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Restocking the European Flat Oyster *Ostrea edulis* in the Solent – A Model for Ecosystem Service Restoration Across Europe

by

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Abstract

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European flat oyster *Ostrea edulis* beds have been reduced by over 90% globally over the last 100 years, through a combination of over-fishing, competition with invasive species, adverse winters and the spread of a lethal invasive parasite, *Bonamia ostreae*, which is now present in most coastal regions of Europe. Popular interest in Ecological Restoration has meant that *O. edulis* broodstock availability has replaced monetary investment as the main limiting factor for oyster restoration in the UK. Concerns associated with limited broodstock include the development of a population with reduced genetic diversity, the survival of *Bonamia*-naive or *Bonamia*-exposed oysters in an environment dissimilar to their own, and further spread of the parasite and consequential disease, bonamiosis. There are many unknowns surrounding effective restoration. This thesis explored the importance of the hydrodynamic environment, broodstock origin and restoration design (re-laying density and elevation from the seabed) on the physiological performance of *O. edulis* to further our understanding of how to restore *O. edulis* to a self-sustaining population. Adult oyster valve movement was influenced by water velocities from 0.19 m s^{-1}, and the threshold for physical displacement of all oyster sizes and weights (from 0.22 m s^{-1}) was found to be lower than many recorded velocities in the Solent. Modelling the trajectory of larvae from given broodstock sites is highly recommended to indicate larval dispersal patterns and identify potential settlement areas where restoration efforts can be focussed. Appreciation of physiological performance of *O. edulis* was achieved by monitoring the metabolic rate, inferred from respirometry, and clearance rate of the oysters in both their local conditions and when acclimated to common aquarium conditions. Growth, body condition, and *B. ostreae* prevalence was thereafter recorded. The Solent is not recommended as a location for restoration evidenced by the poor condition of Solent oysters in comparison to those from Loch Ryan, Scotland, and Galway, Ireland, as well as the decrease in body condition observed in Loch Ryan oysters after 2 years of being re-laid in the Solent. However, the rapid development of a rich and diverse community of organisms observed in association with the oyster gabions deployed in the Beaulieu River should encourage restoration of *O. edulis* to prioritise ecosystem regeneration over commercial oyster production in this area. Undisturbed oyster beds support a rich ecosystem that will directly impact the future biodiversity of coastal areas of UK and Europe. This project identified a potential use for metabolic markers in association with *Bonamia*-infection, and urges further research into combined cellular and molecular approaches to better our understanding of this parasite-host interaction.

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Research Thesis: Declaration of Authorship

Print name: ZOË NATASHA HOLBROOK

Title of thesis: RESTOCKING THE EUROPEAN FLAT OYSTER *OSTREA EDULIS* IN THE SOLENT – A MODEL FOR ECOSYSTEM SERVICE RESTORATION ACROSS EUROPE

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Abbreviations and Definitions

4-IPO: 4-ipomeanol

ABP: Associated British Ports

ACT: β-actin

ADV: acoustic doppler velocimeter

AFDW: ash-free dry weight

BBL: Benthic boundary layer

BMF: Blue Marine Foundation

CA: California

Cathep: Cathepsin B

CBD: Convention on Biological Diversity

cDNA: complementary DNA

Cefas: Centre for Environment, Fisheries and Aquaculture Science

CNRQ: calibrated normalised relative quantity

CoV: coefficient of variation

CPI: cysteine protease inhibitor

DDM: degree decimal minutes

DEFRA: Department for Environment, Food and Rural Affairs

DHC: differential haemocyte count

DIOL: 1,4-Ipomeadiol

DNA: Deoxyribonucleic acid

EEA: European Environment Agency

Ef1-α: elongation factor 1-*α*

ERG: endogenous reference gene

EST: expressed sequence tag

EU: European Union

EUNIS: European Nature Information System

FAO: Food and Agriculture Organization of the United Nations

FPR: false positive rate

G: granulocytes

GAPDH: glyceraldehyde 3-phosphate-dehydrogenase

GBIF: Global Biodiversity Information Facility

GLM: generalised linear model

GOI: gene of interest

H: height

HP: hepatopancreas

IBM: Individual based models
ICES: International Council for the Exploration of the Sea
IFCA: Inshore Fisheries and Conservation Authority
INNS: Invasive non-native species
IQR: inter quartile range
JNCC: Joint Nature Conservation Committee
L: length
L5: 60S ribosomal protein L5
LC: liquid chromatography
LH: large hyalinocytes
LM: linear model
LR: Loch Ryan
LRBAR: Land Rover Ben Ainslie Racing
MA: Massachusetts
MCZ: Marine Conservation Zone
MIQE: Minimal Information for Publication of Quantitative Real-Time PCR Experiments
MPA: Marine Protected Area
MR: microreef
MS: mass spectrometry
N: north
NAD+: nicotinamide adenine dinucleotide
NADP+: nicotinamide adenine dinucleotide phosphate
NADPH: reduced form of NADP+
NBN: National Biodiversity Network
NBSAPs: National Biodiversity Strategies and Action Plans
NGO: Non-governmental organisation
NNS: Non-native species
NOCS: National Oceanography Centre Southampton
NW: north west
OeC1q: C1q
OeEcSOD: extracellular superoxide dismutase
OeFAS: fas-ligand
Oefer: ferritin
OeIAP: inhibitor of apoptosis
Oepepti: Cyclophylin B
OIE: World Organisation for Animal Health
OsHV-1: Oyster herpes-like virus
OSPAR: Convention for the Protection of the Marine Environment of the North-East Atlantic

PC: principal component

PCA: principal components analysis

PCR: polymerase chain reaction

PD: Propagule duration

PLS-DA: partial least squares discrimination analysis

PRR: pattern recognition receptor

PVC: polyvinyl chloride

PVS: phenyl vinyl sulphide

qPCR: quantitative PCR

QPX: Quahog parasite unknown

QSL: quadratic stress law

Re: Reynolds number

RNA: Ribonucleic acid

ROC: receiver operator characteristic

RQI: RNA quality indicator

SAC: Special Area for Conservation

sd: standard deviation

SH: small hyalinocytes

SMR: standard metabolic rate

SOD: superoxide dismutase

Sp.: species (singular)

SPA: Special Protection Area

Spp.: species (plural)

SSSI: Site of Specific Scientific Interest

TBT: Tributyltin

THC: total hemocyte count

TKE: turbulent kinetic energy

TPR: true positive rate

TPT: Triphenyltin

Ubiq: polyubiquitin

UK: United Kingdom

USA: United States of America

WW: wet weight

Chapter 1 *Ostrea edulis*: a species for restoration

1.1 *Ostrea edulis*

The Ostreidae family of 'true' oysters have been a focus for aquaculture since Roman times (Philpots, 1890; Günther, 1897; Council of the European Commission, 1992), yet active fisheries have, within the last 100 years, struggled to maintain healthy and sustainable production of the European flat oyster, *Ostrea edulis*, to meet consumer demand (Laing *et al.*, 2005; Maneiro *et al.*, 2017). In Europe, *O. edulis* was designated a named species in the EU Biodiversity Action Plan in the mid-2000s, as part of a commitment to the International Convention on Biological Diversity (JNCC, 1994, 2007). As a result, a significant increase in *O. edulis* restoration projects has ensued. Although there is a considerable body of literature on the European flat oyster *O. edulis*, there are still substantial gaps in the knowledge in adapting this information from controlled laboratory environments for application to large scale restoration.

The European flat oyster, *O. edulis* (Linnaeus, 1758) naturally occurs in Atlantic Europe and North Africa, from Norway to Morocco, as well as the Mediterranean Sea extending into the Black Sea, and was intentionally introduced into North America and South Africa for culture (Cano *et al.*, 1997; Zaitsev and Alenxandrov, 1998; Airoldi and Beck, 2007; Lallias *et al.*, 2007) (Figure 1.1). *O. edulis* has also self-introduced to Albany Harbour, Western Australia, probably historically via shipping (Morton *et al.*, 2003), and further spread is likely but has not yet been detected (Figure 1.1). At one time the species was amongst the most commercially-important marine resources in European waters (Orton, 1937) and stocks in France, Spain, Ireland, Croatia, Holland and the UK continue to be exploited commercially (Smith *et al.*, 2006; Kamphausen *et al.*, 2011). However, populations of *O. edulis* have been in decline since before the 1970s, and now this species is listed by the Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR) (Haelters and Kerckhof, 2009) as 'threatened or declining'. A combination of factors including overexploitation, disease and extreme climate has reduced the natural *O. edulis* population by over 85% (Beck *et al.*, 2011), consequently reducing an important habitat niche used by many other benthic organism. Oysters are a keystone species that contribute to the control of eutrophication in marine ecosystems (Newell, 1965; Ward and Shumway, 2004; Fulford *et al.*, 2010). They play a major role in dissolved nutrient cycling by removing phytoplankton, suspended solids and organic particles from the water column, and support the development of biodiverse ecosystems. The decline of *O. edulis* represents a loss to European coastal economies not only in terms of food security, but also by affecting the Good Environmental Status of the marine environment as set out by the European Council's Marine Strategy Framework Directive (2008/56/EC). Restoration of *O. edulis* habitat is now being widely discussed across Europe and pilot schemes and trials have been conducted (Pogoda *et al.*, 2019, 2020a).

The significant commercial value of the Eastern oyster, *Crassostrea virginica*, and the Olympia oyster, *Ostrea lurida*, in the USA has encouraged high profile restoration programmes during the last century, (National Research Council, 2004) that have resulted in varying levels of population recovery along the East and West coast of America respectively (National Research Council, 2004; Trimble *et al.*, 2009; White *et al.*, 2009; zu Ermgassen *et al.*, 2013; Bromley *et al.*, 2016b; Schulte, 2017). European flat oyster, *O. edulis*, restoration projects are not a new concept (Günther, 1897; Laing *et al.*, 2006; Smaal *et al.*, 2015) yet continue to be considered 'unsuccessful' (Drinkwaard, 1999; Kamphausen, 2012; Gercken and Schmidt, 2014; Lipcius *et al.*, 2015; Smaal *et al.*, 2015). The current key challenge for *Ostrea* spp. restoration is ensuring project design and management is best suited to achieve a self-sustaining population resilient to pressures including disease, predation, and extreme weather events.

1.2 *Ostrea edulis* distribution

Ostrea edulis is native to Europe but is now found globally in both wild and farmed environments due to extensive translocation during the mid 1800s to late 1900s (Figure 1.1) (Bromley *et al.*, 2016a). This species has been found in most coastal areas of the UK over the last 100 years (Figure 1.2) (GBIF Secretariat, 2019).

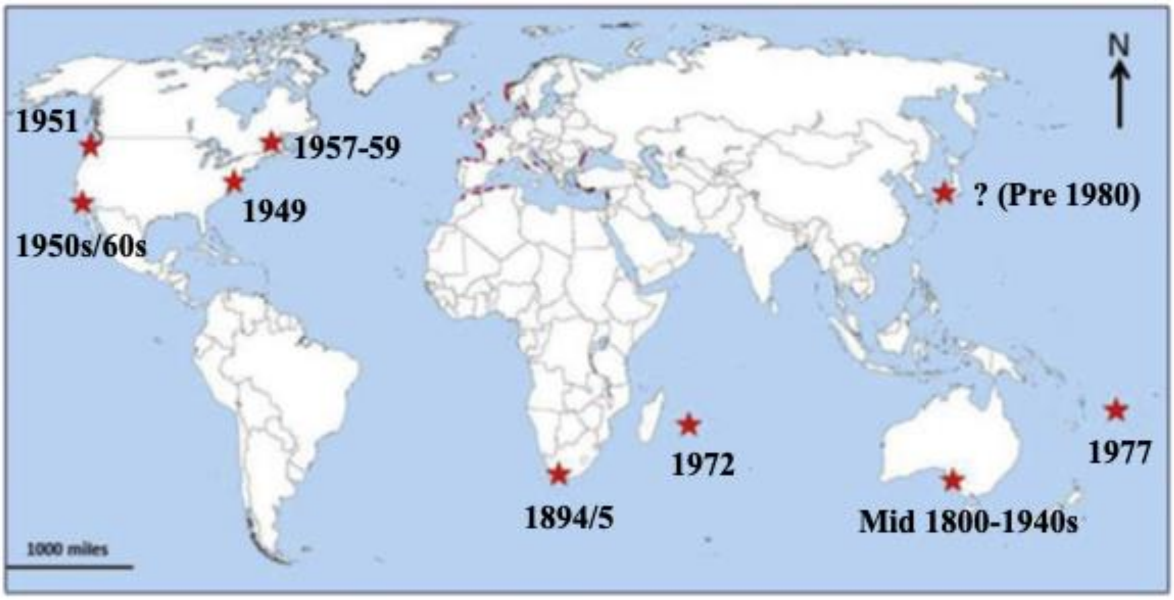


Figure 1.1. Map acquired from (Bromley *et al.*, 2016a) showing locations (red stars) to where *O. edulis* have been translocated to and dates (labelled) of translocation. The question mark indicates an introduction to Japan on an unknown date prior to 1980.

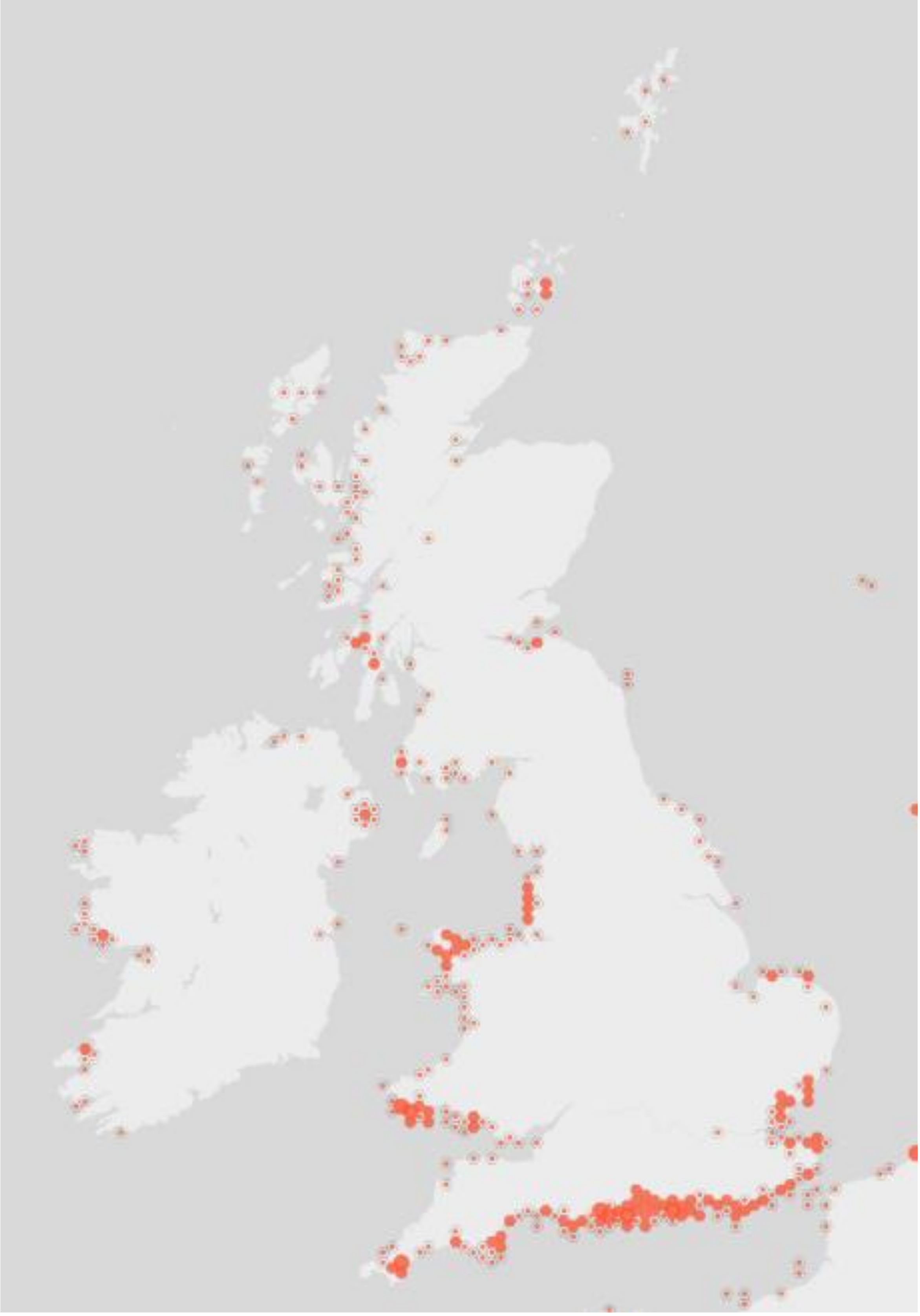


Figure 1.2. UK wild distribution of *O. edulis* (orange) over the last 100 years (1920 – 2020) acquired by the Global Biodiversity Information Facility (GBIF) (GBIF Secretariat, 2019).

1.3 The international decline in the global *Ostrea edulis* population

Excessive harvesting of *O. edulis* beds, and the accidental spread of diseases are two dominant factors that have contributed to a rapid decline in the global population of *O. edulis,* which has now reached over 85% loss of oyster habitat (Jackson *et al.*, 2001; Smith *et al.*, 2006; Smyth *et al.*, 2009; Beck *et al.*, 2011; Grabowski *et al.*, 2012; Gercken and Schmidt, 2014; Long *et al.*, 2017). Additional pressures have included competition with foreign species (such as *Crepidula fornicata* and *Crassostrea*/M*agallana gigas* (Korringa, 1951; Kennedy and Roberts, 1999)), severe weather events (Crisp, 1964; Kennedy and Roberts, 1999; Laing *et al.*, 2005), pollution (e.g. Tributyltin (TBT) – although TBT was introduced after the initial *O. edulis* decline (Smaal *et al.*, 2015)), and climate change (Laing *et al.*, 2005). Overstocking oysters at a high density has also contributed to the decline in *O. edulis* population due to the consequential reduction in available food resources and an increase in disease prevalence, which has implications for restoration and aquaculture efforts (Dame *et al.*, 2002).

1.3.1 Overexploitation

Historical records of oyster landings in the UK show the vast overexploitation of *O. edulis* that has contributed to the decline in British *O. edulis* populations; English and Welsh landings of 1800 tonnes (~27 747 000 oysters) in the year of 1894 (Thorpe, 1896; Laing *et al.*, 2006), massively exceed the 61 tonnes landed in 2016 (Figure 1.3). In 1864, 700 million oysters were consumed in London alone (Cole, 1951), demonstrating the high consumer demand for *O. edulis* (Herbert *et al.*, 2012a). The removal of adult oysters removes the most appropriate and favourable substrate for larval settlement, which consequently reduces recruitment potential. The fishing method ‘dredging’, by dragging a weighted bar and net along the seafloor, is destructive to the seabed and removes more than just oysters (Brumbaugh *et al.*, 2005; Laing *et al.*, 2005; Thorngren *et al.*, 2017; Smyth *et al.*, 2018). Technological advances have increased the efficiency of fishing methods, which are in part to blame for the rapid decline in abundance of *O. edulis* (Ruesink *et al.*, 2005; Long *et al.*, 2017), but even collecting oysters by hand removes habitat, as many invertebrates live on oyster shells and within the reef-like structure they create (Korringa, 1951; Brumbaugh *et al.*, 2005; Grabowski *et al.*, 2005; Smaal *et al.*, 2015).

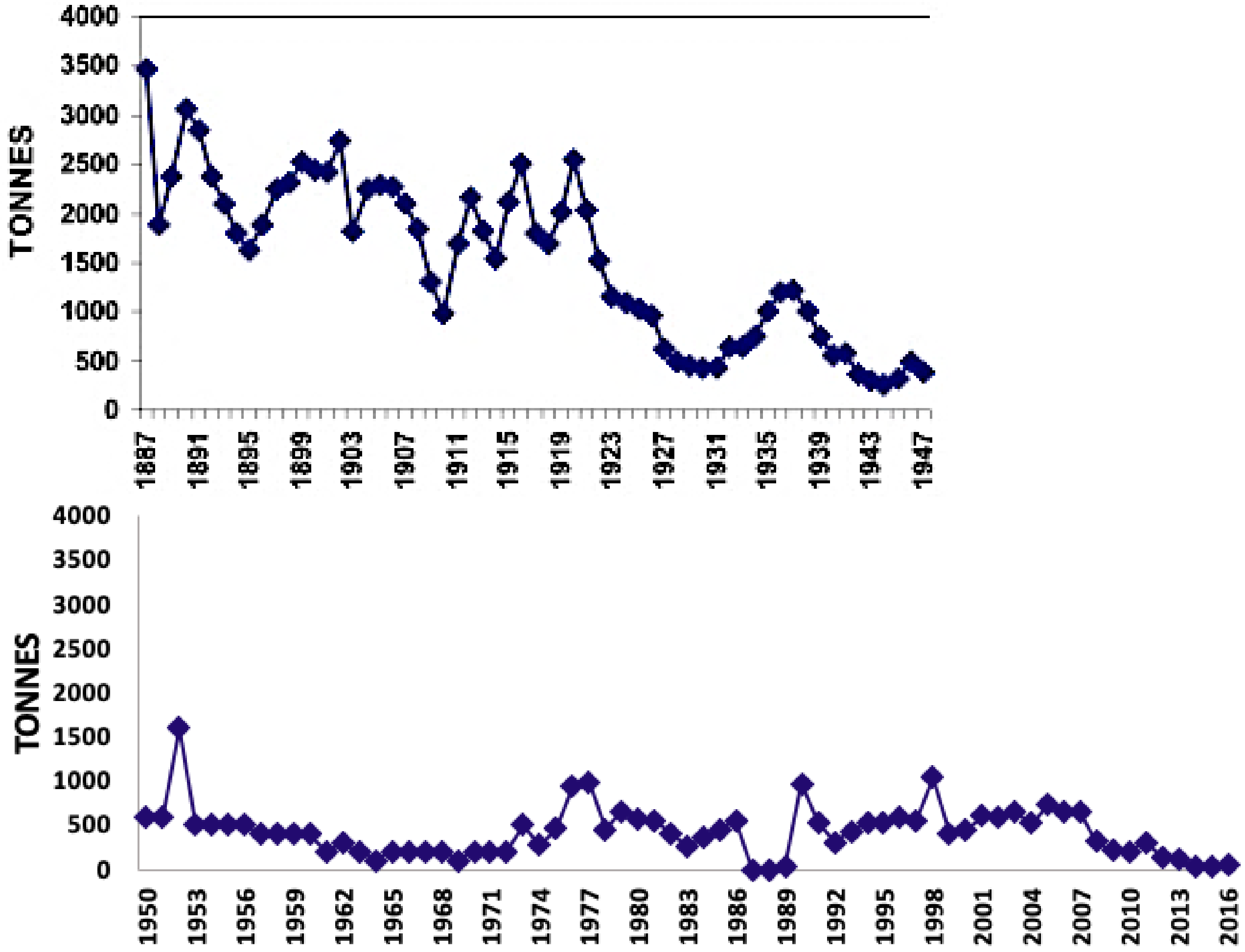


Figure 1.3. Landed tonnage of *O. edulis* in England and Wales between 1887 and 1947 (Laing *et al.*, 2006), and UK tonnage landed in 1950 to 2016 FAO FishStat data archive (FAO, 2006).

1.3.2 The biology of Ostrea edulis recruitment

O. edulis are protandrous hermaphrodites, beginning life as a male and later developing into a female, and then frequently switching between the two (Coe, 1943; Korringa, 1952; Policansky, 1982). Their ability to switch sex, and even reproduce as both male and female, has been observed to occur up to 4 times throughout a single spawning season (July – Sept, Orton 1926) (Orton, 1926; Coe, 1943; Policansky, 1982). Unlike the oviparous *Crassostrea* spp. that release sperm and eggs into the water column for fertilisation, *O. edulis* are larviparous, and only the males release their sperm into the water column (Waller, 1981; Hassan *et al.*, 2018); Females draw free swimming sperm released by the males into their mantle to fertilise their eggs, and retain them for 7-10 days before releasing them as veliger larvae (170-190 µm) (Korringa, 1947; Walne, 1964; Waller, 1981; Laing *et al.*, 2005; Woolmer *et al.*, 2011). Population fitness depends heavily on larvae survival through a 6-15 day pelagic phase to recruitment (Korringa, 1952; Waller, 1981; Lallias *et al.*, 2010). The presence of larvae in the water column may confirm a healthy broodstock (Korringa, 1952), yet environmental factors such as temperature, pH, salinity, light, tidal phase, water quality, predation and available substrate influence recruitment success (Quayle and Newkirk, 1989; Kennedy and Roberts, 1999; Colsoul *et al.*, 2020). Swimming behaviour has been associated with light availability (Cole and Knight-Jones, 1949), salinity and changes with the tidal cycle (Korringa, 1952; Levin, 2006; Woolmer *et al.*, 2011) and settlement will not occur until larvae have found appropriate conditions (Laing *et al.*, 2005). Although all *O. edulis* larvae tend to follow the same life stages (veliger, pediveliger etc), the conditions in which larvae develop impacts the time frame, maturity and probability of survival at each life stage (Korringa, 1952; Walne, 1964; Davis and Calabrese, 1969). *O. edulis* is an euryhaline thermo-tolerant species found in many different environment conditions around Europe (Korringa, 1952; Davis and Calabrese, 1969; Bromley *et al.*, 2016a). Post metamorphosis in their chosen settling location, *O. edulis* are fixed and dependent on their immediate environment (Kamermans *et al.*, 2018). The choice of settling location and subsequent inability to move can dictate the future survival of both the individual and sustainability of the greater population. Despite the vast research on larvae behaviour and development, there are still gaps in our knowledge, largely due to the difficulty in recreating a pelagic phase under laboratory or aquarium conditions (Korringa, 1952; Levin, 2006).

1.3.3 Disease

Molluscs lack a specific immune response and therefore cannot develop an antibody-based long lived immunity to pathogens (Morga *et al.*, 2012; Aranguren and Figueras, 2016). The most historically significant diseases responsible for the most severe oyster mortalities are marteliosis, Denman Island disease, and bonamiosis caused by parasites *Martelia refringens*, *Mikrocytos mackini*, and *Bonamia* spp., respectively (Carnegie *et al.*, 2016). Oyster herpes-like virus (OsHV-1) has caused extensive mortalities amongst the *Crassostrea* spp. but has also been shown to infect *O.*

edulis (when applied by injection) (López Sanmartín *et al.*, 2016), and could pose a future threat to restored populations (Carnegie *et al.*, 2016). Although herpes-like viral infections have previously been found infecting *O. edulis* larvae and spat (Comps and Cochennec, 1993; Renault *et al.*, 2000; da Silva *et al.*, 2005), OsHV-1 has yet to be found naturally occurring in adult individuals.

Mikrocytos mackini is a microcell parasite that infects *Crassostrea* spp., yet several studies have demonstrated the susceptibility of other species (including *O. edulis*) to infection (Bower *et al.*, 1997; Carnegie *et al.*, 2003; Abbott *et al.*, 2011). The only recorded incidence of *Mikrocytos* spp. in the UK was in 2013, when a related species, *Mikrocytos mimicus*, was discovered in a farmed population of *C. gigas* in Norfolk (Hartikainen *et al.*, 2014; Fish Health Inspectorate, 2015). The Paramyxean parasite *Marteilia refringens* can lie dormant in oyster individuals without developing into the full disease (martiliosis) until external conditions such as overcrowding (inducing stress) contributes to an increase in virulence of the pathogen (Laing *et al.*, 2005). *M. refringens* is also yet to be reported in the UK but has been reported in European populations of *O. edulis* since 1970 (Laing *et al.*, 2005; Lallias *et al.*, 2010).

There are four known species within the *Bonamia* family; *B. perspora* and *B. roughleyi* are yet to be found in European waters (Cochennec-Laureau *et al.*, 2003b; Carnegie *et al.*, 2006), but *B. exitiosa* and *B. ostreae* have been reported to affect European *O. edulis* (Pichot *et al.*, 1979; Hine *et al.*, 2001). *B. exitiosa was* originally reported to infect New Zealand's native oyster, *Ostrea chilensis*, and was first detected in Europe (Galacia, Spain) in 2005 (Abollo *et al.*, 2008), and was more recently found infecting *O. edulis* in the Fal estuary, England, in 2010 (Narcisi *et al.*, 2010; Longshaw *et al.*, 2013), and the Solent in 2020 (Helmer *et al.*, 2020). However, *B. ostreae* has so far caused the most significant damage to European *O. edulis* populations and is the species of interest considered within this thesis.

The Haplosporidian parasite *B. ostreae* was first reported in France in 1979 (Pichot *et al.*, 1979; Laing *et al.*, 2005), and was first identified in UK waters in 1982 (Laing & Spencer 2006; Long et al 2017). *B. ostreae* is now present in ten European countries within natural and commercial populations of *O. edulis* (summarised in Table 1.1, Engelsma *et al.*, 2014). For specific dates and locations of *B. ostreae* introductions around Europe, see Lynch *et al.* (2006) and Laing *et al.* (2014) and for USA, see Elston, Farley and Kent (1986) and Feng *et al.* (2013).

Table 1.1. First reports of *Bonamia ostreae* in Europe (and Morocco)

Country	***B. ostreae*** **first identified**	**Reference(s)**
France	1979	(Pichot *et al.*, 1979)
Spain	1980	(Montes and Melendez, 1987; Figueras, 1991)
Netherlands	1980	(Grizel *et al.*, 1988; Van Banning, 1988, 1991)
United Kingdom	1982	(Bannister and Key, 1982; Hudson and Hill, 1991)
Ireland	1987	(Elston *et al.*, 1987; McArdle *et al.*, 1991)
Italy	1990	(Narcisi *et al.*, 2010)
Morocco	2005	(WAHID-Interface, 2005)
Belgium	2008	(WAHID-Interface, 2008)
Norway	2009	(WAHID-Interface, 2009)
Denmark	2014	(Madsen and Thomassen, 2015)
Portugal	-	(OIE, 2019)

The parasite proliferates by developing a parasitophorous vacuole inside host haemocytes, and inhibiting host cell lysis (Hervio *et al.*, 1991); *B. ostreae* can establish itself in haemocytes within 30 minutes, and multiply within 2 hours post contact (Engelsma *et al.*, 2014). At low prevalence, a *B. ostreae* infection can stifle growth and degrade condition of infected individuals, but if the associated disease bonamiosis proliferates then high mortality rates (> 80%, Laing *et al.*, 2005; Culloty and Mulcahy, 2007) occur (Robert *et al.*, 1991; Lallias *et al.*, 2010). Bonamiosis is a notifiable shellfish disease in the UK (Aquatic Animal Health (England and Wales) Regulations, 2009), and transferral of infected stock to non-infected areas is prohibited to stall any further spread of the disease (Woolmer *et al.*, 2011). Once *B. ostreae* has been identified in an area, there is little chance of eradication (Woolmer *et al.*, 2011); *B. ostreae* has been shown to persist in areas re-introduced with oysters after being previously cleared of all adult oysters (hosts) and left for several years (Van Banning, 1985, 1987; Woolmer *et al.*, 2011).

The mechanism of parasite transmission is unknown; *B. ostreae* virulence is thought to be seasonal or affected by environmental conditions (Culloty and Mulcahy, 2007; Engelsma *et al.*, 2010; Hine *et al.*, 2014); multiple studies have found higher levels of *B. ostreae* in October or post summertime (Laing *et al.*, 2014; OIE, 2016). Overcrowding and handling, along with rapid changes in abiotic conditions such as temperature, have resulted in an increase in the prevalence of *B. ostreae* (Hawkins *et al.*, 1992; Laing *et al.*, 2014). This has implications for restoration planning and management. *B. ostreae* has been in Europe for nearly 50 years, yet there are still many gaps in our understanding of its lifecycle and transmission (Laing *et al.*, 2005). Questions on population susceptibility to *Bonamia* spp. are ongoing (Culloty *et al.*, 2004; Narcisi *et al.*, 2010; Engelsma *et al.*, 2014; Vera *et al.*, 2016) and will be discussed further in Chapter 3.

1.3.4 Species associated with the decline in Ostrea edulis production

The Pacific oyster (*Crassostrea gigas*) and American slipper limpet (*Crepidula fornicata*) are two non-native species (NNS) that (as benthic filter feeders) compete with *O. edulis* for space and food (Blanchard, 1997; Herbert *et al.*, 2016). These two species are particularly common in areas that once dominated as *O. edulis* grounds, and although their impact on *O. edulis* survival is contested (Thieltges *et al.*, 2006; Barbier *et al.*, 2017), accidental introductions of foreign species such as these often lead to the suppression of native species less robust to factors such as disease and climate change (Lenz *et al.*, 2011). Pacific oyster *Crassostrea gigas* was imported from British Columbia (USA) to Europe to replenish declining European *O. edulis* populations (Shatkin *et al.*, 1997; Drinkwaard, 1999; Troost, 2010), first to the River Blackwater Essex for culture in 1926 (Utting and Spencer, 1992), and to the Oosterschelde estuary in the Netherlands in 1964. C. *gigas* rapidly became the farmed oyster of choice in many parts of Europe due to its adaptability to new environments and early maturity (Troost, 2010), and the broadcast spawning reproductive method of this species led to its rapid dissemination and settlement into wild populations (Drinkwaard, 1999; Troost, 2010). This shift in active farming efforts, and the adaptability of *C. gigas* to European habitats contributed to the decline in abundance of native species, *O. edulis*. Originally thought to have arrived in Europe via the transport of American oysters (*Crassostrea virginica*) for aquaculture in the 1870s, *Crepidula fornicata* have since established large high-density populations in most historical oyster bed habitats in Europe (Cole, 1951; Barnes *et al.*, 1973; de Montaudouin *et al.*, 1999; McNeill *et al.*, 2010). *C. fornicata* are filter feeders that compete with *O. edulis* for space and food on the sea floor (Korringa, 1951; Barnes *et al.*, 1973; de Montaudouin *et al.*, 1999; Henry and Lyons, 2016) and have the ability to ingest oyster larvae (Laing *et al.*, 2005). The chain structure made up from multiple *C. fornicata* individuals are smooth, further repelling the settlement of *O. edulis* larvae. The effect of these smooth shell structures on the immediate hydrodynamic environment is discussed in Chapter 4.

Sea stars, *Asterias rubens* and *Solaster papposus* are thought to pose a predatory threat to *O. edulis* (Hancock, 1955, 1969; Laing *et al.*, 2014; Bromley *et al.*, 2016b), the former found in large numbers in South Wales (Woolmer *et al.*, 2011). However, Hancock (1955) found that *A. rubens* neglected oysters when barnacles and *C. fornicata* were present, and *S. papposus* selected *A. rubens* if present, thus reducing overall predation pressure from starfish. This prey-shift concept was later reiterated by Le Cam and Viard (2011) who highlighted that while non-native species (such as *C. fornicata* or invasive oyster drill *Ocenebra inornata*) may disrupt an ecosystem, they may become prey and relieve pressure from other native prey groups; Grason and Buhle (2016) found that native cancid crabs had a preference for *O. inornata* over *Ostrea edulis* if present. Crabs often prey on young molluscs but some (cancroid and portunid spp.) have the strength to crush adult bivalve shell (Quayle and Newkirk, 1989). Atlantic Clionid sponges use chemical and mechanical methods to drill through rock and shell for colonisation (Rützler and Rieger, 1973; Carroll *et al.*, 2015). Molluscs can initially repair puncture wounds to their shell and add internal shell material to protect their softer tissue (Quayle and Newkirk, 1989), yet this uses energy that could have been applied to somatic growth (Palmer, 1992; Stefaniak *et al.*, 2005; Carroll *et al.*, 2015). In addition, holes made in the shell are a potential access point for parasites and predators and can negatively affect the condition of an individual (Wesche *et al.*, 1997; Rosell *et al.*, 1999; Carroll *et al.*, 2015). The European common tingle, *Ocenebra erinacea*, and American tingle, *Urosalpinx cenerea*, are gastropods that feed on oysters by drilling a hole in the bivalve shell and using a proboscis to suck out the soft flesh (Carriker, 1981; Laing *et al.*, 2005). *U. cenerea* arrived in Britain with the translocation of American oysters *C. gigas* for cultivation in the 1920s (Cole, 1951) and has since become a pest to English *O. edulis* populations and are thought to provide a more serious threat than native tingle *O. erinacea* (Key and Davidson, 1981). Key and Davidson (1981) suggested oyster drills were responsible for 25% of mortality of young oysters (30 - 45 mm) per year, and that the presence and abundance of tingle populations should be monitored in close association with *O. edulis* populations (Cole, 1951; Laing *et al.*, 2005). The New Zealand and Australian barnacle, *Elminius modestus* has been identified as a potential pest and competitor of settlement space for *O. edulis* (Cole, 1951) since being recognised to have reached British shores by Crisp and Chipperfield (1948) and Knight-Jones (1948).

1.3.5 Shipping and associated pollution

Shipping activity can have a negative effect on the immediate environment by the dissemination dispersal of invasive non-native species (INNS), increased pollution, and increased turbidity and sedimentation. The accidental trans-global distribution of non-native species (NNS) either through ballast water (Ruiz *et al.*, 1992) or hull fouling (Howard, 1995; Gollasch, 2002), is a major issue caused by global shipping, and is a known mechanism of transfer of the oyster parasite *Bonamia ostreae* (Bishop *et al.*, 2006; Feng *et al.*, 2013). INNS often become invasive to their new environment owing to the absence of competition or predators, and this can have a devastating effect on the natural ecosystem (Airoldi and Bulleri, 2011; O’Shaughnessy *et al.*, 2020). The south of England is thought to be a gateway for NNS arriving to Britain from Europe and further afield (Eno *et al.*, 1997; O’Shaughnessy *et al.*, 2020). The slipper limpet, *Crepidula fornicata*, tingle *Urosalpinx cenerea*, and Pacific oyster *Crassostrea gigas*, are three NNS from the United States of America, that are now found in high numbers in the Solent area (Helmer *et al.*, 2019; O’Shaughnessy *et al.*, 2020).

Fouling, caused by the build-up of algae, bacteria and invertebrates such as barnacles and mussels over time on the underwater parts of a vessel, can over time have a detrimental effect on both the vessel and the environment; friction on the hull can impact vessel performance and reduce fuel efficiency, and a fouled hull is corroded by fouling organisms (Abarzua and Jakubowski, 1995). Antifouling paints, used as a deterrent, contain biocides that slowly release over time to slow the process of fouling; in the 1970s, copper was replaced with organotins such as triphenyltin (TPT) and more infamously tributyltin (TBT), which was a highly successful anti-fouling agent (Champ and Seligman, 1996; Langston *et al.*, 2015). Unfortunately, leached TBT residue proved to be toxic to coastal organisms such as oysters and gastropods by disrupting sexual physiology (tingles *Ocenebra erinacea* and *Urosalpinx cenerea*, Féral and Le Gall, 1983; Ellis and Agan Pattisina, 1990; Ronis and Mason, 1996, and dogwhelk *Nucella lapillus*, Gibbs *et al.*, 1987; Langston *et al.*, 2015), and stunting growth and shell development (*O. edulis*, Axiak *et al.*, 1995). Although TBT is thought to take up to 30 years to fully break down (Champ and Seligman, 1996), restrictions on use of TBT since 1989 (Arai *et al.*, 2009) have allowed the worst affected species, such as *N. lapillus*, to recover. *O. erinacea* and *U. cenerea* populations have experienced a revival as well, which ironically has meant these two predatory species now pose a major threat to oyster populations (Laing *et al.*, 2005; Faasse and Ligthart, 2007; Morton, 2011).

Today, over 90% of global trade is transported by sea, which demonstrates the level to which the globalisation of the shipping container (in 1956) has changed our society (Miller, 2012; Walker *et al.*, 2019). Over 53 000 active merchant ships were recorded in 2019 including general cargo ships (31.6%), crude oil tankers (13.9%), and passenger ships (9.0%), and 265 million tonnes of deadweight cargo was transported across the world in the 5152 container ships alone (www.statista.com, 2019). Increased shipping has led to a global development and expansion of ports to accommodate large vessels that need regular maintenance such as dredging deep channels (Erftemeijer and Lewis III, 2006; Miller, 2012). A volume of 0.5 million m^3 was dredged annually between 2005 and 2013 in Southampton water (ABP, 2014). Dredging activity causes a number of consequences for the environment; the active removal of substrate can alter the benthic ecosystem (Newell *et al.*, 1998), dredging activity can lift substrate material (containing contaminants) into suspension causing turbidity at the seabed water interface and reduced water quality (Amado-Filho *et al.*, 2004; Erftemeijer and Lewis III, 2006), and physical changes to bathymetry can alter water velocities and wave conditions (Erftemeijer and Lewis III, 2006). Dredged material from Southampton, Portsmouth and Isle of Wight ports and harbours is deposited at a certified marine deposit site only 13 km offshore from Bembridge on the Isle of Wight at Nab Tower (site WI060) monitored by Cefas (Bolam *et al.*, 2016). High levels of suspended material have been shown to reduce the clearance rate of bivalves, as their available food is diluted with grains of sediment (Wilber and Clarke, 2001), and the settling of this material can cause smothering and restrict larval settlement (Abelson and Denny, 1997). *O. edulis* is more sensitive to turbidity than other oyster species (*C. virginica*), and the reduced energy gain through reduced feeding results in a decrease of growth rate (Utting, 1988; Grant *et al.*, 1990).

1.3.6 Severe weather events

Fluctuations in temperature and salinity are two stressors that can impact the fitness of individuals and populations (Laing *et al.*, 2005; Harvey *et al.*, 2014; Kamermans *et al.*, 2018). Fitness is the ability of an organism to survive and reproduce in their environment, and therefore can provide measure of the health of a population (Orr, 2009). Although reported to successfully grow and breed in seawater with a wide range of temperatures (15 °C - 25 °C +, Korringa, 1956; Fariñas-Franco *et al.*, 2018) and in salinities as low as 16 (Laing *et al.*, 2005), *O. edulis* have demonstrated unfavourable responses such as the reabsorption of eggs and reduced food ingestion under stressed conditions (Loosanoff, 1962). High mortality events such as those seen in the 1920s and 30s (Orton, 1940; Kennedy and Roberts, 1999; Laing *et al.*, 2005) and more recently in the 1960s (Laing *et al.*, 2005) have been correlated to particularly cold weather.

An abnormally cold winter was recorded in the British Isles and parts of Europe from December 1962 to late February 1963, with average air and seawater temperatures 5-6 °C lower than normally seen at this time of year (Crisp, 1964; Van Banning, 1979; Key and Davidson, 1981). Crisp *et al.* (1964) suggested that the cold water restricted the performance of both British or re-laid Brittany oysters (*O. edulis*) and relaid Portuguese oysters (*Crassostrea angulata*) and shell gaping allowed mud and associated bacteria to enter and accumulate in the shell which choked and obstructed feeding of the oysters. As a result, oyster mortalities were 80% in Poole Harbour, 80% in the Helford river and between 70 and 95% on the Essex and Kent coast (Crisp, 1964). The Food and Agriculture Organisation (FAO) subsequently documented a crash in *O. edulis* fisheries across Europe with a record low landing of 1000 tonnes in 1964 and 1967 in comparison to 4000 tonnes in 1960 and 3400 tonnes in 1961; The UK only produced 100 tonnes of *O. edulis* in 1964. Newtown Creek on the Isle of Wight, and the Beaulieu River in the New Forest National Park experienced 100% mortality of their oyster beds as a result of this cold winter. As with bivalves, the mortalities seen in other taxonomic groups were complex and not necessarily a result of the cold water, although temperatures did exceed many tolerance thresholds. Opportunist diseases and physical damage caused by ice are two of many factors attributed to mass mortality events seen as a result of cold weather (Miller, 1855; Orton and Lewis, 1931; Cole, 1940, 1951; Crisp, 1964). With the rapid acceleration of global warming, it is more likely that UK fisheries will be negatively impacted by ‘marine heatwaves’, which have supposedly increased in frequency and duration by 34% and 17%, respectively, over a 91-year period (Oliver *et al.*, 2018). However, abnormally low temperatures and heavy snow brought by “The Beast from the East” (as named by British and Irish) in 2018 reminded us that extremely cold weather events are still likely to occur and put fisheries at risk (Pinnegar *et al.*, 2020).

Host-parasite interactions are impacted by abiotic and biotic factors, which allow each to dominate different niches (Lynch *et al.*, 2014). For example, a high salinity environment would benefit an oyster population by keeping *Martelia refringens* prevalence low (Laing *et al.*, 2005). Similarly, prevalence of disease may decrease with higher temperature, but oyster mortality may be higher (Laing *et al.*, 2014). Current intelligence on these interactions is likely to change as the climate changes as a result of global warming. Warmer seawater may increase dissemination and prevalence of diseases but may provide a platform for a stronger immunity for host organisms. *Bonamia ostreae* is known to be limited by low temperatures, yet seawater temperature is rising and therefore fewer places will be limiting for the parasite (Rowley *et al.*, 2014).

1.4 Potential benefits for the restoration of *Ostrea edulis* beds

Significant alteration of the habitat, and abundance and distribution of associated species, can shift the function of entire ecosystems (Lenihan and Peterson, 1998; Jones *et al.*, 2013; Hicks *et al.*, 2016). The overexploitation of *O. edulis* for consumption and accidental introduction of disease into naïve populations have had a dramatic and irreversible effect on the benthic environment, altering trophic relationships more rapidly than a natural system would (Millar, 1968; Hicks *et al.*, 2016). Fishing restrictions and regulations to control the movement of oysters have been implemented in some areas to protect *O. edulis* habitat from further damage, but this species has not demonstrated a natural revival (Donnan, 2007; Pollock, 2016; Smyth *et al.*, 2018; Allison *et al.*, 2020). It has become apparent that the only way *O. edulis* can be maintained for the future is by human intervention through restoration (Millar, 1968; Laing *et al.*, 2005).

