.* 1998; Balestri *et al.* 2003; Badalamenti *et al.* 2015). *P. oceanica* forms lush dense meadows, the canopies of which can grow to up to 60 cm in height with blades reaching up to one metre (figure 1.6; Molenaar *et al.* 2000).

Figure 1.6 Example of dense *P. oceanica* meadow in the Aegean Sea. Meadow is at less than 5m and blade lengths of approx. 60cm -90cm (Author's own)

The rhizomes of *P. oceanica* demonstrate two distinct growth forms, orthotropic or plagiotropic (figure 1.7, Molenaar *et al.* 2000). Orthotropic rhizomes grow vertically and slowly, allowing the plant to avoid being covered with silt, with straight leaves that can exceed 1m in length (Molenaar *et al.* 2000). These rhizomes branch infrequently, internodes between leaves are short and this growth form is most commonly found in the middle of meadows (Molenaar *et al.* 2000). Plagiotropic rhizomes grow horizontally with shorter, more curved leaves and growth occurs when environmental conditions allow for colonisation of vacant substrate and therefore most often occur on the edges of meadows (Molenaar *et al.* 2000).

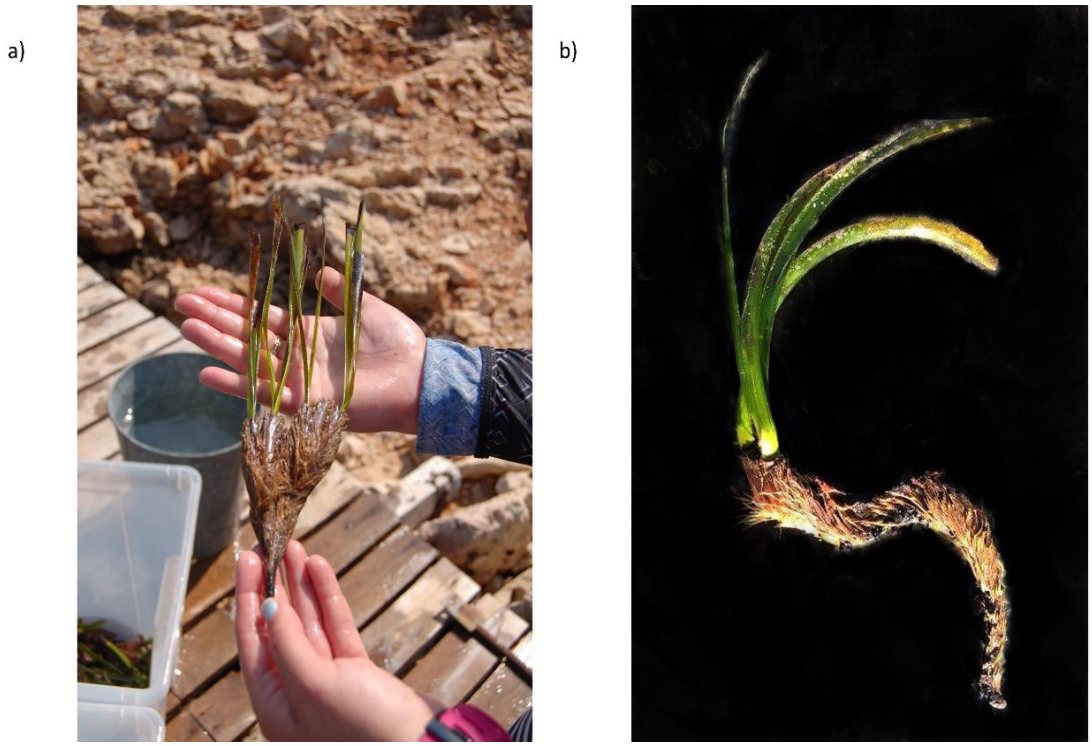


Figure 1.7 Example of *Posidonia oceanica* fragment showing a) orthotropic growth (Image: © de Moraes) and b) plagiotropic growth (image: Ward 2018)

P. oceanica very rarely reproduces sexually, producing flowers occasionally, favouring warm summers, in which case flowering occurs in autumn (Boudouresque *et al.* 2012). The fruits then take six to nine months to ripen, dropping from the plant between May

and July, and have just one seed (Boudouresque *et al.* 2012). *P. oceanica* mainly reproduces vegetatively, or colonises vacant areas by expansion of the plagiotropic rhizomes (Boudouresque *et al.* 2012). Preference for clonal reproduction has resulted in low genetic variability of the species (Boudouresque *et al.* 2012).

1.4 *P. OCEANICA* ECOSYSTEM SERVICES

Ecosystem services provided by *P. oceanica* meadows alone are currently estimated at up to €514 ha^{-1} yr^{-1} (Campagne *et al.* 2014). A considerable number of studies have been carried out into the ecological functioning and importance of *P. oceanica* meadows in the western Mediterranean Sea (e.g. Guidetti, 2000; Barrón *et al.* 2006; Moranta *et al.* 2006; Deudero *et al.* 2008; Lo Iacono *et al.* 2008; Serrano *et al.* 2012; Vassallo *et al.* 2013). While it is generally considered that the trends found in the western region are applicable to the eastern Mediterranean, there is little data to verify this.

1.4.1 Commercial fisheries

P. oceanica meadows are of great importance to marine biodiversity, due to the food and shelter they provide (Bianchi & Morri, 2000). The dense meadows formed by the long blades that are characteristic of this species provide protection, and therefore are nursery grounds, to many species in early life stages (Kalogirou *et al.* 2010). It has been estimated that *P. oceanica* contributes up to €35/ ha each year to fishery resources (Campagne *et al.* 2015), which considering the estimated total area coverage of *P. oceanica* is at 4,350,000 ha in the Mediterranean (Marbà *et al.* 2014); this amounts to an estimate of €152,250,000 per annum. Kalogirou *et al.* (2010), studied the fish assemblages associated with *P. oceanica* meadows on the island of Rhodes (Southern Dodecanese) using seine nets. They found a total of 109,350 fishes from 88 species. Of

these species 23 were seagrass residents and 19 were juvenile migrants (i.e. utilised the meadows as a nursery habitat). They also calculated the biomass of each species caught; seven of the top ten with highest biomass were of commercial value. Of these seven species, three were seagrass residents, *Boops boops* (bogue), *Sphyraena viridensis* (yellow barracuda), and *Sphyraena sphyraena* (European barracuda) and four were juvenile migrants, *Oblada melanura* (saddled seabream), *Sparisoma cretense* (Mediterranean parrotfish), *Sardinella aurita* (round sardinella), and *Pagrus pagrus* (common seabream). Depth was not considered in this study, however, but is an important contributing factor in species distributions and assemblages (Hyndes *et al.* 1999). Invertebrates and cephalopods were also not considered in this study, however, a number of species within these groups are known to be wholly or partially reliant on *P. oceanica* meadows including the squid, *Luligo vulgaris,* (Sanchez *et al.* 2010) and the mollusc *Pinna nobilis* (Coppa *et al.* 2010).

1.4.2 Refuge for protected species

P. oceanica canopies provide species with protection and food. For example, the two European species of seahorses, *Hippocampus hippocampus* and *Hippocampus guttulatus*, are known to prefer seagrass habitats such as *P. oceanica* (Goffredo *et al.* 2017), and both species are listed as near threatened in the Mediterranean on the IUCN red list (Pollom, 2016). Both species are protected by CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna) and Annex II of the Specially Protected Areas and Biological Diversity (SPA/ BD) protocol of the Barcelona Convention (Pollom, 2016). *Hippocampus guttulatus* was shown to forage in *P. oceanica* meadows where macrobenthic fauna is higher during the day time (Kitsos *et al.* 2008; Kalogirou *et al.* 2012). *H. guttulatus* is especially sensitive to habitat destruction due its preference of shallow seagrass meadows that are more prone to

natural and human disturbances (Gristina *et al.* 2017). Due to lack of data across the whole extent of its geographic range, globally the IUCN list these species as data deficient (Gristina *et al.* 2017; Pollom, 2017; Woodall, 2017).

A key species that relies on *P. oceanica* meadows is *Pinna nobilis* (Linnaeus 1758), the noble pen shell (figure 1.8). *P. nobilis* is one of the largest bivalves in the world, growing to lengths of up to 1.2 metres and, like *P. oceanica*, is endemic to the Mediterranean (Katsanevakis & Thessalou-Legaki, 2009). It is largely associated with *P. oceanica* meadows, achieving lifespans of over twenty years (Coppa *et al.* 2010). This species is protected under the EU habitats directive Annex 4, however, it is still showing declines as a result of incidental damages from trawlers or anchoring, collection by divers and the loss of habitat resulting from the regression of *P. oceanica* meadows. *P. nobilis* has known to have a depth range from 0.5 m to 60 m and tends to live sheltered within the seagrass canopy (Hendriks, Deudero, & Tavecchia, 2012).

Figure 1.8. Juvenile *Pinna nobilis* sheltered by a *P. oceanica* meadow. Individual is approx. 16cm in width as widest point. (Author's own)

The anterior section of the mussel is buried in the seabed and anchored to the substratum or among rhizomes and shoots of *P. oceanica* by byssus threads (Vázquez-Luis *et al.* 2014). These byssus threads from *P. nobilis* specifically were the source of an extremely fine and valuable fabric called "sea silk" that was harvested until the 20th century (Katsanevakis & Thessalou-Legaki, 2009; Voultsiadou, Koutsoubas, & Achparaki, 2010). *Pinna nobilis* is known as an ecosystem engineer, providing areas for colonisation of various benthic invertebrate species (Rabaoui *et al.* 2015). The posterior end of the shell that projects into the water column has an inhalant syphon that filters water for food and oxygen (Davenport *et al.* 2011).

More recently *P. nobilis* has come under severe threat from a new parasite, *Haplosporidium pinnae*, that was first detected in 2016 (Darriba, 2017; Vázquez-Luis *et al.* 2017) and described as a new species in 2018 by Catanese *et al.* (2018). The *H. pinnae* parasite seems to exclusively attack *P. nobilis* and was first discovered in the Balearic Islands and south coast of Spain (Vázquez-Luis *et al.* 2017). The parasite attacks the digestive system of the bivalve, preventing proper absorption of food by gut tissues and therefore causing starvation of the infected individual (Catanese *et al.* 2018). Mass mortality events have been seen in increasing numbers across the Mediterranean Sea with up to 100% mortality in regions where the parasite has spread (Catanese *et al.* 2018; Carella *et al.* 2019). In just two years the parasite has spread across to the Aegean Sea and has been responsible for mass mortality on Lesvos Island (Katsanevakis *et al.* 2019).

1.4.3 Carbon Storage

P. oceanica is unique among seagrass species in its ability to capture and store very large amounts of carbon (estimate range from 40-419 kg C_{org} m^{-2}) for millennia (Mateo *et al.* 1997, Lavery *et al.* 2013) and is estimated to store the most organic carbon of all

seagrass species (Fourqurean *et al.* 2012). The majority of this carbon is stored within the extensive and long-lived rhizome mattes where plant detritus and sediment settle and get trapped (Mateo *et al.* 1997, Serrano *et al.* 2012) and show the slowest turnover rates of all species, allowing long term accumulation (Duarte & Chiscano, 1999). Lo Iacono *et al.* (2008) studied the carbon storage of a meadow in the North-Western Mediterranean. Seismo-acoustic imaging was carried out to measure the thickness of the *P. oceanica* mattes, that, when combined with lab analysis of sediment cores enabled them to calculate the total carbon accumulation of the meadow. The carbon accumulation of this meadow was estimated at 7,486 tons across the 60 m^2 meadow present in the survey location. A consistent vertical growth rate of matte reported in the study suggests little disturbance to the matte in the last 6,000 years (Lo Iacono *et al.* 2008). The stability of the system is a significantly contributing factor to the carbon storage potential of the meadows; and means when not physically damaged carbon can be stored by them for thousands of years. It has therefore be suggested that when these meadows are disturbed or destroyed they convert from a carbon sink to a carbon source, and release this once stored carbon back into the ocean-atmosphere CO_2 pool (Fourqurean *et al.* 2012).

Furthermore, areas of the closely related species *Posidonia australis* that had been disturbed by seismic testing have been found to contain 72% lower soil organic carbon stocks than undisturbed seagrass (Macreadie *et al.* 2015). Macreadie *et al.* (2015) took soil samples from within disturbed, recovered and undisturbed seagrass habitats, by hammering corers into the sediment. Lab analysis was then carried out to determine dry bulk density and carbon 14 concentration that was measured using mass spectroscopy. An increase in aerobic heterotrophs also indicated a change in the biogeochemical structure of the disturbed soil. *P. australis* displays many similarities

to the close related *P. oceanica*, including that they are amongst the slowest growing species of seagrass and therefore their recovery from disturbance is slower than most seagrass species (Macreadie *et al.* 2015).

The top 14 cm of *P. oceanica* sediments have been shown as highly organic (up to 69%) and dated up to 15 y BP (Serrano *et al.* 2012). This rapidly decreased to 10% at 52cm (500yrs of burial) before decreasing more slowly to an average of 5% between 52 cm and 475cm (estimated between 530 and 4320 y BP) (Serrano *et al.* 2012).

Serrano *et al.* (2014) compared organic carbon stocks of *P. oceanica* mattes at different depths (2 and 32 metres). They reported fourteen to sixteen times more organic carbon found in the shallow samples than the deep ones, indicating that light availability may be of critical importance when modelling seagrass ecosystem dynamics such as carbon storage and sequestration. Shallow samples were also shown to more be homogeneous while in the deep cores organic content decreased down the length. There were only two cores taken at 32 m depth, and the shallow cores were taken horizontally by inserting the corer into an exposed wall of *P. oceanica* rhizome matte. The comparability of cores taken vertically and those taken from the exposed rhizome wall has not been widely discussed due to rarity of these exposed mattes. There were also no cores taken at intermediate depths, so it is unknown if the relationship is linear or otherwise. Much of the data collected on carbon storage in *P. oceanica* have originated from the western basin (e.g. Barrón *et al.* 2006; Lo Iacono *et al.* 2008; Serrano *et al.* 2012; Vassallo *et al.* 2013) and it is assumed that these trends are consistent across the Mediterranean.

1.5 *P. OCEANICA* DISTRIBUTION

In spite of the reported importance and fragility of *P. oceanica* meadows, there are still considerable portions of coastline in the eastern Mediterranean (43%) that remain unmapped for seagrass presence (Marbà *et al.* 2014, Telesca *et al.* 2015).

Marbà *et al.* (2014) estimated the area of *P. oceanica* in the Mediterranean at up to 4,350,000 ha based on habitable areas. The known area of *P. oceanica* has been calculated at 1,224,707 ha. It has been estimated that 88% of the Western Mediterranean has been mapped for *P. oceanica* meadows (Telesca *et al.* 2015), however of the total known area, 58.3% (713,992 ha) is in the Eastern Mediterranean, despite 58% of the region remaining unmapped (Telesca *et al.* 2015), meaning the estimates of *P. oceanica* by Marbà *et al.* (2014) could be substantially different to reality.

Despite this lack of comprehensive data for the region, there is estimated to be a total known area of 713,992 ha of *P. oceanica* meadows in the eastern Mediterranean, 1.4 times that of the western basin. Telesca *et al.* (2015) took *P. oceanica* distribution data from 263 studies, including reviewed journals, unpublished datasets and EU reports, that were then compiled into a single map showing presence, absence or lack of data (figure 1.9). This emphasises the lack of research carried out in the area and suggests that the eastern basin needs more attention than it has previously been given in the study of this endemic species.

Many western countries, including France and Spain, have already mapped 100% of their coastline for *P. oceanica* (Telesca *et al.* 2015). As of 2011, Greece had just 8% of coastline mapped for *P. oceanica* presence, the lowest of all Mediterranean countries. Of that 8%, there was a total area of 44,939 ha of *P. oceanica* meadow, already more

than some countries that have mapped 100% (Telesca *et al.* 2015). It has been claimed almost 65% of the unmapped potential seagrass areas of the Mediterranean Sea are in Greek waters (Topouzelis *et al.* 2018). Topouzelis *et al.* (2018), have recently developed a satellite mapping method that has been purportedly used to map the majority of the Greek coastline, which will be discussed in more detail in Chapter 3.

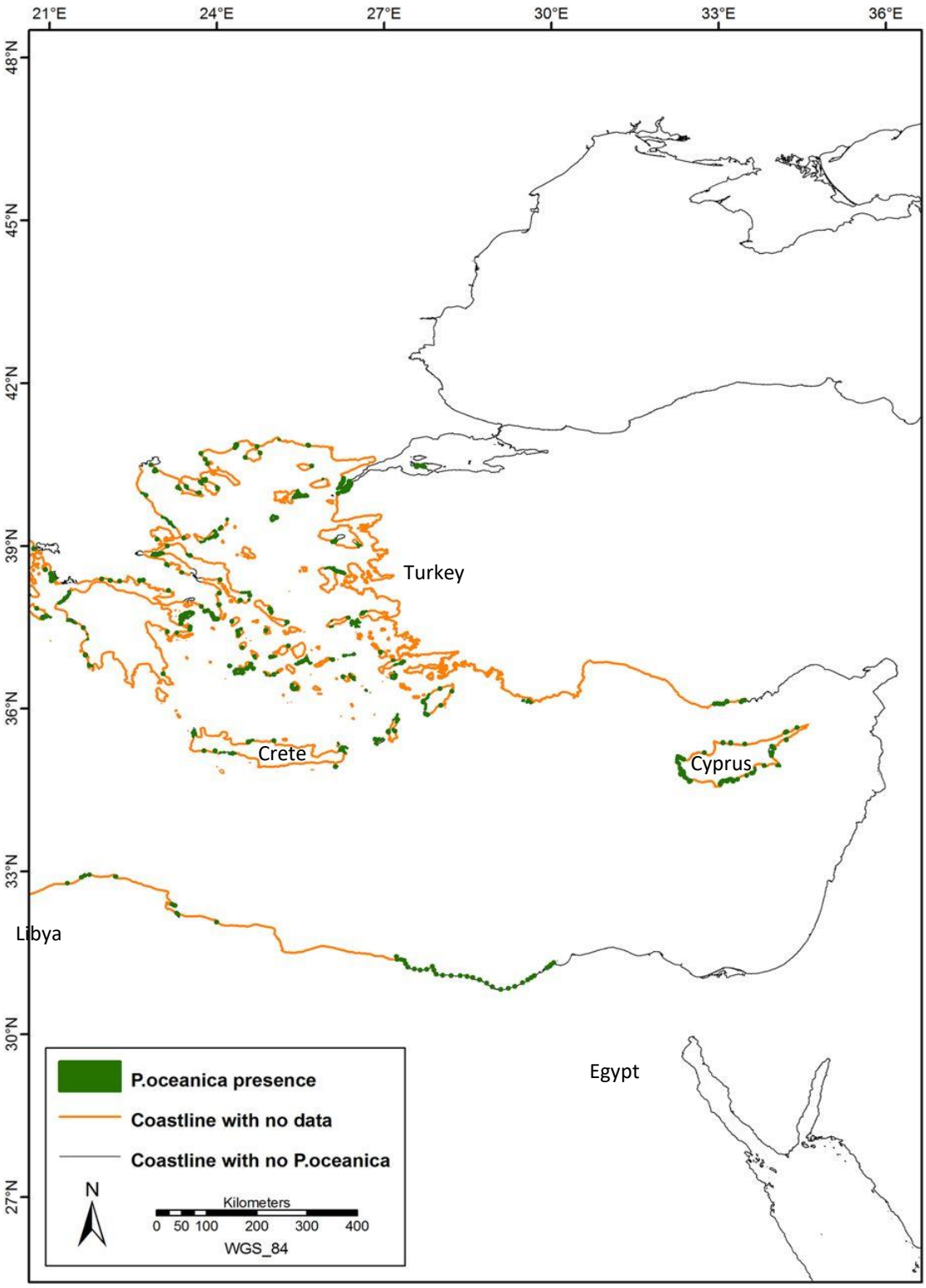


Figure 1.9 Extent of confirmed *P. oceanica* presence (green) and absence (grey) in the E. Mediterranean. Orange indicates coastline with no survey data (from Telesca *et al.* 2015).

Maps of seagrass meadows are important to aid the monitoring of changes in community structure, cover and biomass across the whole system (Hossain *et al.* 2014, Lefebvre *et al.* 2009). These processes interplay across small and large spatial scales meaning physical field survey methods, e.g. diving, are hard to implement and are often time consuming and expensive to achieve on a large scale (Hossain *et al.* 2014, Lefebvre *et al.* 2009). In the case of *P. oceanica* it is also imperative that methods used are suitable for a wide depth range (0-45 m) due to its lower depth limit (Pasqualini *et al.* 1998).

1.6 *P. OCEANICA* DECLINE AND THREATS

Posidonia oceanica meadows are experiencing severe regression, with a loss of the areal extent estimated to be occurring at around -1.7% yr^{-1}, almost twice that of global seagrasses (Waycott *et al.* 2009; Marbà *et al.* 2014). The remaining meadows are experiencing decreasing shoot density, which has been estimated at a rate of about 50% loss since 1990 (Marbà *et al.* 2014). If the current estimated value of ecosystem services provided by *P. oceanica* meadows of €514 ha^{-1} yr^{-1} is correct then, the current models of regression equate to a loss of €2 million per year (Campagne *et al.* 2014).

P. oceanica meadows are protected under the EU Habitats Directive 1992, where they are acknowledged as being a priority habitat in need of designated areas of conservation (Vassallo *et al.* 2013, Campagne *et al.* 2015, Telesca *et al.* 2015). Since 1960 it has been estimated that up to 38% of potential seagrass area has been lost from the Mediterranean (Marbà *et al.* 2014). This decline is caused by numerous factors, many of which are of anthropogenic origin (Boudouresque *et al.* 2009).

The main and most widespread of these stressors are coastal development, pollution and trawling (Boudouresque *et al.* 2009). Coastal development is high in the

Mediterranean due to rapid population growth and popularity with tourists. It destroys *P. oceanica* meadows by direct burial, increased turbidity, hypersedimentation and erosion (Boudouresque *et al.* 2009). The construction of a single harbour in Marseille, France directly destroyed eleven hectares of *P. oceanica* meadows and a further 68 hectares from the plume of turbidity released by the construction (Boudouresque *et al.* 2009). Trawling is one of the most severe causes of *P. oceanica* loss, with a standard trawler uprooting 99,000 - 363,000 shoots ha^{-1} (Boudouresque *et al.* 2009). This leaves behind large expanses of scarred and unvegetated areas throughout meadows; due to the slow growth of *P. oceanica* it can take c.100 years for the damaged meadows to return to normal density (González-Correa *et al.* 2005 Boudouresque *et al.* 2009).

Increases in sea surface temperature (SST) due to warming is also having a significant impact on the meadows (Jordà, Marbà, & Duarte, 2012). Response of *P. oceanica* meadows to sea surface temperature (SST) warming was modelled in the Balearic Islands by Jordà, Marbà, & Duarte (2012). It was estimated that under the current projections of SST increases, *P. oceanica* could be functionally extinct by 2049 ± 10 years due to temperature increases combined with other anthropogenically caused stressors. If all local disturbances of the meadows ceased, i.e. all anthropogenic disturbances stopped, by 2010, then SST warming alone would result in the functional extinction of this species by 2063 ± 13 years. Due to the enclosed nature of the Mediterranean Sea, *P. oceanica* must either adapt to climate change or face extinction, as a northern shift in geographical range is impossible, however, it's slow growth rate and the rarity of sexual reproduction makes adapting unlikely.

Pollution affects the upper and lower depth limits of the meadows and is the cause of significant decline of *P. oceanica* near large urban centres and sewage outputs (Boudouresque *et al.* 2009). Whilst the impact of major marine pollutants on seagrass

habitats have been characterised to some extent (e.g. Ruiz *et al.* 2001; Serrano *et al.* 2011; Cozza *et al.* 2003), the extent and impact of microplastic pollution has not been widely addressed; and remains a significant knowledge gap.

Microplastics have been accumulating in the marine environment, particularly coastal sediments, for the past few decades (Andrady, 2011; Nor, & Obbard, 2014). They are widely recognised as a contaminate of global concern due to bioavailability and harmful impact on many marine species (Coppock *et al.* 2017), such as impairing cognitive function (Crump *et al.* 2020), development of abnormal cells (Wang *et al.* 2019) and tissue damage (Wang *et al.* 2019b). To date there is little research that clearly identifies areas of microplastics accumulation in coastal vegetation excepting a small number of papers on mangrove systems (e.g. Nor, & Obbard, 2014). Seagrass blades in the water column obstruct water flow, resulting in reduced waves and currents within the seagrass canopies causing particles to be deposited (Koch *et al.* 2006). Thus it is possible that under these conditions there is an increase in microplastic deposition and retention within these canopies, potentially making them an important coastal accumulation area for plastic pollution.

1.7 VALUE OF EMERGING TECHNIQUES

There are still important knowledge gaps in seagrass ecosystem services such as contributions to fisheries and coastal protection (Ruiz-Frau *et al.* 2017). There is a need to assess ecosystem services at the correct scale, that ideally would be meadow or landscape level (> 10–100 m^2 level) to quantify different types of animals utilizing seagrass meadows in a different manner (Nordlund *et al.* 2018). In Southeast Asia, Indonesia and the Philippines seagrass meadows have been well studied, including fisheries contributions, however, other countries such as Thailand, Malaysia,

Singapore, Cambodia, etc. are severely lacking data (Ooi *et al.* 2011). Within the research from Indonesia and the Philippines, there has been a distinct focus on backreef meadows, that differ significantly from forereefs, which have so far been neglected in representation in literature (Ooi *et al.* 2011). In the Mediterranean there has been a clear focus on seagrasses in the western basin (e.g. Guidetti, 2000; Barrón *et al.* 2006; Moranta *et al.* 2006; Deudero *et al.* 2008; Lo Iacono *et al.* 2008; Serrano *et al.* 2012; Vassallo *et al.* 2013), while the eastern has been somewhat over-looked. The eastern basin is warmer on average than the western basin and is warming faster due to climate change (Pisano *et al.* 2020). It is therefore clear that trends cannot be extrapolated across the entire region as has been done previously; but also understanding the ecosystem services of the eastern basin seagrasses, where temperatures are warmer than the western basin, can suggest the possible future of ecosystem services in the western basin under rising SST projections.

Understanding the extent of seagrass cover and use of seagrass by ecologically and commercially important species across entire habitat ranges (including geographic location and depth) and lack of this data has resulted in inadequate knowledge of biological diversity for many species and regions (Ward, *et al.* 1999). It is therefore fundamental to increase the capacity and knowledge of survey methods used in these habitats to improve the knowledge of their importance and inform policy decisions. Emerging techniques that have been utilised in other environments can help to progress seagrass research and fill these knowledge gaps to create a more holistic understanding of the ecosystem service, distribution and threats of seagrass ecosystems.

1.8 Aims and Summary of Thesis

The research presented in this chapter demonstrates that new survey methods into the distribution and functioning of seagrass ecosystems are needed to complement existing methods in order to understand the ecological functioning and conservation of seagrass meadows.

Chapter 2, presents results from a novel method for mapping seagrass using down-scan sonar. The method was designed to be accessible to all organisations in terms of skill and cost, encouraging the collection of seagrass and bathymetry data in areas where information on seagrass distribution is limited.

Chapter 3 shows how eDNA can be utilised as a novel survey technique for detecting species presence within seagrass habitats, using the critically endangered bivalve *Pinna nobilis* as a case study. It considers how seagrass canopies may be influencing the movement of eDNA into and out of the system and therefore the suitability of the technique for species that utilise seagrass meadows.

Chapter 4 reports investigations of the influence seagrass canopies may have on the dispersal of microplastics. It uses recently developed techniques to investigate the potential for these habitats to accumulate microplastics within the sediments.

The final chapter provides a synthesis of the research and shows how each technique provides insights that further the understanding of seagrass ecology and conservation.

Chapter 2

An assessment of down-scan sonar as a mapping tool for seagrass habitats

2.1 INTRODUCTION

Seagrasses globally are under increasing stress leading to their degradation and rapid loss, estimated at 110 km ^{2} yr^{-1} since 1980 (Duarte 2002; Waycott *et al.* 2009). *Posidonia oceanica* in the Mediterranean sea is no exception to this, the loss of which was estimated as -1.2% yr^{-1} in 2014 (Marbá *et al.* 2014). In order to include seagrass into successful management plans, it is imperative to understand their distribution (McKenzie *et al.* 2020). Maps of seagrass habitats are vital tools for monitoring changes in distribution, biomass and community structure across the whole seagrass ecosystem (Hossain *et al.* 2014, Lefebvre *et al.* 2009). Seagrasses have a wide depth range with some species found from intertidal ranges to more than 60 metres deep (Kuo & den Hartog 2007). The large spatial scale and depth ranges of some seagrass species mean in water survey methods, such as diving, are hard to implement and are often time consuming and expensive to achieve on a large scale (Hossain *et al.* 2014, Lefebvre *et al.* 2009). In the case of *P. oceanica* it is also imperative that methods used are suitable for a wide depth range (0-45/50m) due to its lower depth limit (Pasqualini *et al.* 1998). There are environmental challenges of seagrass mapping, such as water turbidity and depth, both of which are highly variable in the coastal zone, and make the

observation of seagrass challenging (McKenzie *et al.* 2020). Traditional methods for seagrass mapping include SCUBA / snorkel surveys to carry out a combination of transects and sampling points (McKenzie *et al.* 2001; McKenzie, 2003) and in- situ visual assessments of percentage cover using quadrats (Duarte & Kirkman, 2001). these methods, however, are time consuming with high associated costs (Short & Cole, 2001) and only includes estimations of cover at specific points not the spatial variation of seagrass cover (Veettil *et al.* 2020). As these methods involve extensive field work, in recent years seagrass cover and change has been estimated using remote sensing (Veettil *et al.* 2020).

2.1.1 Remote sensing methods for mapping seagrass

Remote sensing methods have become an imperative supplement to conventional mapping and monitoring methods due to their rapidity, large area coverage and repeatability (Pham *et al.* 2019). Due to advances in technology and variety of remote sensing based tools have been applied for mapping seagrass habitats since the 1990's (Hossain *et al.* 2015). Satellite or airborne remote sensing have been widely used for monitoring and mapping seagrass (Chauvaud *et al.* 1998; Dekker *et al.* 2007; Lyons *et al.* 2011; Pu, *et al.* 2012; & Roelfsema *et al*, 2014). Direct remote sensing methods are based on spectral reflectance of chlorophyll and other leaf components (Qiu *et al.* 2019). Key factors influencing the quality of seagrass remote sensing are the spectral reflectance of seagrasses and attenuation of the useful portion in the electromagnetic spectrum and infrared radiation by its surrounding aquatic environment (Thorhaug *et al.* 2006). There are also many acoustic techniques for seagrass mapping, detection and monitoring, with the most common systems being multibeam echosounder sonar, Side-scan sonar and single beam echosounder (Umit Gumusay *et al.* 2019). Survey grade

systems have high purchase prices and therefore low cost systems are becoming the focus of further research (Umit Gumusay *et al.* 2019).

Ground truth data is vital to classification of input data, validation of the method and assessing conditions of study area (Umit Gumusay *et al.* 2019). Current ground truthing methods often use very labour-intensive surveys carried out using SCUBA or snorkel equipment such as those by Komatsu *et al.* 2003, Lefebvre *et al.* 2009 & Lyons *et al.* 2011). Field data for ground truthing is often collected by snorkel taking photographs a set distance from the seabed (Lyons *et al.* 2011, Pu *et al.* 2012 & Roelfsema *et al.* 2014). These are time consuming and costly, making them unsuitable for large scale studies. Due to this some initial studies into seagrass mapping did not include ground truth data because information by SCUBA or even grab samples is not feasible to collect for large areas of study (Umit Gumusay *et al.* 2019). Other ground truthing methods for acoustic data have included downward facing video alongside the sonar transducer (e.g. Hamana & Komatsu, 2016; Pergent *et al.* 2017), however these are limited by water clarity that can restrict the depth to which images can be acquired. The depth limits for many of these methods, is often shallower than the depth range of *P. oceanica*, and therefore do not cover the entire habitat range being mapped.

2.1.1.1 Satellite mapping of seagrass

Satellite imagery has been used to map seagrass meadows due to the ability to cover large areas (Lefebvre *et al.* 2009 Pham *et al.* 2019). Remote sensing via satellite uses the radiance of the visible bands from the sea floor received by the optical on board sensor (Hossain *et al.* 2015). Light attenuation is the exponential decrease in light intensity with increasing depth, that is a result of absorption and scattering (Zimmerman and Dekker, 2007; Hossain *et al.* 2015). Absorption is mainly caused by

phytoplankton, inorganic and organic matter, coloured dissolved organic matter and the water itself; while scattering is largely caused by inorganic and organic matter and increased with turbidity (Hossain *et al.* 2015). Therefore both atmospheric correction and estimating the light attenuation coefficient are necessary for optical mapping of seagrass communities (Giardino *et al.* 2019). Theoretically any satellite or airborne sensor can detect seagrass reflectance, and the most commonly used visible bands are from multispectral and hyperspectral satellite sensors (Hossain *et al.* 2015).

Cover of eelgrasses (*Zostera marina* and *Zostera noltii*) was assessed over a 140 km^2 area in the Schleswig – Holstein Wadden Sea by Kohlus *et al.* (2020). Sentinel -2 and Landsat - 8 OLI sensors were used with processing following a standardised method by Stelzer (1998) which included atmospheric correction, cloud detection linear spectral unmixing and band combinations for vegetations density assessment using Normalized Difference Vegetation Index. Classification of pixels was performed by decision tree that combined different indices derived from the spectral information of the surfaces. Post processing removed incorrectly classified pixels from the final maps via visual inspection by experts. Ground truthing was carried out using dedicated transect and quadrat (10 × 10 m) ground truth measurements. Dense eelgrass cover (>60% density) was classified with an accuracy of 84% for Landsat-8 and 79% for Sentinel-2 while the seagrass free and sparsely covered areas (0–10% density) were classified with an accuracy of 68% (Landsat-8) and 86% (Sentinel-2). It was thought Sentinel- 2 performed better due to higher spatial resolution of the sensor (10m x 10m). Sentinel-2 had a tendency to underestimate seagrass density while Landsat-8 had a tendency to overestimate. It is important to note that this study observed intertidal seagrass meadows and therefore did not need to correct for light attenuation.

A comparison of mapping performance from four satellites (Landsat-8, Sentinel-2, Ziyuan 3-A and WorldView- 3) was carried out in the Eastern Banks, Moreton Bay, Australia (Kovacs *et al.* 2018). Six species were known to be present in the study site (*Halophila ovalis, Halophila spinulosa, Halodule uninervis, Zostera muelleri* (dominant), *Cymodocea rotundata and Syringodium isoetifolium*) and the water depth was shallow (above 3 m Lowest astronomical tide) with some surrounding waters up to 20m (although it was not made clear if these surrounding waters were surveyed). A semi-automated supervised object-based image analysis (OBIA) method was used (see Roelfsema *et al.* 2014 for detailed methods). Initial course segmentation was used to create image objects. Followed by extraction of shallow and exposed areas based on OBIA theory. Shallow and exposed areas were then sub divided into five individual bank areas via vector layer. Specific spectral bands used can be found in table 1 of Kovacs *et al.* (2018). A nearest neighbour algorithm was used to assign all objects a percentage cover and dominant species class. Field data was collected using georeferenced snorkel photograph transects between 500m and 1,500m. This was used for both calibrating the algorithms and accuracy assessments. All satellites created similar maps of distribution of seagrass species and seagrass cover. WorldView did not map any of the deeper waters (depth not stated). Average accuracy of the four sensors was 66% for species distribution and 57% for seagrass percentage cover. All sensors performed best in the highest seagrass percentage cover category (81% accuracy by Sentienl-2 down to 67% accuracy by Ziyuan 3-A) and worst in the lowest percentage cover, 1 -10% (30% accuracy by Ziyuan 3-A and 21% accuracy by Sentienl-2).

Lyons *et al.* (2011), and Roelfsema *et al*, (2014), also used satellite imagery to map seagrass in in the Eastern Banks, Moreton Bay, Australia. Satellite imagery used from

Quickbird 2 (Lyons *et al.* 2011 & Roelfsema *et al*, 2014), IKONOS and World-View2 (Roelfsema *et al*, 2014), giving a range of resolutions from 2.4m^2 to 30m^2. The maximum depth in the study site was shallow (less than 3m) with deeper areas being masked out during the processing stage. Field data was used to calibrate the algorithms and assess the accuracy of maps produced, and was collected similar to the method above with snorkeller and underwater camera taking GPS reference images of the seabed. Both studies showed that accuracies were lower for percentage cover assessments than they were for species distribution. Roelfsema *et al*, (2014) showed overall accuracies for the object-based species composition maps ranged from 68% to 83% with a median of 77% while for the percentage cover maps this ranged from 48% to 58% with a median of 52%. Lyons *et al.* (2011) did not state an overall accuracy, however they showed user accuracy was lowest in the 70% -100% seagrass density category and suggested this was due to confusion with deep water, although areas deeper than 3m were excluded from the data analysis.

Sagawa *et al.* (2008), used IKONOS satellite imagery to map seagrass meadows in Japan with water depth ranging from 0 – 30m. Unlike most other studies they used Side-scan sonar as a method of validation rather than the labour-intensive snorkel surveys. Satellite images were analysed using a supervised classification with regard to the different bottom-type areas. Three different classes were highlighted: *Zostera caulescens*, *Zostera asiatica*, and sand. Sonar data and satellite images were overlaid using a GIS software to obtain training data and then to classify every pixel with reference to the three classes designed. The authors also collected aquatic video camera data to create three error matrices. They found convincing correspondence between the sonar and satellite derived maps up to a depth of 10m after which the accuracy was very low. The Side-scan sonar had an overall accuracy of 97.3% that

justified their use of this technique as a method for both collection of calibration data and ground truth data.

Satellite mapping does, however, have significant limitations, such as cost for high resolution imagery, image distortion and its loss of accuracy when surveying depths of greater than fifteen metres depending on water clarity (Lefebvre *et al.* 2009). Absorption and scattering increase with depth through the water column, causing an exponential reduction in light intensity, that means that accuracy of optical remote sensing decreases rapidly with depth (Zimmerman & Dekker, 2007). Unlike terrestrial plant ecosystems, seagrass communities are often submerged and hence there are limitations in applying landscape techniques using remote sensing methods to seagrasses (Veettil *et al.* 2020). This is especially pertinent for *P. oceanica* due to its large depth range, although, at shallower depths this technique has been shown to be useful owing to the size of area that can be covered in a short period of time (Piazzi, Acunto, & Cinelli, 2000).

Recently, Topouzelis, *et al*, (2018), used Landsat 8 satellite imagery to map *P. oceanica* around the Greek islands. They claimed that while satellite imagery had previously been shown to provide accurate maps in shallow waters, they were able to map down to 40m in some areas using Landsat 8 images with a 30m resolution. The authors highlighted the need for reliable up-to-date reference data from *in situ* observation, sonars and ROVs to test the accuracy of the maps produced from these satellite images due to current reference data being outdated and unreliable. This lack of reliable reference data hinders the ability to produce accurate maps and particularly in an area that does not have detailed bathymetry data readily available.

2.1.1.2 Acoustic mapping of seagrass

Side-scan or multi beam sonar for acoustic mapping of seagrass ecosystems is another widely used remote sensing method (e.g. Pasqualini *et al.* 1998; Komatsu *et al.* 2003; Sagawa *et al.* 2008; Lefebvre *et al.* 2009; Montefalcone *et al.* 2014). Differences in back scatter of the acoustic signal allows submerged aquatic vegetation to be differentiated from unvegetated benthic substrates (Warren & Peterson, 2007). The larger the difference between these two signals, the higher the canopy height (Lefebvre *et al.* 2009, Warren & Peterson, 2007). Low canopy height or biomass can, however, make it hard to distinguish seagrass from other vegetation or substrate (Hossain *et al.* 2014).

Side-scan sonar and Multibeam sonar from boats have been used to map seagrass (Komatsu *et al.* 2003 & Sagawa *et al.* 2008). In some cases, it has been able to distinguish between two species of *Zostera* due to significant difference in canopy height between species (Sagawa *et al.* 2008). Side-scan sonar has been shown to be a highly accurate method with 97.3% accuracy in Japan, however they experienced misclassification at the boundaries of areas (Sagawa *et al.* 2008). Side-scan sonar was used to map seagrass in Texas in shallow (< 2m), but turbid water, to an accuracy level of 97% (Rahnemoonfar *et al.* 2018). Pergent at al. (2017) used Side-scan sonar for mapping *P. oceanica* meadows between 10 and 50m and an underwater camera fixed to the transducer for reference data. There was, however, no accuracy assessment presented to evaluate the performance of this method at depth. Multibeam sonar was used to assess *P. oceanica* meadows in Malta by Micallef *et al.* (2012), but again despite field data being collect by ROV and SCUBA there was no accuracy assessment given.

However, there are limitations to both these methods. These include depth due to boat draft, often making it hard to map below 3 metres and the slow boat speed making it

highly time consuming (Sagawa *et al.* 2010). In addition, for conservation organisations, the equipment and software needed, i.e. boats and Side-scan/ multibeam sonars, are very expensive and require a lot of training to use, therefore often not a practical solution for ongoing monitoring.

More recently Greene, *et al.* (2018), showed side-scan sonar was a reliable tool for mapping the seagrass *Thalassia testudinium,* in Florida. The cost of the Side-scan sonar array was $1,740 and the set up required a boat that has maintenance and operating costs to consider. The average depth of area mapped was just 0.8m which is just a fraction of the depth range of *P. oceanica*.

To date down-scan sonar has yet to be extensively used for mapping of seagrass meadows. Its smaller size and lower cost in comparison with side-scan and multibeam sonars suggests its potential as a low cost method of mapping. Down-scan sonar in conjunction with BioBase Echosound (cibiobase, Navico, Inc., Minneapolis, MN) for sonar analysis has been shown to be robust in freshwater systems while still being efficient and cost effective (Radomski & Holbrook, 2015), and has been used to map a small area of *Zostera marina* seagrass in the USA (Luczkovich *et al.* 2013). It has yet to be applied to other seagrass species, such as those with a wider depth and habitat range such as *P. oceanica* but has the potential to provide the much-needed reference and bathymetry data required for large-scale reliable satellite mapping.

2.1.2 Chapter Aims

Knowledge of the distributions of key ecosystems, such as seagrass, allows for more effective conservation by providing evidence for management (Tyllianakis *et al.* 2019). Despite *P. oceanica* being one of the most studied species in terms of mapping effort,

the vast majority of this has been concentrated on the western Mediterranean and more information on the distribution in the eastern region is needed (Umit Gumusay *et al.* 2019). A review by Appolloni *et al.* (2020) found the studies on the use of remote sensing for monitoring *P. oceanica* to be limited despite the recognition that it is highly useful technology. It is possible that the many studies on mapping methods identified by Umit Gumusay *et al.* (2019) did not become adapted for long term monitoring effort of *P. oceanica*. This demonstrates the need for an accessible and low cost method that can be used across the entire geographical and depth ranges of *P. oceanica*. Therefore the objectives of this study were

- to test the efficacy of down-scan sonar as a tool for mapping seagrass;
- to characterise the effort and resource required to map locally significant areas of coastal water;
- to assess the accuracy of recently produced seagrass maps for Greek waters.

2.2 METHODS

2.2.1 Study Area

The Aegean Sea is located in the eastern Mediterranean basin lying between Greece and Turkey. It has a negligible tidal range, rarely exceeding 10 cm.

This study was carried out around the Aegean islands of Samos and Lipsi (figure 2.1) with surveys taking place between September 2017 and April 2018. Lipsi, one of the northern Dodecanese Islands (37°18′N 26°45′E), has an area of 16 km^2 and 35 km of coastline that is characterised by a relatively complex bathymetry and range of benthic substrates. Samos, one of the North Aegean Islands (37°45′N 26°50′E), has an area of

477 km^2 and 149 km of coastline. In Samos surveys concentrated on a shallow bay to the southeast of the island, known as Mesokampos Bay, that is characterised by sandy substrates and dense, continuous seagrass meadows (figure 2.1 b). The methods were subsequently trialled at Vroulia Bay, Lipsi (figure 2.1c), where bathymetric conditions are more complex and seagrass cover is patchier. A larger scale trial was subsequently conducted around the majority (70%) of Lipsi island.

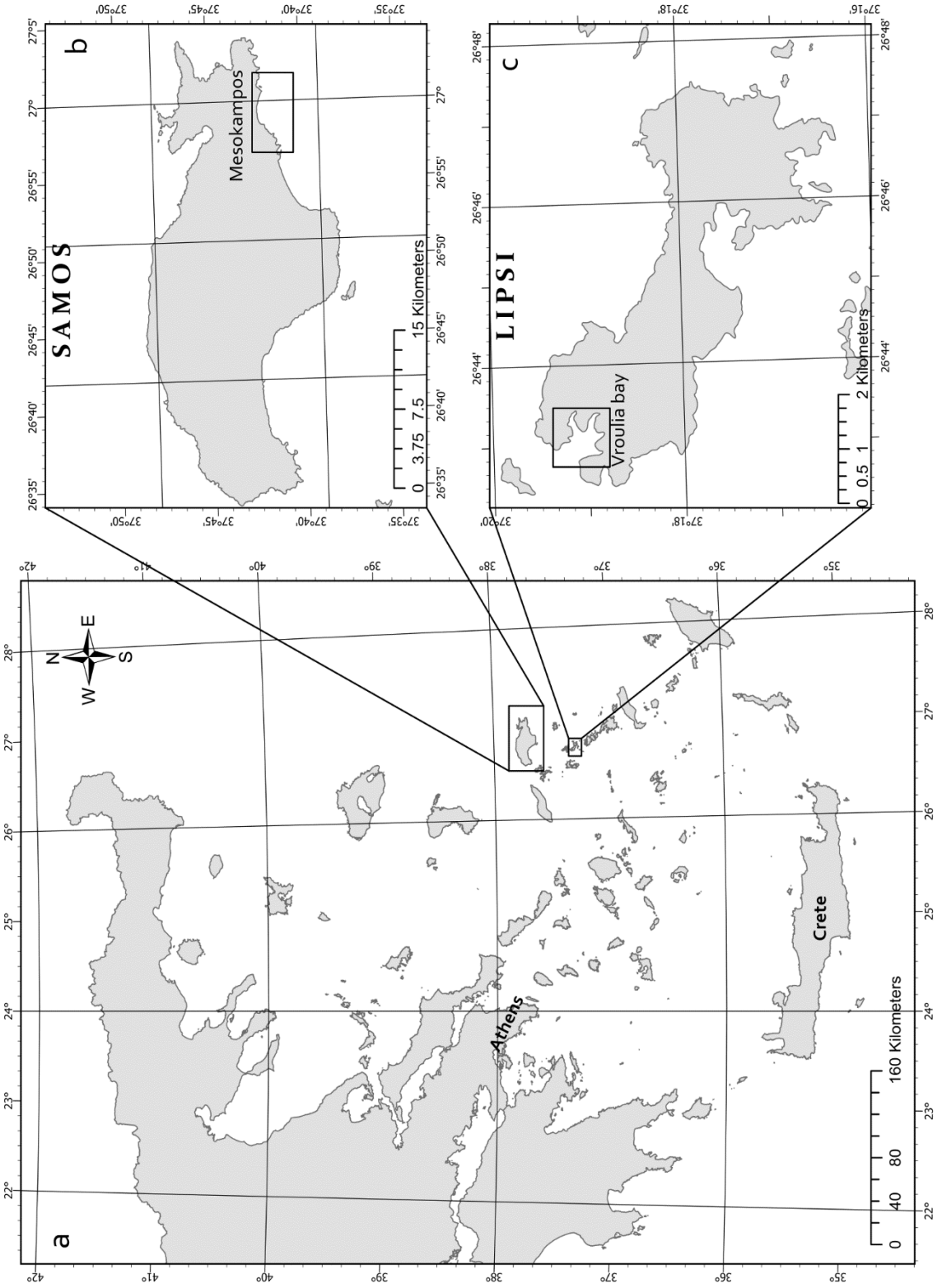


Figure 2.1 a) map of Greece with the locations of Samos and Lipsi outlined in black, b) enlarged map of Samos with the study site of Mesokampos outlined in black, c) enlarged map of Lipsi with the study site of Vroulia Bay outlined in black.

2.2.2 Mapping of *P. oceanica* meadows

2.2.2.1 Sonar mapping

A Lowrance Elite 7 Ti, down-scan sonar (Ping frequency set at 200 KHz and 20 pings per second GPS accuracy of 6m as stated by the sonar estimated position error), was attached to the back of a kayak using a stabilising metal arm and positioned so the transducer was parallel to the sea floor, just below the surface so no offset was required. Survey transects were carried out parallel to the shore 100 metres apart up to a maximum distance of 300 metres offshore. Survey speeds did not exceed 5 km hr^{-1} to maximise data resolution. Mapping was carried out in only calm weather conditions, taking place in winds no greater in strength than a Beaufort 2.

Sonar-derived point-cloud data from each transect were analysed using BioBase cloudbased software (cibiobase, Navico, Inc., Minneapolis, MN) to estimate aquatic vegetation extent and volume to a limit of 1% of the water column. Down-scan sonar transducers emit 10-20 "pings" (i.e signals) per second towards the seabed. The sonar unit evaluates these signals and stores them as point data. This data is uploaded to BioBase and the algorithm interprets the sound waves for bottom location, vegetation presences, and if present at what height the canopy intercepts the signal, given in biovolume. Biovolume (B), that is the percentage of the water column occupied by the vegetation, can be used to estimate vegetation canopy height (C) in meters (m) by:

$$C = (D.B)$$

where *D* is the depth of the water column (m). Data points were visually inspected before processing by BioBase to remove areas with excessive scatter. This scatter was clearly distinguishable from "clean" sonar derived data as shown in figure 2.2. These areas were always over rocky substrates and usually with steep gradients (greater than 15%) and since *P. oceanica* is highly unlikely to be growing in ecologically significant populations in these conditions (Di Maida *et al.* 2013), it was deemed appropriate to remove them to prevent false positives.

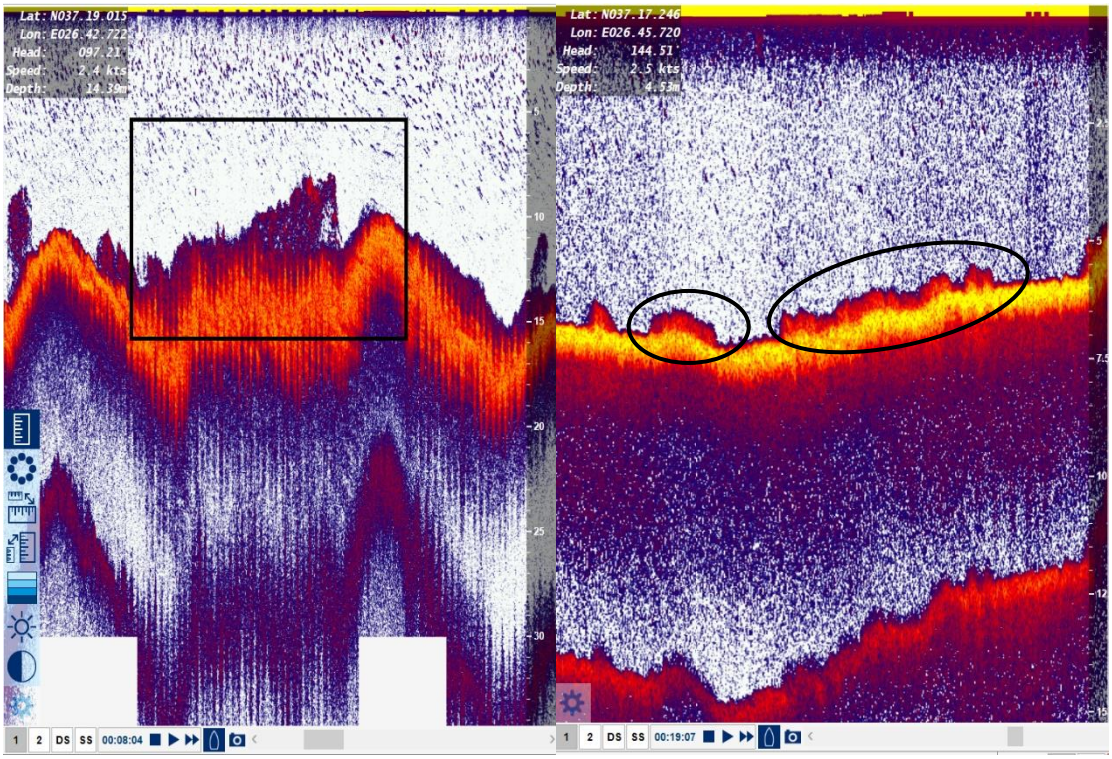


Figure 2.2 Left: Example of sonar data that showed high level of scatter (outlined with black square). These areas were removed to maintain the quality of the data for further processing. Right: area of "clean" sonar data with seagrass outlined in black circles. Sonar data was viewed using Reef Master (version 1.8).

2.2.2.2 Ground Truthing

It was not practical to use snorkelers / divers for this study due to depth at which many of the sample points were located. Ground truth points were allocated along sonar

tracks using an equalised stratified sampling method across depth categories at a minimum of 10 m apart, allowing for the GPS accuracy of ± 6 m.

A heavily-weighted, pyramid-framed quadrat (50cmx 50cm, 1m high) mounted with two digital cameras set for video capture (GoPro Hero 5: 1080p, 60fps, 12 Megapixels), was lowered to the seafloor. The quadrat was heavily weighted to increase sinking speed and prevent movement once resting on the seabed. The resulting video capture analysed for percentage cover and canopy height.

A greater number of ground truthing points were taken in Vroulia Bay compared to Samos due to its wide depth range and more complex topography. It is also much more accessible for repeated surveys over a number of days than many other locations on the island. Sixty-seven ground truth points were taken from directly on the sonar track and a further 46 points were taken from points without sonar data directly associated to test the accuracy of the interpolation of sonar data, making a total of 113. Due to the smaller region in Samos that were mapped at the development stage fewer (40 directly on sonar tracks and 6 from the general area) ground truthing points were carried out.

Only canopy heights observed by both the camera and sonar to be more than 10 cm were classified as present in accordance with the threshold set for the sonar data. Canopy heights of less than 10 cm were considered to be absent of *P. oceanica* to avoid confusion with algae or the non-native seagrass *Halophila stipulacea* (Fornes *et al.* 2006).

2.2.2.3 Interpolation of sonar data

Spatial extent of seagrass was estimated between sonar derived data points using Inverse Distance Weighting (IDW) interpolation using ArcGIS 10.4 (ESRI). Curtarelli *et*

al. (2015), showed IDW produced results of bathymetric interpolation similar to that of kriging. IDW estimates cell values by averaging the values of sample data points in the neighbourhood of each processing cell. Points closest to the centre of the cell being estimated have more weighting in the averaging process. Kriging is a more complex and advanced geostatistical tool that makes use of a spatial continuity measure to calculate the spatial autocorrelation between points at graduated distances. It uses this calculation of spatial autocorrelation to determine the weights that should be applied at various distances. IDW and kriging are similar in that they both form weights from surrounding measured values to predict unmeasured locations, however IDW is a much simpler tool. IDW was therefore used in this work for its simplicity and accessibility.

2.2.3 Statistical Analysis

Confusion matrices, measured using Cohen's kappa coefficient (κ), were used to perform pairwise evaluations of agreement between the presence/absence *in situ* measurements, sonar-derived data and the LANDSAT-derived data presented by Topouzelis *et al.* (2018). A value of κ in the range of 0-0.20 can be classified as slight agreement, 0.21-0.40 as fair, 0.41-0.60 as moderate, 0.61-0.80 as substantial, and 0.81-1 as almost perfect (Landis and Koch 1977). Observed accuracies were also calculated in percentages as recently there have been limitations in kappa statistics highlighted (Pontius & Millones, 2011). All analyses were completed in RStudio (Version 1.2.5019) using the package irr (Gamer *et al.* 2019).

For the classification of canopy heights weighted Cohen's kappa was used. Weighted Cohen's kappa allows for differences between classes to be accounted for in the calculation of the kappa value and are better than standard kappa's for more than two

ordinal categories. For the Lipsi data a linear weighted kappa was used, this gave equal weighting to each group and therefore the penalty for being wrong by 1 or 3 categories is the same. In Mesokampos Bay a quadratic weighted kappa was used as this is a more accurate presentation of the data than standard kappa. A quadratic weighting allowed the penalties between categories to begin mild but get increasingly harsher as the difference between correct and incorrect groups became larger. The quadratic weighting could not be used for the data deriving from Vroulia Bay, Lipsi due to the number categories that contained zero data points, preventing standard errors to be calculated and therefore confidence intervals were also unavailable.

2.3 RESULTS

2.3.1 Mapping Effort

Sonar transects covering a total of 90.4 km were completed across both islands; Mesokampos Bay = 7.2 km, Vroulia bay = 4.3km and around the Lipsi coastline = 83.2 km (which equates to 70% of the entire coastline of the island).