Restoration methods frequently used in the USA for restoration of Eastern oyster *Crassostrea virginica* or Olympia oyster, *Ostrea lurida*, in the USA over the last century such as the deployment of oysters on the seabed or within elevated structures have proven beneficial for *O. edulis* (Kennedy and Roberts, 1999; Laing *et al.*, 2005; Donnan, 2007; Smyth *et al.*, 2018). Increased *O. edulis* recruitment has been attributed to the re-laying of adult oysters (Korringa, 1946; Key and Davidson, 1981), and is thought to be dependent on available substrate (Laing *et al.*, 2006; Smyth *et al.*, 2018, 2020; Allison *et al.*, 2020), although more field studies are required to gather evidence *in situ*. Technical manuals for restoring *Crassostrea* spp. in the USA, that offer a wealth of information and experience, have standardised a restoration ‘best practice’ (Brumbaugh *et al.*, 2005; Baggett *et al.*, 2014; zu Ermgassen *et al.*, 2016). Although the same experience and knowledge are incomplete for *Ostrea* spp., the addition of *O. edulis* to the list of protected species in the Biodiversity Action Plan in the mid-2000s (JNCC, 2007), and to the OSPAR list of ‘threatened or declining’ species in 2008 (Haelters and Kerckhof, 2009), has raised the profile for *O. edulis* restoration efforts, which will help build the necessary information to provide similar technical advice.

The Millennium Ecosystem Assessment, initiated in 2001 by the United Nations, identified four categories under which various ecosystem services could be defined: ‘provisioning’, ‘regulating’, ‘supporting’ and ‘culturally significant’ (Leemans and de Groot, 2005). This concept finds links between ecological function and human well-being (Fisher *et al.*, 2009), and adds value (often monetary) to restoration (Stagl, 2004).

For *O. edulis* on the seabed, *provisioning* services include the benefits for commercial fisheries and aquaculture. Replenishing native oyster fishery stocks is a high priority for many coastal areas to ensure profit for both fisheries and restaurant industries (Brumbaugh *et al.*, 2005).

Regulating services include water quality, the reduction of coastal erosion, and nutrient cycling. Excess nutrients in coastal water bodies, driven by domestic and industrial anthropogenic discharge, can generate (occasionally toxic) algal blooms.(Paerl, 1997). This eutrophication exacerbates the biological demand on dissolved oxygen of the affected water body and can cause mass mortality of marine fauna (Rabalais *et al.*, 2010). High levels of primary production stabilise an ecosystem, providing a greater abundance of food for primary consumers (Seitz *et al.*, 2014). However, the amount of suspended sediment in the water surrounding oyster beds has been shown to affect the clearance rate of *O. edulis* and consequently slow growth rate (Korringa, 1952; Hutchinson and Hawkins, 1992; Laing *et al.*, 2005). Toxic algal blooms that can result in significant accumulation of rotting phytoplankton on the seabed can cause local hypoxia and have been shown to negatively affect oyster beds (Laing *et al.*, 2005). Even small reductions in oxygen concentration can cause stress to individuals and increase susceptibility to disease (Patterson *et al.*, 2014).

Oysters filter water through their gills, ingesting algae, bacteria, detritus and sediment, which reduces the suspended solids in the water column (Korringa, 1952; Orth *et al.*, 2006; Grabowski and Peterson, 2007; Smaal *et al.*, 2015) creating a less turbid environment more appealing to tourists, and to higher trophic and commercially valuable organisms, such as the edible crab (Grabowski *et al.*, 2005; Sawusdee, 2015; zu Ermgassen *et al.*, 2016). A clearer water column allows deeper penetration of light, essential for phytoplankton and seagrass growth (Orth *et al.*, 2006; Wall *et al.*, 2008). *O. edulis* produce faeces and expel additional grit known as pseudo-faeces, both containing high concentrations of organic nitrogen (Grabowski and Peterson, 2007). This elevates the microbial activity of denitrifying bacteria in the sediment increasing the production of nitrogen gas (N) and regulating the cycle of nutrients in the immediate food web (Grabowski and Peterson, 2007; Kellogg *et al.*, 2013). Further research into the intricacies of the elevated microbial activity hosted by an oyster bed is underway (Vivaldi Project, 2016-20, https://www.vivaldi-project.eu).

Coastal erosion, caused by the movement of marine sediment with pressure from wind, waves and tides, can have a devastating effect on coastal ecosystems due to the changes in habitat structure and function (Jones *et al.*, 2013). This natural process is being accelerated by climate change induced sea level rise (Jones *et al.*, 2013). Undisturbed oyster beds are one method thought to minimise and perhaps reverse the effects of coastal erosion, and could provide a natural alternative to concrete coastal defence developments (Meyer *et al.*, 1997; Coen *et al.*, 2007; Grabowski and Peterson, 2007; zu Ermgassen *et al.*, 2016).

Supporting services offered by native oysters include the formation of reef-like habitat for other species. Undisturbed, an oyster population naturally forms a three-dimensional structure composed of multiple oysters attached to one another (Figure 1.4) (Perry *et al.*, 2020). *O. edulis* are rarely seen in this formation now, as once an area has been damaged by fishing activity such as dredging, the habitat needs time to recover (Brumbaugh *et al.*, 2005). Although much of the literature has focussed on vertical reef-building *Crassostrea* spp. (Coen *et al.*, 1999; Breitburg *et al.*, 2000; Ruesink *et al.*, 2005; Grabowski and Peterson, 2007; zu Ermgassen *et al.*, 2015), the morphology of *O. edulis* shells is also known to facilitate shelter and habitat for numerous species (Korringa, 1951; Grabowski *et al.*, 2005; Smaal *et al.*, 2015; Lown *et al.*, 2021).

Figure 1.4. The three-dimensional structure developed from oysters settling on one another in an undisturbed oyster ground (these oysters were retrieved from cages in Hamble Point Marina, the Solent, UK in 2017).

Culturally significant services offered by native oysters are tourism, coastal heritage and community consolidating opportunities. Areas with high *O. edulis* production, such as France, often promote these opportunities as a means of education for the local community and to encourage tourism

(Gercken and Schmidt, 2014). The United States encourage a high level of public involvement in their oyster restoration, engaging school children and adults in volunteer work (DeAngelis *et al.*, 2019). Oyster shell recycling depositories are made accessible to the public with the purpose of collecting enough shells for re-seeding oyster settlement areas with new shell substrate for larvae (Baggett *et al.*, 2014). Raising public awareness to the threat of natural habitat loss is paramount for species protection, but projects with such open access must be carefully monitored to prevent further damage such as the spread of disease.

In order to appreciate some, or any, of these rewards offered by a replenished oyster population, both environmental and economic advantages must be considered, and many of these ecosystem services have yet to be quantified or demonstrated to full effect. Literature on the clearance and retention rate of algae by *Crassostrea* spp. has already supported online calculators (https://oceanwealth.org/tools/oyster-calculator/) in areas such as Chesapeake Bay to propose the benefits and put economic value to the benefits of oyster restoration. A site-specific clearance rate for *O. edulis* is yet to be quantified and few have attempted the difficult task of quantifying the retention of such particulate matter (Møhlenberg and Riisgård, 1978; Wilson, 1980; Nielsen *et al.*, 2017). Stakeholders involved in *O. edulis* restoration, such as the fishing industry, local government agencies and the public, are likely to judge project success from the perspective of their different priorities. It would be normal for an oyster fisherman to judge project success by the abundance of oysters available for landing (a provisioning service), whereas an ecologist may look at changes in species diversity (a supporting service), and a local council may assess water clarity (a regulating service) (Stagl, 2004). Restoration projects have often been weighted heavily towards the enhancement of fisheries by increasing oyster production, but evidence from *C. virginica* suggests that the wealth of Ecosystem Services provided by an undisturbed oyster bed offer more to a coastal ecosystem than individuals for harvest (Paerl, 1997; Grabowski *et al.*, 2012). It is becoming more apparent that a balanced management approach to the whole ecosystem yields the most sustainable benefits for all stakeholders in question (Beck *et al.*, 2011).

1.5 Environmental considerations for the restoration of *Ostrea edulis*

1.5.1 Site selection

Selecting an appropriate site for *O. edulis* restoration is crucial for the long-term fitness of the population in facilitating successful annual reproduction and recruitment, and avoiding mortality either from predation, outbreaks of disease, or excessive fishing activity. Laing and Spencer (2006) identified important environmental considerations for site selection including temperature and salinity, disease and predation, and oxygen concentration and pollution, highlighting the need to acknowledge physical, physiological, biological and chemical factors. Laing and Spencer (2006) also accentuated economic considerations such as fisheries, public accessibility and shellfish cultivation. Established or historic oyster grounds would likely offer suitable environmental conditions to benefit oyster development, such as nutrition and water temperature. In addition, the already occupant oysters or residual shells would provide immediate relief for larvae settlement and promote recruitment (Rodriguez-Perez *et al.,* 2019). Highly industrialised areas offer ease of access to the oysters, but pose a higher risk of pollution, such as marinas where boats may still be leaching remainder toxic TBT anti-fouling paint (Woolmer *et al.,* 2011). Similarly, sites previously exposed to disease (bonamiosis) have often been considered inappropriate for restoration (Kennedy and Roberts, 1999; Robert and Gerard, 1999), due to the slow recovery rate of the population (Laing *et al.*, 2005). However, reports that oysters from *Bonamia*-exposed populations might demonstrate increased tolerance, or even resistance to *Bonamia ostreae*, when compared to naïve oysters (Martin *et al.*, 1993; Naciri-Graven *et al.*, 1998), has led to discussions between restoration practitioners as to the benefits of using *Bonamia*-exposed *O. edulis* populations for restoration (Smaal *et al.,* 2015; Pogoda *et al.,* 2019). The difficulty in detecting *B. ostreae* amongst a population before the infection is so advanced that mortalities occur, remains an issue for large scale restoration (Carnegie *et al.*, 2016). Establishing sanctuary areas, closed to public and commercial fishing activity, is a robust way to ensure restoration is not disturbed by dredging and trawling practices commonly used by more than just oyster fishermen. The use of floating devices such as pontoons, or offshore windfarms would act as sanctuary areas void of fishing pressures, and are consequently gaining interest for use in bivalve aquaculture or restoration (Kamermans *et al.*, 2016, 2018; Buck *et al.*, 2017; Smaal *et al.*, 2017). However, offshore areas often have high wave energy that might challenge the retention of re-laid oysters, and accessibility for monitoring efforts. The hydrodynamic environment has significant effect on physiological and biological factors and is therefore a key consideration for *O. edulis* restoration site selection. Establishing 'ideal site conditions' for *O. edulis* restoration is challenging and is something still under discussion amongst specialist science groups such as NORA (Native Oyster Restoration Alliance) (https://noraeurope.eu). Initiated in 2016, NORA is a platform for a network of *O. edulis* scientists and practitioners to share experience and knowledge on *O. edulis* restoration efforts in (currently 10) European countries. A number of specialists within the NORA community are in the process of developing a publication to address this uncertainty around what is, and what isn't necessary for site selection, and some of the likely factors are listed below (Table 1.2).

Table 1.2. In no particular order, a list of some environmental factors often considered when selecting a site for *O. edulis* restoration and (if known) their suggested range or measurement.

Factor	***O. edulis***	**Reference(s)**
Water depth	≤ 30 m (although *O. edulis* found up to 50 m)	(Pogoda *et al.*, 2019; Pogoda *et al.*, 2020b)
Water temperature	7-27 °C	(Laing and Spencer, 2006; Pogoda *et al.*, 2020b)
Water salinity	25-35	(Laing and Spencer, 2006)
Water pH	5-8	(Cao *et al.*, 2009)
Food availability	Chl-α concentration, 2-3 μg L^{-1}	(Rogan and Cross, 1996)
Substrate type/presence/availability	Firm, preferably bivalve shell	(Laing and Spencer, 2006; Colsoul *et al.*, 2020)
Sedimentation	Low	(Laing and Spencer, 2006)
Water quality (pollutants)	TBT (tributyltin)	(Woolmer *et al.*, 2011)
Nutrient concentration	-	(Rogan and Cross, 1996)
Dissolved oxygen	> 3.5 mg L^{-1}	(Smaal *et al.*, 2017)
Disease status/prevalence	*e.g. Bonamia ostreae*	(Laing and Spencer, 2006; Pogoda *et al.*, 2020b)
Predator presence/abundance	*e.g. Ocenebra erinacea*	(Laing and Spencer, 2006)
Competitor presence/abundance	*e.g. Crepidula fornicata*	(Laing and Spencer, 2006)
Exposure to air, wind & currents	Low, shear stress < 0.6 N m^{-2}, current velocity 0.05 – 0.45 m s^{-1}	(Laing and Spencer, 2006; Pogoda *et al.*, 2020b)

1.5.2 Hydrodynamics

Hydrodynamics are the ‘physics that deal with the motion of fluids and the forces acting on solid bodies immersed in fluids and in motion relative to them’ (Merriam-Webster, no date). As discussed in section 1.3.2, *O. edulis* larvae experience a pelagic phase (6-15 days) prior to settlement and are subject to hydrodynamics in the form of tidal regimes, water currents and wave activity that can influence their settlement location. Similarly, once settled, *O. edulis* are subject to the natural fluctuations of local hydrodynamics. Published contradictions as to the type of hydrodynamics best for *O. edulis* make site choice for restoration projects difficult. Woolmer *et al.* (2011) found oyster beds were mainly in sheltered locations in Swansea Bay and Milford Haven waterway with tidal currents at 1-2 kts and tidal movement of < 0.1 m s^{-1} that would help to retain larvae and reduce smothering by silt. However, Woolmer *et al.* (2011) also suggested that historical oyster reefs as described by Wright (1923, 1932) could have been encouraged by the high tidal range of the Bristol Channel. One key aspect is ensuring restoration efforts avoid economic loss as a result of the local hydrodynamic environment. For example, in 1869, 960 000 oysters re-laid in the Hamble river were swept away by tidal currents (Philpots, 1890). Similarly, larvae are vulnerable to the effects of tidal currents and availability of settlement grounds. The role of hydrodynamics in controlling oyster distribution and settlement location are discussed in Chapter 2.

1.5.3 Deploying cultch for substrate

In removing an oyster from the seabed for consumption, the fishing industry removes both the consumable, and the habitat required for successful recruitment of future populations (Laing *et al.*, 2006; Smyth *et al.*, 2018). *O. edulis* beds and the associated habitat have been depleted by 85% globally (Beck *et al.*, 2011) and restoration projects often require re-laying cultch to replace this lost habitat (Smyth *et al.*, 2018). Successful *O. edulis* recruitment has been reported on many different hard substrates such as tiles coated with lime (Cole and Knight-Jones, 1939; Mann and Powell, 2007; Colsoul *et al.*, 2020), polished marble (Hidu *et al.*, 1975), and compacted shredded automobile tyres (Mann and Powell, 2007). However, research into larval behaviour has revealed a high level of settling selection, with adult oyster shell often thought to be the preferred substrate (Cole and Knight-Jones, 1949; Korringa, 1951; Jonsson *et al.*, 1999; Kennedy and Roberts, 1999; Colsoul *et al.*, 2020; Potet *et al.*, 2021). Larval settlement relies on more than simply the material offered (Fuchs and Reidenbach, 2013), and evidence of a preference for live oysters over abandoned (dead) shells highlights the importance of chemical cues from conspecifics and biofilms in attraction of *O. edulis* larvae for settlement (Cole and Knight-Jones, 1939; Bayne, 1969; Burke, 1986; Tamburri *et al.*, 2008; Woolmer *et al.*, 2011; Mesías-Gansbiller *et al.*, 2013; Smyth *et al.*, 2018, 2020; Rodriguez-Perez *et al.*, 2019).

Much of the literature has affirmed the benefits of laying cultch for successful oyster restoration (Key and Davidson, 1981; Kennedy and Roberts, 1999; Luckenbach, Mann and Wesson, 1999 (and references within); Laing, Walker and Areal, 2005; Smyth *et al.*, 2018). Restoration projects in the United States, (such as Chesapeake Bay, Fisherman’s Island, Virginia) for the Eastern oyster, *Crassostrea virginica*, have encouraged the recycling of oyster shells from restaurants and private consumers to use in the restocking of cultch for oyster larvae settlement (Rothschild *et al.*, 1994; Laing *et al.*, 2005; Nestlerode *et al.*, 2007). This method requires continued effort, and several papers have identified its poor economic return (Coen and Luckenbach, 2000; Mann and Powell, 2007). In addition, there are concerns that cultch aids the transmission of disease to previously non-infected areas (Bushek *et al.*, 2004; Laing *et al.*, 2005; Woolmer *et al.*, 2011; Gercken and Schmidt, 2014), or that the laying of material in question is simply a form of ocean dumping (Beck *et al.*, 2011). Hawkins *et al.* (2005) found cultch-less oysters to have better immune-competence than those attached to either scallop, slipper limpet or cockle shell. This has implications for restoration, as healthier and more resilient oysters might result from oysters laid at a high enough density to provide a natural cultch settlement ground for future generations.

The presence of *Crepidula fornicata* on oyster ground can both benefit and hinder oyster settlement; *C. fornicata* shells provide natural cultch (Key and Davidson, 1981) but their faeces creates ‘mussel mud’, which is known to be less appealing settling ground for oyster larvae (Hawkins *et al.*, 2005; Woolmer *et al.*, 2011). The Solent seafloor is currently covered in a high density of *C. fornicata* shells, yet *O. edulis* recruitment is low, suggesting that the negative influence of *C. fornicata* outweighs the positive. In 1982, extensive dredging of the Fal Estuary in Cornwall, UK, occurred as part of a restoration strategy to remove *C. fornicata* from the seabed before re-laying *O. edulis* individuals (Laing *et al.*, 2005; Woolmer *et al.*, 2011). Regardless, the population abundance of *C. fornicata* in the Fal Estuary has continued to grow (Syvret and Fitzgerald, 2008), and the wild oyster fishery is more likely sustained by the inefficient fishing technique (hand dredge under sail) used by the local oyster men than from previous clearance of *C. fornicata* (Long *et al.*, 2017).

1.5.4 Choice of restoration broodstock

Natural succession through reproduction and recruitment success is essential for any restoration or conservation programme to reduce human intervention or effort in the long term. The choice of broodstock for restoration has implications for the physiological efficiency of an oyster individual, the robustness and sustainability of a population influenced by genetic succession, and the environmental impact of their presence such as the potential spread of disease.

O. edulis have successfully populated coastal regions from 64 ºN in Norway to 32 ºN in Morocco (Bromley *et al.*, 2016a) and have demonstrated survival in a range of temperatures (-1.5 – 35 ºC) and salinities (18 – 40) (Nielsen *et al.*, 2017). Although this demonstrates their ability to acclimate to many different conditions, differences in physiological performance have been shown to occur from these different environments. Korringa (1957) studied the effect of temperature on different stages of the life cycle, and although he found a crucial threshold for ‘reproductive activity’ at 15 ºC and successful larval development only above 17 ºC, he acknowledged that populations from different regions would require different environmental conditions, a theory supported by Wilson and Simons (1985). For example, Korringa (1957) suggested that oysters from Essex would not survive in Scotland as they would not be able to breed, and advised oyster farmers against purchasing oysters from too far afield that might not spawn in their waters. This theory was based on the assumption that oysters would still produce eggs and sperm, but without the trigger to release gametes, oyster recruitment would not take place (Korringa, 1957). Bromley *et al.* (2016) subsequently published a list of the different temperatures (13-25 °C) that induced spawning in populations of *O. edulis* at different latitudes (from Italy to Norway respectively). Further analysis of the effects of broodstock origin on physiological performance of *O. edulis* is discussed in Chapter 3.

The international translocation of *O. edulis* for cultivation has led to a homogenisation of genetic differentiation amongst the global population (Beaumont *et al.*, 2006). Genetic studies have demonstrated mixed reviews as to the heterozygosity of this species across Europe. Several claim that *O. edulis* has low genetic variation (Saavedra *et al.*, 1993; Launey *et al.*, 2002; Sobolewska and Beaumont, 2005; Donnan, 2007), while others claim to detect differences between European regions (Johannesson *et al.*, 1989; Maršić-Lučić, 1999; Sobolewska and Beaumont, 2005; Vera *et al.*, 2016). Restoration projects often rely upon hatchery-reared oyster spat for oyster re-laying due to the need for a certain size (oysters < 50 mm may get washed out to sea) and number (cost efficiency can mean re-laying individuals in high numbers, Bayraktarov *et al.* 2016). However, with current low densities of wild *O. edulis* in Europe, there is a limited source of hatchery seed (Lynch and O'Hely, 2001; Adamson *et al.*, 2018). Oysters with the same origin (ie: from the same hatchery) may be from a limited broodstock resulting in a lower genetic diversity (Gosling, 1982; Hedgecock and Sly, 1990; Gaffney *et al.*, 1992; Saavedra and Guerra, 1996; Saavedra, 1997; Launey and Hedgecock, 2001; Boudry *et al.*, 2002; Sobolewska and Beaumont, 2005; Laing *et al.*, 2005; Appleyard and Ward, 2006; Taris *et al.*, 2007; Lind *et al.*, 2009; Lallias *et al.*, 2010), which ultimately reduces population fitness and ecosystem functionality (Gosling, 1982; Saavedra, 1997; Lynch and O'Hely, 2001; Laing *et al.*, 2005; Lallias *et al.*, 2010);. In addition, there is concern that domesticated organisms might outcompete their wild counterpart thus reducing genetic diversity in the wild (Lallias *et al.*, 2010). An alternative source of oyster spat is pond culture, and large ponds have been shown to retain a high genetic diversity, almost as well as that found in the wild (Lallias *et al.*, 2010). Laing *et al.* (2005) acknowledged that the use of hatchery or pond cultured oysters is often the only way to acquire high numbers of oysters for restoration but suggested an occasional injection of wild individuals to diversify the genetic makeup of the population.

Whilst necessary to protect disease-free populations from the introduction of pathogens, movement restrictions have been considered as a bottleneck to the development of restoration projects across Europe. Organisations have reflected differently on the challenges presented by the moratorium on oyster movements. Some restoration practitioners have proposed benefits in translocating parasite-positive but otherwise healthy stocks, based on the premise that these stocks are likely to reflect the presence of 'resistant' or 'tolerant' oysters (e.g. Smaal *et al.*, 2015; Pogoda *et al.*, 2019). In support of this position, there are reports that oysters from *Bonamia*-exposed populations might demonstrate increased tolerance, or even resistance to *Bonamia ostreae*, when compared to naïve oysters (Martin *et al.*, 1993; Naciri-Graven *et al.*, 1998). da Silva *et al.* (2005) demonstrated that the level of disease resistance varied depending on family and origin of oyster population, finding higher resistance from indigenous individuals. This reinforces Korringa's (1957) recommendation that local spat selection is important for restoration purposes. Specific genes in *Bonamia*-resistant individuals were identified by Morga *et al.* (2012), which could be used to target appropriate *Bonamia*-resistant stock for restoration purposes. *Bonamia*-resistance would benefit *O. edulis* populations and perhaps should be the focus of strategy for restoration projects (Laing *et al.*, 2006; Lallias *et al.*, 2010; Woolmer *et al.*, 2011). Markers of gene resistance are explored in Chapter 4.

1.5.5 Elevating oysters above the seabed

Elevated oyster beds (natural or artificial) can increase water flow across the bivalves (Lenihan, 1999; Schulte *et al.*, 2009; Woolmer *et al.*, 2011). This can help reduce siltation, undesirable for oyster culture (Korringa, 1952; Quayle and Newkirk, 1989; Berghahn and Ruth, 2005). Hanging oysters in nets or on elevated reefs may also reduce biofouling of *C. fornicata* on the population and reduce spatial competition between the species (Woolmer *et al.*, 2011; Sawusdee *et al.*, 2015). Quayle and Newkirk (1989) suggested that oysters grow faster when elevated due to an increase in the food to silt ratio, and Schulte *et al.* (2009) similarly demonstrated maximised growth rate and physiological excellence of American native oysters (*Crassostrea virginica*) in high relief from the seabed. Sawusdee *et al.* (2015) has since demonstrated improved physiological performance of *O. edulis* elevated only 0.4 m from the seabed. Elevated rack culture is a common European method for the culture of different oyster species (such as *Crassostrea gigas*) but has been shown to result in higher prevalence of disease and mortality when used for *O. edulis* culture (Laing *et al.*, 2005). This is unlikely to be due to elevation, and more as a result of the frequent (monthly) monitoring and cleaning required with rack culture due to prevalent fouling by pests such as crabs and starfish, as *O. edulis* is sensitive to excessive handling (Hawkins *et al.*, 2000; Laing *et al.*, 2005). The benefits of elevating oysters from the seabed are clear, yet different locations and environmental conditions present different challenges, highlighting the difficulty in identifying a standard management strategy for restoration projects.

1.5.6 Re-laying density

O. edulis are dioecious brooders, and one obvious ecological constraint for an *O. edulis* population is the density of oysters on the seabed, as large distances between oysters can lead to sperm becoming too dilute for successful fertilisation (Vogel *et al.*, 1982; Denny and Shibata, 1989), which can heavily impact population growth and sustainability. Mature oysters on the seabed provide both broodstock for reproduction purposes, and a natural substrate preferred by settling larvae (see section 1.6.3), both essential for population sustainability (Laing *et al.*, 2005). Schulte *et al.* (2009) found that spat density was directly comparable to adult density, and Korringa (1947) concluded that the abundance of larvae released is directly proportional to the number of mother oysters in the area concerned. More recently, Guy *et al.* (2018) found a positive correlation between oyster density and brooding oysters at a natural site in Ireland, with the most beneficial brood numbers at an oyster density of 1.618 m^{-2}. Although the formation of ‘reefs’ by this species is contested (Fariñas-Franco *et al.*, 2018; Pogoda *et al.*, 2019), an established *O. edulis* population would also provide shelter for a community of benthic organisms (Korringa, 1951; Grabowski *et al.*, 2005; Smaal *et al.*, 2015). Historical evidence shows that re-laying oysters is often required to sustain oyster production (Key and Davidson, 1981). *O. edulis* are currently at a density well below a sustainable number in European waters, and restoration projects require large scale re-laying of adult oysters. However, an increase in population abundance (in the interest of oyster restoration) may cause stress to the population leading to a reduction in production and an increase in the prevalence of bonamiosis (Le Bec *et al.*, 1991; Van Banning, 1991; Héral, 1993; Hawkins *et al.*, 2000; Berghahn and Ruth, 2005; Engelsma *et al.*, 2010; Kamphausen, 2012; Laing *et al.*, 2014). Evidence from a project in Essex led to the conclusion that 10 oysters m^{-2} was the most beneficial oyster density (Laing *et al.*, 2005), but site-specific features (such as a history of *Bonamia ostreae*) would likely influence the development of a restored *O. edulis* population.

1.5.7 Managing pests (Crepidula fornicata)

Crepidula fornicata are often a subject for eradication as part of native oyster restoration. However, the physical removal of *C. fornicata* via harrowing or dredging may disturb *O. edulis* and cause stress from shell damage or handling. Woolmer *et al.* (2011) also highlighted the relatively high cost (£2995, in 2011) simply for an “application to dispose of dead *C. fornicata* at sea” post harrowing. *C. fornicata* are reliant on an oxygenated and nutrient rich environment for survival and smothering a bed of *C. fornicata* by adding a high density of cultch or another species (such as *O. edulis*) is one suggested method of (low cost) competitor eradication (Johnson, 1972; Woolmer *et al.*, 2011). *C. fornicata* have generally been considered a threat to native species, yet recent studies have provided evidence that their impact on the survival native bivalve spp. are not as restrictive as originally thought (Thieltges *et al.*, 2006). Barbier *et al.* (2017) found *C. fornicata* beds to actually enhance bivalve recruitment and species richness, but Lown *et al.* (2021) found the positive linear correlation between *O. edulis* density and abundance of additional epifaunal species to be surpressed when *C. fornicata* are present. *C. fornicata* are now established in most historical oyster bed habitats, and restoration organisations must determine the economic benefit and environmental importance of its eradication for the success of the native oyster.

1.6 Economic considerations for the restoration of *Ostrea edulis*

At the 1992 Rio Earth Summit held by the United Nations, the UK was one of 150 countries to sign the Convention on Biological Diversity (CBD), launched as an urgent appeal to conserve global biodiversity and achieve sustainable development. As a result, the Joint Nature Conservation Committee (JNCC), under the UK Government provided a platform called the National Biodiversity Network (NBN) gateway (since 1997) upon which to upload biological observations from around the UK, essential for evidence-based practices such as policy and decision making in association with restoration. The number of publicly accessible records (biodiversity observations) with a resolution of < 1 km s^{-1} collated in the NBN gateway increased three-fold (10.5 million to 35.2 million) between 2010 and 2017 (http://jncc.defra.gov.uk/page-6073), and these records increased to 130 million by the end of May 2019 as a result of the development of new platform, NBN Atlas. In 2010 in Nagoya, Japan, the UK prepared National Biodiversity Strategies and Action Plans (NBSAPs) to be achieved by 2020 (Defra, 2011). The UK NBSAPs included establishing Marine Protected Areas (MPAs) in excess of 25% of English waters and 'restoring at least 15% of degraded ecosystems as a contribution to climate change mitigation and adaptation' (available at https://www.cbd.int/nbsap/targets/, Defra, 2011). A progress report in January 2019 revealed that by November 2018, nearly 24% of UK water was recorded as part of an MPA (available at https://www.cbd.int/doc/nr/nr-06/gb-nr-06-p1-en.pdf, JNCC, 2019), and funding for biodiversity conservation had increased from an average of £77.4 million per year (2006-2010) to over £180 million in 2015. The protection of global ecological biodiversity has meant more robust environmental targets are being set, and projects such as oyster restoration that offer a wealth of environmental benefits through ecosystem services (discussed in section 1.4) have retained a high profile. Information about historic and existing *O. edulis* restoration in Europe (excluding the UK) can be found in Appendix A (Table A1).

1.7 Existing *Ostrea edulis* restoration in the UK.

Several privately-owned regions of the UK coastline have played a role in maintaining wild *O. edulis* fisheries including Loch Ryan, Scotland, the Beaulieu Estate, England, and the River Fal, Cornwall. Historical regulations and legislation concerning public fishing rights have allowed these areas to develop strategic and bespoke methods for their fishing business. The Rossmore Oyster Company in Loch Ryan is currently the only successful wild fishery in Scotland (Donnan, 2007; Fariñas-Franco *et al.*, 2018) and is carefully managed by a limited number of fishing vessels (Eagling *et al.*, 2015, 2017). Loch Ryan is one of only a few bays containing oyster fisheries in the UK still reported to be *Bonamia*-free (Fariñas-Franco *et al.*, 2018). Loch Ryan has a history of restoration; In the 1880s and 1960s, oysters were sourced from France, Holland, and Essex, and in the 1960s from Brittany, to restock the Loch Ryan seabed (Millar, 1964; Beaumont *et al.*, 2006). Natural spat fall and successful wild stock have since been observed and managed by private land owners (Beaumont *et al.*, 2006; Lallias *et al.*, 2010). The River Fal, England, has sustained its oyster fishery with a law against mechanical oyster dredging (Long *et al.*, 2017); Oysters are harvested with sailing and rowing boats only (Laing *et al.*, 2014).

Understandably, the more publicly accessible waterbodies such as Lough Foyle (Ireland), Thames Estuary (England), and the Solent (England) have witnessed more variability in oyster availability, likely due to more sporadic fishing activity and other forms of tourism. The spread of *B. ostreae* along the south coast of England from 1982 and into Ireland via Cork in the 1980s also contributed to the fluctuations in oyster landings (Elston *et al.*, 1987; Hudson and Hill, 1991; Steins, 1997; Culloty and Mulcahy, 2007; Laing *et al.*, 2014). Lough Foyle reacted to poor oyster recruitment, reduced spawning, late spawning, and outbreaks of *B. ostreae* by restricting fishing rights throughout several recent years (2008-2016), endorsing a maximum of just three days fishing each week (Pollock, 2016). This action consequently protected the fishery and ensured the survival of a small population (Donnan, 2007), although population numbers have not since increased. Attempts have been made to restore *O. edulis* populations in Scotland (Donnan, 2007; Shelmerdine and Leslie, 2009), Ireland (Tully and Clarke, 2012; *CuanBeo*, 2020), the Dutch section of the North Sea (Smaal *et al.*, 2015), Northern Ireland (Roberts *et al.*, 2005), and England (Allison *et al.*, 2020). Information on existing and obsolete *O. edulis* restoration in the UK can be found in Appendix A (Table A2).

1.7.1 The Solent Oyster Restoration Project

Records of active *O. edulis* cultivation in the Solent, in the south of England, date back to the mid 19th Century, in the form of (pond) breeding, re-laying and harvesting oysters (Key and Davidson, 1981). The early Solent fishery (1862-1876) experienced successful spatfalls each year, but oyster numbers were limited due to the overfishing of oysters to replenish deficient oyster beds elsewhere (eg. Bosham Fishery), and a high level of predation from borers or oyster drills (Key and Davidson, 1981). Sanctuary areas were established by the South of England Oyster Company in the 1860s and 70s to protect newly seeded oysters from excessive dredging (Kamphausen, 2012). This activity coincided with the Oyster and Mussels Fisheries Act, 1866, that was passed as a result of the increased demand for oysters in Britain (Philpots, 1890; Key and Davidson, 1981). The first recorded crash of the Solent *O. edulis* population occurred in 1920-21 and is now considered likely to be the result of a protozoan parasite *Hexamita* sp. outbreak (Orton, 1924; Mackin *et al.*, 1951). This halted all *O. edulis* production in the south of England, and subsequent severe winters in 1939/40 and 1946/47 slowed recovery (Cole, 1951). Although early oyster production appeared to suffer from mass mortality events and heavy predation by oyster drills (Key and Davidson, 1981), the Solent became one of the largest fisheries in the UK and in Europe (Davidson, 1976; Key and Davidson, 1981; Helmer *et al.*, 2019). Key and Davidson (1981) provide an extensive overview of the scale of the Solent fishery from 1872 to 1980, when the current Solent fishery peaked, with record landings ~850 tonnes worth £1 million. During that time, fishing pressure increased from 17 fishing vessels (in 1972) to 450 vessels (in 1978), which highlights the rapid escalation of the Solent oyster trade (Key and Davidson, 1981). Since then, an annual stock survey collected by the Southern Inshore Fisheries and Conservation Authority (IFCA) has shown a decline in the Solent *O. edulis* population abundance, reaching a critical low between 2007 and 2013 which ultimately caused the Southern IFCA to place temporary restrictions on the fishery (Kamphausen *et al.*, 2011; Southern IFCA, 2017). Although there are reports of successful reproduction, the low number of remaining oysters have failed to repopulate the Solent to a sustainable density, and consequently the fishery has not recovered. Despite having been infected with *Bonamia* for over 30 years (Laing *et al.*, 2014), the Solent has suffered relatively low mortality rates from bonamiosis (<1%), which could be due to the low oyster density (as a result of low annual recruitment) (Laing *et al.*, 2005, 2014).

Blue Marine Foundation (BMF) (https://www.bluemarinefoundation.com/projects/solent/) is one non-governmental organisation (NGO) and charity that has received much public and commercial support to restore *O. edulis* in the Solent, south England. Initiated in 2016, their restoration project aims to implement a self-sustaining population of *O. edulis* for both fishery and environmental benefit (BMF, 2017). One of BMF's objectives is to reseed the Solent with 5 million oysters over their 5-year project timeline and are conducting research to establish new, less intensive, fishing techniques incorporating semi-aquaculture methods. BMF have already deployed 45 000 oysters onto the seabed and 20 000 into cages hanging from marinas, which are currently acting as broodstock and they plan to lay sediment (cultch) favourable to the native oyster in areas likely to receive larvae for settlement.

1.8 Knowledge gaps in *Ostrea edulis* restoration

A self-sustaining population of *O. edulis* achieved from active restoration is the synergistic assembly of individual decisions on management and development of a restoration project. Disease, pests, substrate and broodstock have been identified as important areas for *O. edulis* restoration to address (Laing *et al.*, 2005; Mann and Powell, 2007; Lipcius *et al.*, 2015), yet quantification for the success within these parameters are yet to be defined. The overall aim of this thesis is to address the gaps in our knowledge of how to restore *O. edulis* to a self-sustaining population. This aim is met by the following objectives:

a) Understanding the importance of hydrodynamics for restoration site selection by analysing both small- and large-scale effects of water flow on *O. edulis* with implications for re-laying adults, juveniles and cultch (Chapter 2)
b) Understanding the effect of broodstock origin on physiological performance with a comparison of physiological efficiency between three UK *O. edulis* populations (Chapter 3)
c) Exploring the potential for molecular and metabolic markers of resistance to pathogenic agent *Bonamia ostreae* (Chapter 4)
d) Testing the effects of re-laying density and elevated restoration (as an exemplar of one method) on habitat and the physiological efficiency of a population of *O. edulis* with a field study (Chapter 5).

Chapter 2 The role of hydrodynamics in controlling oyster distribution and constraining the location of restoration

2.1 Introduction

Appropriate site selection is essential for restoration of a sessile species such as *Ostrea edulis*, as abiotic factors including water temperature, salinity and hydrodynamics can influence the physiological performance of a population (Orton, 1937; Korringa, 1957; Shumway, 1982; Hutchinson and Hawkins, 1992; Haure *et al.*, 1998). Although *O. edulis* have demonstrated phenotypic plasticity surviving in a variety of environments across the globe (Bromley *et al.*, 2016a), restoration attempts should choose sites that offer the oysters favourable abiotic conditions (now and in the future) to reduce stress on their physiological fitness. The restoration of *O. edulis* often requires the re-laying of oysters or cultch to either bolster a depleted natural population or reintroduce the species to an area. For restoration site selection purposes, local hydrodynamics must be considered for the small- and large-scale, direct and indirect effect on the restoration animal. In physics, the term ‘hydrodynamics’ refers to the study of fluid motion and the forces acting on associated submerged solids (Merriam-Webster, no date), such as the flow of water around a free-swimming oyster larva, or the friction found at the seabed/benthic boundary layer (BBL) (Newman, 2017). Hydrodynamics can influence morphology and growth of individuals (Mead and Denny, 1995), can physically displace individual oysters and cultch (Denny, 2006; Joyce *et al.*, 2019), alter oxygen concentrations, nutrient and food availability (Rothschild *et al.*, 1994; Reidenbach *et al.*, 2013), change larval swimming patterns (Fuchs *et al.*, 2017), and impact the level of sedimentation (Rothschild *et al.*, 1994; Colden *et al.*, 2016), which could influence the success of restoration efforts. Coastal water systems are complex, and while tidal patterns can be successfully predicted, features such as storms and flooding create more unpredictable scenarios that need to be taken into consideration (Meyer *et al.*, 1997; Pinnegar *et al.*, 2020). The hydrodynamic environment is a constant feature for *O. edulis* populations, and therefore must be of high priority in the early selection stages of sites for restoration.

2.1.1 The effect of hydrodynamics on the physiology of individuals

One important component of restoration site selection is the effect of the hydrodynamic environment on the physiology of any individuals, as any stress would negatively affect the population as a whole and likely impact the success of the overall restoration. Bivalve molluscs have two shells that form an armour around their softer tissue. The adductor muscle connecting the two shells controls the valve movement, oyster feeding and ventilation can only occur when the shell is open (Hopkins, 1931), but an open shell does not necessarily indicate feeding (Galtsoff, 1928; Morton, 1973). The opening and closing of the shell is a complex phenomenon that is stimulated by a variety of factors including light, water temperature and pH, mechanical stimulation and food availability (Galtsoff, 1928; Higgins, 1980). The observed time an oyster spends open per day has been reported at an average of between 17 and 20 hours (Galtsoff, 1926, 1928). Often found in an estuarine environment with natural daily fluctuations in conditions including turbidity, temperature, and salinity, oysters can control their valve movement to protect their soft tissues from potentially lethal influxes of water (Galtsoff, 1928). Natural shell closures thought to be associated with daily tidal rhythms, temperature or salinity, and changes in food availability have been observed in oysters (Morton, 1971, 1973; Higgins, 1980 and references within), clams (Williams and Pilditch, 1997; Kim *et al.*, 1999), and mussels (Borcherding, 1992). The closure of *Mytilus* spp. shells has also been used as an indicator of poor water quality and potential pollution (Kramer *et al.*, 1989; Borcherding, 1992; Gnyubkin, 2010). The effect of water velocity has not previously been given great consideration as a physical stressor for bivalve molluscs, possibly as they populate both high and low energy environments. If there were an upper water velocity threshold that stimulated the shell closure of *O. edulis* individuals, then this could affect natural feeding and ventilation and have an impact on the oyster's overall physiology. This could have implications for restoration projects aiming to restore a fast-growing population.

2.1.2 The effect of turbulence at the benthic boundary layer (BBL)

The hydrodynamic environment is the continual 3-dimensional transport of seawater containing elements that have an effect on the growth and wellbeing of oysters such as oxygen, nutrients and algae. Turbulence generated from the angular topography of an oyster bed has been shown to influence oyster physiology both positively and negatively (Lenihan *et al.*, 1996; Colden *et al.*, 2016); high water velocities can increase oxygen availability to, and uptake by, the benthic community (Reidenbach *et al.*, 2013), yet can also impact metabolism of both adults and larvae through the higher energy cost associated with feeding and swimming (Walne, 1972; Fuchs *et al.*, 2017). The Eastern oyster, *Crassostrea virginica*, grows in vertical reefs, which have been shown to have a significant effect on water velocity across the reef (Soniat *et al.*, 2004; Colden *et al.*, 2016). Turbulence and the associated shear stress play a role in bringing post pelagic-stage larvae to the seabed for settling and metamorphosis (Wethey, 1986; Mullineaux and Butman, 1991; Fuchs *et al.*, 2007; Whitman and Reidenbach, 2012; Fuchs and Reidenbach, 2013), but the irregularity of this turbulence and shear stress can hinder the attachment of these larvae to an appropriate substrate (Eckman *et al.*, 1994; Crimaldi *et al.*, 2002; Jonsson *et al.*, 2004; Whitman and Reidenbach, 2012). Greater larval recruitment for mussel *Mytilus edulis* (Petraitis, 1990), oyster *Crassostrea virginica* (Whitman and Reidenbach, 2012) and oyster *Ostrea edulis* (Potet *et al.*, 2021) has been observed in areas with high topographic rugosity (and low shear stress) in comparison with areas of low rugosity. The natural crevices and dips formed from the angular shape of oysters provide a variety of shear stress that can positively affect larvae settlement (Eckman, 1990; Mullineaux and Garland, 1993; Soniat *et al.*, 2004; Koehl and Hadfield, 2010; Whitman and Reidenbach, 2012; Potet *et al.*, 2021). However, excessive shear stress can forcibly dislodge young larvae even after initial settlement or lead to larvae choosing to detach themselves from the seabed (Reidenbach *et al.*, 2009; Koehl and Hadfield, 2010; Whitman and Reidenbach, 2012).

The loss of native oyster beds around the UK has resulted in a shift in the benthic community, with invasive slipper limpet, *Crepidula fornicata*, dominating the epibenthos of southern, south western and south eastern UK coastal regions (Rayment, 2008). During feeding, *C. fornicata* secrete a mucus net that likely restricts oyster larvae from settling on their shell (Jørgensen *et al.*, 1984; Shumway *et al.*, 2014). It has been shown that *C. fornicata* occasionally consume their own faeces (Shumway *et al.*, 2014), and it is likely that they ingest oyster larvae, as oysters have been shown to ingest *C. fornicata* larvae (Pechenik *et al.*, 2004). The chains of multiple individuals developed by *C. fornicata* along with their mucus surface layer results in a smoother seabed surface, creating a more laminar flow of water at the benthic boundary layer (BBL) and likely reducing the turbulent mixing found above an oyster bed (Reidenbach *et al.*, 2010; Colden *et al.*, 2016). This has implications for oyster feeding through re-suspension of particles available for consumption, and for larval settlement. Although *C. fornicata* has replaced *O. edulis* as the most dominant benthic species in many UK coastal areas, the literature on hydrodynamics in association with this species is currently limited (de Montaudouin *et al.*, 1999; Moulin *et al.*, 2007). Understanding the changes to the hydrodynamics at the BBL as a result of the shift in benthic community will help identify potential issues with oyster settlement and inform restoration projects aiming to naturally repopulate areas.

2.1.3 Large scale effect of hydrodynamics on the distribution of larvae

While most projects aim to retain any deployed material such as adult oysters and cultch within a selected area, the opposite is true for larvae dispersal. For restoration purposes, a wider dispersal of *O. edulis* larvae would be desirable in order to naturally repopulate a larger area. *O. edulis* larvae spend approximately 10 days in a pelagic phase before settlement (Walne, 1964; Laing *et al.*, 2005), during which they are vulnerable to abiotic and biotic factors such as storms, strong currents, predation or starvation (Korringa, 1952). Larvae are known to possess a certain degree of control in their ‘milling’ movements and choice of settlement location as will be discussed within this chapter (Broekhuizen *et al.*, 2011), but strong currents could have an impact on local recruitment by washing young oysters away (Key and Davidson, 1981).

2.1.4 Modelling larval distribution

In situ observations of the pelagic phase of larvae are challenging due to the individual variety in larvae dispersal including geographical scale and distance travelled, vertical and horizontal trajectory of individuals, and duration of dispersal. An appreciation of the intricacies of oyster reproduction and larval behaviour developed around 100 years ago, with several scientists making advances in the field (Orton, 1920, 1926, 1927b, 1927a; Nelson, 1924; Prytherch, 1934; Cole and Knight-Jones, 1939, 1949). The time of *O. edulis* spawning, larvae release and settlement has been associated with phases of the moon and later with tidal pressure. Orton (1926) was the first to associate larval behaviour with the lunar cycle, with higher numbers of recently spawning larvae observed one week after a full moon. More recent parallels have been drawn between larval behaviour pre-settlement and the lunar tidal system, with higher numbers of larvae seen in surface waters during a flood tide in comparison to an ebb tide (Korringa, 1947; Hidu and Haskin, 1978). Rather than assume this was a feature of light, Korringa (1947) associated similar observed patterns of larval behaviour with tidal pressure. Similar behaviour in response to tidal pressure has been observed in other bivalve molluscs (e.g. *Mytilus* spp. Rao, 1954). *O. edulis* larvae are known to be competent swimmers and have been observed 'bouncing' above the seabed pre-settlement, in their search for an appropriate substrate. Early observations of the 'bouncing' of *O. edulis* before settlement were thought to be solely an activity to find an appropriate substrate (Cole and Knight-Jones, 1939), yet more recent studies by Cranfield (1973) suggest that this swimming and crawling 'bouncing' activity is an integrated part of the first phase of settlement, even when appropriate substrate is available. Cranfield (1973) explained the potential need for this bouncing (frequent but not constant interaction with the substrate) to stimulate the physiological processes required for the cementing process. This crawling behaviour has been seen in many invertebrate larvae as a method of protection from strong hydrodynamics and predators (Shanks, 2009).

Apart from its influence on larval growth during the pelagic phase, temperature is thought to play a less significant role than tidal pressure on the settlement process (Korringa, 1947), but substrate type is thought to have an important role. Unlike barnacles (*Semibalanus* spp.) that can settle on any hard textured surface in the intertidal (Hills *et al.*, 1999), or other motile species (e.g. polychaetes *Hydroides* spp.) that can change their initial chosen settlement location post metamorphosis, *O. edulis* are not able to move location and are therefore highly selective about their settlement substrate. Recent studies have demonstrated that substrate type is less important than surface structure and the presence of biofilms (Smyth *et al.*, 2018; Rodriguez-Perez *et al.*, 2019; Potet *et al.*, 2021).

Models are useful in reducing the time and effort required to track individual larvae, and provide a probable trajectory for the propagule duration (PD) associated with the species (Shanks, 2009). A three-dimensional model approach is essential for integrating the observed information about the larval pelagic phase; vertical swimming behaviour can begin as early as initial release and can dramatically affect horizontal trajectory (Leis, 2006). Eulerian hydrodynamic models (ones using a numerical scheme of partial differential equations) are often made for assessment of the impact of marine developments. These Eulerian models are parameterised with information gathered from multiple years of water movement observations (water currents and tidal sequences) within a specific area, which can be accurately calibrated and successfully predicted and modelled. Lagrangian particle models allow a single unit or particle to be followed within these Eulerian modelled systems, for example to trace a particle of sand within a specific tidal system. Behavioural traits specific to *O. edulis* larvae, such as vertical swimming at certain times, can be modelled in addition to the Lagrangian model and in combination are called individual based models (IBMs). IBMs allow for a more detailed predictor of the pelagic larvae phase. Combining these three model types (Eulerian, Lagrangian and IBM) provides an informed hypothesis of larvae dispersal that incorporates both individual species behaviour and the physical impact of the immediate environment (Willis, 2011).

2.1.5 Hydrodynamics in the experiment site of interest: The Solent

Blue Marine Foundation (BMF) initiated the Solent Oyster Restoration Project (https://www.bluemarinefoundation.com/projects/solent/) in 2016, in an attempt to revive the once thriving *O. edulis* population in the Solent. Various locations were identified as potential restoration sites, and the hydrodynamic environment was a factor for consideration. The topography and bathymetry of the Solent creates a hydrodynamic environment that interacts with tides to give a double high water and a short and vigorous ebb. The English Channel funnels a significant volume of water through its narrow passage (only 34 km wide at the Straits of Dover (Prandle *et al.*, 1993)) and the tides are amplified by shallow water areas and an amphidrome (a point of zero tidal amplitude) near Weymouth that acts as a pivot for the tidal system. Seawater currents in the Solent have been recorded to reach velocities of 4.5 knots (2.32 m s^{-1}) at the western entrance to the Solent (see Table 2.1), and higher current speeds with greater turbulence in the same location are thought to occur (Key and Davidson, 1981; Iriarte and Purdie, 2004). However, these data are from the sea surface; in water channels less than 40 m deep there is a consistently observed pattern to the velocity profile of the water from zero at the bed to a maximum at the surface; this is a logarithmic profile also known as the law of the wall (Willis, 2011). The velocity profile is dependent also on the roughness of the bed and can be characterised by near-bed measurements. Quaresma et al. (2007) observed velocities of 0.59 m s^{-1} at 0.15 m above the seabed along the main channel of Southampton Water (Flood, 1981).

Table 2.1. High water velocities recorded in the Solent.

Water velocity (m s^{-1})	**Location**	**Reference**
0.98	River Itchen leading into the Solent	(URS and Wilson, 2012)
> 2	West Solent	(Dyer, 1971; Cefas, 2001)
2.32	Western entrance to the Solent	(Key and Davidson, 1981; Iriarte and Purdie, 2004)
0.59	0.15 m above the seabed along the main channel of Southampton Water	(Quaresma *et al.*, 2007)

Aim

In collaboration with the present restoration project in the Solent led by Blue Marine Foundation, this chapter presents both observed and modelled experiments to assist in targeting appropriate areas for restoration within the Solent with respect to the local hydrodynamics. Flume experiments were used to quantify the role of small-scale hydrodynamics and the effect of velocity and turbulence on individuals, beds and cultch. A comparison was also made between an *O. edulis* bed and a *C. fornicata* bed allowing inferences to be made that reflect the current Solent benthic environment. Modelled data accounted for the large-scale effect of hydrodynamics on potential restoration sites in the Solent and predicted larvae dispersal trajectory and settlement locations.

Objectives

1. To observe individual oyster valve movement in response to water velocity.
2. To determine critical water current speeds at which displacement of either cultch or juvenile oysters occurs.
3. To compare hydrodynamic features of the benthic boundary layer (BBL) associated with an *O. edulis* bed with that of a *C. fornicata* bed.
4. To determine probable larvae trajectory and settlement location given specific release sites using an individual based model (IBM).

2.2 Methods

2.2.1 Physical metrics

Oyster height (mm), length (mm) and wet weight (g) were considered as appropriate measurements for growth and measured in accordance with (Galtsoff, 1964) (Figure 2.1). Measurement of oyster width (mm) have previously been considered inconsistent with highly variable repeat measurements and was therefore not included in the oyster dimensions in this study (Sheldon, 1968).

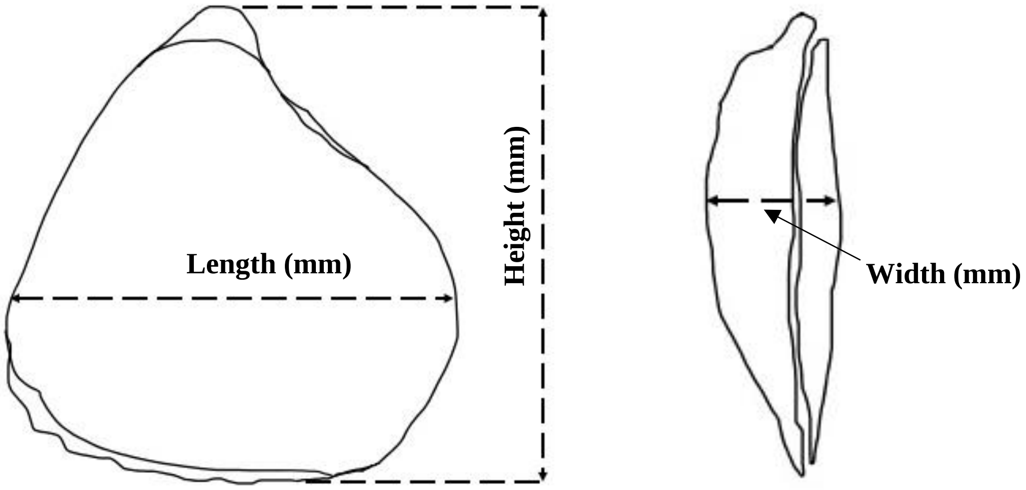


Figure 2.1. Metrics for appropriate measurement (length, height and width) of oyster dimensions (mm), adapted from (Galtsoff, 1964).

2.2.2 Flume experiments

Experiments were conducted with the use of a 2-m-diameter annular flume (the Lab Carousel, Thompson *et al.*, 2004a) made of transparent acrylic. Seawater was paddled anticlockwise around a 0.15 m wide, 0.40 m deep channel running the perimeter of the flume. Water flow was controlled using a movable flume lid with 8 paddles placed equidistantly, powered by an E-track® AC inverter motor (Thompson *et al.*, 2004a), and water speed was measured by lid rotations (Hz). These measurements were converted to m s^{-1}; Experimental flow speeds of 0.06, 0.13, 0.22, 0.31, 0.42 and 0.52 m s^{-1} were used, calculated as the depth average velocity (n = 6) of seawater travelling along a clean flume in a series of calibration runs undertaken prior to the main experiments. Water velocity along the channel (u), perpendicular to the flow (v), and vertically (w) was measured using a downward facing 10 MHz Nortek Vectrino acoustic Doppler velocimeter (ADV) attached to the side of the flume. All ADV measurements were taken after at least 5 minutes of any water flow acceleration to allow the boundary layer time to adjust (Thompson *et al.*, 2004a). No additional sediment was added to the flume, but neutrally buoyant seeding material was occasionally added to the seawater to improve the sensor sensitivity to acoustic backscatter. Sand filtered seawater from the Solent was used for each experiment, which maintained a salinity of 34.0 ± 0.3 (mean ± sd) and room temperature of 17.3 ± 1.0 °C (mean ± sd).

To understand the experimental hydrodynamics in three dimensions, and to fully appreciate the turbulence of the benthic boundary layer (BBL), over 3000 data readings were taken per sample at up to 14 different points on a vertical axis through the water column and averaged to acquire a mean water velocity at each elevation ($\bar{u}$, m s^{-1}). These ADV data could then be used to determine bed shear stress (τ) through the use of the Reynolds number (Re), Turbulent Kinetic Energy (TKE) method, and the Quadratic Stress Law (QSL) to fully characterise the hydrodynamic environment associated with an oyster bed. The Re was calculated for each flume acceleration and for each profile (P1, P2 and P3) when appropriate using the equation:

$$Re = \frac{\bar{U}\,D}{V} \tag{1}$$

Where $\bar{U}$ is the depth averaged velocity (m s^{-1}), *D* is the flume water depth (z = 0.4 m) and *V* is the kinematic viscosity of the seawater, which was calculated at 1.12 x 10^{-6} (Ns m^{-2}, Pa s) at a temperature of 17.3 °C, salinity 34, and density of 1025.01 kg m^{-3} (El-Dessouky and Ettouney, 2002; Park and Olivieri, 2011). TKE was calculated for each elevation from the seabed (z) using the standard empirical formula (Soulsby, 1983; Thompson *et al.*, 2013; Kassem *et al.*, 2015):

$$TKE_z = \frac{1}{2}\,\rho\,(\bar{u}_z^2 + \bar{v}_z^2 + \bar{w}_z^2) \tag{2}$$

where ρ is seawater density (1025.01 kg m^{-3}), and $\bar{u}_z$, $\bar{v}_z$ and $\bar{w}_z$ are the time-averaged velocity components acquired by the ADV at each elevation from the seabed (z). These TKE values could be then be multiplied by the value 0.19 (generally accepted as an empirical coefficient for a range of bottom roughnesses) to infer the bed shear stress (τ) (Soulsby, 1983) as recommended by (Thompson *et al.*, 2004b):

$$\tau_{TKE} = 0.19 \times TKE \tag{3}$$

The Quadratic Stress Law (QSL) represents shear stress (τ) as:

$$\tau = \frac{1}{2}\,\rho\,C_D\,\bar{U}^2 \tag{4}$$

where $\bar{U}$ is the depth averaged velocity. This equation enables the calculation of the drag coefficient (C_D), a measure of friction, which is generally accepted to be a constant number (Thompson *et al.*, 2004a). The hydrodynamic roughness scale length (z_0) could be calculated with the Log Law of the Wall equation (Whitman and Reidenbach, 2012; Chirol *et al.*, 2015):

$$\overline{U}_z = \frac{u^*}{\kappa} \ln \left(\frac{z}{z_0} \right) \tag{5}$$

which is dependent on four assumptions: (1) the flow is unidirectional and steady, (2) the flow is turbulent and rough, (3) the water is clear (which allows the use of the von Kármán constant (κ = 0.41)), and (4) the measurements were taken within the logarithmic part of the boundary layer. Estimates of u* and z_0 were acquired with the use of a linear regression of ln (z) in relation to velocity ($\bar{u}$).

All data was processed in Matlab (see external digital supplementary data S2) and visualised in R using the ggplot2 package (Wickham, 2009). Spikes in the ADV data (caused by distortion in signal frequency) were removed with the use of the de-spiking code (Goring and Nikora, 2002), which takes a polynomial of best fit through data from either side of the spike to interpolate through the spike, and smooth the data.

Experiment I: Individual oyster response

This study used ten adult oysters (mean average sizes: height = 73.87 mm, length = 71.62 mm, wet weight = 91.75 g, n = 10, dimensions were taken in accordance with section 2.2.1) from a batch of oysters from the Solent (Chichester and Langstone Harbours) provided by Blue Marine Foundation on 14/11/2018. These oysters were kept in an outside holding tank at the National Oceanography Centre Southampton with a flow through system pumping unfiltered seawater directly from the Solent beside Empress Docks, and therefore no additional food or sustenance was provided. The seawater salinity remained high at 34.0 ± 0.3, and temperature naturally fluctuated with the environment.

An oyster was placed into the flume and left in low lighting to acclimate and open their valves in an average water flow speed of 0.06 m s^{-1} for 3 hours (Figures 2.2 and 2.3). Loosanoff and Nomejko (1946) demonstrated that *C. virginica* was not influenced by orientation of the oyster with respect to the current direction. For consistency, all native oysters were placed in the flume such that the hinge of the shell was placed downstream (Figure 2.3). Each individual was then exposed to increasing water flow speed of c. 0.02 m s^{-1} every 15 minutes. The time it took for the oyster to close its valves or be physically displaced by the water flow, was recorded. Water velocity was measured at the point of displacement at 10 heights along a vertical plane (0.001, 0.01, 0.02, 0.03, 0.05, 0.07, 0.10, 0.12, 0.15, and 0.18 m above the seabed), to build a 3-dimensional profile of the water flow.

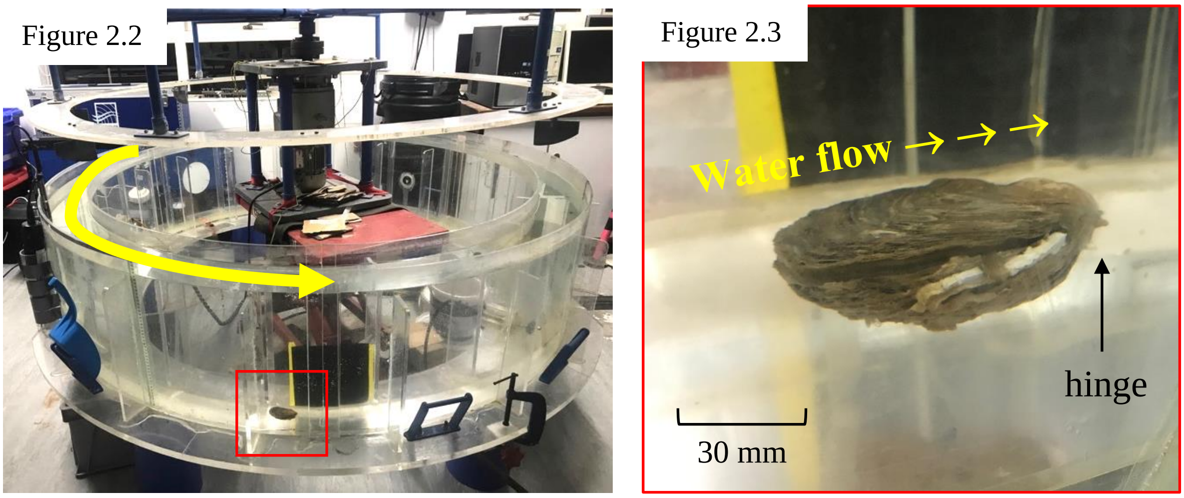


Figure 2.2 and Figure 2.3. The Lab Carousel (as described by Thompson *et al.* (2004a)) (Figure 2.2) and a close up of the single *O. edulis* placed with open valves facing the water flow (Figure 2.3).

Velocity (m s^{-1}) along the vertical plane was measured for the point at which each oyster either closed its valves or was physically displaced. These water velocity data were then cross referenced with the weight of the oyster (g) to assess any correlation between the two.

Experiment II: Juvenile oyster physical displacement

A batch of juvenile oysters was provided by Blue Marine Foundation from Jersey Sea Farm Hatchery on (22/02/2019). The oysters had a collective wet weight (WW) of 0.66 kg (average individual size = 35.6 (H) mm x 32.9 (L) mm, 5.6 (WW) g, n = 37) (Figures 2.4 and 2.5). Oysters were kept in the same outside holding tank as in Experiment I, and not provided with any additional food/sustenance.

Figure 2.4

Figure 2.5

Figure 2.4 and Figure 2.5. Juvenile *O. edulis* from Jersey Sea Farm Hatchery.

The batch of juvenile oysters was placed within the flume seawater channel in a dense layer with height of a single oyster (Figures 2.6). Water flow speed began at 0.06 m s^{-1} and was increased by 0.02 m s^{-1} increments every 15 minutes. The distance of displacement of oyster juveniles, and the water velocity at time of displacement was measured at 10 heights along a vertical plane (0.001, 0.01, 0.02, 0.03, 0.05, 0.07, 0.10, 0.12, 0.15, and 0.18 m above the seabed), to build a 3-dimensional profile of the water flow. This experiment was conducted five times, returning the juvenile oysters into the same starting position for each experiment.

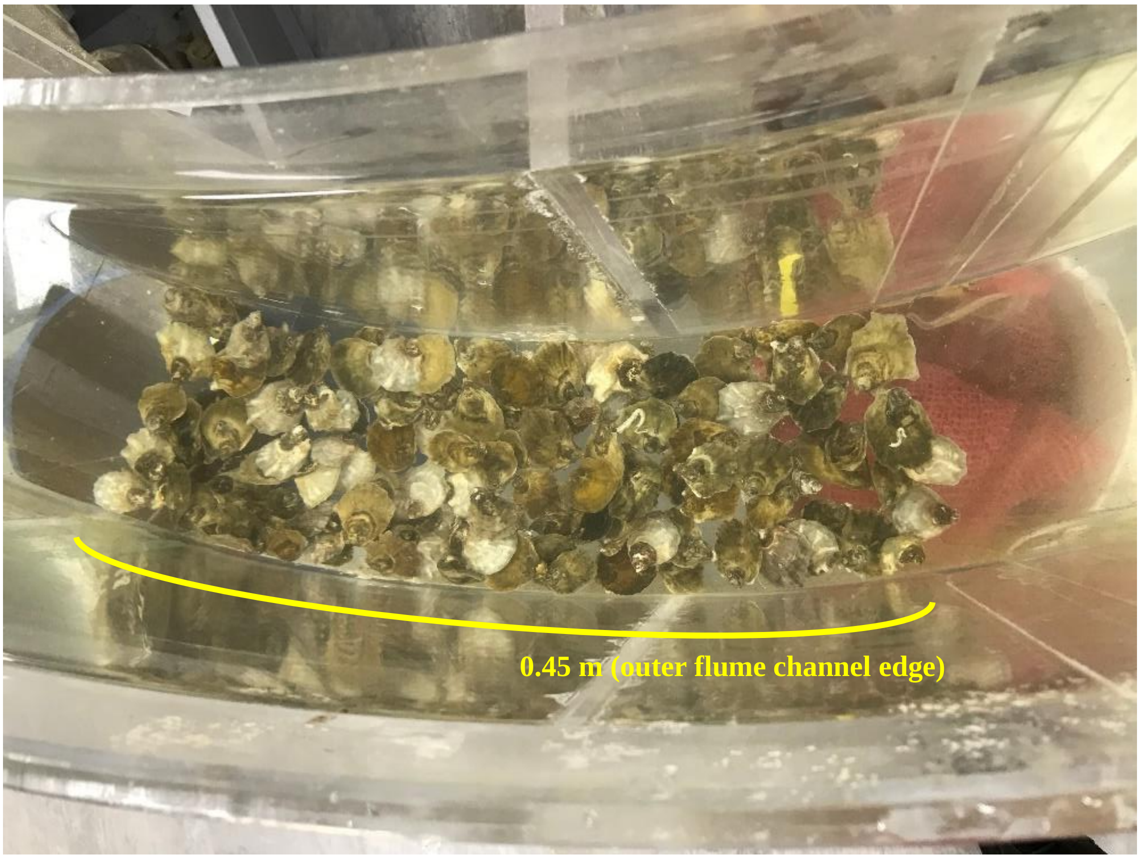


Figure 2.6. Plan view of experiment II starting position with juvenile *O. edulis* positioned along the flume seawater channel in a dense layer with height of a single oyster covering an area of c. 0.06 m^2.

A vertical profile of velocity ($m\ s^{-1}$) was recorded at the moment the juvenile oysters were displaced. The number of oysters displaced was recorded.

Experiment III: Spat on shell physical displacement

Spat-on- (blue mussel) -shell cultch (broken mussel shell) established in ponds at Jersey Sea Farm by Tony Legg (with a collective Wet Weight (WW) of 2.5 kg) was provided by Blue Marine Foundation in February 2019. Each mussel shell had at least one spat settled onto its surface (Figure 2.7). The spat-on-shell was kept within an onion bag in the same outside holding tank as above, thus no additional food/sustenance was provided.

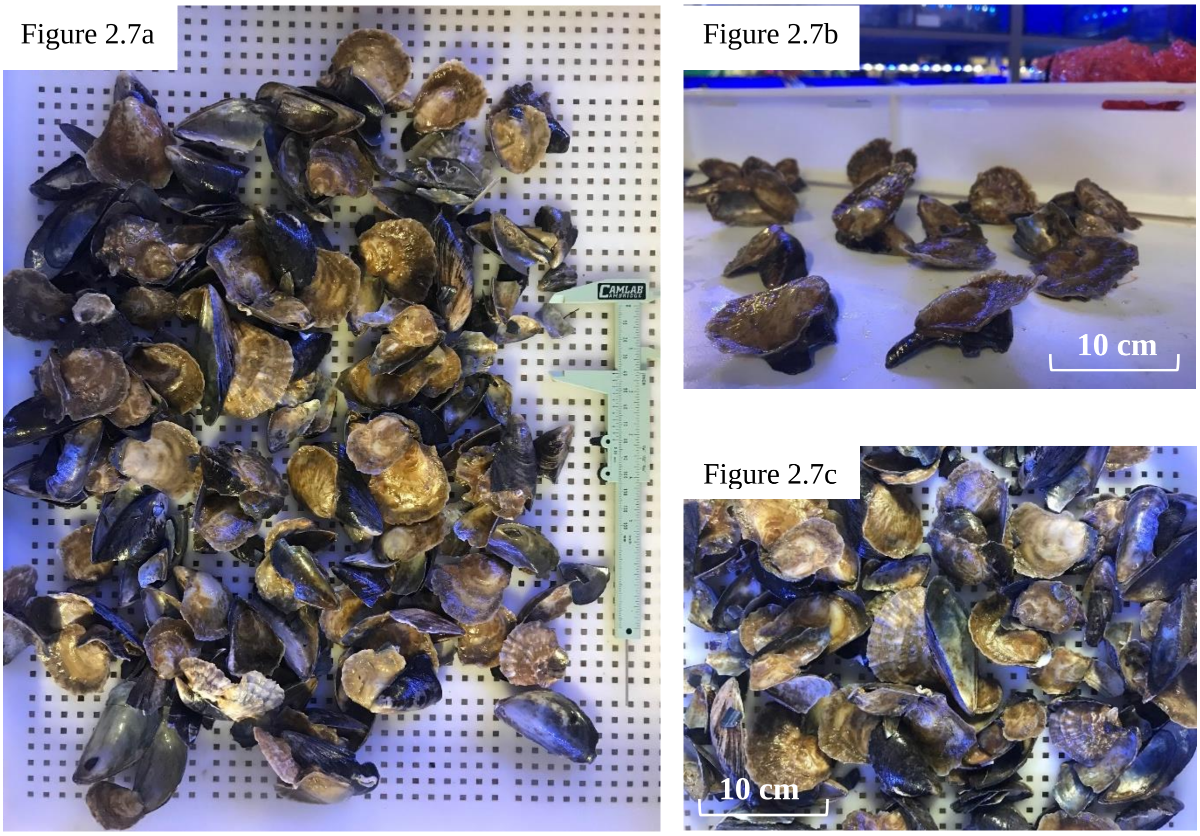


Figure 2.7 (a, b and c). *O. edulis* spat on broken blue mussel (*Mytilus edulis*) shell from Jersey Sea Farm.

Three experiments were conducted, with the spat-on-shell placed into the seawater flume channel in three different experimental designs to consider the effect of height and density on physical displacement associated with hydrodynamic environment. For the first experiment, the spat-on-shell were placed in a high density block up to 0.15 m above the seabed, reaching a length of 0.40 m (measured along the outer edge) along the channel. In experiment 2, spat-on-shell were placed at a shallower depth of 0.05 m high (2-3 shells high), reaching 0.75 m (measured along the outer edge) along the channel for the second experiment, and at one single shell depth, reaching 2.25 m (measured along the outer edge) along the channel for the third experiment (Figure 2.8). The ADV probe was placed 0.75 m (measured along the outer flume channel edge) ahead of the spat-on-shell in order to capture the laminar flow speed around the flume and to avoid any shell-derived boundary layer. Water flow speed was set at 0.06 m s^{-1} to start and then increased in increments of ~ 0.05 m s^{-1} every 480 seconds to allow the water to adjust to laminar flow along the water column. Each experiment was repeated three times with spat-on-shell to its starting position each time. A vertical profile of the water velocity at the time of any displacement was recorded, and shell distribution patterns observed.

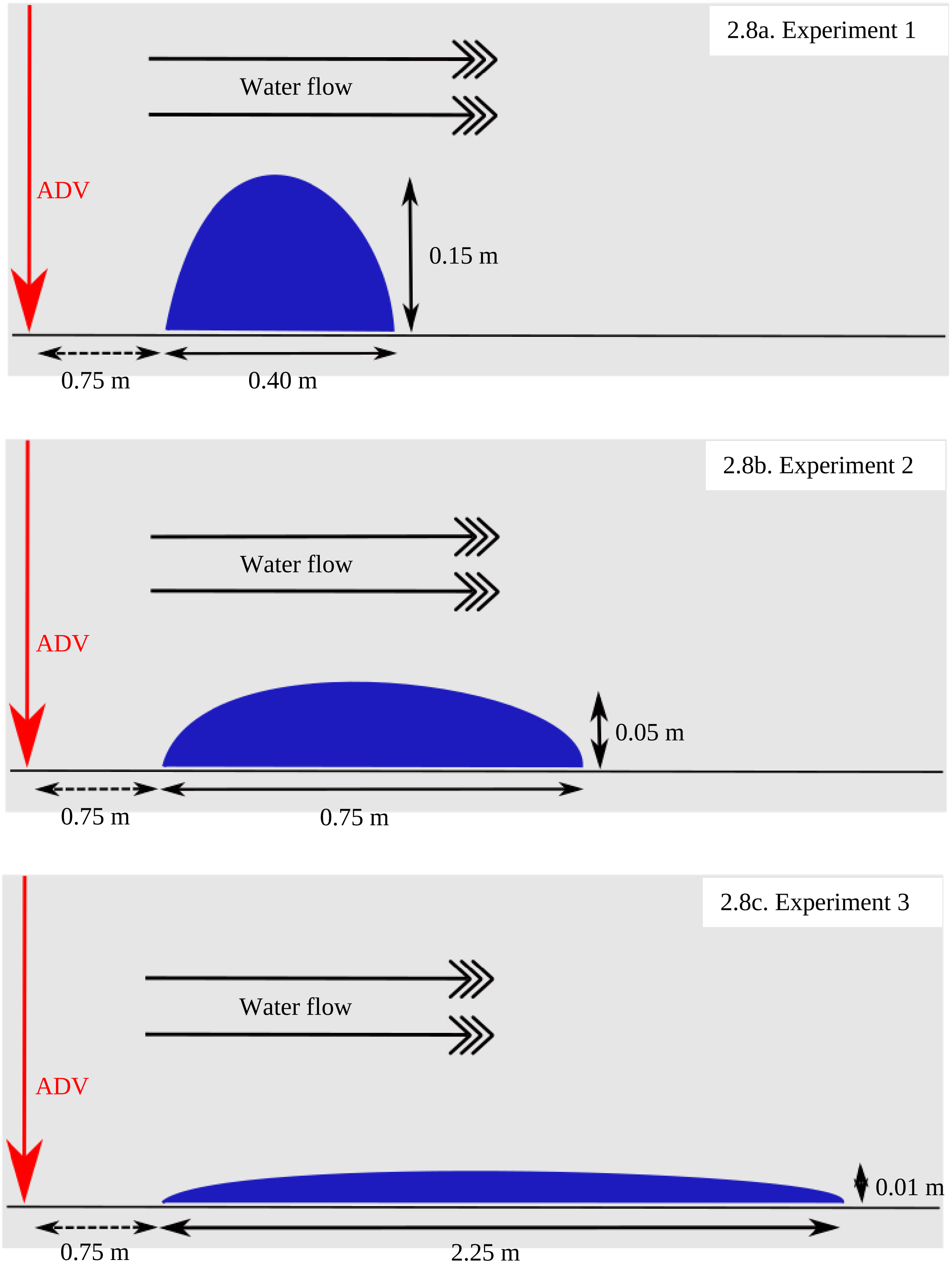


Figure 2.8 (a b and c). Depth and distribution of *O. edulis* spat-on- (mussel, *Mytilus edulis*) -shell (blue) at the start of each of the three experiments (not to scale) at a starting position with (a) 0.15 m shell depth, 0.40 m length, (b) 0.05 m shell depth, 0.75 m length, and (c) 0.01 m shell depth, 2.25 m length.

Patterns of dislodgement were observed, recorded and compared across three experiments. Additional comparisons were drawn between depth of shell material in the starting position.

Experiment IV: Benthic Boundary Layer (BBL) – *Ostrea edulis* bed vs. *Crepidula fornicata* bed

A total of 22 adult oysters was used for this experiment. Twelve adult oysters from the Solent (Chichester and Langstone Harbours) were added to the 10 adult oysters used for Experiment I (see above) (mean sizes: length = 70.53 mm, width = 74.95 mm, wet weight = 85.11 g, n = 22). These oysters were also kept in the same outside holding tank as above and were not given any additional food or sustenance.

All 22 adult oysters were placed into the flume seawater channel in a bed formation of several oysters deep (~ 0.06 m high) reaching 0.40 m along the (outer) length of the channel, covering an area of ~ 0.06 m^2. The experiment could begin immediately as it did not matter whether the oysters had valves open or closed. Water velocity was set and remained constant for each experiment; five different experiments with different water surface flow speeds (0.06, 0.13, 0.31, 0.42, and 0.52 m s^{-1}) were conducted. Data were collected in three vertical velocity profiles, positioned before (P1), directly above (P2), and after (P3) the oyster bed and spaced 0.5 m apart to determine how the oyster bed affected the water velocity, turbulence, shear stress and hence altering the benthic boundary layer (BBL). For each profile, the ADV measured water velocity at up to 7 heights (P1 and P3: 0.003, 0.01, 0.02, 0.05, 0.07, 0.12, and 0.17 m above the seabed, P2: 0.003, 0.01, 0.02, 0.05, 0.07, 0.11 m above the *O. edulis* bed) (Figures 2.9, 2.10 and 2.11).

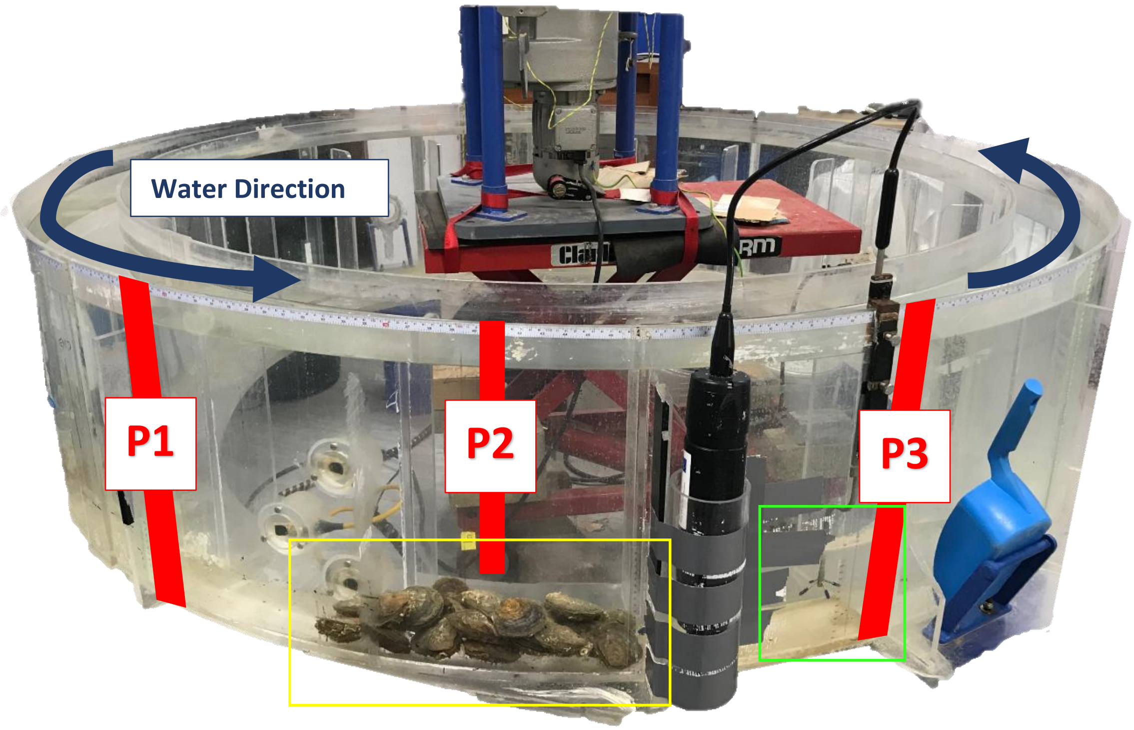


Figure 2.9. Annular flume with positions P1, P2 and P3 referenced in red. Yellow and green box explained below (see Figures 2.10 and 2.11).

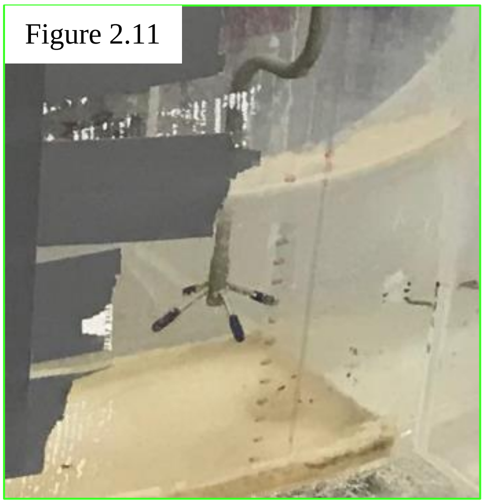


Figure 2.10 and Figure 2.11. *Ostrea edulis* placed into the annular flume at a high density of 367 oysters m^{-2} to create an oyster bed structure (Figure 2.10). ADV probe positioned to take measurement at P3 (Figure 2.11).

A batch of ca. 100 *Crepidula fornicata* in chains of between 2-10 individuals was gathered from the Solent seabed on 8th May 2019 and placed into a holding tank in the National Oceanography Centre aquarium. The holding tank provided a constant flow through of sand-filtered seawater, and therefore additional algae sustenance (a mix of *Isochrysis galbana* and *Tetraselmis suecca* at 40 000 cells ml^{-1}) on alternate weekdays (Monday, Wednesday and Friday) was provided.

In a separate experiment, with the oysters removed, *C. fornicata* were placed into the flume channel in the same format as the oysters (in Figure 2.10), reaching a length of 0.40 m along the channel and a depth of c. 0.05 m from the seabed, covering an area of c. 0.06 m^2 (Figures 2.12 and 2.13). Experiments were conducted as above. Five different experiments with different water surface flow speeds (0.06, 0.13, 0.22, 0.31, and 0.42 m s^{-1}) were conducted to observe change in water velocity, turbulence, and shear stress at the benthic boundary layer (BBL). For each profile (P1, P2, P3), the ADV measured water velocity at up to 14 heights (P1 and P3: 0.003, 0.01, 0.02, 0.03, 0.04, 0.05, 0.07, 0.08, 0.10, 0.11, 0.12, 0.14, 0.15, and 0.17 m above the seabed, P2: 0.003, 0.01, 0.02, 0.03, 0.04, 0.05, 0.07, 0.08, 0.09, 0.10, 0.11, 0.12, and 0.13 m above the *C. fornicata* bed).

Figure 2.12

Figure 2.13

Figure 2.12 and Figure 2.13. *Crepidula fornicata* placed into the annular flume at a high density to create a bed structure. Plan view (Figure 2.12) and side view (Figure 2.13).

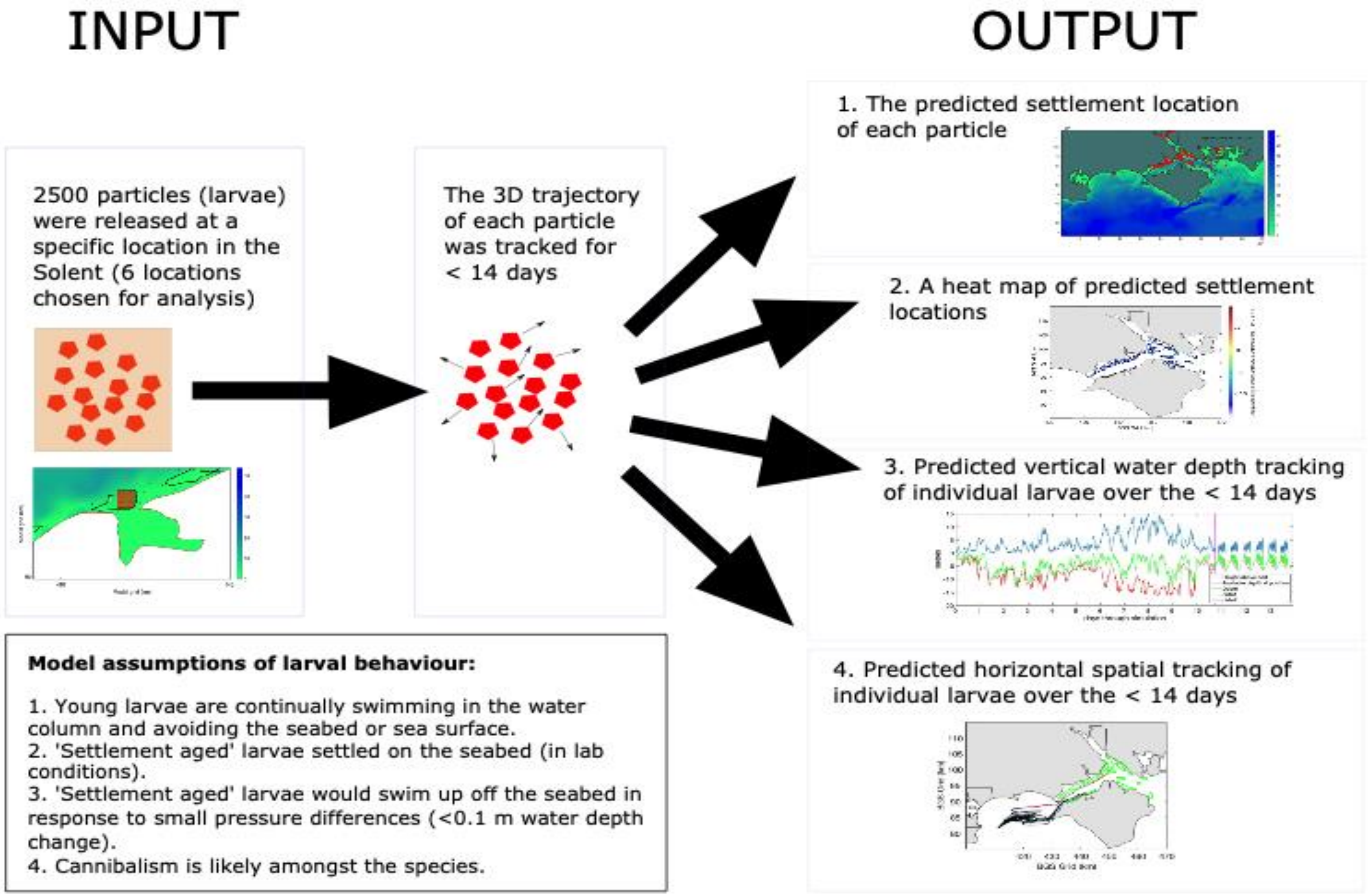
Water velocity, TKE and shear stress was analysed for each vertical profile (P1, P2 and P3). These data were used to calculate the Reynolds number (Re), drag coefficient (C_D), and estimate shear velocity (u*) and roughness scale length (z_0).

2.2.3 Model experiments of large-scale oyster larvae dispersal

A simulation model was used to study the theoretical movement of *O. edulis* larvae around the Solent from given starting locations (Willis *et al.*, 2019). The model was constructed using an existing hydrodynamic model of the Solent area, paired with an individual-based model (IBM) of *O. edulis* larvae. Paired with hydrodynamic models, IBM's have proven useful in helping to establish patterns of larvae dispersal for many different broadcast spawning organisms (Herbert *et al.*, 2012b). The hydrodynamic Eulerian model was built by HR Wallingford using newly surveyed and previously reported bathymetric and flow data with a variable spatial resolution of between c. 100 and 2000 m (Willis *et al.*, 2019). Solent hydrodynamics were simulated using the two-dimensional depth averaged flow model TELEMAC-2D (http://www.opentelemac.org/), similar to the method explained by Herbert *et al.* (2012b). The IBM was designed to simulate the pelagic phase of oyster larvae over the course of < 14 days and predict areas of settlement given a starting location within the Solent. For each model run, 2500 individual larvae individuals were released as Lagrangian particles from a single location, and their 3-dimensional trajectory modelled. Larval behaviour was incorporated into the model upon the following four assumptions: (1) young larvae are continually swimming in the water column and avoiding the seabed or sea surface (Cragg and Gruffydd, 1975; Waller, 1981; Key, 1987), (2) 'settlement aged' larvae settled on the seabed in lab conditions (Cragg and Gruffydd, 1975), (3) 'settlement aged' larvae would swim up off the seabed in response to small pressure differences (< 0.1 m water depth change) (Cragg and Gruffydd, 1975), and (4) cannibalism is likely amongst the species (Cragg and Gruffydd, 1975; Troost *et al.*, 2008; Willis *et al.*, 2019). The model output offered (i) predicted larval settlement location given a starting location, (ii) a heat map of predicted larval settlement locations given a starting location, (iii) predicted vertical water depth tracking of individual larvae throughout the 14 pelagic days, and (iv) predicted horizontal spatial tracking of individual larvae throughout the 14 pelagic days (Figure 2.14).

Predicted settlement locations given a starting location

For each scenario and model run, 2500 particles (larvae) were released simultaneously from a manually drawn square 'zone' within the Solent (e.g. Figures 2.15 and 2.16). Five of the six release zones chosen for each model scenario/simulation were at the active (n = 6) or proposed (n = 2) oyster restoration sites associated with Blue Marine Foundation (Table 2.2, Figure 2.17).



INPUT

2500 particles (larvae) were released at a specific location in the Solent (6 locations chosen for analysis)

The 3D trajectory of each particle was tracked for < 14 days

Model assumptions of larval behaviour:

1. Young larvae are continually swimming in the water column and avoiding the seabed or sea surface.
2. 'Settlement aged' larvae settled on the seabed (in lab conditions).
3. 'Settlement aged' larvae would swim up off the seabed in response to small pressure differences (<0.1 m water depth change).
4. Cannibalism is likely amongst the species.

OUTPUT

1. The predicted settlement location of each particle
2. A heat map of predicted settlement locations
3. Predicted vertical water depth tracking of individual larvae over the < 14 days
4. Predicted horizontal spatial tracking of individual larvae over the < 14 days

Figure 2.14. A flow diagram explaining the simulation model inputs and outputs for larvae trajectory around the Solent.

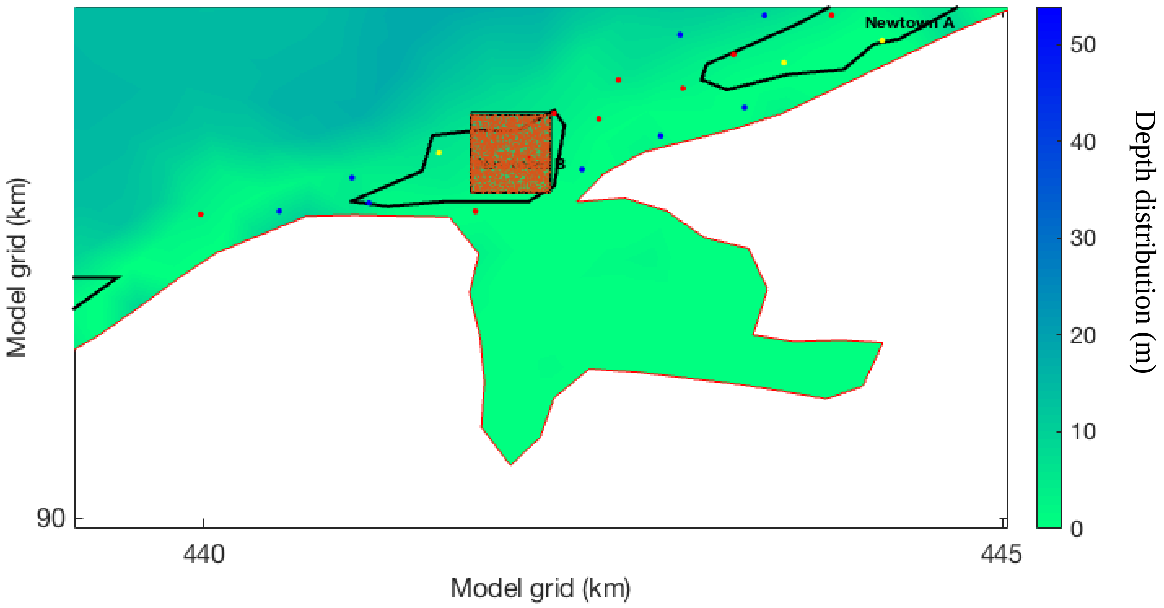


Figure 2.15. A close-up example of a square 'zone' (marked with an orange polygon) chosen for 2500 particles (larvae) to be released at Newtown Creek.