Mapping speeds averaged 2.5 km hr^{-1}, including travel time. IDW analysis provided an overall coverage of 1.94 km^2 in Mesokampos Bay and 7.36 km^2 in Lipsi (figure 2.3), at an average survey efficiency of 20 to 32 hectares hr^{-1}. The areas of Lipsi Island mapped were completed over a period of 6 days consisting of a total 30 boat hours. There were two areas in Lipsi that were unable to be mapped due to safety considerations and

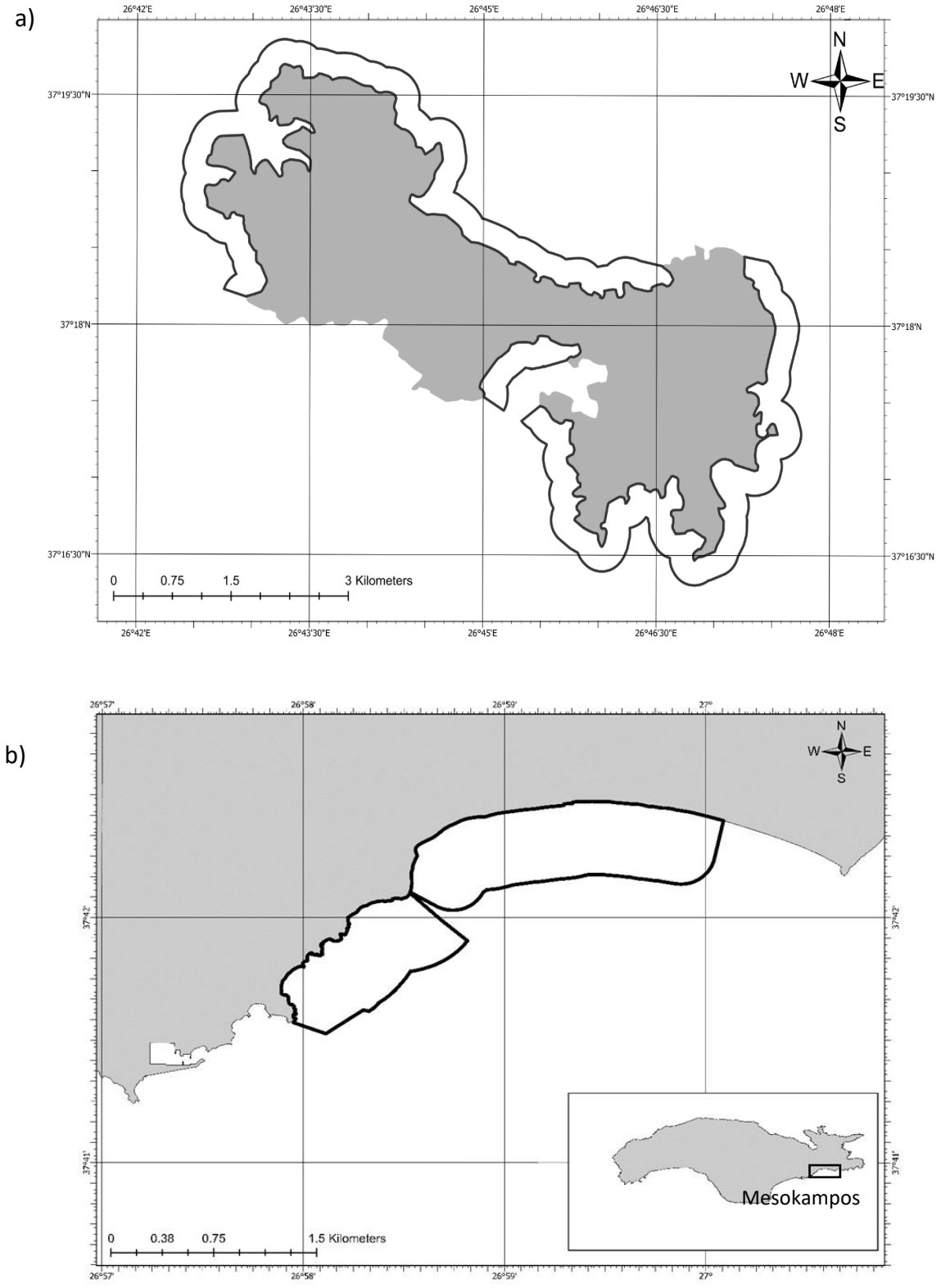


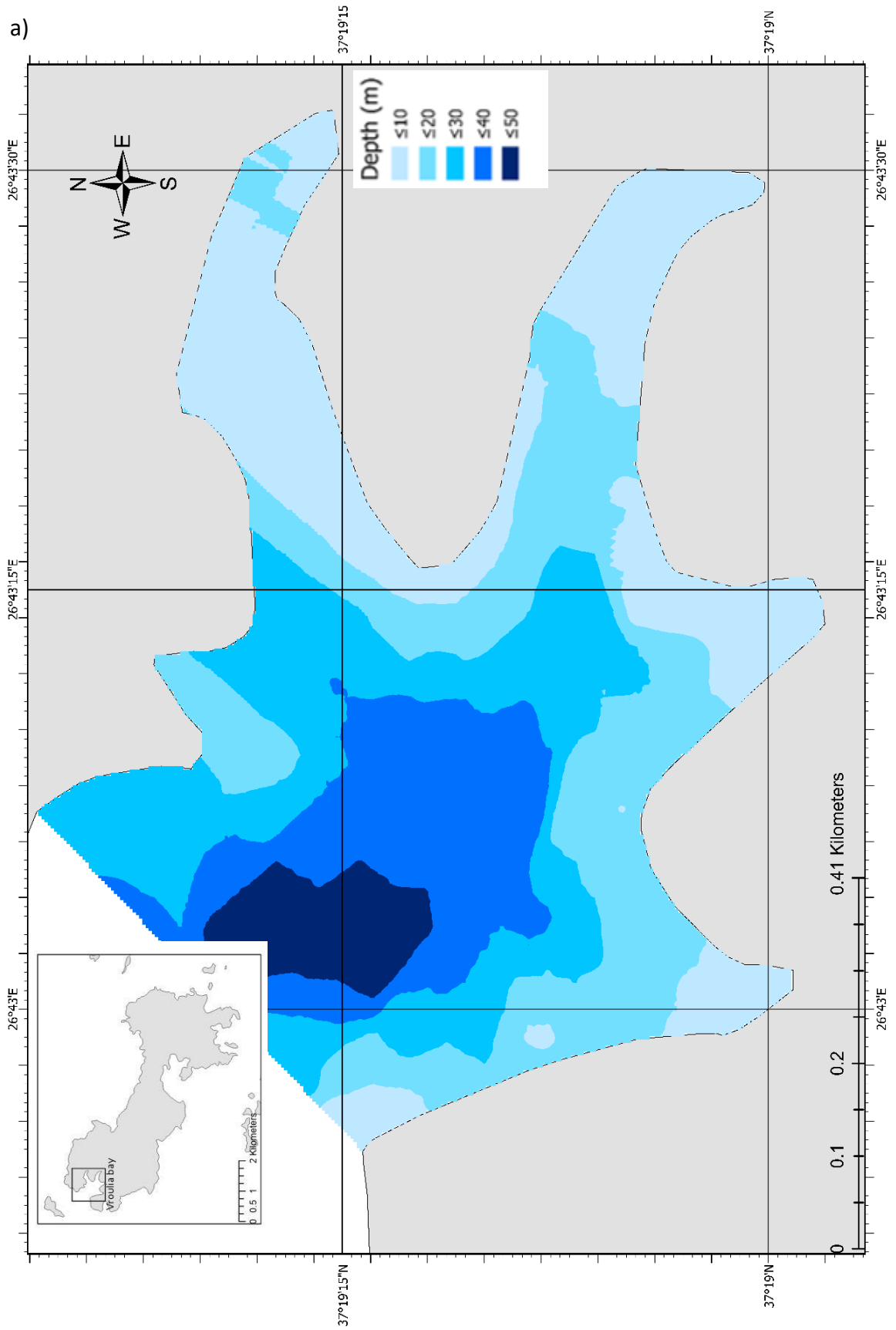
Figure 2.3 Maps of the region covered by sonar in Lipsi a) and Samos b). The location of Mesokampos Bay is outlined in black in the inserted map of Samos

lack of access for kayaks at the time of surveying or because it was a working port with ferries travelling through. In Samos the area mapped took a total six boat hours spread over two days.

Results from the *in situ* survey provide an estimate that seagrass covered 58% of the surveyed area at Mesokampos Bay and 10% in Vroulia Bay. Canopy height was significantly higher in Mesokampos Bay compared to Vroulia (t-test: $t = -3.959$, $df = 157$, $p < 0.001$; mean effect size = 13.469 ± 6.72 cm 95% CI). In Mesokampos bay seagrass was observed at 4.5 m – 12.6 m, while in Vroulia bay seagrass was observed from 2.7 m down to 28.4 m depth.

2.3.2 Sonar derived bathymetry of study area

Bathymetry maps for the study areas show contrasting bathymetric conditions between the two locations and can be seen in (figure 2.4). Vroulia Bay, Lipsi, showed a very complex bathymetry with a steep slope in the centre of the bay, reaching depths of up to 50m. Samos on the other hand showed very consistent bathymetry with a steady slope to a maximum depth of 20m.


a)
37°19'15"
37°19'N
26°43'30"E
26°43'15"E
26°43'E
37°19'15"N
N
S
E
W
Depth (m)
≤10
≤20
≤30
≤40
≤50
0.41 Kilometers
0
0.1
0.2
Vroulia bay
0 0.5 1 2 Kilometers

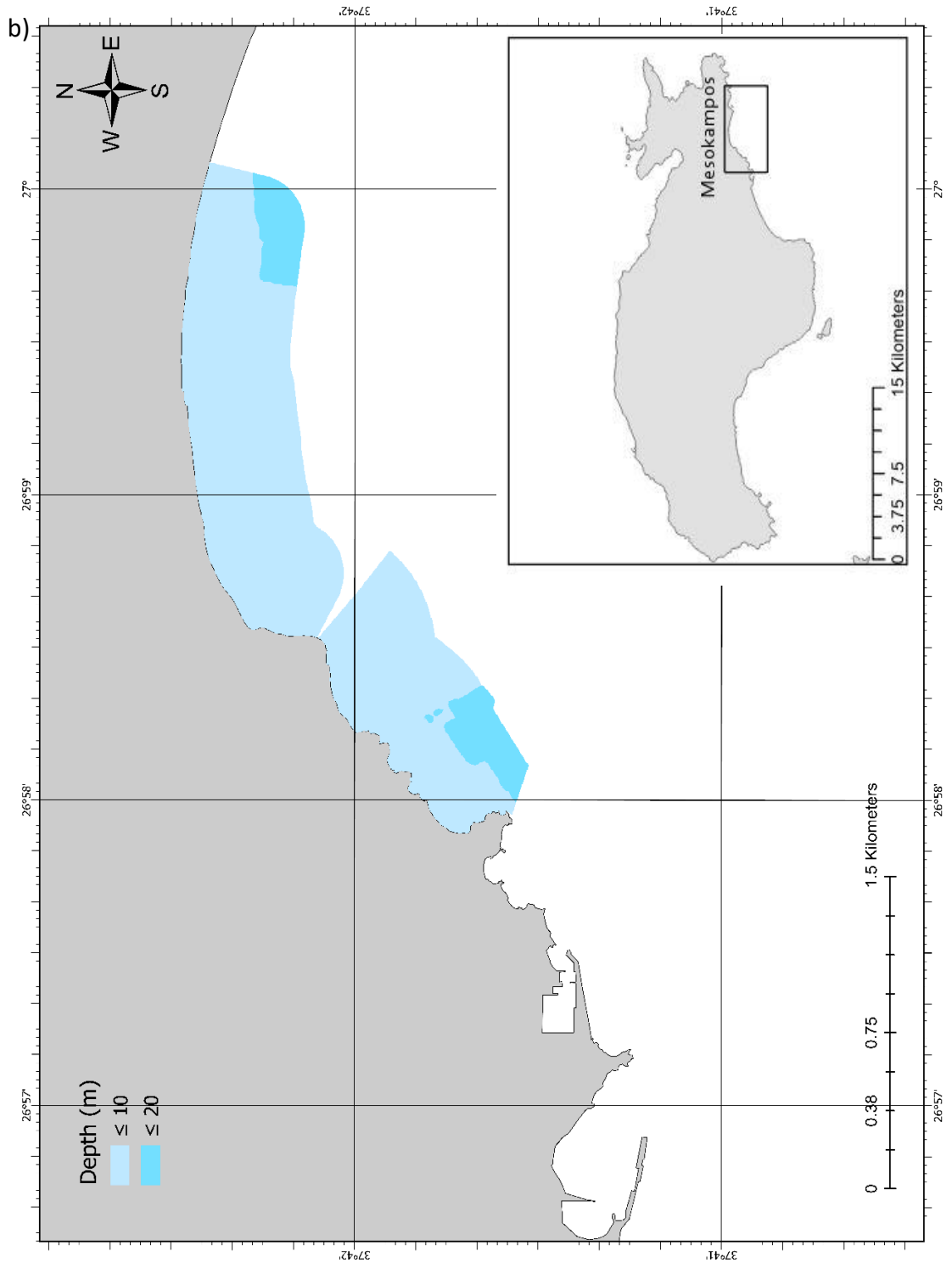


Figure 2.4 Bathymetry depth profiles for Vroulia Bay, Lipsi a) and mapped regions in Mesokampos, Samos b). Each contour represents a 10m depth change, starting with the palest blue for 0-10m and the darkest for 40m-50m

2.3.3 Accuracy of Presence/Absence data

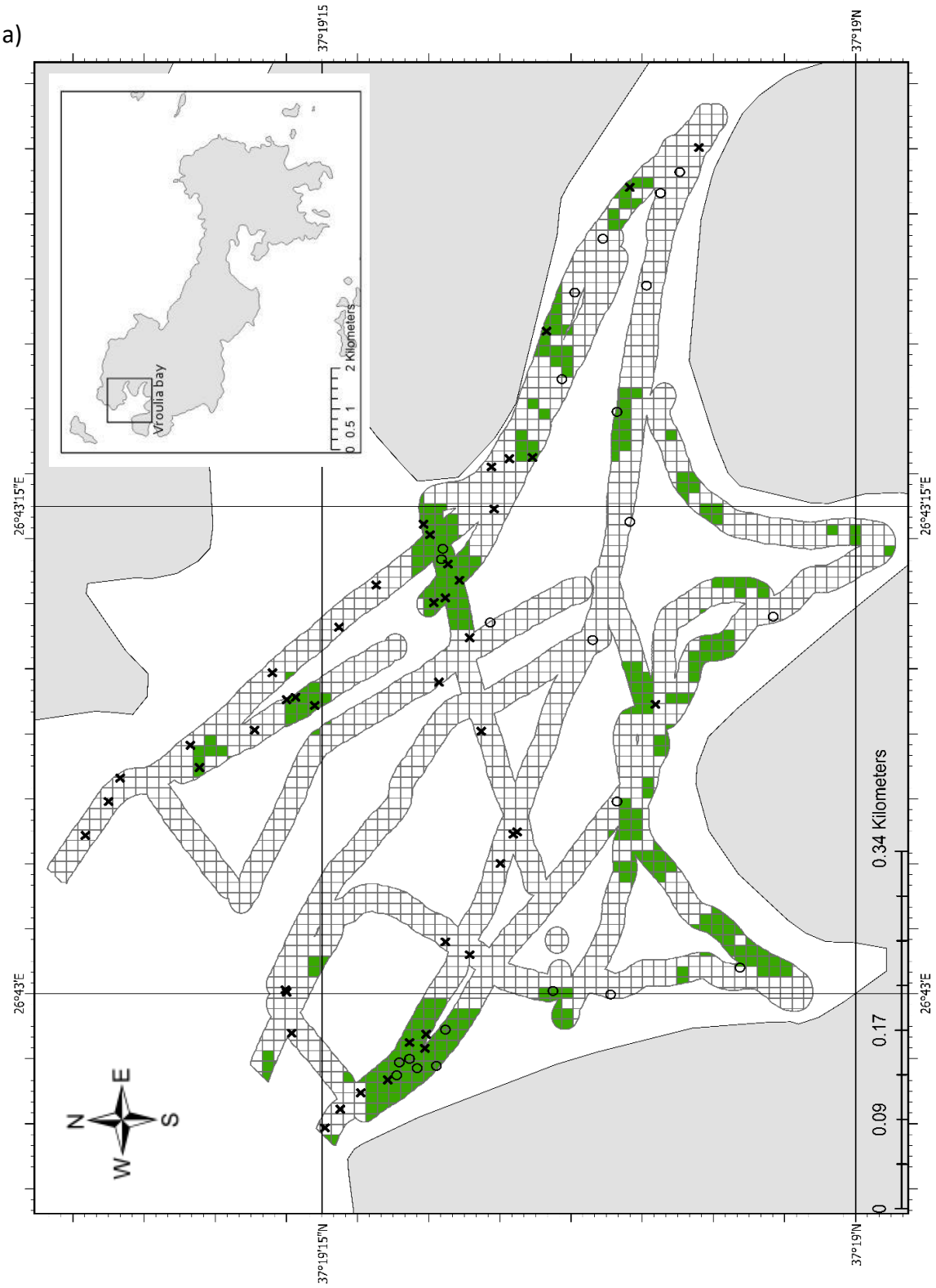
2.3.3.1 Sonar derived data

Observed accuracy for sonar data alone for the presence or absence of seagrass was 64% in Vroulia, Lipsi, and 83% in Mesokampos, Samos (figure 2.5, table 2.1). At Mesokampos $\kappa = 0.622$ (95% C.I. = 0.369, 0.874) and was significantly different to zero ($p < 0.0001$), while in Vroulia κ was lower (κ=0.237; 95% C.I. = 0.001, 0.481) and was just significantly different from zero ($p = 0.050$) (table 3.1).

Table 2.1: Confusion matrices for Vroulia Bay, Lipsi and Mesokampos, Samos of observed *P. oceanica* data against sonar track data *P. oceanica*

			Sonar	
			absent	**present**
Observed	**Lipsi**	**absent**	30	14
		present	10	13
	Samos	**absent**	11	4
		present	3	11

a)


37°19'15
37°19'N
26°43'15"E
26°43'E
37°19'15"N
37°19'N
Vroulia bay
0 0.5 1 2 Kilometers
0 0.09 0.17 0.34 Kilometers
N
E
S
W

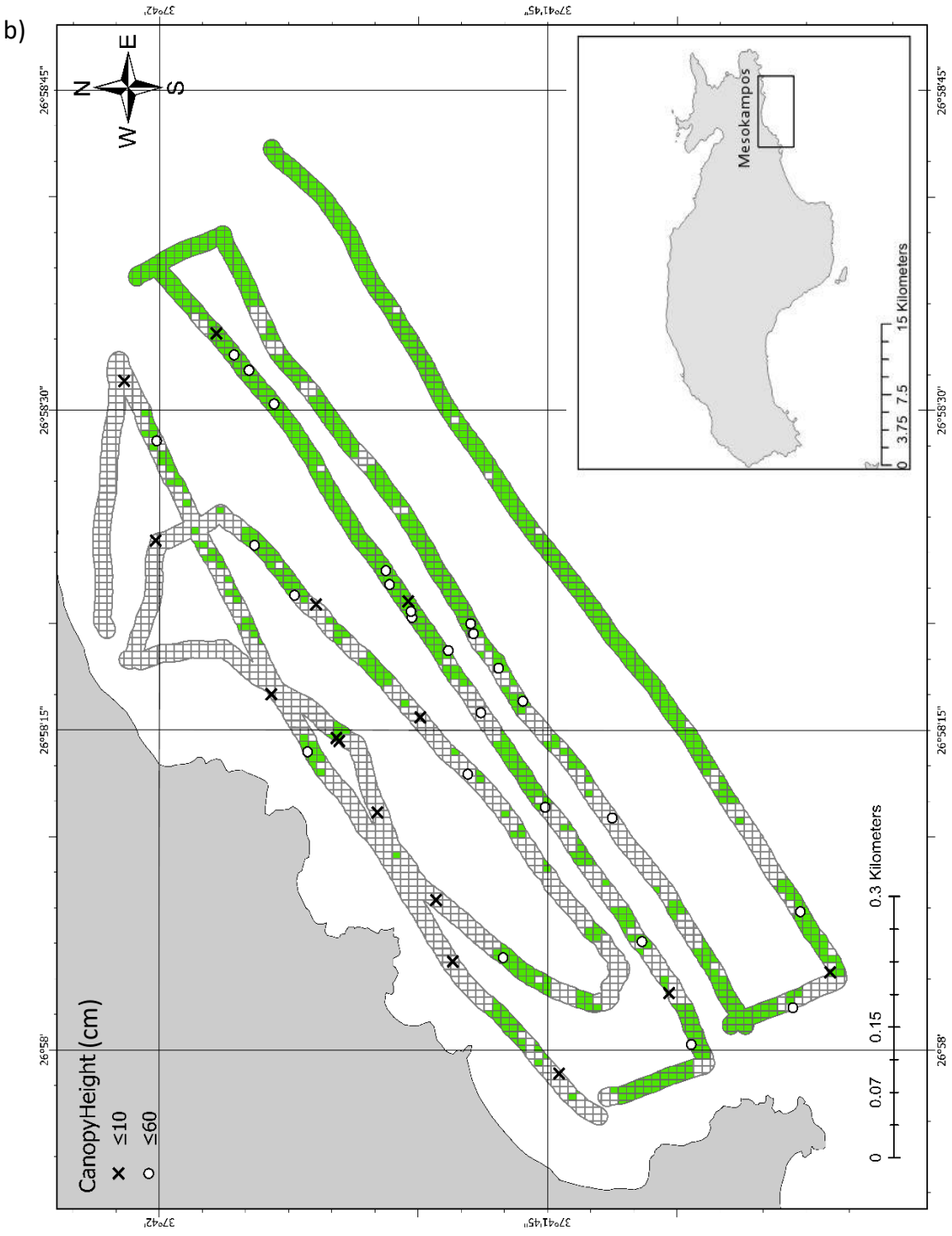


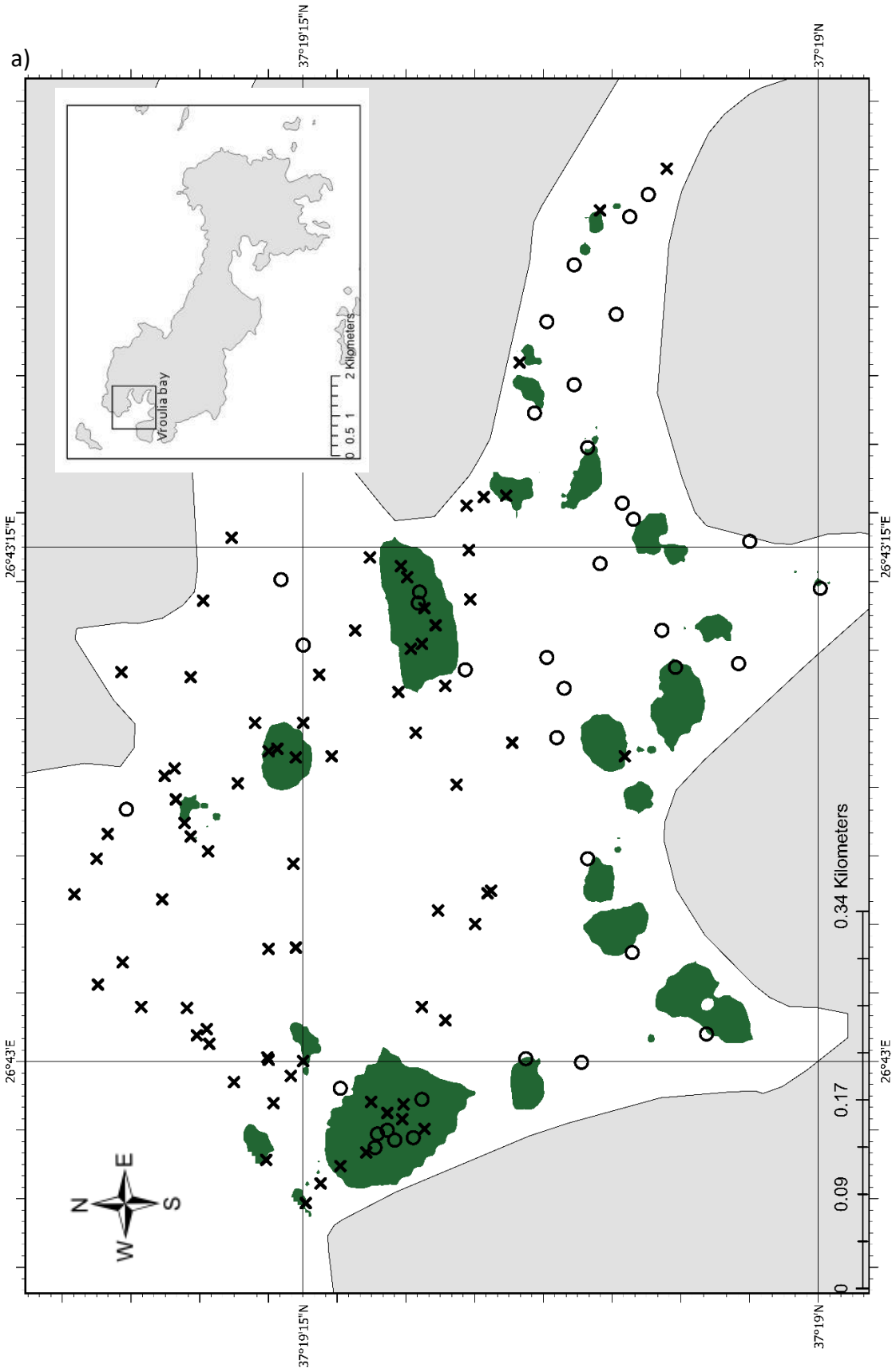
Figure 2.5 maps of sonar track data and observed presence/ absence in a) Vroulia Bay, Lipsi and b) Mesokampos, Samos. Sonar derived presence absence is shown using the coloured grid, green squares signify seagrass presence, no colour, shows absence. Ground truthed data is depicted by the crosses for seagrass absence and the circles for seagrass presence.

2.3.3.2 Interpolated sonar data

The observed accuracy for the detection of seagrass by sonar-derived data and interpolated using IDW interpolation in ArcGIS was 85% in Mesokampos Bay and 68% in Vroulia. At Mesokampos κ = 0.669 (95% C.I. = 0.445, 0.894) and was significantly different to zero ($p < 0.001$) while in Vroulia κ was lower (κ = 0.256; 95% CI= 0.068, 0.444), but still significantly different from zero ($p = 0.006$). These results can be found in full in table 2.2 and figure 2.6

Table 2.2: results of confusion matrices for Vroulia Bay, Lipsi and Mesokampos, Samos of observed *P. oceanica* data against sonar interpolated *P. oceanica*

			Sonar Predicted	
			absent	**present**
Observed	**Lipsi**	**absent**	60	16
		present	20	17
	Samos	**absent**	13	4
		present	4	26


a)
37°19'15"N
37°19'N
26°43'15"E
26°43'E
Vroulia bay
0 0.5 1 2 Kilometers
0 0.09 0.17 0.34 Kilometers
N
E
S
W

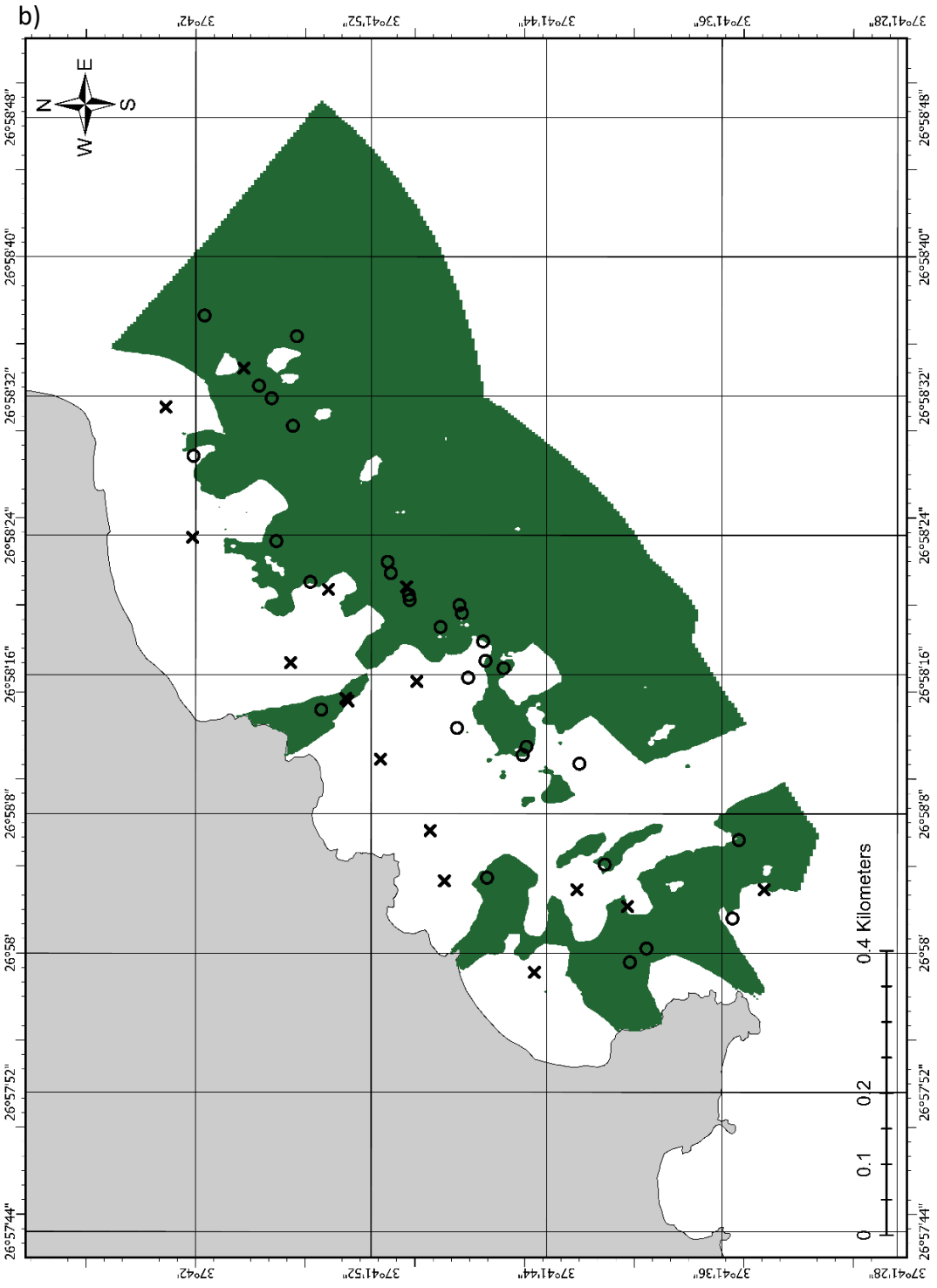


Figure 2.6 maps of interpolated sonar data and observed presence/ absence in a) Vroulia Bay, Lipsi and b) Mesokampos, Samos.. Sonar derived presence absence is shown using green to signify seagrass presence, no colour, shows absence. Observed data is depicted by the crosses for seagrass absence and the circles for seagrass presence.

2.3.4 Accuracy of down-scan sonar data in measuring measure canopy height

2.3.4.1 Sonar derived classification data

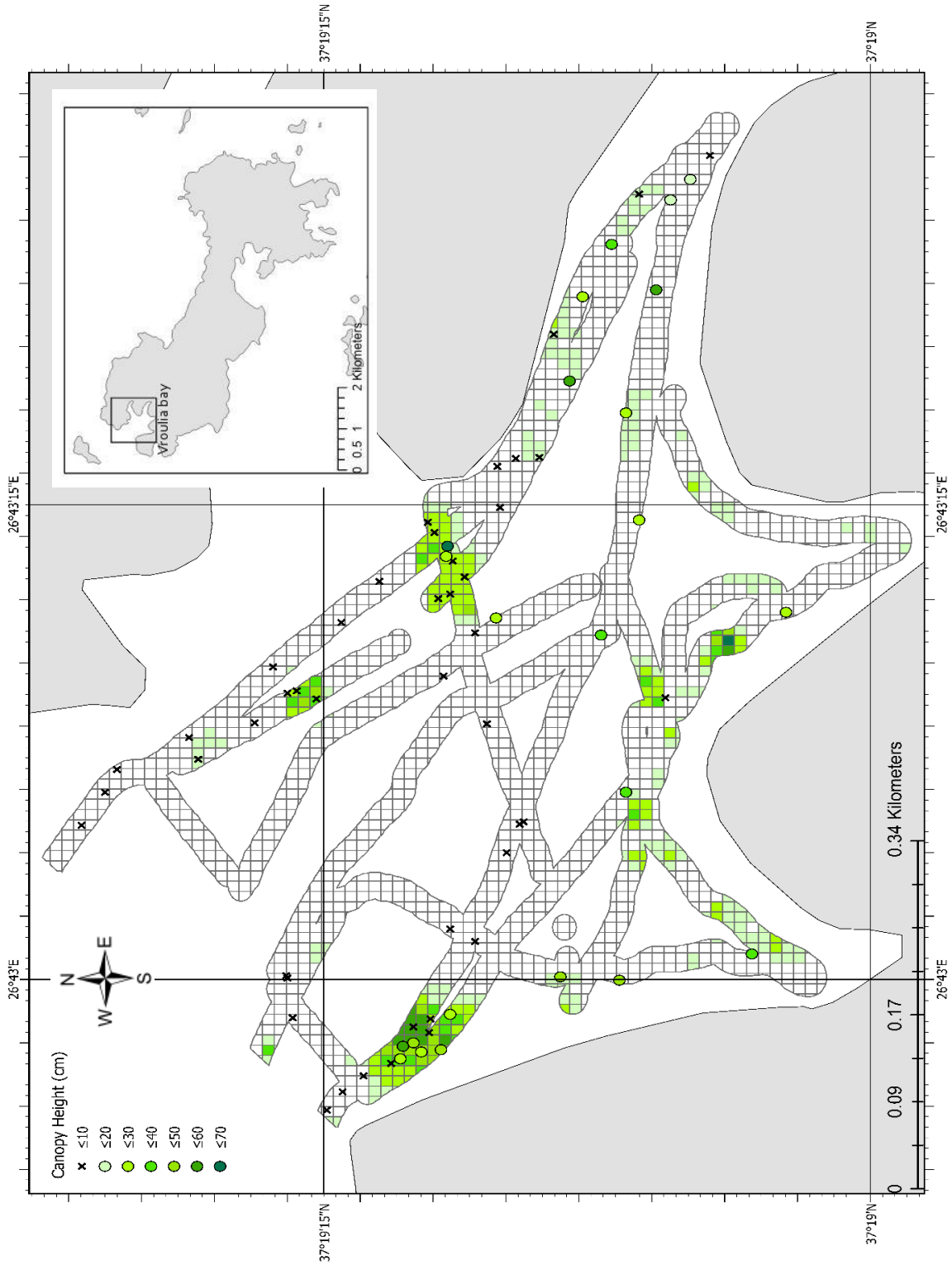
Observed accuracy for sonar data alone when estimating seagrass canopy height was 52% in Vroulia, Lipsi, and 48% in Mesokampos, Samos (figure 2.7, table 2.3 & 2.4). At Mesokampos κ= 0.611 (95% C.I. = 0.3054, 0.9176, p = <0.000) (table 2.4), while in Vroulia κ was lower (κ=0.197; 95% C.I. = 0.000, 0.4067, p = 0.0294) (table 2.3). Despite the lower observed accuracies these were still more than was expected by chance in both Mesokampos and Vroulia Bay.

Table 2.3 results of confusion matrices for Vroulia Bay, Lipsi of observed *P. oceanica* data against directly sonar derived *P. oceanica.* Highlighted in grey are cells in which sonar estimation agreed with the observed canopy height.

		Sonar						
Observed		**≤10**	**≤20**	**≤30**	**≤40**	**≤50**	**≤60**	**≤70**
	≤10	30	2	7	2	2	1	0
	≤20	2	0	0	0	0	0	0
	≤30	4	1	2	1	1	0	0
	≤40	2	2	0	0	0	0	0
	≤50	1	1	0	0	2	0	0
	≤60	1	1	0	0	0	1	0
	≤70	0	0	1	0	0	0	0

Table 2.4 results of confusion matrices for Mesokampos, Samos of observed *P. oceanica* data against directly sonar derived *P. oceanica.* Highlighted in grey are cells in which sonar estimation agreed with the observed canopy height.

		Sonar					
Observed		**≤10**	**≤20**	**≤30**	**≤40**	**≤50**	**≤60**
	≤10	11	3	0	0	0	1
	≤20	0	0	0	0	0	0
	≤30	1	9	3	0	0	0
	≤40	2	1	0	0	0	0
	≤50	0	1	2	0	3	0
	≤60	0	0	0	1	0	2


37°19'15"N
37°19'N
26°43'15"E
26°43'E
Vroulia bay
0 0.5 1 2 Kilometers
N
E
S
W
Canopy Height (cm)
≤10
≤20
≤30
≤40
≤50
≤60
≤70
0 0.09 0.17 0.34 Kilometers

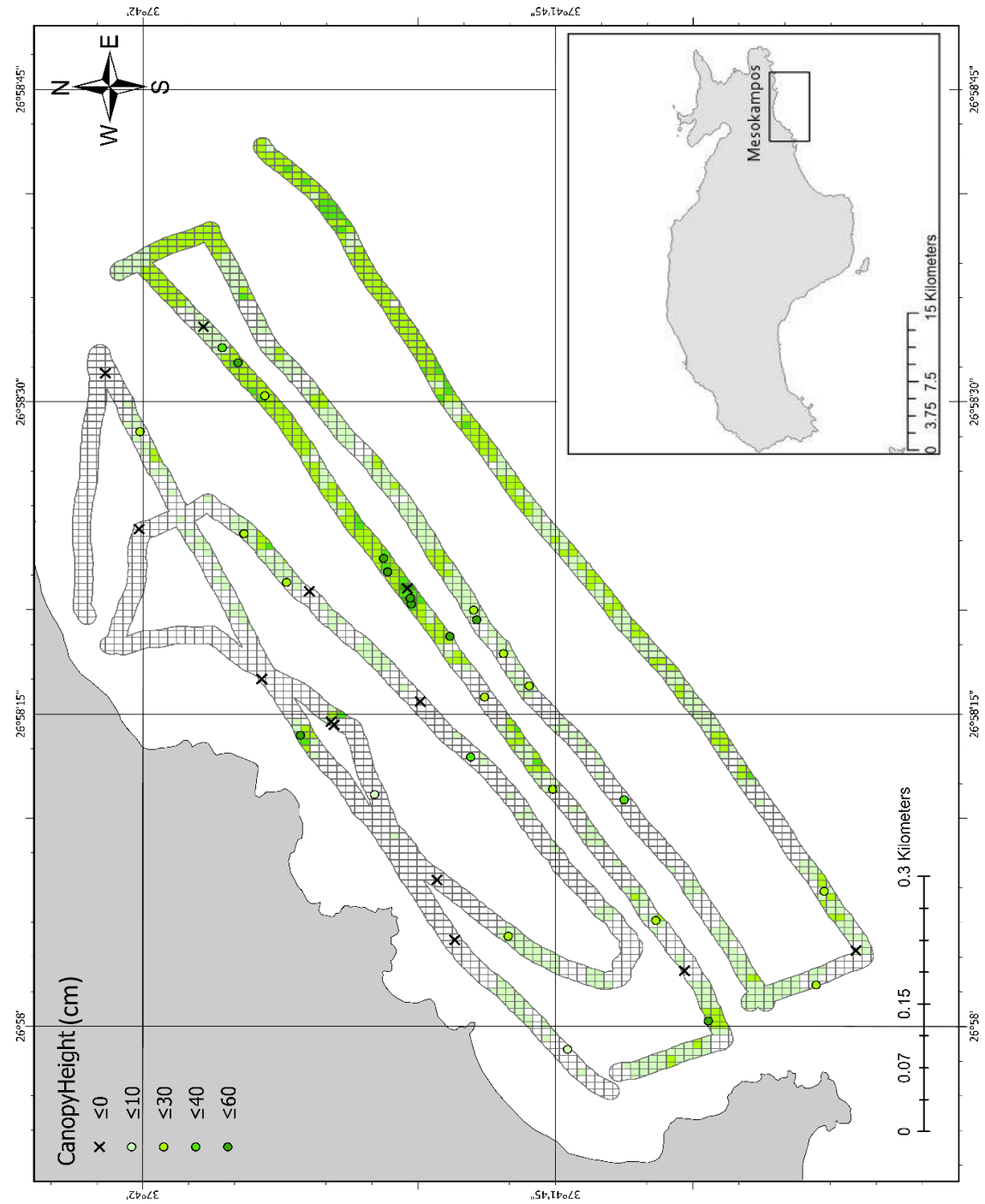


Figure 2.7 maps of sonar track data and observed presence/ absence in a) Vroulia Bay, Lipsi and b) Mesokampos, Samos.. Sonar derived seagrass is shown using the coloured grid, green squares of various shades represent estimated canopy heights. Observed data is depicted by the crosses for seagrass absence and the circles for various shades for seagrass canopy height.

2.3.4.2 Interpolated classification sonar data

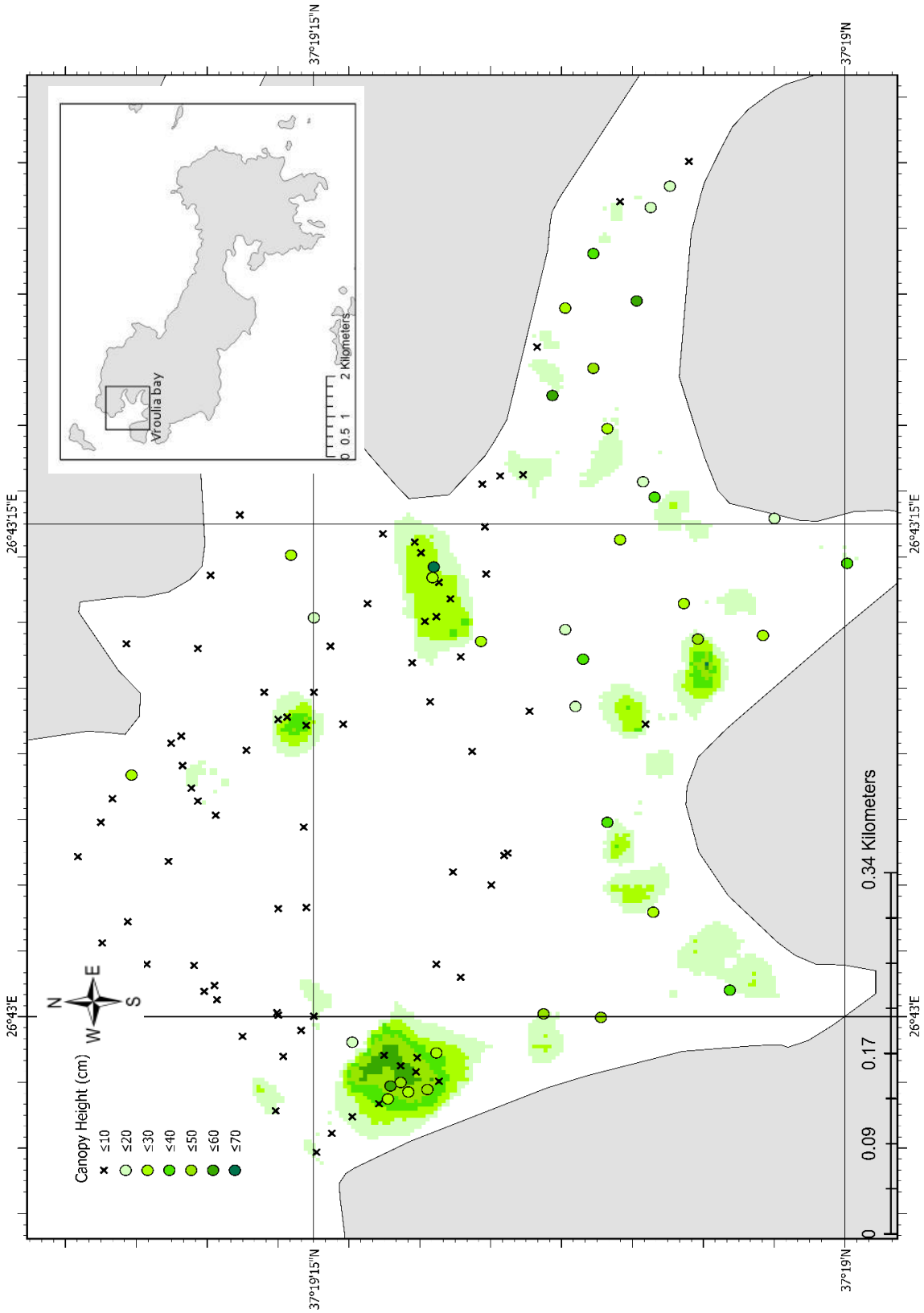
Observed accuracy for the sonar data interpolated using IDW interpolation in ArcGIS when estimating seagrass canopy height was 58% in Vroulia, Lipsi, and 38% in Mesokampos, Samos (figure 2.8, table 2.5). At Mesokampos κ= 0.467 (95% C.I. = 0.1634, 0.7714), while in Vroulia κ was lower (κ=0.188; 95% C.I. = 0.0236, 0.3523) (table 2.6). Despite the lower observed accuracies these were still more than was expected by chance in both Mesokampos (18% expected) and Vroulia Bay (50%).

Table 2.5. results of confusion matrices for Vroulia Bay, Lipsi of observed *P. oceanica* data against sonar interpolated *P. oceanica.* Highlighted in grey are cells in which sonar estimation agreed with the observed canopy height.

	Sonar interpolated							
Observed		≤10	≤20	≤30	≤40	≤50	≤60	≤70
	≤10	60	2	7	3	2	2	0
	≤20	8	0	0	0	0	0	0
	≤30	7	2	2	1	0	1	0
	≤40	2	4	0	0	0	0	0
	≤50	2	2	0	0	3	0	0
	≤60	1	1	0	0	0	1	0
	≤70	0	0	1	0	0	0	0

Table 2.6. results of confusion matrices for Mesokampos, Samos of observed *P. oceanica* data against sonar interpolated *P. oceanica.* Highlighted in grey are cells in which sonar estimation agreed with the observed canopy height.

	Sonar interpolated							
Observed		≤10	≤20	≤30	≤40	≤50	≤60	≤70
	≤10	13	2	0	0	1	0	0
	≤20	0	1	0	0	0	0	0
	≤30	2	10	2	0	0	0	0
	≤40	2	2	0	0	0	0	0
	≤50	0	2	2	1	2	0	0
	≤60	0	0	1	1	1	0	0
	≤70	0	1	0	0	0	0	0


37°19'15"N
37°19'N
26°43'15"E
26°43'E
Vroulia bay
0 0.5 1 2 Kilometers
N
E
S
W
Canopy Height (cm)
≤10
≤20
≤30
≤40
≤50
≤60
≤70
0 0.09 0.17 0.34 Kilometers

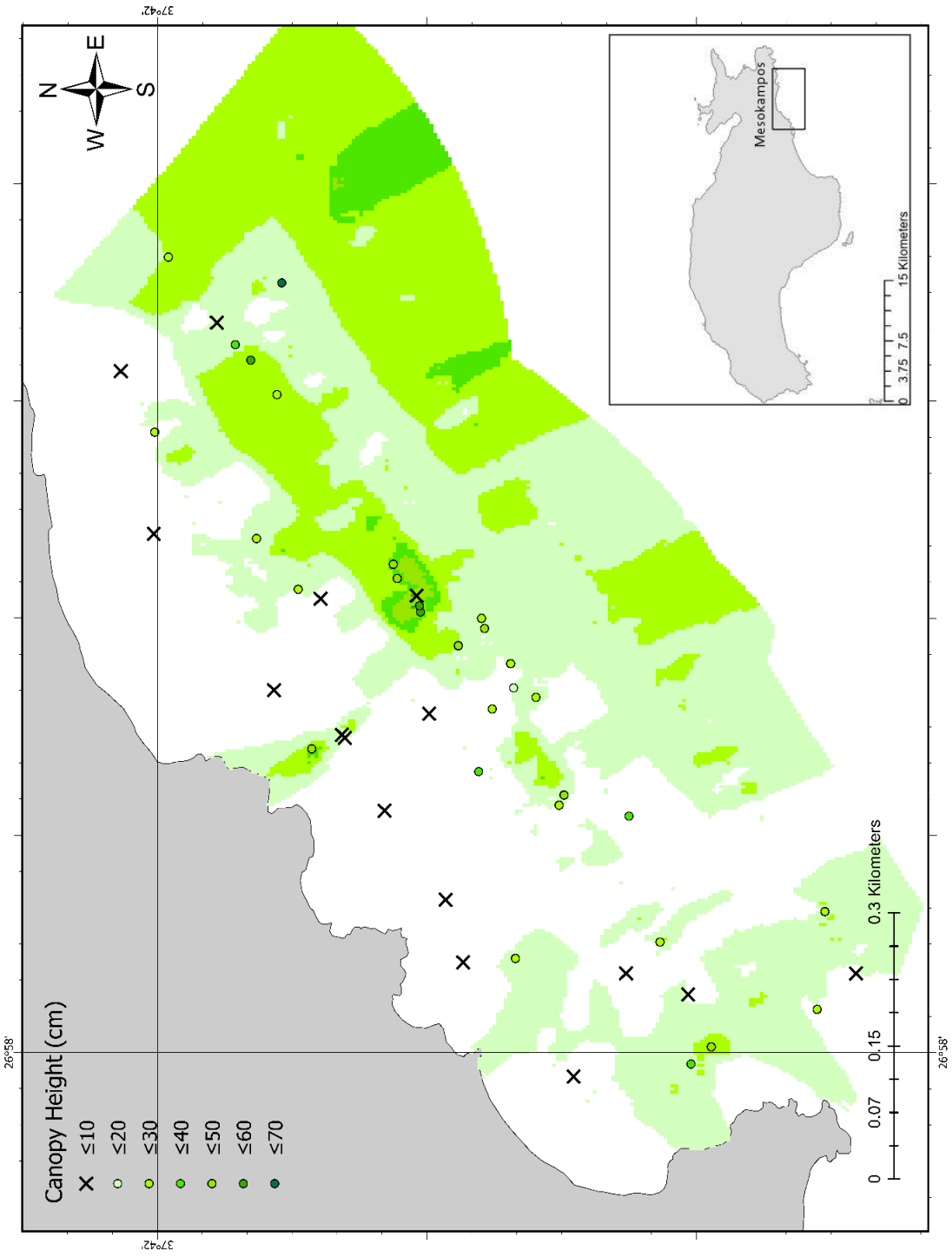


Figure 2.8 maps of interpolated sonar data and observed seagrass estimated canopy height in a) Vroulia Bay, Lipsi and b) Mesokampos, Samos. Sonar derived seagrass is shown using various shades of green to represent estimated canopy heights. Observed data is depicted by the crosses for seagrass absence and the circles for various shades for seagrass canopy height.

2.3.5 Sonar derived maps of Lipsi Island

The 83.2 km of kayak transects covered 70% of the entire coastline of Lipsi island. This equated to mapping an area of 7.36km^2 once interpolation of sonar data had taken place. The sonar method estimated there to be 53 hectares of seagrass in the coastal waters around the Island of Lipsi, that equates to covering 7.19% of the available benthos. As shown in figure 2.9, there is more seagrass present in the south east coastal area of Lipsi and very little present in the south west.

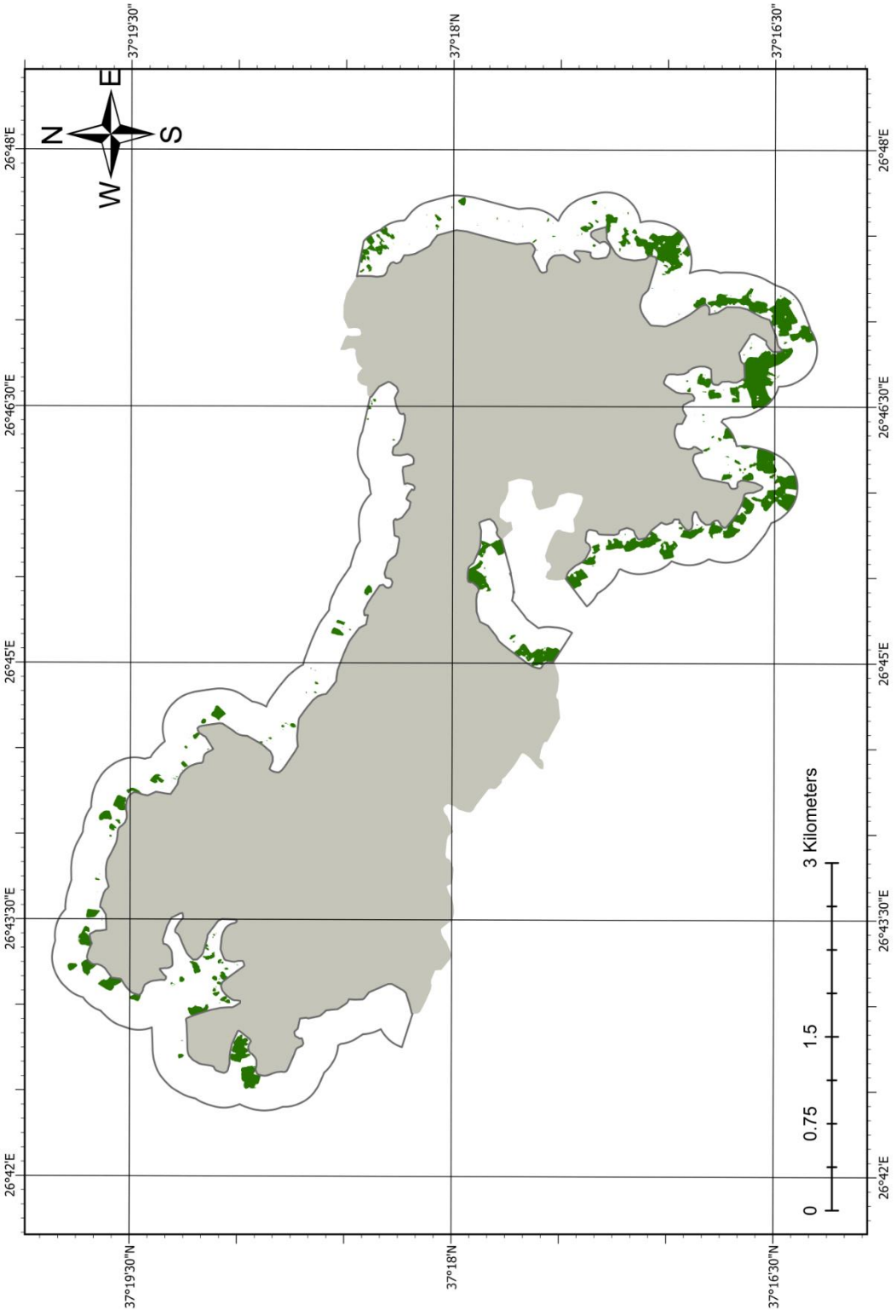


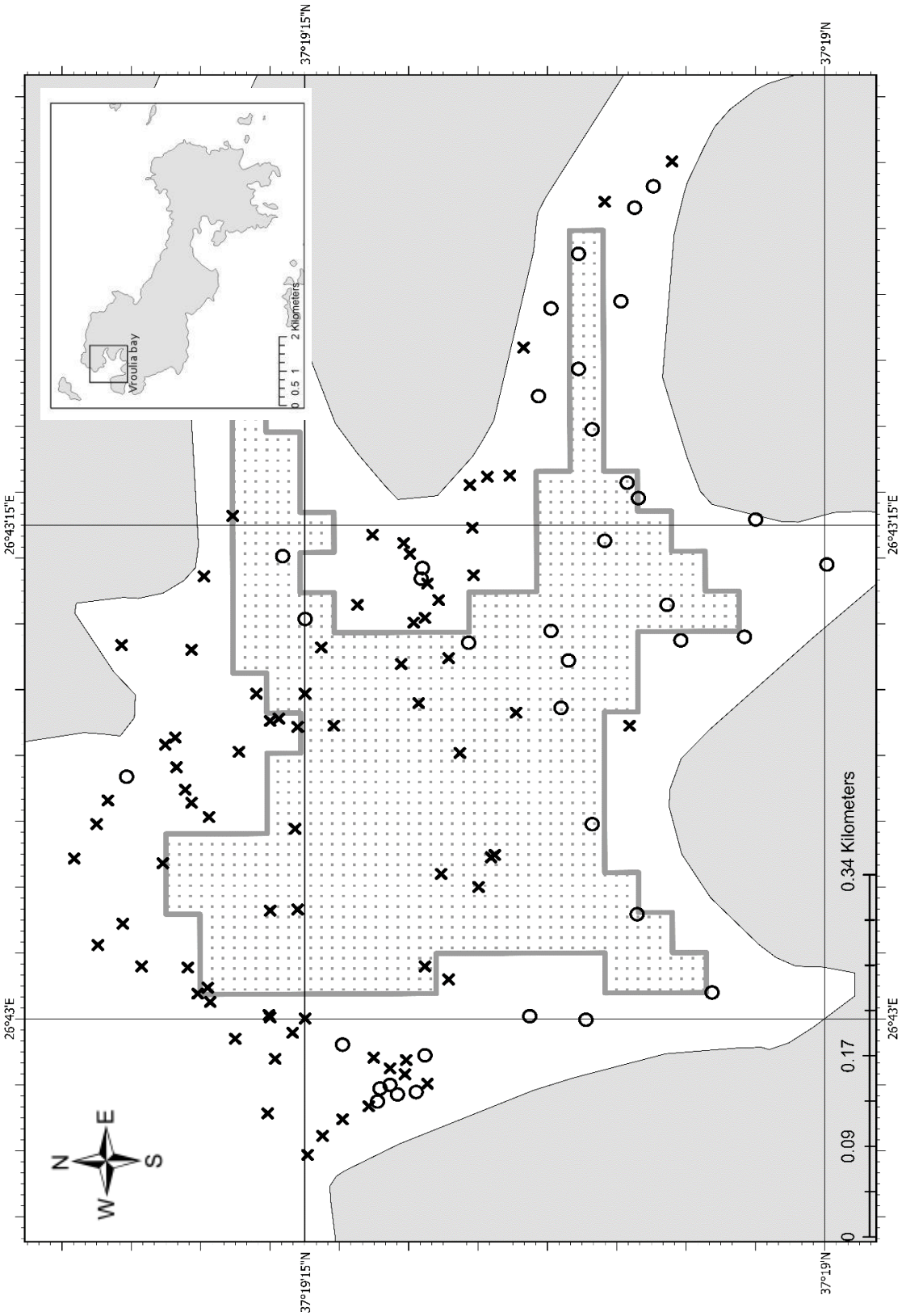
Figure 2.9 sonar derived seagrass presence around the island of Lipsi. Seagrass presence was defined in this case as any vegetation over 10cm in height.

2.3.6 Comparison of Sonar and Satellite maps

Sonar-derived data were more accurate than satellite-derived data (figure 2.10). The satellite-derived map was over 10% less accurate in Mesokampos Bay, showing an observed accuracy of 67% and κ = 0.200 (95% CI= 0.482, -0.0822). In Vroulia Bay, the two methods performed similarly with the sonar being 5% more accurate than the satellite observed accuracy of 63% and κ = 0.147 (95% CI= 0.336, -0.042). Satellite maps were not significantly different to chance in either location based on our observed data (p = 0.145 in Mesokampos and p = 0.341 in Vroulia). Furthermore, the satellite-derived data produced considerably more false positives for seagrass cover than the sonar-derived data (Mesokampos: 23.9% vs 8.6%; Vroulia: 19.4% vs 16.8%) (table 2.7).

Table 2.7 results of confusion matrices for Vroulia Bay, Lipsi and Mesokampos, Samos of observed *P. oceanica* data against satellite mapped *P. oceanica*

			Satellite Predicted		**Sonar Predicted**	
			absent	**present**	**absent**	**present**
Observed	**Lipsi**	**absent**	58	18	60	16
		present	23	14	20	17
	Samos	**absent**	5	11	13	4
		present	4	26	4	26


37°19'15"N
37°19'N
26°43'15"E
26°43'E
Vroulia bay
0 0.5 1 2 Kilometers
N
E
S
W
0 0.09 0.17 0.34 Kilometers

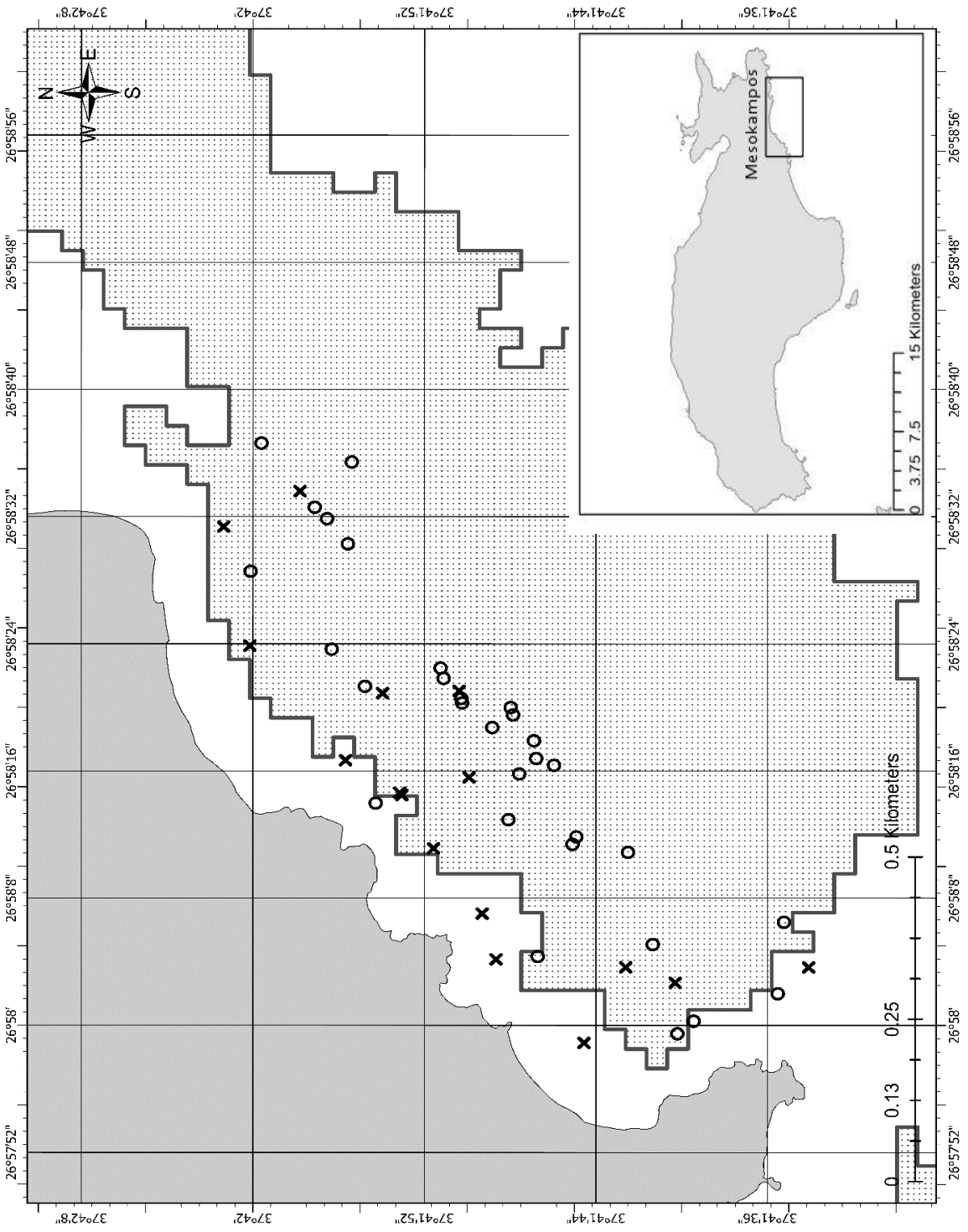
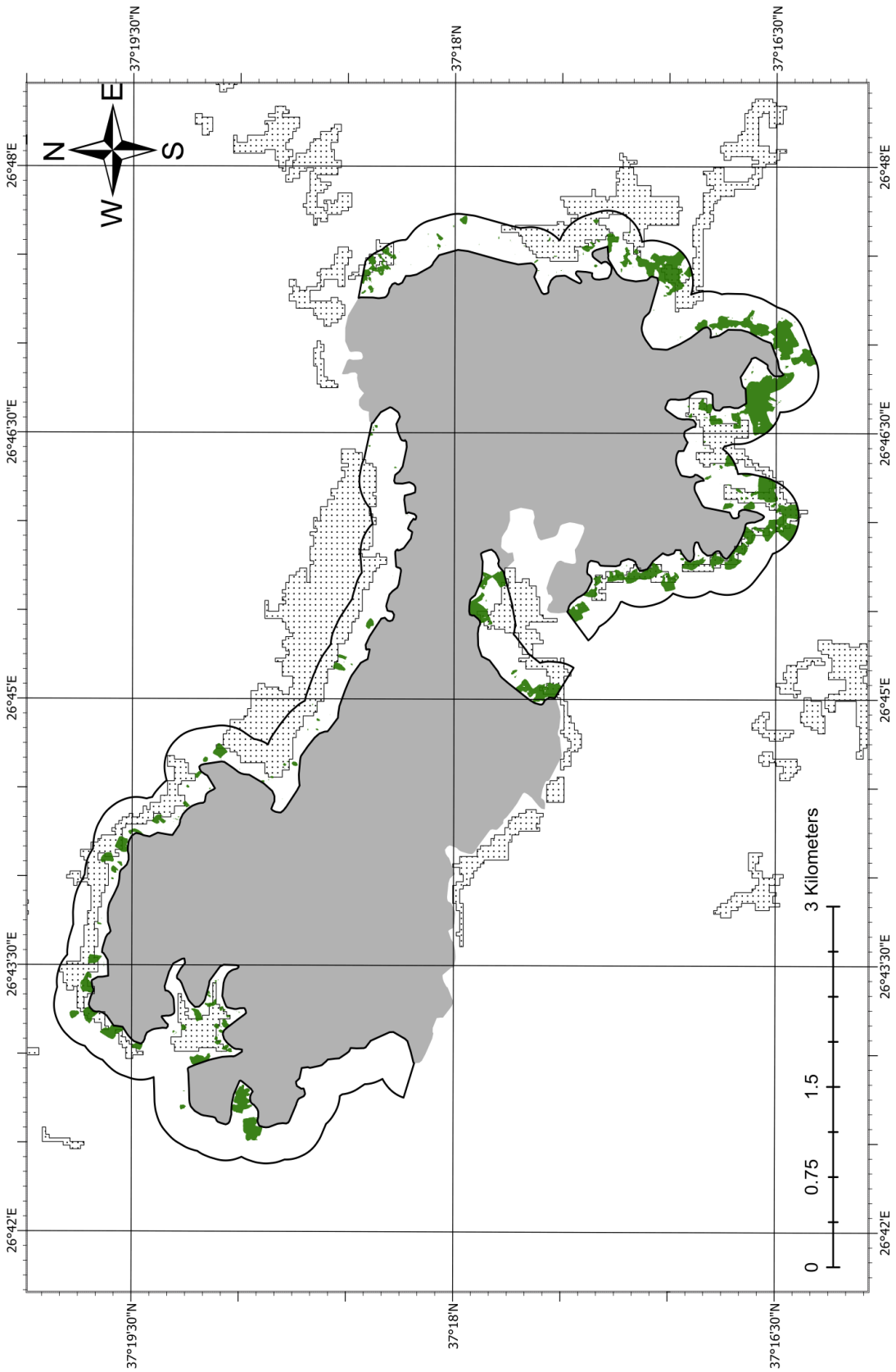


Figure 2.10 Maps showing satellite predicted seagrass from Topouzelis *et al.* (2018) and observed seagrass presence and absence in a) Vroulia Bay, Lipsi and b) Mesokampos, Samos.. Observed presence is denoted by hollow circles, while observed absence by crosses. Satellite presence is outlined in grey.

The extent of seagrass cover predicted by the two methods differs considerably (figure 2.11). Our study estimates that 53 hectares of seagrass are found in the coastal waters off the island of Lipsi (7.19% of the available benthos); 71% less than the 184 hectares reported by Topouzelis *et al.* 2018. In Samos our study estimated 58 hectares of seagrass (29.8% of available benthos), 49% less than Topouzelis *et al.* 2018 estimated this to be 114 hectares. The two methods only overlapped on 24.43 hectares of estimated seagrass in Lipsi and 53.15 hectares in Samos.


37°19'30"N
37°18'N
37°16'30"N
26°48'E
26°46'30"E
26°45'E
26°43'30"E
26°42'E
N
S
E
W
0
0.75
1.5
3 Kilometers

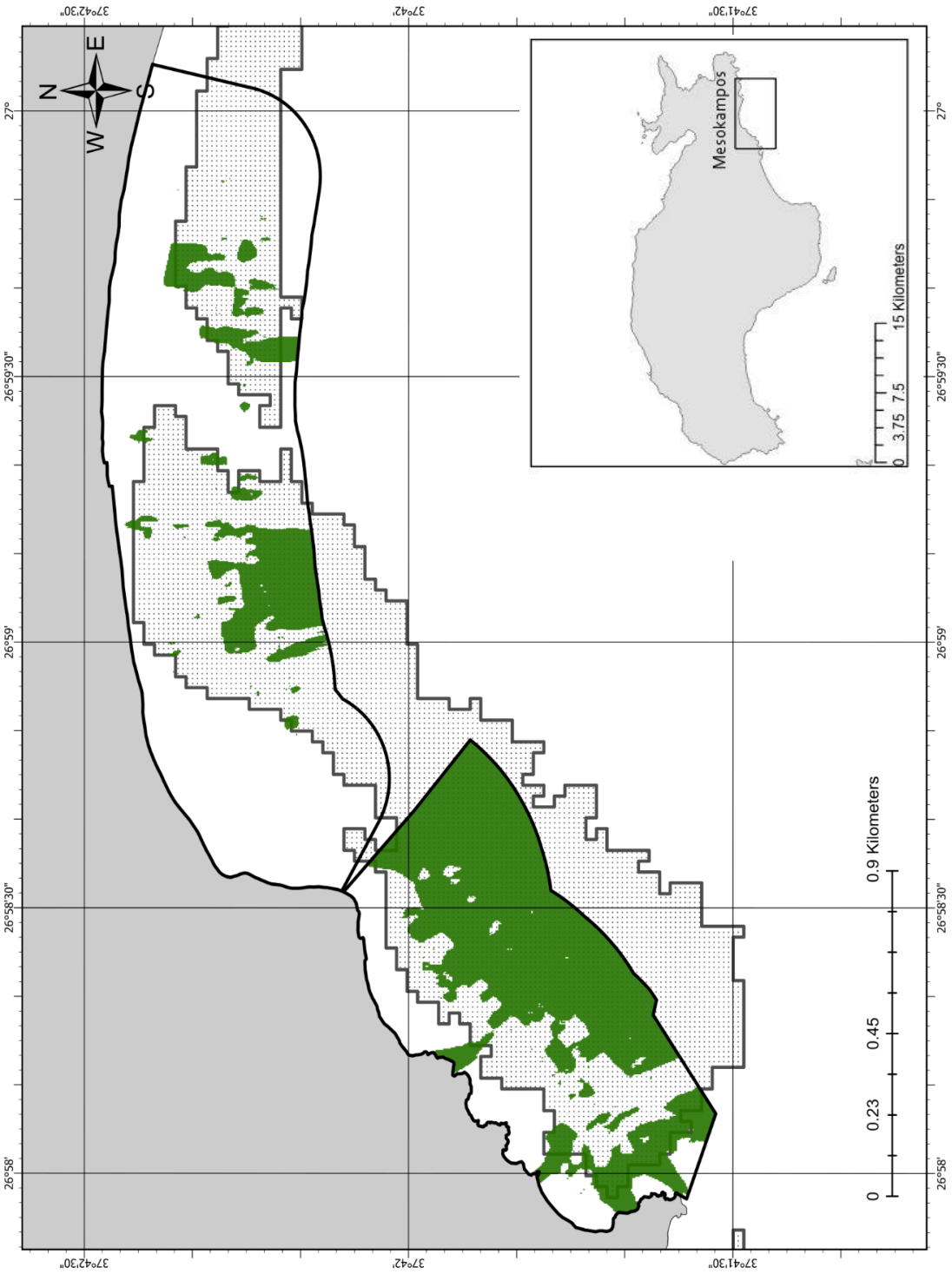


Figure 2.11 Comparison of satellite derived seagrass maps and sonar derived in Lipsi and Samos. Satellite presence is outlined in grey overlaid on the sonar derived presence (dark green) and absence (no colour).

2.4 DISCUSSION

2.4.1 Using Down imaging sonar for mapping seagrass

Down imaging sonar can provide a simple, low-cost tool that can be used to map seagrass extents particularly in areas where meadows are dense and continuous. To date sonar has only been proven to map seagrasses in waters down to 16m (Sagawa *et al.* 2008), however, we demonstrate its potential to map down to 28.4 m that was the deepest point in this work were seagrass was confirmed through both the ground truthing and sonar derived data. The data suggest that down-scan sonar imaging can be an effective tool for a number of survey and monitoring tasks. Luczkovich *et al.* (2013) found the BioBase tool in combination with an EchoSounder sonar to have an accuracy of 77% for mapping submerged aquatic vegetation, although no depth range was given for this study. This is between the observed accuracy of the two survey sites in this study, Samos (85% observed accuracy) and Lipsi (68% observed accuracy). Luczkovich *et al.* (2013) was investigating the seagrass species *Zostera marina*, that demonstrates the application of this technique is possible for multiple seagrass species.

Use of this sonar method can be applied at the local scale very easily to map and subsequently monitor seagrass presence over time. It has been identified that 27% of countries within seagrass bioregions lack data at even the presence or absence scale, that is a consequence of seagrass inhabiting deep or turbid waters (McKenzie *et al.* 2020). Sonar methods such as the one presented here can assist with filling these gaps where satellite mapping is unreliable. This is applicable in, not only the eastern Aegean where the method was tested, but the United Kingdom in which seagrass remains poorly mapped (Mc Kenzie *et al.* 2020). In the UK *Zostera marina* is a common species (Foden & Brazier, 2007), that could use this method where water is too turbid for

reliable satellite mapping. The ease of this method means it can be applied on a yearly basis to monitor seagrass presence over time for signs and rates of decline. Depth is a major limitation of satellite mapping preventing data collection from the whole seagrass habitat range (Veettil *et al.* 2020). Sonar mapping is not as constricted to shallow water and therefore has the potential to map the entire depth range of seagrass species, providing a better basis for conservation and management plans.

Sagawa *et al.* (2008) demonstrated Side-scan sonar as a technique for ground truthing satellite data, proving it reliable enough to use for calibration of algorithms and validation of maps. Better reference data is need for the Greek regions in order to progress satellite mapping potential and provide the most accurate maps possible of seagrass cover (Topouzelis *et al.* 2018). This method has been demonstrated as a viable method for mapping seagrass at the local scale and therefore could be used to provide this much needed reference data in order to map the rest of the Greek territorial waters using satellite in areas as accurately as possible. Drone imagery has also been developed as a tool for mapping coastal seagrass (Ventura *et al.* 2016), providing another potential source of large scale data collection to be used in conjunction with both satellite and sonar methods.

Sagawa *et al.* (2008) reported an accuracy of 97.3% using Side-scan sonar for mapping seagrass up to a depth of 16 metres, which is a higher accuracy than demonstrated by the sonar method in this study, however their depth range was smaller and there was no mention of bathymetry complexity. While their survey area had depth greater than 16m due to their ground truthing method (SCUBA) only data up to 16m was cross referenced with in field data.

The observed accuracy of the two survey sites varied in this study, Samos (85% observed accuracy) and Lipsi (68% observed accuracy). The differences between the accuracy at the two locations were likely result of increased patchiness at the Lipsi study site. Samos was shown to have a significantly higher average canopy height than Lipsi indicating a healthier and more continuous meadow.

Where seagrass coverage is patchier, the accuracy decreases but our data were still significantly more accurate than chance as shown in Vroulia where κ=0.237 (C.I. = 0.001, 0.481, p = 0.050). We suggest that the decreased accuracy is a combination of three factors. Firstly, sonar data have been shown to lose accuracy when mapping across interfaces between habitat types (Sagawa *et al.* 2008). Secondly, sonar data were mapped at a resolution of 10 square metres whereas ground truthing measurements were made across a small percentage of this area (0.25 sq m); in patchy environments there is a greater probability that the *in situ* measurement is not truly representative of the majority of the 10 sq m sonar-surveyed area. There is also a possible additive GPS error when considering that different GPS points were taken for the location of sonar data, the point of *in situ* measurements and the actual landing site of the camera. False positives made up 21% of ground truthing points in Lipsi and false negatives comprised 15% of these incorrect points. Meanwhile in Samos 14% of the incorrect points were false positives and 10% false negatives. Finally, in our study area, as elsewhere (Longstaff & Dennison, 1999), increased patchiness was related to decreased shoot density and canopy height. The canopy height observed in Lipsi was significantly lower than in Mesokampos ($p < 0.001$), and Lipsi was visibly more patchy than the continuous meadow observed in Samos.

Observed accuracy of classification of seagrass into canopy height classes was consistently lower for both Samos and Lipsi. Accuracy in Samos dropped to 38% when the classification data was interpolated, down from 85% when the presence/ absence data was interpolated. The difference between accuracies were less marked in Lipsi, down 10% from 68% to 58% for interpolated presence/ absence. Sonar track data were also far less accurate when classifying canopy heights in Samos than the presence/ absence data. This accuracy dropped from 83% in Samos for the presence absence study, to 48% when classifying canopy height. Lower accuracy of classification data could be a result of the dense seagrass canopies blocking the acoustic signal from reaching the seabed. The data suggest that in this case the BioBase algorithms need optimising to allow biomass of seagrass to be estimated from sonar data. It also suggests that while it cannot be used reliably for health assessments it can give a reliable overall presence assessment for areas.

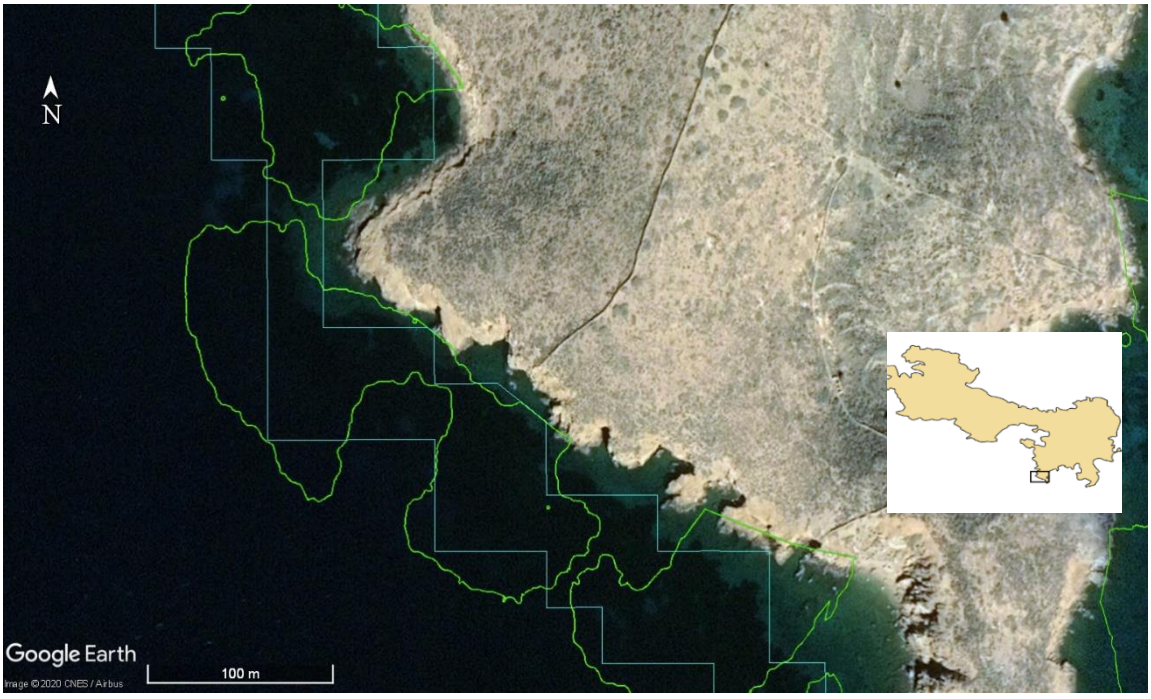
2.4.2 Comparative accuracy of sonar and satellite mapping

Topouzelis *et al.* (2018) shows significant differences in spatial extent of seagrass compared to the sonar derived maps presented here. Around the coast of Lipsi in the study areas, sonar estimated seagrass cover to be 71% less than Topouzelis *et al.* (2018). In Samos the differences were not as marked, however, still clear. In both survey locations down imaging sonar derived seagrass maps had a higher observed accuracy than satellite derived maps. Furthermore sonar derived maps produced κ values that were significantly better than chance, while satellite derived maps did not. Satellites performed better in Samos than Lipsi however it was still over 10% less accurate than the sonar derived maps in this location. The differences in seagrass estimations, especially around the entire coast of Lipsi, are stark and worrying. If the

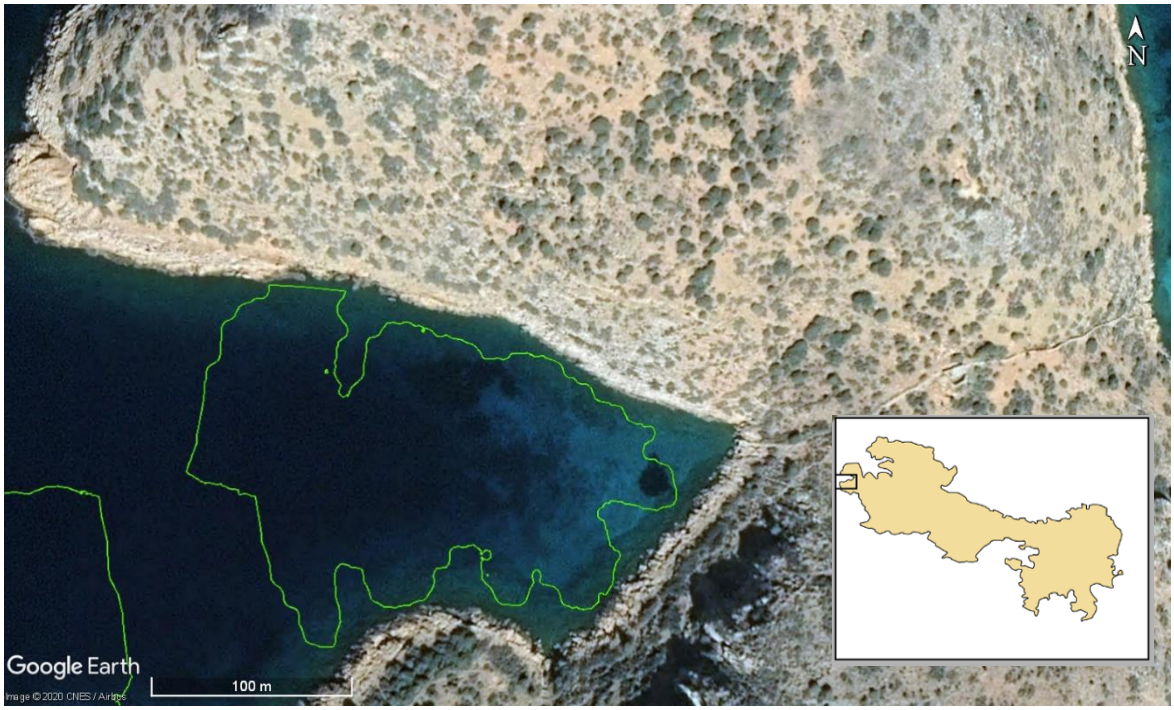
trends are consistent across other areas then Greek decision making may be based on highly error-prone data.

The maps created by both methods were briefly compared to freely available Google Earth Pro satellite imagery, using the estimated seagrass extent exported from Arc GIS Pro (figure 2.12). This was carried out as a third test for each method with independently collected data. Due to the poor quality of these images only the nearshore areas can be properly compared and only in some regions of the island. These images confirmed that while some areas showed agreement with satellite derived maps (figure 2.12 a), many showed clear areas where satellite mapping missed (figure 2.12 b & c) or overestimated seagrass cover (figure 2.12 d).

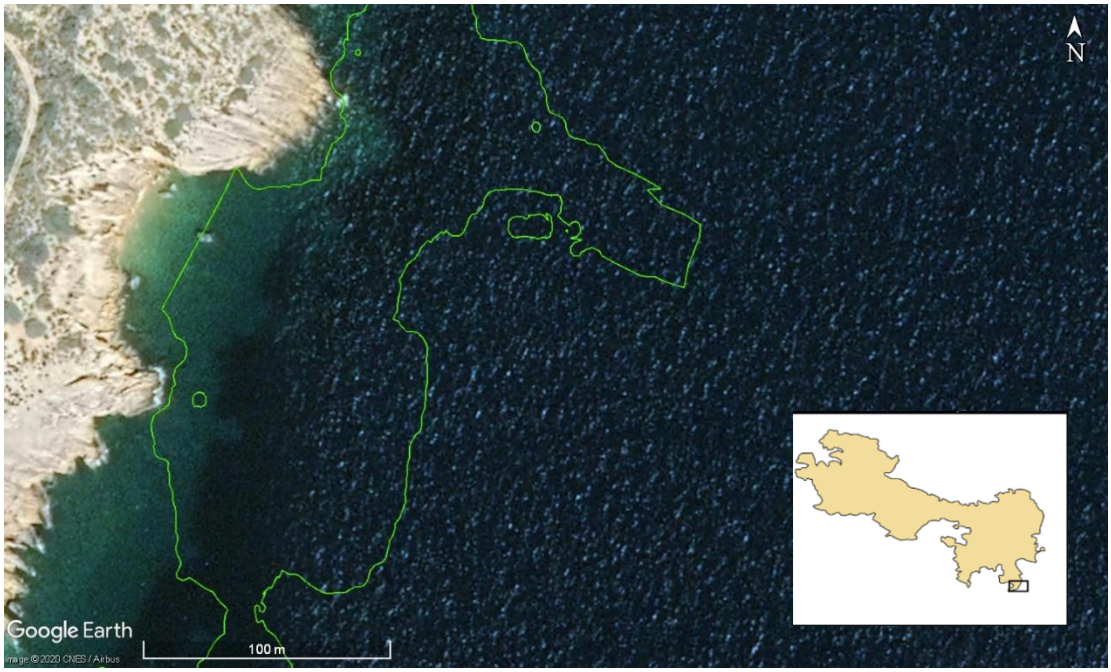
a)



b)



c)



d)

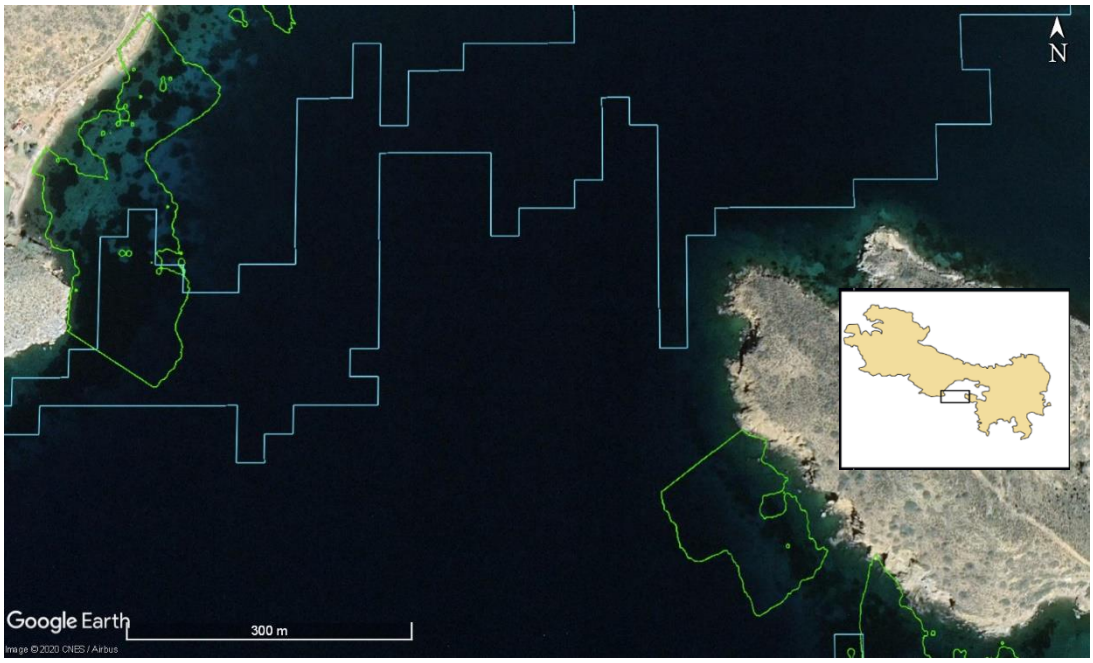


Figure 2.12 Comparison of sonar estimated seagrass presence (green outline) and satellite estimated (blue outline) against freely available satellite imagery in Google Earth Pro from 2019.

The clearest difference between the two methods was visible in Vroulia bay, Lipsi. The decreased accuracy of satellite mapping was due to the methods allocation of a large seagrass meadow in the centre of Vroulia bay which the sonar found to be bare of significant seagrass cover. From the bathymetry maps produced in this study using the sonar data there is a distinct slope in the centre of Vroulia down to depth of 50m. Topouzelis *et al.* (2018), satellite mapping estimations classify this deep water to be seagrass, however our ground truthing observations demonstrated no *P. oceanica* coverage in that area of the bay. Topouzelis *et al.* (2018), did not collect their own ground truth data and instead used reference maps that dated from 1998-2001, 15 years before the satellite mapping study was carried out. This use of external data is efficient in theory, however the authors were not familiar with the study areas, which for method development it a vital part of understanding if results are representative.

Topouzelis *et al.* (2018), claim to map seagrass from satellite imagery with mean accuracy of 76.3% across 62 sites. However, 29% of these sites had an accuracy of less than 69%, with 15% of sites having an accuracy of less than 50% and a lowest accuracy of 29.54%. The authors declare the mapping method to be capable of mapping to depths of 40m based off one site for which they had accurate bathymetry. The other sites were mapped using bathymetry from the Hellenic Military Geographical Service (HMGS). This bathymetry data did not provide detailed fine scale resolution, that was noted when finding bathymetry for this study, most notably classifying Vroulia bay to be a depth of 20m or less. From the comparison between both sonar mapping and ground truthing data collected here, with the satellite mapping, satellites should still be used with extreme caution when mapping deep seagrass meadows due to the greater likelihood of false positive results.

The bathymetry of Mesokampos bay shows a considerably more gradual slope to that of Vroulia bay in Lipsi that has a deceptively steep gradient reaching depths of over 50m in the centre of bay. Satellite derived data is known to have a depth limit for the reliable mapping of *P. oceanica* of about 15m -20m (Fornes *et al.* 2006), which is likely to contribute to the decreased accuracy in Vroulia Bay. There is a lack of fine scale bathymetric data for the Greek seas, this hampers the use of this satellite imagery for seagrass mapping and may lead to overestimations of seagrass coverage in unexpectedly deep coastal water. Therefore caution is imperative when using satellite imagery for measuring changes in seagrass cover where detailed bathymetry is not available.

2.4.3 Cost and effort of kayak borne sonar mapping

Forty hours mapping resulted in seagrass coverage maps for 9.3 km^2 of coastal habitat across Samos and Lipsi, an overall data acquisition rate of 23 ha per hour. This compares favourably with the rate of acquisition for SCUBA and snorkel-based surveys, that are considerably slower and not recommended for areas larger than 1 km^2 (McKenzie, Finkbeiner, & Kirkman 2001). Whilst kayaks can survey across much greater spatial range than SCUBA or snorkel, there are limitations on their use, particularly in remote areas, that impact on all three methods but are less impactful on boat-borne sonar and remote sensing methods. These include access to suitable launching sites, adverse weather conditions, hazards from shipping and having to maintain a certain proximity to a coastline for health and safety reasons. Two areas of the Lipsi coastline were not mapped due to safety considerations and lack of access for kayaks at the time of surveying or safety issues due to a working port with ferries and consistent boat traffic.

Kayak-borne down-scan sonar is a considerably more cost-efficient survey method compared to boat-borne side imaging sonar. On the Lowrance website a Lowrance Elite 7 Ti sonar, including a down-scan transducer as used in this method, costs £530. A single two-man sea kayak can be purchased for around £300 and a second hand iPad mini can be bought for £145 including the waterproof case. Finally, an unlimited subscription to CiBioBase costs £1,729 (converted to pounds from USD). The total start-up cost of this method is £2,700.41. This start-up cost includes the unlimited subscription to BioBase. Operational costs are also low. This is a significantly lower outlay than that required to deploy a research vessel fitted with side imaging sonar and means that our method will be much more affordable for many research and

conservation teams. A low cost Side-scan sonar starts at around £1,510 (Kaeser, Litts, & Wesley Tracy, 2013), which is over double the cost of the middle range down-scan sonar used in this method, that was priced at £530. Sea kayaks cost approximately £300 each, have negligible maintenance costs and have no running costs, when compared with the costs of purchasing, running and maintaining a boat this is substantially more cost effective for NGOs.

2.5 CONCLUSIONS

Overall down-scan sonar and Biobase Echosound work well at mapping seagrass at the presence/ absence level on gradually sloping sea floors where seagrass typically grows. The cost of the method is much lower than those used previously and requires far less technical skill from surveying groups making it more accessible to non- profit organisations to map the *P. oceanica* meadows around the Greek Islands. As our method provides both fine-scale bathymetric data and estimates of seagrass cover, it offers a good complementary method for use with other remote sensing tools. The method provides a low cost, reliable and accessible way to carry out the much needed mapping of a protected habitat in a region where there is very little known about the location of *P. oceanica*. Future research should focus on the ability of down-scan sonar in combination with BioBase to assess seagrass presence at depth, to assess different species of seagrass and its ability to distinguish species in mixed meadows. There is also important research to be carried out on this method in regards to its ability to map smaller species of seagrass.

Chapter 3

Environmental DNA in seagrass habitats: an emerging tool for assessing species presence

3.1 INTRODUCTION

Seagrass meadows support a rich diversity of marine species because of the food and refuge they provide (Goffredo *et al.* 2017; Jackson *et al.* 2015; Tuya *et al.* 2014; Vlachopoulou *et al.* 2013). The distribution and specific ecological interactions of these species are difficult to ascertain, and complete genetic, species, ecosystem and ecological process inventories are rarely available (Ward *et al.* 1999). Other habitats, congeneric species or biogeographical data are used as surrogates of biodiversity distribution in seagrass habitats (Topouzelis *et al.* 2018, Ward *et al.* 1999). The most common survey method for studying the abundance of marine species is underwater visual census (UVC). UVC has very variable levels of accuracy depending on species response to diver presence and experience of surveyors (MacNeil *et al.* 2008). It is also usually limited to the upper reaches of the water column and therefore may bias our understanding of coastal ecology towards shallower areas.

Despite the difficulties of gaining robust data about ecological interactions in marine environments, it is clear that the endemic Mediterranean seagrass *Posidonia oceanica* provides food and refuge to many species, making them of significant importance to

marine biodiversity and the local economy of the region (Bianchi & Morri, 2000). The species that seagrass wholly or partly support, include those of commercial and ecological importance across taxonomic groups including fish (e.g. *Mullus surmuletus* (striped red mullet) (Moranta *et al.* 2006), *Mullus barbatus* (red mullet), *Sardina pilchardus* (European pilchard), and *Sardinella aurita* (round sardinella) (Kalogirou *et al.* 2010)); cephalopods (e.g. *Sepia officianalis* (common cuttlefish) (Cardonpa *et al.* 2007) and *Octopus vulgaris* (Ulaş *et al.* 2019)) and bivalves notably the noble pen shell, *Pinna nobilis* (e.g. Basso *et al.* 2015).

Species distributions are often poorly known, which makes marine reserves or priority areas difficult to define because of inadequate knowledge of biological diversity (Ward, *et al.* 1999). To fill these gaps in knowledge and better inform policy makers, accurate and robust ecological data is necessary to understand species locations and the diversity of an area. Species inhabiting *P. oceanica* meadows have been shown to exhibit clear vertical zonation of certain species, for example for polychaetes 38 species have been exclusively found on *P. oceanica* blades and 60 species exclusively within the rhizomes (Gambi *et al.* 1992). Emerging techniques that are capable of accurately and precisely assessing the presence of species within seagrass canopy, with less effort than UVC would provide better biodiversity assessments of the systems. Environmental DNA is an emerging technology that may have application in this area, but a better understanding is needed into how eDNA may move around a seagrass ecosystem to assess how useful it can be as a monitoring tool. *Pinna nobilis* is a key species that inhabit *P. oceanica* meadows, for which monitoring is vitally important. If eDNA based techniques can identify the presence of species in seagrass beds it could

transform the understanding, monitoring and protection of seagrass meadow communities.

3.1.1 Pinna nobilis

3.1.1.1 *P. nobilis* ecology

Pinna nobilis (figure 3.1) is an important bivalve endemic to the Mediterranean (Katsanevakis & Thessalou-Legaki 2009). The shell of *Pinna nobilis* has a distinct triangular shape, characterised by a much wider surface area on the lateral side while a much narrower area on the dorso-ventral side (García-March *et al.* 2007). *P. nobilis* is thought to be important to the ecosystem it inhabits; and it has been referred to as an ecosystem engineer (Rabaoui *et al.* 2015). The most notable service it provides is filtration of organic matter that it filters through the inhalant syphon filtering water for food and oxygen (Davenport *et al.* 2011). A single *P. nobilis* adult has been shown to clear a sixty litre tank of suspended detritus in less than an hour (Trigos *et al.* 2014). A dense population of *P. nobilis* could significantly contribute to maintaining clear waters

Figure 3.1 Deceased adult *Pinna nobilis* on edge of *P. oceanica* meadow in Samos, Greece. (Authors own)

and recycling of organic matter from detritus (Trigos *et al.* 2014). It also increases the variety of environments and provides a surface for colonisation by other benthic species in what would otherwise be a soft-bottom area with little colonisation opportunities (Rabaoui *et al.* 2015).

P. oceanica meadows can reduce the effect of drag forces on *P. nobilis* shells sheltered within the canopy (Hendriks, *et al.* 2011), and it is considered that this is the primary reason for their close association with *P. oceanica* meadows (Basso *et al.* 2015). Hendriks *et al.* (2011), demonstrated the concept of wave attenuation of *P. oceanica* meadows in respect to *Pinna nobilis*. Flow speed, shell size and meadow density all had significant effects on drag attenuation. At low flow speeds of between 0.05 and 0.10ms^{-1} medium sized *P. nobilis* shells (15cm shell width) fully situated within the canopy experienced up to a 47% reduction in drag forces, however, no clear attenuating effect was seen at higher velocities (0.25–0.35ms^{-1}). For larger shells (19cm shell width) only dense meadows attenuated drag forces on shells causing an up to 56% decrease in drag forces. Small shells (8cm shell width) had such small drag forces experienced initially that no attenuation was observed. They showed that shells

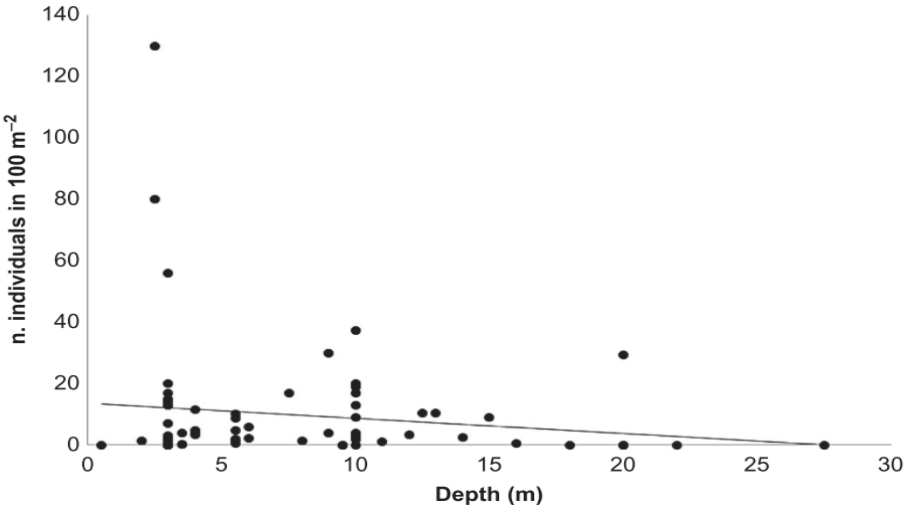


Figure 3.2 *Pinna nobilis*, population density (individuals per 100 m2) along a depth gradient (from Basso et al 2015).

under 0.019m^2 surface area are protected from large drag forces inside the meadow, i.e. shells that do not protrude above the seagrass canopy.

In addition *P. oceanica* meadows have been shown to effectively trap particles from the flow of water (Hendriks *et al.* 2008), resulting in increased food supply for *P. nobilis* (Basso *et al.* 2015). The dense network of robust rhizomes and roots formed in the *P. oceanica* mattes provides a structure to which the *P. nobilis* can anchor itself via the byssus threads (Basso *et al.* 2015). Another benefit of *P. oceanica* meadows as a habitat, is the ability of *P. nobilis* to anchor to the substrate by compression of the basal part of the shell as it becomes compressed and embedded within the matte as it grows (Basso *et al.* 2015). In a review by Basso *et al.* (2015) of publications related to the distribution of *P. nobilis*, they were found most frequently in *P. oceanica* meadows, with 27% of the reports and an average of 8 ± 2 individuals 100 m^{-2}, with higher densities in the first 10-12m (figure 3.2). These data were collected using a variety of survey methods, such as quadrats, transects or concentric circles, however, all were carried out using in water divers. As stated in the review, these methods pose their own limitations such as dive time or access and therefore generating reliable estimations of *P. nobilis* populations can be time consuming or problematic.

3.1.1.2 *P. nobilis* reproduction

P. nobilis reach sexual maturity around two years of age (Basso *et al.* 2015) and spawning takes place from June to September (Prado *et al.* 2020). *P. nobilis* are asynchronous successive hermaphrodites in which differing degrees of sexual maturity are observed between male and female dominant stages, with each type of gamete being broadcast sequentially into the water column during the spawning

period (Prado *et al.* 2020). Little is known about the dispersal capacity of this species due to the lack of information on mortality of larval (dispersing stage) and juvenile phases (Basso *et al.* 2015).

3.1.1.3 *P. nobilis* genetic variation

Sanna *et al.* (2013) showed moderate genetic structuring in *P. nobilis* across the Mediterranean Sea. While there was a low genetic divergence among haplotype and haplogroups, showing a likely common origin of the species, there were at least two genetically divergent populations. These could be grouped in to the western Mediterranean (inc. Sardinian- Corsican region, Elba Island and Sicily) and the eastern Mediterranean (the Aegean Sea and Tunisian coast). The authors detected a significant pattern of genetic structuring along the west east direction, with samples from the Aegean seas and Tunisian coasts being genetically differentiated from the remaining samples (figure 3.3).

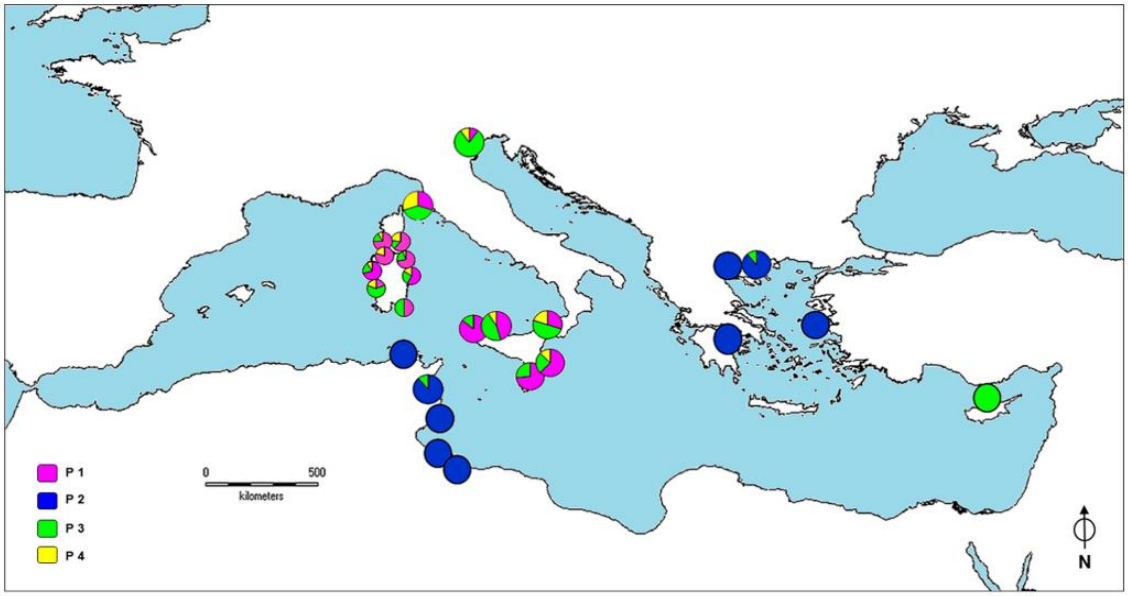


Figure 3.3 COI dataset: Bayesian cluster distribution. Frequency distribution of the four groups of haplotypes P1, P2, P3, and P4, for *Pinna nobilis* as evidenced by Bayesian analysis over the Mediterranean map (Sanna *et al.* 2013).

Katsares *et al*, (2008), investigated *P. nobilis* genetic diversity specifically in the Aegean Sea. There was again shown to be very high haplotypic diversity (0.6667-0.900) in all studied populations of *P. nobilis,* and 14 different haplotypes were found across 25 samples. Chios Island was shown to have the highest diversity with four different haplotypes from five samples. Epanomi was the second highest with six haplotypes from eight samples, and Aggelochori had three haplotypes from nine samples. The Corinthiakos gulf had the lowest diversity, with only one haplotype present in the three samples that were the same as haplotypes from Aggelochori. The location of these sites in relation to one another is shown in figure 3.4.

Figure 3.4 Location of sample sites used in the Katsares et al 2008 study on genetic diversity of *P. nobilis* in the Aegean region (Katsares et al 2008).

3.1.1.4 *P. nobilis* decline

Loss of seagrass habitats is likely to lead to a decline in populations of *P. nobilis* (Öndes *et al.* 2020). *P. oceanica* meadows are the primary preferred habitat type for *P. nobilis*, however these meadows are experiencing serious decline (Marbá *et al.* 2014). As a result, recruitment and hydrodynamic protection of juveniles could be negatively affected, leading to a decrease in *P. nobilis* (Basso *et al.* 2015). Furthermore, *P. nobilis* have been exploited by humans since Egyptians and Romans developed fabric from its byssus threads, called sea-silk, which was very high value and sold to only the wealthier social classes, and also for decoration and food (Basso *et al.* 2015). Although sea-silk is far less common now, *P. nobilis* are still under threat from human activities, in particular poaching, pollution and anchoring (Öndes *et al.* 2020). It has also been shown that fishing operations, such as illegal trawling in shallow water and purse seining over benthic habitats result in the by-catch of *P. nobilis* (Öndes *et al.* 2020). The

greatest threat to this species at present is the newly characterised parasite, *Haplosoridium pinnae*, that targets *P. nobilis* and has caused mass mortality events across the Mediterranean (Cabanellas-Reboredo *et al.* 2019).

The Hellenic seas are known to have long been home to an important population of these bivalves (Katsares *et al.* 2008), and until recently it had been thought that they were not affected by the *H. pinnae* parasite that had decimated populations in the western region. With the confirmation of the *H. pinnae* arrival in Lesvos and the subsequent mass mortality event of *P. nobilis* (Katsanevakis *et al.* 2019), it has become more important than ever to locate and monitor remaining populations.

3.1.2 Marine survey techniques

A wide variety of survey methods are used to study marine biodiversity (Costello *et al.* 2017). The majority of biodiversity surveys are carried out using underwater visual census (UVC), however, while being the most common method for these studies, the data often shows high levels of variation, and accuracy can depend on species (Edgar *et al.* 2004). UVC is a broad term used to define direct visual sampling by snorkellers or SCUBA divers, e.g. Strip transect, Line Transects, Point Counts and Rapid Visual techniques (for further details on these specific type of UVC see review by Murphy & Jenkins, (2010)). UVC methods show a distinct bias towards larger species that are identifiable by eye or swim close to the observation point (Costello *et al.* 2017) and are biased by observer experience (Murphy & Jenkins, 2010). A common and well-known error of the UVC method is imperfect detectability (Monk, 2014). Imperfect detectability is the inability of surveyors to detect all individuals and/or species in the survey area, leading to uncertainty in an observed zero and negatively biased

estimated of species occurrence (Dorazio, 2012, Katsanevakis *et al.* 2012). Imperfect detection is particularly an issue when dealing with environments that have rare or cryptic species (White *et al.* 2020).

Underwater videos are an alternative to UVC surveys and include Remote Underwater Video (RUV), Baited remote underwater videos (BRUV), TOWed Video (TOWV) and Diver Operated Video (DOV) (Mallet and Pelletier, 2014). However, issues with these methods include variable sampling effort as a result of current velocities and bait plume (Taylor *et al.* 2013), selectivity towards species that respond positively to bait (Cappo 2010), long observation and image analysis process and large data set management (Mallet and Pelletier, 2014).

Benthic trawls and grabs are other common survey methods for biological monitoring of benthic communities (Jørgensen *et al.* 2011). There are, however, biases associated with both of these methods. Benthic trawls can miss small individuals that can swim under the footrope and species specific behavioural responses (Somerton *et al.* 2007). Grab sampling on the other hand are difficult to use in coarser sediments where, the pebble sizes prevent proper closure of grabs and can only sample areas of around 0.05–0.25 m^2 per grab (Jørgensen *et al.* 201).

UVC surveys are the typical method of quantifying for *Pinna nobilis* populations (Basso *et al.* 2015), however they are no exception to imperfect detectability, and Hendriks *et al.* (2012), showed that the probability of detection reached a maximum of 71.4% for the largest individuals, but was just 34.9% for the smallest. This shows a bias towards detection of larger individuals leading to an under estimation of juveniles and small shells.

Another known issue with UVC surveys is the misidentification of species. Misidentification of species is thought to be the most likely cause of low accuracy when the UVC is carried out by non-experts (Hassell *et al.* 2013). *Pinna rudis* is a congenic species to *Pinna nobilis* (Vázquez-Luis *et al.* 2017), that occupies a similar habitat. *P. nobilis* juveniles, which often have rougher shells than that of the adults, can be confused with *P. rudis* that has a similar morphology. *P. rudis* tends to prefer rocky substrates and has a considerable smaller maximum size then *P. nobilis,* as demonstrated in figure 3.5 (Cosentino & Giacobbe, 2006). A method of combating issues with identification is through the use of extracellular/ environmental DNA (Wood *et al.* 2019).

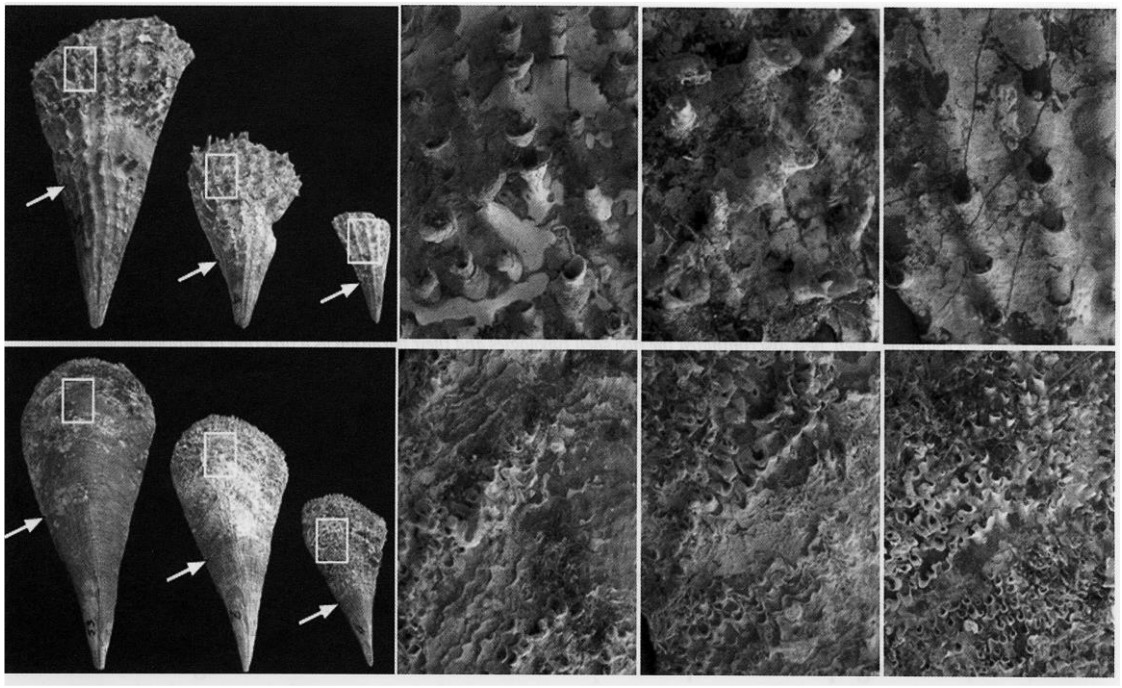


Figure 3.5 Shell morphology of *Pinna rudis* (top) and *Pinna nobilis* (bottom). Image from (Cosentino & Giacobbe, 2006)

3.1.3 Environmental DNA

Environmental DNA (eDNA) is an emerging surveying technique that is being used with increasing regularity for a range of freshwater and marine species (e.g. Ficetola *et al.* 2008; Foote *et al.* 2012; Mauvisseau *et al.* 2017; Xia *et al.* 2018; Weldon *et al.* 2020). eDNA refers to DNA that can be extracted from environmental samples rather than directly from the source species. It can be detected in concentrations as low as 1 copy mL^{-1} (Turner *et al.* 2015) via the release of DNA from macro-organisms into the environment through mucus, faeces, urine and carcasses (Ficetola *et al.* 2008). The method exploits an advantage of aquatic environments that sloughed tissues are suspended in the aqueous environment making it possible to detect DNA from even rare organisms that are often particularly difficult to detect using traditional methods (Jerde *et al.* 2011).

When compared to standard survey methods, eDNA can be a relatively inexpensive method that can improve detection probabilities while being non-invasive to the survey organism once established and allows for cost-effective screening across wide geographical areas (Currier *et al.* 2018; Weldon *et al.* 2020). It can also avoid the physical and ethical problems associated with potentially destructive benthic sampling techniques such as bottom trawls or grabs (Costello *et al.* 2017).

While generally used to define species presence/absence, the concentrations of recovered eDNA can, in some circumstances, provide information on relative population size (e.g. Takahara *et al.* 2012; Matsuhashi *et al.* 2016) or ecology. Environmental DNA has been shown as a useful tool to detect spawning aggregation of species such as the Japanese eel, *Anguilla japonica*, helping to guide visual method (e.g.

underwater camera) to better understand their ecology (Takeuchi *et al.* 2018). Bracken *et al.* (2019) reported an increase in concentrations of lamprey, *Petromyzon marinus*, eDNA during spawning events due to release of gamete and aggregations of individuals; once spawning events finished eDNA concentrations decreased. Similarly Tillotson *et al.* (2018) demonstrated an increase in eDNA concentrations during spawning events of sockeye salmon, *Oncorhynchus nerka*, that decreased towards the end of the season.

In water, DNA diffuses rapidly from its source, meaning theoretically the presence of a specific organism can be detected across relatively large distances (Rees *et al.* 2014), however significant questions remain about the rates and factors affecting degradation (Barnes *et al.* 2014) and diffusion (Lacoursière-Roussel & Deiner, 2019). Pilliod *et al.* (2014), could not detect eDNA from caged salamanders more than 5m downstream in a free-flowing river system. Flow rates of this system were between 0.50-0.57 ms^{-1} and therefore reached the 5m sampling point in just 2.5 seconds assuming perfect flow and no degradation, settling or substrate adsorption. Jane *et al.* (2015), detected eDNA from caged trout 239.5m downstream, showing that increased water flow rate decreased the effect distance had on eDNA concentration. There is still a clear need to better understand the mechanics of eDNA diffusion, dilution and transportation within the environment (Lacoursière-Roussel & Deiner, 2019). Understanding these mechanics is important because increased transport results in a higher probability of detection over a wider area whereas decreased transport implies increased specificity.