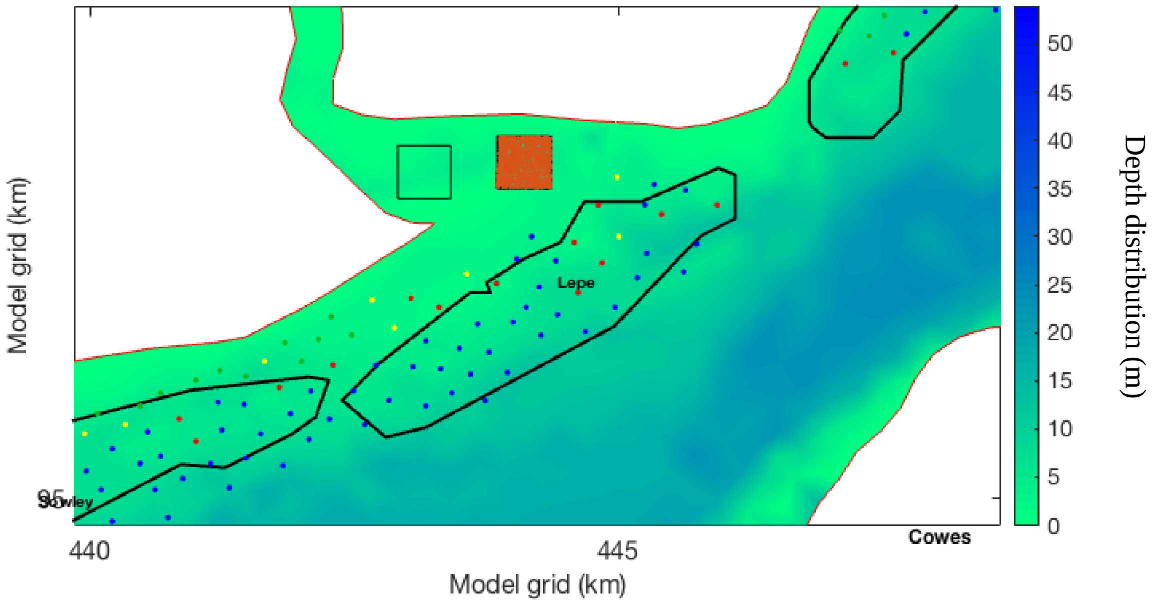


Figure 2.16. A close-up example of a square 'zone' (marked with an orange polygon) chosen for 2500 particles (larvae) to be released in the Beaulieu River.

Table 2.2. Chosen 'zones' for model simulated release of 2500 particles (larvae) within The Solent and their restoration status (active or proposed) at the time of research. The five active or proposed zones for restoration were locations associated with the Solent Oyster Restoration Project led by Blue Marine Foundation.

Location	***Latitude (decimal degrees)***	***Longitude (decimal degrees)***	***Site for restoration***
Land Rover Ben Ainslie Racing (LRBAR)	50.792386°	-1.107047°	active
Hamble Point	50.853023°	-1.311018°	active
Saxon Wharf	50.913097°	-1.379214°	active
Beaulieu River	50.781667°	-1.383056°	proposed
Newtown Creek	50.730000°	-1.410000°	proposed
Lymington	50.745000°	-1.518000°	N/A

Figure 2.17. Locations of active (3, red) and proposed (2, yellow) sites for restoration (at the time of study) for Blue Marine Foundation's Solent oyster restoration project and one potential natural source of larvae (blue). Map created in Google Earth Pro.

Heat maps of predicted settlement locations given a starting location

Information on the historical distribution of *O. edulis* populations in the Solent collected from previous literature was digitised into the model (Key & Davidson 1981; Palmer and Firmin 2011). The heat map feature layered predicted larval settlement density from the model output onto the digitally mapped representation of previous *O. edulis* populations to determine how modelled distribution compared with historical data. This was achieved with the use of a kernel density estimation, by creating a grid over the final distribution map and counting the number of larvae in each grid cell to determine the density across the Solent. To provide a smooth estimate of probability of settlement a Gaussian spreader (of extent 25 cells) was used to give each larva a finite chance of being in the adjacent grid cell, and a smaller but still finite chance of the larva being in the further adjacent grid cell. The model output was presented on a log scale because it was felt the output patterns were more informative across the modelled area, as a logarithmic scale serves to highlight and differentiate all areas of predicted settlement including those with a very low associated probability. Statistical comparisons between final dispersion kernals (predicted densities of larval settlement) was not possible as many particles left the model area, and having left were removed from the model, whereas in the real situation, larvae that leave the modelled area may well return and add to the actual settlement due to the rhythmic nature of tidal currents.

Predicted vertical water depth tracking of individual larvae throughout the 14 pelagic days

The modelled particles (larvae) were given a growth rate randomly selected from a normal random distribution with a mean that ensured the larvae grew from a given initial size of 160 μm to 300 μm after 10 days (Willis *et al.*, 2019), similar to the maturation time assumed for Broekhuizen *et al.*'s (2011) model of the settlement of *Ostrea chilensis* larvae. At this point, larval behaviour shifted from pelagic swimming in the upper water column (a random walk chosen from ‘milling’ speeds reported by Cragg and Gruffydd (1975) with neutral buoyancy) to behaviour associated with settlement. This settlement behaviour allowed the larvae to ‘bounce’ along the seabed in their search for an appropriate substrate for settlement as observed by Cranfield (1973), and Cragg and Gruffydd (1975), with swimming speeds observed of *C. virginica* larvae by Hidu and Haskin (1978), which depended on larvae size (straight hinged larvae (75 μm) moved at 0.5 m h^{-1} and eyed larvae (300 μm) swam 30 m h^{-1}). Similar models have assumed a bouncing of larvae before settlement for the Chilean flat oyster *O. chilensis* (Broekhuizen *et al.*, 2011) and for Eastern oyster *C. virginica* (Mann, 1988). Cranfield (1973) suggested that the settlement process of both *C. virginica* and *O. edulis* could be considered one and the same due to earlier observations for both species showing similar traits. The simulated frequency of the ‘bounce’ was based on the tides and associated water pressure (Cragg and Gruffydd, 1975; Hidu and Haskin, 1978); on a flood current, when water levels and hydrostatic pressure were increasing, larvae would bounce upwards, and then sink with the ebb tide. The depth at which the individual larvae could be found reported in the output every hour and depended on the total depth of water available to them; larvae tended to use all the water depth available to them until c. 10 days, when they were more likely to be found nearer the seabed due to the input parameters of buoyancy, swimming speed and settlement behaviour (e.g. Figures 2.18 and 2.19). The choice of settlement location was further dependent on data from past *O. edulis* population density recorded from dredge surveys and on former maps of the area. Thus, the model incorporated a probability of settlement for each part of the Solent. The model employed a uniform random number generator at each time that a model larva was in a position to settle. At each model step, new random numbers were generated for each modelled larva, all uniformly distributed between 0 and 1. Each grid square of the model has a fixed settlement probability assigned to it, again between 0 (no chance of settlement) and 1 (definite settlement immediately). Those larvae whose newly assigned random number was less than the grid square assigned settlement probability settled at that grid square while those whose newly assigned number was higher than the settlement probability did not settle and carried on being advected by the currents. In this way, more particles were likely to settle at the grid squares with higher probability of settlement, than those with lower probabilities. If the randomly generated number was within the settlement range (from 0 to the settlement probability) then the larvae stopped bouncing and settled. This was designed to model the patterns of settlement within known areas of high settlement potential, as the final patterns are dependent on the strength and direction of prevailing currents and the overall size of the area. This method of calculation is a good analogue to the natural situation because each larva in reality will have a similar probability of settlement, dependent on location. Even in the most likely areas for settlement some larvae will not settle immediately, and some, albeit very few, will settle in the most unlikely areas. These rules were designed specifically to be as simple as possible and for each fixed probability of settlement produce a Poisson Distribution of settlement over any time interval. The Poisson Distribution is appropriate here because it is a natural distribution often seen in nature and is discrete and positive with a minimum number of variable parameters. Poisson Distribution also has a long tail which means that rare but possible events can be captured, such as a few larvae crossing an ideal settlement location without settling. The likelihood of this scenario and the possible effects of this type of situation can be captured in the model by perhaps increasing the overall number of modelled units until the probability of rare targets being hit can be calculated.

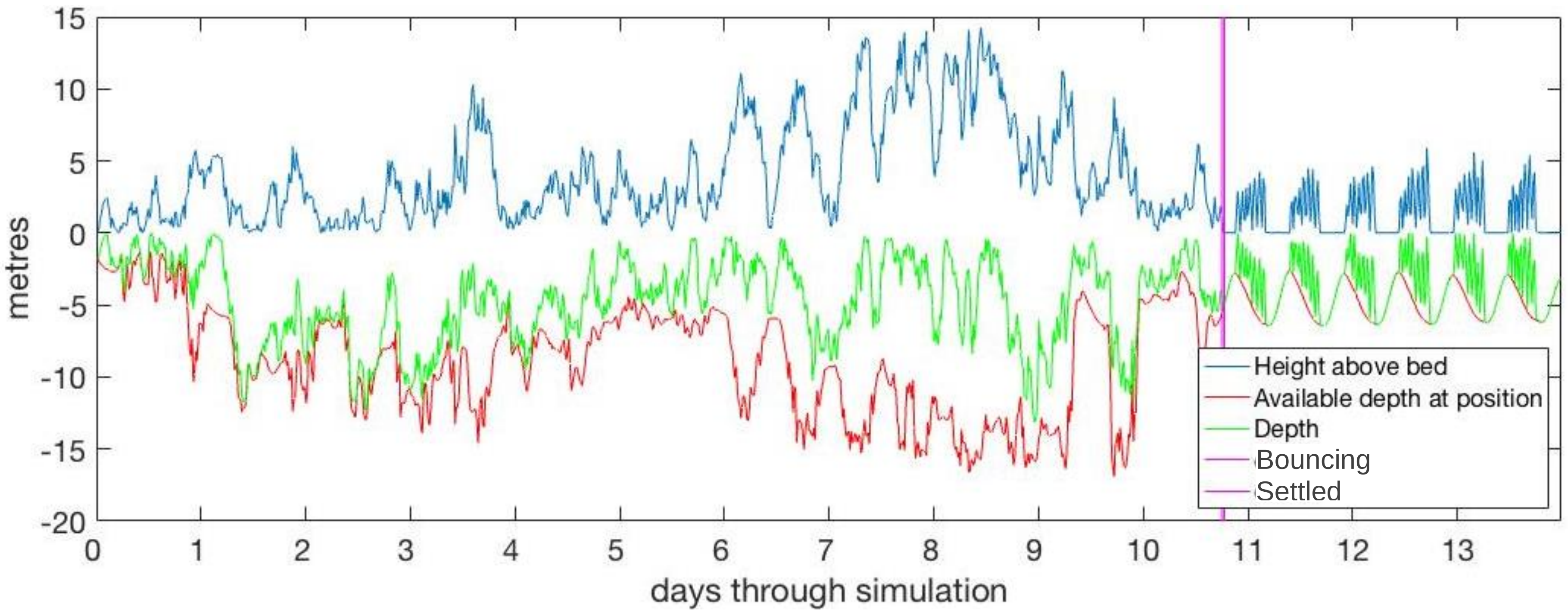


Figure 2.18. An example of the predicted vertical trajectory of one individual particle (larvae) (of a total 2500) within the < 14 day assumed pelagic phase (this individual was released from the Hamble River). Modelled larvae moved throughout the water column for c. 10 days visible from the interaction between the depth of the larvae (green line) with the available depth (red line). There are two magenta lines just before day 11, that are so close together to be indistinguishable. These lines show that the modelled larva in this case settled almost immediately after beginning the settlement bounce phase. After settlement the larva stopped all lateral movement and the pattern after day 11 shows the available depth changing smoothly with the tide at that fixed location. The output data continue to show the modelled larvae bouncing, but this is for model diagnostic information only. Modelled settlement occurred at the magenta line just before day 11.

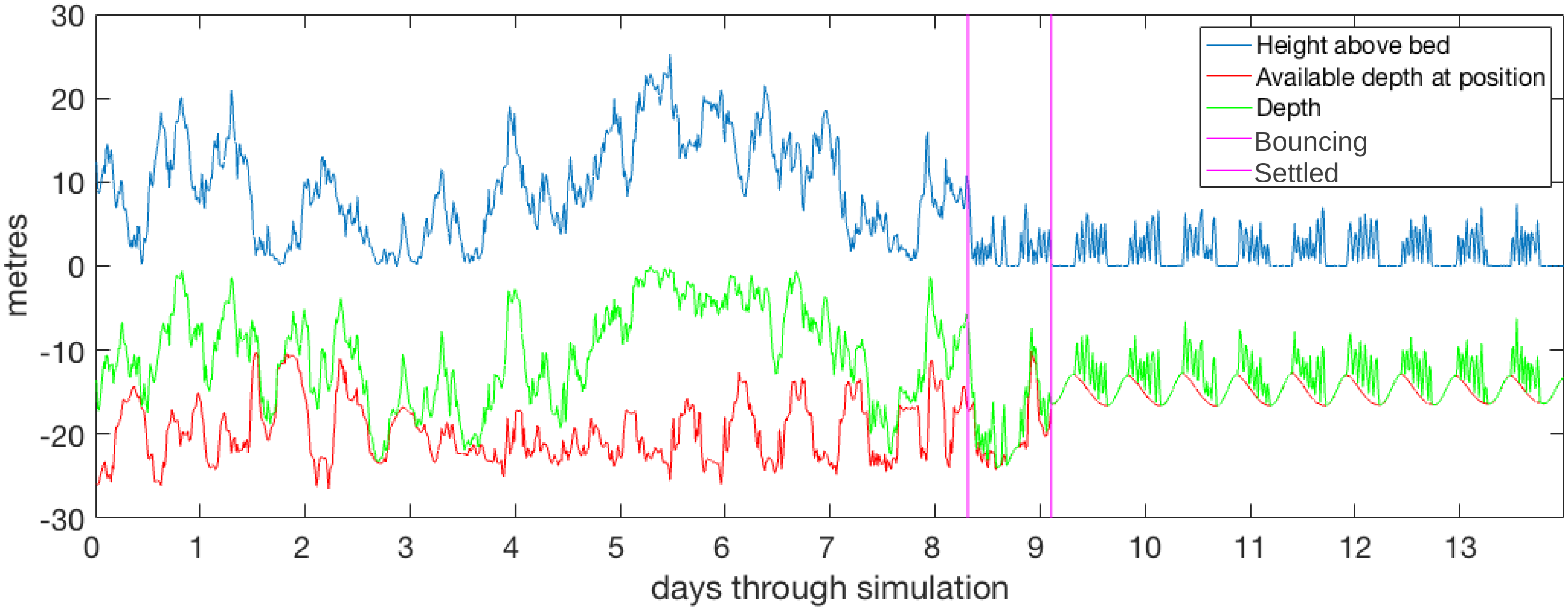


Figure 2.19. An example of the predicted vertical trajectory of one individual particle (larvae) (of a total 2500) within the < 14 day assumed pelagic phase (this individual was released from LRBAR). Modelled larvae moved throughout the water column for c. 10 days visible from the interaction between the depth of the larvae (green line) with the available depth (red line). Just after day 8 the lefthand magenta line shows when the settlement phase started. The modelled larva moves to the bed and bounces during the incoming tide, and remains fixed during the outgoing tide, and it can be seen that this causes it to move to a shallower area as the red line trends upward between day 8 and day 9. The larva settles just after day 9 as shown by the second magenta line, presumably having reached any area of appropriate depth. After settlement the larva stopped all lateral movement and the pattern after day 9 shows the available depth changing smoothly with the tide at that fixed location. The output data continue to show the modelled larvae bouncing, but this is for model diagnostic information only. Modelled settlement occurred at the magenta line just after day 9.

Predicted horizontal spatial tracking of individual larvae throughout the 14 pelagic days

The method for modelling larvae ‘milling’ using both the hydrodynamic model and a passive drifting Lagrangian particle model was explained by (Herbert *et al.*, 2012b). These methods have been applied to track the larvae of several marine invertebrates including the Manila clam, *Ruditapes philippinarum* (Herbert *et al.*, 2012b), and brachyurans including *Lophopanopeus* spp. and *Pachygrapsus crassipes* (DiBacco *et al.*, 2001; Morgan *et al.*, 2009) using observed data on propagule duration (PD), distance travelled, and larvae behaviour (Shanks, 2009 and references within). The model used for this study followed Mead (2004, 2008) and the recommendations of the International Council for the Exploration of the Sea (ICES) working group on ‘Physical – Biological Interactions’, and the ICES working group on ‘Recruitment Processes’ (North *et al.*, 2009). The velocities of the particles was calculated using the water velocities with Runge Kutta fourth order integration (Willis, 2011). A total of 2500 particles (larvae) were released from a chosen starting position within the Solent for each model run and were assumed to travel a unique trajectory. The horizontal trajectory of individual larvae could be tracked from the point of release to their chosen settlement location with a position reported every hour. The model predicted that some larvae would remain close to the release zone (Figure 2.20), some would travel far but remain within the Solent (Figure 2.21), while others would travel longer distances outside the Solent channels before returning to a settlement site within the Solent (Figure 2.22). Although the passive drift larval model showed larvae travelling long distances, only those that returned to the Solent to settle at an appropriate depth and near appropriate substrate were registered and counted by the model. Those that travelled further were assumed to have been lost to depth. This was explained in Shanks (2009) with the description of larvae with long PD actually not necessarily travelling much further than those with a shorter PD; “PD is a poor predictor of dispersal potential” (Shanks, 2009).

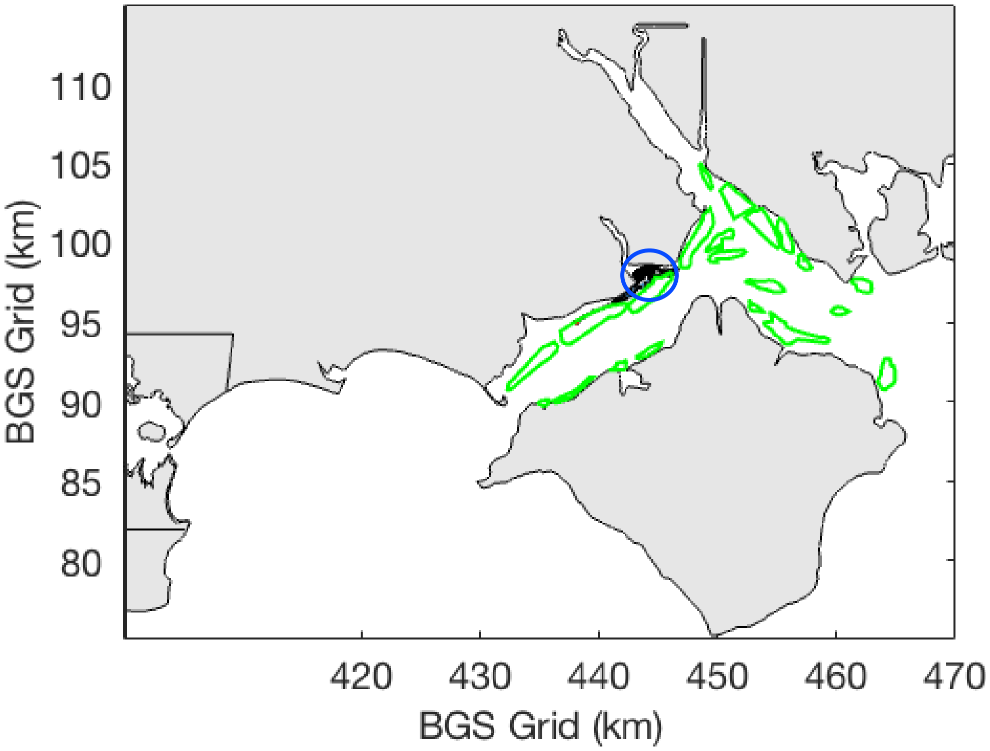


Figure 2.20. The predicted horizontal trajectory of one particle (larvae) (e.g. starting location is Beaulieu River, marked with a blue circle (see Table 2.2 for exact location)) and following a trajectory very close to the release zone. A total of 2500 larvae were released with each model run, and each larva was assumed to follow a unique trajectory for <14 days before settling. The black lines represent the horizontal movement. The lime green areas mark the historical beds likely to be most appropriate for *O. edulis* larval settlement.

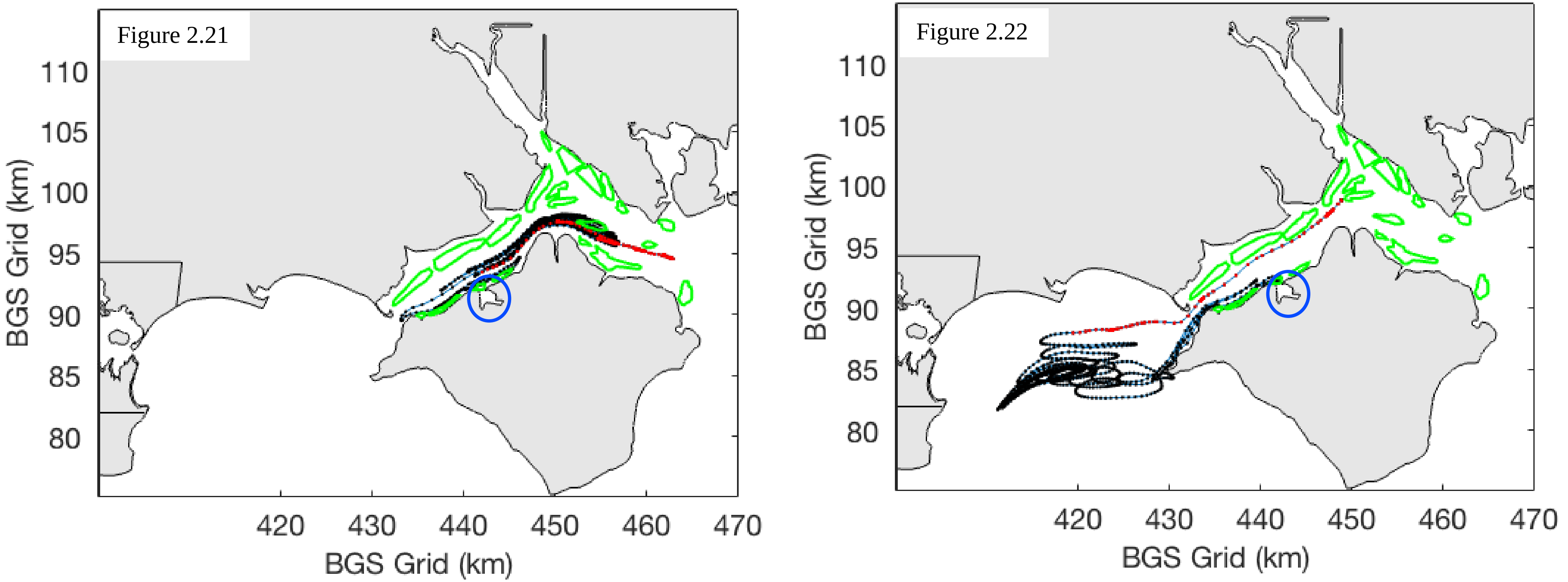


Figure 2.21 and Figure 2.22. The predicted horizontal trajectory of two particles (larvae) when given a starting location in (e.g.) Newtown Creek, Isle of Wight marked with a blue circle (see Table 2.2 for exact location) and following a trajectory inside (Figure 2.21) and outside (Figure 2.22) the Solent. A total of 2500 larvae were released with each model run, and each larva was assumed to follow a unique trajectory for <14 days before settling. The black lines represent the horizontal movement and the red lines represent the ‘bouncing’ movement towards the end of the pelagic phase. The lime green areas mark the historical beds likely to be most appropriate for *O. edulis* larval settlement.

To summarise, each Chapter objective and respective experiment is listed below (Table 2.3).

Table 2.3. A summary of the Chapter objectives and respective experiments.

Objective	**Experiment number and name**	
To observe individual oyster valve movement in response to water velocity	1	Individual oyster response
To determine critical water current speeds at which displacement of either cultch or juvenile oysters occurs	2	Juvenile oyster physical displacement
	3	Spat on shell physical displacement
To compare hydrodynamic features of the benthic boundary layer (BBL) associated with an *O. edulis* bed with that of a *C. fornicata* bed	4	Benthic Boundary Layer (BBL) – *Ostrea edulis* bed vs. *Crepidula fornicata* bed
To determine probable larvae trajectory and settlement location given specific release sites using an individual based model (IBM)	5	Predicted settlement locations given a starting location
	6	Heat maps of predicted settlement locations given a starting location

2.3 Results

2.3.1 Flume experiments

Experiment I: Individual oyster response

Oysters were observed closing their valves at water velocities as low as 0.19 m s^{-1} with an average of 0.29 m s^{-1} ± 6.0 (mean ± sd, n = 10) (Table 2.3). For some oysters, displacement from their starting position did not prompt them to close their valves. The depth-averaged water velocity (Ū, m s^{-1}) at which oyster valve closed showed a weak yet positive correlation ($R^2 = 0.61$, $P = 0.0046$) to individual oyster weight (g) (Figure 2.23).

Table 2.4. Depth averaged water velocity (Ū, m s^{-1}) at which 10 adult oysters either closed their valves or were physically displaced.

Oyster dimensions			**Water velocity (Ū) (m s^{-1})**	
Height (mm)	*Length (mm)*	*Wet weight (g)*	*At which the oyster was displaced*	*At which the oyster closed its valves*
79.6	70	118.07	0.26	0.31
76.4	76.6	120.96	0.26	0.36
81.8	78.6	131.90	0.26	0.39
72.8	67.2	87.94	0.31	0.31
75.1	70.5	95.44	-	0.26
69.5	87.1	71.59	0.26	0.26
73.8	76.4	73.02	0.23	0.23
70.5	58.7	76.85	-	0.31
69.1	66.5	73.75	-	0.19
70.1	64.6	67.99	-	0.26

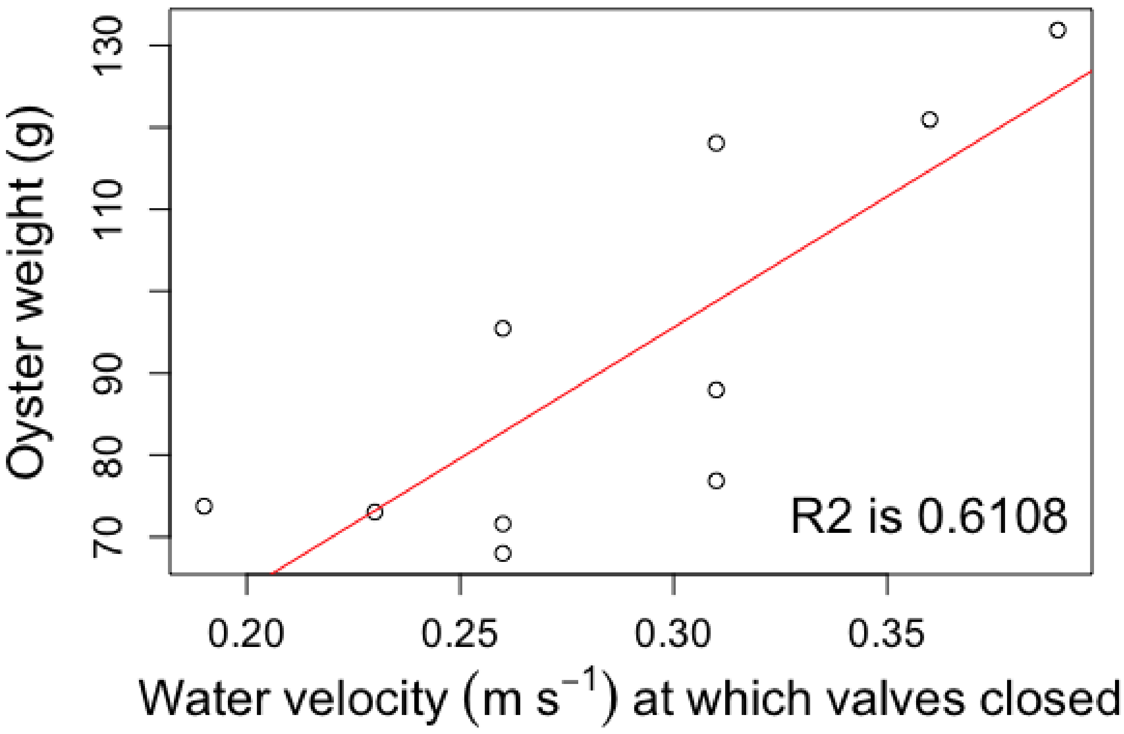


Figure 2.23. The correlation between oyster weight (g) and the water velocity (m s^{-1}) at which the individuals closed their valves.

Experiment II: Juvenile oyster physical displacement

Juvenile oysters were similarly displaced with water velocities from 0.22 m s^{-1} (Table 2.5). A small number of oysters were displaced during each run and the distance and pattern of displacement was noted (Figure 2.24).

Table 2.5. Depth averaged water velocities ($\bar{U}$, m s^{-1}) at which the juvenile oysters were displaced during the five experiments in order of water velocity, and the distance of displacement (m).

Depth averaged water velocity ($\bar{U}$, m s^{-1})	**Number of juvenile oysters displaced**	**Distance of displacement (m)**
0.22	1	0.02
0.31	5	0.10
0.31	1	0.16
0.42	1	0.25
0.52	2	Freely moving around the flume

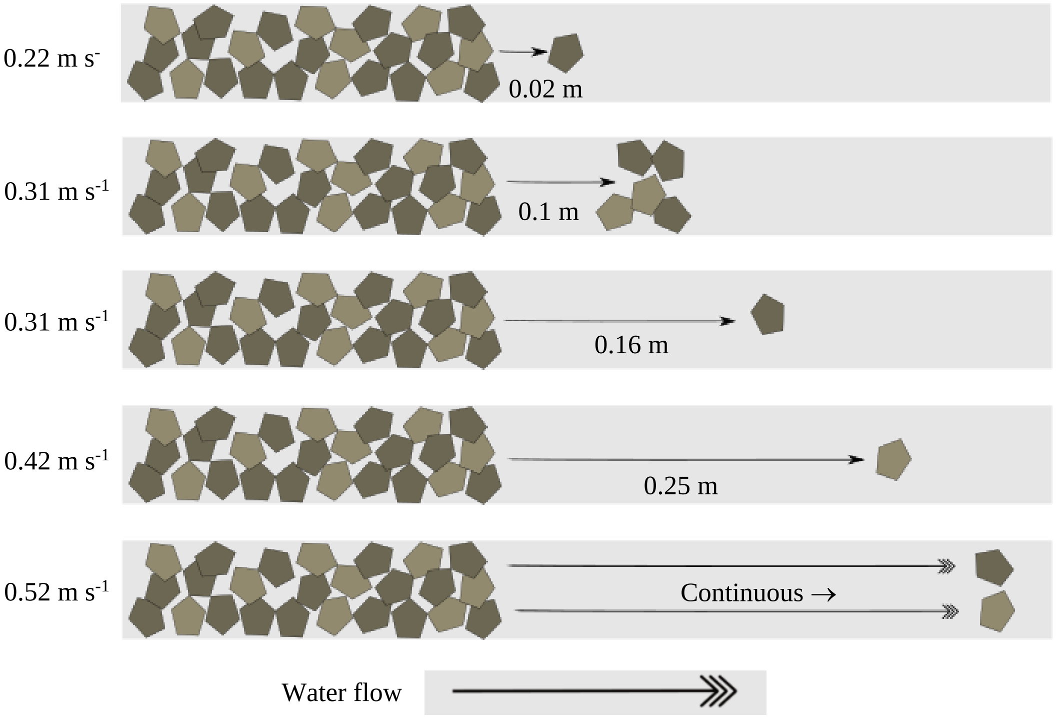


Figure 2.24. Plan view pattern of juvenile oyster displacement at the depth averaged water velocities ($\bar{U}$, m s^{-1}). Brown pentagons represent oysters and the arrows represent direction of flow. Single headed arrow represent displacement. Three-headed arrows represent continuous movement.

Experiment III: Spat on shell physical displacement

The displacement of spat on shell depended on initial formation (shell depth). Spat-on-shell laid at 0.01 m shell depth, were displaced at a vertically averaged flow of 0.49 m s^{-1} (z = 0-0.1 m, n = 6), and spat-on-shell at 0.05 m shell depth were displaced at a vertically averaged flow of 0.44 m s^{-1} (z = 0-0.1 m, n = 6) (Figure 2.25). The spat-on-shell that started at 0.05 m shell depth were displaced in two sections; A small clump of spat-on-shell was separated from the rest of the spat-on-shell with the flow, and the shell pile was made shallower at one end with the flow (Figures 2.26 and 2.27). Spat-on-shell layered at 0.15 m shell depth remained in its starting formation and was not moved by the fastest experimental water velocity for this study (0.52 m s^{-1}).

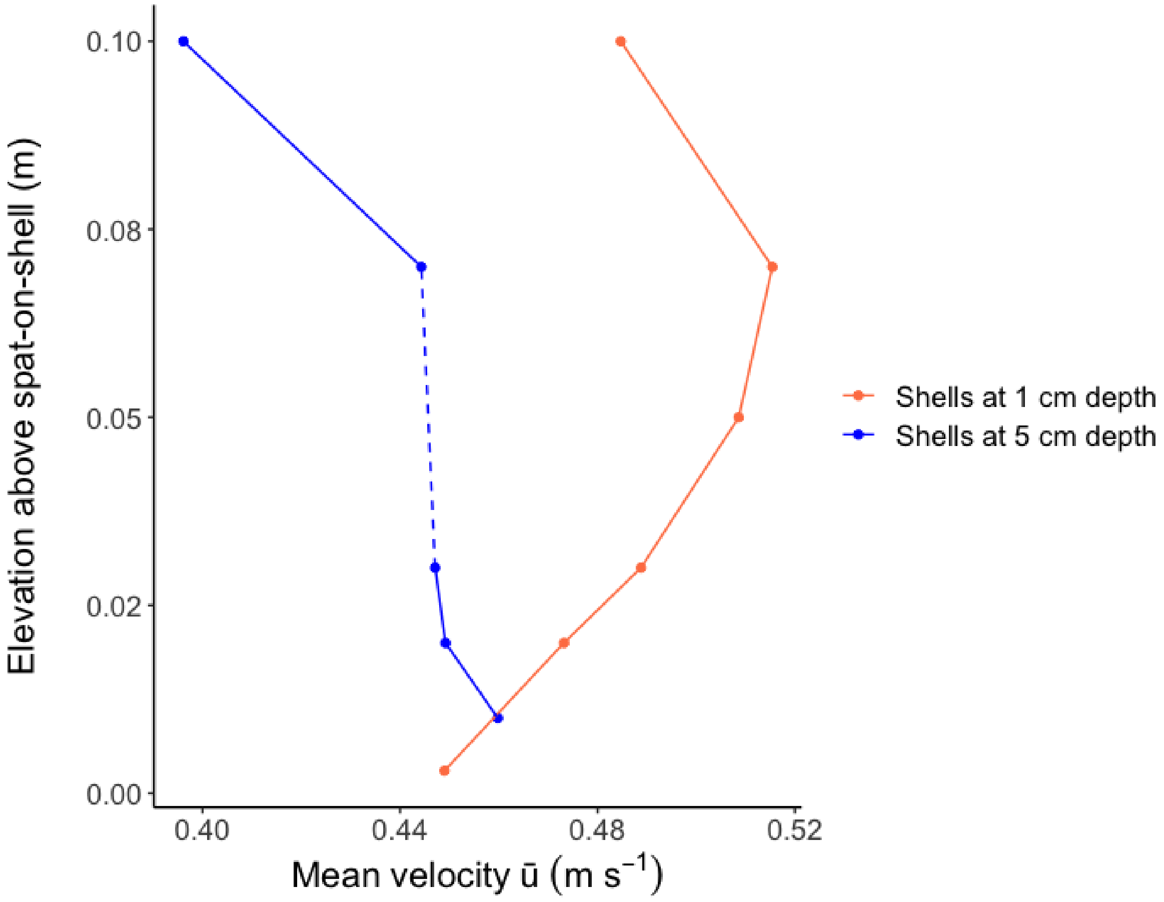


Figure 2.25. Mean water velocity at which the spat-on-shell were displaced for shells in a starting depth of 0.01 m above the bed (orange) and shells with a starting depth of 0.05 m above the bed (blue). Over 3000 data readings were taken per sample at each point on the vertical axis through the water column and averaged to acquire this mean water velocity at each elevation (ū, m s^{-1}). Irregular data were removed and replaced with a dashed line to connect the measured vertical height points (circles).

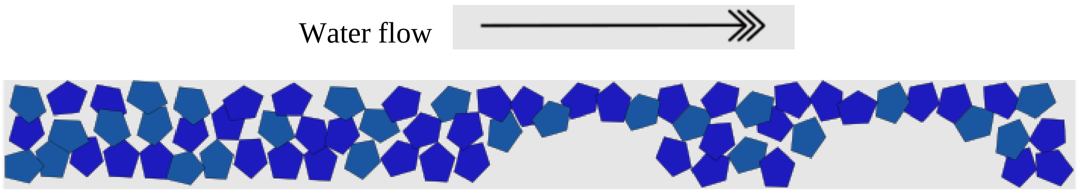


Figure 2.26. Plan view of the pattern of final spat-on-shell displacement with starting position of 0.01 m shell depth. Blue pentagons represent the displacement of spat-on- (mussel, *Mytilus edulis*) -shell, and arrow represents the water flow.

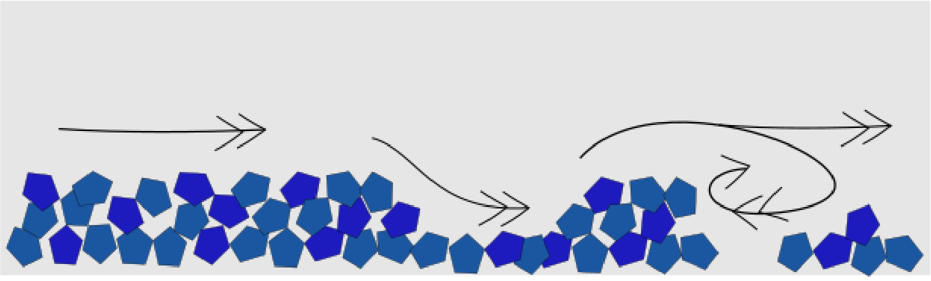


Figure 2.27. Side-on view of the pattern of final spat-on-shell displacement with starting position of 0.05 m shell depth. Blue pentagons represent the spat-on- (mussel, *Mytilus edulis*) -shell and the associated displacement, and arrows represent the predicted water flow (as turbulence was not measured here) as theorised by Vogel (1996) where substrate rugosity increases the penetration of eddies at the surface that divide the substrate.

Experiment IV: Benthic Boundary Layer – *Ostrea edulis* bed vs. *Crepidula fornicata* bed

The *O. edulis* bed caused a reduction in water velocity within the first 0.01-0.02 m of the water column at P2, and an increase in velocity almost immediately above that (Figure 2.28), as would be expected for a well-formed boundary layer (Soniat *et al.*, 2004). The same effect was not observed with the *C. fornicata* bed, and flow remained relatively constant at all three tested positions (P1-P3) (Figure 2.29). A small increase in speed could be seen at P3 (post *C. fornicata* bed) at the lowest water speed (0.06 m s^{-1}), shown in blue (Figure 2.29). Irregular data were removed from the analysis and referenced with dotted lines connecting the measured vertical height points (circles) in each figure. The water flow at P3 (post *O. edulis* bed) shows a slower recovery of water velocity below 0.12 m in comparison to above 0.12 m. Shear stress (Pa) was greatly increased at position P2 above the *O. edulis* bed and remained high after the bed (P3) (Figure 2.30). The experiment at 0.42 m s^{-1} (pink line) demonstrated the shear stress expected to be seen above a rough surface, with two spikes in the flow (at 0.07 m and 0.11 m, above the *O. edulis* bed, Figure 2.30). Naturally, the stress was not very high in the laminar flow at P1, but gentle spikes in turbulence could be seen at the slower experimental velocities (0.06 and 0.13 m s^{-1}, Figure 2.30). Stress above the *C. fornicata* bed was over three times smaller than that seen above the *O. edulis* bed but was more varied between experiments (Figure 2.31). The water directly above the *C. fornicata* bed showed a slight increase in shear stress, and evidence of increased stress was seen in the higher water velocity experiments (0.31 m s^{-1} and 0.42 m s^{-1}, Figure 2.31). The peaks between 0.12 and 0.14 m above the *C. fornicata* bed (Figure 2.31) could represent some eddy formations occurring, but the scale at which this was happening was greatly reduced in comparison to the oyster bed. The drag coefficient (C_D), roughness length scale (z_0) and shear velocity (u*) were calculated from the Reynolds number (Re), shear stress (t_{TKE}), and average velocity ($\bar{u}$) according to 2.2.2 (detail can be found in Appendix B). C_D was not significantly different between an *O. edulis* bed and a *C. fornicata* bed (LM, $F_{1,170}$ = 0.1842, P = 0.6683).

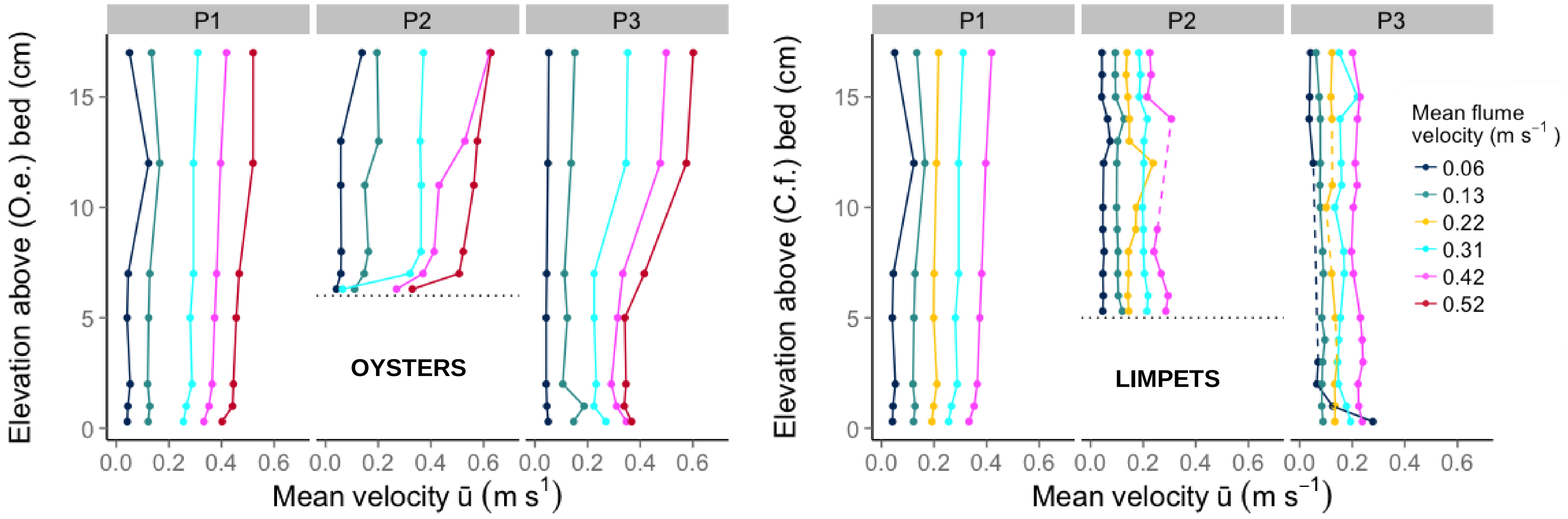


Figure 2.28 and Figure 2.29. Mean water velocity (ū, m s^{-2}) 0.5 m upstream from (P1), above (P2), and 0.5 m after (P3) an *O. edulis* (oyster) bed (Figure 2.28) and a *Crepidula fornicata* (limpet) bed (Figure 2.29) at the 6 experimental water flows. The top of the bed is marked with dotted lines. Irregular data were removed and replaced with dashed lines to connect the measured vertical height points (circles).