3.1.4 Seagrass hydrodynamics

Seagrasses have been shown to play an important role in manipulating the hydrodynamics of their surrounding water column, particularly in reducing wave energy. *Posidonia oceanica* forms particularly dense and high canopies (figure 3.6) and therefore has a larger effect on water column hydrodynamics than some of the smaller

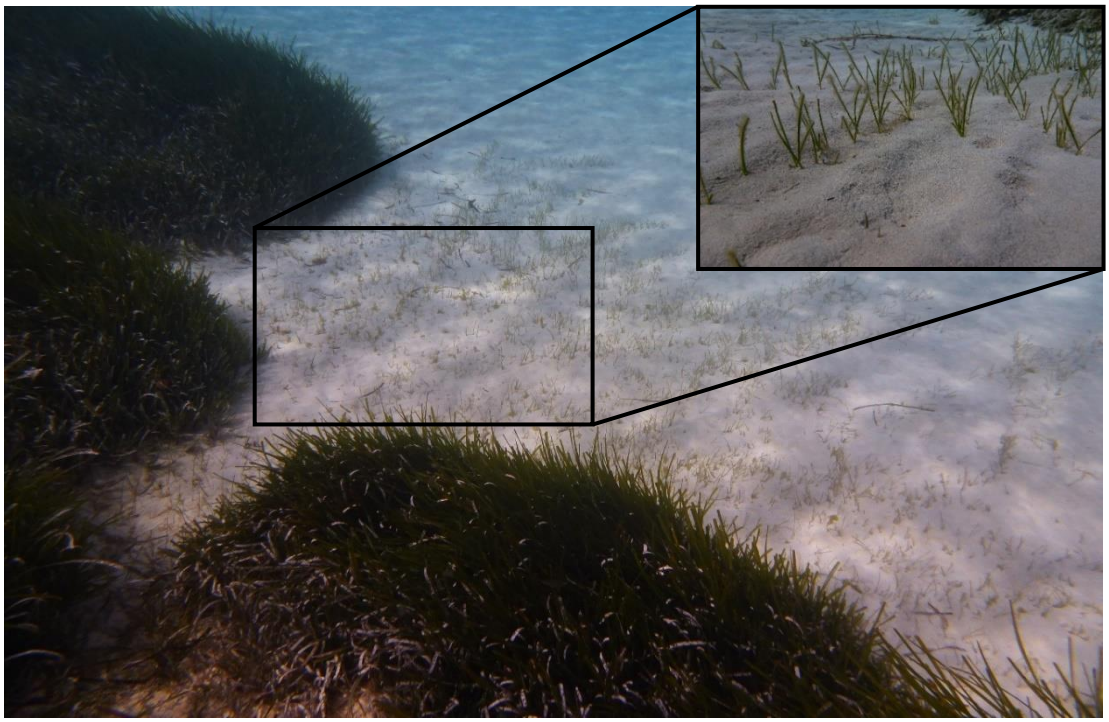


Figure 3.6 *Cymodocea nodosa* meadow (inset) extending from the boundary of *Posidonia oceanica* meadow showing difference in physiology in Lipsi, Greece. (Author's own)

seagrass species (Manca *et al.* 2012).

Stratigaki *et al.* (2011), conducted flume experiments on artificial *P. oceanica* meadows demonstrating a 35% reduction in wave height over meadows with a density of 360 stems m^{-2}. If this density was halved, there was still more than a 20% reduction in wave height over the meadow. The authors also showed that water velocity was significantly reduced within the meadow, with this effect being more pronounced in the denser meadow. When the submergence ratio was increased (i.e. a larger proportion of the water column occupied with seagrass), horizontal velocities above the canopy were

increased due to the interaction between the wave base and the moving vegetation, whilst inside the canopy these velocities were reduced. Vertical velocities were reduced both above and inside the canopy and the higher the submergence ratio the more significant this decrease was.

Manca *et al.* (2012), also mimicked *P. oceanica* seagrass in a flume to test its ability to reduce wave energy and wave induced flow. They showed wave height decay was two orders of magnitude higher over a seagrass canopy compared to bare sandy substrate under the same conditions. Both regular and irregular waves increased in wave height at the meadows edge before decreasing inside the meadow and tests with the densest canopies (360 shoots m^{-2}) and highest submergence ratio showed the greatest reduction. In- canopy flow was measured at two sites, one near the edge and the other further into the meadow. Near the edge of the meadows flow was reduced by 12.1% while further into the meadow this value increased to 58.7%, in the high density meadow (360 shoots m^{-2}). This wave induced flow inside the meadow was 3% larger in the dense meadow then those in the lower density meadow (180 shoots m^{-2}).

eDNA survey techniques may offer opportunities to expand the reach of regular biotic survey of seagrass ecosystems beyond the confines of standard methods. As seagrass meadows are noted for their influence on water movement (refer to section 3.1.1), it is important to understand eDNA dynamics around seagrass meadows before such a method can be rigorously applied. The extent of diffusion within seagrass meadows and between seagrass and non-seagrass areas will influence the spatial extent which may be represented by a positive eDNA detection.

3.1.5 Chapter Objectives

Conditions within the canopy result in damped wave action and a trapping effect caused by the leaves and rhizomes, favouring the accumulation of fine particles, creating areas enriched with organic matter (De Falco *et al.* 2000). Dejean *et al.* (2011), also stated that eDNA has a tendency to persist in the environment bound to organic or inorganic particles. This binding of DNA to organic matter and a lack of sediment resuspension suggests seagrass meadows may influence the dispersal of eDNA from organisms that inhabit them. Understanding the dispersal and accumulation of eDNA in vegetated systems is important in assessing its potential for the detection and monitoring of cryptic or rare species. Cryptic species are often those which are most prone to the issues of imperfect detectability in surveys (as discussed in section 3.1.2). Development of a molecular technique could help detect these species more reliably than standard surveys alone. There is currently no published literature available on the influence coastal vegetation may have on the detection and /or dispersal of eDNA. In order to optimise these methods, there needs to be more understanding of the dynamics of eDNA interactions within aquatic vegetation to optimise sampling protocols.

The following objectives will be addressed in this chapter:

1. Development and assessment of an environmental DNA technique for the detection of *Pinna nobilis,*
2. Characterisation of *Pinna nobilis* environmental DNA as a monitoring method in spawning and non-spawning season,

3. Determination of the influence of seagrass (*Posidonia oceanica*) vegetation on the dispersal of environmental DNA from a point source.

3.2 METHODS

3.2.1 Study Site and Identification of Focal Individual

This study took place in Vroulia Bay on Lipsi Island, Greece (figure 3.7). Vroulia is a sheltered bay in the north east of Lipsi, with known presence of both *P. oceanica* and *P. nobilis*.

The parameters considered for survey location included a sheltered bay (figure 3.8), regular calm conditions, restricted public access, no urbanisation. Areas meeting these criteria were initially surveyed via snorkel, to a maximum depth of 5m, to identify an isolated *P. nobilis* individual on the edge of a seagrass meadow in order to allow sampling above both bare and vegetated substrate.

eDNA samples were taken from locations near the same focal individual during two sampling periods: spawning and non-spawning. Spawning season samples were taken in late August 2018 during which time spawning was observed in the bay and late September 2018 once spawning activity had ceased.

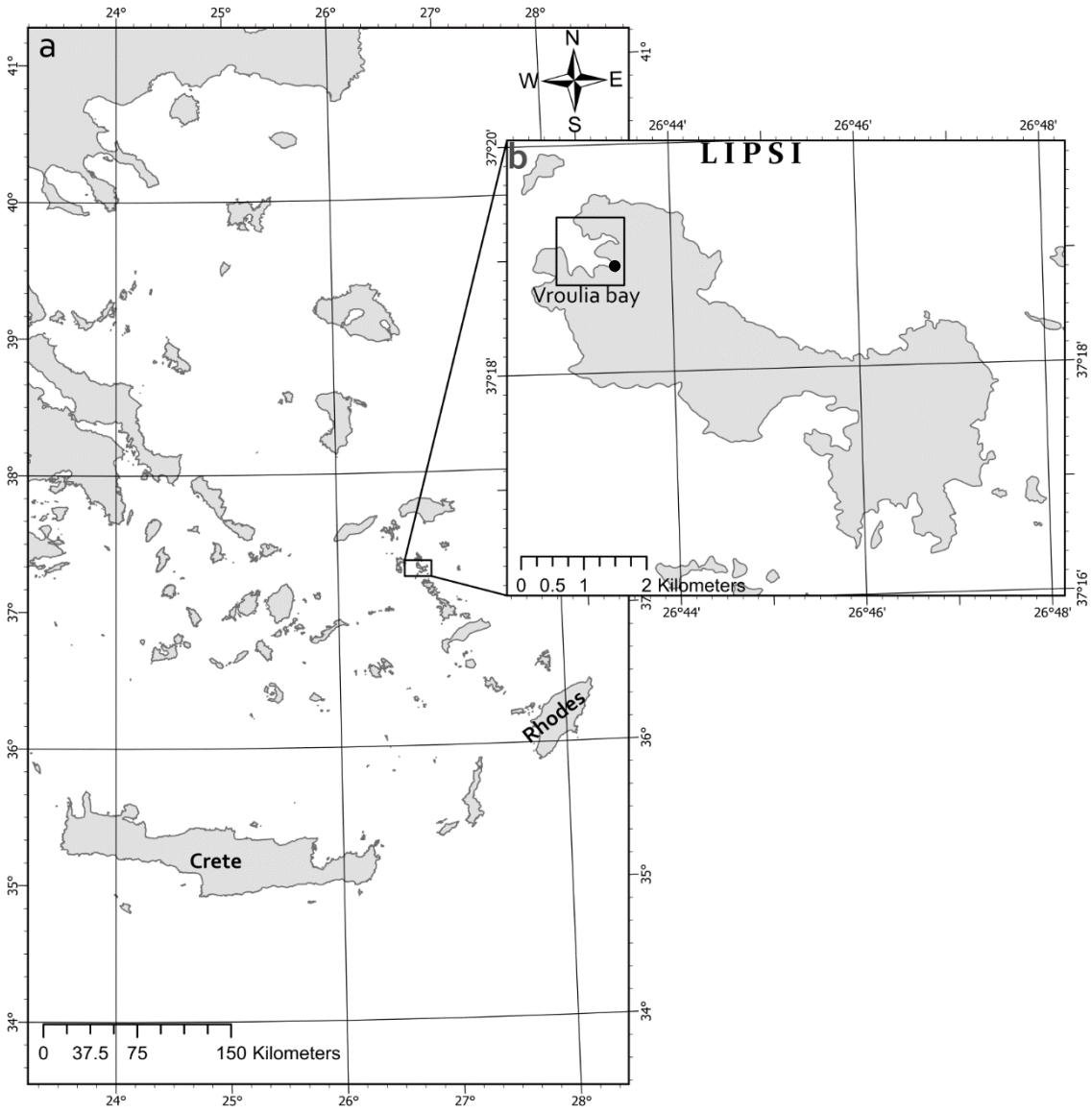


Figure 3.7 a) location of Lipsi (indicated by a black square) in Greece and b)Location of Vroulia Bay on Lipsi. Vroulia is outlined with a black square, and the location of the survey within the bay is indicated by the black dot.

Figure 3.8 Aerial image of Vroulia Bay taken from the mouth of the bay looking back toward shore, demonstrating no urbanisation to the area, limited access route and sheltered nature of the bay. Image © Villemange 2017, reproduced with permission.

3.2.2 eDNA Sampling

A large (19 cm maximum width) *P. nobilis* individual was located near the edge of a *P. oceanica* meadow. From a central point (the *P. nobilis*), three transects were laid, all of 20m (figure 3.9). In the second sampling season (non-spawning), a further point was added to Transect 3 (T3) to test for an interaction with another *P. nobilis* individual located after the first sampling period approximately 10 m from the end of T3 (figure 3.10). This resulted in one point at 25m on this transect only.

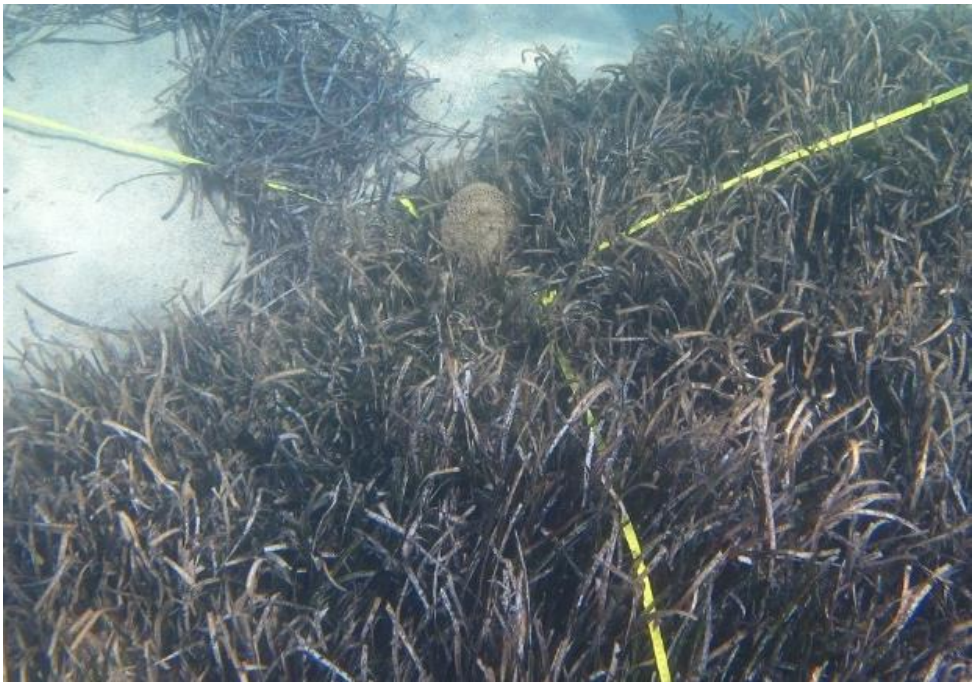


Figure 3.9 *P. nobilis* individual surveyed with the three transects (20-25m) radiating out (B. Quintana 2018)

A 10-minute settling period was observed after transect placement to allow for any water disturbance to settle, to minimise any influence on eDNA presence in water samples or create an artificially mixed environment. Water samples were collected by free diving and using 2.5 L high density polyethylene bottles at five metre intervals. All samples were collected using sealed sample bottles taken to a depth of 10cm above the seabed where they were opened and allowed to fill before being sealed and returned to the surface. Sample collection always started at the transect end furthest from the *P. nobilis* individual, working towards the focal individual (where DNA concentrations were expected to be highest) to minimise water disturbance. The average water depth for the sample area was 2.7m (min: 2.5m - max: 3.0m). During the spawning season a further water sample was collected from the upper 0.5 m of the water column

approximately 15 m from the *P. nobilis* to see if detection from surface samples were possible.

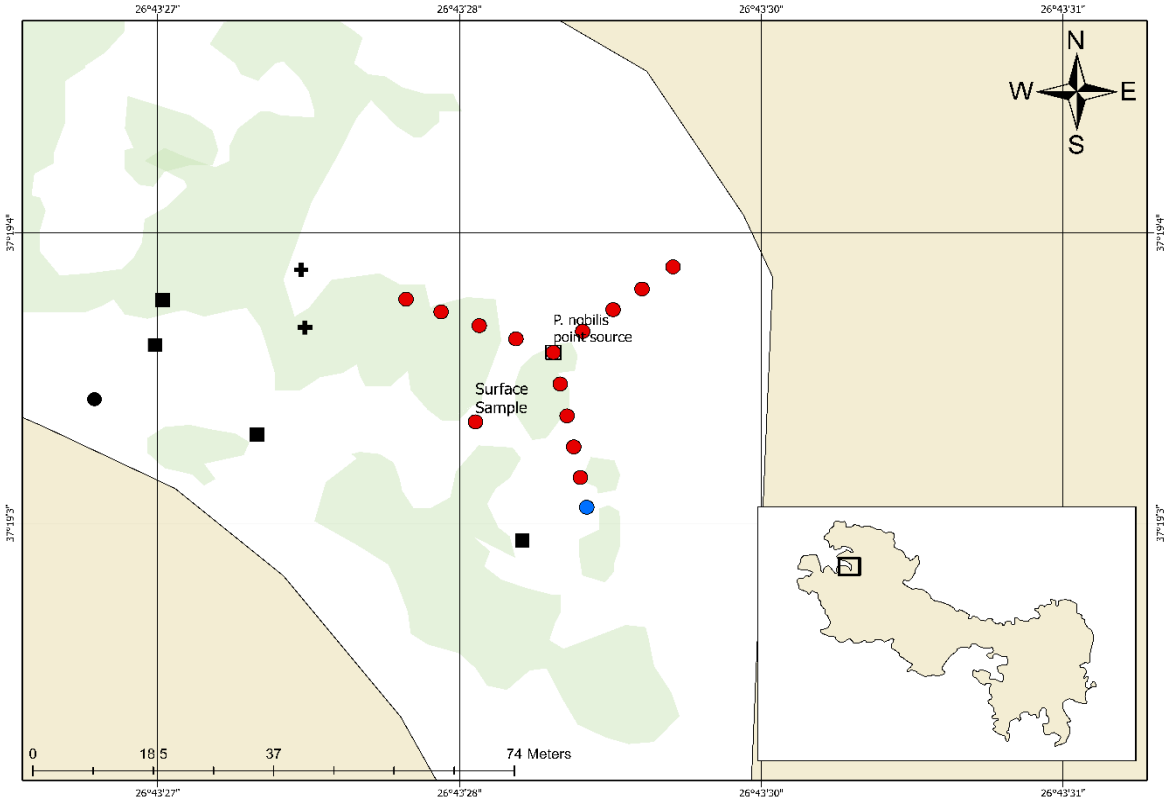


Figure 3.10 Study area showing the locations of *P. nobilis* in the sampling area observed through snorkel surveys. Black squares represent living adult *P. nobilis*; black crosses represent dead *P. nobilis*; the black circle represents suspected juvenile *P. nobilis*. The *P. nobilis* individual used as a point source for this study is at the convergence point of all three transects. Spawning season sampling points are shown in red. Non-spawning season sampled at the same points with an added sampling point shown in blue to test for the influence of the second *P. nobilis* individual.

Environmental variables including water column depth, seagrass percentage cover, shoot density, and blade length were collected at each sampling point. Water column depth was measured using a Suunto D4i dive computer. Shoot density and percentage cover were measured using a 20cm x 20cm quadrat with blade length measured in cm using a tape measure following an adapted Seagrass Watch protocol from McKenzie *et al.* (2003).

After collection, water samples were transported to shore and kept cool in a storage container filled with seawater (ambient SST) and covered with a towel to prevent sun exposure whilst sample collection was completed. Once all samples were collected,

they were transported to the laboratory for processing within two hours and stored at 4 ^{o}C until filtration. All samples were processed within eight hours of initial collection.

3.2.3 Sample Filtration

Water samples were well mixed by inversion before two litres of sample water was filtered through 0.22 µm Sterivex Filter Units (Merck Millipore) using a peristaltic pump. Filtrate was measured using a two litre Griffin beaker to ensure equal volumes of water were processed for each sample. Tap water was filtered in the same way between samples as procedural blanks (filtration controls) to test for cross contamination during the filtration process.

Cross contamination of DNA was avoided by bleach cleaning all filtration equipment prior to use and between each filtration, first in 10% bleach solution for ten minutes, then 5% bleach solution for ten minutes and finally in water to remove residual bleach traces for another ten minutes. This cleaning process was also carried out on tubing between uses and the work bench cleaned with 10% bleach and covered with new paper covers between samples (Renshaw *et al.* 2015). During a pilot sampling season (May 2018) the method was trialled and samples processed to ensure there were no issues with cross-contamination. Following analysis of these procedural blanks, the disinfectant protocol (using 10% bleach) was found to be effective and therefore a single procedural blank (tap water) was conducted during the second survey (September 2018) after the seventh sample (i.e. in the middle of all samples). Unexpected power cuts on the island at the time of sample processing led to prioritisation of collected samples to ensure that all filtration took place within eight

hours of initial sample collection minimising DNA decay and following the method outlined in Weldon *et al.* (2020).

After each sample was filtered, 1.4ml of Longmire's buffer, to act as a preservative buffer (100ml of 1M Tris HCl at pH8, 200ml of 0.5M EDTA at pH8, 2ml of 5M NaCl, made up to 975ml with distilled water, 25ml of 20% SDS), was added to the Sterivex Filter Unit using a needle, before the filter housing was sealed with Parafilm and autoclave tape. Longmire's buffer has been shown to prevent the decay of DNA without the need for freezing (Williams, Huyvaert & Piaggio, 2016). Each filter was placed into a sealed 50 ml Falcon tube and stored at room temperature prior to shipping. Samples were shipped back to the UK within two weeks of collection for molecular analysis at University of West of England, Bristol. Prior to DNA extraction samples were stored at room temperature, DNA was extracted from all filters within two months of collection.

3.2.4 Design of Pinna *nobilis* qPCR primer and probe

All qPCR primers and probes were designed by the author following the MIQE guidelines (Bustin *et al.* 2009). Species-specific primers were designed for *P. nobilis* mitochondrial cytochrome c oxidase subunit I (COI) gene using NCBI Primer blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast) and visually optimised for qPCR using the MIQE guidelines. Five *P. nobilis* sequences (accession numbers EF536842, EF536843, EF536844, EF536845 and EF536846), obtained from NCBI gene bank were used for primer design aligned to generate a consensus sequence using NCBI Primer blast. There is a large, well-known genetic diversity of *P. nobilis* populations in the

Hellenic seas (Katsares *et al.* 2008). DNA sequences were chosen due to their source location of Xios Island (122 km north of Lipsi), the closest sequences available to the survey location. This was an important factor in choosing sequences because it meant they were most likely to be a similar haplotype compared to those from the north of Greece such as Thessaloniki. The probe was designed using the PrimerQuest Tool from Integrated DNA Technologies (https://eu.idtdna.com/pages/tools) using the same sequence set as was used for the primer design. Resulting primer and probe sequences are shown in table 3.1.

Primer specificity was investigated *in silico* using the BLAST tool https://blast.ncbi.nlm.nih.gov/Blast) from NCBI. Results of this showed no possible risk of amplification with other *Pinna spp* or non-target organisms. These primers showed 100% specificity to *Pinna nobilis* sequences from both the northern Aegean (Xios, Aggeloxori and Epanomi) and also from the Tunisian coast.

Table 3.1 Details of primer and probe sequences for Pinna nobilis, PnobCO1F is the forward primer and PnobCO1R is the reverse primer. The primers and probe amplify a 150bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (CO1) gene

Primer name	**Primer and probe sequences (5' – 3')**	**PCR product size (bp)**	**T_m (^oC)**	**GC content (%)**
PnobCO1F	5'-GCTTATTTTAGGGCCGCCAC-3'	150	59.6	55
PnobCO1R	5'-CCCGCCAAATGTAAATAAGCCT-3'	150	59.3	45
PnobCO1P	5'-CCGGATGCCGGCTGAAGACTAGGGCTCCTG-3'	N/A	69.4	N/A

Primer specificity was then determined *in vitro* using a synthetic mini gene taken from the alignment of sequences from Xios island. Using this mini gene, amplification was possible using the primer probe set initially designed. Swabs from a living *P. nobilis* individual were taken and the DNA extracted and amplified using the primers and probe. PCR amplicons from this reaction were sequenced and then checked against the NCBI gene bank. These results showed 100% match and query cover for *P. nobilis* in the northern Aegean. This sequence was as follows:

TTAGGTGAATCGCCACAATGTCCGGATGCCGGCTGAAGACTAGGGCTCCTGTTCTTTGAAGAGTTGGATTTTT
AGGCTTATTTACATTTGGCGGG

Swabs from the live *P. nobilis* individual yielded relatively low levels of DNA in the reference sample and so for this reason the synthetic gene was used to make the standards used in the qPCR reaction.

3.2.5 DNA Extraction

Total DNA was extracted from filter units using a modified phenol:chloroform:isoamyl alcohol DNA extraction method (Renshaw *et al.* 2015). The addition of lysis buffer was omitted due to the use of Longmire buffer in the preservation step. DNA extractions were performed in duplicate, providing procedural replicates, by dividing samples into two extractions, by dividing the filters and recovered Longmire's buffer. Longmire's buffer was extracted from the filters using sterile syringes and decanted into sterile 2 ml microcentrifuge tubes and the volume recovered recorded. The Sterivex filter cartridges were dismantled and the filter membrane removed. The filter membrane was cut into two equal part using a sterile scalpel with each half added to a separate 2 ml microcentrifuge tube, containing the recovered Longmire's buffer. Extraction

controls containing ddH_2O were prepared alongside the environmental samples at the time of processing to identify any extraction based cross-contamination. Extractions took place over a series of days and therefore each day had a respective extraction control. The extraction controls were processed simultaneously with the environmental samples.

DNA extraction was achieved by adding four microlitres of 400 µg ml^{-1} proteinase K (Sigma Aldrich, UK) for each 100 µl of Longmire's buffer and incubated overnight at 55°C. Phenol:chloroform:isoamyl (24:24:1, Sigma Aldrich, UK) was added to the centrifuge tubes in equal volume to that of the Longmire's and incubated at room temperature for 30 minutes, vortexing every ten minutes to remove residual particles from the membrane. Centrifuge tubes were centrifuged at 13,000 RCF, at 4°C, for 5 minutes. The upper aqueous layer was pipetted off into a new sterile 1.5 ml microcentrifuge tube and an equal volume of ice cold isopropan-2-ol (Sigma Aldrich, UK) added. Tubes were gently inverted, then centrifuged in 4°C at 13,000 RCF. Isopropan-2-ol was removed, leaving the DNA pellet adhered to the side of the centrifuge tube. The pellet was washed with 500 µl of 100% ethanol (Sigma Aldrich, UK) and centrifuged for five minutes at 4°C. This process was repeated with 70% ethanol before the pellet was dried on a hot plate at 55°C and rehydrated with 100µl of sterile Tris-EDTA (TE) buffer (Sigma Aldrich, UK), before freezing at -20°C where they were stored until qPCR analysis.

3.2.6 Sequencing of PCR amplicons

The specificity of the qPCR primers and probes were further validated by conducting confirmatory sequencing of environmentally-derived PCR amplicons of 27% (n = 8) of

the 30 water samples collected. The amplicons were purified using a QIAquick PCR Purification Kit (Qiagen Inc.) before sequencing using Source BioSciences Sequencing service (Source BioScience, Nottingham, UK). Overall there were very low levels of DNA present in the samples (maximum of $4.14.x10^{-7}$ ng μl^{-1} and minimum of 5.50×10^{-10} ng μl^{-1}) and during PCR clean up DNA is lost and sequencing is unlikely to work if concentrations are too low. Therefore, PCR products that amplified the greatest quantity of DNA were chosen preferentially to increase the success rate of returning a good quality sequence read. Sequences retrieved were aligned using Geneious Prime software (version 2019.2.3) and taxonomy assigned using the National Center for Biotechnology Information (NCBI) database using Geneious Prime software (version 2019.2.3).

3.2.7 Quantitative PCR (qPCR) analysis

To determine the concentration of *P. nobilis* eDNA, quantitative PCR (qPCR) was performed using a StepOne-Plus™ Real-Time PCR system (Life Technologies, Foster City, USA). All qPCR reactions were set up in a total volume of 20 µl consisting of 10 µl of 2x qPCRBIO Probe Mix Hi-ROX (PCR Biosystems Ltd, London, UK), 0.8 µl of each primer (10 pmol μl^{-1}), 0.4 µl of probe (10 pmol μl^{-1}), 1 µl of extracted template DNA and 7 µl of ddH_2O. For known standard or negative reactions, the template DNA was replaced with 1 µl of known standard or ddH_2O respectively. PCR master mix (i.e. all reagents other than the extracted samples) was made in a separate lab to the extraction procedure. All equipment and ddH_2O used for the reactions were sterilised by exposure to UV light for 30 minutes before use to prevent contamination. The qPCR was performed, in replicates of six for each extraction, with an initial two minute denaturation step at 95˚C, followed by 40 cycles of denaturation at 95˚C for 5 seconds

and annealing at 65˚C for 20 seconds. Each 96-well plate included seven positive standards, replicated in triplicate, of *P. nobilis* synthetic DNA (due to the protected status of *P. nobilis* it was not possible to collect flesh samples as a direct standard) and three PCR controls that contained the PCR master mix and ddH_2O. The seven positive standards were generated from a 1 in 10 serial dilution of a synthetic *P. nobilis* COI gene, ranging from 0.01 ng μl^{-1} to 1x 10^{-8} ng μl^{-1}. The inclusion of positive controls enabled seven standard curves (figure 3.11) to be generated from which *P. nobilis* DNA concentration was estimated. The three negative controls acted as qPCR controls with the addition of ddH2O instead of DNA template. Quantitative PCR inhibition was ruled out by the addition of 1 µl of a known standard of the synthetic *P. nobilis* CO1 gene to two randomly selected environmental samples and run alongside the standard curves to ensure amplification was not inhibited. A water sample was recorded as positive for *P. nobilis* if one or more of the six qPCR replicates of a sample was positive. Positive

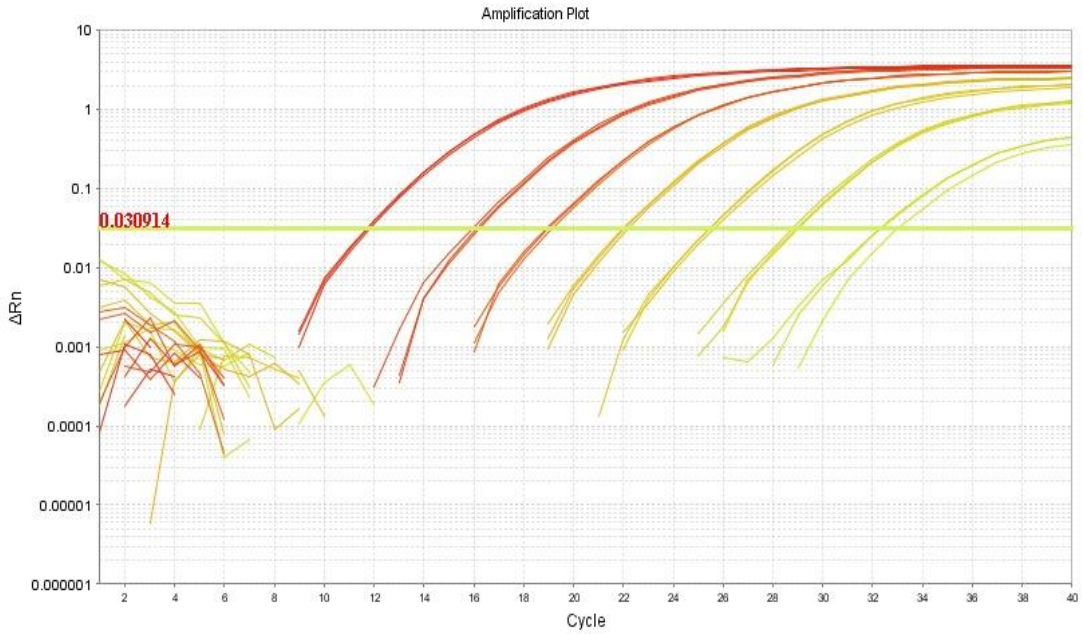


Figure 3.11 Amplification plot of standards used in qPCR analysis. Seven standards were used staring at 0.01ng μl^{-1} (far left amplification) and descending in a 1 in 10 serial dilution to a lowest concentration of 1x 10^{-8} ng μl^{-1} (far right amplification). The threshold is depicted by a green line.

amplification was classified as amplifications that passed a threshold, which is set automatically by the StepOne Plus Software (version2.3).

3.2.8 Statistical analysis

DNA copy number was calculated using the following equation:

$$\text{Number of copies} = \frac{\text{DNA concentration (ng/µl) x [6.022 x 1023]}}{\text{length of template (bp) x [1x109] x 650}}$$

The equation assumes a molar mass of each base pair to be 650 (g mol^{-1}) bp^{-1}, then the only input required is the length of DNA which in this case was 150 bp. The influence of distance from point source, seagrass blade length and sampling season (spawning or non-spawning) on detection probability was tested using two binomial generalised linear models with logit link using the LME4 package (version 1.1-23, Bates *et al.* 2020). Binomial GLMs were used with the detection of *P. nobilis* (i.e. 1 for detection of any concentration and 0 for no detection), this was due to the amount of variation in the results and the number of zeros in non-spawning season. The first model tested for the main effects and interaction between season and distance. The second model tested for the main effects and interaction between season and blade length. Odds ratios were then calculated for the significant factors, that show the size and direction of effect (Rita & Komonen, 2008). This analysis was conducted for the complete data set and each season independently. In each of the three models, factors were serially deleted depending on significance until only the significant ones remained in the model. All statistical analyses were carried out in RStudio (Version 1.2.5019).

3.3 RESULTS

3.3.1 Confirmed Amplification of *Pinna nobilis* DNA

Quantitative PCR demonstrated the newly developed environmental DNA method to be highly sensitive, detecting concentrations of *Pinna nobilis* DNA as low as $5.5x\ 10^{-10}$ ng μl^{-1}, equivalent to one copy per µl. The highest concentrations of DNA amplified were $4.14\ x\ 10^{-07}$ ng μl^{-1} (1327 – 3304 copies μl^{1}) detected at 5 m from the point source on Transect 2 during spawning season. At the source (i.e *P. nobilis* individual) during spawning season a concentration of $3.75\ x\ 10^{-8}$ ng μl^{-1} (97 - 1340 copies μl^{-1}) was found compared to $8.89\ x\ 10^{-9}$ ng μl^{-1} (5 – 92 copies μl^{-1}) at the same sampling point in non-spawning season. During spawning season the lowest eDNA concentration found was $4.02\ x\ 10^{-09}$ ng μl^{-1} (10 - 104 copies μl^{-1}) at 5m from the *P. nobilis* point source on transect 3. During non-spawning season, the lowest DNA concentration was $5.50\ x\ 10^{-10}$ ng μl^{-1} (1 - 5 copies μl^{-1}) that was also at 5m from the point source on transect 3, while the highest concentration was at the source. These results are shown in full in table 3.2.

Overall the greatest number of positive amplifications were seen directly next to the *P. nobilis* point source. Spawning season had amplifications at all sampling points and had consistently high numbers of amplifications along all three transects. In non-spawning season, amplifications were consistently low across all three transects with a clear hot spot at the source of eDNA. The heats maps for amplifications in both spawning and non-spawning season are shown in figure 3.12.

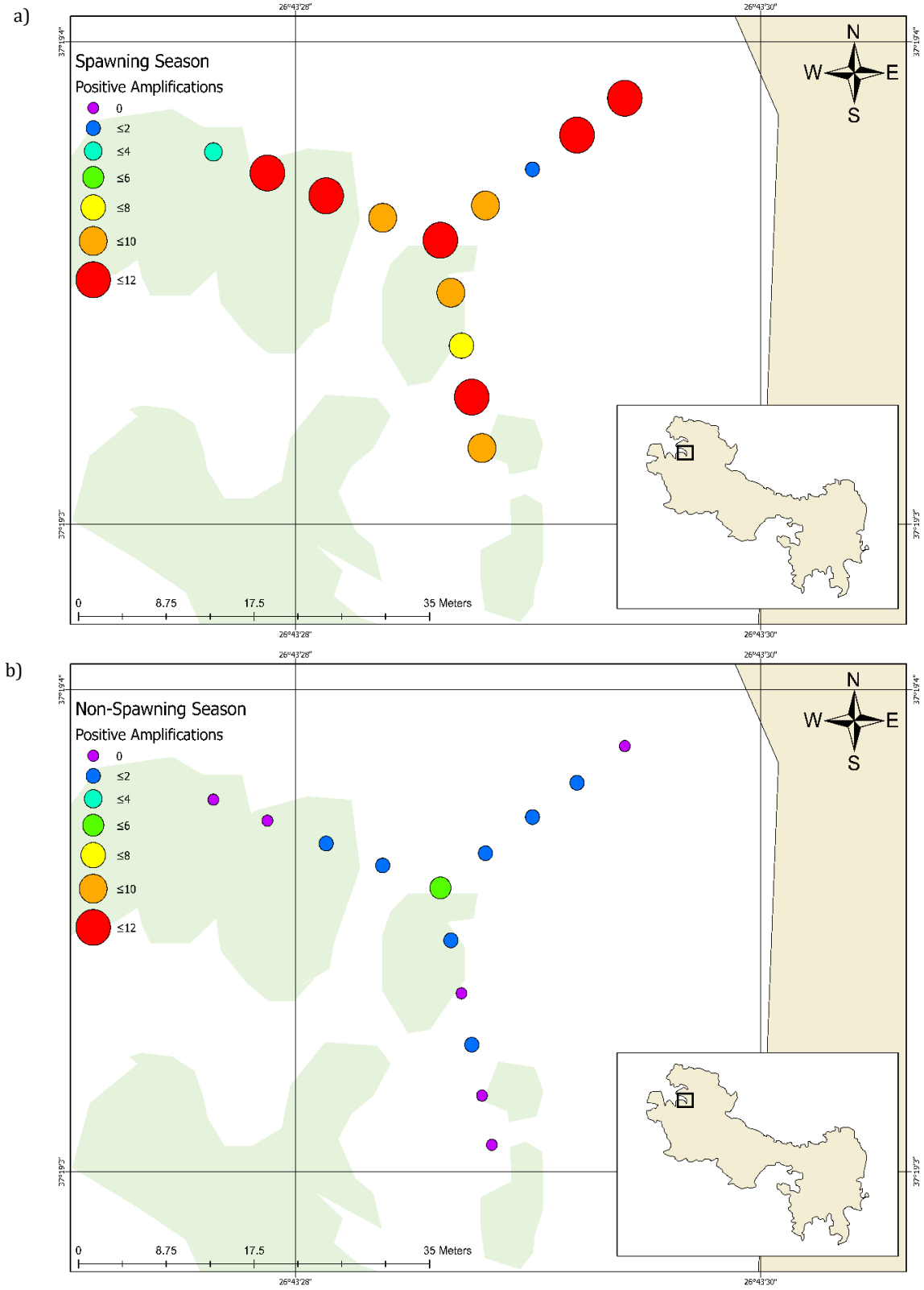


Figure 3.12 Heat maps showing the total positive amplifications in qPCR at each sampling point from a) Spawning season and b) non-spawning season.

Table 3.2 Summary of qPCR analysis for *P. nobilis* eDNA including distance from point source, average blade length, the Ct value, total positive amplifications during qPCR and eDNA concentration (ng μl^{-1}). Blade length is the average blade length of *P. oceanica* seagrass at each sampling point in cm. samples that were sequenced are indicated by * for ones that were unsuccessful and ** those which sequenced successfully.

Season	Distance from focal individual (m)	Blade length (cm)	No. positive amplifications	Mean C_T value	C_T SD	Range of DNA copies μl^{-1}	eDNA Conc. (ng μl^{-1}) (SD)
****Spawning**	0	17	12	31.45	0.96	97- 1340	3.75×10^{-8} (4.24×10^{-8})
Spawning	5	7	9	34.16	1.04	20- 83	6.62×10^{-9} (5.03×10^{-9})
**** Spawning**	5	0	10	27.40	0.29	1327- 3304	4.14×10^{-7} (6.59×10^{-8})
*** Spawning**	5	28	9	33.27	0.86	10- 104	4.02×10^{-9} (3.36×10^{-9})
Spawning	10	14	11	33.47	1.41	10- 146	1.12×10^{-8} (7.89×10^{-9})
Spawning	10	0	2	33.76	0.39	18- 26	2.65×10^{-8} (8.99×10^{-10})
Spawning	10	13	8	33.11	1.06	5- 74	5.51×10^{-8} (3.15×10^{-9})
Spawning	15	24	11	28.33	0.17	982- 1797	2.27×10^{-7} (2.44×10^{-8})
Spawning	15	0	12	29.76	0.30	198- 402	2.99×10^{-8} (8.95×10^{-9})
Spawning	15	0	12	29.84	0.21	547- 753	7.27×10^{-8} (1.30×10^{-8})
*** Spawning**	15 (surface)	0	10	34.86	1.09	59- 92	6.43×10^{-9} (4.24×10^{-9})
Spawning	20	39	4	33.76	1.17	25- 100	7.81×10^{-9} (5.85×10^{-9})
Spawning	20	0	11	31.56	1.06	30- 198	1.39×10^{-8} (1.19×10^{-8})
Spawning	20	21	10	31.75	0.60	104- 342	3.36×10^{-8} (1.26×10^{-8})

****Non-spawning**	0	15	5	33.68	0.89	5- 92	8.89×10^{-9} (5.84×10^{-9})
Non-spawning	5	23	2	36.78	1.18	1- 5	5.96×10^{-10} (4.33×10^{-10})
**** Non-spawning**	5	0	1	35.45	0	0- 7	1.26×10^{-9}
Non-spawning	5	13	2	35.72	1.06	1- 5	5.50×10^{-10} (3.85×10^{-10})
*** Non-spawning**	10	13	2	35.76	0.16	5- 6	1.03×10^{-9} (1.14×10^{-10})
Non-spawning	10	0	1	34.66	0	0- 6	1.03×10^{-9}
Non-spawning	10	15	0	0	0	0	0
Non-spawning	15	33	0	0	0	0	0
Non-spawning	15	0	1	34.47	0	0- 7	1.18×10^{-9}
*** Non-spawning**	15	0	1	36.56	0	0- 6	9.95×10^{-10}
Non-spawning	20	37	0	0	0	0	0
Non-spawning	20	0	0	0	0	0	0
Non-spawning	20	0	0	0	0	0	0
Non-spawning	25	0	0	0	0	0	0

All controls were negative for DNA amplification with the exception of a single positive amplification (1/3 qPCR replicates) from an extraction control taken during processing of spawning season samples. DNA amplification was repeated (n=6) on the extraction control and amplification was confirmed (2/9 qPCR replicates. The three samples potentially impacted by the extraction control contamination were corrected for contamination by subtraction had the average concentration of the contamination replicates (i.e. $4.92x\ 10^{-10}$ ng μl^{-1}) from the DNA concentrations calculated from the qPCR results of these samples. This correction made negligible difference to these sample, considering the uncorrected average eDNA concentrations were 3.80×10^{-8} ng μl^{-1}, 3.41×10^{-8} ng μl^{-1}, and 7.32×10^{-8} ng μl^{-1}, and demonstrated and very low level of contamination when compared to the amounts in the samples. All other controls were negative.

To validate primer specificity qPCR products from eight positive samples were sequenced to confirm the specificity of the eDNA assay (29% of 28 samples). In addition qPCR products from the contaminated lab control (n=1) were also sent for sequencing. Four of the eight samples were successfully sequenced, returning a clean sequence for assigning taxonomic identity. It is likely the samples that were not successfully sequenced, contained insufficient DNA for successful sequence reaction. Initial DNA concentration of PCR products sent for sequencing were already low due to the high sensitivity of the assay ($9.95x\ 10^{-10}$ – 4.14×10^{-7} ng ul^{-1}), with further DNA loss likely to occur during PCR, with kits recovering up to 95% of DNA fragments during cleaning (Qiagen.com). The successfully sequenced products were aligned and taxonomy assigned to the consensus sequence to *Pinna nobilis* isolates from the Greece region (100% match identity, 100% query coverage, sequence accession numbers:

DQ448216, DQ448217, EF536827-EF536829, EF536833, EF536834, EF536837, EF536842, EF536845 and EF536846). This confirms that DNA amplifications using this novel qPCR assay are specific to *Pinna nobilis*.

3.3.2 Seasonal variation in *P. nobilis* detection

Of 146 positive amplifications in total, 131 positive amplifications were generated from samples taken in the spawning season, with at least two replicates amplifying from each sample (figure 3.13). By contrast the non-spawning season yielded just fifteen positive amplifications across all samples taken during that sampling period, with a maximum of five positive amplifications in a single sample. These results from both models suggest that, overall, eDNA was 8.7 times more likely to be detected from water samples in spawning season compared to non- spawning season ($F_{24,26}$ = 6.4, p = 2x 10^{-16}; 95% CI = 6.11 - 12.64; figure 2.11).

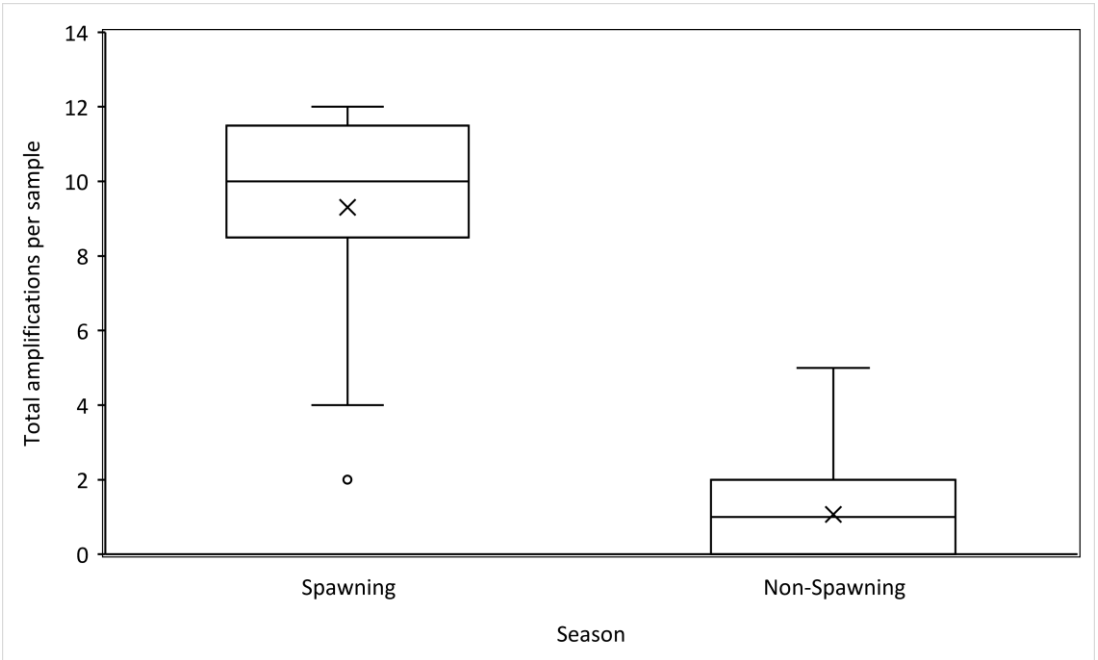


Figure 3.13 the total number of positive amplifications from the qPCR analysis of *P. nobilis* eDNA in Spawning and non-spawning season.

3.3.3 Effect of Distance on eDNA detection

There was a significant interaction between season and distance from the focal individual ($F_{24,26}$ = 2.8, p= 0.0066). This showed that the effect of distance was different between the two sampling seasons.

Likelihood of detection decreased by 10% with each increasing 1m away from the *P. nobilis* individual ($F_{12,13}$ = 9.7, p = 0.000179; 95%CI = 0.8553, 0.9523) during non-spawning season. Using the model it is possible to predict the distance at which, during non- spawning season, detection is extremely unlikely to occur. Based on these data, in non-spawning season by 20m away from the *P. nobilis* point source the probability of detection becomes very low (figure 3.14).

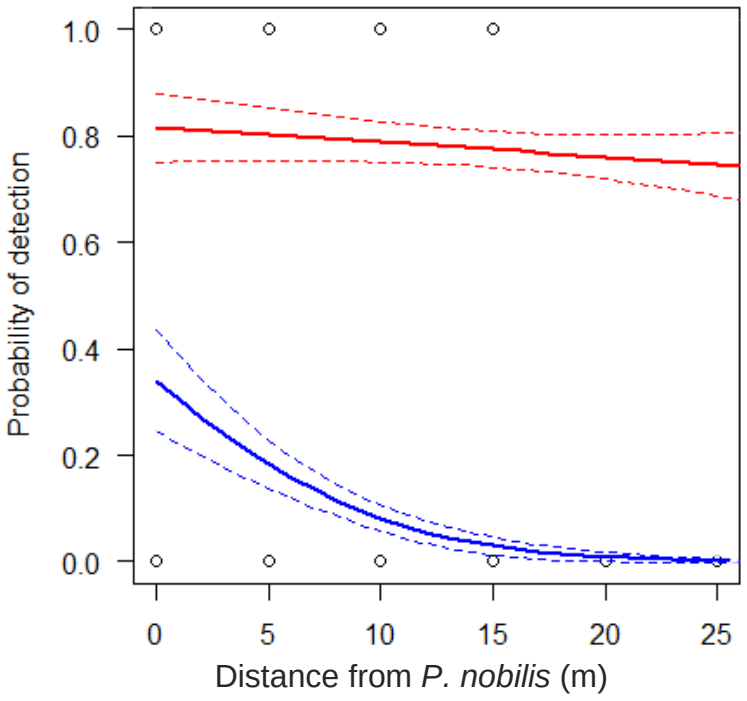


Figure 3.14 The probability of detection in spawning (red) and non-spawning (blue) against distance from the focal *Pinna nobilis* individual which was assumed to be the sole source of eDNA in this system.

Distance had no effect on the probability of detection during spawning season ($F_{12,13}$ = 0.98, p = 0.513526). Based on this model probability of detection starts at an estimated 80% and never drops below approximately 70% in the 20m distance surveyed (figure 3.14). There is a slight decline in probability seen in spawning season (figure 3.14), however this was not significant.

3.3.4 Effect of seagrass on eDNA detection

When only the data from spawning season as modelled in the binomial GLM the only significant factor was seagrass blade length. Seagrass blade length decreased the probability of detecting *P. nobilis* eDNA by 5% with each 1 cm increase in blade length of seagrass ($F_{12,13}$ = 3.5, p= 0.01902; 95% CI= 0.9062, 0.9957). This means increasing seagrass blade length decreases the likelihood of detection eDNA from a point source.

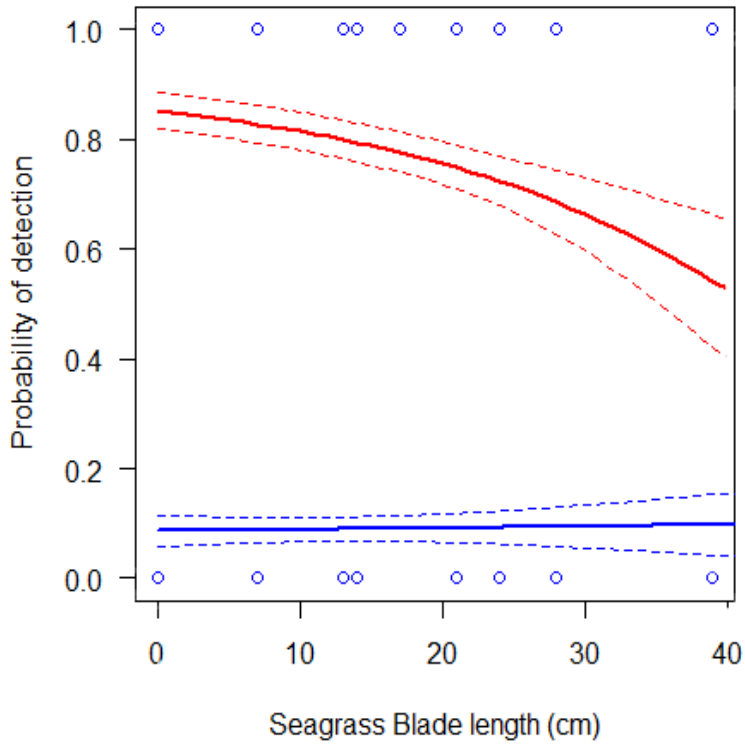


Figure 3.15 Visualisation of model of probability of detection in spawning (red) and non-spawning (blue) against seagrass blade length.

As with the previous model, it can used to predict the probability of detection based on seagrass blade length. This probability never drops below 50% even in the areas of higher seagrass canopy (> 30-40 cm) (figure 3.15).

Seagrass blade length had no effect on the probability of detection during non-spawning season ($F_{12,13}$= 0.5, p = 0.8641). Based on this model, the probability of detection remained consistently low across all seagrass canopy heights (figure 3.15).

3.4 DISCUSSION

3.4.1 Development of a validated eDNA method for detecting *P. nobilis*

To our knowledge, this is the first time that an eDNA protocol has been developed for *Pinna nobilis*. Environmental DNA has been shown here to be a sensitive method for the detection of *P. nobilis*. This method can be used without the need for visual identification, using the specified qPCR primer and probe combination. The method is sensitive picking up quantities as low as 5.50×10^{-10} ng μl^{-1} (or 1 copy μl^{-1}), so large quantities of DNA are not needed to be present in the environment to allow for positive detection.

P. nobilis surveys have already been shown to suffer from imperfect detectability and a bias toward larger individuals (Hendriks, Deudero, & Tavecchia, 2012). UVC surveys can be time consuming, expensive and requiring of experienced expert surveyors to be accurate (Edgar *et al.* 2004), furthermore, surveying at depth it much harder to achieve using UVC. A simple water sampling and qPCR technique such as the one presented here can assist with this monitoring programme reducing the time and manpower

needed to identify the locations of remaining populations, avoiding difficulties of differentiating this species visually with similar species such as *Pinna rudis*.

The development of this tool is particularly timely given an urgent need to survey and monitor *P. nobilis* populations. *P. nobilis* has recently been classed as critically endangered by the IUCN red list due to the mass mortality events caused by the *Haplosporidium pinnae* parasite (Kersting *et al.* 2019). The IUCN have stated there is an urgent need for regional monitoring programmes especially areas of the south Mediterranean Sea where data is lacking (Kersting *et al.* 2019).

3.4.2 Determinants of *P. nobilis* eDNA detectability

The sampling season was a key factor in the detectability of *P. nobilis* with the likelihood of detection increased by 8.7 times during spawning season. In this study *P. nobilis* were observed to be spawning in August, consistent with the Spanish Mediterranean (Richardson *et al* 1999), however there has been no such research as yet into the specific spawning season in Aegean waters (Katsanevakis, 2007). This finding has implications in the utilisation of eDNA methods for *P. nobilis* and other marine bivalve species that broadcast their gametes during spawning. Detectability of all species is likely to increase during spawning season due to the release of gametes into the water column increasing the amounts of DNA present in the environment. Bracken *et al.* (2019), demonstrated the use of eDNA sampling during spawning season, using *Petromyzon marinus,* to get a "snapshot" of fish species presence and was the ideal opportunity to monitor distribution and habitat use due to the increased chance of detecting the target species. Tillotson *et al.* (2018), showed that eDNA quantities can vary significantly day to day during the spawning season of salmon

(*Oncorhynchus nerka*), and they suggested that these may even vary hourly and therefore should be used with caution if the aim is to study biomass from eDNA quantification, which was not attempted here. Little is known yet on the details of the mechanisms that control *P. nobilis* mass spawning events (Prado *et al.* 2020), however spawning season is known to span late summer into early autumn (Basso *et al.* 2015) and has been shown to coincide with temperatures of 20°C (Deudero *et al.* 2017).

Detectability did not alter significantly with distance from the *P. nobilis* during spawning season over the twenty-metre survey distance. This is likely to be a result of the increased eDNA presence in the water from gamete release; and may have been further magnified by released gametes from the presence of other adult *P. nobilis* individuals in the study area. During non-spawning season, however, detectability was strongly associated with distance to the focal individual, with the probability of eDNA detection at twenty metres being extremely low.

To date there has been very little investigation of dispersion of eDNA in marine ecosystems. Understanding patterns in eDNA dispersal is important to understanding the spatial specificity of this monitoring method. The conditions in Vroulia bay, where the study was conducted, are generally very still with little water movement, therefore eDNA will move via passive diffusion only. When combined with the significant decrease in the amount of eDNA being released during non-spawning season we have shown this results in a very specific survey area being sampled.

The interplay of water movement and the amounts of eDNA released are important for determining eDNA detection. eDNA transport has been studied extensively in river systems, assessing the distance moved downstream, not the passive dispersal

investigated here when flow rates are negligible. Pilliod *et al.* (2014) detected eDNA from caged salamanders at five metres but not at fifty metres, they suggested this was likely due to low density of individuals introduced the area, however there were no intermediate sampling points so the reduction in detection was not modelled. Many other studies on eDNA, however, have shown detectability at large distances, for example Deiner & Altermatt (2014) showed invertebrate eDNA from two different species (*Daphnia longispina & Unio tumidus*) was able to be detected at 9.1 and 12 kilometres distance from their respective populations. It is important to note that all of these studies took place in freshwater river or stream systems, and therefore direct comparisons in eDNA dispersal are problematic.

Jane *et al.* (2015) suggested a general interaction between flow and distance from the source on the detection of eDNA. They suggested at low flows, settling of fine particulate organic matter happens quicker and over shorter distances than with increased flow rates, removing the cells from the water column. This may help to explain why, when there was a low density of eDNA in the water column during non-spawning season, distance was a controlling factor to determine detectability.

The sampling season being used will determine the research question able to be addressed when using eDNA to study *P. nobilis*. Detection rates were substantially higher during spawning season and therefore can provide a low-resolution indication to the presence or absence of *P. nobilis* within a study area with relatively little effort needed. More work is needed to assess the spatial scales over which this would be relevant. It may be unable to determine accurately the size of the population and the location of individuals. Some eDNA studies have shown links between biomass and eDNA concentration (e.g. Weldon *et al.* 2020; Sassoubre *et al.* 2016; Takahara *et al.*

2012), but these links should be studied further in relation to spawning seasons. It could also miss any immature juveniles that are not spawning yet as sexual maturity is not reach until 2 years of age (Basso *et al.* 2015), however will confirm the presence of an actively spawning population.

When used during non-spawning season, eDNA can be used to either confirm the identification of specific *P. nobilis* individuals non-invasively or provide a more precise estimate of the location of *P. nobilis* populations. Fine scale water samples taken during non-spawning season, if positive, will show there are *P. nobilis* within twenty metres, therefore narrowing the search radius of visual methods. In contrast water samples during spawning season can identify a population of *P. nobilis* in an area but no individuals need to be sighted.