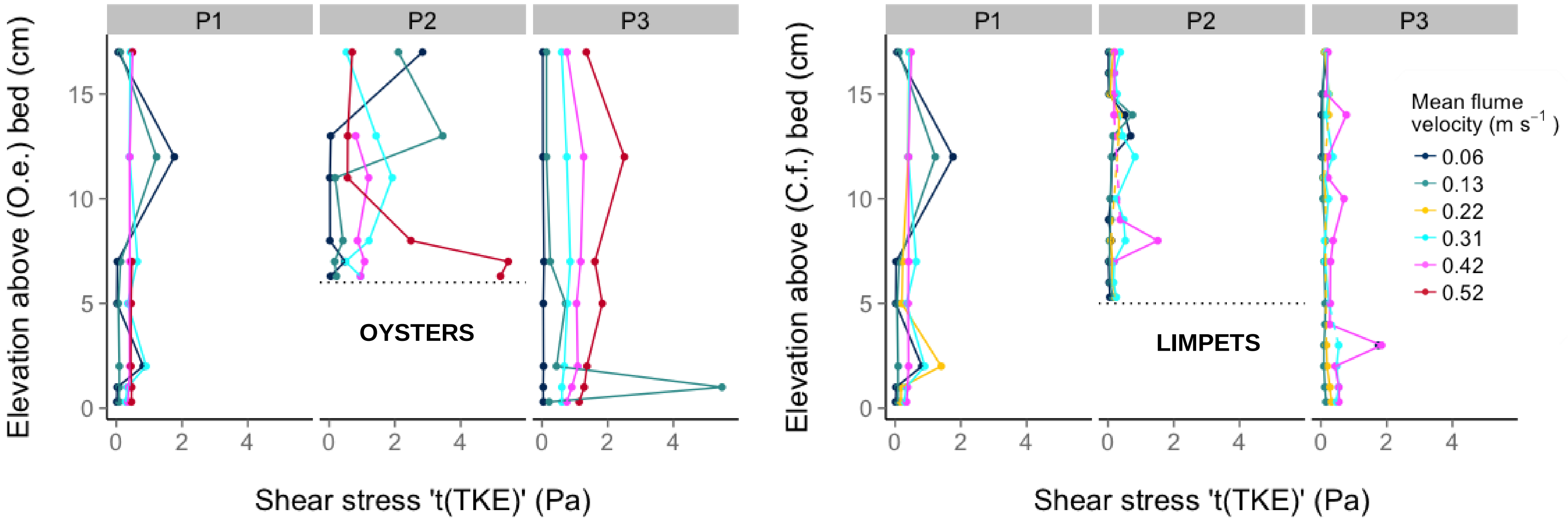


Figure 2.30 and Figure 2.31. Shear stress ‘τ_{TKE}’ (Pa) 0.5 m upstream from (P1), above (P2), and 0.5 m after (P3) an *Ostrea edulis* (oyster) bed (Figure 2.30) and a *Crepidula fornicata* (limpet) bed (Figure 2.31). Dotted lines mark the top of the bed. Irregular data were removed and replaced with dashed lines to connect the measured vertical height points (circles).

The relationship between shear stress (τ_{TKE}) (Pa) and mean velocity (ū, m s^{-1}) was similar to that seen by (Thompson *et al*., 2004a, 2004b), with an increase in stress with increasing flow velocities (Figure 2.32). Higher stress was seen in association with the *O. edulis* bed at profiles P2 and P3 in comparison to the *C. fornicata* bed, especially at higher velocities, but the scatter in the results obscures any clear definition between the two beds.

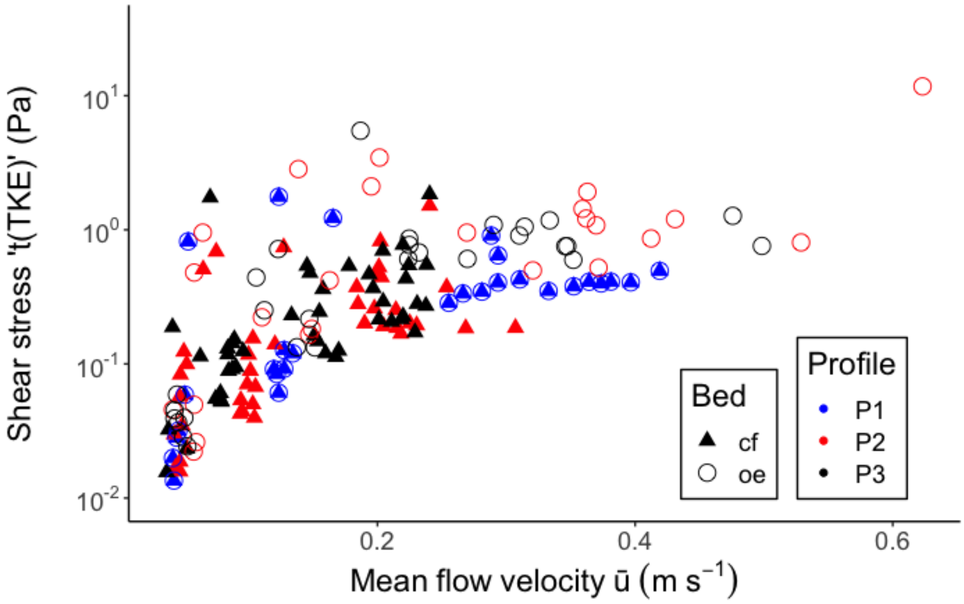


Figure 2.32. Shear stress (τ_{TKE}) (Pa) in relation to mean flow velocity (ū) (m s^{-1}) above a *Crepidula fornicata* (cf) bed (triangle), and an *Ostrea edulis* (oe) bed (circle). Colours represents the position of measurement (prior to (P1) (blue), above (P2) (red), and after (P3) (black) the bed in question. Only experimental velocities at which both *O. edulis* and *C. fornicata* were both tested were included (0.06, 0.13, 0.31 and 0.42 m s^{-1}).

2.3.2 Hydrodynamic trajectory model output (Willis *et al*., 2019)

Predicted settlement locations given a starting location.

The trajectory of 2500 particles (larvae) was reported over a predicted <14 day pelagic phase before settlement (e.g. Figure 2.33a-d). The predicted settlement locations of larvae were tested with six experimental release zones within the Solent (Saxon Wharf, Hamble River, Beaulieu River, Newtown Creek, Lymington, and LRBAR, see Table 2.2). The pattern of larval distribution around the Solent was very different depending on release zone and showed that some starting locations were more dispersive than others. Lymington, the Beaulieu River, and Newtown Creek (Figures 2.33d, 2.34 and 2.35 respectively) were highly dispersive release zones, while Saxon Wharf, the Hamble River, and LRBAR (Figures 2.36, 2.37 and 2.38 respectively) were more retentive.

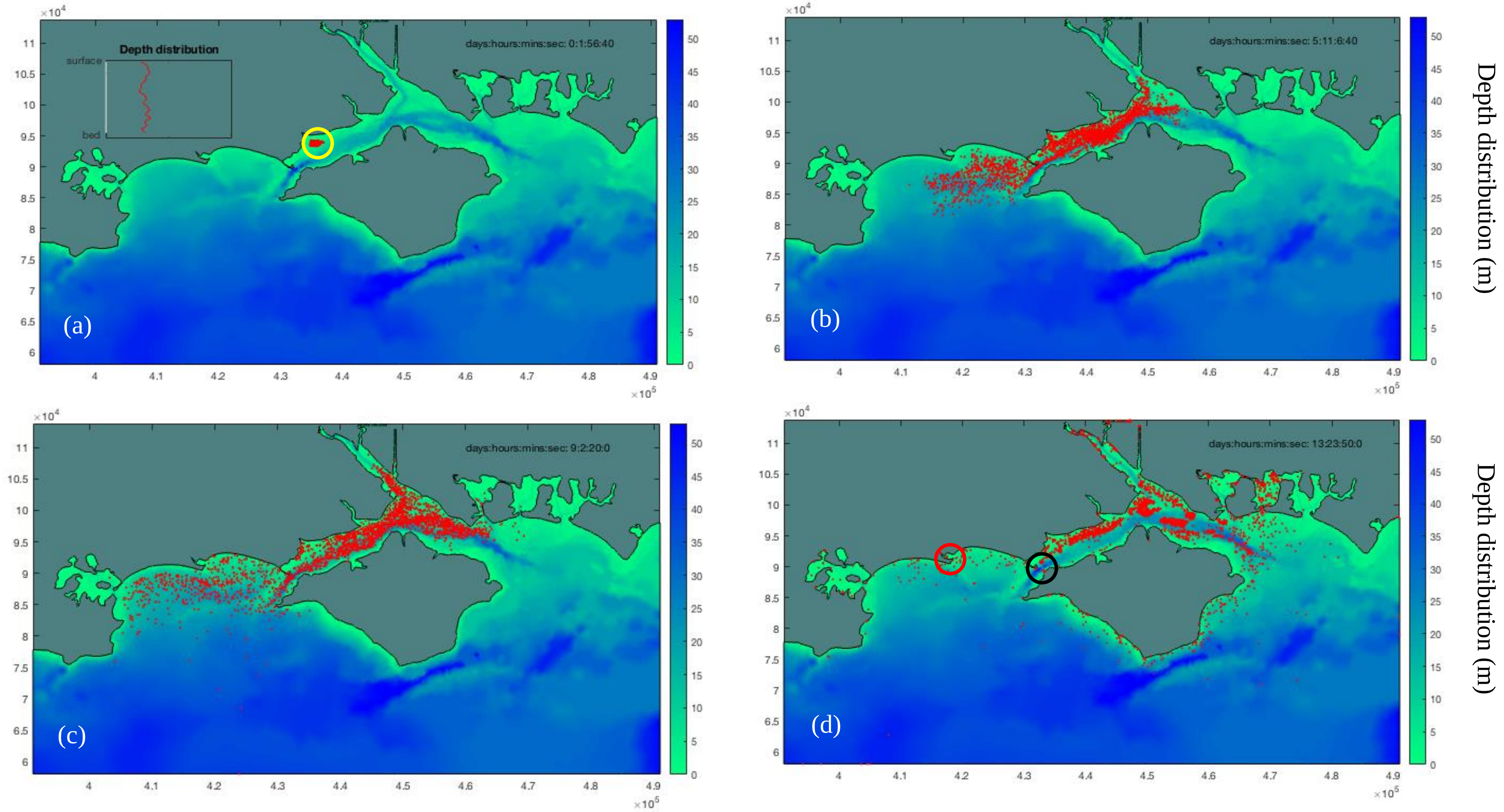


Figure 2.33 (a-d). Modelled trajectory of 2500 *Ostrea edulis* larvae (red dots) around the Solent with an example starting location in Lymington (marked with a yellow circle in Figure 2.33a). Panel (a) shows the particles after nearly 2 hours on day 1 (b) after 5 days, (c) after 9 days, and (d) after 13 days at their final destination. The larvae were strongly retained within the western Solent channel, but a small proportion exited the Needles Channel (marked with a black circle in 2.33d) towards Christchurch Harbour (marked with a red circle). The background and blue colour bar represent water depth (m).

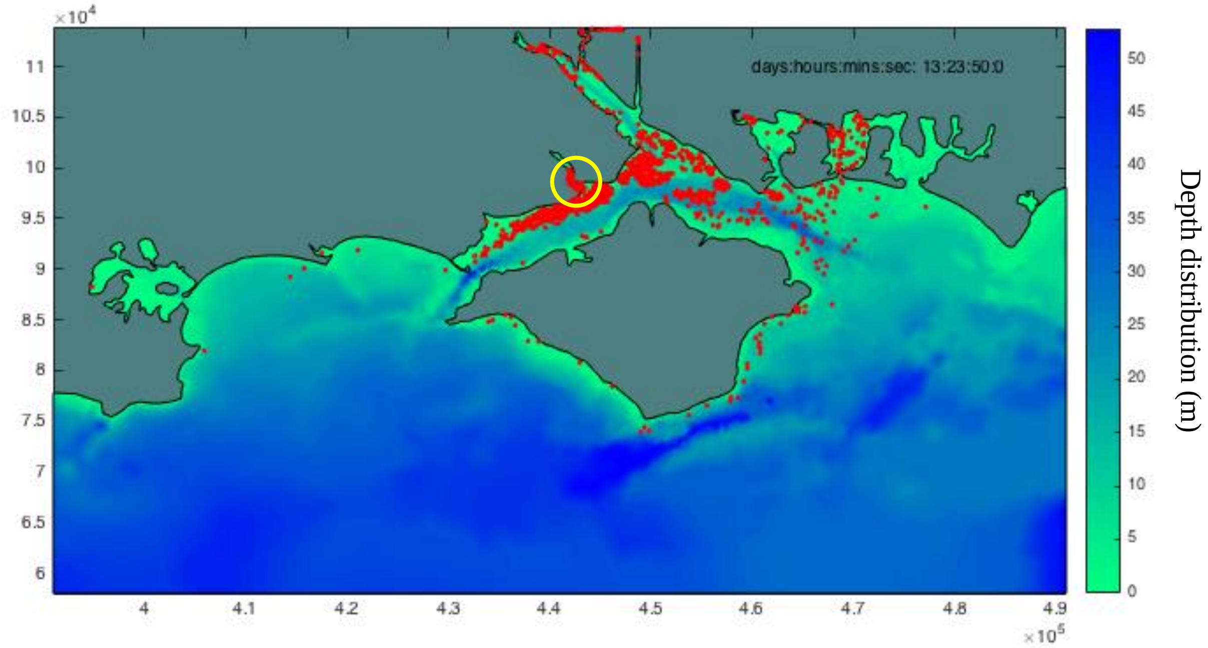


Figure 2.34. Model output of 2500 particles (larvae) (red dots) after < 14 days with a starting location in the Beaulieu River (yellow circle) demonstrating a dispersive environment.

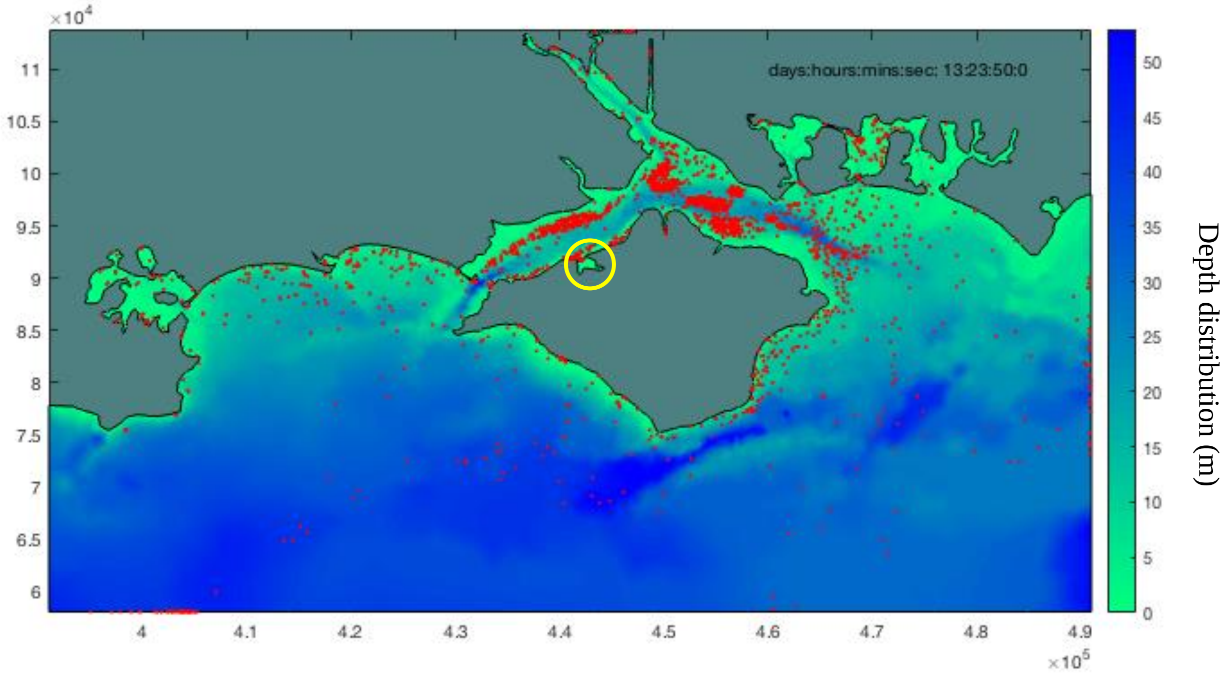


Figure 2.35. Model output of 2500 particles (larvae) (red dots) after < 14 days with a starting location at Newtown Creek (yellow circle) demonstrating a dispersive environment.

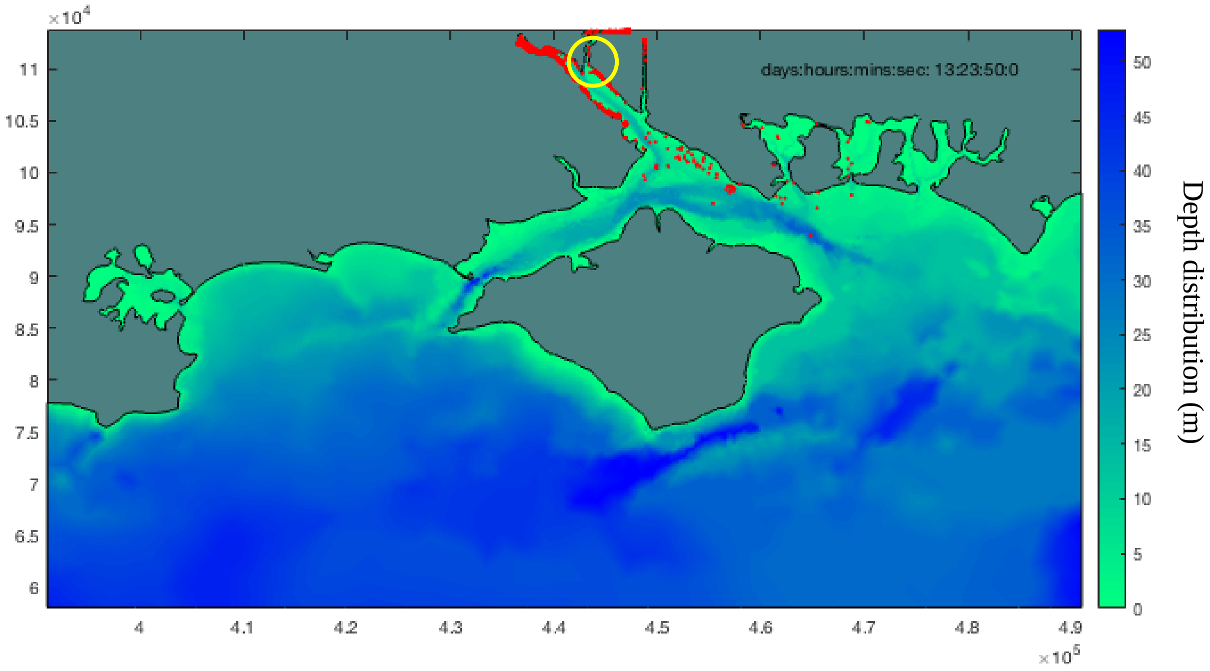


Figure 2.36. Model output of 2500 particles (larvae) (red dots) after < 14 days with a starting location in Saxon Wharf (yellow circle) demonstrating a retentive environment.

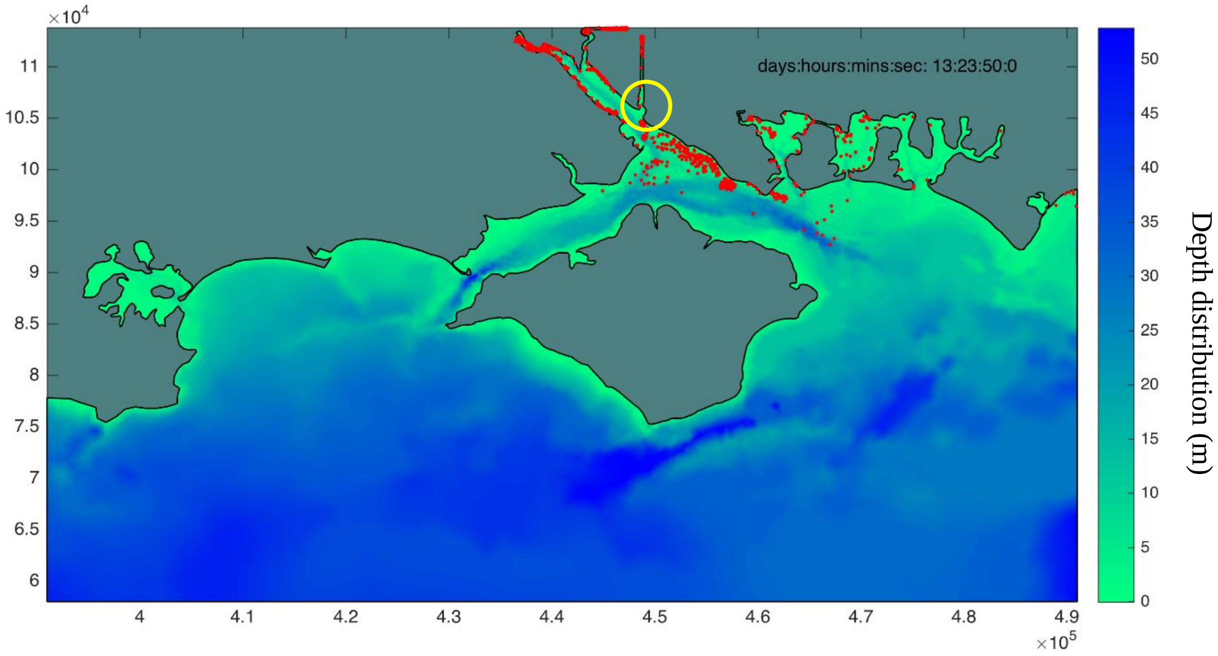


Figure 2.37. Model output of 2500 particles (red dots) after < 14 days with a starting location in the River Hamble (yellow circle) demonstrating a retentive environment.

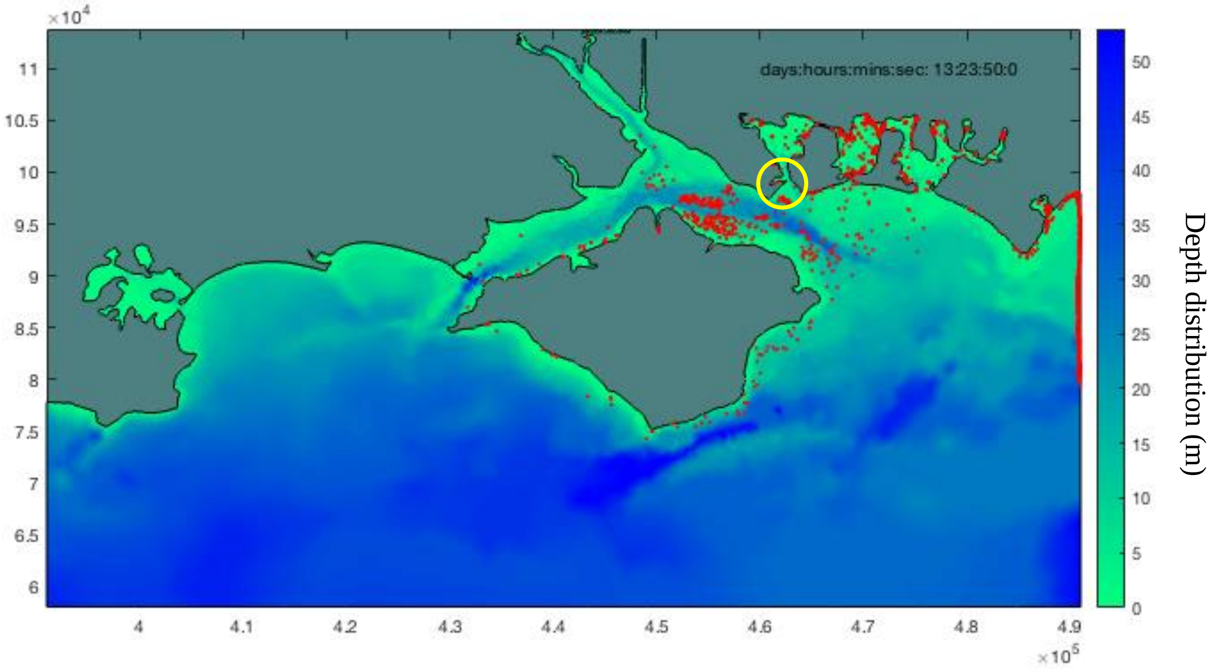


Figure 2.38. Model output of 2500 particles (larvae) (red dots) after < 14 days with a starting location at LRBAR (yellow circle) demonstrating a retentive environment.

Heat maps of predicted settlement locations given a starting location.

The model predicted areas in the Solent in which the larvae were most likely to settle after the < 14-day pelagic phase and formatted these data into heat maps. The areas and density at which larvae settled were highly dependent on starting location. A similar large range and high density was seen with a release zone in Lymington and Beaulieu (Figures 2.39 and 2.40). Larvae released from Lymington were predicted to thinly cover a large area across the Solent (Figure 2.41), while those from Saxon Wharf thinly covered a very small area (Figure 2.42). A very low density of larvae from the Hamble River and LRBAR were predicted to settle along the Eastern channel of the Solent covering a small area (Figures 2.43 and 2.44, respectively). However, the model perimeters were limited to the Solent only and did not account for larvae dispersed to Langstone Harbour or Chichester Harbour. Should the model be further expanded to include these bays, a higher density of predicted larvae settlement might be seen in those areas. These data showed that the model predicted larvae settlement locations were similar to the locations of historical oyster beds (marked in black).

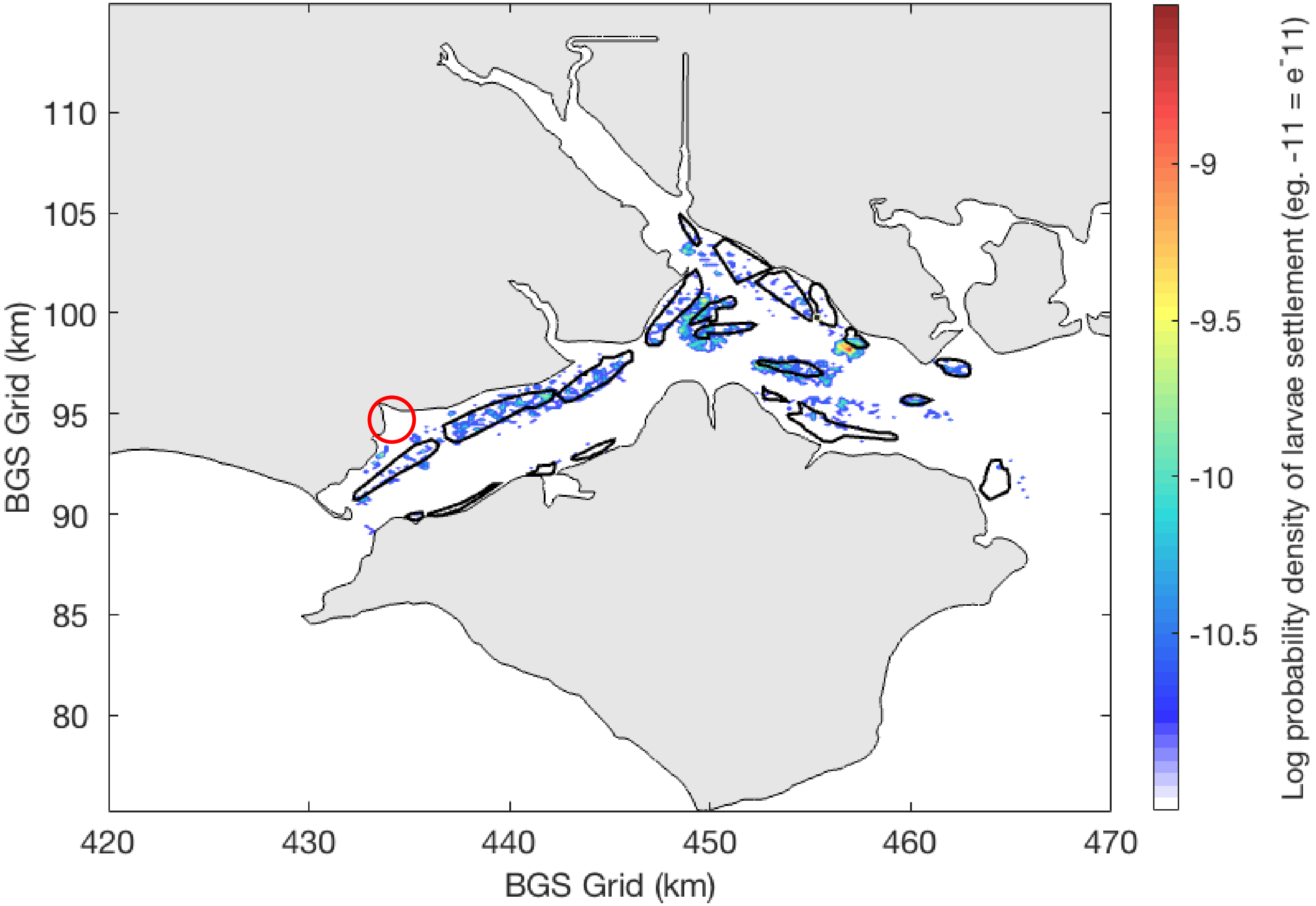


Figure 2.39. Model output of log density settlement scale of 2500 particles after < 14 days with a starting location in Lymington (red circle).

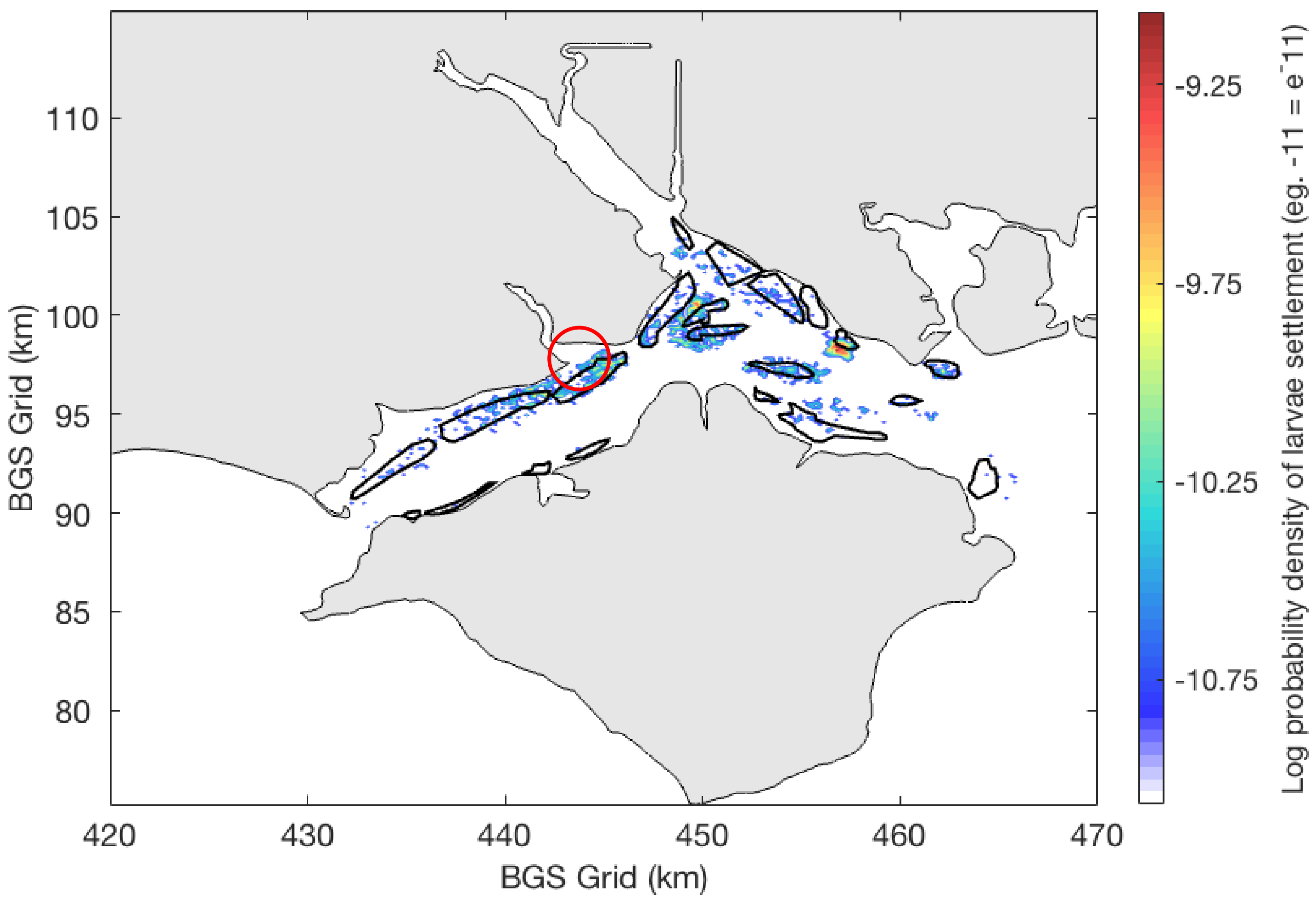


Figure 2.40. Model output of log density settlement scale of 2500 particles after 14 days with a starting location in the Beaulieu River (red circle).

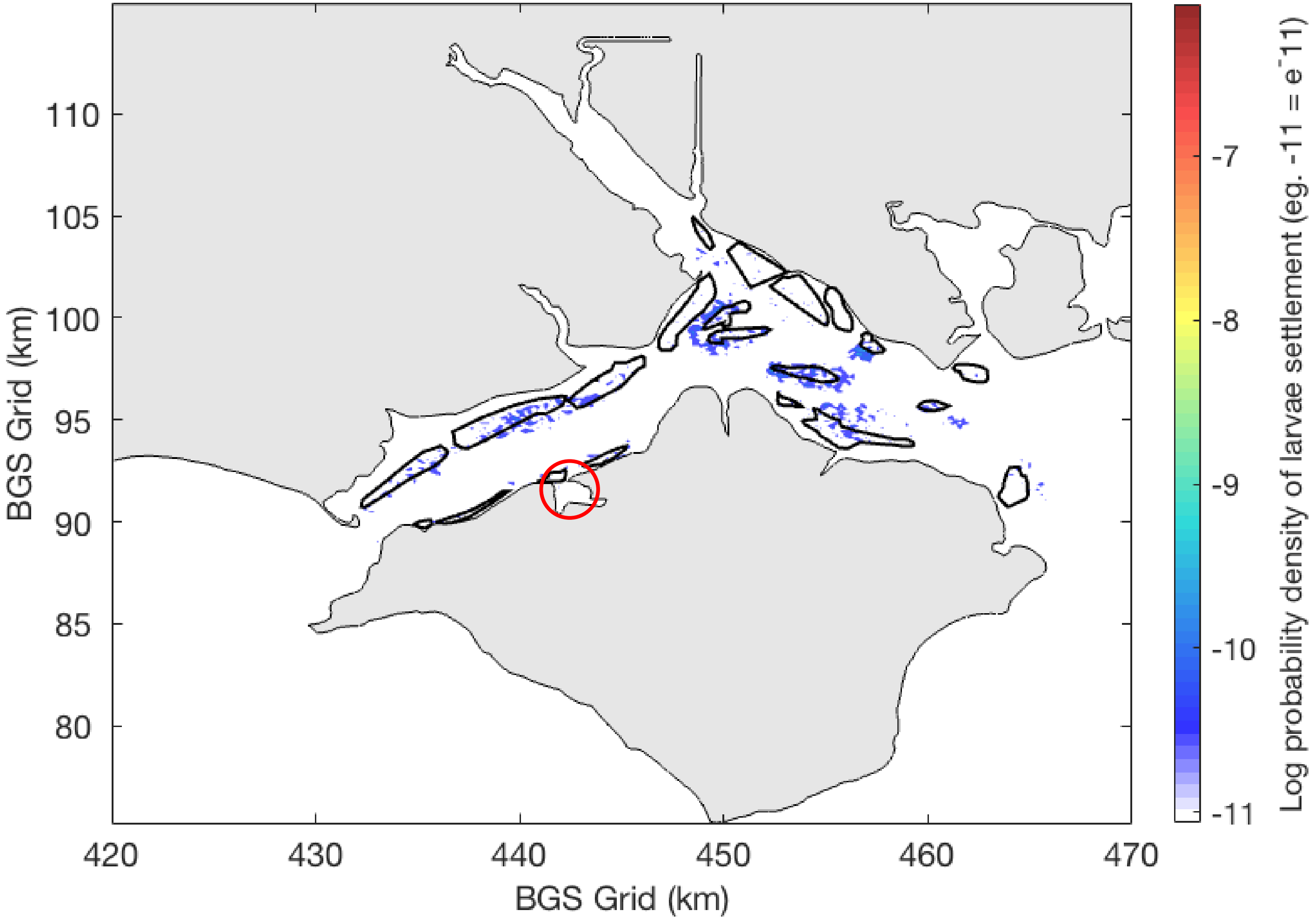


Figure 2.41. Log density settlement scale of 2500 particles after < 14 days with a starting location in Newtown Creek (red circle).

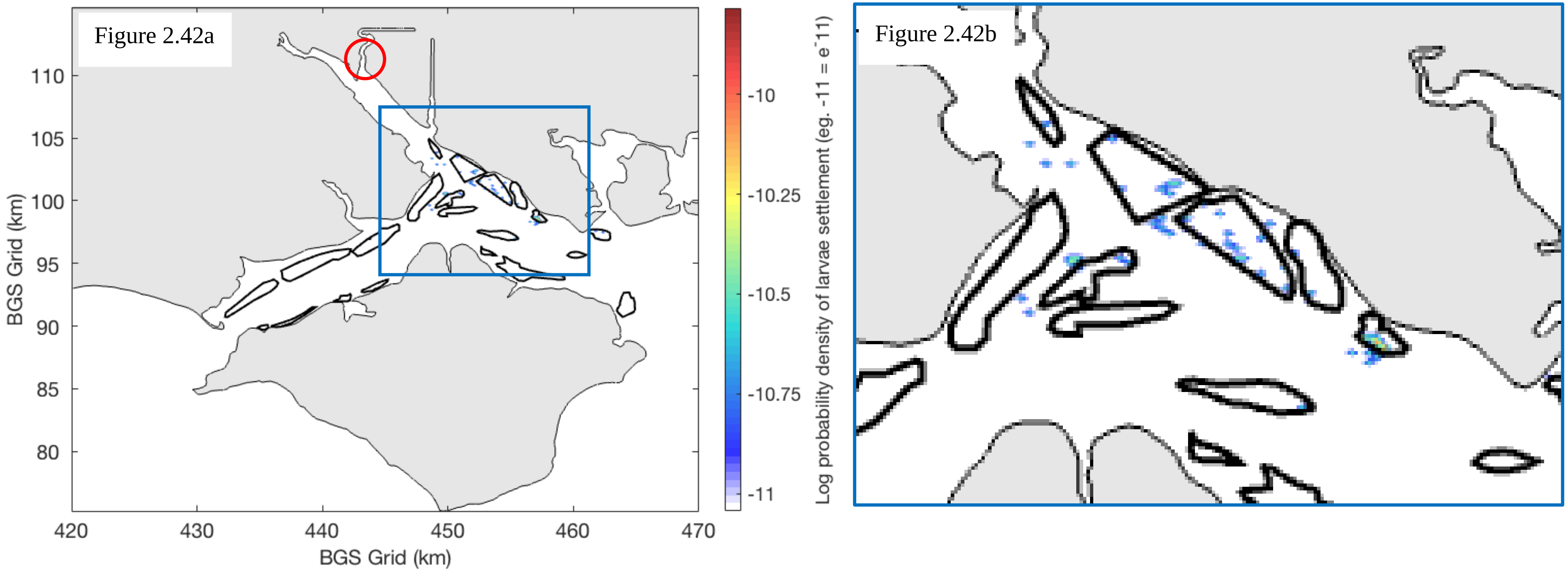


Figure 2.42 (a and b). Model output of log density settlement scale of 2500 particles after < 14 days with a starting location in Saxon Wharf (red circle in 2.42a), and close up view (Figure 2.42b).

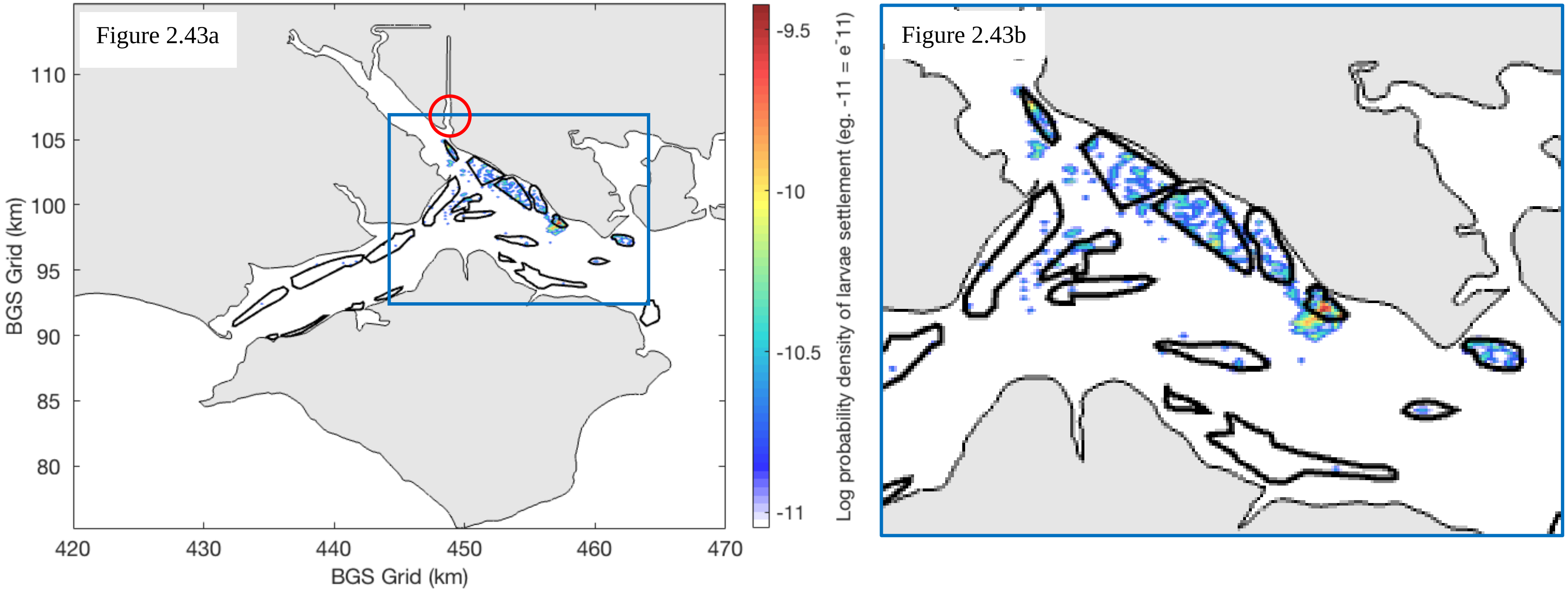


Figure 2.43 (a and b). Model output of log density settlement scale of 1200 particles after 14 days with a starting location in the River Hamble (red circle in 2.43a), and close up view (Figure 2.43b).

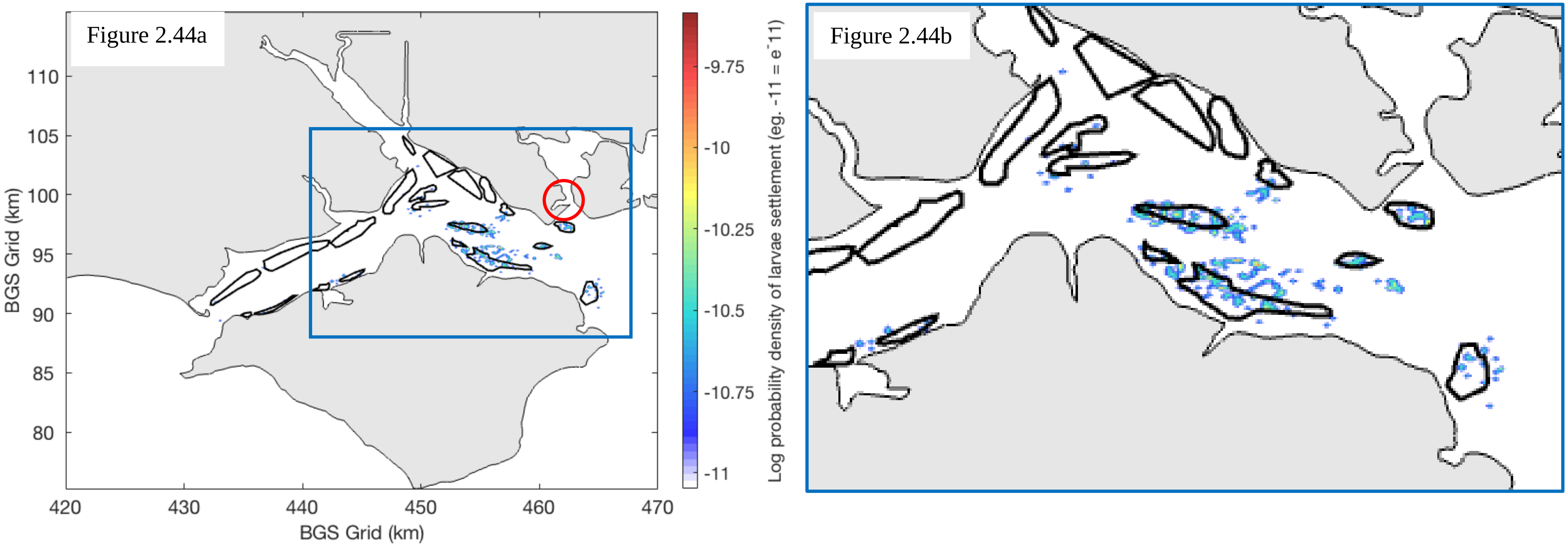


Figure 2.44 (a and b). Model output of log density settlement scale of 2500 particles after < 14 days with a starting location in LRBAR (red circle in 2.44a), and close up view (Figure 2.44b).

2.4 Discussion

The aims of this work were to quantify the role of small-scale hydrodynamics and the effect of water velocity on *Ostrea edulis* adults, juveniles and cultch with the use of flume experiments, and to model predictions of larval dispersal and settlement on a large scale across the Solent. An appreciation of the importance of hydrodynamics in association with an *O. edulis* bed has implications for restoration site selection purposes. The effect of the hydrodynamic environment on adult and juvenile *O. edulis* were observed with the use of a seawater flume operated between 0.06 and 0.52 m s^{-1}, the highest of which is similar to the water velocity previously recorded at 0.15 m from the seabed (0.59 m s^{-1}) at the western entrance of the Solent (Quaresma *et al.*, 2007). The first experiments tested the water velocities at which adult oysters closed their valves and at which adults, juvenile oysters, and spat-on-shell were physically displaced. The hydrodynamic envioronment associated with an *O. edulis* bed was then compared with that associated with a *Crepidula fornicata* bed using the same flume system.