Environmental DNA also has the potential to be a really powerful tool for studying species presence at depth. As shown in section 3.1.1.1 about *P. nobilis* ecology, individuals of this species are more often found in the first 10- 12m (Basso *et al.* 2015). All of the reviewed studies collected data using SCUBA divers. Some studies have also stated the potential depth limit of *P. nobilis* as 60m (Katsanevakis 2006) which is beyond the range of standard UVC. As discussed in section 3.1.2 UVC methods have limitations, that include dive time and training. The level of expertise and specialist equipment required, such as rebreathers, to survey at depth, combined with the extra risks and expense these activities preclude deep diving as a suitable method for widespread UVC surveys beyond 20-25 metres in depth. This is why the level of information that we have about the distribution and ecology of many coastal species, including *P. nobilis* and indeed *P. oceanica*, is confined to the shallower parts of their range.

3.4.3 eDNA in Seagrass ecosystems

Seagrass blade length was a significant factor, in determining eDNA detectability during spawning season. For each 1 cm increase in blade length probability of detection decreased by 5%. Suggesting the more seagrass there is the less likely the eDNA is to be detected. This could be due to the seagrass canopy obstructing the flow of water from the *P. nobilis* individual, aiding the settling of eDNA out of the water column, and/or eDNA adhering to seagrass blades.

Seagrass meadows, and in particular *P. oceanica* meadows, have been shown to increase sedimentation (Manca *et al.* 2012). eDNA can be removed from the water column by binding to particulate matter which then becomes incorporated into substrates such as clay (Turner *et al.* 2014). Capacity for binding is dependent on particle size with smaller particles having a higher capacity to bind to DNA (Levy-Booth *et al.* 2007). Buxton *et al.* (2017), suggested water samples from an environment with organic sediment types can result in false negatives due to this process of binding and incorporation with the sediment.

Here we did not test the eDNA content of the sediments, only the water above the sediments within the canopy or on bare sand. Turner *et al.* (2015) showed eDNA persistence in sediments was much greater than in water samples. They found 8-1800 times more eDNA in the sediments than water and showed it persisted for up to 3 months when in sediments. The increased sedimentation rates in seagrass meadows and reduced resuspension of fine particles, could be causing the eDNA particles to be stored in the sediments, reducing the detection of eDNA from the water column.

Environmental DNA can be developed for other species key species that interact with seagrass meadows such as the sea horses *Hippocampus hippocampus* and *Hippocampus guttulatus.* Both species are classed as globally data deficient on the IUCN red list due to lack of data across the whole extent of its geographic range (Gristina *et al.* 2017; Pollom, 2017; Woodall, 2017).

3.5 CONCLUSION

This study has developed a sensitive eDNA protocol for detecting *P. nobilis* and demonstrated the differences in detectability between spawning and non-spawning season. This tool has the power to progress effective monitoring of *P. nobilis* distribution across their entire habitat range and at various spatial scales. The data and models presented here highlight the need for further research into the impacts aquatic vegetation have on the movement and dispersal of eDNA from a point source. Our results demonstrated seagrass blade length decreased the likelihood of detection, however, the mechanics of this are still unknown. It is possible that reduction in water flow within the canopy reduces the diffusion of eDNA when at low densities becoming trapped in the seagrass canopy preventing it from dispersing far from the point source and leading to false negative results. Increased sedimentation rates within in seagrass canopies could be increasing the rate of eDNA incorporation into the sediments, removing it from the water column. This suggests that, in non-spawning seasons, positive eDNA amplifications can provide a provide a precise estimate of location, especially when the *P. nobilis* are in low flow environments. *P. oceanica* meadow has been shown to be the preferred habitat for these bivalves along with many species of commercial and ecological importance at some stage in their life cycle (Kalogirou *et al.* 2010, etc). In order to develop eDNA as a monitoring tool for these species, or any

species that utilises aquatic vegetation understanding these mechanics of dispersal is vital in the application of eDNA. More research is needed to understand vertical movement of eDNA out of seagrass meadow and how eDNA from outside the seagrass meadow permeates the canopy.

Further study would be needed to see if there was an association with *P. nobilis* biomass / population with eDNA quantity as quantifications were not used in this study. There is also a need to understand how this mechanism would work in areas of seagrass with different flow rates, as if riverine systems are used as a model it would suggest with increased flow, detection could happen over greater distances.

Chapter 4

Effects of seagrass meadows on microplastic deposition.

4.1 Introduction

Seagrass meadows play an important role in manipulating the hydrodynamics of their surrounding water column, markedly reducing wave energy (Fonseca & Cahalan, 1992; Stratigaki *et al.* 2011). *Posidonia oceanica* forms very dense, high canopies and therefore has a larger baffling effect than the smaller seagrass species (Stratigaki *et al.* 2011; Manca *et al.* 2012). Dense canopies of *P. oceanica*, comprising of 360 shoots/m^2, have been shown to reduce wave height above a meadow by up to 35% (Stratigaki *et al.* 2011), and reduced flow in the middle of the canopy by up to 58.7% (Manca *et al.* 2012). These influences on fluid hydrodynamics as a result of seagrass presence has also been shown to increase sedimentation rates and modify sediment composition within the meadow (Zhang *et al.* 2020; van Katwijk *et al.* 2010; De Falco *et al.* 2000), potentially leading to the accumulation of pollutants within the sediments.

4.1.1 Seagrass modification of sediments

Analysis of the grain sizes of sediments present within an aquatic system can provide important information about the hydrological conditions in that location (Murray &

Thieler, 2004). Sediment grain size is often measured using the Phi Scale and from this sediment sorting, skewness and kurtosis can be calculated. Sorting demonstrates the level of variability of sediment sizes in a sample, whereas skewness and kurtosis describe the shape of distributions (Joanes, and Gill 1998). Skewness describes if the data stretches in one direction more than the other (i.e. extreme right skewness or extreme left skewness) while kurtosis measures the height of the peak (i.e. flatness of the distribution peak) (Groeneveld & Meeden, 1984).

The level of sorting occurring in sediments is predominantly influenced by the energy of the environment from which the sediments are collected (De Falco *et al.* 2000). Sorting demonstrates the level of variability of sediment sizes in a sample. Coarser particles tend to be deposited as soon as flow intensity diminishes sufficiently for a particular size fraction to settle out as gravity begins to dominate vertical diffusion (Murray & Thieler, 2004). Well-sorted sediment indicates less variation in grain size and is more likely to be associated with high-energy environments where vertical diffusion plays a more dominant role than gravity, separating large particles from small. Poorly sorted sediments indicate mixed sediments and therefore a large degree of variation in sediment grain sizes. These poorly sorted sediments are associated with low energy environments where gravity is the dominating force.

De Falco *et al.* (2000) showed seagrass meadows influenced the accumulation of fine particles due to the modified hydrological conditions within the canopy. These modifications included dampened wave action, trapping effects of the leaves and rhizomes and changes to hydrodynamics at the sediment-water interface. Zhang *et al.* (2020) modelled particle deposition using synthetic seagrass designed on *Zostera marina* shoots. The authors varied the *ah* (a nondimensional measure of vegetation

density that is a function of shoot density, blade number and blade length) and the velocity, finding that denser meadows (*ah* = 0.67) retained fine particles at much higher rates than bare bed at flow velocity of 0.16ms^{-1}. By comparison, less dense meadows (*ah* = 0.12) showed the same level of particle retention as bare bed under the same flow velocities. A similar relationship has been reported from *Zostera marina* meadows measured *in situ* in the North Sea (van Katwijk *et al.* 2010). Dense meadows (>160 shoots m^{-2}) again showed a significant increase in fine sediment fractions compared to unvegetated areas. Fractions between 16 and 63 μm (medium and coarse silts) contributed 10.5% of the sediment composition in unvegetated areas but significantly more (18.3%) in vegetated areas. Furthermore, densely covered meadows in sandy exposed sites showed clear muddification (i.e. increase in fine particles and /or organic matter). These factors indicate *P. oceanica* increases the sorting coefficient of the associated sediments.

De Falco *et al.* (2000) demonstrated the ability of *P. oceanica* to affect the sedimentary patterns of the seafloor in the Gulf of Oristano, Italy. Muddy sands and coarser grain size fractions dominated sediment samples from a meadow with over 50% shoot coverage. In contrast sediments from a seagrass-free gap through the meadow, had fewer coarser grain sizes and were dominated by fine sands. *P. oceanica* sediments had a high sorting coefficient (σ_φ = 2.7 ± 0.4) indicating they were very poorly sorted. By contrast, sediments from a seagrass free gap through the meadow were less muddy and better sorted, with a lower sorting coefficient (σ_φ = 1.4 ± 0.3).

Once settled within seagrass habitats, sediments have a reduced propensity to resuspend into the water column. Terrados & Duarte (2000) provided experimental evidence of reduced particle resuspension within *P. oceanica* meadows by tracking the

movement of tracer particles. The percentage loss of tracer particles from within a seagrass meadow (38.0% - 97.8%) was significantly lower than outside it (62.9% - 99.3%), albeit with high levels of variability. The authors also reported that water motion did not significantly differ between sampling stations in *P. oceanica* meadows and those at non-vegetated comparison locations, and therefore suggested that tracer particle loss was not directly related to water motion. It should be noted that the meadow used in this study was located at a depth of 15 metres, which may explain the reduced effect of *P. oceanica* on water movement. Gacia & Duarte (2001) also demonstrated reduced sediment resuspension within *P. oceanica* canopies. The authors found, on average, a three-fold reduction in resuspension at vegetated sites when compared with adjacent unvegetated sites. *P. oceanica* significantly reduced erosion in the meadow by restricting resuspension to the upper 1 mm of sediments rather than the upper 3 mm as observed in unvegetated sites.

Microplastics are small pieces of plastics that are less than 5 mm in size (Duncan *et al.* 2018; Thompson *et al.* 2009). Sediments from seagrass meadows were shown by De Falco *et al.* (2000) to be dominated by fine sands (125–250 μm), so there is a potential for microplastics of similar sizes to be trapped along with the sediments.

It is possible that under conditions of increased sedimentation and lower sorting, such as those found in seagrass meadows, particularly *P. oceanica*, there is an increase in microplastic deposition and retention. This could potentially make seagrass ecosystems an important area for the coastal accumulation of plastic particles. Microplastics have previously been shown to have no clear relationship to specific sediment grain size fractions when individual fraction sizes are considered (Urban-Malinga *et al.* 2020; Alomar *et al.* 2016; Nor & Obbard, 2014), this type of analysis

hasn't yet been modelled as a continuous scale. Microplastics have been shown to have some relation with sediment sorting, with Zobkov & Esiukova (2017) finding microplastic concentrations increased with increased sediment sorting in a lagoon in the Baltic sea.

4.1.2 Microplastics in marine environments

Microplastics can be categorised as either primary microplastics - that are purposefully manufactured, such as those associated with cosmetic products, preproduction nurdles, and fibres from clothing - or secondary microplastics, that are derived from the breakdown of larger macroplastics in the environment by physical abrasion and UV radiation (Barnes *et al.* 2009, Martin *et al.* 2017, Duncan *et al.* 2018).

Microplastics are widely recognised as a contaminant of global concern due to their bioavailability and potentially harmful impact on a wide array of marine species (Clark *et al.* 2016; Coppock *et al.* 2017). Many laboratory and field studies have reported a wide range of organisms suffering from lethal or sub-lethal effects as a result of exposure to microplastic pollution (see Guzzetti *et al.* 2018 for a review). For example, Crump *et al.* (2020), recently demonstrated the impacts of microplastics on shell selection in the hermit crab, *Pagurus bernhardus*. Hermit crabs exposed to plastic showed impaired shell selection, making them less likely to make contact and enter optimal shells. The authors suggested this could be due to microplastics impairing cognitive function and subsequently disrupting hermit crab behaviour. Microplastics have also been shown to influence the behaviour of copepods, such as *Calanus finmarchicus,* that was shown to experience shifts in prey selection, feeding, lipid

accumulation and moulting in the presence of nylon microparticles (Cole *et al.* 2019). Brine shrimp, *Artemia parthenogenetica,* have been observed to develop abnormal intestinal epithelial cells after exposure to polystyrene microplastics, possibly affecting nutrition absorption and energy metabolism (Wang *et al.* 2019). In Medeka, *Oryzias melastigma*, polystyrene microplastics caused the decrease of gonadosomatic index (GSI) in females and both the hepatosomatic index (HSI) and GSI in males (Wang *et al.* 2019b). These microplastics were also shown to cause obvious oxidative stress and tissue damages along with the disruption to the reproductive endocrine system in different ways between sexes. Microplastics have also been found in the intestines and stomachs of marine mammals, however, their direct impact is still unknown at this trophic level (e.g. Hernandez-Milian *et al.* 2019; Nelms *et al.* 2019).

Further to their purported direct effects, microplastics have also been shown to be a vector of pathogenic bacteria such as *Vibrio spp* (Kirstein *et al.* 2016) and *Aeromonas spp* (Viršek, *et al.* 2017), harmful chemicals, antibiotics, metals and harmful algal bloom (HAB) species (Naik, *et al.* 2019). Brennecke *et al*, (2016) demonstrated the ability of microplastics to adsorb potentially toxic heavy metals such as copper and zinc. Jinhui, *et al.* (2019), subsequently showed the heavy metals adsorbed to these microplastics can then impact the growth of the seahorse, *Hippocampus kuda,* reducing body length, body weight, condition factor, specific growth rate and survival rate.

While there is a growing body of evidence that microplastics are present in the marine environment and are almost universally distributed (e.g. Claessens, *et al.* 2011; Alomar *et al.* 2016; Alimba & Faggio, 2019) there are some significant knowledge gaps. To date there is little research that clearly identifies and characterises the influence of coastal vegetation on microplastic distribution despite the importance of these habitats as low

energy refuges for biodiversity (but see Hazimah, Nor, & Obbard, 2014, Li *et al.* 2018, & Garcés-Ordóñez, *et al.* 2019 for analyses of microplastics in mangrove systems). The next section reviews the limited research that has been undertaken on microplastics in seagrass habitats to date.

4.1.3 Microplastics in seagrass systems

The first published report of microplastic pollution in seagrass (Goss *et al.* 2018) demonstrated microplastic contamination of seagrass blades of the Caribbean species *Thalassia testudinum* . Microplastics were adhered to 75% of blades studied; of these 81% were microfibres, 16% were beads, and 3% were plastic chips. Furthermore, the authors documented a clear, albeit non-significant, trend for increasing microplastic accumulation on seagrass blades with a higher epibiont cover. There have been numerous examples of species inhabiting the seagrass meadows, such as molluscs, grazing the epiphytes from the seagrass blades (see review by Orth & Van Montfrans, 1984). There has also been evidence of macrograzers preferentially grazing on seagrass blades with a high epiphyte load due to increased nutrition (Tomas *et al.* 2005). Examples of this include the six-spine leatherjacket (*Meuschenia freycineti*) and the yellow-finned leatherjacket (*Meuschenia trachylepis*) on *Posidonia australis* blades (Wressnig & Booth, 2007), and *Paracentrotus lividus* on *Cymodocea nodosa* blades (Jiménez-Ramos *et al.* 2018). Due to this grazing preference and the relationship shown in Goss *et al.* (2018), seagrass is a potential vector for microplastics to enter the food chain.

Huang *et al.* (2020), investigated microplastic accumulation within sediments in *Enhalus acoroides* (Tape Seagrass) meadows located in two bays in China. In both meadows microplastic densities were significantly higher in vegetated sites than bare

ones. The authors found the enrichment index (or pollution load) of microplastics was 2.1 and 2.9 in the bays and the most abundant shape of microplastic was fibres comprising an average 58.6% ± 16.0% across all sites.

Similarly in Orkney, Scotland, the accumulation of microplastics in *Zostera marina* meadows has been documented (Jones *et al.* 2020). The number of microplastics recovered from seagrass sediments was significantly higher than sediments from outside the seagrass meadow within the sediment controls. There was an average of 300 particles ± 30 SE microplastics found per kg of dry weight sediment inside the seagrass meadow, while from bare sand it was 110 particles kg^{-1} ± 20 SE. Furthermore, the authors also suggested that seagrass grazers were more susceptible to ingesting microplastics due to the increased number of microplastics found within the bodies of seagrass-associated biota (4.50 per individual ± 0.96) compared to bare sediment associated biota (1.60 per individual ± 0.32).

As *P. oceanica* has been shown to have some of the greatest impacts of any seagrass on hydrological movements, it follows that it represents a highly effective trap for microplastics, accumulating them within the rhizome matte. A better understanding of how microplastics interact with marine vegetated sediment could mean seagrass preservation / restoration might have a localised impact on removing microplastics from the environment. It also means that species that inhabit seagrass habitats might have greater exposure to microplastics and their leachates than other species. This can then highlight areas where seagrass conservation and restoration can play a role in microplastic removal or where seagrass loss might lead to the resuspension of large quantities of accumulated microplastics. The remaining seagrass meadows, especially in the Aegean, are likely to be on relatively sparsely populated stretches of coast.

Research focusing on meadows near dense human habitation may skew our understanding of the scale of the problem.

4.1.4 Chapter Objectives

The objectives of this chapter are:

1. To identify whether microplastic particles are accumulating within an isolated *P. oceanica* seagrass meadow in the Eastern Aegean Island of Lipsi,
2. To understand the spatial dynamics of microplastic accumulation within seagrass meadows and the environmental factors responsible.

4.2 Experimental Design

4.2.1 Study Area

The study was conducted at Elena Bay, a small bay located within Lipsi Bay on the Greek Island of Lipsi (figure 4.1), in May 2018. Lipsi is one of the Northern Dodecanese Islands (37°18′N 26°45′E), and has a local population of around 700 people concentrated around Lipsi Bay.

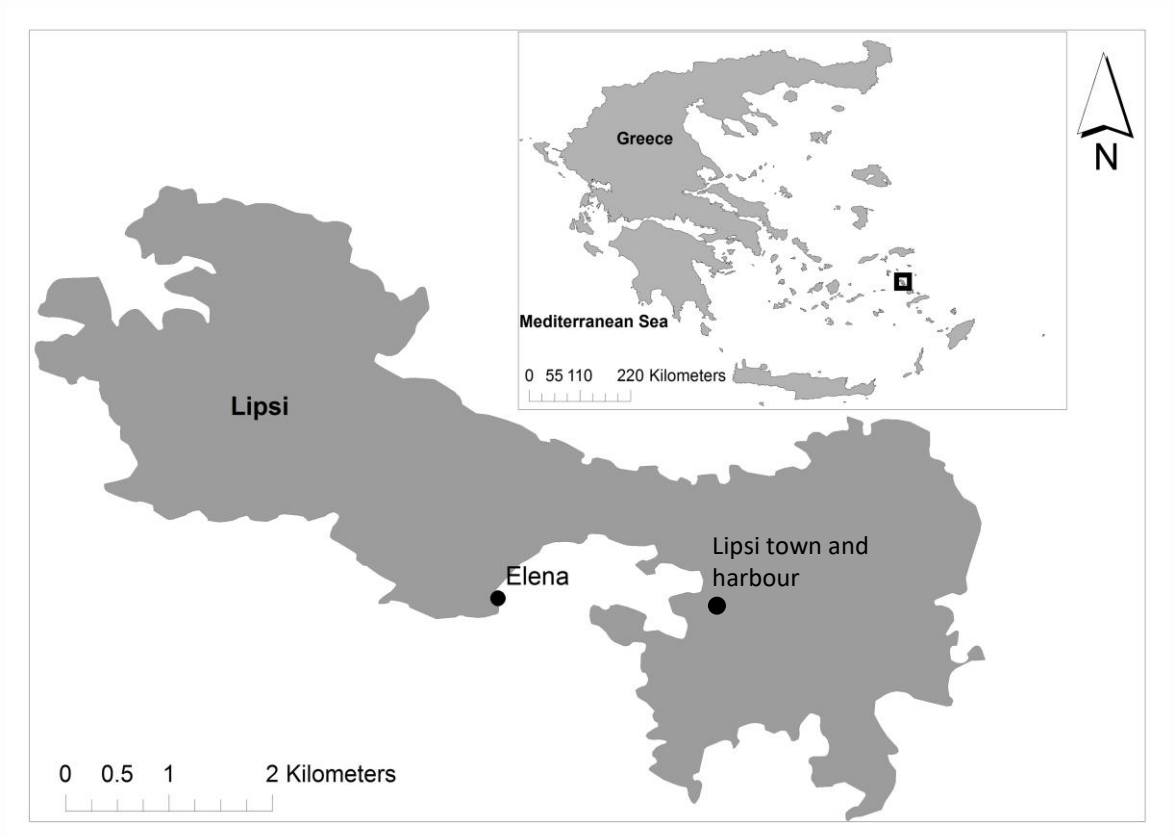


Figure 4.1 Location of Lipsi in the Aegean Sea and the sampling site, Elena Bay (marked with a black dot), on Lipsi. The map also highlights the location of Lipsi town and harbour (black dot) in relation to the sampling site

Elena Bay does not receive a high footfall of tourists even during peak times (pers obs.), however it is exposed to regular ferry and boat traffic as it is near the access route to the harbour. This site was selected due to the presence of accessible, dense seagrass meadows alongside non-vegetated substrates, allowing samples from vegetated and

non-vegetated environments to be collected in the same area. It is was also assumed to be representative of the conditions of many seagrass meadows on small Greek islands.

4.2.2 Sample collection

Sediment samples were collected at five metre intervals along four 50 metre transects, of which three passed through an approximately 50 x 20 metre seagrass meadow. The transects were placed 10 metres apart. The shallowest waters (c. 2 m from the shoreline) of the study site were characterised by bare rocky substrates devoid of sediment, so transects commenced at point nearest the shore where sediments had started to accumulate.

At each sampling location, three 100 ml samples of sediment were collected in 125 ml glass jars that had been cleaned with distilled water and securely sealed. The jars remained sealed until they reached the seabed where they were opened and sediment collected. Jars were resealed before returning to the surface to prevent loss of sediments and minimise mixing with water column. A further three sample jars were rinsed and filled with the distilled water used for cleaning as a contamination control for the washing process. All sample jars were then sealed with Parafilm around the lids to secure them in place and shipped back to the UK for analysis.

Environmental variables were also collected at each sampling point. Percentage seagrass cover was estimated using a 50 x 50 cm quadrat and overall meadow health was measured using standard methods from McKenzie *et al.* (2003), Mayot *et al.* (2006) and (Pergent, *et al.* 1995).

4.2.3 Microplastics extraction

Sediment samples were dried in aluminium containers covered with aluminium foil at 55°C for 72 hours. Sediment Microplastic Isolation (SMI) units were then used to density separate the microplastics from the sediments adapting the method of Coppock *et al* (2017). In this study, 50 g of dried sediment from each sample was added to a floatation solution of 700 ml of high density (1.2g cm^{-3}) NaCl. All NaCl solutions were filtered with 10 µm filters to remove any possible contaminants before use. NaCl was preferred in this study as opposed to higher-density ZnCl, as recommended by Coppock and colleagues (2017), due to it creating an intense effervescent reaction with seagrass sediments in a preliminary study leading to loss of sample.

Sediments were mixed using a magnetic stir plate for five minutes, following this any air bubbles were released by three sharp pulses of the stir plate. Samples were left to settle overnight in the SMI units. Supernatant was filtered through 30 µm nylon mesh and any large pieces of organics, such as fragments of rhizomes or shells, were washed with 50% ethanol solution onto the filter before removal. SMI units were disassembled and washed thoroughly after each use with ultra-pure water, paying particular attention to the ball valves. The units were then reassembled and filled with fresh, filtered NaCl solution for 10 minutes to remove environmental contaminants. Dampened nylon filters were left exposed, in Petri dishes as settlement controls. These controls were carried out with each sample being processed. Separation controls were run through the SMI units after every four samples using filtered high-density NaCl solution. All SMI units were kept covered where possible with foil lids. The use and movement of plastics in the analysis laboratory were minimised, cotton lab coats were worn at all times and work was carried out in a laminar flow cabinet.

4.2.4 Microplastic identification

Each filter was analysed under a Ceti Steddy Trinocular Stereo Microscope at x45 magnification. The colour and type of suspected microplastics (i.e. fragment or fibre) was noted and each one was moved to a glass fibre filter and labelled for future analysis. No beads or films were identified from the sediment.

A 20% sub-sample of suspected microplastics were analysed to identify polymer type using Fourier Transform Infrared (FT-IR) and Attenuated Total Reflectance (ATR-IR) Spectroscopy at Plymouth Marine Laboratory. This subsample was selected randomly using a random number generator, with the exception of two fragments, that were specifically chosen as particles suspected of being contamination from shaving on the SMI ball valve caused by coarser sand particles. This analysis provided identification of particles as plastic and identified the polymer type. FT-IR spectra were compared against a spectral database from a number of polymer libraries to give match quality scores. Spectra matches with a score of ≥70% or considered to have reliable spectral matches during visual inspection were accepted (Nelms *et al.* 2018).

Filters from the settlement controls and separation controls were counted under the microscope and totalled. These particles were removed from the total suspected particles found in environmental sample filters processed on the same day.

4.2.5 Particle size analysis

Following microplastic extraction, the remaining sediment sample was used for particle size analysis. Samples were sieved into large fractions (greater than 1000 μm) and small fractions (less than 1000 μm), giving the percentage composition of large

and small fractions. Following Serrano *et al.* (2012) a subsample of sediments were used for detailed particle sizing. Organic compounds were removed via combustion at 550°C for 8 hours as the use of acid would have removed the smaller carbonate particles that make up a large proportion of these sediments.

Once organics had been removed, samples were prepared for further particle size analysis by being mixed to a paste with 5% Calgon and suspended in water. Particle sizes were measured in a Malvern Mastersizer (Malvern Instruments Ltd., Malvern, UK) with an RF-300 lens. Samples were added until the device read between 5-15% obstruction of the optical laser before commencing analysis. Triplicate readings were taken for each sample giving the percentage composition of the sediment according to the Phi (φ) scale, mean particle size, median particle size, sorting coefficient (σ_φ) and skewness. σ_φ is a value given to each sample to represent the level of sorting present in the sediments (Stoner, 1980). The sorting coefficient is a measure of the uniformity of grain sizes which is represented by the standard deviation of phi values and can be calculated using the equation;

$$\sigma_{\varphi} = \frac{\varphi 84 - \varphi 16}{4} + \frac{\varphi 95 - \varphi 5}{6.6}$$

Where $\varphi 84$, $\varphi 16$, $\varphi 95$, and $\varphi 5$ represent the 84^{th}, 16^{th}, 95^{th} and 5^{th} percentiles respectively. Level of sorting can be categorised using the σ_φ value according to Folk (1974, cited by Blair & McPherson, 1999), into the 7 categories shown in table 4.1.

Table 4.1: Level of sorting classified in to categories according to σ_φ values (Folk, 1974, cited by Blair & McPherson, 1999)

Sorting coefficient (σ_φ)	Category
0.00 - 0.35	Very well sorted
0.35 - 0.50	Well sorted
0.50 - 0.70	Moderately well sorted
0.70 - 1.00	Moderately sorted
1.00 - 2.00	Poorly sorted
2.00 - 4.00	Very poorly sorted
> 4.00	Extremely poorly sorted

As the Mastersizer was unable to accurately measure particles larger than 1000 µm and the unavailability of sieves larger than 1000 µm, percentage composition of larger particle fractions were unable to be manually measured which truncated the sediment composition values. Estimates of median particle size, sorting (σ_φ), and skewness were calculated for the whole sediment sample by assuming the data followed a log normal distribution. This distribution fitted the data up to $\varphi \geq 0$ that had been measured using the particle sizer and hence was used to model the distribution of the larger fractions. From visual inspection of the samples it was assessed that no sediment grains were found at sizes greater than -4 φ (16 mm). The R packages fitdistrplus (Delignette-Muller & Dutangand, 2015) and truncdist (Novomestky & Nadarajah, 2016) were used to calculate the parameters of the distribution using the assumptions of a truncated log normal distribution at a lower bound of 4 (i.e. truncated at the left hand side of the distribution). Distributions were modelled for each sediment sample jar and the coefficients of these modelled distributions were then used to estimate the actual sorting coefficient (σ_φ), mean, median and skewness of each complete sample (including large fractions).

4.2.6 Statistical analysis

Microplastics and sediment data were spatially visualised using Arc GIS Pro (ESRI 2020). Data were interpolated between sampling points using an inverse distance weighted (IDW) interpolation.

The effect of sediment characteristics, seagrass and distance from shore on the accumulation of microplastics was investigated using a generalised linear mixed model (GLMM) with a negative binomial family and log link. Analyses were carried out using the R package glmmADMB (Skaug *et al.* 2016; Fournier *et al.* 2012). Outputs of the model were checked for normally distributed residuals, homogeneity of variance, outliers and overdispersion.

To avoid problems of collinearity between independent variables in the sediment composition data set, a Principle Component Analysis (PCA) was carried out using R packages ade4 (Dray & Dufour, 2007) and factoextra (Kassambara & Mundt 2020) to create independent variables that described the sediment conditions. Variables included in the PCA were percentage composition of size fractions (i.e. clay, silt, coarse silt, very fine sand, fine sand, medium sand and coarse sand), sorting (σ_φ), median particle size and skewness. This created two principle components that were used in the model to investigate the effect of sediment composition on microplastics accumulation. R packages ggfortify (Tang *et al.* 2016) and ggplot2 (Wickham, 2016) were used to visualise the distribution of principles components. Two separate models were performed, one investigating sediment composition effects and the other testing the effect of the interaction between seagrass and distance from shore, the different tests were carried out because seagrass presence was likely to be affecting sediment composition and therefore the principle components were not independent of seagrass

presence. All statistical tests were carried out using R version 4.0.0 (R Core Team, 2020).

4.3 RESULTS

4.3.1 Seagrass environment

The seagrass meadow sampled had an average seagrass cover of 99% ± 0.74 (95% CI), an average shoot density of 509 shoots m^{-2} ± 22.87 (95% CI), and an average blade length of 60.13 cm ± 2.49 (95% CI). The meadow is therefore classified as a dense meadow (according to Pergent *et al.* 1995). There were some extremely sparse areas extending from the meadow with an average cover of 2.77% ± 1.31 95% CI, an average shoot density of 66 shoots m^{-2} ± 24.07 (95% CI) (classified as a semi-meadow), and an average blade length of 4.93 cm ± 1.86 95% CI. As might be expected, both seagrass shoot density (Spearman's rank correlation: $R = 0.960$; $p < 0.001$; figure 4.2a) and blade length ($R = 0.954$, $p < 0.000$; figure 4.2b) were positively correlated with percentage cover of seagrass.

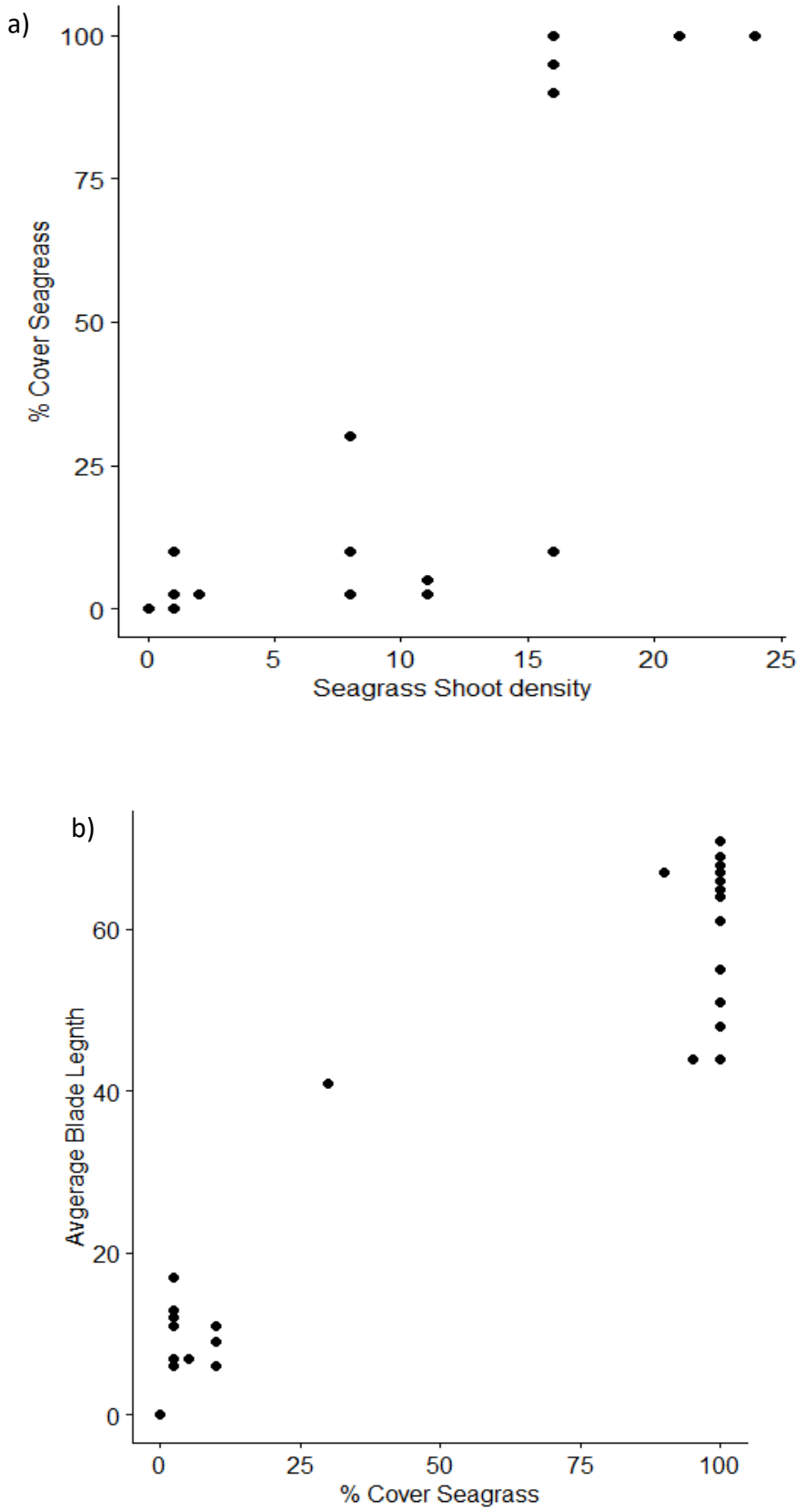


Figure 4.2 The relationship between a) seagrass shoot density and percentage cover of seagrass and b) percentage cover of seagrass and average blade length (cm).

Median particle size (in φ) of the small fractions showed significant positive correlation with seagrass percentage cover ($p < 0.0015$, Spearman's correlation coefficient = 0.274), indicating the overall decrease in particle size with increased seagrass cover (figure 4.3).

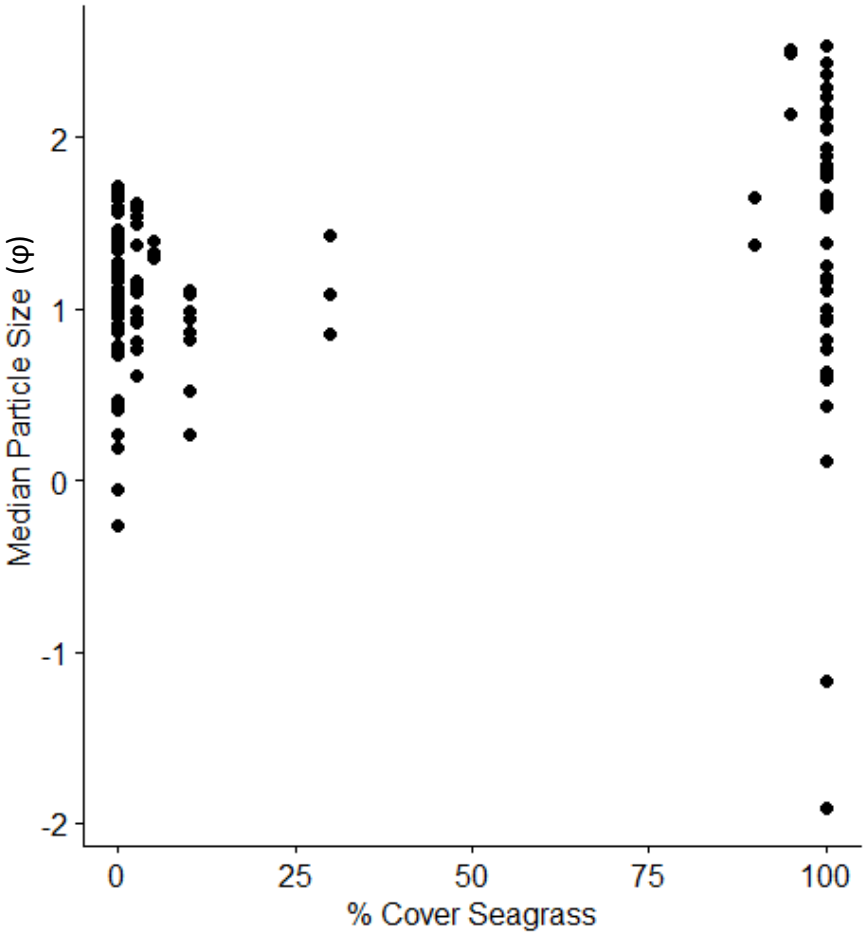


Figure 4.3 Scatter plot showing percentage (%) cover seagrass against median particle size (φ)

There was a strong positive relationship between seagrass cover and the sorting coefficient, σ_φ ($R = 0.6385$, $p < 0.000$), suggesting greater sorting of sediments outside the seagrass meadow (figure 4.4).

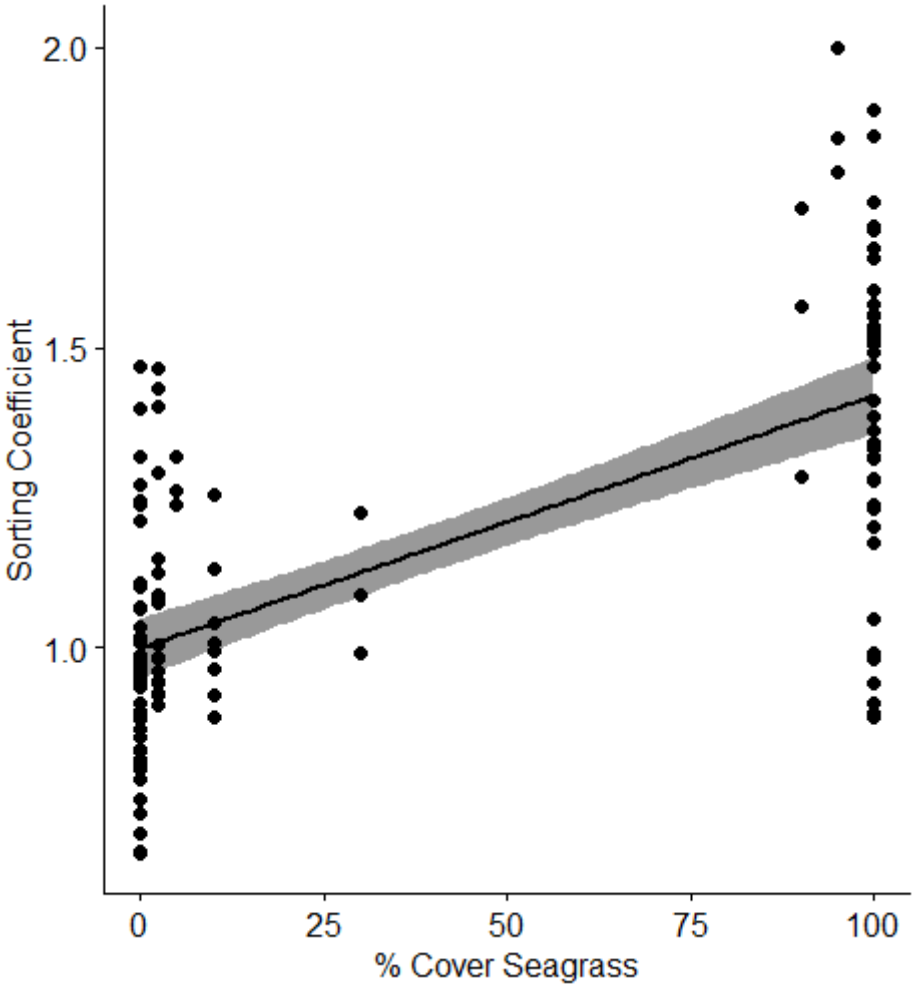


Figure 4.4 Scatter plot showing percentage (%) cover seagrass against sorting coefficient (σ_φ)

4.3.2 Confirmed presence of microplastics

Three sediment samples were collected at each of the eleven sampling points along four transects equating to a total of 132 sediment samples. Microplastics were found in 117 of the 132 sediment samples (87%) with a total of 624 suspected microplastics before contamination on controls were removed (see figure 4.5 for examples). A total

contamination of 44 microplastics (1.2 particles per filter ± 0.8, 95% CI) were found, most of which were fibres (39 fibres and 5 fragments). After contamination was accounted for and removed from all corresponding environmental samples there were 448 suspected microplastics. On average there were 4.42 ± 3.37 (95% CI) particles per 50 g dry weight sediment inside the seagrass meadow and 4.78 ± 4.7 (95% CI) per 50 g dry weight outside the meadow. Of the suspected microplastic particles extracted over 99% (n = 445) were identified as plastic fragments, with less than 1% present as fibres (n = 3). All fragments were likely to have been of a secondary nature (i.e. coloured and angular).

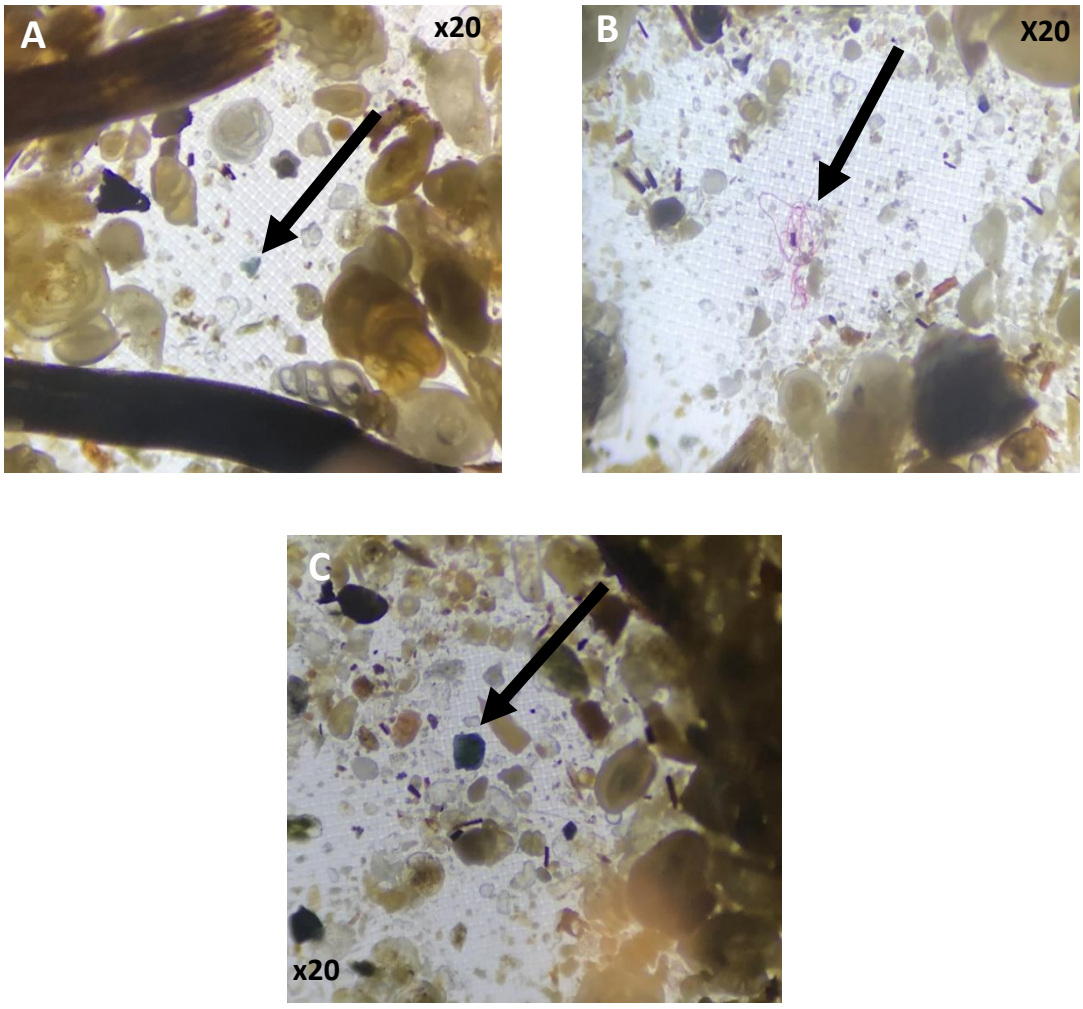


Figure 4.5 Stereo microscope images of suspected microplastic in sediment samples from Elena Bay, Lipsi. A: a blue fragment at x20 magnification, B: Red fibre at x20 magnification, C: A blue fragment at x20 magnification. All microplastics are indicated by a black arrow marking their location.

A 20% subsample (n = 89) of suspected microplastics were tested using FT-IR and AT-IR spectroscopy. Of that subsample 80% (n = 71) were confirmed as microplastics including eleven different polymers (table 4.2).

Table 4.2: summary of plastic types recovered as microplastic fragments (total 68) from sediment samples in Lipsi, Greece. All microplastics were confirmed as plastic using FT-IR and AT-IR. Microplastics with a spectral match of 70% or greater and those considered to have reliable spectral matches during visual inspection were accepted. Densities taken from Omnexus (2020).

Polymer type	No. fragments recovered	% fragments recovered	Plastic density (g cm^{-3})
Poly (methyl methacrylate) (Acrylic)	7	10.3%	1.10-1.20
Diallyl phthalate (DAP)	3	4.4%	N/A
Nitrile	1	1.5%	1.04-1.06
Nylon (polyamide)	2	2.9%	1.01-1.19
Plasticiser	2	2.9%	N/A
Polyacetal	5	7.4%	1.41-1.42
Polycarbonate	1	1.5%	1.10-1.52
Polyester (PEST)	5	7.4%	1.27-1.40
Polyethylene (PE)	31	45.6%	0.89-0.98
Polypropylene (PP)	7	10.3%	0.9-1.05
Polyvinyl chloride (PVC)	4	5.9%	1.15-1.7

The 18 non-plastic fragments were identified as glass (44%, n = 8), pyridinium dichromate (PDC) (44%, n = 8), brewers' yeast (6%, n = 1) and diammonium phosphate (6%, n = 1). Of the suspected microplastic fibres, all three analysed were confirmed as plastic with two of the fibres being rayon (1.53 g cm^{-3}) while the final one was identified as polyester.

4.3.3 Spatial Distribution of Microplastics

There was no clear accumulation of microplastics within or around the edges of the seagrass meadow (figure 4.6). There is, however, a distinct channel down the centre of the meadow from which very few (< 3) microplastics were recovered, along with another area of little accumulation on the edge of the survey area. There are also some small areas of clear microplastic accumulation, although, these do not seem to correspond with seagrass presence.

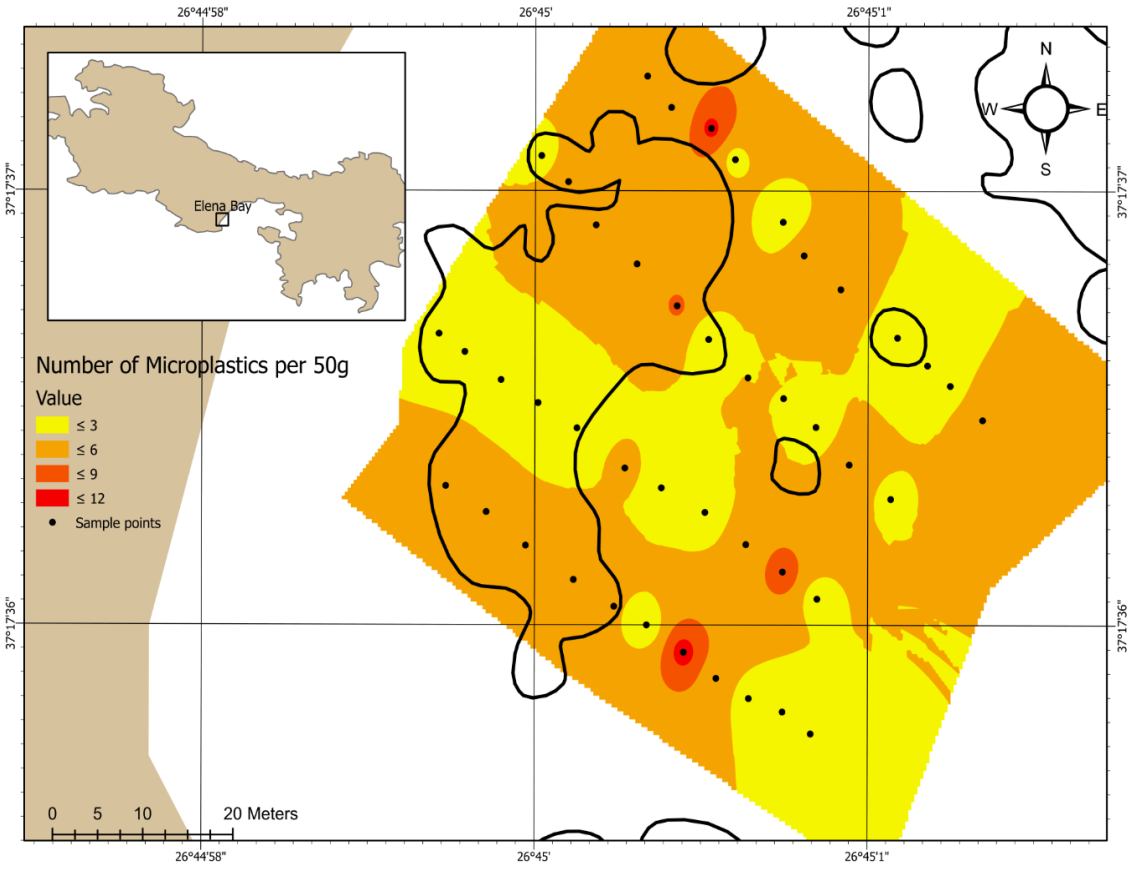


Figure 4.6 distribution of microplastics in the area surveyed. Seagrass meadows are outlined in black. Colours represent the number of microplastics per 50g. Specific sampling points are shown by dots crossing the seagrass meadow into bare sand

4.3.4 Sediment composition

Small fractions, less than 0 φ (<1000 μm), made up an average of 78.5% by weight of the sediment samples. These fractions contained an average 2.67% organic content. Of the small fractions the median particle size was 445.38 μm. The most abundant grain size fraction across all samples was medium sand (1 φ) with an average of 33.88% composition of the small fractions (table 4.3).

Table 4.3: overall percentage composition of fractions within the small fractions (less than 0ϕ grain size)

Fraction name	Particle size range (φ)	% Composition of small fractions
% Clay	≤8	0.296
% Silt	8-5	2.063
% Coarse Silt	5-4	1.516
% Very Fine Sand	4-3	3.854
% Fine Sand	3-2	17.019
% Medium Sand	2-1	33.883
% Coarse Sand	1-0	29.680

The mean sorting coefficient (σ_φ) across all samples was 1.15 ± 0.06 (95% CI), indicating that generally sediments were poorly sorted. Where seagrass cover was ≥ 90%, σ_ϕ was 1.4 ± 0.08 (95% CI) while in areas of seagrass cover of < 90%, which in this study comprised of values from 30%-0%, σ_ϕ was 1.01 ± 0.04 (95% CI) (figure 4.7). While the sorting coefficient was significantly higher in the seagrass meadow (p = 0.00184) (i.e. more seagrass less sorted sediments), both are still classified as poorly sorted.

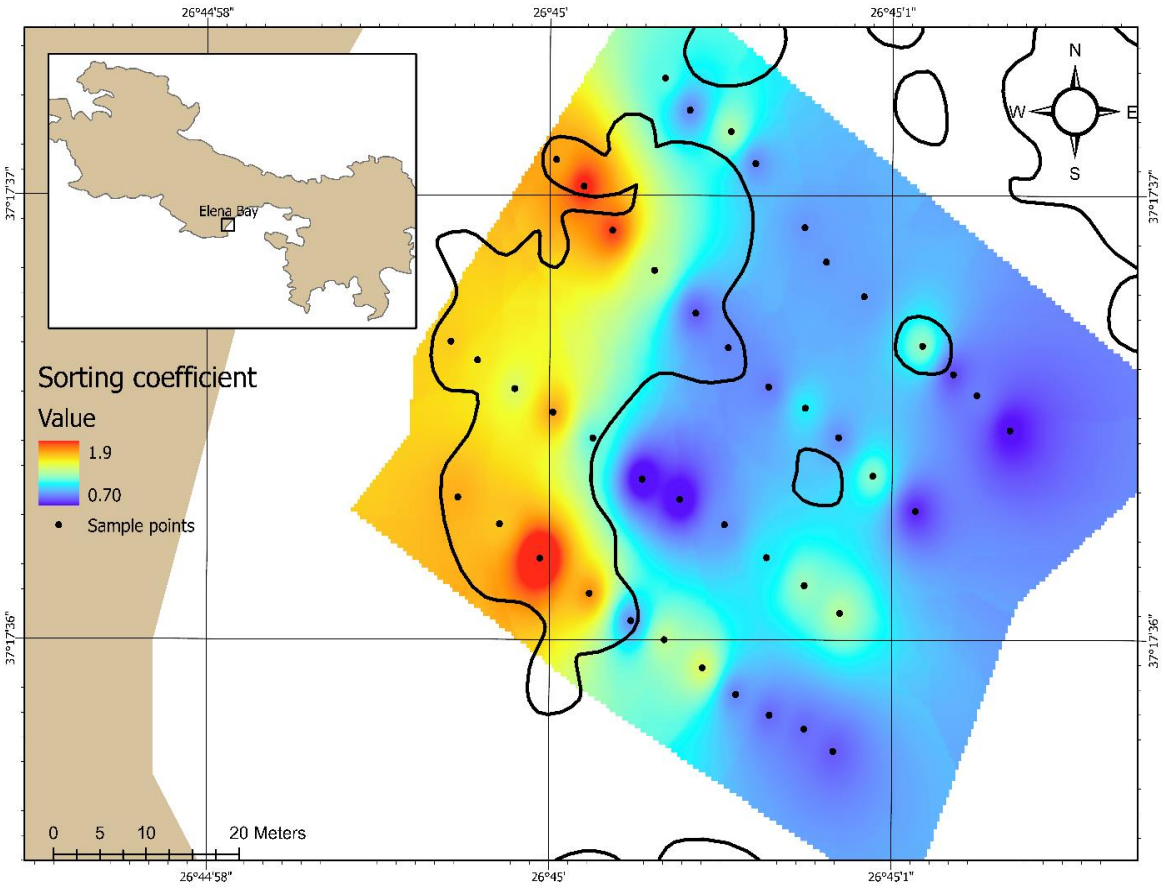


Figure 4.7 Spatial representation of the sorting coefficient (σ_φ) across the survey area. Seagrass meadows are outlined in black and blue represents areas of low σ_φ value while red represents areas of higher σ_φ. Specific sampling points are shown by dots crossing the seagrass meadow into bare sand. The brown shaded area represents the coastline.

There was also a clear accumulation of clay and silt sediment fractions along the left shore side of the seagrass meadow (figure 4.8).

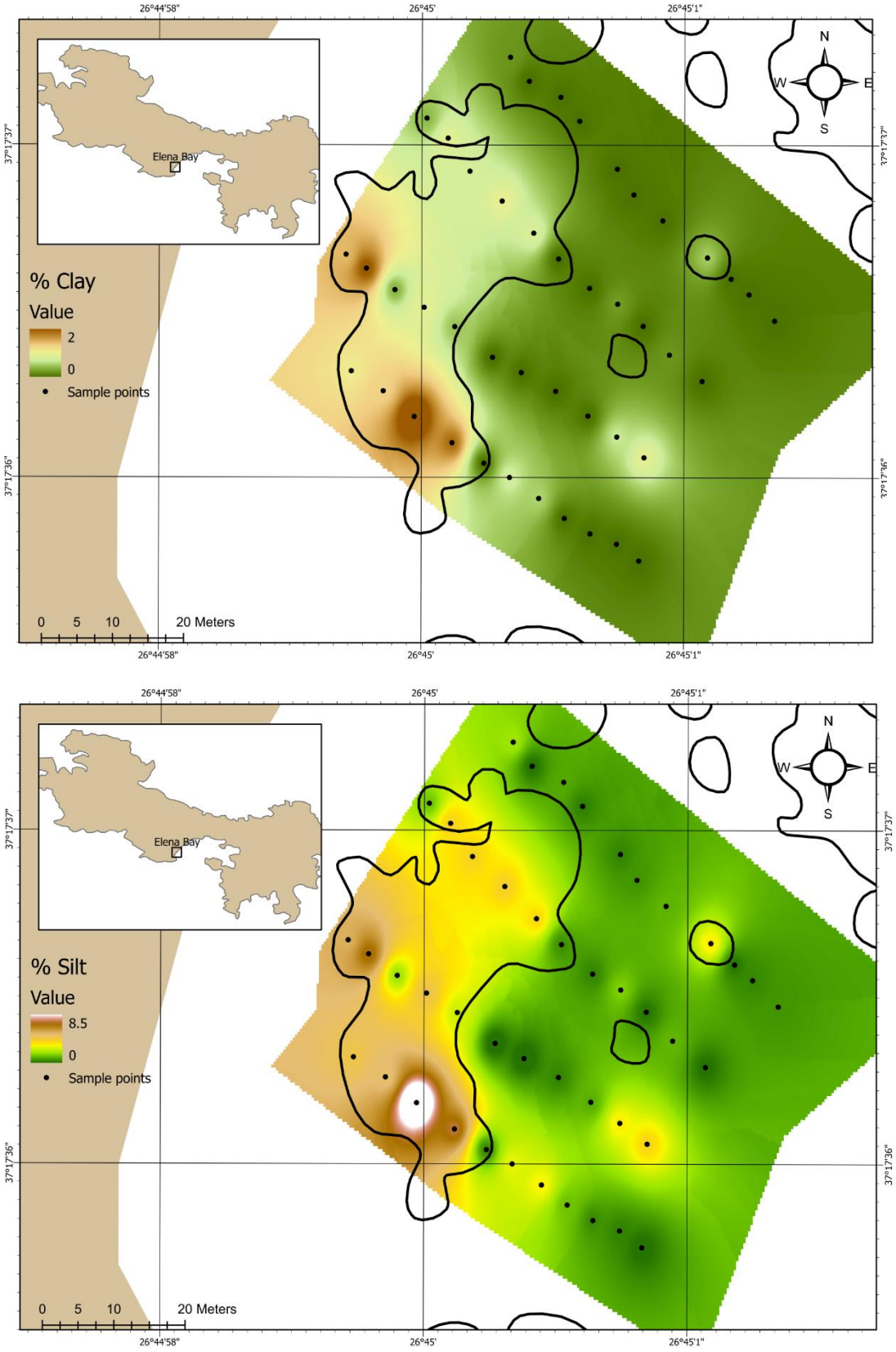


Figure 4.8 percentage contribution of a) clay and b) Silt sediments fractions across the survey area. Seagrass meadows are outlined in black and the brown shaded area represents the coastline.

4.3.5 Environmental determinants of microplastic distribution

Principle components analysis was carried out to provide independent descriptors of the sediment conditions. The first two principle components (PCs) of the sediment data combined explained 83.86% of the variance in the sediment data set, 52.80% (SediPC1) and 31.07% (SediPC2), of the variance (figure 4.9).

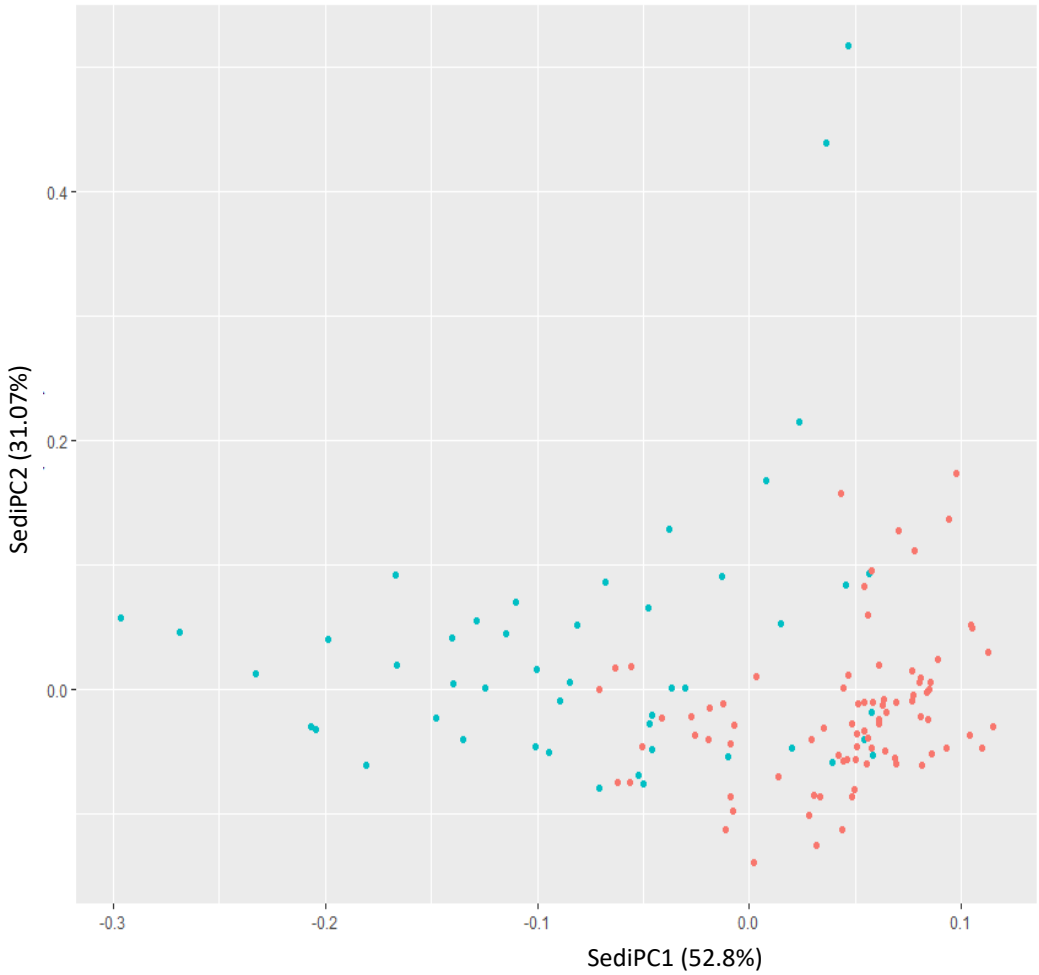


Figure 4.9 relationships between principle components 1 and 2 and seagrass cover. Data points from within the seagrass meadow area are shown in blue and non-meadow in red.

SediPC1 was predominantly comprised of the variables from the sediment fractions, excluding medium sand fraction, and σ_ϕ. All these variables contributed to over 10% of the variance in this component. Increasing values of SediPC1 are associated with an

increasing percentage of coarse sand and decreasing percentages of the smaller fractions (phi > 2) and a lower sorting coefficient (more sorted sediments). The second principle component, SedPC2, was mostly comprised of the variables medium sand skewness and median particle size, all of which contributed to over 10% of the variance (table 4.4 and figure 4.10). Increasing values of SediPC2 are associated with increasing percentage of large fractions and decreasing percentage of fine sand.

Table 4.4 summary of eigenvectors and percentage contributions to of each variable to the two principle components retrieved from the PCA.

Variable	Eigenvectors for SediPC1	% contribution to SediPC1	Eigenvectors for SediPC2	% contribution to SediPC2
% Clay	-0.3703	13.5	0.0420	0.5
% Silt	-0.3844	14.5	0.0349	0.5
% Coarse Silt	-0.3650	13.0	-0.0032	0.0
% Very Fine Sand	-0.3760	14.0	0.0389	0.5
% Fine Sand	-0.3303	10.5	-0.1961	4.0
% Medium Sand	0.0034	0.0	-0.4068	16.0
% Coarse Sand	0.3297	10.5	-0.0339	0.0
% large fractions (0ϕ- -3ϕ)	0.1652	2.5	0.4411	19.5
Sorting (σ_ϕ)	-0.3083	9.5	0.2381	0.0
Median Particle size	-0.2766	7.5	-0.3257	10.5
Skewness	-0.1294	2.0	0.4642	22.0
Kurtosis	-0.0843	0.0	0.4664	22.0

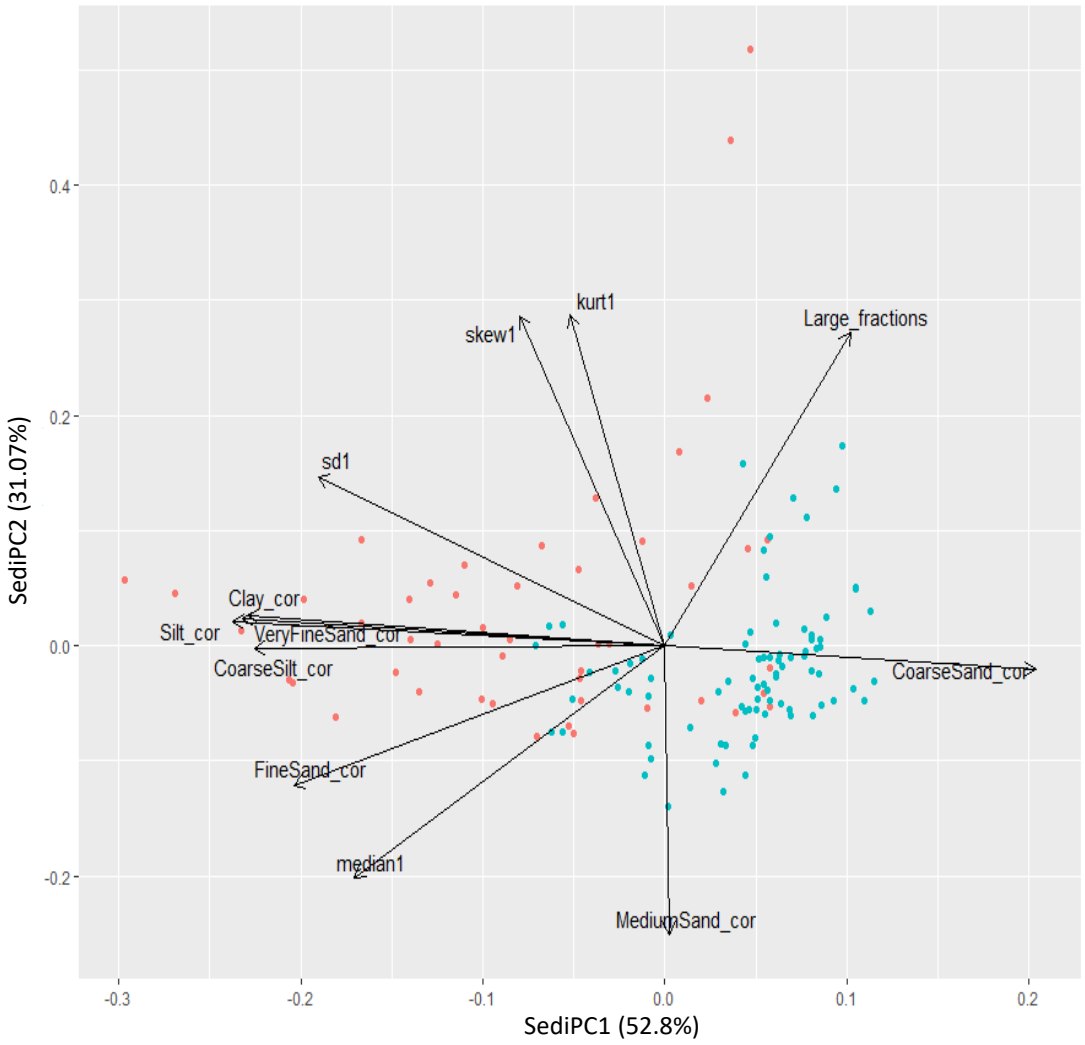


Figure 4.10 Eigenvalues and direction of relationship between all variables entered into the PCA and the resulting Principle components. Data points from within the seagrass meadow area are shown in blue and non-meadow in red.

The first negative binomial model tested for the interaction between sediment composition and microplastics accumulation. SediPC1 was significantly negatively associated with the number of microplastics ($F_{1,40}$ = 7.08; p = 0.01). This correlation was negative showing that as SediPC1 increased, i.e. as the percentage contribution of smaller particle fractions decreased, the number of microplastics decreased (figure 4.11a). SediPC2 was not significantly associated with any changes in microplastic load ($F_{1,40}$ = 0.00; p = 0.998; figure 4.11b). SediPC1 was contributed to mostly by the sediment fractions, excluding medium sand, and σ_ϕ. The largest of these contributions

came from the percentage of silt in a sample. figure 4.12 illustrates the relationships between these small sediment fractions, along with σ_ϕ and microplastics. All these variables showed a positive correlation with microplastics.

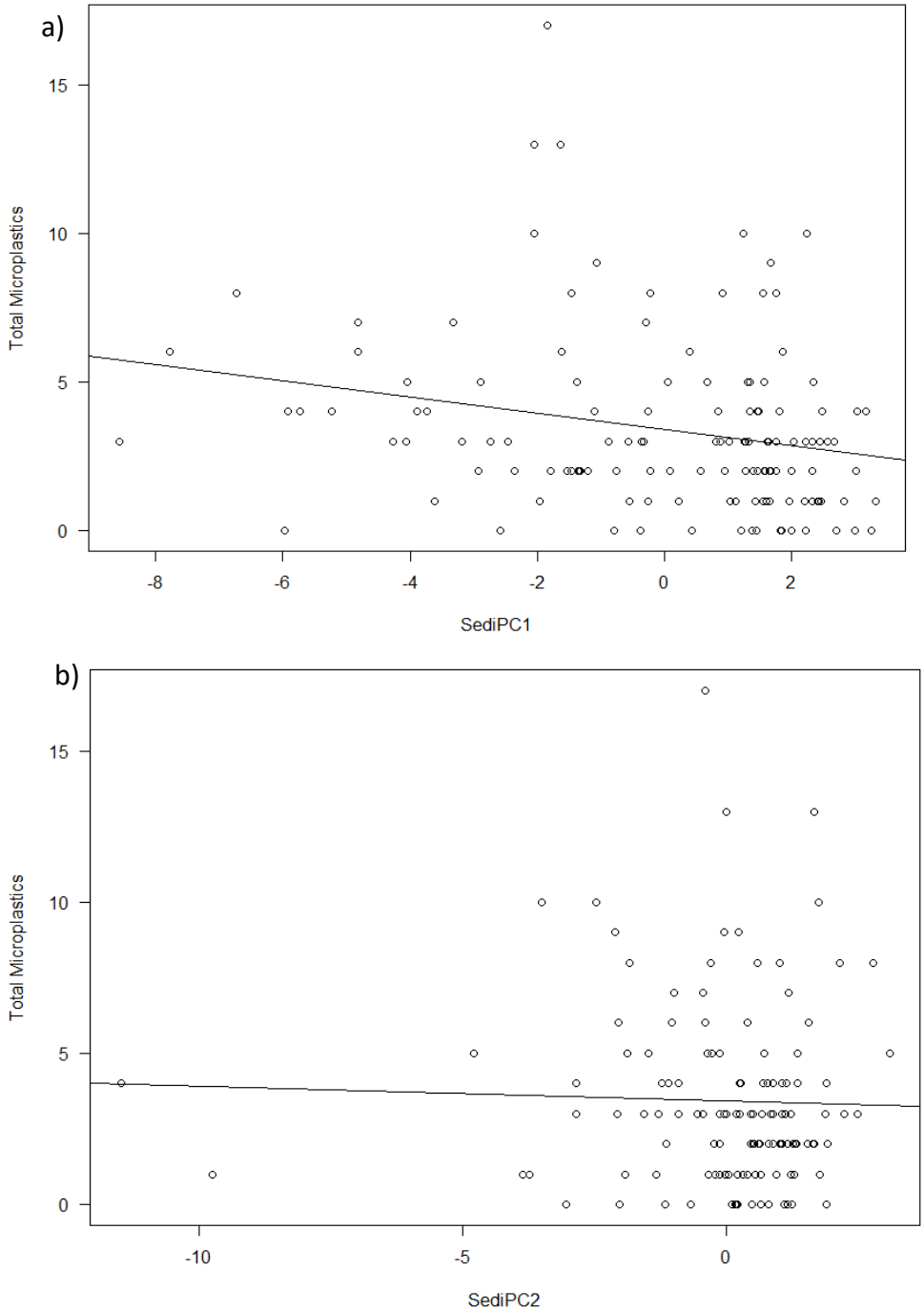


Figure 4.11 relationships of SediPC1 (a) and SediPC2 (b) with total microplastics. SediPC1 is associated with an increasing percentage of coarse sand and decreasing percentages of the smaller fractions (phi > 2) and a lower sorting coefficient (more sorted sediments). Increasing values of SediPC2 are associated with increasing percentage of large fractions and decreasing percentage of fine sand

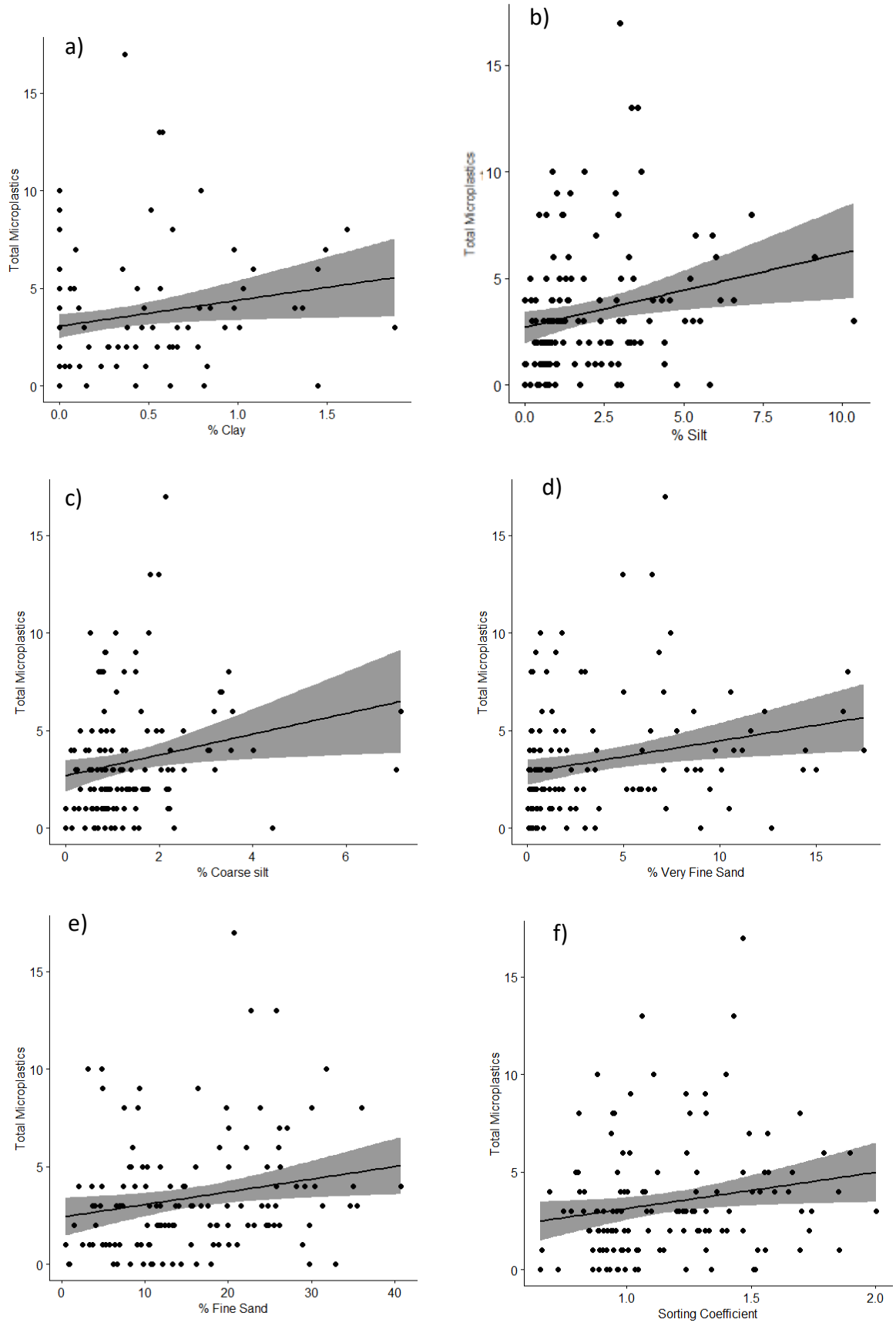


Figure 4.12 relationships between total microplastic loading per sample and a) percentage (%) clay, b) % silt, c) % coarse silt, d) % very fine sand, e) % fine sand and f) sorting coefficient σ_ϕ.

The second GLMM assessed the effect of seagrass cover and distance from shore on the accumulation of microplastics. Neither distance from shore ($F_{1,40}$ = 1.28; p = 0.264) nor seagrass percentage cover ($F_{1,40}$ = 0.193; p=0.663) (figure 4.13) had a significant impact on the number of microplastics recovered; nor was the interaction between the two variables significant ($F_{1,40}$ = 0.00; p=0.988).

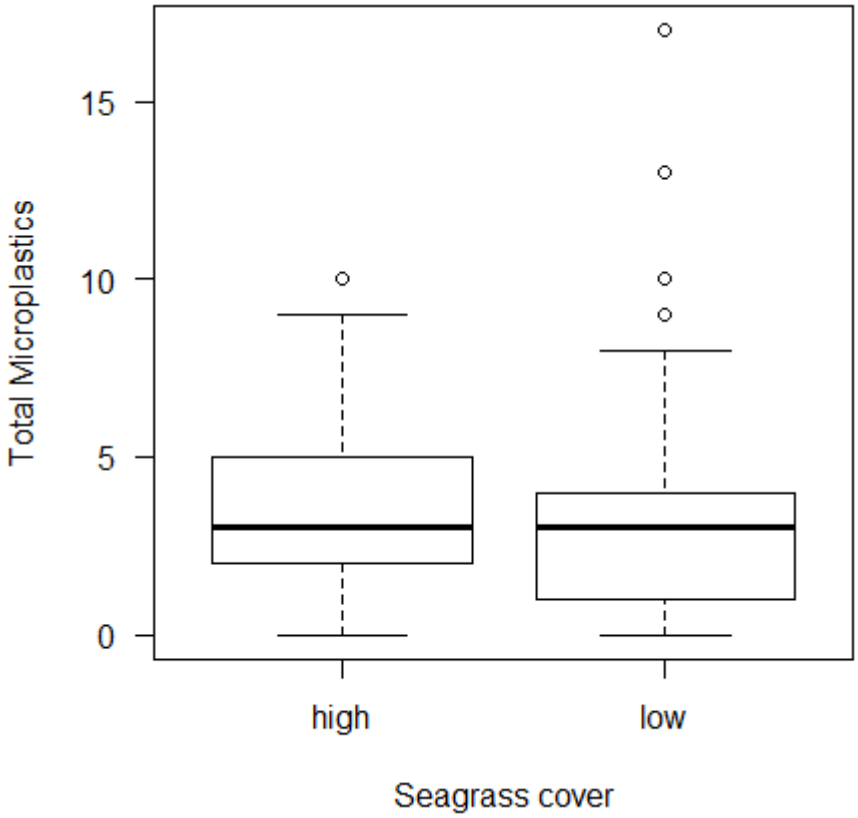


Figure 4.13 relationships between total microplastic loading and seagrass cover. High seagrass cover was defined as over 40%.

4.4 DISCUSSION

4.4.1 Microplastic presence and morphology

Initial analysis focused on extraction and characterisation of any microplastics present within seagrass sediment samples collected. The results presented here confirm the presence of microplastics in 88% of sediment samples taken from Elena Bay, a small isolated bay on the small Greek island of Lipsi, with 22.1 ± 16.9 (95% CI) particles kg^{-1} within the seagrass meadow and 22.4 ± 23.7 (95% CI) particles kg^{-1} outside the meadow. In contrast with previous studies investigating microplastics in seagrass sediments, microplastic concentrations found here are substantially lower. In Orkney, Scotland, there was an average of 300 ± 30 SE particles kg^{-1} microplastics found inside the seagrass meadow, while from bare sand it was 110 kg^{-1} ± 20 SE particles kg^{-1} (Jones *et al.* 2020). In two bays studied in Hainan Island, China, 196.7 ± 16.1 particles kg^{-1} and 780.2 ± 147.0 particles kg^{-1} were found in vegetated sites whilst unvegetated sites contained 93.3 ± 15 and 267.1 ± 60.5 particles kg^{-1} (Huang *et al.* 2020). Both studies recovered substantially more microplastics per kg dry weight than were found here in either the vegetated or unvegetated sampling points.

Surface water samples were not taken during this study so only the sediment loading of microplastics can be considered in detail. The Aegean Sea is generally a very low energy environment with an estimated average wave power of < 5 k Wm^{-1}, while areas in the western Mediterranean are much higher energy environments with 15 – 20 k Wm^{-1} (Mork *et al.* 2010). Buoyant microplastics are known to be transported in the direction of ocean currents and wave action (Zhang, 2017). This suggests the input of microplastics from external sources should be minimal for islands in the eastern Aegean, such as Lipsi, as wave energy is generally low, indicating that direct terrestrial

sources may be the main contributors to microplastics loading. These terrestrial sources are likely to be minimal for an island like Lipsi, especially in isolated locations like Elena Bay.

If microplastic loading is predominately coming from terrestrial sources, these results suggest that Lipsi may not be as heavily contaminated by plastics as other coastal regions and seagrass meadows. The island of Lipsi is not densely populated (estimated 700-800 inhabitants), despite increases in footfall during the summer months due to tourism. Elena Bay itself is not easily accessible, therefore rarely sees bathers or tourists, even during the peak season. This is in contrast to both bays studied in Hainan Island, China (Huang *et al.* 2020) which were both surrounded by residential areas. Although Orkney is not densely populated, currents surrounding the archipelago are much stronger than those around Lipsi potentially transporting oceanic microplastics to the islands and accounting for the increased concentrations reported by Jones *et al.* (2020). In addition to these location-based factors, Lipsi also has effective recycling and water treatment systems in place (Brebbia *et al.* 2006). The combination of these factors could mean that the area surveyed has a low level of microplastic loading from the land, this would support our low concentrations of sediment microplastics recovered.

Overall microplastic fragments were substantially more common (n = 445) than fibres (n = 3), making up over 99% of all particles identified, with fibres making up less than 1%. This is similar to the findings of Alomar, *et al.* (2016), which found fragments to be the most abundant microplastic morphology in four of their six sediment sampling locations in the Balearic Islands. The authors found fragments were more than sixty percent more abundant than fibres at sites within a marine protected area (MPA) while

in unprotected and more urbanised sampling locations fibres were more abundant (> 60%) than fragments. Microplastics found in populated areas outside of the MPA were attributed to sewage outputs while those found inside the MPA were suggested to have been fragmented from larger pieces of plastic transported into the area by strong currents or winds. Therefore, it could be suggested from results of this study, that Elena Bay may actually reflect more closely the conditions of an MPA, demonstrating fragments to be the more common microplastic type (Alomar *et al.* 2016).

In addition, wastewater discharge has been shown to be primarily composed of microplastic fibres (Sutton *et al.* 2016), due to the amount of shedding of these particles from textiles in domestic washing machines, that has been shown to produce > 1900 fibres per wash (Browne *et al.* 2011). It is therefore possible that a combination of the low permanent population density of Lipsi and an effective wastewater treatment system account for the lower proportion of fibres in coastal waters around Lipsi when compared with other areas previously studied.

As with this study, fragments were reported to be more common than fibres at a site in the River Thames (Horton *et al.* 2017). It was reported that these fragments were both angular and coloured, much like the ones reported in this study, Horton *et al*. (2017) suggested that they were therefore most likely to be locally derived secondary microplastics rather than artificial fibres introduced to the system by sewage effluent. This further supports the reasoning that there is very little wastewater input to the survey area, and therefore potentially explains the lack of fibres found in Elena Bay.

This is contrast to the findings of many previous studies that isolated more fibres than fragments in marine sediment samples (e.g. Mistri *et al.* 2018; Martin *et al.* 2017; Claessens *et al.* 2011). Martin and colleagues (2017) state that fibres were exclusively

recovered from the samples taken from the sediment-water interface in the Irish continental shelf, this could suggest the tendency for fragments to settle out of the water column, therefore appearing be more prevalent in sediments, whilst fibres are likely to be more prone to resuspension. Goss *et al.* (2018) has demonstrated microplastics adhering to the blades of seagrass *Thalassia testudinum*, it is likely that this process would be similar with blades of *P. oceanica* in the meadow studied here, which could result in fibres, that have been resuspended, becoming incorporated into the epiphytic community of seagrasses.

When the ratio of fragments to fibres isolated from seagrass meadows is considered it does not appear to be as consistent as it is in bare sediment. Huang *et al.* (2020), found a higher proportion of fragments (43.1% ± 4.8% of the overall composition of microplastics) than fibres at one of the two vegetated sites studied, while Jones *et al.*(2020), found significant variation in microplastic morphology between bare sand and seagrass sediment. Seagrass meadows have been shown to trap larger plastic debris, accumulating up to 14 bag fragments ha^{-1} (Balestri, *et al.* 2017). These larger macroplastics breakdown into fragments (Thompson *et al.* 2004) which therefore could be responsible for depositing microplastic fragments directly into the seagrass system. This suggests fragments isolated from seagrass sediments in this study (99% of all microplastics) may be as a result of macroplastic break-down or lateral transport of secondary microplastics from the wider marine environment.

4.4.2 Polymer types

Proportions of polymers found in Elena bay samples are comparable in relative concentrations to other published studies. The most common polymer type identified was polyethylene, PE, (45.6% of all fragments analysed). PE is extremely common, used

primarily for packaging (i.e. bottle, bags containers etc.) and is therefore a microplastic that has been shown to have high abundance both worldwide and in the Mediterranean Sea (Gago *et al.* 2018; White *et al.* 2018). PE has also been found to be the most abundant microplastic identified in collections from the North and Baltic seas between 2013 and 2014, where it represented between 40-55% of particles (Kirstein *et al.* 2016). Similarly, Vianello *et al.* (2013), found PE to be the most common polymer type in sediments from a Venice lagoon. It is therefore unsurprising it constituted nearly half of the polymer fragments identified in this study. PE also has a low density, 0.89-0.98 g cm-3 (Omnexus, 2020), which means it was substantially less dense than the NaCl solution (1.2 g cm^{-3}) used to separate the microplastics from the sediments, therefore, the recovery rate of this particular fragment was likely to be higher than other denser polymers. Marine debris of densities less than that of seawater (1.02 g cm^{-3}) tend to float or remain suspended in the water column, therefore low density microplastics are less likely to sink and settle into sediments (Graca, *et al.* 2017). Lobelle & Cunliffe, (2011), demonstrated that biofilm formation on PE can result in the sinking of debris within three weeks and therefore may help to explain the presence of such high concentrations of this low density microplastic in sediments.

The next most common polymers found in this study were Polypropylene (PP) and Poly (methyl methacrylate) (acrylic), both constituting 10.3% of the fragments identified. PP is another low density microplastic, 0.85-0.92 g cm^{-3} (Omnexus, 2020), and commonly found in marine samples. Vianello *et al.* (2013) and Kirstein *et al.* (2016) found it as the second most common (34.1% and 14% - 20% respectively) microplastic polymer identified. Acrylic has a slightly higher density (1.16-1.20 g cm^{-3}, Omnexus, 2020) than polypropylene and was found to account for 10% of particles analysed in studies by Zhang *et al.* (2019) and Lusher *et al.* (2015), in line with the findings

presented here. Both PE and PP have been shown to adsorb polychlorinated biphenyls (PCBs) and Polycyclic aromatic hydrocarbons (PAHs) on their surface, both of which have toxicological effects on marine life (Hirai *et al.* 2011).

Polyvinyl chloride (PVC) was also found in this study, although at a lower incidence than PE and PP (n = 4, 5.9% of fragments identified). PVC fragments are of particular importance in determination of microplastic loadings on aquatic habitats as they have a high capacity to accumulate heavy metals (Cu and Zn) from antifouling paints (Brennecke *et al*, (2016). Concentrations of these metals on microplastics have been recorded up to 800 times higher than surrounding water concentrations and have the potential to accumulate these heavy metals (Brennecke *et al.* 2016). As discussed in section 4.1.2, these heavy metals can have a toxic effect on marine life. Further research is required to understand whether heavy metal accumulation is occurring on Mediterranean microplastics and to what extent these may be impacting on marine species.

4.4.3 Seagrass meadow characteristics

Traditional seagrass survey techniques (described in section 4.2.2) were used to assess the state and health of the seagrass meadow itself in order to inform the interpterion of microplastic data and understand microplastic interactions within the seagrass meadow sampled. In the meadow studied, there was an average seagrass percentage cover of 99% ± 0.74 95% CI, an average shoot density of 509 shoots m^{-2} ± 22.87 95% CI, and an average blade length of 60.13 cm ± 2.49 95% CI. Using the scale described in Pergent, *et al*, (1995), this meadow can therefore be classed as Type II, or a dense meadow. As expected, there was a significant correlation between shoot density and increasing seagrass percentage cover, that is typical of a healthy seagrass meadow.

There was also a significant correlation between seagrass percentage cover and the percentage of organic matter found in the small sediment fractions (less than 1000 μm). Again, this relationship is expected in a normally functioning seagrass meadow, as De Falco *et al.* (2000) demonstrated, the trapping of smaller particles by *P. oceanica* rhizomes leads to higher rates of organic matter retained within the meadows. The data from this study also demonstrated that median particle size decreased with seagrass cover, which is again, to be expected due to the trapping effect of the rhizomes (Zhang, *et al.* 2020). All of these relationships indicate that the seagrass meadow was functioning as a healthy meadow, therefore it can be assumed, that it is representative of other *P. oceanica* meadows in similar geographic locations.

Another significant relationship demonstrated here between seagrass and sediment was between the percentage cover of seagrass and the sorting coefficient ($R = 0.6385$, $p < 0.000$). As discussed (section 4.1.1), better sorted sediments have been reworked by water currents and/or wave action, creating a sediment with more consistent particle sizes and a lower σ_ϕ value (De Falco *et al.* 2000). Less sorted sediments are associated with lower energy environments and therefore are not reworked as much, retaining a more varied mixture of particle sizes, resultant in a higher σ_ϕ value. The results presented here reveal an increase in the σ_ϕ with increasing seagrass cover. This shows that sediments from outside the meadow were generally better sorted than those inside the meadow. Both sampling areas were classified as poorly sorted overall, however, with only marginal differences in σ_ϕ, 1.4 ± 0.08 (95% CI) in the seagrass meadow and 1.01 ± 0.04 (95% CI) outside the boundary of the meadow, indicating both environments experience similar energy levels.

4.4.4 Microplastic distribution and seagrass interactions

Seagrass cover and distance from shore had little effect on microplastic accumulation in the seagrass meadow studied here. This contracts to other research into the impacts of seagrass on the accumulation of microplastics. Jones *et al.* (2020), and Huang *et al.* (2020), both demonstrated significantly increased concentrations of microplastics in seagrass sediments compared to bare sand substrate. The overall microplastics input to study areas in previously published literature could have been significantly higher than that of the one studied here, as discussed in the previous sections (4.4.2, 4.4.3). Additionally, neither study investigated *P. oceanica* as studied here in Greece. Jones *et al.* (2020), studied *Zostera marina,* while Huang *et al.* (2020) *Enhalus acodoides.* Both these seagrass species form dense canopies, similar to *P. oceanica*, however they lack the dense, woody rhizome mattes *P. oceanica* form. A direct comparison between the effects of different seagrass species on sedimentation has never been carried out so the differences in these dynamics is hard to quantify.

SediPC1 (predominantly comprised of the variables from the sediment fractions, excluding medium sand fraction, and σ_φ) was the only significant factor in determining microplastic distribution found by the GLMMs. The variables contributing to this factor were predominantly comprised of silt, very fine sand, clay, coarse silt and σ_φ (i.e. sorting coefficient). This relationship between SediPC1 and microplastics suggests a tendency for microplastics to accumulate in lower energy environments and is in agreement with the study by Vianello *et al.* (2013) in Venice. Initially in this study the lower energy environments were assumed to be the seagrass meadows themselves, however this was later contradicted by the sediment distribution observed which revealed very limited differences between vegetated and unvegetated.

This potential microplastic loading in low energy environments has potential implications for many coastal ecosystems as low energy environments are often refuges for vulnerable species and ecologically important habitats including coral reefs in tropical regions (Johansen *et al.* 2007), and mangroves (Sheaves, 2005). These coastal habitats, including seagrass meadows, provide habitats for many species of commercial and ecological importance such as the endangered species *Pinna nobilis,* that inhabits Mediterranean seagrass meadows (Hendriks *et al.* 2011).

The low energy environment of the overall sample area could be a result of the local shape of the coastline or meadows further offshore slowing water movement. This would make the presence of the small meadow inconsequential to the overall deposition of microplastics in the local area due to minimal impact on flow rates. Wider scale surveys are therefore needed to understand the cumulative effect of seagrass trapping microplastics as these boundary seagrass ecosystems could potentially be at a higher risk from microplastic exposure.

A study conducted by Alomar *et al.* (2016) has shown grain size to have no significant influence on microplastic distribution (Alomar *et al.* 2016) in contrast to results of this work. For the model created in this study factors, including Phi and multiple grain fractions, were combined to create one overall factor against which microplastic distribution was modelled. This was done as the chosen variables were closely linked and influencing each other, therefore couldn't be reliably separated as independent variables. In this study related variables were grouped using the PCA. It would also suggest when looking at Alomar *et al.* (2016), that broader grain size fractions are more likely to demonstrate a significant relationship with microplastics meaning the overall

sediment composition is a factor in microplastics distribution rather than individual fractions, therefore acting at a broader scale than previously thought.

Although differences in the degree of sorting (σ_ϕ) from within the seagrass meadow and beyond its boundary were minimal they were still significant, with both classified as poorly sorted. During the analysis of sediment grain size distribution data it was noted that there is an ephemeral river discharging into the seagrass area surveyed (figure 4.13). When this ephemeral river is flowing, the immediate coastal system including the study area, is likely to gain both energy and an influx of sediment, particularly of the smaller size fractions such as clay and silt in the north western shore-side edge of the seagrass meadow (refer to figures 4.7 and 4.8 in section 4.3.4). With little to no anthropogenic development in the ephemeral river catchment it is unlikely to be a source of microplastics. This is evidenced by microplastic distribution results, that did not show any increase in microplastics where the river discharges into the survey area (figure 4.6).

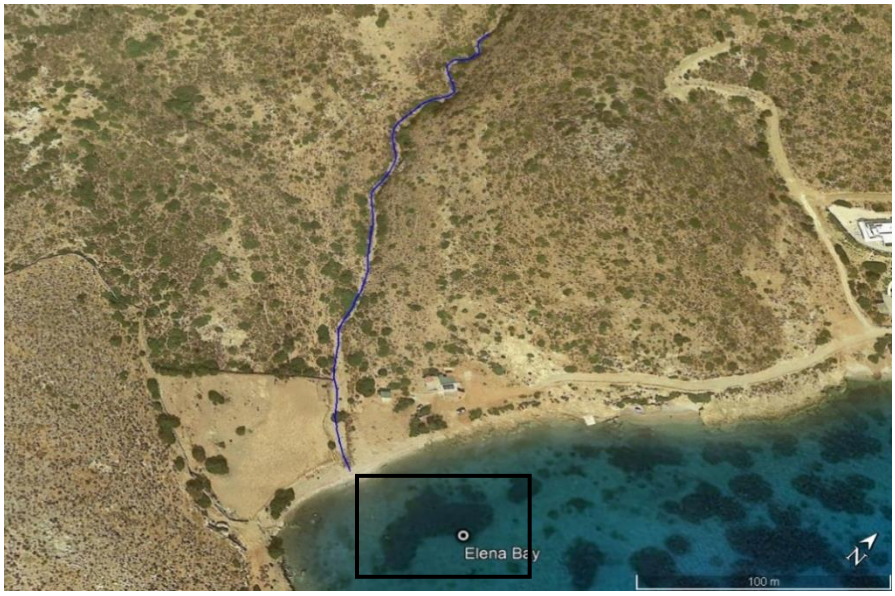


Figure 4.13: Satellite image of Elena bay with ephemeral river highlighted in Blue and the seagrass meadow studied outlined by a black box. (Image: Google Earth pro)

Seagrass in this location is key for trapping sediment, and should these sediments have been higher in microplastics loading, a potentially important barrier to prevent them reaching the wider marine ecosystem. Previous studies by Jones *et al.* (2020), and Huang *et al.* (2020) demonstrate the ability of some seagrass meadows to accumulate microplastics. These meadows are likely acting as a barrier, accumulating the microplastics before they can be dispersed into the wider marine environment. Loss of seagrass ecosystems from these areas is more likely, as they experience increased anthropogenic stress, however may have previously unconsidered impacts as a microplastic sink.

As discussed, there was no significant difference between microplastic accumulation inside or outside of the seagrass meadow. During sample collection there was observational evidence of dead seagrass matte below the surface layer of sediment, this extending out from the meadow into what was otherwise considered to be bare sand substrate as it showed no sign of living vegetation (pers. obs.). In addition, sampling points beyond the meadow boundary did have periodic, sparse seagrass cover (2.77% ± 1.31 95% CI), shoot density (66 shoots m^{-2} ± 24.07 95% CI) (classified as a semi-meadow), and blade length (4.93 cm ± 1.86 95% CI). *P. oceanica* meadows, just as the one studied here, are known to be regressing across the region (Telesca *et al.* 2015). It is therefore possible that this particular meadow has recently regressed and died off leaving behind the densely packed mattes, known to be the storage location of much of the carbon within seagrass systems (Fourqurean *et al.* 2012). Microplastics isolated from bare-substrate samples in this study could therefore be the result of microplastic accumulation over previous decades when seagrass cover was present at these sampling points, before die off occurred, (since at least the 1970's, Andrady 2011). If this were the case, microplastics would not yet have been resuspended into the water

column and could have potentially skewed our results by simulating a seagrass meadow whilst little living biomass was actually present at the time of sampling. However, when aerial imagery (Google Earth Pro) was investigated there was little evidence for this, therefore it is possible that the matte may be remnants of a die off event that occurred pre-2008, as this is the oldest satellite imagery available from Google Earth.

In addition, the meadow studied was not completely isolated from other live seagrass and is surrounded by further meadows off shore, at greater depths and larger than the one sampled here. These meadows were not surveyed due to safety concerns, and therefore restricted the survey area to that which was safely accessible by snorkel. It is therefore possible that microplastics could also be deposited from the water column within these deeper meadows further off shore which would be encountered first by incoming currents. These meadows could therefore be acting as filters further off shore accumulating deposited microplastic particles before they come into contact with the meadow studied here.

4.4.5 Methodological considerations

As touched on previously (section 4.4.2), the density of the salt solution used for the SMI extraction of microplastics could have influenced the density of microplastics recovered from sediment samples. The density of NaCl used was 1.2gcm^{-3}, less than the density would have been if zinc chloride (ZnCl, 1.5 g cm^{-3}) had been used (as recommended by Coppock *et al.* (2017)). NaCl was used in this research as prolonged effervescence was experienced when ZnCl was mixed with sediment samples collected. The lower density of NaCl could mean only low density microplastics (< 1.2gcm^{-3}) were recovered from sediment samples and those that were of a higher density, e.g. high-

density polyethylene, which reaches densities of up to 1.28g cm^{-3} (Li *et al.* 2018), or those microplastics that had increased density though biofouling (Long *et al.* 2017), remained in the sediments. Despite this potential limitation, some high-density microplastic polymers were recovered from the samples such as Polyester (PEST, 1.39-1.44 g cm^{-3}) and Polyacetal (1.41-1.43 g cm^{-3}). Both polymer types constituted 7.5% of the particles identified in this study despite their higher densities. It is possible these microplastics were adhered to organics that were more buoyant than the salt solution and therefore became suspended at the surface during the density separation step. These organic particles were then washed with ethanol during the filtration stage that potentially displaced the microplastic fragments onto the filter. It is therefore likely that the proportion of these polymer types recovered in were only a fraction of the true proportion present in the sediment samples. The SMI unit was an ideal tool for separation of microplastics from marine sediments and can therefore easily be applied to sediments from other seagrass habitats in different regions. Some development is needed into the ideal suspension media for seagrass sediments, as saturated NaCl solution is likely to miss the higher density microplastics, however ZnCl was not appropriate for seagrass sediments.

4.5 Conclusions

This study is the first to assess the relationship between coastal vegetation, *P. oceanica*, and microplastics in the Mediterranean. Microplastic concentrations quantified from sediments in Lipsi (22.3 ± 20.3, 95% CI, particles kg^{-1}) were found to be lower than in other coastal regions studied previously, this is likely a reflection on the low permanent population density and urbanisation on the island. Fragments were significantly more

common (99% of samples) than fibres, suggesting a locally derived secondary source of microplastics pollution and a low waste-water input into the survey area. It is likely that in low energy environments there is very little dispersal of microplastics away from the point source, this has potentially negative implications for species living in areas of high microplastic input but low energy, as their exposure is much greater.

The seagrass *P. oceanica* was not found to have a significant impact on the dispersal of microplastics in this instance. Sediment variables, such as the sorting coefficient and the smaller grain size fractions (i.e. silt, very fine sand and clay) were found to have a combined effect in influencing the accumulation of microplastics. There are likely to be further environmental variables beyond the scope of this study, such as water flow, surrounding seagrass presence and off-shore currents which also have an effect of the accumulation rates of microplastics in coastal vegetation.

Chapter 5

Final Discussion

5.1 Final Discussion

The overall aim of this thesis was to investigate the potential for emerging methods to increase our knowledge and understanding of seagrass systems, using a case study of *Posidonia oceanica* in the Aegean Sea. Seagrass habitats are both ecologically and economically important but under threat from numerous anthropogenic stressors. It is therefore vital that we develop and apply novel tools to allow for a much greater understanding of seagrass distribution, ecology and ecosystem function (Chapter 1). This thesis has presented three emerging survey methods in the context of novel seagrass research. All methods have the potential to further our knowledge of these systems and can be applied in various situations and conditions depending on the aims of research. Down-scan sonar mapping can inform interested stakeholders on changes to the seagrass distribution and health e.g. regression or recovery, while investigating the microplastic content of seagrass sediments may give an indication of local or wider pollution levels. Environmental DNA surveys can then be used to monitor critical species that are at risk from changes to meadow distribution and health. There is also potential for these methods to be used in conjunction with each other to overcome the logistical challenges of surveying and monitoring these habitats, for example, sonar mapping can be used to locate deep water meadows while eDNA could then be used to survey the meadows for rare or elusive species presence without the safety risks or logistical challenges. This would provide a better understanding of community

structure changes within deeper seagrass meadows compared to shallow or intermediate depth meadows, currently challenging and expensive to do.

Table 5.1 Summary of techniques used in this thesis, including the advantages and elements of improvements for each.

Technique	Advantages	Elements for improvement
Kayak-borne down-scan sonar mapping	• Rapid acquisition of data across large areas compared to visual survey techniques. • Easy to use. • Inexpensive set up & low maintenance costs. • Potential to provide data in deep or turbid water.	• Alternative vehicles which allow faster survey speeds or greater survey range from shore. • Improved data clean up and analysis algorithms allowing for increased accuracy in areas of patchy seagrass and/or complex bathymetry.
Environmental DNA	• Non-invasive. • Species specific. • Possibility of surveying at different spatial scales. • Potential to detect cryptic or rare species.	• Characterisation of impact of biomass on DNA availability. • Development of robust assays to allow precise spatial determination of individuals / populations. • Development of in-field testing kits to reduce costs and requirement for access to laboratory..
SMI extraction	• Easy to use. • Portable. • Small sample size required. • Non-destructive to microplastics. • Low cost.	• Further development to enable use of ZnCl with seagrass sediments • Further development needed for efficient use with highly organic soil.

Seagrass research can also benefit from further investigations into how emerging techniques from other disciplines not studied here can be applied to these vital ecosystems. Many species of seagrass are facing an uncertain future, with declines not only being seen regionally, but also on a global scale. There are now emerging techniques into the viability of transplanting and replanting methods for various seagrass species (e.g. Bastyan *et al.* 2008; Ward *et al.* 2020), however these methods are still early in development and replanted meadows are still susceptible to damage,

therefore good management and practices and enforced protection are key to the preservation of seagrasses (Ward *et al.* 2020). By clearly understanding the role seagrass systems play in supporting critical species, ecosystem services and their interaction with marine pollutants their protection will be easier to justify to policy makers. In addition to this better mapping of seagrass meadows will make regulations easier to implement. The technologies and methods presented in this thesis can provide the scientific evidence required to justify the protection of seagrasses to policy makers and governing bodies.

5.1.1 Rapid low-cost seagrass mapping techniques

Although seagrass meadows are facing decline from anthropogenic disturbance, accurate distribution assessments and conservation efforts are hampered by a lack of presence/absence data for most species. Therefore, work undertaken for this thesis focused on the development and validation of a kayak-borne survey method for low cost and rapid mapping of *P. oceanica,* using down-scan sonar and demonstrating the rapid acquisition of data across 9.32 km^2 of coastal water (Chapter 2). This novel application of down-scan sonar is of particular relevance to scenarios where monitoring needs to be conducted at an intermediate scale with limited financial input, common for conservation-based projects. Although mapping accuracy reduced as seagrass coverage become patchier, accuracy was consistently more significant than chance. Sonar data has previously been shown to lose accuracy when mapping across interfaces between habitat types (Sagawa *et al.* 2008) and therefore it is unsurprising that accuracy declined in the patchier seagrass environments.

Between the two test sites from this thesis, down-scan sonar with BioBase analysis had an observed accuracy of 85% in Samos and 68% in Lipsi. This is comparative to the

findings of Luczkovich *et al.* (2013) who found the BioBase tool in combination with a down-scan EchoSounder sonar to have an accuracy of 77% for mapping submerged aquatic vegetation. In contrast to the satellite mapping by Topouzelis *et al*. (2018), results of this study highlighted stark differences between seagrass extent estimated by satellite and sonar mapping methods, casting doubt on the accuracy of the national level assessment based on analysis of satellite-derived data (Topouzelis *et al*. 2018).

Sonar derived maps produced κ values that were significantly better than chance, while the satellite derived maps did not. The decreased accuracy of satellite mapping was due to the allocation of a large seagrass meadow in the centre of Vroulia bay which the sonar found to be bare of significant seagrass cover. From the bathymetry maps produced in this study from the sonar data there is a distinct slope in the centre of Vroulia down to a depth of fifty metres. Topouzelis *et al.* (2018), did not collect their own ground truth data and instead used reference maps that dated from 1998-2001, 15 years before the satellite mapping study was carried out. *P. oceanica* in the Mediterranean is known to be undergoing regression (-1.7% yr^{-1}) (Marbà *et al.* 2014) and therefore it's likely Topouzelis *et al.* (2018) were using out of date and inaccurate data for both calibrating the algorithms and accuracy assessments. This highlights the importance of remote sensing studies having access to high-quality ground truthing data to ensure their results are accurate and truly representative of the study area.

Satellite mapping of seagrass has shown higher accuracies than presented by Topouzelis *et al.* (2018) in shallow water (see section 2.1.1.1 for examples). These studies used calibration and accuracy assessment data which was collected during a similar time period and maintained a limit of detection of less than 15 m water depth.

Presence/absence classification was consistently more accurate in both test sites than classifications for canopy height with accuracy dropping to 38% and 58% in Samos and Lipsi respectively. Due to the lack of accurate existing maps available, particularly at depths of over 10 metres, presence/absence data would still be sufficient to inform policy decisions and provide baseline maps of seagrass extent on which long term monitoring can be based. The sonar method developed here is low effort and can be applied at the local scale quickly and easily to subsequently monitor seagrass presence over time. The ease of this method means it can be applied on an annual basis for routine monitoring of seagrass distribution over time for both signs and rates of regression.

A low cost side-scan sonar developed by Kaeser *et al.* (2013) is priced at approximately £1,510. Prices as of July 2020 indicate that mid-range down-scan sonars, such as the one used in this study, are approximately 30% of the cost of the cheapest side-scan sonars. This sonar can be mounted onto sea kayaks, as presented in this thesis, providing a more cost-effective alternative to boat mounted mapping methods particularly important for NGOs. Furthermore, down-scan sonars are more regularly used by recreational sailing craft than side-scan, opening up the possibility that this method could be adapted for a citizen science approach to seagrass monitoring. Most large-scale citizen science projects provide long term monitoring data (Dickinson *et al.* 2020), which is crucial for understanding seagrass regression. Citizen science has been shown to improve conservation efforts by building scientific knowledge, whilst also inspiring and educating the public through direct involvement and understanding (McKinley *et al.* 2017). However, the low speed is recommended by BioBase for accurate data collection (<5 km hr^{-1}) and may be a barrier to citizen science, as most recreational craft travel at speeds greater than this.

Kayak-mounted sonar is also limited by the distance from shore it is safe to cover and is slower than satellite-based mapping. It is therefore recommended that these methods be used in conjunction with each other. Down-scan sonar can provide much needed ground truthing data from which satellite mapping becomes more accurate. To date, sonar has only been proven to map seagrasses at up to 16 m depth (Sagawa *et al.* 2008), however, this work demonstrates it's potential to map at up to 28.4 m depth (deepest point of seagrass presence confirmed through both ground truthing and sonar derived data). The accuracy of sonar at greater depth intervals was not tested here due to a lack of seagrass presence at the deepest points in Vroulia Bay, however this shows promise and support for future work.

5.1.2 Use of Environmental DNA in seagrass ecosystems

Seagrass meadows support a rich biodiversity of marine species that utilise the food and refuge they provide (Goffredo *et al.* 2017; Jackson *et al.* 2015; Tuya *et al.* 2014; Vlachopoulou *et al.* 2013). Using the Critically Endangered bivalve *P. nobilis,* a seagrass specialist, as a model species, this work provides the first evidence that eDNA techniques can be used as a highly sensitive detection method for monitoring endangered or elusive species presence within and around seagrass meadows at both the macro- and micro-scale (Chapter 3). In addition, this chapter provides the first known study into how aquatic vegetation, influences eDNA dispersal from a point source.

This thesis also demonstrated the ability of this method to detect copies of eDNA from surface water samples, thus negating the necessity of free diving or expensive SCUBA equipment for future sample collection in some circumstances.

Imperfect detectability is a known error of any UVC or underwater video survey, for example when surveying *P. nobilis* there is a marked bias towards the detection of larger individuals (Hendriks *et al.* 2012). Underwater video surveys are limited to species within the field of view and are sometimes hard to identify to species level (Mallet and Pelletier, 2014). eDNA monitoring can also suffer from imperfect detectability, as demonstrated by the results of Chapter 3, however the determinates of detectability under various environmental conditions is still largely unknown.

For pelagic or motile species angling surveys are another alternative survey method that can provide good estimations of species, but more importantly generate detailed size data, allowing for weight measurements and assessment of specimen health (Willis *et al.* 2000). However, there are problems associated with this survey method including the variation in catchability of species (Arreguín-Sánchez, 1996) and size selectivity of the various methods (i.e. traps nets and hooks) (Millar & Fryer, 1999). This survey technique is not appropriate for bivalves and there is also the invasive nature of this method (Mallet and Pelletier, 2014), that has ethical implications. Environmental DNA can offer increased accuracy as well as a non-invasive and more cost effective alternative or accompaniment to traditional survey methods (e.g. Wheldon *et al.* 2020).

The likelihood of detection of *P. nobilis* using eDNA was 8.7 times higher during spawning season than non-spawning season (95% CI = 6.1 - 12.6) and, unlike non-spawning season, detection probability was unaffected by distance from the point

source. During non-spawning season the probability of detection dropped to zero by a distance of twenty metres from the source. Whilst it has been shown that eDNA taken during spawning season can provide a "snapshot" of bivalve species presence (Bracken, *et al.* 2019), the results presented in this work extend this finding to suggest that multiple surveys at different times can be used to identify species presence at different spatial scales, potentially to individual seagrass beds or even within them.

Outside of spawning season less DNA is likely to be released from an individual *P. nobilis* when compared with spawning season. Results therefore suggest that during the spawning a significant increase in DNA released by the bivalve masked this decreasing trend with distance. If sampling during the spawning season was expanded to cover a larger distance it's possible a similar trend would be observed if given sufficient distance from individual *P. nobilis.*

During the spawning season, seagrass blade length was shown to significantly impact eDNA detectability, with detectability decreasing with increasing blade length. This could be due to the attenuating effect of seagrass on water movement, reducing water flow and therefore eDNA dispersal. It could also be a result of eDNA binding to organic particles and their subsequent removal from the water column due to sedimentation (Buxton *et al.* 2017). Environmental DNA has been shown to aggregate to fine organic particles (Turner *et al.* 2014), these smaller particles are known to settle out of the water column quicker when water flow is slower reducing dispersal of eDNA from the point source (Jane *et al.* 2015). Chapter 4 demonstrated a higher proportion of organic matter within the seagrass meadow as well as a significant decrease in median particle size with increasing seagrass cover. These sediment characteristics are therefore likely

to promote the incorporation of eDNA into the sediments, removing it from the water column.

To our knowledge this is the first study to create an eDNA monitoring protocol for a marine bivalve of conservation concern. It has been estimated that 30%– 34% of marine and freshwater invertebrates are data deficient, including those which have attributes making them at higher risk of extinction, such as restricted distribution (Collier *et al.* 2016). Marine bivalve species which are vulnerable to declining populations such as Giant Clams, *Tridacna spp*, and have not been assessed by the IUCN since 1996 (Wells, 1996). There is clearly a need for species distribution mapping and population assessment of these at risk species to provide conservation evidence and facilitate their protection.

5.1.3 Microplastic pollution in coastal vegetation

Microplastics have been highlighted in recent years as a significant and abundant marine pollutant but still little is understood about the deposition and accumulation processes in coastal vegetation, including seagrass meadows. This thesis applied emerging isolation techniques to explore the presence and abundance of microplastics in the context of a remote *P. oceanica* meadow (Chapter 4). Fine-scale mapping demonstrated that the influence of seagrass on microplastic deposition and retention is likely to be complex and highly dependent on both the source of microplastic pollution and patterns of water movement. Whilst there is emerging evidence that microplastics can accumulate in vegetated coastal habitats, data from this research suggests that this is not universally the case and that the local context is important.

Microplastic fragments were substantially more common (n = 445) than fibres (n = 3), making up over 99% of the particles identified compared to less than 1% fibres. Huang *et al.* (2020), found a similar relationship at one of the seagrass vegetated sites studied whilst Jones *et al.* (2020) found no difference in microplastic morphology. Sites with a higher fragment proportion have been suggested to have low sewage effluent exposure with the major source instead, being locally derived secondary microplastics (Horton *et al.* 2017; Alomar *et al.* 2016; Sutton *et al.* 2016). Microplastic fibres were the most common morphology isolated across all sites studied on microplastic content of mangrove sediments in the Persian Gulf (Naji *et al.* 2019), however details on anthropogenic influences to these sites was not discussed. Microplastic fibres were mainly detected in mangrove sediments from areas near the river estuaries, bays, harbours or areas of tourism within the Guangdong and Fujian provinces, while fragments were detected more uniformly throughout sampling locations (Zhou *et al.* 2020). This corresponds to the low population density of Lipsi and relatively remote location of the survey area. This suggests microplastic morphology in coastal vegetation is highly influenced by anthropogenic activity.