2.4.1 Adult closing valves and adult and juvenile oyster displacement

Adult oysters were observed closing their valves at flume water velocities from 0.19 m s^{-1} with an average of 0.29 m s^{-1} ± 6.0 (mean ± sd, n = 10). Oysters feed and ventilate their gills with their valves open, and respond to threats of predators, or changes in abiotic conditions such as temperature and salinity by closing their valves. Although oysters have demonstrated tidal and diurnal patterns in the opening and closing of their valves, closing their valves restricts respiring or feeding, which has an impact on their physiology. These data suggest that restoration site choice must consider the local water velocities to ensure oysters can ventilate and feed efficiently. This study let the oysters relax at low speed (0.06 m s^{-1}) for 3 h before study and only monitored where the oyster closed its valves. Further study might complement this work by continuing the constant velocity to see if and when the oyster reopened after acclimation.

Displacement of adult oysters occurred at water velocities from 0.23 m s^{-1} with an average of 0.26 m s^{-1} ± 0.026 (mean ± sd, n = 6) and this displacement velocity was positively correlated (adjusted R^2 = 0.61) with the wet weight (WW) of each adult oyster (Figure 2.23). Juvenile oysters were displaced at water velocities from 0.22 m s^{-1}. At the highest flume speed of 0.52 m s^{-1} juvenile oysters were swept around the flume in a continuous motion. The Solent is a high energy hydrodynamic environment with recorded current surface water velocities of 2.32 m s^{-1} (Key and Davidson, 1981; Iriarte and Purdie, 2004), and wave power is likely to have an additional (untested in this study) impact on the oyster’s grounding. Despite having been displaced at a water velocity of 0.26 m s^{-1}, three oysters did not close their valves until the water had reached higher velocities of 0.31, 0.36 and 0.39 m s^{-1}, which suggests water velocity has more influence than physical movement on the oyster’s stimulus to close its valves.

Restoration that requires re-laying must consider hydrodynamic influence, as displacement of oysters would be detrimental to any project. Any choice of restoration site in the Solent must therefore be carefully considered alongside the choice of oyster for restoration; if the technique is laying oysters freely onto the seabed, they must be at a mass that would be able to withstand the local hydrodynamics and resist displacement.

These experiments showed that the threshold for oyster displacement at all sizes and weights were not particularly high, and often much lower than reported velocities in the natural Solent benthic system (0.59 m s^{-1} at 0.15 m from the seabed, Quaresma *et al.*, 2007), suggesting that restoration projects laying juvenile oysters (< 65 g) should ensure the oysters are either contained (within a weighted bag or cage) or relaid within areas with natural water velocities < 0.22 m s^{-1}, while adult oysters (> 65 g) could be more securely relaid onto the seabed in an area with water velocities < 0.26 m s^{-1}. However, all experiments were conducted in a bare flume without any additional substrate, and the addition of sand or mud might have created friction to counter the potential displacement of the oysters. The Lab Carousel flume used for experiments provided a consistent flow of water, allowing a detailed analysis of the effect of specific velocities and stress on individuals and beds as a whole. Irregular fluctuations in water velocities such as wave gusts experienced in the natural environment are likely to exacerbate the observed results (Fredriksson *et al.*, 2010), and further studies would benefit from recording the effect of water velocity on individuals in situ to complement the above study.

2.4.2 Spat on shell displacement

The displacement of spat-on-shell depended on the initial starting formation of the shell; the most secure shell formation that did not experience any displacement at the highest water velocities was the deepest one with multiple spat-on-shell layered to a depth of 0.15 m. The naturally 3-dimensional spat-on-shell created an interlocking system due to the irregular shapes, and the layering effect created a more robust structure less likely to move with a high water velocity environment than a single (0.01 m depth) or double layer (0.05 m depth) of the same material. Layering spat-on-shell to a substantial thickness would be effective for retaining oysters at/in a high energy site/environment, but further analysis as to how the physiology of oysters might be affected within the lower layers should be studied before this should be applied for restoration purposes.

2.4.3 Oyster (Ostrea edulis) bed v limpet (Crepidula fornicata) bed

The differences in hydrodynamics associated with an *O. edulis* bed and a *C. fornicata* bed were immediately clear from a comparison of how the species altered the water velocity above the beds (P2) and after the beds (P3) (Figures 2.28 and 2.29). At the higher experimental water velocities (0.31, 0.42 and 0.52 m s^{-1}), *O. edulis* slowed the water velocity immediately above the bed to almost half of the depth averaged speed, while the same velocities were not altered by the *C. fornicata* bed that allowed the water to flow freely over the surface of their smoother shells. This could have implications for recruitment; a slower water velocity immediately above an oyster bed might allow larvae more control over settlement on adult oyster shell without the hindrance of stronger currents (Korringa 1940). The absence of a deceleration in current above the *C. fornicata* bed could flush any potential settling *O. edulis* larvae, while facilitating *C. fornicata* larvae to settle. *C. fornicata* currently dominates the Solent seabed with abundances reported at 84 to 306 limpets m^{-2}, while only 2 oysters were found in the same entire study (Helmer *et al.*, 2019). Although the limpets ultimately provide a large proportion of hard substrate and potential settling ground in the Solent, the difference in hydrodynamics above the *C. fornicata* bed might negatively impact the settlement of native oyster larvae. The slowing of water immediately above the oyster bed (< 0.02 m) due to the rugosity of the oyster bed meant the volume of water had to squeeze through a narrower channel (0.02 – 0.11 m (depth)), which increased the water velocity in this channel. The shear stress profiles showed a marked contrast between the two beds; the oyster bed resulted in higher stresses (three times in magnitude) by comparison to the stresses associated with the limpet bed (Figures 2.30 and 2.31). Both beds created a turbulent environment, accentuated by peaks in the shear stress along each vertical profile that suggested eddies were being formed, where pockets of water spiral and flow against the current. The highest experimental flume speeds (0.42 and 0.52 m s^{-1}) resulted in the highest shear stress above the beds of both species, as expected. At 0.5 m after the *C. fornicata* bed (P3), only at the highest speed experiment (0.42 m s^{-1}) did the flow not recover to a more vertically uniform stress state. Although the oysters created a much higher shear stress above (P2) and after (P3) the bed, this profile was more uniform throughout and the stress appeared to be more evenly distributed with depth.

The Reynolds numbers (Re) and drag coefficient (C_D) in association with an oyster bed were higher than those associated with a limpet bed, which represents a more turbulent water body. Turbulence is present in constant water flow as a result of seabed topography and the eddy formation around this topography. Whitman and Reidenbach (2012) reported a higher drag coefficient (C_D) above a *Crassostrea virginica* bed (C_D = 0.019) than that found above whelk shell restoration (C_D = 0.011), oyster shell restoration (C_D = 0.009), and mud (C_D = 0.004). Their theory was that reef height was positively correlated with C_D. This study corroborates their finding and suggests shell orientation plays an important role, as no difference in C_D was seen between the subjects of this study (*O. edulis* and *Crepidula fornicata*), both of which tend to lie closer and flatter on the seabed than *Crassostrea virginica*. One factor that has not been accounted for throughout this study is the impact of wave movement that could apply a more significant force on larvae at the BBL. Moments of random high velocity water flow and unpredictable water movements of high momentum could be detrimental to oyster larvae settlement by allowing no pre-warning of force onto the substrate and attachment phase (Whitman and Reidenbach, 2012).

Use of the flume allowed for a sensitive analysis of the hydrodynamic environment at the seabed using established physics that can be scaled up and applied to large scale restoration efforts. However, random events *in situ* such as storm surges and wave activity at the seabed, which would have a significant effect on the hydrodynamic environment, could not be accounted for in the flume experiments. Similarly, sediment, which is known to play a role in the hydrodynamics at the seabed, was not added to the flume and any friction that may have influenced the physical displacement of the oysters was therefore not accounted for in the analysis. The flume experiments in this study highlight the need for further investigation into the effect of *C. fornicata* shells on the hydrodynamic environment, and the implications for the development of *O. edulis* beds for restoration.

2.4.4 Larvae dispersal

These flume experiments were complemented with the output from an individual-based model (IBM) partnered with a hydrodynamics model of the Solent. The IBM was programmed to predict the horizontal and vertical trajectory of larvae around the Solent and density likelihood of settlement areas given starting release ‘zones’. The six chosen release zones were bays or rivers leading into the Solent, five of which were active or proposed sites for *O. edulis* restoration in association with Blue Marine Foundation (BMF) at the time of study. The release sites in the Western channel (Lymington, Newtown Creek and the Beaulieu River) were highly dispersive (Figures 2.33, 2.34 and 2.35), while those along the central and eastern channel (Saxon Wharf, Hamble, and LRBAR) were more retentive (Figures 2.36, 2.37 and 2.38). However, model boundaries may have restricted the visual representation of where the larvae were likely to settle given these starting locations (Siegel *et al.*, 2003). Both a dispersive and retentive environment could be beneficial for restoration purposes. A dispersive restoration site may benefit a restoration project aiming to naturally repopulate a wider area and create a higher number of settlement locations. A retentive environment would benefit an oyster farmer or sanctuary area where the main purpose is sustaining a population more locally. Choosing a starting larval pump location that will direct the natural dispersal of larvae to an appropriate settling location could dramatically improve chances for the self-sustainability of the population. With more information of larval behaviour and consequent improvements to hydrodynamic models, restoration projects can re-lay adults in locations strategically chosen for their larval dispersion trajectory to a (natural or prepared) settlement location with appropriate conditions and substrate. This has been attempted already in Wales: Smyth *et al.* (2018) concluded that buying expensive cultch and re-laying would not stimulate restoration if hydrodynamics and habitat type had not been considered. The modelled distribution of settlement sites in this study matched historical beds, demonstrating the importance in historical trends for consideration in choice of restoration sites. Hydrodynamic observations and habitat mapping must therefore be considered when considering sites for successful restoration. The number of larvae is also proportionate to the number of broodstock (Korringa, 1947), and therefore an area that re-lays a high number of adult oysters will likely produce a significant amount of larvae.

2.4.5 Use of models to inform restoration

Passive drift models tend to over-predict the distance of propagule travel during pelagic phase (Shanks, 2009), but the model used for this study predicted larval dispersal and settlement using observations from laboratory studies of *O. edulis* larval behaviour such as the bouncing technique (Cragg and Gruffydd, 1975), and existing data of Solent hydrodynamics (Herbert *et al.*, 2012b; Willis *et al.*, 2019). Tracking individual larvae *in situ* poses a challenge in large volumes of water such as the Solent, and models are frequently used to enable further understanding of larval trajectories. Several factors known to influence larvae development and settlement such as water quality, temperature, salinity (Waller, 1981), storm events (Shanks, 2009), nutrition, and substrate biofilms (Rodriguez-Perez *et al.*, 2019) were not included in the model used for this study. These factors would be a welcome addition to any further *O. edulis* larvae dispersal models.

2.4.6 Conclusion

Site selection is incredibly important for the future of the restoration project as oysters will be impacted by both the immediate small-scale, and large-scale hydrodynamic environment. *Ostrea edulis* adults, juveniles and cultch are sensitive to water velocity, and locations and density of larvae settlement is subject to the dispersiveness of their release zone. Further analysis of oyster valve movement and the long-term effect on feeding regimes would benefit this research.

Chapter 3 Physiological performance of *O. edulis* from three sub-populations within the UK: Implications for choice of broodstock origin

3.1 Introduction

3.1.1 The translocation of oysters

Due to the extent of loss of *Ostrea edulis* beds in the UK and Europe, restoration requires the re-laying of adult oysters both to repopulate the water column with larvae, and to replace the lost shell substrate, both essential for recruitment. Increased understanding for the valuable benefits of oysters to an ecosystem has meant grants and funding to support projects have become less of a limitation to restoration than finding appropriate broodstock for re-laying. The ability to withstand being out of water for several days and successful acclimation to new environments has meant oysters have been extensively moved around the world for both aquaculture and seabed culture (Bromley *et al.*, 2016a). One unfortunate consequence of this oyster translocation has been the facilitation of a rapid spread of haplosporidian parasite *Bonamia ostreae* that is the main constraint for *Ostrea* spp. cultivation across many parts of Europe (Grizel *et al.*, 1987; Culloty *et al.*, 2001; Culloty and Mulcahy, 2007; da Silva *et al.*, 2008). As explained in Chapter 1, *B. ostreae* attacks the immune system of oysters by targeting the haemocytes where it can multiply and proliferate, and subsequently can develop into the disease bonamiosis, which can result in high mortality rates of > 80% (Robert *et al.*, 1991; da Silva *et al.*, 2005; Laing *et al.*, 2005; Lallias *et al.*, 2010).

To control the spread of *B. ostreae*, Europe implemented strict biosecurity measures (EC Council Directive 2006/88/EC) that restrict the translocation of *O. edulis* stock. However, while addressing the challenge of *Bonamia*, this biosecurity practice reduces the already limited availability of appropriate *O. edulis* for restoration broodstock purposes. With only three *O. edulis* hatcheries registered in the UK (Adamson *et al.*, 2018), and only one of those (Morecombe Bay) offering *O. edulis* spat, the majority of both restaurant and restoration demand in the UK is being met by a handful of wild fisheries: Thames Estuary, the Solent, the River Fal, the west coast of Scotland and Lough Foyle (OSPAR, 2015).

3.1.2 The effect of broodstock origin on physiological performance

Like other molluscs, *O. edulis* is a cold blooded euryhaline ecto-thermo-conformer, with no body temperature control but with the ability to rapidly adjust to changing temperature and salinity through physiological adaptations at a cellular level (Rodhouse, 1978). Choice of settlement location is crucial for long term survival of the adult oyster, as metamorphosed spat remain sessile and are subject to their immediate environment. It is likely that oyster feeding patterns will be location- and environment-specific, and therefore influenced by oyster origin. Although *O. edulis* have demonstrated high plasticity with successful settlement in a range of habitats from Norway to Morocco (Bromley *et al.*, 2016a), their physiological profile has been shown to be highly variable depending on location and environment (see 1.6.5), which suggests that choice of broodstock origin may influence the development of the oyster population. In their comparison of 19 populations of *O. edulis* along the Atlantic and Mediterranean coastlines, Saavedra *et al.* (1995) established that although these groups of oysters likely once possessed similar genetic profiles, they can now be recognised as unique or cryptic species owing to the history of their geographical environments. Geographically segregated physiological differences could be hereditary, or a result of their local environment and likely a combination of both (Ahmed, 1975; Levinton and Lassen, 1978).

Physiological metrics associated with the aforementioned concerns for restoration include oyster clearance rate, respiration rate, growth rate, body condition, haemocyte profile, and parasite presence. Newell, Johson and Kofoed (1977) observed that clearance rate was the most influential physiological process on body condition (scope for growth) and reproduction. Respiration is essentially energy expenditure (Newell *et al.*, 1977), and therefore it could be argued that increased respiration rate indicates a reduction in physiological efficiency. Thermal acclimation stress has been shown to affect the metabolic rate and clearance rate of *O. edulis* (Newell *et al.*, 1977; Haure *et al.*, 1998; Eymann *et al.*, 2020); Respiration and clearance rates have been shown to increase with temperature. Haure *et al.* (1998) found that clearance rate increased with temperatures until it peaked at 17 °C before slowly declining in activity up to 30 °C. The same study also demonstrated that less energy (10 J L^{-1}) was required to sustain an oyster with a 1 g dry weight at higher temperature (30 °C) than at lower temperature (12-13 J L^{-1} energy required between 10 °C and 17 °C) (Haure *et al.*, 1998), which explains why oysters tend to grow faster in warmer conditions (Davis and Calabrese, 1969; Mann, 1979). Standard metabolic rate (SMR) can be inferred from respiration of oysters and this usually gives an insight into the energy required for physiological mechanisms such as reproduction & somatic tissue growth (Lemasson *et al.*, 2018). Growth and reproduction can occur if energy gain (ie. food intake) outcompetes energy losses (such as metabolism or respiration) (Warren and Davis, 1967; Newell *et al.*, 1977). Increased metabolic rate is expensive for the individual owing to increased use of energy, and therefore the ability to control this even in warmer waters could demonstrate higher physiological efficiency. Body condition is measured by weight ratio of soft tissues to shell tissue (Lucas and Beninger, 1985). This index provides much information into the physiological status of an individual; a low ratio reveals a high soft tissue content and a thinner shell, which suggests an abundance of energy is being invested in somatic growth, whereas a high ratio reveals low soft tissue content and a heavier shell which might suggest defence against predation or lack of available food for somatic growth. In a fishery context, faster growing soft tissue is more desired for production.

Unlike vertebrates, and many other invertebrates, bivalve molluscs rely less upon their haemolymph for the transport of oxygen to their tissues and more on the associated immune properties, which makes haemolymph an appropriate target for analysing the oyster immune system in response to parasite *B. ostreae* (Jones, 1983). Oyster haemocytes can be divided into two cell types (granulocytes and agranular hyalinocytes) and counted in three sub-populations: granulocytes (GR) with visible cytoplasmic granules (~10 µm), and hyalinocytes, both small (~5 µm) (SH) and large (~15 µm) (LH) lacking granules (Auffret, 1989; Chagot *et al.*, 1992; Hauton *et al.*, 1998; Xue *et al.*, 2000; da Silva *et al.*, 2008; Morga *et al.*, 2009; Comesaña *et al.*, 2012). Haemocytes circulate in an oyster via an open system oxygenated in the gills and mantle before returning to the heart via the nephridium (Jones, 1983). Oyster haemocytes have been shown to have a half-life of 22 days, and are continuously produced in high numbers (at least 10^6 cells per 10 g) (Jemaá *et al.*, 2014), demonstrating the efficiency of cell regeneration amongst this taxa (Bodnar, 2009). Parasite *B. ostreae* attacks the haemocytes of *O. edulis* and has been shown to control host immune cellular functions in order to persist within the infected individual (Mauel, 1984; Cheng, 1987). Symptoms reported in *Bonamia*-infected *Ostrea* spp. include black, emarginated & frayed gills (Dinamani *et al.*, 1987; Kroeck and Montes, 2005), and a change in circulating haemocyte ratios; Cochennec-Laureau *et al.* (2003a) found a higher number of large agranular haemocytes (large hyalinocytes) in infected oysters, and lower number of granulocytes in *Bonamia*-susceptible oysters; data supported by da Silva *et al.* (2008) and Comesaña *et al.* (2012). Further evidence of the negative effect of *B. ostreae* on *Ostrea* spp. include stunted oyster growth and body condition, and a reduced reproductive potential (Robert *et al.*, 1991; Montes *et al.*, 2003). If the disease bonamiosis has developed to a later stage, oysters will exhibit shell gaping, which is often a prelude to death.

3.1.3 Metabolic profiling as a method to understand physiology

The metabolome is the collection of highly complex molecular compounds (<1000 Da) called metabolites that control metabolism in all biological cells (Tikunov *et al.*, 2010). Profiling the metabolome offers insight into the functioning of cells and can therefore offer further investigation into the physiological functionality of an individual (Tikunov *et al.*, 2010; Cao and Wang, 2016). For example, a metabolome profile can help to identify stress induced feeding patterns that could lead to stunted reproduction or somatic growth (Lemasson *et al.*, 2018). Viant, Rosenblum and Tjeerdema (2003) were able to differentiate between the metabolomes of healthy, growth stunted and diseased red abalone, *Haliotis rufescens*, which suggests specific metabolome markers could be developed to assess the physiological fitness of individuals and measure the effects of environmental stressors on a population. Metabolomics has already shown promise in aiding the aquaculture industry with the use of biomarkers that can identify problems in cultivation (hatchery production, nutrition, and disease), its progress aided by the fact that most metabolites are not species specific (Alfaro and Young, 2018). Analysis of the metabolome of Pacific oyster, *Crassostrea gigas*, revealed an affiliation between glycogen content and energy production; oysters with a high glycogen content have a better coping mechanism with stress (Li *et al.*, 2017). Often used for ecotoxicology studies, metabolomics is a relatively new field of science for understanding the connection between cellular activity and physiological functionality (Cao and Wang, 2016), and only recently has the metabolome of *O. edulis* begun to be explored in relation to differences seen in physiological performance (Eymann *et al.*, 2020).

3.1.4 The concept of 'physiological races'

Different *O. edulis* populations can be thought of as 'physiological races' (Loosanoff and Nomejko, 1951; Korringa, 1956; Wilkins and Mathers, 1973; Ahmed, 1975), owing to the highly varied response to environmental pressures such as disease seen in populations with only a small geographical divide (Culloty *et al.*, 2004; Laing *et al.*, 2005). Laing *et al.* (2005) suggested that due to their adaptation to faster growth, the Solent oysters were in a lower physiological condition than those in Scotland. In a smaller scale comparison, Sawusdee (2015) found the condition index of oysters from Poole Harbour to be significantly higher than those from Solent and Chichester Harbour populations - a distance of only 50 miles. With at least five active large-scale restoration projects currently running in the UK (see Appendix A, Table A2), a detailed analysis into the effect of oyster origin on physiological performance would offer constructive wisdom for application in choice of restoration broodstock.

Aim

The aim of this research was to determine the importance of broodstock origin on physiological performance and establish if the oyster metabolome could discriminate between populations. In order to retrieve the most information on the physiological diversity of *O. edulis* populations around the UK, oysters from three wild fisheries were considered for comparison: *Bonamia*-free Loch Ryan (Scotland), *Bonamia*-exposed Solent (England) and *Bonamia*-exposed Galway (Ireland). Seasonal effects were considered owing to the effect of temperature on physiological performance.

Objectives

1. To compare the physiological performance (clearance and respiration rate) of *O. edulis* from Ireland, Scotland and England throughout the four seasons, first at their local conditions and then at aquarium conditions.
2. To compare the haemolymph composition and general body condition of *O. edulis* from Ireland, Scotland and England throughout the four seasons, first at their local conditions and then once acclimated to aquarium conditions.
3. To quantify the burden of parasite *B. ostreae* in oysters using an OIE-recognised identification method with primers as developed by Ramilo *et al.* (2013).
4. To establish if metabolomics gives us the power to discriminate between oyster populations.

3.2 Methods

3.2.1 Husbandry

Oysters

Oysters were acquired quarterly, in batches of 30-50 individuals from Ireland (The RedBank Food Company, Galway Bay), Scotland (Rossmore Oysters, Loch Ryan) and England (Portsmouth & Langstone harbours in the Solent) (dates in Table 3.1).

Table 3.1. Dates of oyster arrival to the National Oceanography Centre

	Loch Ryan		*Galway*		*Solent*	
Winter	Batch F	28/02/2018	Batch E	15/01/2018	Batch D	21/11/2017
Spring	Batch J	08/05/2018	Batch G	09/04/2018	Batch H	18/04/2018
Summer	Batch K	06/09/2018	Batch L	11/09/2018	Batch M	24/09/2018
Autumn	Batch N	05/11/2018	Batch O	15/11/2018	Batch P	14/11/2018

Oysters were collected using oyster dredges and sent to the National Oceanography Centre Southampton (NOCS) via post in either wooden or polystyrene crates and were out of water for < 48 h. Individuals were then gently scrubbed to remove unwanted epifauna and tagged with numbered fragments of elastic band glued onto their shell. Individuals from Galway and Loch Ryan were kept at a density of 40-100 oysters m^{-2} in tanks of sand filtered seawater from the Solent, maintained at their local temperature and salinity (which remained constant at 33) at the time of harvest (Table 3.2). Oysters from the Solent were kept in an outside tank with a flow through system with water directly from the Southampton Water. All oysters were left to acclimate to their new environment for at least 3 days before experiments began. Oyster tanks were cleaned every three days. Oyster dimensions (height (mm), length (mm) and wet weight (g)) were taken in accordance with (Galtsoff, 1964) (see Figure 3.1).

Table 3.2. Seawater temperature of oyster holding tanks. Oysters were first kept at the same temperature from which they came (Local), and were then brought up to aquarium conditions (Aqua) post initial physiological experiments. In the spring, oysters were only kept for initial physiology experiments at local conditions.

		Loch Ryan		***Galway***		***Solent***	
Winter	***Local***	Batch F	8.7 °C ± 0.15	Batch E	10.9 °C ± 0.2	Batch D	7.4 °C ± 0.17
	Aqua		15.8 °C ± 0.28		14.4 °C ± 0.72		14.5 °C ± 0.29
Spring	***Local***	Batch J	10.6 °C ± 0.07	Batch G	11.4 °C ± 0.15	Batch H	11.6 °C ± 0.41
	Aqua		-		-		-
Summer	***Local***	Batch K	18.9 °C ± 0.92	Batch L	19.7 °C ± 0.27	Batch M	17.7 °C ± 0.05
	Aqua		17.0 °C ± 0.14		16.7 °C ± 0.11		17.1 °C ± 0.11
Autumn	***Local***	Batch N	10.7 °C ± 0.32	Batch O	11.2 °C ± 0.05	Batch P	12.4 °C ± 0.05
	Aqua		17.6 °C ± 0.48		16.9 °C ± 0.00		17.1 °C ± 0.16

Feeding

Oysters were fed alternate days with a mixed diet of algae; *Isochrysis galbana*, *Tetraselmis suecca* and *Phaeodactylum tricornutum* were added to each tank at a concentration of 40 000 cells ml^{-1} as suggested by Walne (1970, 1972) for optimum growth. During experiments, oysters were fed 40 000 cells ml^{-1} each day to reduce any starvation effect on clearance rates (sensu Riisgård *et al.*, 2003, 2006; Nielsen *et al.*, 2017), but not fed 24 h before each experiment to reduce the possibility of confounding results with faeces or free algae particles (Wiegert, 1968; Rodhouse, 1978; Lemasson *et al.*, 2018).

3.2.2 Population scale study

The respiration rate and clearance rate of six oysters from each origin (Loch Ryan, Galway and the Solent) were observed at their local conditions and then sacrificed for further analysis of the haemolymph and soft tissue. The right valve was disconnected from each oyster using an oyster knife, surgical blade and safety glove, accessing through the hinge and taking care not to damage the soft tissues (Howard and Smith, 1983). The remaining live oysters were acclimated to the NOCS aquarium conditions over the course of one month with daily water temperature changes of no more than 1 °C. Once acclimated, respiration and clearance rate of a further six oysters were measured in the aquarium conditions.

Clearance rate

Six 1 L buckets were filled with 800ml of seawater and placed into a water bath at the required temperature. Each bucket containing a magnetic flea was placed above a magnetic stirrer to ensure continuous circulation of seawater. Individual oysters were placed on a platform within each bucket and left for three hours to acclimate (Sawusdee, 2015). A known concentration of algae (c. 35 000 cells ml^{-1} *Isochrysis galbana* and c. 35 000 cells ml^{-1} *Phaeodactylum tricornutum*) was then added to each bucket. Samples of water (20-25 ml) were collected from each bucket at Time (T) = 0, 60 and 120 minutes. Algal concentration was acquired by calculating fluorescence using a Cytosense flow cytometer (CytoBuoy) and analysed using Cytoclus4 software (Dubelaar and Gerritzen, 2000). Clearance rates were calculated using Coughlan's (1969) equation:

$$CR = V\ x\ \frac{\ln C_1 - \ln C_2}{T} \tag{6}$$

CR	Clearance rate (L h^{-1} g AFDW^{-1})	V	Water volume in bucket (L)
C_1	Initial algae concentration (algal cells ml^{-1})	T	Time (h)
C_2	Final algae concentration (algal cells ml^{-1})		

Standard metabolic rate inferred from respiration rate

Respiration rate was measured as a proxy for standard metabolic rate (Lemasson *et al.*, 2018). Individual oysters were placed into 1 L sealed air-tight chambers and submerged into a fresh water bath to maintain the desired temperature. Each was attached to a peristaltic pump pumping seawater through the container at a continual rate of 3 L h^{-1}. Salinity and temperature were measured at the start and end of each experiment, and air pressure data were acquired from Southampton Weather forecast (www.southamptonweather.co.uk), which is updated every 5 seconds. The oysters were left to acclimate within their chambers for 3 hours before the start of the experiment (Sawusdee, 2015). Oxygen concentration (mg L^{-1}) in each chamber was then measured for another two hours using a Fibox 3TM fibre-optic oxygen meter (PreSens; Germany). One control chamber containing no oyster was monitored to control for bacterial respiration. Oxygen consumption was analysed using the calculation from Widdows and Johnson (1988):

$$RR = (ct_0 - ct_1 - C)\ x\ \frac{V_c - V_a}{1000}\ x\ \frac{60}{(t_1 - t_0)} \tag{7}$$

RR	Respiration rate (ml O_2 h^{-1} g $AFDW^{-1}$)	60	time between readings (minutes)
ct_0	Oxygen saturation at the start (mg L^{-1})	ct_1	Oxygen saturation at the end (mg L^{-1})
V_c	Volume of chamber (ml)	V_a	Volume of animal (ml)
t_1	Starting time (minutes)	t_0	End time (minutes)
C	difference in control oxygen saturation during the experiment		

All clearance rate and respiration rate calculations were corrected to the ash-free dry weight (AFDW) of individual oyster tissue. This was obtained by drying oyster tissue in an 80 ˚C oven for 48 h (Mo and Neilson, 1994), then ashing it in a 500 ˚C furnace for 5 hours (Rodhouse, 1978), weighing it at each interval.

Assessment of circulating haemocyte population

Haemolymph was extracted from the adductor muscle sinus using a 1 ml disposable syringe with Luer-Lok™ tips. A 25 µl sample of haemolymph was diluted to 50% with a sterile marine saline (Schlieper, 1972) to prevent clotting, and then transferred onto a Neubauer haemocytometer and left for 10 minutes for the cells to settle. Using Nomarski Contrast Interference Optics on an Olympus microscope (400x magnification), haemocytes were then divided into two cell types (granulocytes and hyalinocytes), and counted in three sub-populations: granulocytes, small hyalinocytes, and large hyalinocytes. The total haemocyte count (THC) and count by differentiation (DHC) were used for analysis.

Body condition

Post experiment, the condition of each oyster was calculated using a body index from dried body tissue and shell (Lucas and Beninger, 1985):

$$Condition\ index\ (CI) = \frac{Dry\ tissue\ weight\ (g)\ x\ 100}{Dry\ shell\ weight\ (g)} \tag{8}$$

Tissue samples

Tissue samples were then taken from the hepatopancreas (HP) and gill (G) regions (Figure 3.1), flash frozen in liquid nitrogen and placed in both -20 ˚C and -80 ˚C for later deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) analysis respectively.

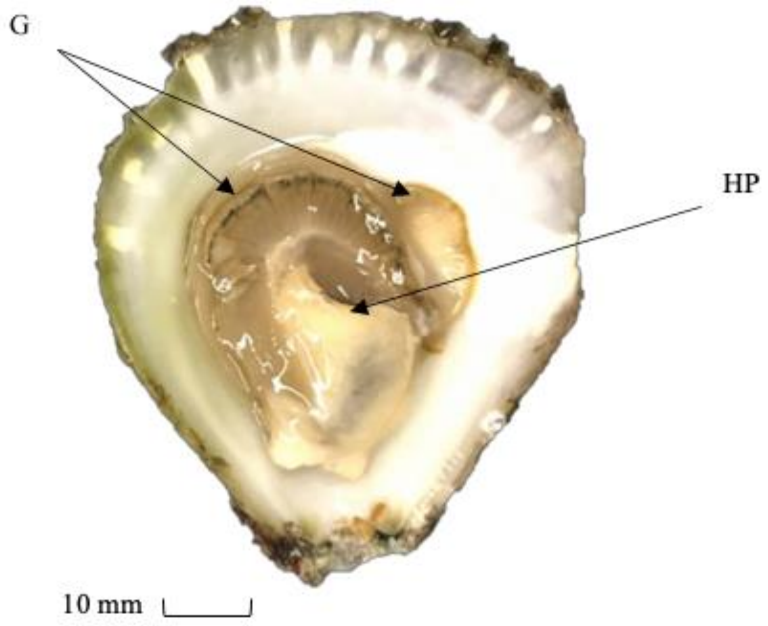


Figure 3.1. Oyster with location of tissues taken for analysis: hepatopancreas (HP) and gill (G) tissue taken for DNA/RNA analysis.

Bonamia ostreae burden

Bonamia ostreae infection was identified with the use of polymerase chain reaction (PCR) followed by gel electrophoresis. DNA was extracted from gill tissues using QIAGEN™ DNeasy Blood and Tissue Kit, with the knowledge that gill tissues are rich in haemocytes (Jemaá *et al.*, 2014). Where present, parasite DNA was then amplified using PCR with 30 cycles heating to 95 ºC (1 minute), cooling to 59 ºC (1 min), and warming to 72 ºC (1 min). The primers used to target *B. ostreae* were *BOSTRE* f: TTA CGT CCC TGC CCT TTG TA, *BOSTRE* r: TCG CGG TTG AAT TTT ATC GT, as developed by Ramilo *et al.* (2013); the forward primer targeting the 18S rDNA gene, and reverse primer targeting the ITS1 region of the species. Amplified DNA fragments from *B. ostreae* were size fractionated by gel electrophoresis and identified after staining with an intercalating DNA binding dye ethidium bromide before visualisation on a transilluminator. Positive and negative controls were used for each gel, and presence of *B. ostreae* was identified through amplicon size (208 bp) (Ramilo *et al.*, 2013). Only the oysters from the local experiments were tested for the presence of *B. ostreae* to ensure only *B. ostreae* infections acquired from the wild (and not the NOCS aquarium) were represented.

Metabolic profiling

Hepatopancreas tissue samples flash frozen and kept at -80 °C were packaged in dry ice and sent to the McCullagh Metabolomics Laboratory for untargeted Metabolomics, Department of Chemistry at the University of Oxford for analysis.

Each sample was analysed using up to three separate Liquid Chromatography–Mass Spectrometry (LC-MS/MS) methods using two LC systems (Thermo Scientific ICS-5000+ ion chromatography system and a Dionex Ultimate 3000) coupled directly to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI II electrospray ionisation source (Thermo Fisher Scientific, San Jose, CA, USA) (Walsby-Tickle *et al.*, 2020). Each of the three methods (ion exchange (IC-MS/MS), C18-reverse phase chromatography (C18-MS/MS), and derivatised C18-reverse phase chromatography (dC18-MS/MS)) used a 5 µL partial loop injection of each sample. The ion exchange (IC-MS/MS) method performed chromatographic separation of the molecules using a Dionex IonPac AAS11-HC column (2 x 250 mm^2, 4 µm; Dionex, Sunnyvale, CA, USA) with an inflow rate of 0.25 mL min^{-1}. The C18-MS/MS method used a CORTECS T3 C18 column (2.1 x 100 mm^2, 1.6 µm; Waters, Milford, MA, USA) with a flow rate of 0.3 mL min^{-1} and total run time of 18 minutes. The dC18-MS/MS method used a Waters AccQ-Tag column (2.1x100mm) with a flow rate of 0.5mL/min and total run time of 9.5 minutes. All raw data was processed using Progenesis QI (Waters, Elstree, UK), and all ions with a coefficient of variation (CoV) > 30% were removed. This was to ensure only the most discrete peaks with a single point were considered for analysis while the rest were rejected as noise (Walsby-Tickle *et al.*, 2020).

IC-MS/MS analysis: Collected hydrophilic compounds, total features analysis = 432, significant features = 21, false discovery rate = 0.016.

C18-MS/MS analysis: Collected hydrophobic compounds, total number of features=4402, significantly different features = 2, false discovery rate = zero.

dC18-MS/MS analysis: Analysed 1° and 2° amines, total number of features = 7407, significant features = 4, false discovery rate = 0.003.

3.2.3 Statistical analysis

All statistical analyses were conducted in the R environment (R Core Team, 2018) with packages ggbiplot, lsmeans, ggplot2 and devtools (Wickham, 2009; Vu, 2011; Lenth, 2016; Wickham *et al.*, 2018). A linear model (LM) or generalised linear model (GLM) was fitted to the data where appropriate.

For the metabolomics analysis, four pieces of data were collected for each ion measured by each of the three methods (IC-MS/MS, C18-MS/MS and dC18-MS/MS): retention time, accurate mass, abundance and fragmentation patter. These were then clustered using a principal components analysis (PCA) to remove the dimensions and distil all the data while retaining the variability. Partial least squares discrimination analysis (PLS-DA) was then used to maximise the separation between groups of samples by removing the outlier measure (one of the four above) that least conformed to the model. This PLS-DA technique is achieved by creating new axes that maximise the distance between the means, while minimising the scatter of the groups. Permutation analysis was finally used to determine whether PLS-DA was an appropriate analysis technique to determine the validity of this analysis method for each of the three methods (IC-MS/MS, C18-MS/MS and dC18-MS/MS).

3.3 Results

Morphological measurements (shell height (mm) (H), length (mm) (L), and wet weight (g) (WW)), and physiological measurements (clearance rate, respiration rate and oyster condition) were acquired from six oysters from each origin (Loch Ryan, Galway and the Solent). Measurements were taken first at their local conditions (temperature and salinity), and then post 1-month acclimation to observe their adaptation to the NOCS aquarium. Acclimation data were not successfully collected in spring due to unavoidable faults in the aquarium, and therefore only data from winter, summer and autumn were considered at aquarium conditions.

3.3.1 Oyster physical metrics

There was significant interaction between origins in regression explaining height (mm) and wet weight (g) with a steeper relationship seen between Loch Ryan and the Solent (*P* = 0.029), and no detectable differences between Loch Ryan and Galway (*P* = 0.533) (Figure 3.2).

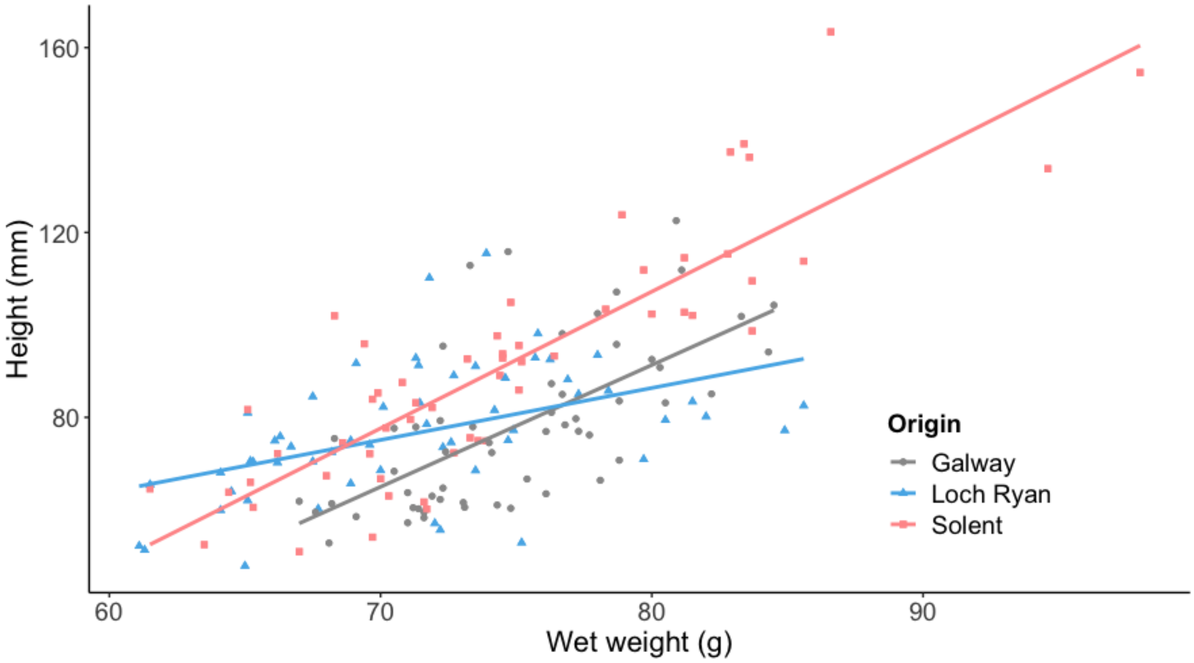


Figure 3.2. Size metrics (length (mm) vs wet weight (g)) of oysters (n = 56) from Galway (grey spheres), Loch Ryan (blue triangles) and The Solent (pink squares) used for comparative analysis.

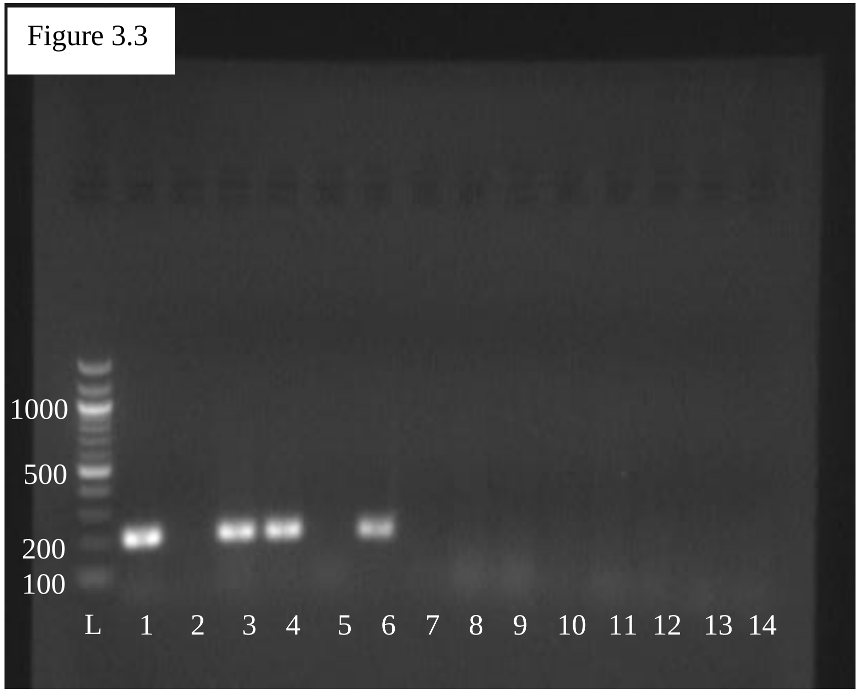
The difference in height and wet weight of the oysters used for analysis between oyster populations was not statistically significant (LM, $F_{2,69}$ = 1.84, *P* = 0.17, and LM, $F_{2,69}$ = 2.23, *P* = 0.12 respectively, n = 24) (Table 3.3). Oyster size was therefore not a complicating factor for consideration; the ash free dry weight (AFDW) of all oysters was 2.716 ± 8.57 g (mean ± sd). The width of the oysters was statistically different (LM, $F_{2,69} = 8.35$, $P = 0.001$), but this was not a concern as oyster shape was not considered an important factor for this study.

Table 3.3. Size metrics of Loch Ryan, Galway and Solent oysters used for comparison (n = 24): height, length and wet weight. Minimum (Min), maximum (Max), average (Mean), and standard deviation (sd).

	Height (mm)				Length (mm)				Wet weight (g)			
	Min	**Max**	**Mean**	**sd**	**Min**	**Max**	**Mean**	**Sd**	**Min**	**Max**	**Mean**	**sd**
Loch Ryan	61.1	85.6	71.46	5.56	52.8	83.8	69.90	7.35	51.3	115.5	78.70	13.93
Galway	58.6	84.5	74.48	5.32	66.6	90.0	76.58	6.97	52.8	122.6	80.94	18.60
Solent	63.3	98.0	74.37	7.37	61.9	96.4	75.22	7.54	11.9	163.4	88.15	28.84

3.3.2 Parasite burden

The DNA used to determine *B. ostreae* presence was extracted from oyster gill tissue that had been kept at -20 ºC after being retrieved from oysters within 10 days of their arrival to the NOCS. Presence of *B. ostreae* was determined using BOSTRE primers as designed by Ramilo *et al.* (2013) using amplicon size (208 bp, examples shown in Figure 3.3 and Figure 3.4). *B. ostreae* was not present in any of the Loch Ryan oysters but was found present in 25% of the Galway oysters (n = 24), and in 33.33% of the Solent oysters (n = 24). These samples were taken after the experiments at local conditions, and within 10 days of arrival to the NOCS. Acclimated oysters were not tested for the presence of *B. ostreae*.



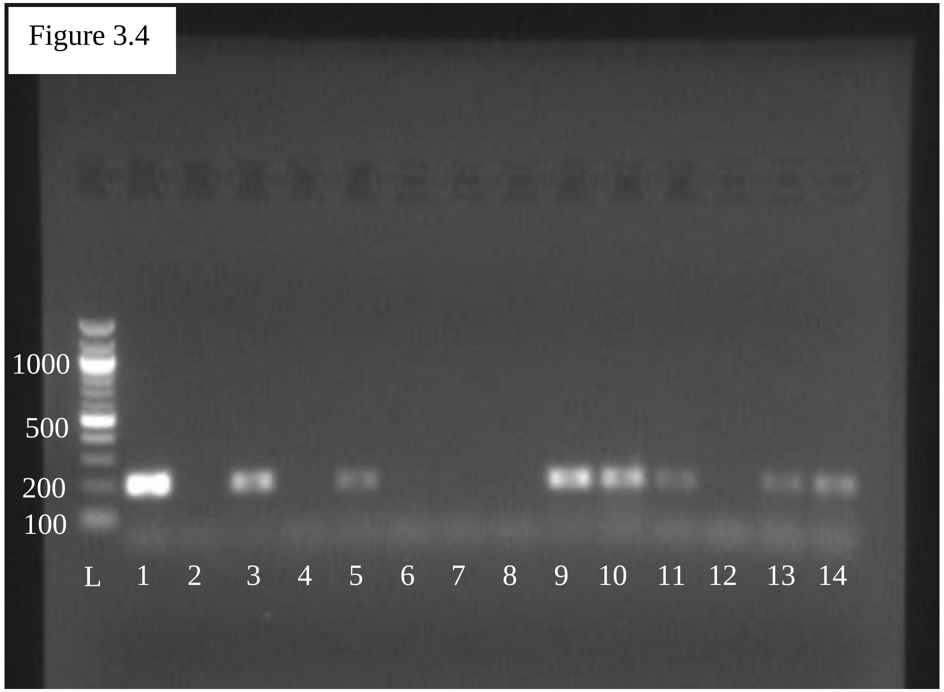


Figure 3.3 and Figure 3.4. Agarose gel electrophoresis after PCR assay performed to identify presence of *Bonamia ostreae* using primer pair BOSTRE-F/BOSTRE-R. In Figure 3.3, Lanes: (1) *B. ostreae* positive control, (2) negative control (MillieQ^{TM} ultrapure water), (3, 4 and 6) *Ostrea edulis* infected with *B. ostreae*, (5, 7-14) *O. edulis* not infected with *B. ostreae*, (L) 100 bp reference ladder. In Figure 3.4: Lanes: (1) *B. ostreae* positive control, (2) negative control (MillieQ^{TM} ultrapure water), (3, 5, 9-11, 13, 14) *O. edulis* infected with *B. ostreae*, (4, 6-8, 12) *O. edulis* not infected with *B. ostreae*, (L) 100 bp reference ladder.