Emerging literature is exploring the potential direct and indirect impacts of microplastics on marine ecosystems but there is still a lack of understanding of the full implications of this anthropogenic pollutant. Microplastics are known to adsorb toxic chemical such as PCBs, PAHs and heavy metals (Hirai *et al.* 2011; Brennecke *et al.* 2016). Additionally, microplastic contamination has been shown to affect the microbial communities of sediments and the functioning of that community (Seeley *et al.* 2020). This means the presence of microplastic in the environment in Lipsi could be having an, as yet, unknown knock on effect on the marine species that live or utilise both the sediments and water column.

Seagrass cover and distance from shore had no significant effect on microplastic accumulation in Lipsi according to the data and models presented here, although these findings are contrary to other recent seagrass studies (Jones *et al.* 2020, Huang *et al.* 2020). Recent work has also started to explore other coastal vegetation but as yet very little is known yet about the ability of coastal vegetation types, including kelp forests and salt marshes, to trap or capture marine microplastics. For example, in mangrove sediments microplastic abundance has been found to be up to 8.5 times higher than mangrove free sediments (Zhou *et al.* 2020). Whilst in salt marshes macroplastics have been shown to degrade into microplastics, however the fate of these particles, once released remains unknown (Weinstein *et al.* 2016). Contrasting and limited findings, such as these, are common in spatial based studies and indicative of emerging bodies of research, demonstrating a need for further, far more extensive surveys and experimental approaches to address these gaps in knowledge.

Recently published studies however, have not modelled sediment composition with seagrass or any other coastal vegetation. Sediment distribution presented here suggests the seagrass meadow studied is trapping sediments effectively, so if microplastics were present within the water column they are likely to become trapped within the seagrass sediment. Lack of microplastic concentrations within the water is the most likely reasoning for the relationships found in this study. Sediment variables such as the sorting coefficient and smaller grain size fractions (i.e. silt, very fine sand and clay), when combined into independent principle components, also had a combined effect on the accumulation of microplastics. Alomar *et al.* (2016) found no such relationship between sediment grain sizes and microplastics (Alomar *et al.* 2016), however in that analyses grain sizes were only treated as independent variables and therefore subject to problems of collinearity, reducing the statistical significance of the

analysis. This suggests the interaction between microplastics and sediment grain size could be occurring at a broader scale than previously considered. It is therefore possible that overall sediment characteristics are in fact the determinates of microplastic accumulation rather than individual grain size fractions.

5.2 Final Conclusion

The findings presented in this thesis demonstrate the progression of seagrass ecosystem research that can be achieved by using emerging survey methods. These emerging techniques can offer new insights into the role of seagrass on the detectability of key species and therefore the application of monitoring particular species. These emerging techniques can provide new avenues for developing existing methods, improving the accuracy of mapping or species monitoring. These approaches can also shine light on the role seagrass has on the deposition, accumulation and processing of marine pollutants. Progressing research in this way will help to inform both policy decisions on the management and conservation of seagrass meadows and the practicalities of using emerging techniques in this unique, yet widespread ecosystem.

5.3 Key findings

1. Down-scan sonar represents a quick and cost-effective technique for mapping of seagrass at intermediate spatial scales and can be deployed to ground truth larger scale satellite-based mapping techniques.

2. Environmental DNA has been shown to be used successfully as a sensitive and non-invasive survey method in seagrass systems with variations in scale and sampling season to broaden or focus research questions.

3. *P. oceanica* has been found not to play a significant direct role in the accumulation of microplastics in an isolated bay. Sediment analysis suggests that microplastic accumulation in near shore seagrass beds may be highly localised around terrestrial sources of plastic pollution and requires further study.

4. Emerging survey techniques can enhance our understanding of seagrass meadow distribution and ecosystem services with potential to inform policy and conservation efforts.

5.4 Recommendations For Future Work

5.4.1 Remote sensing for seagrass mapping

5.4.1.1 Combining down-scan sonar and satellite research methods

With less than 10% of the Greek coastline mapped there is a clear need for an accurate mapping method which can be applied on a largescale. This thesis presented the successful application of kayak mounted down-scan sonar to map *P. oceanica* meadows with greater accuracy at distinguishing between deep water and seagrass meadows than satellite mapping used by Topouzelis *et al.* (2018). However, this down-scan technique is limited by scale as it cannot be used at the same spatial scales as satellite mapping. Accurate ground truthing data is required to train satellite mapping algorithms to recognise seagrass from satellite imagery (Topouzelis *et al.* 2018). Down-scan sonar provides a tool that can collect this required data quickly and at low cost, therefore work is needed to investigate in some detail the complimentary use of both down-scan sonar and satellite images to co-create seagrass maps. The use of sonar would not only provide accurate estimations of seagrass presence but also bathymetry data for the coastal regions where seagrass grows. These techniques used together

have the potential to become a powerful tool for seagrass mapping, and cover all areas of *P. oceanica* habitat range.

Satellite mapping for the areas of Lipsi and Samos should be redone using the method from Topouzelis *et al.* (2018) combined with information from down-scan sonar to assess the ability of coarse-resolution data to calibrate and assess the accuracy of Landsat imagery. Sonar would provide accurate depth profile for regions, and therefore the depth at which this method is reliable can be accurately assessed.

5.4.1.2 In depth testing of mapping across varied study sites

Sonar data have been shown to lose accuracy when mapping across interfaces between habitat types (Sagawa *et al.* 2008). In this study, Lipsi had a very complex bathymetry, with patchy seagrass cover, while Samos had a simpler bathymetry and continuous seagrass cover. Expanding the test sites for this method to areas of intermediate bathymetric complexity and seagrass health would further understand the limits of accuracy for the down-scan and BioBase technique. This is important for the future application of the method and understanding for which regions and species of seagrass it is an appropriate survey method.

5.4.1.3 Accuracy of mapping various species

Side-scan sonar has been able to distinguish between two species of *Zostera* due to significant difference in canopy height between species (Sagawa *et al.* 2010). Down-scan sonar was not tested in this respect or the potential of BioBase algorithms to process the sonar data in that level of detail. It would therefore be useful for the future application of this technique in other survey regions to understand the capacity to

differentiate species in mixed meadows or to map species of seagrass that form lower canopy heights than those of *P. oceanica*.

Down-scan sonar is a potential seagrass mapping tool for various species of seagrass. From the results here it performs better in areas with dense, tall seagrass canopies and therefore may be better suited to larger seagrass species. *Zostera marina* has already been shown as a viable species for this mapping technique by Luczkovich *et al.* (2013). *Z. marina*, like *P. oceanica*, forms dense canopies up to 1m. Other species that should be investigated with this method include, *Thalassia testudinum*, *Posidonia australis,* and *Enhalus acoroides*, all of which have thick leaves, and form tall canopies of continuous meadows. Many species of seagrass require more information on their distribution, down-scan sonar with use of the BioBase tool has the potential, with further testing, to provide this data. It is therefore important to develop methods which can map these species across their entire habitat ranges, including at depth or in turbid water.

There is still much to learn about seagrasses growth and morphology at depth and therefore, until more research has been carried out in these areas this method should be used with caution. During the drop camera work for Chapter 2 it was noted some sparse but tall vegetation was growing at depth in Vroulia Bay. The morphology of this species was similar to that of *Halophilia stiulacea*, which is a tropical species alien to the Mediterranean and typically has a very small canopy height of approximately 10cm, growing horizontally, flush to sea bed. The vegetation seen at 37m in Vroulia bay however was much larger than this reaching vertical hights of approximately 60cm and has yet to be identified. Should *H. stipulacea* show differing morphologies at shallow and deep zones, this will contradict current knowledge on the species and have

implications on which species are appropriate for mapping at depth using sonar technology.

5.4.2 Development of eDNA for key species

The potential of eDNA to monitor other key species within seagrass habitats should also be investigated. Once there is an improved understanding of the interactions between seagrasses in various environmental conditions and eDNA movement, other target species for monitoring should be considered. Many species of commercial and ecological importance utilise seagrass meadows at some stage in their life cycle (Kalogirou *et al.* 2010 and sections 1.4.1 & 1.4.2) Many of these species are mobile and therefore may enter the seagrass canopy periodically for grazing, predation, or shelter. Species of ecological importance which are lacking data, are often rare or elusive which makes monitoring them using existing techniques laborious and expensive. Studying the presence of eDNA of these species will help to understand how eDNA could be used in the future to aid monitoring programs and develop more robust tools to inform protective legislation. Key species that interact with seagrass meadows, such as the sea horses *Hippocampus hippocampus* (short-snouted seahorse) and *Hippocampus guttulatus* (Long-snouted seahorse), are classed as globally Data Deficient on the IUCN red list due to lack of data across the whole extent of their geographic range (Gristina *et al.* 2017; Pollom, 2017; Woodall, 2017). An eDNA monitoring tool for these species could help fill these gaps in knowledge and help management of populations.

Deep seagrass meadows are at more risk from rising sea levels and eutrophication if they are unable to migrate meadow limits to maintain sufficient sunlight for photosynthesis. In order to give these meadows and their associated communities better protection they must be better understood. Environmental DNA can provide

information in species distributions with depth and therefore this important element of future research. Taking water samples from seagrass meadows at depth will allow a greater understanding of species zonation and utilisation of seagrass meadows.

Another key species that would benefit from a quick and effective monitoring method would be *Haplosporidium pinnae*. *H. pinnae* is the parasite responsible for the mass mortality events seen in *P. nobilis* populations across the Mediterranean (Cabanellas-Reboredo *et al.* 2019). This novel parasite was first described in 2018 by Catanese *et al.* (2018), and there are still large gaps in the knowledge of its life cycle and transmission. When the parasite was first described, the only conclusive identification of the infection was the removal of dying or very recently dead *P. nobilis* specimens, which then required dissection and histological analysis (Catanese *et al.* 2018). More recently a real time PCR technique was developed and tested, however this still requires tissue samples from suspected infected *P. nobilis* individuals (López-Sanmartín *et al.* 2019). *P. nobilis* is now classed as critically endangered on the IUCN red list (Kersting *et al.* 2019), and as a result of this critical condition, authorisation to collect tissue samples from *P. nobilis* may be under more critical review to avoid stress to the individual. Development in optimising the PCR method from López-Sanmartín *et al.* (2019), to apply to water samples would provide a non-invasive alternative to confirm *H. pinnae* infections in a given area.

5.4.3 Further understanding of DNA dispersal in seagrass meadows

In order to understand these influences of seagrass on eDNA in more detail and further develop the monitoring technique in the wider ecosystem, further research should be carried out on the movement of eDNA in seagrass ecosystems . Using artificial seagrass systems such as those often used in flow experiments will allow for these interactions

to be studied in more detail under controlled conditions. Tank experiments will allow for changes in flow velocity or direction to be studied, as well as interactions between DNA dispersal and various shoot densities and/or canopy heights. Estimations of the impacts seagrass canopies have on biomass approximations from eDNA quantities can also be carried out in these conditions and eDNA decay rates within seagrass canopies and sediments can be better understood. Along with differing flow rates, study of the vertical movement of eDNA between the seagrass canopy and water column would further our understanding of these dynamics. In Chapter 3, the horizontal distance was studied in detail, and while the surface sample was taken during spawning season to investigate the potential of surface sampling, any vertical gradients have not yet been studied. This will allow for better understanding on if species which reside solely inside the meadow can be detected from water samples taken outside of the meadow boundary and if species which never entre the canopy can be detected from water samples taken from within it. Understanding these interactions is key to using eDNA as a monitoring tool accurately and efficiently across all habitats.

While the results of Chapter 3 demonstrated a decrease in eDNA detection with seagrass blade length, only water samples were taken for analysis. To develop further our understanding of eDNA interactions with seagrass habitats sediment samples should be analysed for eDNA presence. The retention of eDNA within sediments has been shown to surpass that of the water column (Turner *et al.* 2015), with eDNA being amplified from sediment samples up to 4600 years old (Anderson-Carpenter *et al.* 2011). Sediment cores from within seagrass meadows can be taken and the sedimentary layers dated to show the time frame in which the eDNA would have been incorporated into the sediments. Due to the stability of seagrass sediments (Gacia & Duarte, 2001), these meadows could be a vital source of eDNA for many different uses,

such as monitoring of historical species extent, or testing for the presence of rare of cryptic species over time. There have also been no studies into the possibility of eDNA adhering to the seagrass blade themselves and therefore this could be a key avenue of future research to understand the fate of eDNA within the seagrass meadow.

Seagrass may attenuate the spread of eDNA due to reductions in water flow (section 3.1.4), particularly in the non-spawning season, and therefore might provide more clustered concentrations of DNA that are useful in determining spatial location of individuals. This may be particularly useful in determining whether *P. nobilis* are in habitats below the depth limits of UVC.

Posidonia oceanica seagrass, which is the focus of this thesis, is a large seagrass with long lived rhizomes and it is not uncommon for shoots to reach up to 1m or more (Fourqurean *et al.* 2012; Kuo & den Hartog 2007), however most seagrasses are considerably smaller in canopy heights with shoots of 1-20 cm (Kuo & den Hartog 2007). It is therefore important to compare the influences these various species may have on movement and trapping of eDNA. In this context, all aspects of the seagrass habitat should be investigated such as the water within or around the seagrass canopy and the sediments, *P. oceanica* sediments are particularly stable among seagrass species. Relationships should not be assumed to be identical across all species of seagrass due to the morphological differences, so to fully understand the dynamics of eDNA across seagrass systems a range of species must be investigated.

Studies into eDNA are looking into the correlation between qPCR DNA concentration and biomass (e.g. Takahara *et al.* 2012; Sassoubre *et al.* 2016; Weldon *et al.* 2020), therefore using eDNA in place of traditional sampling methods to inform population densities. Before these techniques can be used in this respect, in seagrass systems,

there needs to be further understanding of the interactions. If eDNA does not disperse uniformly throughout the meadow or prevents eDNA molecules from entering the system/ leaving the system, seagrass resident species or species that never enter the canopy; these will all require different approaches when it comes to monitoring. If these factors are not accounted for, it could lead to over or under estimations of populations and therefore misinform management strategies.

5.4.4 Microplastic loading of Lipsi Island

To investigate this further, a study of the microplastics concentrations in surface water and water column samples should be taken from around the island of Lipsi. Potential sources of microplastics into the coastal areas of Lipsi, such as the ephemeral river mentioned in section 4.4.4. This will also elucidate the reasons for the overall low microplastic loading of the sediments in the survey area compared to other sediment samples from the Mediterranean, if water column microplastic were low, sediment microplastic content would also be expected to be lower.

5.4.5 Holistic approach to microplastics in seagrass ecosystems

While the scope of the study in Chapter 4 did not extend past the sediments associated with the *P. oceanica* meadow, it has been previously shown that microplastics also adhere to the seagrass blades themselves (Goss *et al.* 2018; Jones *et al.* 2020). There has been no study on the microplastic adherence rates to *P. oceanica* blades or the incorporation into epiphytes associated with *P. oceanica*. Jones *et al.* (2020) found microplastics in most of the biota studied, although a difference between microplastic content of biota associate with seagrass and those from outside the meadow was not tested. Remy *et al.* (2015) reported presence of artificial fibres in organisms inhabiting detritus from *P. oceanica* leaf litter, however no relationship was identified. Organisms

associated with the seagrass meadow should be investigated for differences in microplastics accumulation compared with those in unvegetated areas. A comprehensive study is recommended into the microplastic loading across the whole seagrass habitat, including the seagrass above and below ground biomass, sediments and water column inside and outside the canopy. This can be done using in situ samples from all aspects of the seagrass meadow. Sediment cores can also be taken and the layers dated to assess any changes to microplastic deposition over time. It would also be beneficial to compare areas of high and low microplastic loading to test if the relationship between seagrass changes as microplastic loading increases or decreases. This would increase our understanding of how microplastics are moving though food chains and the exposure of seagrass associated species.

5.4.6 Seagrasses as pathogen control species

Microplastics have been shown to be a vector of both human and fish pathogens (Kirstein *et al.* 2016; Viršek *et al.* 2017). In contrast seagrasses have previously been shown to reduce the number of bacterial pathogens in seawater samples (Lamb *et al.* 2017). There have also been a small number of lab-based studies on the antimicrobial properties of seagrass, focusing mainly on individual extracts, such as hexane, chloroform and methanol, (e.g. Kumar *et al.* 2008; Qi *et al.* 2008; Mayavu *et al.* 2009; Kannan *et al.* 2010; Kannan *et al.* 2012). Should seagrasses play a role in the control of pathogenic bacteria that adhere to microplastics, this will have key implications to the management of these habitats. It is important to study the potential of seagrass species to reduce the pathogen adhering to the microplastics that travel through or settle in seagrass habitats, as this could be as yet unknown ecosystem service provided by the meadows. Seagrass often grow in low energy coastal waters that are also popular

bathing waters. The meadows in these areas could also be providing a natural trapping or cleaning method for microplastics in coastal bathing waters demonstrating an added benefit of seagrass presence. This would promote their protection and restoration at touristic beaches, in addition to the known ecosystem services such as carbon sequestration and support of commercial fisheries.

References

Alimba, C.G. and Faggio, C., (2019). Microplastics in the marine environment: Current trends in environmental pollution and mechanisms of toxicological profile. *Environmental Toxicology and Pharmacology*, 68, pp.61-74.

Almanza, V., Buschmann, A.H., Hernández-González, M.C. and Henríquez, L.A., (2012). Can giant kelp (*Macrocystis pyrifera*) forests enhance invertebrate recruitment in southern Chile? *Marine Biology Research*, 8(9), pp.855-864.

Alomar, C., Estarellas, F., & Deudero, S. (2016). Microplastics in the Mediterranean Sea: Deposition in coastal shallow sediments, spatial variation and preferential grain size. *Marine Environmental Research*, 115(January), 1–10.

Anderson-Carpenter, L. L., McLachlan, J. S., Jackson, S. T., Kuch, M., Lumibao, C. Y., & Poinar, H. N. (2011). Ancient DNA from lake sediments: Bridging the gap between paleoecology and genetics. *BMC Evolutionary Biology*, 11(1), 1–15.

Andrady, A.L., (2011). Microplastics in the marine environment. Marine pollution bulletin, 62(8), pp.1596-1605.

Arreguín-Sánchez, F., (1996). Catchability: a key parameter for fish stock assessment. *Reviews in Fish Biology and Fisheries*, *6*(2), pp.221-242.

Badalamenti, F., Alagna, A., & Fici, S. (2015). Evidences of adaptive traits to rocky substrates undermine paradigm of habitat preference of the Mediterranean seagrass *Posidonia oceanica*. *Scientific Reports*, 5, 8804.

Baker, S., Paddock, J., Smith, A. M., Unsworth, R. K. F., Cullen-Unsworth, L. C., & Hertler, H. (2015). An ecosystems perspective for food security in the Caribbean: Seagrass meadows in the Turks and Caicos Islands. *Ecosystem Services*, 11, 12–21.

Balestri, E., Cinelli, F., & Lardicci, C. (2003). Spatial variation in *Posidonia oceanica* structural, morphological and dynamic features in a north-western Mediterranean coastal area: a multi-scale analysis. *Marine Ecology Progress Series*, 250, 51–60.

Balestri, E., Menicagli, V., Vallerini, F., & Lardicci, C. (2017). Biodegradable plastic bags on the seafloor: A future threat for seagrass meadows? *Science of the Total Environment,* 605–606, 755–763.

Barbier, E. B., Hacker, S. D., Kennedy, C., Koch, E. W., Stier, A. C., & Silliman, B. R. (2011). The value of estuarine and coastal ecosystem services. *Ecological Monographs,* 81(2), 169–193.

Barnes, D. K. A., Galgani, F., Thompson, R. C., & Barlaz, M. (2009). Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society B: Biological Sciences,* 364, 1985–1998.

Barrón, C., Duarte, C., Frankignoulle, M., & Borges, A. (2006). Organic carbon metabolism and carbonate dynamics in a Mediterranean seagrass (*Posidonia oceanica*), meadow. *Estuaries and Coasts,* 29, 417–426.

Basso, L., Vázquez-Luis, M., García-March, J., Deudero, S., Alvarez, E., Vicente, N., Duarte, C., & Hendriks, I. (2015) The Pen Shell, *Pinna nobilis*: A Review of Population Status and Recommended Research Priorities in the Mediterranean Sea. In: Barbara E. Curry, editor, *Advances in Marine Biology,* Vol. 71, Oxford: Academic Press, pp. 109-160.

Bastyan, G.R. and Cambridge, M.L., (2008). Transplantation as a method for restoring the seagrass *Posidonia australis*. *Estuarine, Coastal and Shelf Science*, 79(2), pp.289-299.

Blair, T. C., & McPherson, J. G. (1999). Grain-size and textural classification of coarse sedimentary particles. *Journal of Sedimentary Research*, 69(1), 6–19.

Bianchi, C., & Morri, C. (2000). Marine Biodiversity of the Mediterranean Sea : Situation , Problems and Prospects for Future. *Marine Pollution Bulletin*, 40, 367–376.

Blandon, A., & Zu Ermgassen, P. S. E. (2014). Quantitative estimate of commercial fish enhancement by seagrass habitat in southern Australia. *Estuarine, Coastal and Shelf Science,* 141, 1–8.

Boudouresque, C. F., Bernard, G., Pergent, G., Shili, A., & Verlaque, M. (2009). Regression of Mediterranean seagrasses caused by natural processes and anthropogenic disturbances and stress: A critical review. *Botanica Marina*, 52, 395–418.

Boudouresque, C.F., Bernard, G., Bonhomme, P., Charbonnel, E., Diviacco, G., Meinesz, A., Pergent, G., Pergent-Martini, C., Ruitton, S. and Tunesi, L., (2012). *Protection and conservation of* Posidonia oceanica *meadows*. RAMOUGE and RAC/ SPA publisher. Tunis, 1- 202.

Bracken, F. S. A., Rooney, S. M., Kelly-Quinn, M., King, J. J., & Carlsson, J. (2019). Identifying spawning sites and other critical habitat in lotic systems using eDNA "snapshots": A case study using the sea lamprey *Petromyzon marinus* L. *Ecology and Evolution,* 9(1), 553–567.

Brebbia, C. A., Tiezzi, E., & Conti, M. E. (2006). *Management of Natural Resources, Sustainable Development and Ecological Hazards* (Vol. 99). Wit Press

Brennecke, D., Duarte, B., Paiva, F., Caçador, I., & Canning-Clode, J. (2016). Microplastics as vector for heavy metal contamination from the marine environment. *Estuarine, Coastal and Shelf Science,* 178, 189–195.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T. and Thompson, R., (2011). Accumulation of microplastic on shorelines worldwide: sources and sinks. *Environmental Science & Technology*, 45(21), pp.9175-9179.

Buxton, A.S., Groombridge, J.J. and Griffiths, R.A., (2017). Is the detection of aquatic environmental DNA influenced by substrate type?. *PLoS One*, 12(8), p.e0183371.

Cabaço, S., & Santos, R. (2012). Seagrass reproductive effort as an ecological indicator of disturbance. *Ecological Indicators*, 23, 116–122.

Cabanellas-Reboredo, M., Vázquez-Luis, M., Mourre, B., Álvarez, E., Deudero, S., Amores, Á., Addis, P., Ballesteros, E., Barrajon, A., Coppa, S., Garcia- March, J., Giacobbe, S., Gimenez Casalduero, F., Hadjioannou, L. *et al.* (2019). Tracking a mass mortality outbreak of pen shell *Pinna nobilis* populations: A collaborative effort of scientists and citizens. *Scientific Reports,* 14, 13355.

Campagne, C. S., Salles, J. M., Boissery, P., & Deter, J. (2014). The seagrass *Posidonia oceanica*: Ecosystem services identification and economic evaluation of goods and benefits. *Marine Pollution Bulletin,* 97(1–2), 391–400.

Cappo, M., (2010). Development of a baited video technique and spatial models to explain patterns of fish biodiversity in inter-reef waters (Doctoral dissertation, James Cook University).

Cardona, L., López, D., Sales, M., De Caralt, S. and Díez, I., (2007). Effects of recreational fishing on three fish species from the *Posidonia oceanica* meadows off Minorca (Balearic archipelago, western Mediterranean). *Scientia Marina*, *71*(4), pp.811-820.

Carella, F., Aceto, S., Pollaro, F., Miccio, A., Iaria, C., Carrasco, N., Prado, P. & De Vico, G. (2019). A mycobacterial disease is associated with the silent mass mortality of the pen shell *Pinna nobilis* along the Tyrrhenian coastline of Italy. *Scientific Reports*, 9(1), 1–12.

Carlson, P. R. J., Yarbro, L. A., Madley, K., Arnold, H., Merello, M., Vanderbloemen, L., McRae, G., & Durako, M. J. (2003). Effect of el niño on demographic, morphological, and chemical parameters in turtle-grass. *Environmental Monitoring and Assessment*, 81, 393–408.

Catanese, G., Grau, A., Valencia, J. M., Garcia-March, J. R., Vázquez-Luis, M., Alvarez, E., Deudero, S., Darriba, S., Carballal, M., & Villalba, A. (2018). *Haplosporidium pinnae sp*. nov., a haplosporidan parasite associated with mass mortalities of the fan mussel, *Pinna nobilis*, in the Western Mediterranean Sea. *Journal of Invertebrate Pathology*, 157(March), 9–24.

Chan, K.M., Shaw, M.R., Cameron, D.R., Underwood, E.C. and Daily, G.C., (2006). Conservation planning for ecosystem services. *PLoS Biology*, *4(11)*, p.e379.

Chauvaud, S., Bouchon, C. and Maniere, R., (1998). Remote sensing techniques adapted to high resolution mapping of tropical coastal marine ecosystems (coral reefs, seagrass beds and mangrove). *International Journal of Remote Sensing*, 19(18), pp.3625-3639.

Christianen, M. J. A., van Belzen, J., Herman, P. M. J., van Katwijk, M. M., Lamers, L. P. M., van Leent, P. J. M., & Bouma, T. J. (2013). Low-Canopy Seagrass Beds Still Provide Important Coastal Protection Services. *PLoS One*, 8(5).

Claessens, M., Meester, S. De, Landuyt, L. Van, Clerck, K. De, & Janssen, C. R. (2011). Occurrence and distribution of microplastics in marine sediments along the Belgian coast. *Marine Pollution Bulletin,* 62(10), 2199–2204.

Clark, J.R., Cole, M., Lindeque, P.K., Fileman, E., Blackford, J., Lewis, C., Lenton, T.M. and Galloway, T.S., (2016). Marine microplastic debris: a targeted plan for understanding and quantifying interactions with marine life. *Frontiers in Ecology and the Environment*, *14*(6), pp.317-324.

Cole, M., Coppock, R., Lindeque, P.K., Altin, D., Reed, S., Pond, D.W., Sørensen, L., Galloway, T.S. and Booth, A.M., (2019). Effects of nylon microplastic on feeding, lipid accumulation, and moulting in a cold water copepod. *Environmental Science & Technology*, *53*(12), pp.7075-7082.

Collier, K.J., Probert, P.K. and Jeffries, M., (2016). Conservation of aquatic invertebrates: concerns, challenges and conundrums. *Aquatic Conservation: Marine and Freshwater Ecosystems,* 26(5), pp.817-837.

Collins, K. J., Suonpää, a. M., & Mallinson, J. J. (2010). The impacts of anchoring and mooring in seagrass, Studland Bay, Dorset, UK. *Underwater Technology*, 29(3), 117–123.

Cosentino, A., & Giacobbe, S. (2006). Shell ornament in *Pinna nobilis* and *Pinna rudis* (Bivalvia: Pteriomorpha). *Journal of Conchology*, *39*(2), 135.

Coppa, S., Guala, I., Lucia, G. A. De, Massaro, G., & Bressan, M. (2010). Density and distribution patterns of the endangered species *Pinna nobilis* within a *Posidonia oceanica* meadow in the Gulf of Oristano (Italy). *Journal of the Marine Biological Association of the United Kingdom*, 90, 885–894.

Coppock, R.L., Cole, M., Lindeque, P.K., Queirós, A.M. and Galloway, T.S., (2017). A small-scale, portable method for extracting microplastics from marine sediments. *Environmental Pollution*, 230, pp.829-837.

Costanza, R., d'Arge, R., De Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'neill, R.V., Paruelo, J. and Raskin, R.G., (1997). The value of the world's ecosystem services and natural capital. *Nature,* 387(6630), pp.253-260.

Costello, M. J., Basher, Z., McLeod, L., Asaad, I., Claus, S., Vandepitte, L., Bates, A. (2017). Methods for the study of marine biodiversity. *In The GEO Handbook on Biodiversity Observation Networks* (pp. 129–163). Springer.

Cozza, R., Iaquinta, A., Cozza, D. and Ruffolo, L., (2013). Trace metals in *Posidonia oceanica* in a coastal area of the Ionian Sea (Calabria, Italy). *Open Journal of Ecology, 3 (2).* 102-108.

Crain, C.M., Halpern, B.S., Beck, M.W. and Kappel, C.V., (2009). Understanding and managing human threats to the coastal marine environment. *Annals of the New York Academy of Sciences*, *1162*(1), pp.39-62.

Crump, A., Mullens, C., Bethell, E.J., Cunningham, E.M. and Arnott, G., (2020). Microplastics disrupt hermit crab shell selection. *Biology Letters*, *16*(4), p.20200030.

Currier, C. A., Morris, T. J., Wilson, C. C., & Freeland, J. R. (2018). Validation of environmental DNA (eDNA) as a detection tool for at-risk freshwater pearly mussel species (Bivalvia: Unionidae). *Aquatic Conservation: Marine and Freshwater Ecosystems,* 28(3), 545–558

Curtarelli, M., Leão, J., Ogashawara, I., Lorenzzetti, J., & Stech, J. (2015). Assessment of Spatial Interpolation Methods to Map the Bathymetry of an Amazonian Hydroelectric Reservoir. *ISPRS International Journal of Geo-Information,* 4, 220–235.

Darriba, S. (2017). First haplosporidan parasite reported infecting a member of the Superfamily Pinnoidea (*Pinna nobilis*) during a mortality event in Alicante (Spain, Western Mediterranean). *Journal of Invertebrate Pathology*, 148(May), 14–19.

Davenport, J., Ezgeta-Balić, D., Peharda, M., Skejić, S., Ninčević-Gladan, Ž., & Matijević, S. (2011). Size-differential feeding in *Pinna nobilis* L. (Mollusca: Bivalvia): Exploitation of detritus, phytoplankton and zooplankton. *Estuarine, Coastal and Shelf Science,* 92(2), 246–254.

De Falco, G., Ferrari, S., Cancemi, G., & Baroli, M. (2000). Relationship between sediment distribution and *Posidonia oceanica* seagrass. *Geo-Marine Letters*, 20(1), 50–57.

Deiner, K., & Altermatt, F. (2014). Transport Distance of Invertebrate Environmental DNA in a Natural River. *PLoS One,* 9(2).

Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Taberlet, P., & Miaud, C. (2011). Persistence of Environmental DNA in Freshwater Ecosystems. *PLoS One,* 6, 8–11.

Dekker, A., Brando, V., Anstee, J., Fyfe, S., Malthus, T. and Karpouzli, E., (2007). Remote sensing of seagrass ecosystems: use of spaceborne and airborne sensors. In *Seagrasses: Biology, Ecology and Conservation* (pp. 347-359). Springer, Dordrecht.

Delignette-Muller ML and Dutang C (2015), fitdistrplus: An R Package for Fitting Distributions. *Journal of Statistical Software*, 64(4), 1-34

Deudero, S., Morey, G., Frau, A., Moranta, J., & Moreno, I. (2008). Temporal trends of littoral fishes at deep *Posidonia oceanica* seagrass meadows in a temperate coastal zone. *Journal of Marine Systems*, 70, 182–195.

Deudero, S., Grau, A., Vázquez-Luis, M., Álvarez, E., Alomar, C. and Hendriks, I.E., (2017). Reproductive investment of the pen shell *Pinna nobilis* Linnaeus, 1758 in Cabrera National Park (Spain). *Mediterranean Marine Science*, *18*(2), pp.271-284.

Dewsbury, B. M., Bhat, M., & Fourqurean, J. W. (2016). A review of seagrass economic valuations: Gaps and progress in valuation approaches. *Ecosystem Services*, 18, 68–77.

Dickinson, J.L., Zuckerberg, B. and Bonter, D.N., (2010). Citizen science as an ecological research tool: challenges and benefits. *Annual review of ecology, evolution, and systematics,* 41, pp.149-172.

Dorazio, R. M. (2012). Predicting the Geographic Distribution of a Species from Presence-Only Data Subject to Detection Errors. *Biometrics,* 68, 1303–1312.

Dorenbosch, M., Nagelkerken, I., & van der Velde, G. (2006). Seagrass beds and mangroves as nurseries for the threatened Indo-Pacific Humphead wrasse, *Cheilinus undulatus*

and Caribbean Rainbow parrotfish, *Scarus guacamaia*. *Biological Conservation*, 129, 277–287.

Dray S and Dufour A (2007). The ade4 Package: Implementing the Duality Diagram for Ecologists. *Journal of Statistical Software*, 22(4), pp. 1-20.

Duarte, Carlos M., & Chiscano, C. L. (1999). Seagrass biomass and production: a reassessment. *Aquatic Botany,* 65, 159–174.

Duarte, C.M., & Kirkman , H. (2001). Methods for the measurements of seagrass abundance and depth distribution. *Global Seagrass Research Methods*, p. 140 -153

Duarte, Carlos M. (2002). The future of seagrass meadows*. Environmental Conservation,* 29, 192–206.

Duarte, C. M., Middelburg, J. J., & Caraco, N. (2005). Major role of marine vegetation on the oceanic carbon cycle. *Biogeosciences,* 2, 1–8.

Duncan, E.M., Broderick, A.C., Fuller, W.J., Galloway, T.S., Godfrey, M.H., Hamann, M., Limpus, C.J., Lindeque, P.K., Mayes, A.G., Omeyer, L.C. and Santillo, D., (2019). Microplastic ingestion ubiquitous in marine turtles. *Global Change Biology*, *25*(2), pp.744-752.

Duffy, J. E. (2006). Biodiversity and the functioning of seagrass ecosystems. *Marine Ecology Progress Series,* 311, 233–250.

Edgar, G., Barrett, N., & Morton, A. (2004). Biases associated with the use of underwater visual census techniques to quantify the density and size-structure of fish populations. *Journal of Experimental Marine Biology and Ecology*, 308, 269–290.

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters,* 4(4), 423–425.

Foden, J. and Brazier, D.P., (2007.) Angiosperms (seagrass) within the EU water framework directive: a UK perspective. *Marine Pollution Bulletin*, 55(1-6), pp.181-195.

Fonseca, M. S., & Cahalan, J. A. (1992). A preliminary evaluation of wave attenuation by four species of seagrass. *Estuarine, Coastal and Shelf Science,* 35(6), 565–576.

Foote, A. D., Thomsen, P. F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L. A., Salling, A., Galatius, A., Orlando, L., & Gilbert, M. T. P. (2012). Investigating the Potential Use of Environmental DNA (eDNA) for Genetic Monitoring of Marine Mammals. *PLoS One,* 7(8), 2–7.

Fornes, A., Basterretxea, G., Orfila, A., Jordi, A., Alvarez, A., & Tintore, J. (2006). Mapping Posidonia oceanica from IKONOS. *ISPRS Journal of Photogrammetry and Remote Sensing,* 60, 315–322.

Fournier DA, Skaug HJ, Ancheta J, Ianelli J, Magnusson A, Maunder M, Nielsen A, Sibert J (2012). "AD Model Builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models." Optimum. *Methods Software*, 27, 233-249.

Fourqurean, J. W., Duarte, C. M., Kennedy, H., Marbà, N., Holmer, M., Mateo, M. A., Apostolaki, E., Kendrick, G., Krause-Jensen, D., McGlathery, K., & Serrano, O. (2012). Seagrass ecosystems as a globally significant carbon stock. *Nature Geoscience,* 5, 505–509.

Gacia, E., & Duarte, C. M. (2001). Sediment retention by a Mediterranean Posidonia oceanica meadow: The balance between deposition and resuspension. *Estuarine, Coastal and Shelf Science,* 52(4), 505–514.

Gago, J., Carretero, O., Filgueiras, A. V., & Viñas, L. (2018). Synthetic microfibers in the marine environment: A review on their occurrence in seawater and sediments. *Marine Pollution Bulletin*, 127, 365–376.

Gambi, M.C., Lorenti, M., Russo, G.F., Scipione, M.B. and Zupo, V., (1992). Depth and seasonal distribution of some groups of the vagile fauna of the Posidonia oceanica leaf stratum: structural and trophic analyses. *Marine Ecology*, *13*(1), pp.17-39.

Gamer, M., Lemon, J., Fellows, I., & Singh, P. (2019). irr: Various Coefficients of Interrater Reliability and Agreement. R package version 0.84.1. https://CRAN.R-project.org/package=irr

Garcés-Ordóñez, O., Castillo-Olaya, V. A., Granados-Briceño, A. F., Blandón García, L. M., & Espinosa Díaz, L. F. (2019). Marine litter and microplastic pollution on mangrove

soils of the Ciénaga Grande de Santa Marta, Colombian Caribbean. *Marine Pollution Bulletin,* 145, 455–462

García-March, J. R., Pérez-Rojas, L., & García-Carrascosa, A. M. (2007). Influence of hydrodynamic forces on population structure of *Pinna nobilis* L., 1758 (Mollusca: Bivalvia): The critical combination of drag force, water depth, shell size and orientation. *Journal of Experimental Marine Biology and Ecology,* 342(2), 202–212.

Giardino, C., Brando, V.E., Gege, P., Pinnel, N., Hochberg, E., Knaeps, E., Reusen, I., Doerffer, R., Bresciani, M., Braga, F. and Foerster, S., (20190. Imaging spectrometry of inland and coastal waters: state of the art, achievements and perspectives. *Surveys in Geophysics*, 40(3), pp.401-429.

Goffredo, S., Piccinetti, C., & Zaccanti, F. (2017). Volunteers in Marine Conservation Monitoring : a Study of the Distribution of Seahorses Carried Out in Collaboration with Recreational Scuba Divers. *Conservation Biology,* 18(6), 1492–1503.

González-Correa, J.M., Bayle, J.T., Sánchez-Lizaso, J.L., Valle, C., Sánchez-Jerez, P. and Ruiz, J.M., (2005). Recovery of deep *Posidonia oceanica* meadows degraded by trawling. *Journal of Experimental Marine Biology and Ecology*, 320(1), pp.65-76.

Goss, H., Jaskiel, J. and Rotjan, R., (2018). *Thalassia testudinum* as a potential vector for incorporating microplastics into benthic marine food webs. *Marine Pollution Bulletin*, *135*, pp.1085-1089.

Graca, B., Szewc, K., Zakrzewska, D., Dołęga, A., & Szczerbowska-Boruchowska, M. (2017). Sources and fate of microplastics in marine and beach sediments of the Southern Baltic Sea—a preliminary study. *Environmental Science and Pollution Research,* 24(8), 7650–7661.

Greene, A., Rahman, A. F., Kline, R., & Rahman, M. S. (2018). Side-scan sonar: A cost-efficient alternative method for measuring seagrass cover in shallow environments. *Estuarine, Coastal and Shelf Science,* 207(April), 250–258.

Gristina, M., Cardone, F., Desiderato, A., Mucciolo, S., Lazic, T. and Corriero, G., (2017). Habitat use in juvenile and adult life stages of the sedentary fish *Hippocampus guttulatus*. *Hydrobiologia*, 784(1), pp.9-19.

Groeneveld, R.A. and Meeden, G., (1984). Measuring skewness and kurtosis. *Journal of the Royal Statistical Society: Series D (The Statistician)*, 33(4), pp.391-399.

Grol, M. G. G., Grol, M. G. G., Dorenbosch, M., Kokkelmans, E. M. G., & Nagelkerken, I. (2008). Mangroves and seagrass beds do not enhance growth of early juveniles of a coral reef fish. *Marine Ecology Progress Series*, 366, 137–146.

Guidetti, P. (2000). Differences Among Fish Assemblages Associated with Nearshore *Posidonia oceanica* Seagrass Beds , Rocky – algal Reefs and Unvegetated Sand Habitats in the Adriatic Sea. *Estuarine, Coastal and Shelf Science*, 50, 515–529.

Guzzetti, E., Sureda, A., Tejada, S. and Faggio, C., (2018). Microplastic in marine organism: Environmental and toxicological effects. *Environmental Toxicology and Pharmacology*, *64*, pp.164-171.

Hassell, N. S., Williamson, D. H., Evans, R. D., & Russ, G. R. (2013). Reliability of Non-Expert Observer Estimates of the Magnitude of Marine Reserve Effects. *Coastal Management,* 41(4), 361–380.

Heck, K. L., Hays, G., & Orth, R. J. (2003). Critical evaluation of the nursery role hypothesis for seagrass meadows. *Marine Ecology Progress Series,* 253, 123–136.

Hendriks, I. E., Sintes, T., Bouma, T. J., & Duarte, C. M. (2008). Experimental assessment and modelling evaluation of the effects of the seagrass *Posidonia oceanica* on flow and particle trapping. *Marine Ecology Progress Series*, 356, 163–173.

Hendriks, I. E., Cabanellas-Reboredo, M., Bouma, T. J., Deudero, S., & Duarte, C. M. (2011). Seagrass Meadows Modify Drag Forces on the Shell of the Fan Mussel *Pinna nobilis*. *Estuaries and Coasts,* 34(1), 60–67.

Hendriks, I. E., Deudero, S., & Tavecchia, G. (2012). Recapture probability underwater: predicting the detection of the threatened noble pen shell in seagrass meadows. *Limnol. Oceanography: Methods*, 10, 824–831.

Hernandez-Milian, G., Lusher, A., MacGabban, S., & Rogan, E. (2019). Microplastics in grey seal (*Halichoerus grypus*) intestines: Are they associated with parasite aggregations? *Marine Pollution Bulletin,* 146, 349–354.

Hirai, H., Takada, H., Ogata, Y., Yamashita, R., Mizukawa, K., Saha, M., … Ward, M. W. (2011). Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Marine Pollution Bulletin,* 62(8), 1683–1692.

Horton, A. A., Svendsen, C., Williams, R. J., Spurgeon, D. J., & Lahive, E. (2017). Large microplastic particles in sediments of tributaries of the River Thames, UK – Abundance, sources and methods for effective quantification. *Marine Pollution Bulletin,* 114(1), 218–226.

Hossain, M. S., Bujang, J. S., Zakaria, M. H., & Hashim, M. (2014). The application of remote sensing to seagrass ecosystems: an overview and future research prospects. *International Journal of Remote Sensing,* 36(1), 61–114.

Huang, Y., Xiao, X., Xu, C., Perianen, Y. D., Hu, J., & Holmer, M. (2020). Seagrass beds acting as a trap of microplastics - Emerging hotspot in the coastal region? *Environmental Pollution,* 257, 113450.

Hughes, A. R., Williams, S. L., Duarte, C. M., Heck, K. L., & Waycott, M. (2009). Associations of concern: Declining seagrasses and threatened dependent species. *Frontiers in Ecology and the Environment,* 7, 242–246.

Hyndes, G.A., Platell, M.E., Potter, I.C. and Lenanton, R.C.J., (1999). Does the composition of the demersal fish assemblages in temperate coastal waters change with depth and undergo consistent seasonal changes? *Marine Biology*, 134(2), pp.335-352.

Infantes, E., Orfila, A., Simarro, G., Terrados, J., Luhar, M., & Nepf, H. (2012). Effect of a seagrass (*Posidonia oceanica*) meadow on wave propagation. *Marine Ecology Progress Series,* 456, 63–72.

Jackson, J.B., Kirby, M.X., Berger, W.H., Bjorndal, K.A., Botsford, L.W., Bourque, B.J., Bradbury, R.H., Cooke, R., Erlandson, J., Estes, J.A. and Hughes, T.P., (2001). Historical overfishing and the recent collapse of coastal ecosystems. *Science*, 293(5530), pp.629-637.

Jackson, E. L., Rees, S. E., Wilding, C., & Attrill, M. J. (2015). Use of a seagrass residency index to apportion commercial fishery landing values and recreation fisheries expenditure to seagrass habitat service. *Conservation Biology,* 29(3), 899–909.

Jane, S. F., Wilcox, T. M., Mckelvey, K. S., Young, M. K., Schwartz, K., Lowe, W. H., & Letcher, B. H. (2015). Distance , flow and PCR inhibition : eDNA dynamics in two headwater streams. *Marine Ecology Resources,* 15, 216–227.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, 4(2), 150–157.

Jiménez-Ramos, R., Egea, L.G., Vergara, J.J. and Brun, F.G., (2018). Nutrient load and epiphytes are drivers of increased herbivory in seagrass communities. *Marine Ecology Progress Series*, 599, pp.49-64.

Jinhui, S., Sudong, X., Yan, N., Xia, P., Jiahao, Q., & Yongjian, X. (2019). Effects of microplastics and attached heavy metals on growth, immunity, and heavy metal accumulation in the yellow seahorse, *Hippocampus kuda* Bleeker. *Marine Pollution Bulletin,* 149, 110510.

Joanes, D.N. and Gill, C.A., 1998. Comparing measures of sample skewness and kurtosis. *Journal of the Royal Statistical Society: Series D (The Statistician)*, 47(1), pp.183-189.

Johansen, J. L., Fulton, C. J., & Bellwood, D. R. (2007). Avoiding the flow: Refuges expand the swimming potential of coral reef fishes. *Coral Reefs,* 26(3), 577–583.

Jones, K. L., Hartl, M. G. J., Bell, M. C., & Capper, A. (2020). Microplastic accumulation in a *Zostera marina* L. bed at Deerness Sound, Orkney, Scotland. *Marine Pollution Bulletin*, 15, 110883.

Jordà, G., Marbà, N., & Duarte, C. M. (2012). Mediterranean seagrass vulnerable to regional climate warming*. Nature Climate Change,* 2, 821–824.

Jørgensen, L.L., Renaud, P.E. and Cochrane, S.K., (2011). Improving benthic monitoring by combining trawl and grab surveys*. Marine pollution bulletin*, 62(6), pp.1183-1190.

Kaeser, A., Litts, T., & Wesley Tracy, T. (2013). Using low cost Side-Scan sonar for benthic mapping throughout the lower Flint River, Georgia, USA. *River Research and Applications,* 29, 634–644.

Kalogirou, S., Corsini-Foka, M., Sioulas, A., Wennhage, H., & Pihl, L. (2010). Diversity, structure and function of fish assemblages associated with *Posidonia oceanica* beds in an area of the eastern Mediterranean Sea and the role of non-indigenous species. *Journal of Fish Biology,* 77, 2338–2357.

Kalogirou, S., Wennhage, H. and Pihl, L., (2012). Non-indigenous species in Mediterranean fish assemblages: Contrasting feeding guilds of *Posidonia oceanica* meadows and sandy habitats. *Estuarine, Coastal and Shelf Science*, 96, pp.209-218.

Kannan, R.R.R., Arumugam, R. and Anantharaman, P., (2010). Antibacterial potential of three seagrasses against human pathogens. *Asian Pacific Journal of Tropical Medicine*, 3(11), pp.890-893.

Kannan, R.R.R., Arumugam, R. and Anantharaman, P., (2012). Chemical composition and antibacterial activity of Indian seagrasses against urinary tract pathogens. *Food chemistry,* 135(4), pp.2470-2473.

Kassambara, A and Mundt, F (2020). factoextra: Extract and Visualize the Results of Multivariate Data Analyses. R package version 1.0.7. https://CRAN.R-project.org/package=factoextra

Katsanevakis, S. (2006) Population ecology of the endangered fan mussel *Pinna nobilis* in a marine lake. *Endangered Species Research* 1, 51– 59.

Katsanevakis, S., (2007). Growth and mortality rates of the fan mussel *Pinna nobilis* in Lake Vouliagmeni (Korinthiakos Gulf, Greece): a generalized additive modelling approach. *Marine Biology*, 152(6), pp.1319-1331.

Katsanevakis, S., & Thessalou-Legaki, M. (2009). Spatial distribution, abundance and habitat use of the protected fan mussel *Pinna nobilis* in Souda Bay, Crete. *Aquatic Biology,* 8(1), 45–54.

Katsanevakis, S., Weber, A., Pipitone, C., Leopold, M., Cronin, M., Scheidat, M., Doyle, T., Buhl-Mortensen, L., Buhl-Mortensen, P., D'Anna, G., de Boois, I., Dalpadado, P., Damalas, D., Fiorentino, F., Garfola. *et al.* (2012). Monitoring marine populations and communities: Methods dealing with imperfect detectability. *Aquatic Biology,* 16(1), 31–52.

Katsanevakis, S., Tsirintanis, K., Tsaparis, D., Doukas, D., Sini, M., Athanassopoulou, F., Kolygas, M., Tontis, D., Koutsoubas, D., & Bakopoulos, V. (2019). The cryptogenic parasite *Haplosporidium pinnae* invades the Aegean Sea and causes the collapse of *Pinna nobilis* populations. *Aquatic Invasions,* 14.

Katsares, V., Tsiora, A., Galinou-mitsoudi, S., & Imsiridou, A. (2008). Genetic structure of the endangered species *Pinna nobilis* (Mollusca: Bivalvia) inferred from mtDNA sequences. *Biologia*, 63, 412–417.

Kersting, D., Benabdi, M., Čižmek, H., Grau, A., Jimenez, C., Katsanevakis, S., Öztürk, B., Tuncer, S., Tunesi, L., Vázquez-Luis, M., Vicente, N. & Otero Villanueva, M. (2019). *Pinna nobilis*. The IUCN Red List of Threatened Species 2019

Kirkman, H., (1978). Decline of seagrass in northern areas of Moreton Bay, Queensland. *Aquat. Bot*. 5, 63–76.

Kirstein, I. V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., & Gerdts, G. (2016). Dangerous hitchhikers? Evidence for potentially pathogenic *Vibrio spp.* on microplastic particles. *Marine Environmental Research*, 120, 1–8.

Kitsos, M. S., Tzomos, T., Anagnostopoulou, L., & Koukouras, A. (2008). Diet composition of the seahorses, *Hippocampus guttulatus* Cuvier, 1829 and *Hippocampus hippocampus* (L., 1758) (Teleostei, Syngnathidae) in the Aegean Sea. *Journal of Fish Biology,* 72(6), 1259–1267.

Koch, E., Ackerman, J., Verduin, J., & Keulen, M. (2006). Fluid dynamics in seagrass ecology - from molecules to ecosystems. In *Seagrasses: Biology, Ecology and Conservation* (pp. 193–225). Springer.

Kohlus, J., Stelzer, K., Müller, G. and Smollich, S., (2020). Mapping seagrass (*Zostera*) by remote sensing in the Schleswig-Holstein Wadden Sea. *Estuarine, Coastal and Shelf Science*, p.106699.

Komatsu, T., Igarashi, C., Tatsukawa, K., Sultana, S., Matsuoka, Y., & Harada, S. (2003). Use of multi-beam sonar to map seagrass beds in Otsuchi Bay on the Sanriku Coast of Japan. *Aquatic Living Resources,* 16, 223–230.

Kovacs, E., Roelfsema, C., Lyons, M., Zhao, S. and Phinn, S., (2018). Seagrass habitat mapping: how do Landsat 8 OLI, Sentinel-2, ZY-3A, and Worldview-3 perform? *Remote Sensing Letters,* 9(7), pp.686-695.

Kuo, J., & den Hartog, C. (2007). Seagrass morphology, anatomy, and ultrastructure. In *SEAGRASSES: BIOLOGY, ECOLOGYAND CONSERVATION* (pp. 51-87). Springer, Dordrecht.

Kumar, C.S., Sarada, D.V., Gideon, T.P. and Rengasamy, R., (2008). Antibacterial activity of three South Indian seagrasses, *Cymodocea serrulata*, *Halophila ovalis* and *Zostera capensis*. *World Journal of Microbiology and Biotechnology*, 24(9), pp.1989-1992.

Lacoursière-Roussel, A. and Deiner, K., (2019). Environmental DNA is not the tool by itself. *Journal of fish biology*. 1– 4.

Lamb, J.B., Van De Water, J.A., Bourne, D.G., Altier, C., Hein, M.Y., Fiorenza, E.A., Abu, N., Jompa, J. and Harvell, C.D., (2017). Seagrass ecosystems reduce exposure to bacterial pathogens of humans, fishes, and invertebrates. *Science*, 355(6326), pp.731-733.

Landis, J., & Koch, G. (1977). The Measurement of Observer Agreement for Categorical Data. *Biometrics,* 33, 159-174

Lavery, P. S., Mateo, M. Á., Serrano, O., & Rozaimi, M. (2013). Variability in the Carbon Storage of Seagrass Habitats and Its Implications for Global Estimates of Blue Carbon Ecosystem Service. *PLoS ONE,* 8(9).

Lefebvre, A., Thompson, C. E. L., Collins, K. J., & Amos, C. L. (2009). Use of a high-resolution profiling sonar and a towed video camera to map a *Zostera marina* bed, Solent, UK. *Estuarine, Coastal and Shelf Science,* 82, 323–334.

Levy-Booth, D.J., Campbell, R.G., Gulden, R.H., Hart, M.M., Powell, J.R., Klironomos, J.N., Pauls, K.P., Swanton, C.J., Trevors, J.T. and Dunfield, K.E., (2007). Cycling of extracellular DNA in the soil environment. *Soil Biology and Biochemistry*, *39*(12), pp.2977-2991.

Li, J., Zhang, H., Zhang, K., Yang, R., Li, R., & Li, Y. (2018). Characterization, source, and retention of microplastic in sandy beaches and mangrove wetlands of the Qinzhou Bay, China. *Marine Pollution Bulletin,* 136, 401–406.

Lobelle, D., & Cunliffe, M. (2011). Early microbial biofilm formation on marine plastic debris. *Marine Pollution Bulletin,* 62(1), 197–200.

Lo Iacono, C., Mateo, M. A., & Guasch, L. (2008). Very high-resolution seismo-acoustic imaging of seagrass meadows (Mediterranean Sea): Implications for carbon sink estimates. *Geophysical Research Letters*, 35, 1–5.

Longstaff, B. J., & Dennison, W. C. (1999). Seagrass survival during pulsed turbidity events: the effects of light deprivation on the seagrasses *Halodule pinifolia* and *Halophila ovalis*. *Aquatic Botany*, 65(1-4), 105-121

López-Sanmartín, M., Catanese, G., Grau, A., Valencia, J. M., García-March, J. R., & Navas, J. I. (2019). Real-Time PCR based test for the early diagnosis of *Haplosporidium pinnae* affecting fan mussel *Pinna nobilis. PLoS ONE,* 14(2), 1–12.

Luczkovich J, Sprague M, Krahforst CS, Walsh J, Carpenter D (2013) Acoustics and estuarine ecology: using active and passive methods to survey the physical environment, vegetation and animals in North Carolina's coastal estuaries. *Proceedings of Meetings on Acoustics* 19:005006

Lusher, A. L., Tirelli, V., O'Connor, I., & Officer, R. (2015). Microplastics in Arctic polar waters: The first reported values of particles in surface and sub-surface samples. *Scientific Reports,* 5(1), 1–9.

Lyons, M., Phinn, S., & Roelfsema, C. (2011). Integrating Quickbird Multi-Spectral Satellite and Field Data: Mapping Bathymetry, Seagrass Cover, Seagrass Species, and Change in Moreton Bay, Australia in 2000 and 2007. *Remote Sensing,* 3, 42–64.

Macreadie, P. I., Trevathan-tackett, S. M., Skilbeck, C. G., Sanderman, J., Curlevski, N., Jacobsen, G., & Seymour, J. R. (2015). Losses and recovery of organic carbon from a seagrass ecosystem following disturbance. *Proceedings of the Royal Society B,* 282.

Marbà, N., Díaz-Almela, E., & Duarte, C. M. (2014). Mediterranean seagrass (*Posidonia oceanica*) loss between 1842 and 2009. *Biological Conservation,* 176, 183–190.

Martin, J., Lusher, A., Thompson, R. C., & Morley, A. (2017). The Deposition and Accumulation of Microplastics in Marine Sediments and Bottom Water from the Irish Continental Shelf. *Scientific Reports,* 7(1), 1–9.

Mateo, M. A., Romero, J., Pérez, M., Littler, M., & Little, D. (1997). Dynamics of Millenary Organic Deposits Resulting from the Growth of the Mediterranean Seagrass *Posidonia oceanica*. *Estuarine, Coastal and Shelf Science,* 44, 103–110.

Mayavu, P., Sugesh, S. and Ravindran, V.J., (2009). Antibacterial activity of seagrass species against biofilm forming bacteria. *Research Journal of Microbiology*, 4(8), pp.314-319.

McKinley, D.C., Miller-Rushing, A.J., Ballard, H.L., Bonney, R., Brown, H., Cook-Patton, S.C., Evans, D.M., French, R.A., Parrish, J.K., Phillips, T.B. and Ryan, S.F., (2017). Citizen science can improve conservation science, natural resource management, and environmental protection. *Biological Conservation*, 208, pp.15-28.

McKenzie, L. J., Finkbeiner, M. A., & Kirkman, H. (2001). Methods for mapping seagrass distribution. *Global seagrass research methods*, 101-121

McKenzie, L.J., (2003). Guidelines for the rapid assessment and mapping of tropical seagrass habitats. Department of Primary Industries. The State of Queensland, pp.17-18.

McKenzie, L., Nordlund, L.M., Jones, B.L., Cullen-Unsworth, L.C., Roelfsema, C.M. and Unsworth, R., (2020). The global distribution of seagrass meadows. Environmental Research Letters.

Mcleod, E., Chmura, G. L., Bouillon, S., Salm, R., Björk, M., Duarte, C. M., Lovelock, C., Schlesinger, W., & Silliman, B. R. (2011). A blueprint for blue carbon: toward an improved understanding of the role of vegetated coastal habitats in sequestering CO2. *Frontiers in Ecology and the Environment,* 9, 552–560.