3.3.3 Clearance rate

Oyster clearance rates at local conditions were between 0.02 and 4.68 L h^{-1} g $AFDW^{-1}$. The interaction between oyster origin and season had a statistically significant effect on oyster clearance rate (GLM, $F_{6,66}$ = 5.806, P = 8.11e-05) (Figure 3.5). Galway oysters demonstrated the highest clearance rate in autumn, although this season had the greatest variability. A significantly reduced clearance rate was seen in Solent oysters during the spring in comparison to other seasons. Infection with *B. ostreae* had no effect on clearance rate at local conditions (GLM, $F_{1,60}$ = 1.664, P = 0.202).

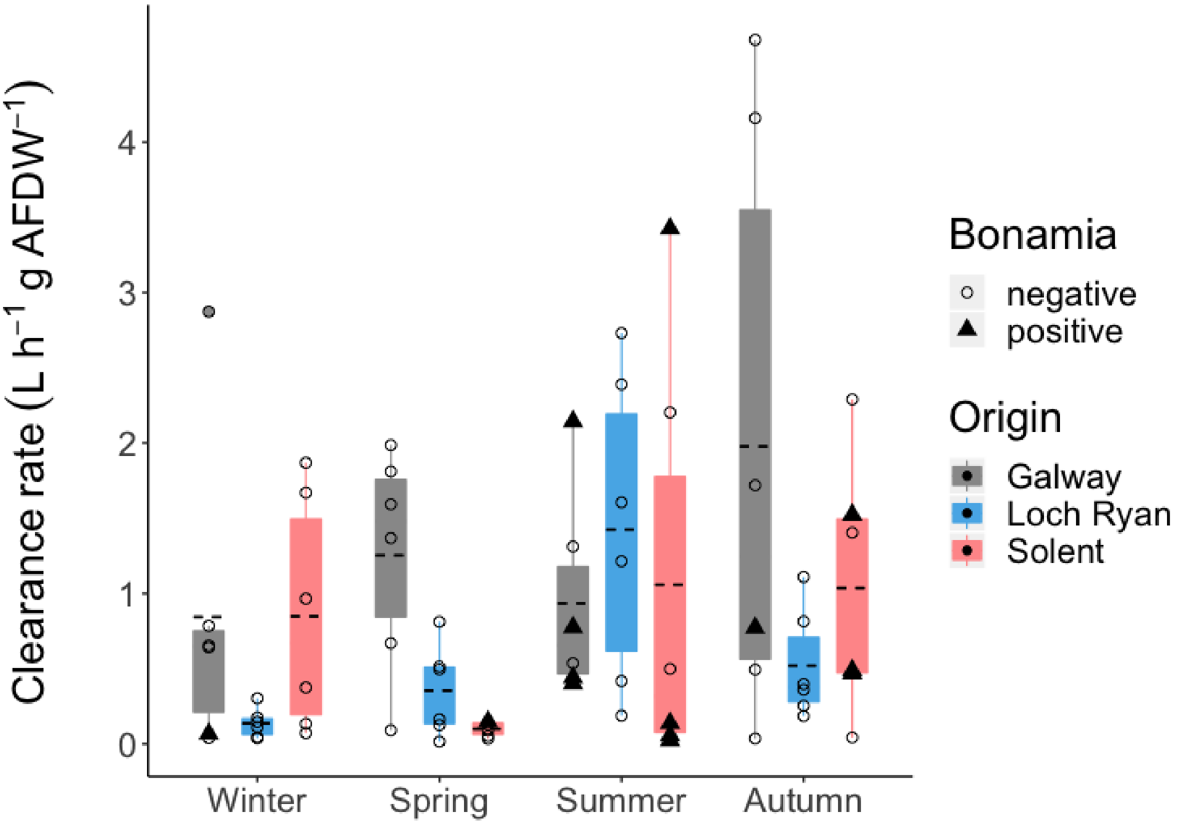


Figure 3.5. Clearance rate of oysters from Galway, Loch Ryan and the Solent at their local conditions throughout the four seasons. Individual oysters (n = 6 per experiment) are represented with circles (negative for *B. ostreae*) and triangles (positive for *B. ostreae*) and mean is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

3.3.4 Standard metabolic rate inferred from respiration rate

Oyster respiration rates at local conditions were between 0.002 and 2.50 mg O_2 h^{-1} g $AFDW^{-1}$ (Figure 3.6). The interaction between oyster origin and season explained statistically significant amounts of variation in respiration rate at local conditions (GLM, $F_{6,66}$ = 11.92, P = 9.182e-09). Infection with *B. ostreae* had no statistically significant effect on respiration of oysters at local conditions (GLM, $F_{1,60}$ = 0.06, P = 0.807).

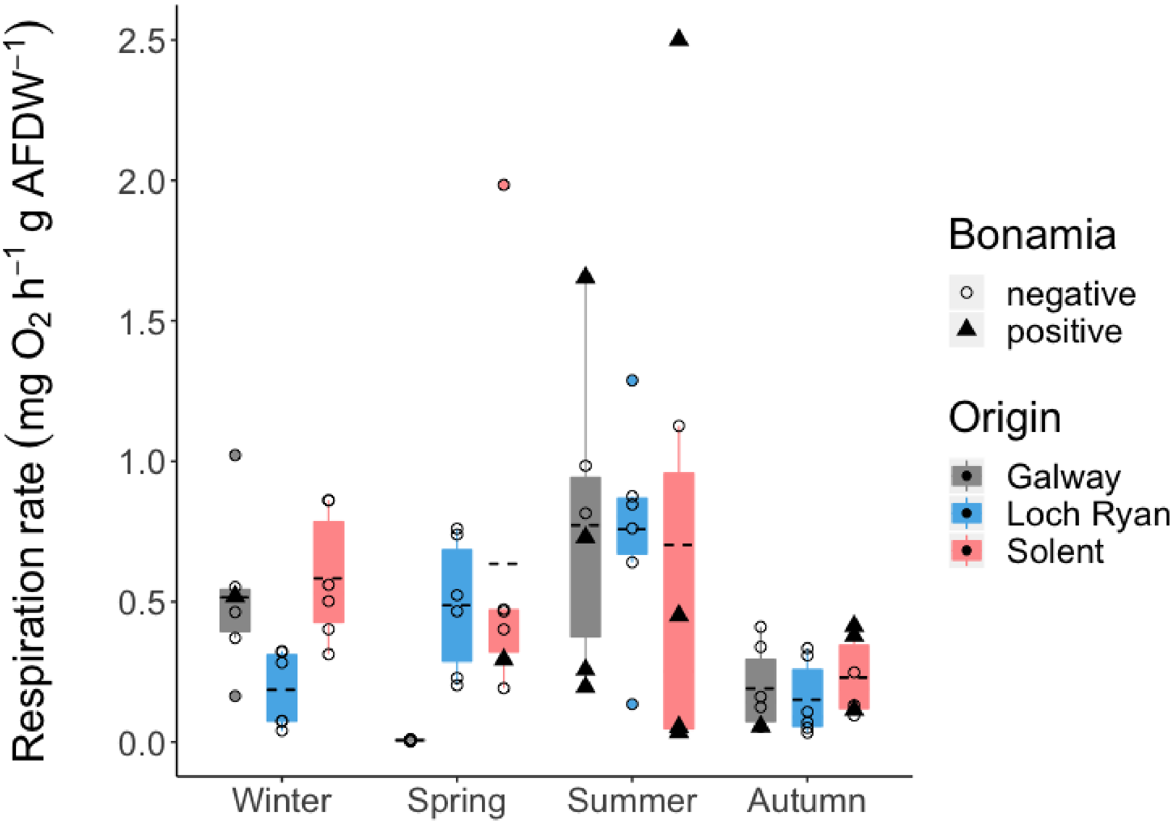


Figure 3.6. Respiration rate of oysters from Galway, Loch Ryan and the Solent at their local conditions throughout the four seasons. Individual oysters (n = 6) are represented with circles (negative for *B. ostreae*) and triangles (positive for *B. ostreae*) and mean is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

3.3.5 Assessment of circulating haemocyte population

The total haemocyte count (THC) and differential haemocyte count (DHC) of small hyalinocytes (SH), large hyalinocytes (LH) and granulocytes (G) were compared between oysters from each origin (Galway, Loch Ryan and the Solent), between seasons, and between oysters with and without a *B. ostreae* infection. All analysis was conducted on oysters at local conditions.

THC was significantly affected by oyster origin (GLM, $F_2 = 3.432$, $P = 0.037$) (Figure 3.7) and season (GLM, $F_3 = 4.967$, $P = 0.003$) (Figure 3.8). A Tukey's post hoc test revealed that the mean THC of the Galway oysters (5.79×10^6, standard deviation (sd) = 3.50×10^6 haemocytes ml^{-1}) was significantly different to that of the Solent oysters (3.95×10^6, sd = 3.19×10^6), and between spring (6.83×10^6, sd = 4.86×10^6 haemocytes ml^{-1}) and autumn (4.35×10^6 , sd = 1.58×10^6 haemocytes ml^{-1}), and spring and winter (3.71×10^6, sd = 1.85×10^6 haemocytes ml^{-1}) (Table 3.4).

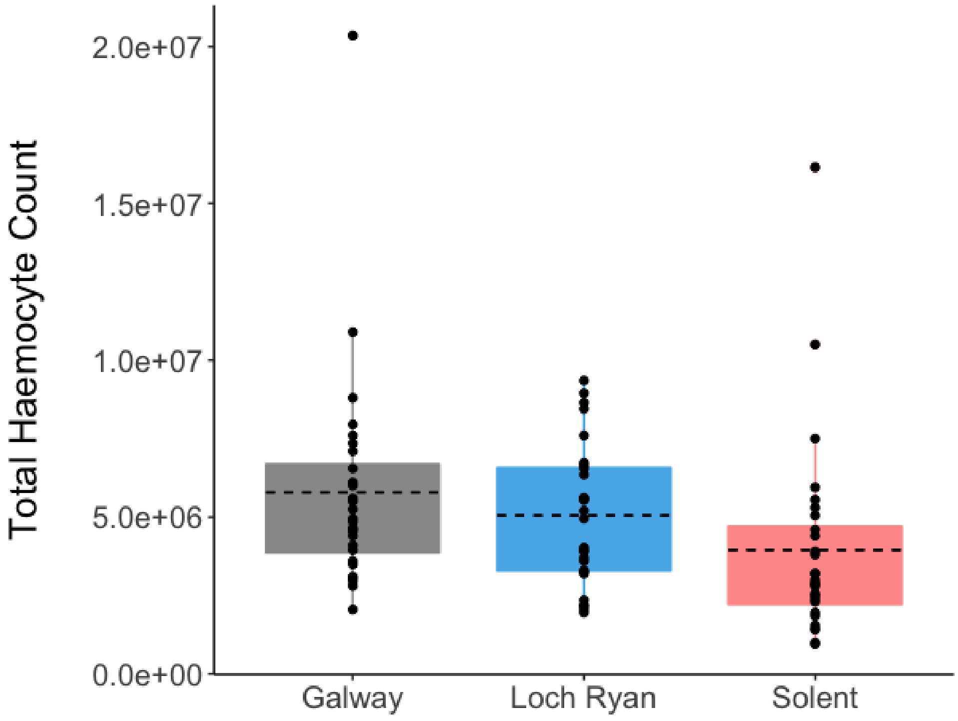


Figure 3.7. Difference in Total Haemocyte Count (THC) was significant between oysters from Galway and the Solent. Mean values for each Origin are represented with a dashed line. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

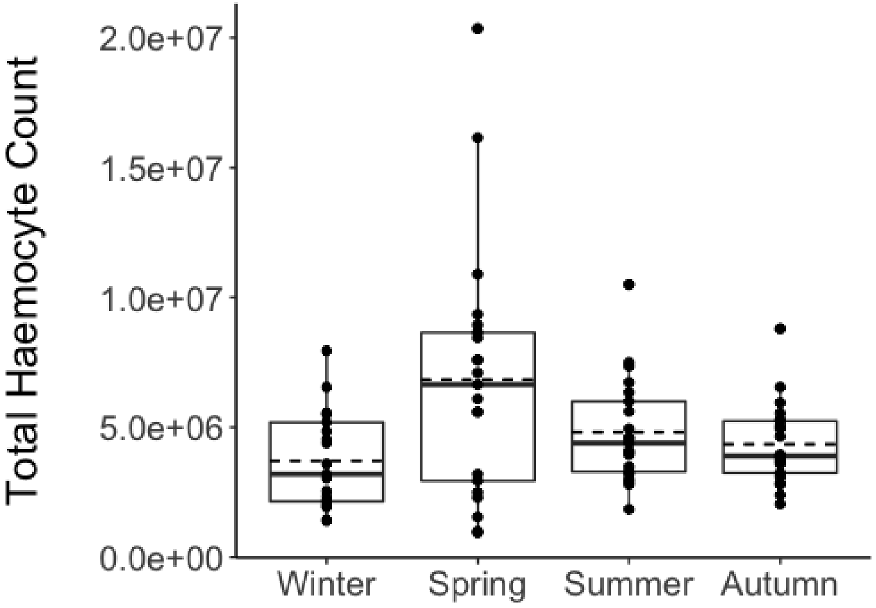


Figure 3.8. Difference in Total Haemocyte Count (THC) was significant between spring and autumn, and spring and winter. Mean values for each season are represented with a dashed line. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

Table 3.4. A Tukey's post hoc test with a confidence level of 0.95 revealed significant differences in the mean total haemocyte count (THC) between the Galway and Solent oysters, and between spring and autumn, and spring and winter.

	Mean (haemocytes ml^{-1})	Standard deviation (sd) (haemocytes ml^{-1})	*P* value
Galway	5.79 x 10^6	3.50 x 10^6	0.031
Solent	3.95 x 10^6	3.19 x 10^6	
Spring	6.83 x 10^6	4.86 x 10^6	0.048
Autumn	4.35 x 10^6	1.58 x 10^6	
Spring	6.83 x 10^6	4.86 x 10^6	0.004
Winter	3.71 x 10^6	1.85 x 10^6	

The effect of season on circling large hyalinocytes (LH) was different for each origin (GLM, $F_{6,78}$ = 3.065, P = 0.010) (Figure 3.9). Similarly, the effect of season on the number of circling granulocytes (G) was dependent on origin (GLM, $F_{6,77}$ = 3.087, P = 0.010) (Figure 3.10).

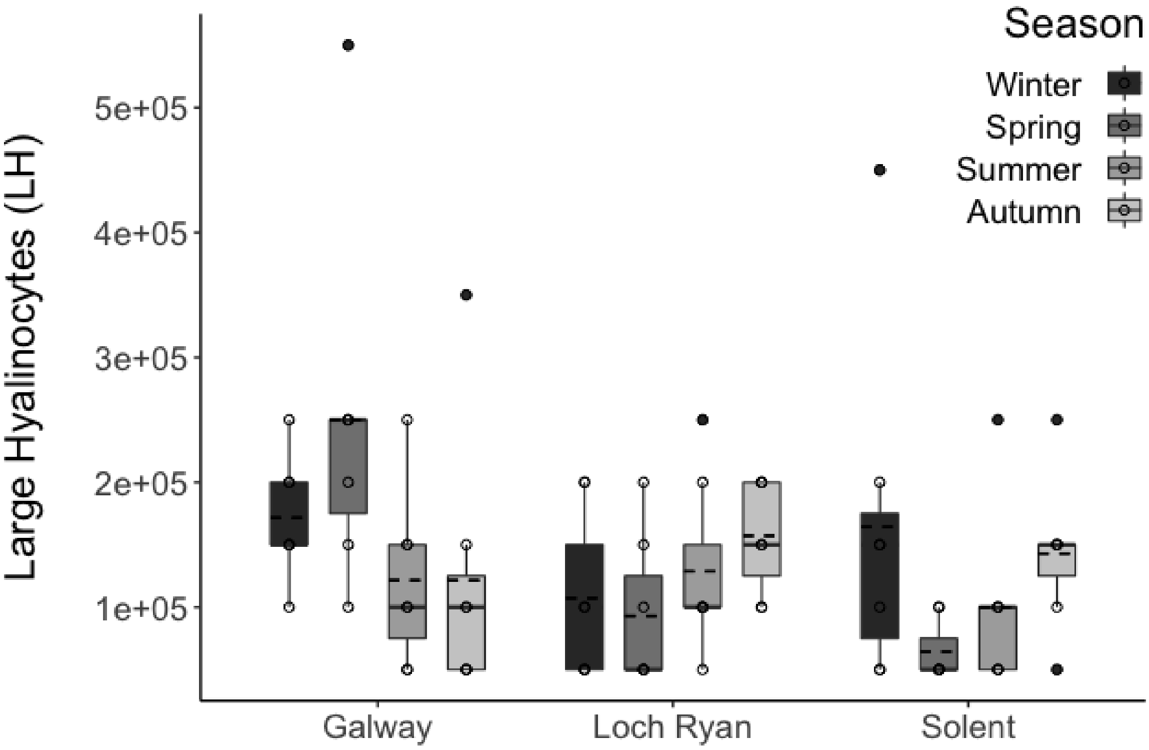


Figure 3.9. The difference in circling large hyalinocytes (LH) between origins was dependent on season. Individual oysters (n = 6) are represented with circles and mean is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

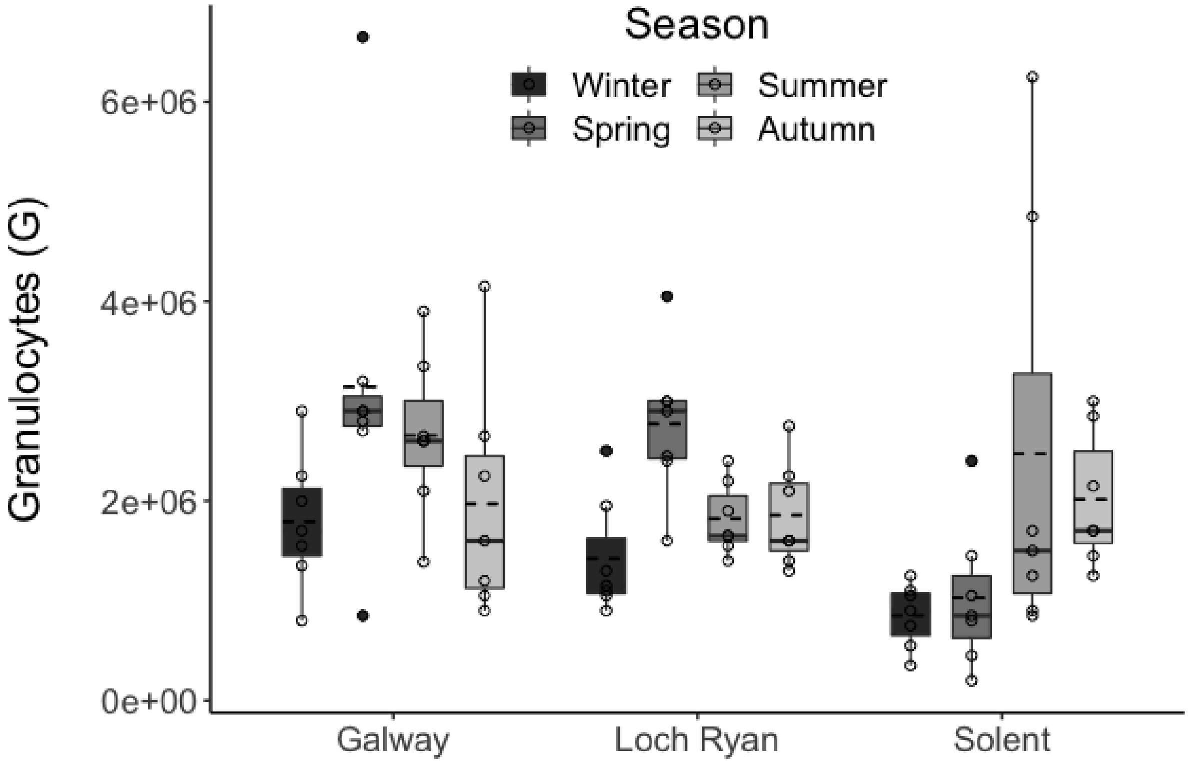


Figure 3.10. The difference in circling granulocytes (G) between origins was dependent on season. Individual oysters (n = 6) are represented with circles and mean is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

Season had a statistically significant effect on the number of small hyalinocytes (SH) (GLM, $F_{3,83}$ = 5.815, P = 0.001) (Figure 3.11). A Tukey's post hoc test identified that the most significant difference in SH occurred in spring and the comparison between this season with each of the others; mean SH in spring (4.38 x 10^6, standard deviation (sd) = 3.64 x 10^6 SH ml^{-1}) was significantly higher than in autumn (2.26 x 10^6, sd = 8.48 x 10^5 SH ml^{-1}), summer (2.38 x 10^6, sd = 1.22 x 10^6 SH ml^{-1}) and winter (2.21 x 10^6, sd = 1.26 x 10^6 SH ml^{-1}) (Table 3.5).

Oysters infected with *B. ostreae* showed no significant difference in THC or DHC in comparison to those without a *B. ostreae* infection.

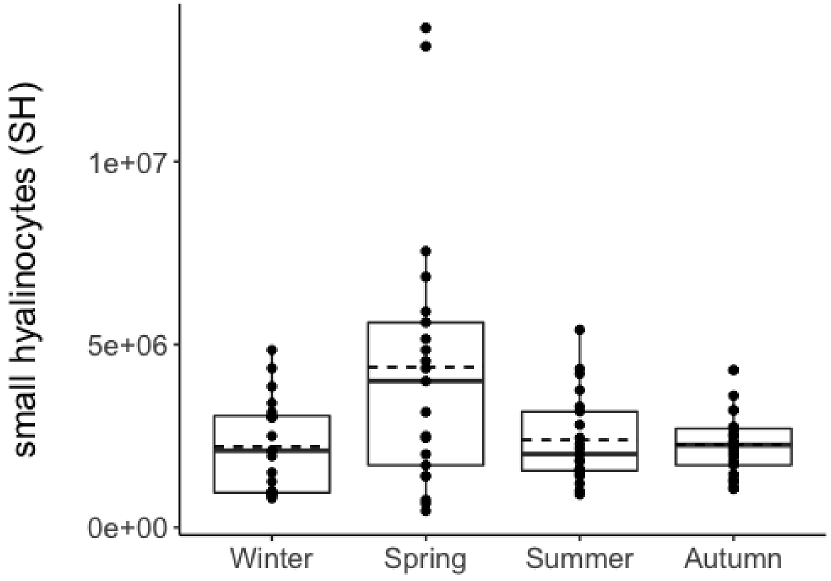


Figure 3.11. Season had a significant effect on the differential haemocyte count (DHC) of small hyalinocytes (SH). Mean values are represented with a dashed line, median values are represented with a solid line. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

Table 3.5. A Tukey's post hoc test with a confidence level of 0.95 revealed significant differences in the mean small hyalinocyte (SH) count between the Galway and Solent oysters, and between spring and autumn, and spring and winter.

	Mean (small hyalinocytes ml^{-1})	Standard deviation (sd) (small hyalinocytes ml^{-1})	*P* value
Autumn	2.26×10^6	8.48×10^5	0.014
Spring	4.38×10^6	3.64×10^6	
Spring	4.38×10^6	3.64×10^6	0.026
Summer	2.38×10^6	1.22×10^6	
Spring	4.38×10^6	3.64×10^6	0.010
Winter	2.21×10^6	1.26×10^6	

3.3.6 Body condition

Oyster condition was statistically significantly different between oysters from different origins (GLM, $F_{2,67}$ = 35.08, P = 4.64e-11) (Figure 3.12) and between seasons (GLM, $F_{3,68}$ = 3.43, P = 0.022) (Figure 3.13). Oysters from Loch Ryan clearly had the best body condition of the three populations tested (Figure 3.14). Infection with *B. ostreae* did not have a statistically significant effect on oyster body condition at local conditions (GLM, $F_{1,66}$ = 1.617, P = 0.208).

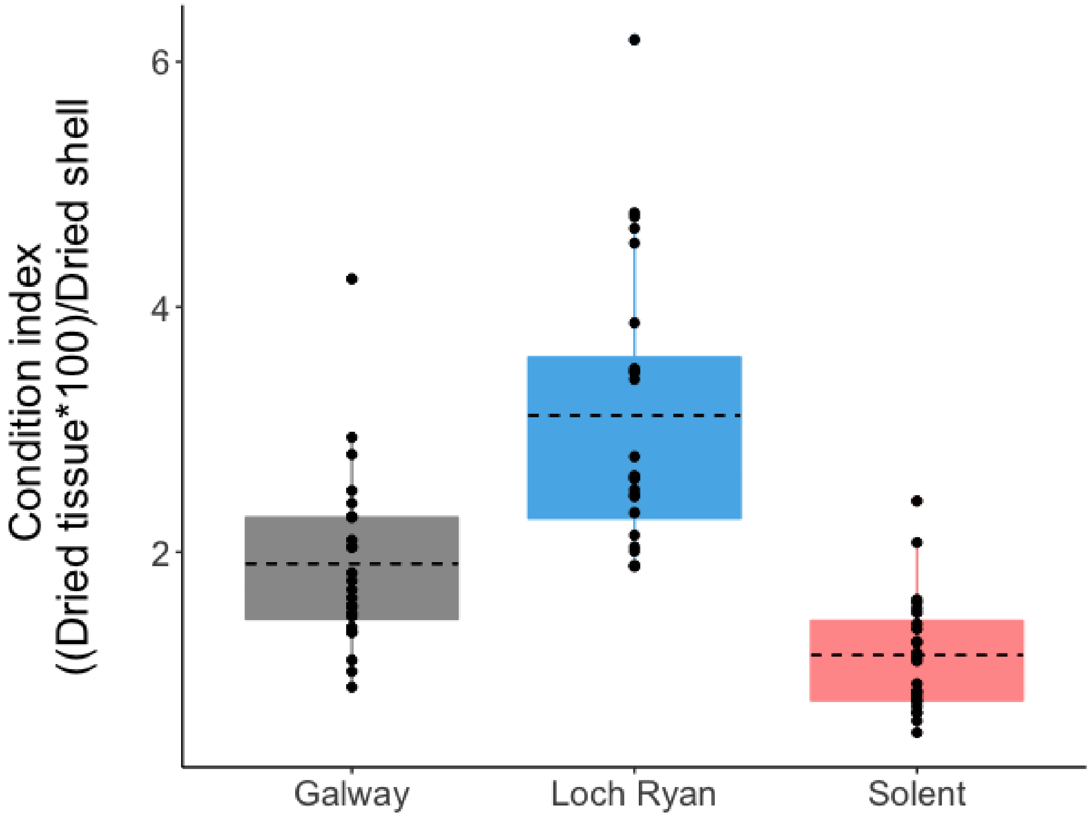


Figure 3.12. Oyster condition index was significantly affected by origin (GLM, $F_{2,67}$ = 35.08, P = 4.64e-11). Mean values are represented with a dashed line. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

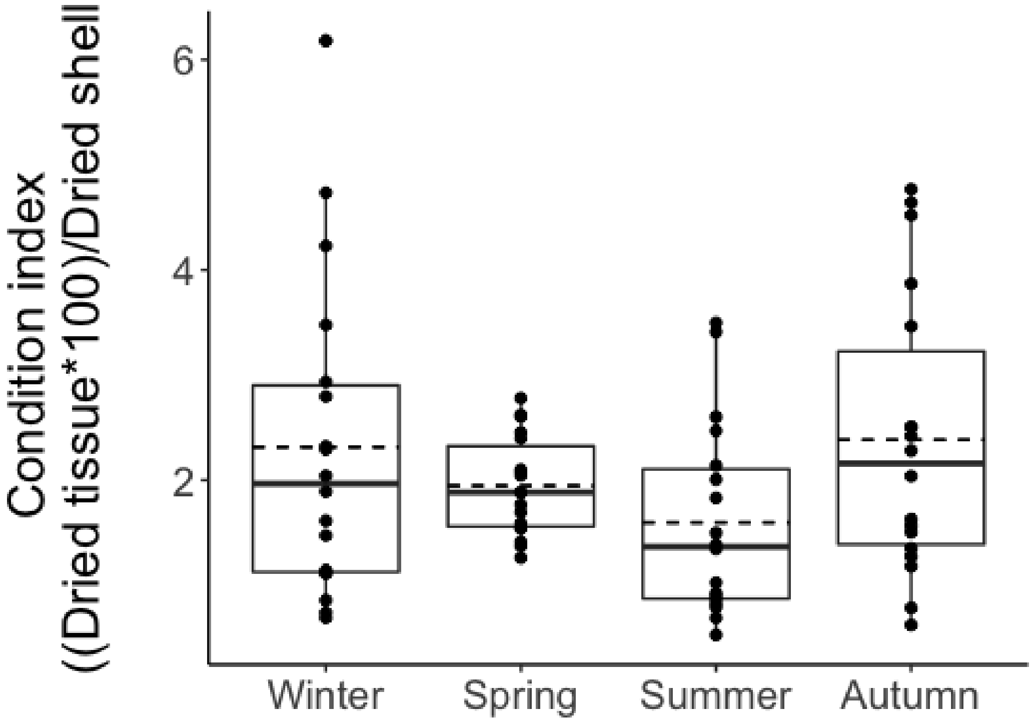


Figure 3.13. Oyster condition was statistically significantly different between seasons (GLM, $F_{3,68}$ = 3.43, P = 0.022). Mean values are represented with a dashed line. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

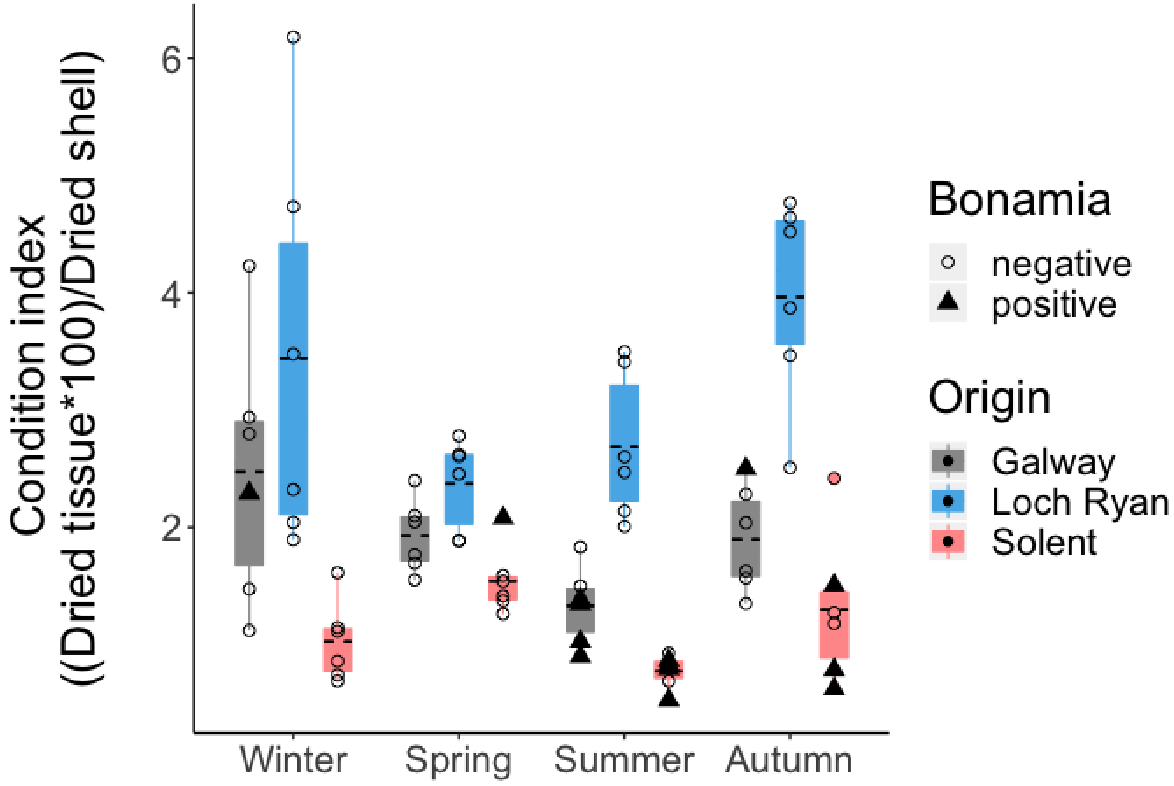


Figure 3.14. Body condition index of oysters from Galway, Loch Ryan and the Solent at their local conditions throughout the four seasons. Individual oysters (n = 6) are represented with circles (negative for *B. ostreae*) and triangles (positive for *B. ostreae*) and mean average is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

Galway and Loch Ryan oysters followed a recognisable pattern with lower condition during spring and summer months, when energy was being directed to spawning and reproduction and less to somatic tissue growth. A Tukey's post hoc test identified that there were statistically significant differences in oyster condition between all three populations, and that statistically significant seasonal differences fell between summer and autumn, and between summer and winter (Table 3.6). Oysters were in the worst condition in the summer.

Table 3.6. A Tukey’s post hoc test with confidence level of 0.95 revealed significant differences in oyster condition between oysters from each origin, and between summer and autumn and between winter and summer.

	Mean (condition index ((dried tissue * 100) / dried shell)	**Standard deviation (sd) (condition index ((dried tissue * 100) / dried shell)**	***P* value**
Galway	1.906	0.730	0.0003
Loch Ryan	3.115	1.156	
Galway	1.906	0.730	< 0.0001
Solent	1.159	0.473	
Loch Ryan	3.115	1.156	< 0.0001
Solent	1.159	0.473	
Winter	2.314	1.522	0.0682
Summer	1.600	0.917	
Summer	1.600	0.917	0.0302
Autumn	2.384	1.335	

3.3.7 Metabolic profiling

Four sets of data (retention time, accurate mass, abundance and fragmentation patter) were collected for each ion measured by the three methods used to establish the metabolic profile for each oyster: ion exchange (IC-MS/MS), C18-reverse phase chromatography (C18-MS/MS), and derivatised C18-reverse phase chromatography (dC18-MS/MS) (see 3.2.2) (Walsby-Tickle *et al.*, 2020). A principal components analysis (PCA) was performed to combine these data for each individual, and a partial least squares discrimination analysis (PLS-DA) was used to maximise the separation between oyster origins (Loch Ryan, Galway and Solent) for each of these three extraction analysis methods. Whether grouped by season (Figure 3.15a and 3.15b) or population (Figures 3.16a and 3.16b), the metabolomic profile of the populations was different. The significant differences in metabolites between populations and seasons were visualised with a heatmap (Figure 3.17). Regions of interest were highlighted in coloured boxes for further analysis (close up images of these regions can be found in Appendix C (Figures C1, C2 and C3).

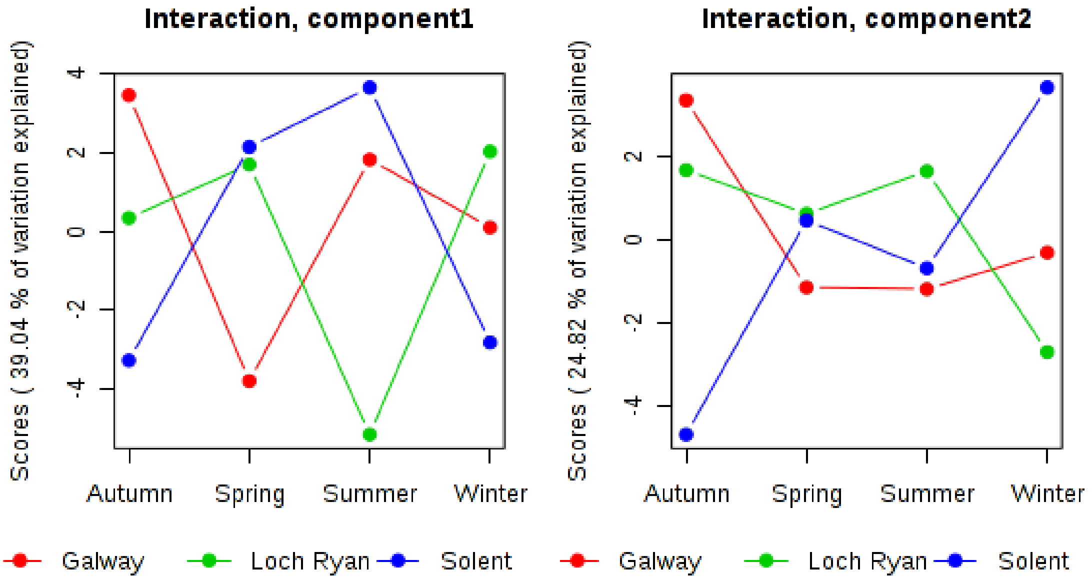


Figure 3.15 (a and b). The effect of season on the metabolic profile of *O. edulis* individuals depended on oyster origin: Galway (red), Loch Ryan (green) or Solent (dark blue). Both plots representing either component 1 (Figure 3.15a) or component 2 (Figure 3.15b) derived from principal components analysis (PCA) accounted for 63.86% of variation amongst the data. Figures created at the University of Oxford.

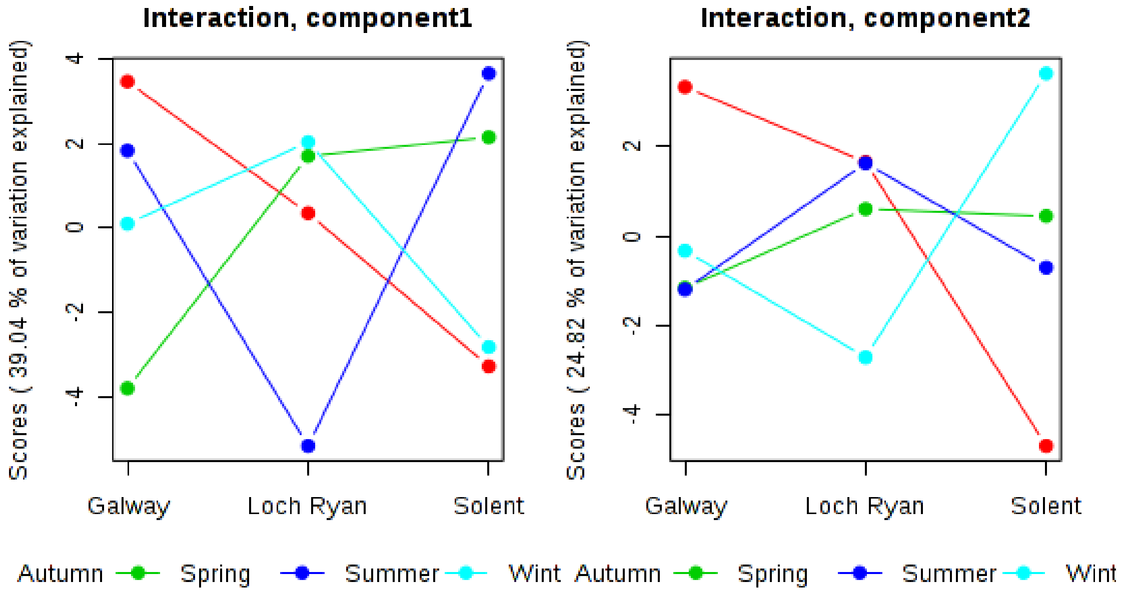


Figure 3.16 (a and b). The effect of oyster origin on the metabolic profile of *O. edulis* individuals depended on season: autumn (red), spring (green) summer (dark blue), or winter (cyan blue). Both plots representing either component 1 (Figure 3.16a) or component 2 (Figure 3.16b) as derived from a principal components analysis (PCA) account for 63.86% of variation amongst the data. Figures created at the University of Oxford.

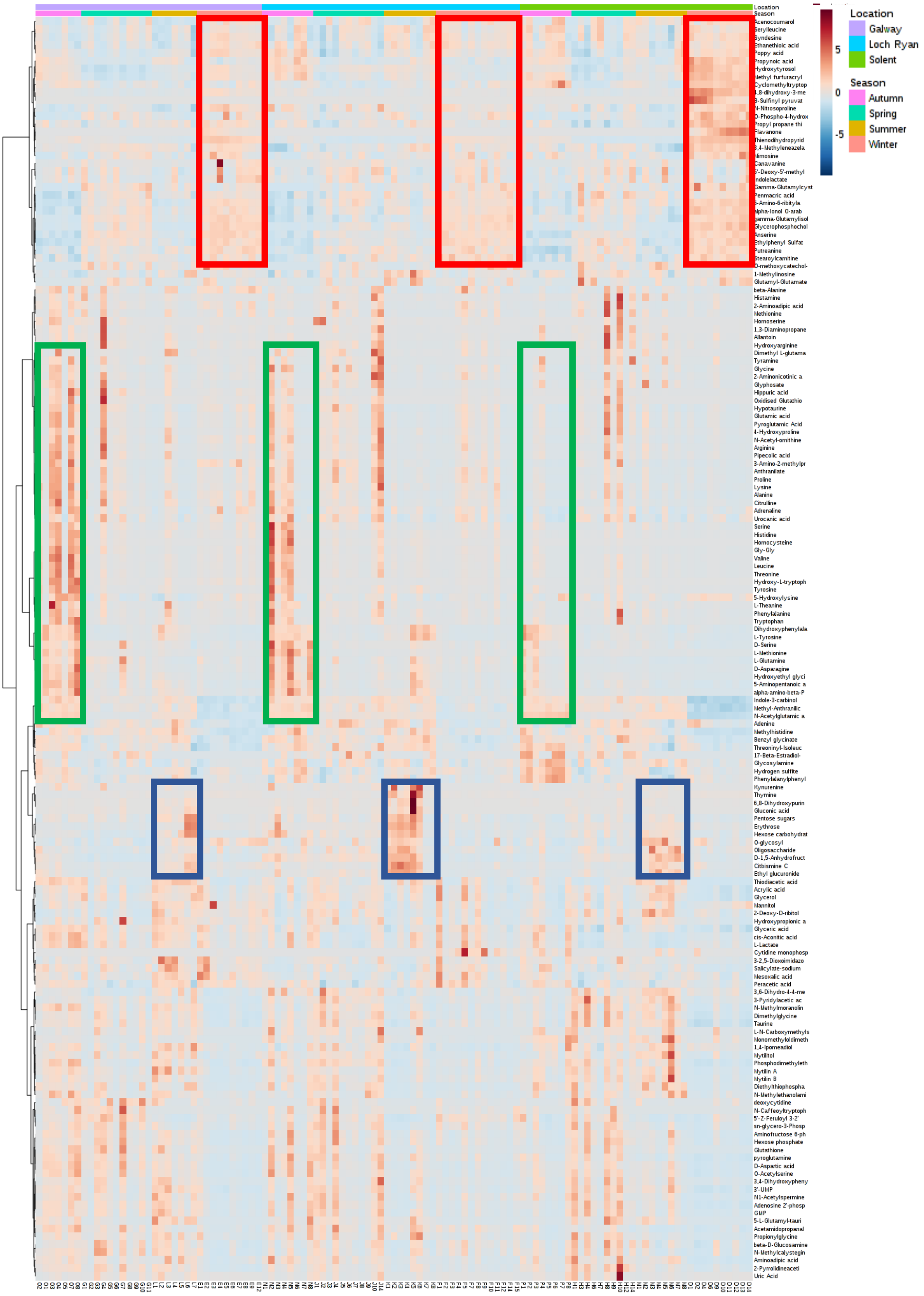


Figure 3.17. A heatmap of specific ions and their expression observed in the metabolic profile of each oyster. Letters and numbers along the x axis represent oyster batch (see Table 3.1) and specific oyster respectively. Three groups highlighted with (a) red, (b) green, and (c) blue rectangles showed significant differences in expression of specific metabolites between oyster populations.

Three regions of molecular compounds were highlighted with (a) red, (b) green, and (c) blue rectangles due to significant differences in their expression between oyster populations (Figure 3.17). Ion compounds within (a) the red rectangles (Table 3.7) were significantly represented at a much higher concentration in the Solent population in comparison to the Galway or Loch Ryan population during the winter (Figure 3.17a, and Figure C1 in Appendix C). Ion compounds in (b) the green rectangles (Table 3.8) were represented at a much higher concentration in the Galway and Loch Ryan population in comparison to the Solent population during the autumn (Figure 3.17b, and Figure C2 in Appendix C). Ion compounds in (c) the blue rectangles (Table 3.9) were represented at a significantly higher concentration in the Loch Ryan population in comparison to the Solent or Galway population during the summer (Figure 3.17c, and Figure C3 in Appendix C). Understanding the roles of these ions may indicate which metabolites could be used as biomarkers for physiological functionality of oyster individuals & populations.

Table 3.7. The 31 named ions (within the red rectangle, Figure 3.17a) significantly represented at a much higher concentration in the Solent population in comparison to the Galway or Loch Ryan populations during the winter.