MacNeil, M. A., Graham, N. A. J., Conroy, M. J., Fonnesbeck, C. J., Polunin, N. V. C., Rushton, S. P., Chabanet, P., McClanahan, T. R. (2008). Detection heterogeneity in underwater visual-census data. *Journal of Fish Biology,* 73(7), 1748–1763.

Mallet, D. and Pelletier, D., (2014). Underwater video techniques for observing coastal marine biodiversity: a review of sixty years of publications (1952–2012). *Fisheries Research*, *154*, pp.44-62.

Manca, E., Cáceres, I., Alsina, J. M., Stratigaki, V., Townend, I., & Amos, C. L. (2012). Wave energy and wave-induced flow reduction by full-scale model *Posidonia oceanica* seagrass. *Continental Shelf Research*, 50–51, 100–116.

Matsuhashi, S., Doi, H., Fujiwara, A., Watanabe, S. and Minamoto, T., (2016). Evaluation of the environmental DNA method for estimating distribution and biomass of submerged aquatic plants. *PloS one*, *11*(6), p.e0156217.

Mauvisseau, Q., Parrondo, M., Fernández, M. P., García, L., Martínez, J. L., García-Vázquez, E., & Borrell, Y. J. (2017). On the way for detecting and quantifying elusive species in the sea: The *Octopus vulgaris* case study. *Fisheries Research*, 191(March), 41–48.

Mayot, N., Boudouresque, C.F., & Charbonnel, E. (2006). Changes over time of shoot density of the Mediterranean seagrass *Posidonia oceanica* at its depth limit. *Biologia marina mediterranea*, 13(4).

Micallef, A., Le Bas, T.P., Huvenne, V.A., Blondel, P., Hühnerbach, V. and Deidun, A., (2012). A multi-method approach for benthic habitat mapping of shallow coastal areas with high-resolution multibeam data. *Continental Shelf Research*, 39, pp.14-26.

Milazzo, M., Badalamenti, F., Ceccherelli, G., & Chemello, R. (2004). Boat anchoring on *Posidonia oceanica* beds in a marine protected area (Italy, western Mediterranean): Effect of anchor types in different anchoring stages. *Journal of Experimental Marine Biology and Ecology,* 299, 51–62.

Millar, R.B. and Fryer, R.J., (1999). Estimating the size-selection curves of towed gears, traps, nets and hooks. *Reviews in Fish Biology and Fisheries*, *9*(1), pp.89-116.

Mistri, M., Infantini, V., Scoponi, M., Granata, T., Moruzzi, L., Massara, F., … Munari, C. (2018). Microplastics in marine sediments in the area of Pianosa Island (Central Adriatic Sea). *Rendiconti Lincei,* 29(4), 805–809.

Molenaar, H., Barthélémy, D., De Reffye, P., Meinesz, A., & Mialet, I. (2000). Modelling architecture and growth patterns of *Posidonia oceanica*. *Aquatic Botany,* 66, 85–99.

Monk, J. (2014). How long should we ignore imperfect detection of species in the marine environment when modelling their distribution? *Fish and Fisheries,* 15, 352–358.

Montefalcone, M. (2009). Ecosystem health assessment using the Mediterranean seagrass *Posidonia oceanica*: A review. *Ecological Indicators*, 9(4), 595–604.

Montefalcone, M., Rovere, A., Parravicini, V., Albertelli, G., Morri, C. and Bianchi, C.N., (2014). Reprint of "Evaluating change in seagrass meadows: A time-framed comparison of Side-scan Sonar maps". *Aquatic Botany*, 115, pp.36-44.

Moranta, J., Palmer, M., Ruiz, A., Morey, G., & Morales-nin, B. (2006). Multi-scale spatial variability in fish assemblages associated with *Posidonia oceanica* meadows in the Western Mediterranean Sea. *Estuarine, Coastal and Shelf Science*, 68, 579–592.

Mork, G., Barstow, S., Kabuth, A. and Pontes, M.T., (2010, January). Assessing the global wave energy potential. *In International Conference on Offshore Mechanics and Arctic Engineering* (Vol. 49118, pp. 447-454).

Mtwana Nordlund, L., Koch, E. W., Barbier, E. B., & Creed, J. C. (2016). Seagrass ecosystem services and their variability across genera and geographical regions. *PLoS ONE,* 11(10), 1–23.

Murphy, H. M., & Jenkins, G. P. (2010). Observational methods used in marine spatial monitoring of fishes and associated habitats: A review. *Marine and Freshwater Research,* 61, 236–252.

Murray, A. B., & Robert Thieler, E. (2004). A new hypothesis and exploratory model for the formation of large-scale inner-shelf sediment sorting and "'rippled scour depressions.'". *Continental Shelf Research*, 24, 295–315.

Naik, R. K., Naik, M. M., D'Costa, P. M., & Shaikh, F. (2019, December 1). Microplastics in ballast water as an emerging source and vector for harmful chemicals, antibiotics, metals, bacterial pathogens and HAB species: A potential risk to the marine environment and human health. *Marine Pollution Bulletin*, *149*, p.110525.

Naji, A., Nuri, M., Amiri, P. and Niyogi, S., (2019). Small microplastic particles (S-MPPs) in sediments of mangrove ecosystem on the northern coast of the Persian Gulf. *Marine pollution bulletin,* 146, pp.305-311.

Nelms, Sarah E., Galloway, T. S., Godley, B. J., Jarvis, D. S., & Lindeque, P. K. (2018). Investigating microplastic trophic transfer in marine top predators. *Environmental Pollution*, 238, 999–1007.

Nelms, S.E., Barnett, J., Brownlow, A., Davison, N.J., Deaville, R., Galloway, T.S., Lindeque, P.K., Santillo, D. and Godley, B.J., 2019. Microplastics in marine mammals stranded around the British coast: ubiquitous but transitory?. *Scientific Reports*, *9*(1), pp.1-8.

Nor, N. H. M., & Obbard, J. P. (2014). Microplastics in Singapore's coastal mangrove ecosystems. *Marine Pollution Bulletin,* 79, 278–283.

Nordlund, L.M., Jackson, E.L., Nakaoka, M., Samper-Villarreal, J., Beca-Carretero, P. and Creed, J.C., (2018). Seagrass ecosystem services–What's next?. *Marine pollution bulletin,* 134, pp.145-151.

Novomestky, F and Nadarajah, S (2016). truncdist: Truncated Random Variables. R package version 1.0-2. https://CRAN.R-project.org/package=truncdist

Omnexus- the material selection platform. Accessed June 2020 at https://omnexus.specialchem.com/polymer-properties/properties/density

Öndes, F., Kaiser, M. J., & Güçlüsoy, H. (2020). Human impacts on the endangered fan mussel, *Pinna nobilis. Aquatic Conservation: Marine and Freshwater Ecosystems*, 30(1), 31–41.

Ooi, J.L.S., Kendrick, G.A., Van Niel, K.P. and Affendi, Y.A., (2011). Knowledge gaps in tropical Southeast Asian seagrass systems. *Estuarine, Coastal and Shelf Science*, 92(1), pp.118-131.

Orth, R.J. and Van Montfrans, J., (1984). Epiphyte-seagrass relationships with an emphasis on the role of micrograzing: a review. *Aquatic Botany*, 18(1-2), pp.43-69.

Orth, R. J., Carruthers, T. J. B., Dennison, W. C., Duarte, C. M., Fourqurean, J. W., Heck, K. L., Hughes, R., Kendrick, G., Kenworthy, W., Olyarnik, S., Short, F., Waycott, M., & Williams, S. L. (2006). A Global Crisis for Seagrass Ecosystems. *Bioscience,* 56, 987–996.

Pasqualini, V., Pergent-martini, C., Clabaut, P., & Pergent, G. (1998). Mapping of *Posidonia oceanica* using Aerial Photographs and Side-scan Sonar : Application off the Island of Corsica (France). *Estuarine, Coastal and Shelf Science,* 47, 359–367.

Paul, M., & Amos, C. L. (2011). Spatial and seasonal variation in wave attenuation over Zostera noltii. *Journal of Geophysical Research: Oceans*, 116(8), 1–16.

Pergent, G., Pergent-Martini, C., & Boudouresque, C. F. (1995). Utilisation de l'herbier a Posidonia oceanica comme indicateur biologique de la qualite milieu littoral en Mediterrenee etat des connaissance. *Mesogee,* 54, 3–27

Pergent, G., Bazairi, H., Bianchi, C. N., Boudouresque, C. F., Buia, M. C., Calvo, S., Clabaut, P., Harmelin-Vivien, M., Mateo, M., Monteflcone, M., Morri, C., Orfanidis, S., Pergent-Martini, C., Semroud, S. *et al.* (2014). Climate change and Mediterranean seagrass meadows : A synopsis for environmental managers. *Mediterranean Marine Science,* 462–473.

Pergent, G., Monnier, B., Clabaut, P., Gascon, G., Pergent-Martini, C. and Valette-Sansevin, A., (2017). Innovative method for optimizing Side-Scan Sonar mapping: The blind band unveiled. *Estuarine, Coastal and Shelf Science*, 194, pp.77-83.

Pham, T.D., Xia, J., Ha, N.T., Bui, D.T., Le, N.N. and Tekeuchi, W., (2019). A review of remote sensing approaches for monitoring blue carbon ecosystems: mangroves, seagrasses and salt marshes during 2010–2018. *Sensors,* 19(8), p.1933.

Piazzi, L., Acunto, S. and Cinelli, F., (2000). Mapping of *Posidonia oceanica* beds around Elba Island (western Mediterranean) with integration of direct and indirect methods. *Oceanologica acta*, 23(3), pp.339-346.

Pilliod, D. S., Goldberg, C. S., Arkle, R. S., & Waits, L. P. (2014). Factors influencing detection of eDNA from a stream-dwelling amphibian. *Molecular Ecology Resources*, 14(1), 109–116

Pisano, A., Marullo, S., Artale, V., Falcini, F., Yang, C., Leonelli, F.E., Santoleri, R. and Buongiorno Nardelli, B., (2020). New Evidence of Mediterranean Climate Change and Variability from Sea Surface Temperature Observations. *Remote Sensing*, 12(1), p.132.

Pollom, R. 2016. *Hippocampus hippocampus*. The IUCN Red List of Threatened Species 2016: e.T10069A90866381. Downloaded on 05 July 2020.

Pollom, R. 2017. *Hippocampus guttulatus*. The IUCN Red List of Threatened Species 2017: e.T41006A67617766. https://dx.doi.org/10.2305/IUCN.UK.2017-3.RLTS.T41006A67617766.en. Downloaded on 06 July 2020.

Pontius Jr, R. G., & Millones, M. (2011). Death to Kappa: birth of quantity disagreement and allocation disagreement for accuracy assessment. *International Journal of Remote Sensing,* 32(15), 4407-4429

Prado, P., Andree, K. B., Trigos, S., Carrasco, N., Caiola, N., García-March, J. R., Tena, J., Fernandez-Tejedor, M., & Carella, F. (2020). Breeding, planktonic and settlement factors shape recruitment patterns of one of the last remaining major population of *Pinna nobilis* within Spanish waters. *Hydrobiologia*, 847(3), 771–786.

Pu, R., Bell, S., Meyer, C., Baggett, L., & Zhao, Y. (2012). Mapping and assessing seagrass along the western coast of Florida using Landsat TM and EO-1 ALI / Hyperion imagery. *Estuarine, Coastal and Shelf Science,* 115, 234–245.

Qi, S.H., Zhang, S., Qian, P.Y. and Wang, B.G., (2008). Antifeedant, antibacterial, and antilarval compounds from the South China Sea seagrass *Enhalus acoroides*. *Botanica Marina*, 51(5), pp.441-447.

Qiagen.com https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/dna-clean-up/qiaquick-pcr-purification-kit/?clear=true#orderinginformation. Accessed 09/07/2020

Qiu, F., Chen, J.M., Croft, H., Li, J., Zhang, Q., Zhang, Y. and Ju, W., (2019). Retrieving leaf chlorophyll content by incorporating variable leaf surface reflectance in the PROSPECT model. *Remote Sensing*, 11(13), p.1572.

Rabaoui, L., Belgacem, W., Ben Ismail, D., Mansour, L., & Tlig-Zouari, S. (2015). Engineering effect of *Pinna nobilis* shells on benthic communities. *Oceanologia*, 57(3), 271–279.

Radomski, P., & Holbrook, B. (2015). A comparison of two hydroacoustic methods for estimating submerged macrophyte distribution and abundance : A cautionary note. *Journal of Aquatic Plant Management,* 53, 151–159

Rahnemoonfar, M., Rahman, A.F., Kline, R.J. and Greene, A., (2018). Automatic seagrass disturbance pattern identification on sonar images. *IEEE Journal of Oceanic Engineering,* 44(1), pp.132-141.

R Core Team. (2019). R: A language and environment for statistical computing (Version 4.0.0). Retrieved from http://www.R-project.org

Richardson, C.A., Kennedy, H., Duarte, C.M., Kennedy, D.P. and Proud, S.V., (1999). Age and growth of the fan mussel *Pinna nobilis* from south-east Spanish Mediterranean seagrass (*Posidonia oceanica*) meadows. *Marine Biology*, 133(2), pp.205-212.

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, 51(5), 1450–1459.

Remy, F., Collard, F., Gilbert, B., Compère, P., Eppe, G., & Lepoint, G. (2015). When Microplastic Is Not Plastic: The Ingestion of Artificial Cellulose Fibers by Macrofauna Living in Seagrass Macrophytodetritus. *Environmental Science and Technology,* 49(18), 11158–11166.

Renshaw, M. A., Olds, B. P., Jerde, C. L., Mcveigh, M. M., & Lodge, D. M. (2015). The room temperature preservation of filtered environmental DNA samples and assimilation into a phenol-chloroform-isoamyl alcohol DNA extraction. In Molecular Ecology Resources (Vol. 15, pp. 168–176). Blackwell Publishing Ltd.

Rita, H., & Komonen, A. (2008, February). Odds ratio: an ecologically sound tool to compare proportions. *Annales Zoologici Fennici*, 45, 66-72.

Roelfsema, C. M., Lyons, M., Kovacs, E. M., Maxwell, P., Saunders, M. I., Samper-villarreal, J., & Phinn, S. R. (2014). Multi-temporal mapping of seagrass cover, species and biomass : A semi-automated object based image analysis approach. *Remote Sensing of Environment,* 150, 172–187

Ruiz, J.M., Pérez, M. and Romero, J., (2001). Effects of fish farm loadings on seagrass (*Posidonia oceanica*) distribution, growth and photosynthesis. *Marine Pollution Bulletin*, 42(9), pp.749-760.

Ruiz-Frau, A., Gelcich, S., Hendriks, I.E., Duarte, C.M. and Marbà, N., (2017). Current state of seagrass ecosystem services: research and policy integration. *Ocean & Coastal Management,* 149, pp.107-115.

Sagawa, T., Mikami, A., Komatsu, T., Kosaka, N., Kosako, A., & Miyazaki, S. (2008). Mapping seagrass beds using IKONOS satellite image and Side-scan sonar measurements : a Japanese case study. *International Journal of Remote Sensing*, 1161, 281–291.

Sanchez, P., Demestre, M. and De Juan, S. (2010). Trophic relationship between *Loligo vulgaris* and *Posidonia oceanica* in Catalan waters (NW Mediterranean). Rapp. Comm. int. Mer Médit., 39. 656

Sanna, D., Cossu, P., Dedola, G. L., Scarpa, F., Maltagliati, F., Castelli, A., Franzoi, P., Lai, T., Cristo, B., Curini-Gallentti, M., Francalacci, P., & Casu, M. (2013). Mitochondrial DNA Reveals Genetic Structuring of *Pinna nobilis* across the Mediterranean Sea. *PLoS ONE,* 8(6).

Sassoubre, L.M., Yamahara, K.M., Gardner, L.D., Block, B.A. and Boehm, A.B., (2016). Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environmental science & technology*, *50*(19), pp.10456-10464.

Seeley, M.E., Song, B., Passie, R. and Hale, R.C., (2020). Microplastics affect sedimentary microbial communities and nitrogen cycling. Nature communications, 11(1), pp.1-10.

Serrano, O., Mateo, M.A., Dueñas-Bohórquez, A., Renom, P., López-Sáez, J.A. and Cortizas, A.M., (2011). The Posidonia oceanica marine sedimentary record: A Holocene archive of heavy metal pollution. Science of the Total Environment, 409(22), pp.4831-4840.

Serrano, O., Mateo, M. A., & Brugués, R. J. (2012). Characterization of soils beneath a *Posidonia oceanica* meadow. *Geoderma,* 185–186, 26–36.

Serrano, O., Lavery, P.S., Rozaimi, M. and Mateo, M.Á., (2014). Influence of water depth on the carbon sequestration capacity of seagrasses. Global Biogeochemical Cycles, 28(9), pp.950-961.

Sheaves, M., (2005). Nature and consequences of biological connectivity in mangrove systems. Marine Ecology Progress Series, 302, pp.293-305.

Short, F.T. and Coles, R.G. eds., (2001). Global seagrass research methods. Elsevier.

Short, F., Carruthers, T., Dennison, W., & Waycott, M. (2007). Global seagrass distribution and diversity: A bioregional model. *Journal of Experimental Marine Biology and Ecology,* 350, 3–20.

Skaug H, Fournier D, Bolker B, Magnusson A, Nielsen A (2016). Generalized Linear Mixed Models using 'AD Model Builder' R package version 0.8.3.3

Somerton, D.A., Munro, P.T. and Weinberg, K.L., (2007). Whole-gear efficiency of a benthic survey trawl for flatfish. *Fishery Bulletin,* 105(2), pp.278-291.

Stelzer, K., (1998). Acquisition of the sediment distribution of the Schleswig Holstein Wadden Sea using multispectral remote sensing data. PhD Thesis. *University Trier.*

Stoner, A. W. (1980). The Role of Seagrass Biomass in the Organization of Benthic Macrofaunal Assemblages. *Bulletin of Marine Science*, 30, 537–551

Stratigaki, V., Manca, E., Prinos, P., Losada, I. J., Lara, J. L., Sclavo, M., Amos, C., Caceres,I., & Sánchez-Arcilla, A. (2011). Large-scale experiments on wave propagation over Posidonia oceanica. *Journal of Hydraulic Research*, 49, 31–43.

Sutton, R., Mason, S. A., Stanek, S. K., Willis-Norton, E., Wren, I. F., & Box, C. (2016). Microplastic contamination in the San Francisco Bay, California, USA. Marine Pollution Bulletin, 109(1), 230–235.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H. and Kawabata, Z.I., (2012). Estimation of fish biomass using environmental DNA. *PloS One*, *7*(4), p.e35868.

Takeuchi, A., Watanabe, S., Yamamoto, S., Miller, M.J., Fukuba, T., Miwa, T., Okino, T., Minamoto, T. and Tsukamoto, K., (2019). First use of oceanic environmental DNA to study the spawning ecology of the Japanese eel *Anguilla japonica*. *Marine Ecology Progress Series*, 609, pp.187-196.

Tang, Y., Horikoshi, M and Li, W. "ggfortify: Unified Interface to Visualize Statistical Result of Popular R Packages." *The R Journal* 8.2 (2016): 478-489

Taylor, M.D., Baker, J. and Suthers, I.M., (2013). Tidal currents, sampling effort and baited remote underwater video (BRUV) surveys: are we drawing the right conclusions? *Fisheries Research*, 140, pp.96-104.

Telesca, L., Belluscio, A., Criscoli, A., Ardizzone, G., Apostolaki, E. T., Fraschetti, S., Salomidi, M. (2015). Seagrass meadows (*Posidonia oceanica*) distribution and trajectories of change. *Scientific Reports*, 5, 12505.

Terrados, J., & Duarte, C. M. (2000). Experimental evidence of reduced particle resuspension within a seagrass (*Posidonia oceanica* L.) meadow. *Journal of Experimental Marine Biology and Ecology,* 243(1), 45–53.

Thompson, R.C., Moore, C.J., Vom Saal, F.S. and Swan, S.H., (2009). Plastics, the environment and human health: current consensus and future trends. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), pp.2153-2166.

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W., McGonigle, D. and Russell, A.E., (2004). Lost at sea: where is all the plastic? *Science*, *304*(5672), p.838.

Thorhaug, A., Richardson, A.D. and Berlyn, G.P., (2006). Spectral reflectance of *Thalassia testudinum* (Hydrocharitaceae) seagrass: low salinity effects. *American Journal of Botany*, 93(1), pp.110-117.

Tillotson, M. D., Kelly, R. P., Duda, J. J., Hoy, M., Kralj, J., & Quinn, T. P. (2018). Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. *Biological Conservation*, 220, 1–11.

Tomas, F., Turon, X. and Romero, J., (2005). Effects of herbivores on a *Posidonia oceanica* seagrass meadow: importance of epiphytes. *Marine Ecology Progress Series*, 287, pp.115-125.

Topouzelis, K., Makri, D., Stoupas, N., Papakonstantinou, A., & Katsanevakis, S. (2018). Seagrass mapping in Greek territorial waters using Landsat-8 satellite images. *International Journal of Applied Earth Observation and Geoinformation*, 67, 98–113.

Trigos, S., García-March, J. R., Vicente, N., Tena, J., & Torres, J. (2014). Utilization of muddy detritus as organic matter source by the fan mussel *Pinna nobilis*. *Mediterranean Marine Science*, 15(3), 667–674.

Turner, C. R., Barnes, M. A., Xu, C. C. Y., Jones, S. E., Jerde, C. L., & Lodge, D. M. (2014). Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods in Ecology and Evolution,* 5(7), 676–684.

Turner, C. R., Uy, K. L., & Everhart, R. C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. *Biological Conservation*, 183, 93–102.

Tuya, F., Haroun, R., & Espino, F. (2014). Economic assessment of ecosystem services: Monetary value of seagrass meadows for coastal fisheries. *Ocean and Coastal Management,* 96, 181–187.

Tyllianakis, E., Callaway, A., Vanstaen, K., & Luisetti, T. (2019). The value of information: Realising the economic benefits of mapping seagrass meadows in the British Virgin Islands. *Science of the Total Environment,* 650, 2107–2116.

Ulaş, A., Göktürk, D. and Gül, B., (2019). Preferences of the Common Octopus *Octopus vulgaris* Cuvier, 1797 (Cephalopoda: *Octopodidae*) to Artificial Nests Placed in Different Habitats at Urla Islands, Aegean Sea, Turkey. *Acta Zoologıca Bulgarıca*, *71*(3), pp.453-462.

Umit Gumusay, M., Bakirman, T., Tuney Kizilkaya, I., Onur, N., & Onur Aykut, N. (2019). A review of seagrass detection, mapping and monitoring applications using acoustic systems. *European Journal of Remote Sensing,* 52, 1–29.

Urban-Malinga, B., Zalewski, M., Jakubowska, A., Wodzinowski, T., Malinga, M., Pałys, B. and Dąbrowska, A., (2020). Microplastics on sandy beaches of the southern Baltic Sea. *Marine Pollution Bulletin*, 155, p.111170.

van Katwijk, M. M., Bos, A. R., Hermus, D. C. R., & Suykerbuyk, W. (2010). Sediment modification by seagrass beds: Muddification and sandification induced by plant cover and environmental conditions*. Estuarine, Coastal and Shelf Science,* 89(2), 175–181.

Vassallo, P., Paoli, C., Rovere, A., Montefalcone, M., Morri, C., & Bianchi, C. N. (2013). The value of the seagrass *Posidonia oceanica:* A natural capital assessment. *Marine Pollution Bulletin*, 75, 157–167.

Vázquez-Luis, M., Banach-Esteve, G., Álvarez, E., & Deudero, S. (2014). Colonization on *Pinna nobilis* at a marine protected area: extent of the spread of two invasive seaweeds . *Journal of the Marine Biological Association of the United Kingdom*, 94(5), 857–864.

Vázquez-Luis, M., Álvarez, E., Barrajón, A., García-March, J. R., Grau, A., Hendriks, I. E., Jimenez, S., Kersting, D., Moreno, D., Perez, M., Ruiz, J., Sanchez, J., Villalba, A., & Deudero, S. (2017). S.O.S. *Pinna nobilis*: A Mass Mortality Event in Western Mediterranean Sea. *Frontiers in Marine Science*, 4, 1–6.

Veettil, B.K., Ward, R.D., Lima, M.D.A.C., Stankovic, M., Hoai, P.N. and Quang, N.X., (2020). Opportunities for seagrass research derived from remote sensing: A review of current methods. *Ecological Indicators*, 117, p.106560.

Vianello, A., Boldrin, A., Guerriero, P., Moschino, V., Rella, R., Sturaro, A., & Da Ros, L. (2013). Microplastic particles in sediments of Lagoon of Venice, Italy: First observations on occurrence, spatial patterns and identification. *Estuarine, Coastal and Shelf Science,* 130, 54–61.

Viršek, M. K., Lovšin, M. N., Koren, Š., Kržan, A., & Peterlin, M. (2017). Microplastics as a vector for the transport of the bacterial fish pathogen species *Aeromonas salmonicida*. *Marine Pollution Bulletin*, 125(1–2), 301–309

Vlachopoulou, E. I., Wilson, A., & Miliou, A. (2013). Disconnects in EU and Greek fishery policies and practices in the eastern Aegean Sea and impacts on *Posidonia oceanica* meadows. *Ocean and Coastal Management*, 76, 105–113.

Voultsiadou, E., Koutsoubas, D., & Achparaki, M. (2010). Bivalve mollusc exploitation in Mediterranean coastal communities: an historical approach. *Journal of Biological Research – Thessaloniki,* 13, 35–45.

Wan, Z., Wang, C., Zhou, J., Shen, M., Wang, X., Fu, Z. and Jin, Y., (2019). Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. *Chemosphere*, *217*, pp.646-658.

Wang, Y., Zhang, D., Zhang, M., Mu, J., Ding, G., Mao, Z., Cao, Y., Jin, F., Cong, Y., Wang, L. and Zhang, W., (2019). Effects of ingested polystyrene microplastics on brine shrimp, *Artemia parthenogenetica*. *Environmental Pollution*, *244*, pp.715-722.

Wang, J., Li, Y., Lu, L., Zheng, M., Zhang, X., Tian, H., Wang, W. and Ru, S., (2019b). Polystyrene microplastics cause tissue damages, sex-specific reproductive disruption and transgenerational effects in marine medaka (*Oryzias melastigma*). *Environmental Pollution*, 254, p.113024.

Ward, T. J., Vanderklift, M. A., Nicholls, A. O., & Kenchington, R. A. (1999). Selecting marine reserves using habitats and species assemblages as surrogates for biological diversity. *Ecological Applications*, 9(2), 691–698.

Ward, E., Meek, S., Gordon, D., Cameron, T., Steer, M., Smith, D., Miliou, A. and Tsimpidis, T., (2020). The use of storm fragments and biodegradable replanting methods allows

for a low-impact habitat restoration method of seagrass meadows, in the eastern Aegean Sea. *Conservation Evidence*, 17.

Warren, J., & Peterson, B. J. (2007). Use of a 600-kHz Acoustic Doppler Current Profiler to measure estuarine bottom type, relative abundance of submerged aquatic vegetation, and eelgrass canopy height. *Estuarine, Coastal and Shelf Science,* 72, 53–62.

Waycott, M., Duarte, C. M., Carruthers, T. J. B., Orth, R. J., Dennison, W. C., Olyarnik, S., Calladine, A., Fourqurean, J., Heck, K., Hughes, R., Kendrick, G., Kenowrthy, W., Short, F., & Williams, S. L. (2009). Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *Proceedings of the National Academy of Sciences, USA,* 106(30), 12377–12381.

Weinstein, J.E., Crocker, B.K. and Gray, A.D., (2016). From macroplastic to microplastic: Degradation of high-density polyethylene, polypropylene, and polystyrene in a salt marsh habitat. *Environmental Toxicology and Chemistry*, 35(7), pp.1632-1640.

Weldon, L., O'Leary, C., Steer, M., Newton, L., Macdonald, H., & Sargeant, S. L. (2020). A comparison of European eel *Anguilla anguilla* eDNA concentrations to fyke net catches in five Irish lakes. *Environmental DNA,* edn3.91.

Wells, S. 1996. *Tridacna rosewateri*. The IUCN Red List of Threatened Species 1996: e.T22139A9362805. https://dx.doi.org/10.2305/IUCN.UK.1996.RLTS.T22139A9362805.en Downloaded on 09 July 2020.

White, S., Faulk, E., Tzilkowski, C., Weber, A., Marshall, M. and Wagner, T., (2020) Predicting fish species richness and habitat relationships using Bayesian hierarchical multispecies occupancy models. *Canadian Journal of Fisheries and Aquatic Sciences,* 77(3), pp.602-610.

Wickham. H (2016) ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York

Williams, K.E., Huyvaert, K.P. and Piaggio, A.J., 2016. No filters, no fridges: a method for preservation of water samples for eDNA analysis. *BMC Research Notes*, 9(1), pp.1-5.

Willis, T.J., Millar, R.B. and Babcock, R.C., 2000. Detection of spatial variability in relative density of fishes: comparison of visual census, angling, and baited underwater video. *Marine Ecology Progress Series*, 198, pp.249-260.

Wood, S.A., Pochon, X., Ming, W., von Ammon, U., Woods, C., Carter, M., Smith, M., Inglis, G. and Zaiko, A., 2019. Considerations for incorporating real-time PCR assays into routine marine biosecurity surveillance programmes: a case study targeting the Mediterranean fanworm (*Sabella spallanzanii*) and club tunicate (*Styela clava*). *Genome*, 62(3), pp.137-146.

Woodall, L. 2012. *Hippocampus hippocampus*. The IUCN Red List of Threatened Species 2012: e.T10069A17338787. https://dx.doi.org/10.2305/IUCN.UK.2012.RLTS.T10069A17338787.en Downloaded on 05 July 2020

Wressnig, A. and Booth, D.J., 2007. Feeding preferences of two seagrass grazing monacanthid fishes. *Journal of Fish Biology*, 71(1), pp.272-278.

Wu, N., Zhang, Y., Li, W., Wang, J., Zhang, X., He, J., Li, J., Ma, Y. and Niu, Z., (2020). Co-effects of biofouling and inorganic matters increased the density of environmental microplastics in the sediments of Bohai Bay coast. *Science of the Total Environment*, p.134431.

Xia, Z., Zhan, A., Gao, Y., Zhang, L., Haffner, G. D., & MacIsaac, H. J. (2018). Early detection of a highly invasive bivalve based on environmental DNA (eDNA). *Biological Invasions,* 20(2), 437–447

Zhang, C., Zhou, H., Cui, Y., Wang, C., Li, Y., & Zhang, D. (2019). Microplastics in offshore sediment in the Yellow Sea and East China Sea, China. *Environmental Pollution,* 244, 827–833.

Zhang, H., (2017). Transport of microplastics in coastal seas. *Estuarine, Coastal and Shelf Science*, 199, pp.74-86.

Zhang, J., Huang, X., & Jiang, Z. (2014). Physiological responses of the seagrass *Thalassia hemprichii* (Ehrenb.) Aschers as indicators of nutrient loading. *Marine Pollution Bulletin,* 83(2), 508–515.

Zhou, Q., Tu, C., Fu, C., Li, Y., Zhang, H., Xiong, K., Zhao, X., Li, L., Waniek, J.J. and Luo, Y., (2020). Characteristics and distribution of microplastics in the coastal mangrove sediments of China. *Science of The Total Environment*, 703, p.134807.

Zimmerman, R.C. and Dekker, A.G., (2007). Aquatic optics: basic concepts for understanding how light affects seagrasses and makes them measurable from space. In *Seagrasses: Biology, Ecology and Conservation* (pp. 295-301). Springer, Dordrecht.

Appendix I: Publications

[1] Ward, E., Meek, S., Gordon, D., Cameron, T., Steer, M., Smith, D., Miliou, A. and Tsimpidis, T., 2020. The use of storm fragments and biodegradable replanting methods allows for a low-impact habitat restoration method of seagrass meadows, in the eastern Aegean Sea. *Conservation Evidence*, 17.

The use of storm fragments and biodegradable replanting methods allows for a low-impact habitat restoration method of seagrass meadows, in the eastern Aegean Sea

Emma A. Ward[1,2]*, Sarah K. Meek[1,3], Dean M. Gordon[1], Tom C. Cameron[2], Mark D. Steer[3], David J. Smith[2], Anastasia Miliou[1] & Thodoris Tsimpidis[1]

[1] *Archipelagos Institute of Marine Conservation, P.O. Box 42 Pythagorio 83 103 Samos, Greece*
[2] *School of Life Sciences, University of Essex, Wivenhoe Park, Colchester, CO4 3SQ, UK*
[3] *Centre for Research in Biosciences, University of the West of England, Coldharbour Lane, Bristol, UK, BS16 1QY, UK*

SUMMARY

Seagrasses are important marine ecosystems but are vulnerable to physical damage from anthropogenic activities such as anchoring and trawling. Replanting damaged areas can represent a viable restoration strategy, yet current methods rely on the removal of plants from existing meadows and in some cases the use of non-sustainable planting materials. In this paper, we present evidence of a sustainable replanting strategy. Storm fragments of the endemic Mediterranean seagrass, neptune grass *Posidonia oceanica* were collected from the shore and shallow water, both the plagiotropic and orthotropic (horizontal and vertical) growth forms were then replanted using one of two biodegradable materials, coconut fibre pots or bamboo stakes, to secure them to the seafloor. Establishment of plagiotropic fragments were increased by bamboo anchorage ($\bar{x}$ = 89% SE ± 0%) compared to orthotropic storm fragments ($\bar{x}$ = 66.5% SE ± 6.5%). By contrast a coconut fibre method resulted in greater establishment of orthotropic fragments ($\bar{x}$ = 79% SE ± 7%) compared to plagiotropic ($\bar{x}$ = 51% SE ± 11%). Fragments showed some blade growth, but little shoot growth after 15 months. The fragment shoot and blade growth did not differ between the plagiotropic or orthotropic fragments replanted by bamboo stakes or coconut fibre pot. Our results suggest that the use of storm fragments and biodegradable anchoring materials constitutes a viable, non-destructive replanting technique in seagrass restoration. Furthermore success can be increased by selecting a growth-form appropriate planting method.

BACKGROUND

Despite seagrass meadows' ability to provide high value ecosystem services, including supporting commercial fisheries (Heck *et al.* 2003), nutrient cycling (Orth *et al.* 2006) and sediment stabilisation (Waycott *et al.* 2009), there has been a global decline in seagrass habitat since the 1970s (Orth *et al.* 2006). The positive feedback provided by seagrass ecosystem services, such as reduced turbidity, may promote the resilience of alternative stable states once seagrass is lost and explain why restoration techniques have historically had varied success (van der Heide *et al.* 2007). However, successful seagrass meadow restoration has been shown to not only restore seagrass cover, but also the ecosystem services they provide, such as carbon sequestration (Greiner *et al.* 2013). Given that seagrass regression may be caused by numerous factors, many of which are anthropogenic in origin (Boudouresque *et al.* 2009), restoration strategies may need to respond to distinct stressors.

The endemic Mediterranean seagrass, neptune grass *Posidonia oceanica* provides ecosystem services that are estimated at up to €514 ha^{-1} $year^{-1}$ (Campagne *et al.* 2015). *P. oceanica* meadows are protected under the EU Habitats Directive 1992, where they are acknowledged as being a priority habitat requiring designated areas of conservation (Campagne *et al.* 2015).

*corresponding author: emmaalice25@gmail.com

Protection is also afforded through the EU Common Fisheries Policy (EC 1626/94, 1994), which prohibits trawling (Lachopoulou *et al.* 2013) and the use of towed fishing gear over areas of *P. oceanica* (1967/2006). Direct physical disturbance is particularly detrimental to the survival of this slow-growing species. Due to such a slow growth rate (rhizome extension rates are just 1-6 cm yr^{-1}) *P. oceanica* is particularly vulnerable to physical damage, such as that caused by anchoring or illegal trawling in the meadows; in the long term even small boats using low-impact anchors can have detrimental consequences (Milazzo *et al.* 2004) as recovery can take hundreds of years (Marbà *et al.* 1996).

Research has suggested there may be potential for using storm fragments for replanting, rather than donor meadows. During the winter storm fragments of *P. oceanica* wash ashore and form onshore banquettes. Collecting such fragments before they desiccate allows the material to be utilised for restoration. There are several advantages of using this technique over traditional methods, including greater availability with lower collection efforts, with significantly less impact on existing populations (Balestri *et al.* 2010). Of the three techniques used for seagrass restoration (seeds, shoots and bare roots with sediment intact and bare roots with shoots) (Davis & Short 1997), bare root with shoots are the most appropriate for replanting from storm fragments. Storm fragments are either

ISSN 1758-2067

planted with materials to secure them directly to the seafloor, or with materials that assist in stabilising the surrounding sediment. Previous transplanting research used a mixed approach in the selection of materials used to secure fragments directly onto the seafloor: whilst some have favoured biodegradable materials, such as bamboo pegs (Davis & Short 1997), others have utilised non-degradable, and potentially polluting, materials such as plastic-coated steel wire hooks (Bastyan & Cambridge 2008). In light of increasing problems of marine pollution, including plastics, replanting techniques should avoid methods that use such materials (Bastyan & Cambridge 2008) in favour of using biodegradable materials to support storm fragments. The aim of our study was to test whether storm fragments planted with biodegradable materials can provide an effective and sustainable method for restoring areas showing signs of physical damage (e.g. anchor scars).

ACTION

Seagrass fragment collection

Posidonia oceanica storm fragments were collected from February to April 2017 at three southerly sites in Samos, Greece (37°45′N 26°50′E). Loose fragments were collected from the shoreline or by snorkelers up to a depth of 5 m. Collection only took place once at the largest site, but more continuously at the two small sites near the research base, as collection at these two sites was simply to replenish the stock of viable storm fragments. Fragments were deemed viable if the blades exhibited no zones of necrosis and the rhizome length was a minimum of 5 cm. Both *P. oceanica* growth forms (plagiotropic and orthotropic) were collected (Figure 1) with a larger proportion of orthotropic fragments available. After collection, fragments were immediately deposited into containers of seawater then transferred to large transparent containers (4 boxes of 50 x 40 x 30 cm). Collection of further storm fragments was limited by the available storage, as approximately 60 fragments were stored in each container, to prevent overcrowding and shading. The seawater was changed every 1-2 days until transportation to the replanting site in April, at which point any fragments no longer deemed viable were discarded.

Replanting site

The fragments were all transported on 10th April 2017 by ferry to Lipsi Island approximately 88 km south of Samos, for replanting at Vroulia Bay, NW Lipsi (37°18′N 26°45′E). Vroulia is a sheltered bay, with limited boat traffic and occasional anchor pressure. Between arrival and replanting (24 – 43 days), the fragments were stored in the same transparent containers. These were covered with mesh and submerged in Vroulia Bay to allow for a period of acclimatisation. Two replanting sites at 4.5 m and 8 m depths were identified: an L-shaped scar within the seagrass bed and a concave indent into the seagrass bed.

Figure 1. Orthotropic fragment on site in Vroulia for pre-replanting measurements (left, photo © K. R. de Moraes) and plagiotropic fragment during health check (right, photo © E. A. Ward).

Seagrass replanting

Replanting was carried out 6th, 21st and 23rd May 2017 over three one-hour scuba dives. Prior to replanting, growth form, number of shoots, number of blades and maximum blade length for each fragment were noted. The fragments were planted 5 cm apart (Molenaar & Meinesz 1995) in four rows of six fragments on six 25 x 15 cm grids. Plagiotropic growth forms were placed preferentially on the outside of the grids, to replicate rhizome positioning in natural seagrass meadows. Plagiotropic rhizomes were planted with the horizontal rhizome orientated out from the centre of the grids (after Molenaar & Meinesz 1995).

Two biodegradable anchoring methods were used, coconut fibre plant pots and bamboo shoots (Figure 2). For method one, each fragment was pressed into the top 2 cm of sediment and secured with two pieces of bamboo (approximately 15 cm segments) inserted on either side of the fragment to form an inverted “V”. For the second method the top 5 cm layer of sediment was removed to allow for the placement of coconut fibre trays that formed a perimeter (35 x 25 cm) around the 25 x 15 grid of storm fragments. The plant pots were inverted and an incision made between each row of pots to allow the rhizomes to penetrate into the sediment below.

ISSN 1758-2067

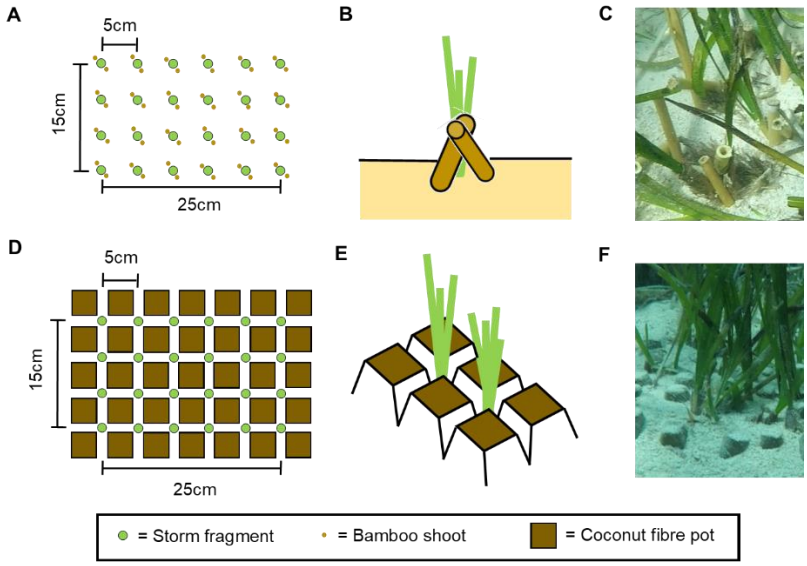


Figure 2. A and **D** Storm fragments are planted in four rows of six to form 15 x 25 cm grids for both replanting methods. **B** and **C,** Bamboo shoots are inserted either side of the fragment to form an inverted "V". **E** and **F,** Coconut fibre pots are inverted and covered in sediment to act as a sediment stabiliser.

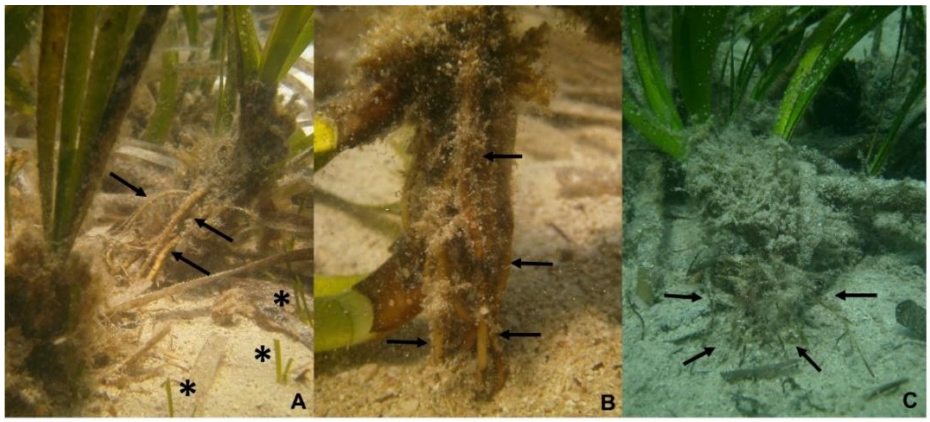


Figure 3. Replanted fragments after 15 months, arrows indicate new root growth, ***** indicates colonisation of the seagrass species, *Cymodocea nodosa*. **A** and **B** fragments at 4.5m depth. **C** fragment at 8m depth. All photos © E. A. Ward.

The trays were covered with the sediment to leave the rhizome partially buried with the shoots emerging above. *In situ* photo documentation was carried out with two GoPro Hero5 cameras (Figure 2C and 2F).

Fragments were monitored after fifteen months on the 23rd and 24th August 2018 during two one-hour scuba dives where the same measurements were noted for each fragment. *In situ* photo documentation was carried out with a Sealife DC1400 (Figure 3).

Data analysis

Generalised Linear Mixed Models (GLMM), with logit link function, accounting for binomial distribution and nesting accounted for as a random effects term were used to determine the statistical significance of factors that impacted fragment establishment (Bolker *et al*. 2008). Replant method (bamboo stake and coconut fibre pots), storm fragment growth form (plagiotropic and orthotropic) and depth (site 1 at 4.5 m and site 2 at 8 m depth) were initially included as fixed factors, including any interaction between them. However, as the deep bamboo planted fragments were likely impacted by recreational boating damage, the likelihood of storm fragment establishment between planting methods could not be determined for depth, this made it inappropriate to include depth as a fixed factor within our model for predicting fragment establishment across both replant methods. Therefore, the data for the bamboo stake planted storm fragments at the deep site were removed from the establishment data analysis. The full model was therefore:

Establishment ~ growth form + method + growth form * method + (1|Block.ID)

To determine the statistical significance of each main term and the interactions they were removed from the model and compared to the more complex model using maximum likelihood (Laplace approximations) to test our *a priori* hypotheses (Crawley 2007).

ISSN 1758-2067

While not ideal, due to the damage to our experimental site, to predict the expected fragment establishment due to differences in the depth of replant site, a GLMM model was refit to the coconut fibre method data across both depths. Therefore, replant method was not included as a fixed factor in this model. The same stepwise model simplification methods were undertaken as above to determine the retention of factors, depth and growth form, within the maximal model for the likelihood of establishment.

The fragments, that were used for the bamboo and coconut replant methods, prior to planting into grids, were not statistically different from each other in terms of maximum blade length ($t_{(142)}$ = 0.75065, p = 0.4541), number of blades ($t_{(142)}$ = 0.0967, p = 0.9231) and number of shoots ($t_{(142)}$ = 0.35396, p = 0.7239) – therefore we analyse the data, for the change in growth from the start to the end of the experiment.. We used ANOVA to determine the statistical significance of replant method (bamboo stake and coconut fibre pots) and storm fragment growth form (plagiotropic and orthotropic) on the change in maximum blade length, number of blades and number of shoots. We examined the residuals of each model for excessive patterning or deviations from normality and all were sound. All statistical analysis was completed using R version 3.5.1.

CONSEQUENCES

A total of 144 *P. oceanica* storm fragments were replanted, 96 at 4.5 m and 48 at 8 m depth, in six grid formations. Fifteen months later, when the sites were resurveyed evidence of a large physical disturbance (presumed anchor drag) to the grid replanted by bamboo method at 8 m was observed. The five remaining grids planted across both sites showed no signs of external physical disturbances.

There was a significant interaction between the planting method and seagrass fragment growth form ($Z_{1,5}$ = -2.751, p < 0.01) (Figure 4). The plagiotropic storm fragments planted with bamboo stakes had a higher percentage of fragment establishment (raw data $\bar{x}$ = 89% SE ± 0%), than the orthotropic fragments planted by the same method ($\bar{x}$ = 66.5% SE ± 6.5%) (Table 1). By contrast, the plagiotropic storm fragments planted by the coconut fibre method had a lower percentage of establishment ($\bar{x}$ = 51% SE ± 11%) compared to the orthotropic fragments planted by the same method ($\bar{x}$ = 79% SE ± 7%). The success of establishment was not influenced by the depth (4.5 vs. 8 m) at which fragments were planted ($Z_{1,3}$ = -0.333, p = 0.739), nor was establishment influenced by an interaction between the growth form and the depth which fragments were planted ($Z_{1,3}$ = -1.376, p = 0.1688), but this is only using the storm fragments that were replanted by the coconut fibre method.

The number of blades decreased amongst the surviving plagiotropic and orthotropic fragments of both the coconut fibre (plagiotropic $\bar{x}$ = -3 SE ± 4 blades, orthotropic $\bar{x}$ = -2 SE ± 2 blades) and bamboo stake method 15 months after planting (plagiotropic $\bar{x}$ = -4 SE ± 4 blades, orthotropic $\bar{x}$ = -2 SE ± 1 blades) and there was no significant difference in the decrease in blade numbers between the fragment growth forms planted by either method ($F_{(3,8)}$ = 0.07114, p = 0.9738). The maximum blade length decreased amongst the surviving plagiotropic coconut fibre ($\bar{x}$ = -4.6 SE ± 1.2 cm), orthotropic coconut fibre ($\bar{x}$ = -5.8 SE ± 1.6 cm) and plagiotropic bamboo ($\bar{x}$ = -0.7 SE ± 3.8 cm) planted fragments, whilst the orthotropic bamboo planted fragments marginally increased in maximum blade length ($\bar{x}$ = 2.8 SE ± 4.7 cm). However, there was no significant difference in the change in maximum blade length between the replant methods and fragment growth form 15 months after planting ($F_{(3,8)}$ = 1.527, p = 0.2806). The surviving fragments showed marginal to no change in the number of shoots for the orthotropic and plagiotropic fragments planted by both the coconut fibre (plagiotropic $\bar{x}$ = 0 SE ± 0.1 shoots, orthotropic $\bar{x}$ = 0 SE ± 0.2 shoots) and bamboo stake method (plagiotropic $\bar{x}$ = -1 SE ± 0.5 shoots, orthotropic $\bar{x}$ = 0 SE ± 0.3 shoots) and there was no significant difference between the shoot growth for the orthotropic and plagiotropic growth forms planted by both replant methods ($F_{(3,8)}$ = 2.39, p = 0.1443). Overall the fragments showed little blade growth and shoot growth after 15 months. The overall change in blade and shoot growth from the start to the end of the experiment did not vary between the orthotropic and plagiotropic fragments planted by either the bamboo or coconut fibre method.

No quantitative data concerning root growth were recorded as this would have disturbed the fragment colonisation process, but visual evidence suggested new root growth had occurred (Figure 3). It was also noted that at the time of replanting the fragments were planted within an L-shaped scar on a patch of bare sand and 15 months later alongside the replanted fragments the seagrass little neptune grass, *Cymodocea nodosa*, had begun to colonise (Figure 3).

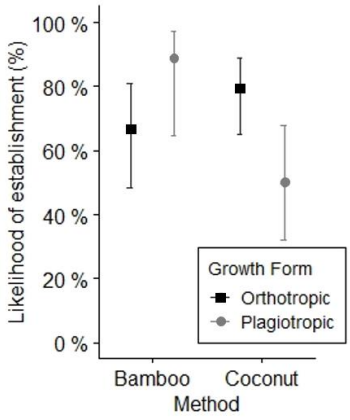


Figure 4. The modelled likelihood of establishment by orthotropic and plagiotropic *P. oceanica* storm fragments under different replanting methods. Error bars represent 95% confidence intervals of the modelled mean fragment establishment.

ISSN 1758-2067

Table 1. Number of fragments planted by method and growth form with fragment establishment 15 months after planting. Grid 3 establishment excluded, due to physical disturbance.

Replant Method	Fragment Growth Form	Grid	Fragments Planted	Fragments Established	Establishment (%)
Bamboo Stakes	Plagiotropic	1	9	8	89
		2	9	8	89
		3	9	-	-
	Orthotropic	1	15	9	60
		2	15	11	73
		3	15	-	-
Coconut Fibre Pots	Plagiotropic	4	10	3	30
		5	9	5	56
		6	9	6	67
	Orthotropic	4	14	10	71
		5	15	14	93
		6	15	11	73

DISCUSSION

This study provides strong evidence to support the use of storm fragments as a suitable material for seagrass replanting in the Mediterranean (Balestri *et al.* 2010), negating the need to use donor meadows for provision of fragments which causes further damage to healthy meadows (Pereda-Briones *et al.* 2018). Our findings demonstrate that small areas of bare sand surrounded by seagrass, such as areas of physical damage caused by anchors, could be restored effectively using planted fragments, even in the case of the slow growing *P. oceanica.* The success of storm fragment replantation is dependent on the growth form of available fragments. In this study, a higher proportion of orthotropic storm fragments were collected, therefore using coconut fibre would have enabled better establishment of this fragment type. However, using plagiotropic fragments, even if these only represent a smaller proportion of the storm fragments collected, is important as horizontal growth by plagiotropic fragments may better assist in the colonisation of bare substrate surrounding the replanted areas. As plagiotropic fragments have improved establishment when replanted using bamboo, a mixed replanting approach is recommended between fragment growth forms.

Whilst there was little evidence of blade and shoot growth, fragment establishment combined with visual evidence of root growth suggests the redistribution of nutrient content to new roots (Balestri *et al*. 2010), which assists the stabilisation of the sediment (Christianen *et al*. 2013). Sediment stabilisation created by replanting – although not measured - may have created conditions that enabled *Cymodocea nodosa* to colonise alongside the storm fragments. These fragments therefore have the potential to assist in sediment re-stabilisation of scar areas and persist once any bamboo or coconut fibre materials have fully biodegraded, contributing to the establishment of multispecies seagrass meadows. Whilst this study highlights the positive potential in replanting strategies, the optimum conservation management strategy would be to prevent physical disturbances, such as anchoring or anchor drags. This could be achieved through the creation of anchor-free zones or provision of semi-permanent buoy-based anchors. Storm fragments are highly susceptible to damage and loss, similar to the existing seagrass beds and evidenced even in a small area during this study.

ACKNOWLEDGEMENTS

The respective funding bodies provided financial support to enable the work to be completed in association with Archipelagos Institute of Marine Conservation; Emma A. Ward, The Erasmus+ Programme, European Region Action Scheme for the Mobility of University Students; Dean M. Gordon, The Hellenic National Agency of Erasmus Plus in the field of youth –Youth and Lifelong Learning Foundation. We also thank for their technical assistance with SCUBA diving logistics: Karlos R. de Moraes, Ruben D. Moya and Leah K. Brinch-Iversen. Mention should go to GoPro for their contribution of two GoPro Hero5 cameras.

ISSN 1758-2067

REFERENCES

Balestri, E., Vallerini, F. & Lardicci, C. (2010) Storm-generated fragments of the seagrass *Posidonia oceanica* from beach wrack –A potential source of transplants for restoration. *Biological Conservation*, **144**, 1644-1654.

Bastyan, G.R. & Cambridge, M.L. (2008) Transplantation as a method for restoring the seagrass *Posidonia australis*. *Estuarine, Coastal and Shelf Science*, **79**, 289-299.

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H. & White, J. S. (2008) Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology and Evolution*, **24,** 127-135.

Boudouresque, C. F., Bernard, G., Pergent, G., Shili, A., & Verlaque, M. (2009). Regression of Mediterranean seagrasses caused by natural processes and anthropogenic disturbances and stress: A critical review. *Botanica Marina*, **52**, 395–418.

Campagne, C. S., Salles, J. M., Boissery, P., & Deter, J. (2015) The seagrass *Posidonia oceanica*: Ecosystem services identification and economic evaluation of goods and benefits. *Marine Pollution Bulletin*, **97**, 391–400.

Christianen, M. J. A., van Belzen, J., Herman, P. M. J., van Katwijk, M. M., Lamers, L. P. M., van Leent, P. J. M. & Bouma, T. J. (2013) Low-canopy seagrass beds still provide important coastal protection services. *PLoS ONE*, **8**. doi:10.1371/journal.pone.0062413

Crawley, M. J. (2007) *The R Book*. Chichester, John Wiley and Sons.

Davis, R. & Short, F. (1997) Restoring eelgrass, Zostera marina L., habitat using a new transplanting technique: The horizontal rhizome method. *Aquatic Botany*, **59**, 1-15.

Greiner, J. T., McGlathery, K. J., Gunnell, J. & McKee, B. A. (2013) Seagrass restoration enhances "Blue Carbon" sequestration in coastal waters. *PLoS ONE*, **8**. doi:10.1371/journal.pone.0072469

Heck Jr. K. L., Hays, G. & Orth, R.J. (2003) Critical evaluation of the nursery role hypothesis for seagrass meadows. *Marine Ecology Progress Series*, **253**, 123-136.

Lachopoulou, E.I., Wilson, A.M. & Miliou, A. (2013) Disconnects in EU and Greek fishery policies and practices in the eastern Aegean Sea and impacts on Posidonia Oceanica meadows. *Ocean and Coastal Management*, **76**, 105-113.

Marbà, N., Duarte, C. M., Cebrián, J., Gallegos, M. E., Olesen, B., & Sand-Jensen, K. (1996) Growth and population dynamics of Posidonia oceanica on the Spanish Mediterranean coast: elucidating seagrass decline. *Marine Ecology Progress Series*, **137**, 203-213.

Milazzo, M., Badalamenti, F., Ceccherelli, G., & Chemello, R. (2004) Boat anchoring on *Posidonia oceanica* beds in a marine protected area (Italy, western Mediterranean): Effect of anchor types in different anchoring stages. *Journal of Experimental Marine Biology and Ecology*, **299**, 51–62.

Molenaar, H. & Meinesz, A. (1995) Vegetative Reproduction in *Posidonia oceanica*: Survival and Development of Transplanted Cuttings According to Different Spacings, Arrangements and Substrates. *Botanica Marina*, **38**, 313-322.

Orth, R.J., Carruthers, T.J.B., Dennison, W.C., Duarte, C.M., Fourqurean, J.W., Heck Jr, K.L., Hughes, A.R., Kendrick, G.A., Kenworthy, W.J., Olyarnik, S., Short, F.T., Waycott, M. & Williams, S.L. (2006) A Global Crisis for Seagrass Ecosystems. *BioScience*, **56**, 987-996.

Pereda-Briones, L., Tomas, F., & Terrados, J. (2018) Field transplantation of seagrass (Posidonia oceanica) seedlings: Effects of invasive algae and nutrients. *Marine pollution bulletin*, **134**, 160-165.

van der Heide, T., van Nes, E.H., Geerling, G.W., Smolders, A.J.P., Bouma, T.J. & van Katwijk, M.M. (2007) Positive Feedbacks in Seagrass Ecosystems: Implications for Success in Conservation and Restoration. *Ecosystems*, **10**, 1311-1322.

Waycott, M., Duarte, C. M., Carruthers, T. J. B., Orth, R. J., Dennison, W. C., Olyarnik, S., Calladine, A., Fourqurean, J., Heck Jr, K., Hughes, A., Kendrick, G., Kenworthy, W., Short, F., & Williams, S. L. (2009). Accelerating loss of seagrasses across the globe threatens coastal ecosystems. *Proceedings of the National Academy of Sciences*, **106**, 12377–12381.

ISSN 1758-2067