1	acenocoumarol	16	thien dihydropyridymine
2	serylleucine	17	3,4-methyleneazela
3	syndesine	18	mimosine
4	ethanethioic acid	19	manavanine
5	poppy acid (meconic acid)	20	5’-deoxy-5’-methylthioadenosine
6	propynoic acid	21	indolelacetate
7	hydroxytyrosol	22	gamma-glutamyl cysteine
8	methyl furfuracryl	23	penmacric acid
9	cyclo methyl tryptophan	24	5-amino-6-(D-ribitylamino)uracil
10	4,8-dihydroxy-3-methyl-3,4-dihydro-1(2H)-naphthalenone	25	alpha-lonol 0-arabinose
		26	gamma-glutamylisol
11	b-sulfinyl pyruvate	27	glycerophosphocholine
12	n-nitrosoproline	28	anserine
13	o-phospho-4-hydroxy-L-threonine	29	ethylphenyl sulfate
14	propyl propane thiosulfonate	30	putreanine
15	flavenone	31	stearoylcarnitine

Table 3.8. The 48 named ions (within the green rectangle, Figure 3.17b) significantly represented at a much higher concentration in the Galway and Loch Ryan populations in comparison to the Solent population during the autumn.

1	hydroxyarginine	17	anthranilate	33	5-hydroxylysine
2	dimethyl L-glutamate	18	proline	34	L-theanine
3	tyramine	19	lysine	35	phenylalanine
4	glycine	20	alanine	36	tryptophan
5	2-aminonicotinic acid	21	citrulline	37	dihydroxyphenylalanine
6	glyphosate	22	adrenaline	38	L-tyrosine
7	hippuric acid	23	urocanic acid	39	D-serine
8	oxidised glutathione	24	serine	40	L-methionine
9	hypotaurine	25	histidine	41	L-glutamine
10	glutamic acid	26	homocysteine	42	D-asparagine
11	pyroglutamic acid	27	gly-gly	43	hydroxyethyl glycine
12	4-hydroxyproline	28	valine	44	5-aminopentanoic acid
13	n-acetyl-ornithine	29	leucine	45	alpha-amino-beta-p
14	arginine	30	threonine	46	indole -e-carbinol
15	pipecolic acid	31	hydroxy-L-tryptophan	47	methyl-anthranilic
16	3-amino-2-methyl-propanol	32	tyrosine	48	N-acetylglutamic acid

Table 3.9. The 12 named ions (within the blue rectangle, Figure 3.17c) significantly represented at a much higher concentration in the Loch Ryan population in comparison to the Galway or Solent populations during summer.

1	kynurenine	7	hexose carbohydrate
2	thymine	8	i-glycosyl
3	6,8-dihydroxypurine	9	oligosaccharide
4	gluconic acid	10	D-1,5-anhydrofructose
5	pentose sugars	11	citbismine c
6	erythrose	12	ethyl glucuronide

3.3.8 Acclimation to aquarium conditions

The remaining oysters were brought up to aquarium conditions over the course of 1 month (black asterisks, Figure 3.18). Clearance rate, respiration rate and body condition were then observed in six of these oysters from each origin to see if their physiological performance had been altered by the change in temperature.

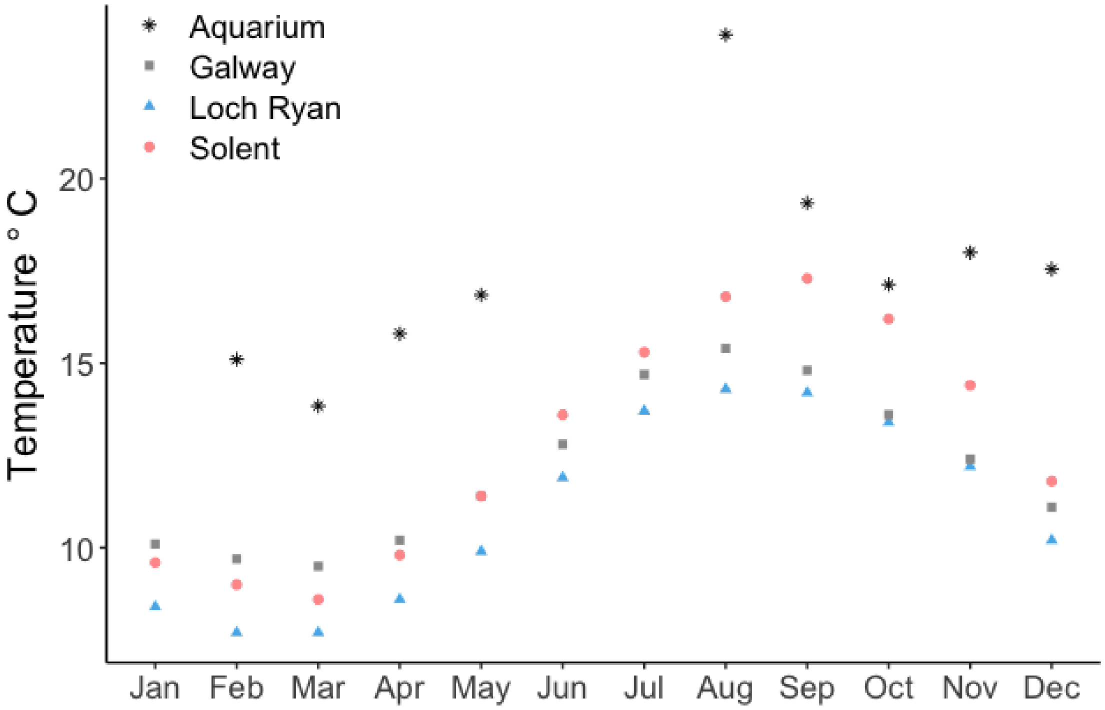


Figure 3.18. Mean seawater temperature in the NOCS aquarium (black asterisks) in 2018, Galway (grey squares), Loch Ryan (blue triangles) and the Solent (pink circles). Data acquired from www.seatemperature.org [Accessed 14th September 2019].

Clearance rate

There was a significant interaction between season, origin and condition (GLM, $F_{4,94} = 5.104$, $P = 0.0009$). Of the three populations around the UK, Loch Ryan oysters demonstrated the most consistently low clearance rate (Figure 3.19). Clearance rate of Solent oysters was much higher during summer months post acclimation to aquarium tank conditions.

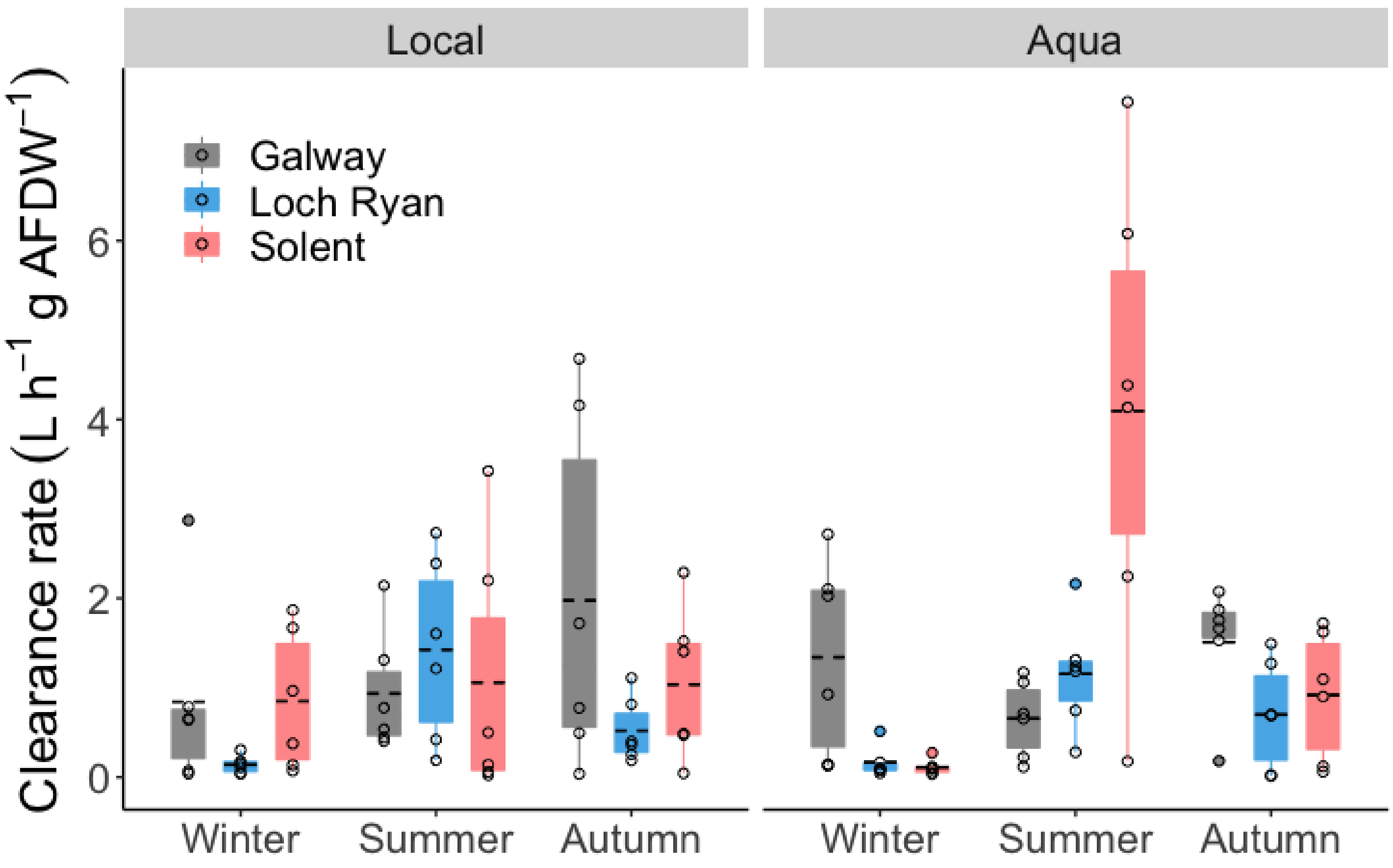


Figure 3.19. Clearance rate of oysters grouped by oyster origin, season and holding condition (local and aquarium conditions). Individual oysters (n = 6) are represented with circles and mean average is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

Respiration rate

The acclimation of the oysters to aquarium conditions removed the effect of season on respiration rate as the oysters became accustomed to the more constant conditions (GLM, $F_{2,104} = 8.29$, $P = 0.0005$) (Figure 3.20). Origin did not have an effect on respiration rate (GLM, $F_{2,102} = 0.76$, $P = 0.471$), which can be seen in the raw data (Figure 3.21).

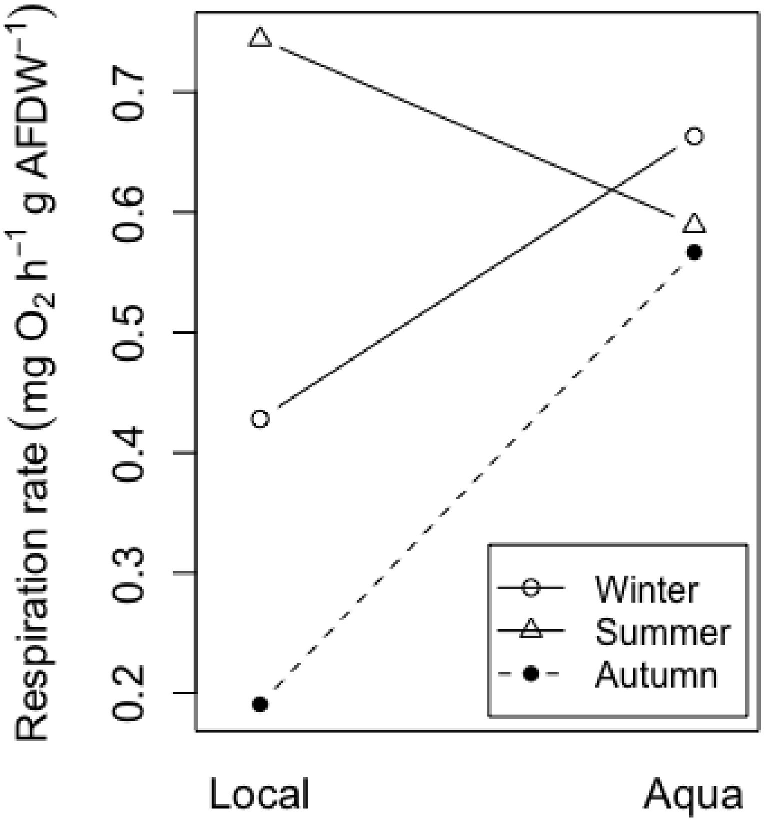


Figure 3.20. Respiration rate was affected by the interaction between season and holding condition (local and aquarium conditions) (GLM, $F_{2,104} = 8.29$, $P = 0.0005$); The acclimation of the oysters to aquarium conditions removed the effect of season. Marks (circles and triangles) represent the mean, n = 18.

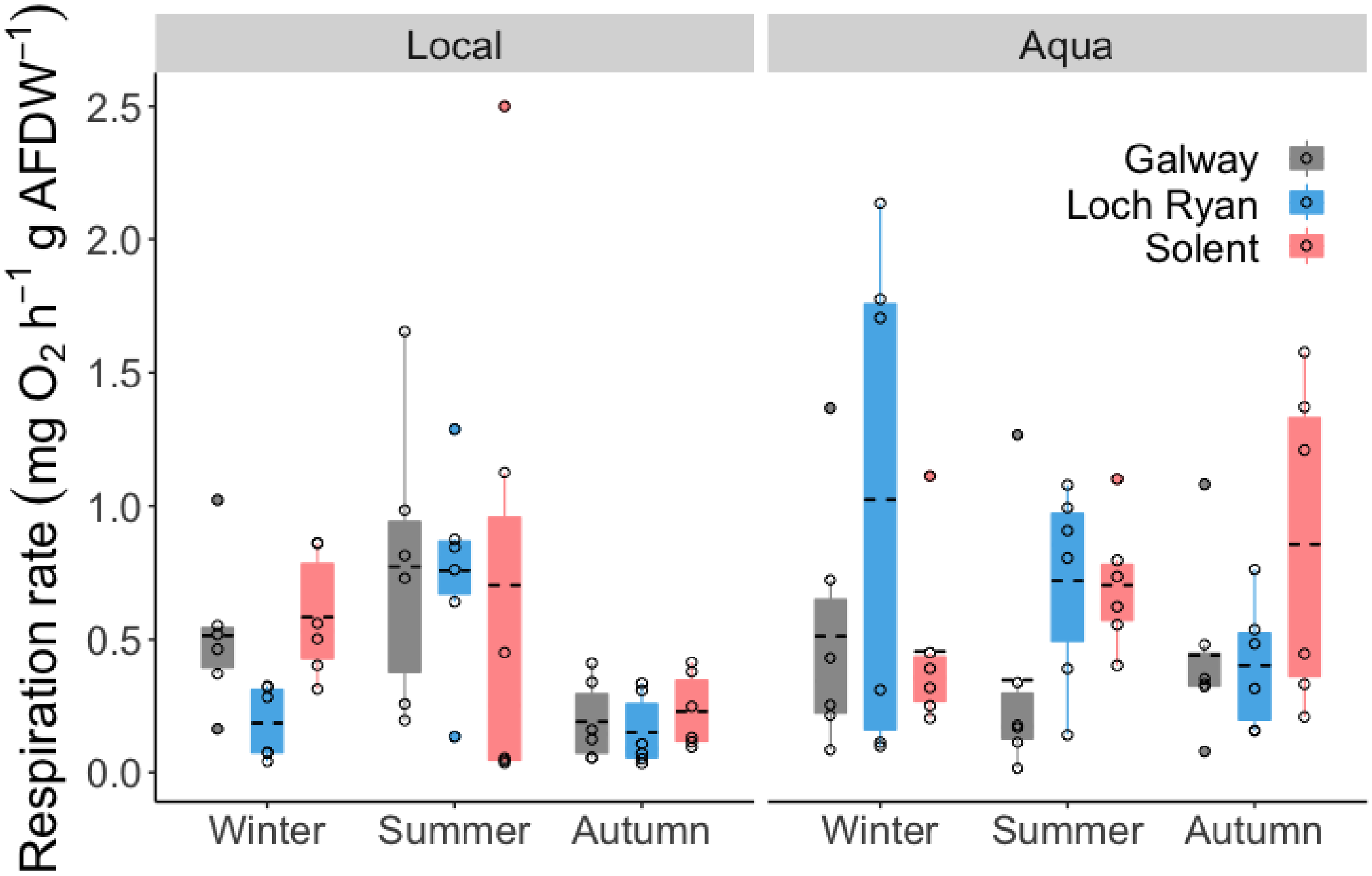


Figure 3.21. Respiration rate of oysters grouped by oyster origin, season and holding condition (local and aquarium conditions). Individual oysters (n = 6) are represented with circles and mean average is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

Oyster condition

Statistically significant differences in variance of oyster body condition was observed between holding conditions (GLM, $F_{1,103} = 7.026$, $P = 0.009$) (Figure 3.22). This was complicated by the effects of origin (GLM, $F_{2,104} = 76.153$, $P < 0.0001$) and season (GLM, $F_{2,104} = 3.538$, $P = 0.033$) (Figure 3.23); condition of Loch Ryan and Galway were significantly reduced post acclimation, but Solent oysters remained at low condition irrespective of holding condition.

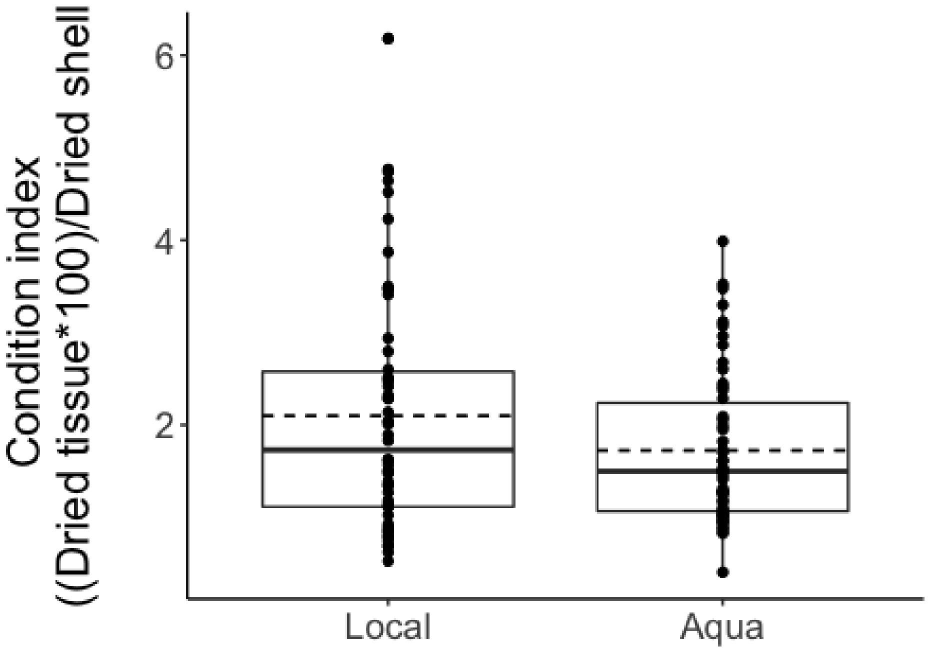


Figure 3.22. Statistically significant differences in oyster body condition (condition index) could be explained by holding conditions; body condition was significantly better when the oysters first arrived to NOCS and were kept at local conditions than post acclimation to aquarium conditions. (GLM, $F_{1,103} = 7.026$, $P = 0.009$).

Overall, the oysters were in significantly better condition when kept in seawater at the temperature and salinity of their origin (Local), than after spending c. 1 month in the aquarium in seawater filtered from the Solent (Aqua) (Figure 3.23). Suggested outliers marked as isolated circles (over 1.5 times the interquartile range) show the spread of data within the small sample size.

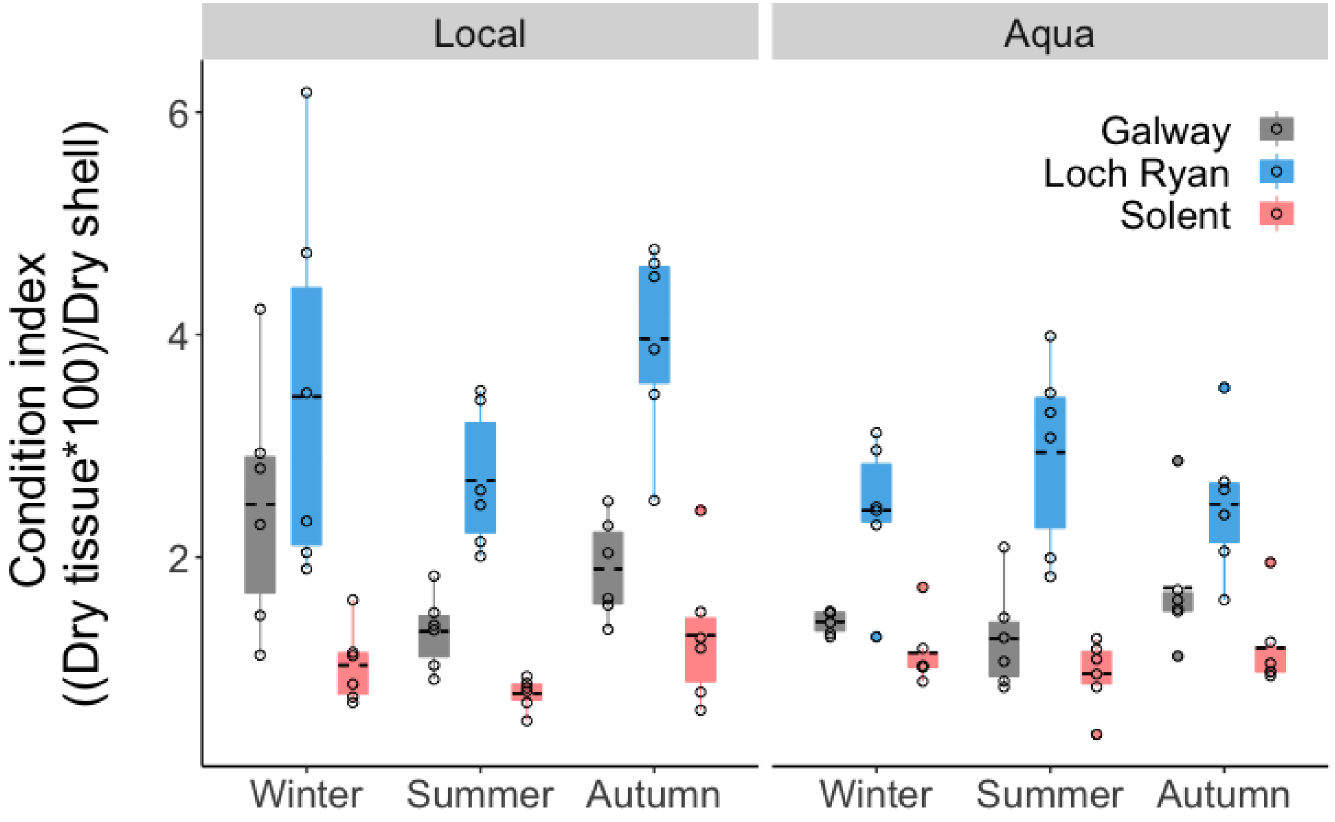


Figure 3.23. Condition index of oysters grouped by oyster origin, season and holding condition (local and aquarium conditions). Individual oysters (n = 6) are represented with circles and mean average is represented with dashed lines. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

3.4 Discussion

O. edulis are poikilothermic osmoconformers and therefore their physiology is strongly determined by their environment, but significant variation in physiological attributes has been seen in regions as close as 50 miles apart (Sawusdee, 2015). Re-laying broodstock is a key element of native oyster restoration, and sourcing oysters that will physiologically thrive in the local environment is essential for establishing a healthy self-sustaining population. Previous studies have suggested that oysters from the immediate environment are likely to be the most successful (Loosanoff and Nomejko, 1951; Korringa, 1957), but with multiple *O. edulis* restoration projects advancing in the UK and in Europe, local oyster spat could become a limiting factor for projects (Bromley *et al.*, 2016a). This study compared the physiological performance of *O. edulis* from three wild UK fisheries; Galway (Ireland), Solent (England) (both *Bonamia*-exposed) and Loch Ryan (Scotland) (*Bonamia*-free). Clearance and respiration rates were acquired from six oysters from each origin over the four seasons (winter, spring, summer and autumn) at their local conditions before molecular analysis of their haemocyte count and parasite (*B. ostreae*) infection status and their metabolome profile was analysed. A further six oysters from each origin and during each season were similarly tested after acclimation to aquarium conditions over the course of one month.

3.4.1 Physiological performance at local conditions

Effect of *Bonamia ostreae* infection

Use of PCR identified the presence of *B. ostreae* in the Galway and Solent populations; 1/4 of the Galway oysters and 1/3 of the Solent oysters were infected (n = 24). Loch Ryan, as assumed, was *Bonamia*-free and no parasite was detected. *B. ostreae* infection did not have a statistically significant effect on either clearance or respiration rates. Previous studies with *Crassostrea* spp. have attributed an increase in total haemocyte count (THC) or change to the differential haemocyte ratio to pathogen infection (da Silva *et al.*, 2008). Farley (1968) observed a higher number of agranulocytes in *C. virginica* infected with the parasite *Haplosporidium nelsoni* (MSX), and La Peyre *et al.* (1995) found increased THC in *C. gigas* infected with *Perkinsus marinus*. However, in that same study, *C. virginica* was not similarly affected by the same parasite (*P. marinus*) and no change in their THC was observed (La Peyre *et al.*, 1995). Haemocytes are the resource actively fighting foreign pathogenic agents (Culloty and Mulcahy, 2007), and therefore it would make sense that a higher number of said resources would benefit a disease-challenged immune response. However, external environmental conditions (such as salinity) and haemocyte locomotion is proposed to be more important than haemocyte abundance for immunity (Fisher and Newell, 1986; Chu *et al.*, 1993). This study similarly found no influence of *B. ostreae* infection on total, or differential, haemocyte count, which highlights the intricate complexity of haemolymph functionality that cannot be explained by haemocyte abundance alone. Low cell counts might also be a function of an existing infection; one technique of bivalve mollusc immune defence is the flocking migration of haemocytes to infected tissue thereby removing them from general haemolymph circulation, and potentially from identification (Allam and Raftos, 2015). Use of careful detection methods (that reflect the disease in question) are crucial in order to gain reliable information from the abundance or concentration of circulating haemocytes in relation to immune response.

Effect of Season

Season had an effect on the condition of the oysters; oyster condition was significantly better in autumn and winter than it was in the summer. Similar fluctuations have been seen in *O. edulis* in Europe (Pogoda *et al.*, 2011). *O. edulis* oocytes are rich in lipids and protein and successful gamete development is dependent upon available energy from nutrients and lipid reserves acquired from feeding (Orton, 1926; Joyce *et al.*, 2013). Glycogen, lipids and proteins must be metabolized to support gametogenesis, and therefore brooding is costly to a female *O. edulis* and limits the amount of energy available for somatic growth and general upkeep (Hassan *et al.*, 2018). The *O. edulis* spawning season is dependent on temperature, but this temperature stimulus can vary between location (Mann, 1979; Ruiz *et al.*, 1992; Bromley *et al.*, 2016a). Spawning tends to occur in the summer (June through August, Walne, 1970), and individuals forfeit their somatic growth and general condition immediately prior to, and throughout this period, in order to invest more energy into developing gametes (Mathieu and Lubet, 1993). If lipid rich algae is abundant during gametogenesis, and individuals are able to store a higher glycogen concentration, then oysters will remain in better condition with less detriment to their body weight, growth and survival (Mathieu and Lubet, 1993; Burke *et al.*, 2008). A natural fluctuation in condition of many marine mollusc bivalves as a result of changes in season or available nutrition has been well documented (Mann, 1979; Ruiz *et al.*, 1992; Mathieu and Lubet, 1993; Joaquim *et al.*, 2008); the Loch Ryan, Galway and Solent oysters in this study demonstrated a degradation of condition during the summer, post spawning. Production of spermatozoa is less costly to the oyster, which is why all *O. edulis* spend the first few years as a male (Joyce *et al.*, 2013).

The average total haemocyte count (THC) seen in the oysters reflect values reported in the literature (da Silva *et al.*, 2008; Comesaña *et al.*, 2012; Sawusdee *et al.*, 2015). A significantly higher THC was seen in Spring in comparison to autumn and winter (Figure 3.8). The lowest THC was recorded in winter, which is supported by previous literature that has linked an increase in oyster THC with temperature (Chu and La Peyre, 1993; da Silva *et al.*, 2008). da Silva *et al.* (2008) suggested that fluctuations in THC were likely to be in relation to physiological requirements such as gonad development. Cells that formulate the bivalve haemolymph are produced continuously in a process called haemaopoiesis, but the haemopoietic organs responsible for this process in several species, including *O. edulis*, have yet to be described (Jemaá *et al.*, 2014; Dyachuk, 2016). Highly individualised and complex structures have been described in other bivalve molluscs, and it is thought that adult bivalves may possess multiple sites of specialist cells that produce haemocytes in response to neighbouring cells (Cheng, 1981; Hartenstein, 2006; Tirapé *et al.*, 2007; Dyachuk, 2016); Jemaá *et al.* (2014) observed hematopoiesis occurring in a folded collection of somatic cells amongst the gill of *C. gigas*. Whether the production of differential cell types is regulated and altered depending on individual oyster immune state is yet to be determined. Agranulocytes (small and large hyalinocytes) have been found to contain a higher diversity of protein than granulocytes, and possess their own unique peptides (Xue *et al.*, 2000). Xue *et al.* (2000) highlighted that the different proteins represent different cell functionality in immune response, and evidence of similarity between small and large hyalinocytes could support the theory that small hyalinocytes (SH) simply represent a developmental stage of either large hyalinocytes (LH) or granulocytes (Cheng, 1981; Xue *et al.*, 2000). The significant seasonal effect on abundance of LH and granulocytes was dependent on oyster origin, which suggests that haemopoiesis of these specific haemocytes are not driven by biological events such as spawning, but indicate population differences in rate of haemopoiesis or maturation of small hyalinocytes. Culloty and Mulcahy (2007) suggested that agranular cells such as the large hyalinocytes might play a role in parasite survival and development, which would suggest they are more vulnerable to the parasite mechanisms that neutralise the host immune response. This theory supported (Cochennec-Laureau *et al.*, 2003a) who reported that *B. ostreae* was more prevalent in LH than in granulocytes, and was seen by da Silva *et al.* (2008) who saw a marked increase in LH in *Bonamia*-challenged oysters. Changes in environmental conditions such as temperature and salinity are highly influential on the behaviour and functionality of bivalve haemocytes (McCormick-Ray and Howard, 1991); haemocyte locomotion has been shown to increase with temperature and decrease with salinity (Fisher and Newell, 1986; Fisher, 1988a). The process of gametogenesis and spawning has also been shown to alter phagocytic activity, which further substantiates the importance of quantifying seasonal fluctuations in any analysis of oyster immunity (Auffret and Oubella, 1994; Fisher *et al.*, 1996; da Silva *et al.*, 2008; Kortet and Vainikka, 2008). Similar to results seen by da Silva *et al.* (2008), this study observed a significantly higher abundance of SH in spring compared to the other seasons (Figure 3.11). This increase in SH prior to the spawning period (known to require large amounts of energy from the parent oyster), could be a physiological mechanism to strengthen the parent immune system prior to exposure to pathogens and other environmental pressures in its weaker state post spawning (Kortet and Vainikka, 2008).

Effect of Origin

Oyster origin had an effect on total haemocyte count (THC); Solent oysters had significantly lower THC than the Galway oysters (Figure 3.7). Seawater temperatures (known to influence THC) tended to be slightly warmer in the Solent than in Galway, but it is difficult to determine whether a difference of (max) 2.6 °C in temperature would have such a significant effect on the haemocytes. Several studies have highlighted the significant influence of salinity on the abundance of haemocytes (Fisher and Newell, 1986; Chu *et al.*, 1993), but the variation in THC of oysters from

Galway and Solent cannot be explained by salinity as THC remained similar at both locations. Effects of temperature and salinity on haemocyte activity are well documented, but effects of notable ancillary factors such as nutrition and seawater pH are less so (van der Knaap *et al.*, 1983; Fisher, 1988b, 1988a). Previous research has demonstrated oyster respiration rate to increase in warmer water (Hutchinson and Hawkins, 1992), which was seen by all three populations at their local conditions (Figure 3.6). However, Loch Ryan and Solent oysters showed highly variable respiration rates post acclimation to aquarium conditions in the winter and autumn respectively, which suggests that their adaptability to the warmer environment depended on the individual (Figure 3.21).

The condition of oysters was significantly different between all origins; Loch Ryan oysters were in the best condition, and Solent oysters were in the worst condition. The condition index used has been favoured in many biological studies by taking the ratio of soft tissue to shell material (Lucas and Beninger, 1985). Low condition index represents a larger shell in comparison to the soft tissue. Shell production requires considerable energy that could otherwise be used for reproduction and somatic growth (Palmer, 1981, 1992; Rosell *et al.*, 1999; Stefaniak *et al.*, 2005). Regional (population) level differences in *O. edulis* condition and growth rates have been previously described (Culloty *et al.*, 2004; da Silva *et al.*, 2005). da Silva, Fuentes and Villalba (2005) observed lower growth rates in *Bonamia*-exposed populations than in *Bonamia*-naïve populations within the same region, which supports the data from this study as Loch Ryan with the highest observed condition index is a *Bonamia*-free population. Although presence of *B. ostreae* did not show any significant effect on individual oyster condition (see above), the presence of the parasite amongst the population may have driven a population-wide reduction in growth rate as a result.

Additional environmental factors could also be responsible for a thicker shell; the increase in oyster shell weight in relation to soft tissue weight indicated by the condition index could represent a form of defence against increased predation, which has been seen in *O. edulis* infected with boring sponges *Cliona celata* and *C. viridis* (Hoeksema, 1983; Rosell *et al.*, 1999), and in other species (Stefaniak *et al.*, 2005). Although *O. edulis* shell weight is positively correlated to shell strength (Lemasson and Knights, 2019), a thicker but more structural latticed shell developed in response to pressure from boring organisms is unlikely to be stronger than a solid shell structure (Stefaniak *et al.*, 2005). Increased pollution such as antifouling agent TBT, and high water currents have been shown to increase the shell thickness of *O. edulis* (Axiak *et al.*, 1995; Newkirk *et al.*, 1995), both of which are more prevalent in the Solent than in Loch Ryan.

The metabolic profiling established that metabolomics does give us the power to discriminate between oyster populations (Figures 3.15 and 3.16). Significant differences in expression of specific metabolites between populations were evident in winter, autumn, and summer (Figure 3.17a, 3.17b and 3.17c respectively). Several metabolites that were more highly expressed in the Solent population than in the Galway and Loch Ryan population during winter (n-nitrosoproline, flavenone, canavanine, and indolelactate) are not naturally produced by marine invertebrates, or are synthesised from the diet (e.g. production of indolelactate requires tryptophan acquired from algae (Wang and Douglas, 1999)). A number of metabolites that showed significantly higher expression in the Solent population during this time are also commonly found in pesticides and insecticides (acenocoumarol, ethanethioic acid, n-nitrosoproline, flavenone, and canavanine), which could feature in antifouling paint (EPA, 1980; Mitri *et al.*, 2009; Bredebach *et al.*, 2011; Gómez-Canela *et al.*, 2014). Although methyl furfuracryl is found in small quantities in the cytoplasm of all eukaryotes, it is also a product in faeces and wastewater and could have been acquired by the oysters through sewage outlets in the Solent (Mehrjouei *et al.*, 2014). The Solent's busy shipping activity and human population could be leaching a higher concentration of biocides and pollution into the environment than evident in Galway or Loch Ryan, which may contribute to the low body condition of these Solent oysters (section 3.3.6). Mimosine and 5'-deoxy-5'-methyl(adenosine) are both protein and DNA inhibitors that can arrest the natural cell cycle (Conti *et al.*, 2002; Buro *et al.*, 2010). Disruption to DNA and cellular function is detrimental to an organism, often leading to disease, and the higher concentration of these metabolites seen in the Solent oysters could also explain the lower body condition of these oysters in comparison to Galway and Loch Ryan oysters.

Several essential metabolites that need to be acquired from diet, or increased in quantity by diet (hypotaurine, glycine, lysine, valine, leucine, threonine, tyrosine, 5-hydroxylsine, phenylalanine, tryptophan, dihydroxyphenylala, L-tyrosine, and L-methionine) were seen in very low concentrations in the Solent population in comparison to the Galway and Loch Ryan populations in autumn specifically, but also throughout all seasons (Figure 3.17b) (Welborn and Manahan, 1995; Wang and Douglas, 1999; Genchi, 2017; Hui *et al.*, 2018). These metabolites are important for mitochondrial function (lysine, 5-hydroxylsine (Hui *et al.*, 2018)), muscle growth and tissue repair (valine, leucine, (Jørgensen, 1979; Diniz *et al.*, 2014)), larval growth (threonine (Katayama *et al.*, 2016)), and intracellular signalling (threonine, tyrosine, phenylalanine, dihydroxyphenylala, L-tyrosine, L-methionine (Jørgensen, 1979; Genchi, 2017)). If (as the data suggest) these essential metabolites are available at high concentration in Galway and Loch Ryan, then restoration efforts should be focussed in these regions rather than in the Solent where the dietary source of this metabolites is restricted.

Metabolites known to be important neurotransmitters (hydroxyarginine, dimethyl L-glutamate, tyramine, glycine, hypotaurine, glutamic acid, arginine, adrenaline, urocanic acid, phenylalanine, dihydroxyphenylala, and L-glutamine) or precursors to neurotransmitters (hydroxy-L-tryptophan) were expressed at low concentrations in the Solent oysters in comparison to the Galway and Loch Ryan oysters in the autumn (Anderson and Stephens, 1969; Usherwood, 1977; Jørgensen, 1979;

Manahan, 1983; Anderson and Burris, 1987; Wafford and Sattelle, 1989; Rodríguez *et al.*, 1993; Kimura *et al.*, 2003; Beyoglu *et al.*, 2012; Curran and Chalasani, 2012; Ripps and Shen, 2012; Diniz *et al.*, 2014; Bauknecht and Jékely, 2017; Roeder, 2020) (Figure 3.17b). Neurotransmitters have been shown to play an important role in essential bivalve physiological processes including larvae development and metamorphosis (Ellis and Kempf, 2011; Mesías-Gansbiller *et al.*, 2013; Cragg, 2016), gill cilia activity and associated feeding rates (Stephens and Prior, 1992; Beiras and Widdows, 1995; Carroll and Catapane, 2007), and regulation of heart and siphon activity (Erdmann, 1935; Kuwasawa and Hill, 1997; Ram *et al.*, 1999; Siniscalchi *et al.*, 2004).

Cell function in response to the immediate environment (such as osmoregulation) is dependent on intracellular and extracellular signalling pathways. Hypotaurine is a precursor to taurine, which is essential for osmoregulation and metamorphosis in molluscs (Welborn and Manahan, 1995). Although seen in high concentration in the Galway and Loch Ryan populations during autumn, the expression of this metabolite (hypotaurine) was almost non-existent in the Solent population. Several other metabolites essential for controlling cellular osmolarity (pipecolic acid, proline, and alanine) were similarly seen in very low concentrations in the Solent population (Figure 3.17b), and this disruption to osmoregulation function could be influencing the condition of these oysters.

In summer, oysters from Loch Ryan expressed a higher concentration of metabolites associated with DNA, sugars, and lipids (kynurenine, thymine, pentose sugars, hexose carbohydrate, l-glycosyl, oligosaccharide, and D-1, 5-anhydrofruct(ase)) than oysters from either Galway or the Solent (Kuksis, 2003; Martínez-Quintana and Yepiz-Plascencia, 2012; Davis and Liu, 2015; Li and Ju, 2017; Goto *et al.*, 2018; Savitz, 2020) (Figure 3.17c). High concentrations of the components of DNA (thymine, pentose sugars) and metabolites that generate energy in the form of nicotinamide adenine dinucleotide (NAD+) (such as kynurenine) are evidence of healthy and active cellular function (Davis and Liu, 2015). Molecular transport across a cell membrane are highly dependent on lipids and sugars, and the high concentration observed in Loch Ryan oysters would benefit this cellular function. Kyrunines are a key immune system regulator with antimicrobial and anti-inflammatory properties (Savitz, 2020), and D-1,5- anhydrofructase also has a role in immune response by inhibiting inflammasomes, which are intracellular multiprotein complexes that are triggered by presence of pathogens or stress (Li and Ju, 2017; Goto *et al.*, 2018).

The Solent oysters are clearly in a worse condition to the Galway or Loch Ryan oysters. Further investigation into the specific ions and metabolites that differentiate these populations is highly recommended for a more holistic understanding of how metabolomics can help identify the physiological traits of UK *O. edulis* populations.

Interaction between treatments

The effect of oyster origin on clearance rate and respiration rate at local conditions depended on the season. The clearance rates seen in this study fall at the lower end of the range previously recorded for *O. edulis* in similar studies (Walne, 1972; Newell *et al.*, 1977; Rodhouse, 1978; Shumway *et al.*, 1985; Baud *et al.*, 1997; Haure *et al.*, 1998), but the given range is large and dependent on various different experiment conditions that add complexity to any direct comparison (Nielsen *et al.*, 2017). Variation in *O. edulis* clearance rate has been attributed to factors such as algae concentration and species (Hildreth, 1980; Wilson, 1983; Shumway *et al.*, 1985; Hutchinson and Hawkins, 1992), experimental design (Galtsoff, 1926; Drinnan, 1964; Bayne *et al.*, 1976; Hildreth, 1976; Jørgensen, 1976), and abiotic conditions (Newell *et al.*, 1977; Hutchinson and Hawkins, 1992; Haure *et al.*, 1998). It is well documented that clearance rate increases with temperature (Walne, 1972; Newell *et al.*, 1977; Rodhouse, 1978; Buxton *et al.*, 1981; Hutchinson and Hawkins, 1992; Baud *et al.*, 1997; Eymann *et al.*, 2020), but in this study, each oyster population demonstrated a unique seasonal pattern in their clearance rate, not driven by temperature. Only the Loch Ryan oysters demonstrated this recognised pattern with higher clearance rates seen in spring and summer than in autumn and winter. Galway oysters demonstrated the highest clearance rates in Autumn, but the highly variable response from each Galway individual during this season suggests an individual factor is in effect and would need further examination. The Solent oysters maintained a similar mean clearance rate throughout winter, summer and autumn, but reduced to significantly low clearance rates in spring. Sytnik and Zolotnitskiy (2014) similarly observed natural seasonal fluctuations in *O. edulis* clearance and respiration rate and proposed that these physiological processes were independent to the effect of temperature in molluscs. The broad geographical domain of *O. edulis* ranging from Morocco to Scandinavia indicates a natural ability to adapt to varying temperatures and salinities (Bromley *et al.*, 2016a), and this study indicates that different regions and populations possess unique mechanisms to cope with thermal pressure on their physiological function.

The effect of oyster origin on the number of granulocytes (differential haemocyte count (DHC)) depended on season; in comparison to the oysters from Galway and Solent, Loch Ryan oysters had reduced number of granulocytes in all seasons apart from winter, when the number of granulocytes was increased. Granulocytes are known to be heavily involved in phagocytosis (La Peyre *et al.*, 1995; Chu, 2000; Dyachuk, 2016), and show more cellular locomotion than agranulocytes (Fisher, 1988a; McCormick-Ray and Howard, 1991). Therefore, a higher proportion of circulating granulocytes might boost oyster immunity (da Silva *et al.*, 2008). Morga *et al.*, (2009) observed a higher concentration of granulocytes in oysters infected with live *B. ostreae* than those naïve to or with an absence of the parasite, which is reflected in the data in this study; it is possible that the Loch Ryan oysters had less demand for the phagocytic activity offered by granulocytes due to the absence of *B. ostreae*. However, contradictory reports that find reduced percentage of granulocytes in *O. edulis* infected with *B. ostreae* (da Silva *et al.*, 2008) reinforce the concept that *O. edulis* populations around the UK and in Europe must be considered as individual physiological races with different immune responses (Culloty *et al.*, 2004).

The host – parasite interaction is not stable and fluctuations in haemocyte activity and abundance are likely to occur with fluctuations in parasite virulence. *B. ostreae* has demonstrated an ability to neutralise the *O. edulis* immune response in order to multiply and proliferate, either by developing a parasitophorus vacuole to protect itself from destructive enzymes, or by restricting apoptosis of infected host cells (Mauel, 1984; Cheng, 1987; Ferrandon, 2009; Råberg *et al.*, 2009). In addition, parasite virulence and host immunity are heavily dependent on the environment, which is evident from increased mortality of infected oysters during warmer months (Snieszko, 1974; da Silva *et al.*, 2008; Engelsma *et al.*, 2010; Laing *et al.*, 2014).

3.4.2 Adaptation or phenotypic plasticity?

The lab acclimation study was to determine if the effects seen were local adaptation over generations, or whether they were simply plasticity of the phenotype that would disappear when the oysters were held at common conditions. After the initial physiological experiments at local conditions, each oyster batch was acclimated to aquarium conditions over the course of 1 month (which included a temperature change that ranged between 14.4 – 17.6 °C, Table 3.2) with daily water temperature changes of no more than 1 °C. Clearance, respiration rates, condition and haemolymph were similarly measured from these acclimated oysters. It was not possible to collect oyster physiological performance at aquarium conditions during spring, and therefore all spring data were removed from this acclimation physiology comparison study. Oyster condition was significantly reduced after acclimation to common conditions (Figure 3.21), which suggests that the oysters were experiencing some form of stress or deficiency that their local wild environments provide, such as a specific type of algae or a different concentration of nutrients. This highlights the importance of origin for oyster development; local oysters translocating to a restoration area would avoid this stress of acclimation to a foreign water body or change in diet. Further studies might consider observing the changes to oyster growth between populations of local and translocated oysters for fishery and production purposes. Clearance rates post acclimation were also standardised across origins, apart from the Solent oysters during the summer that demonstrated a large variety in response reaching rates up to 7.6 L h^{-1} g $AFDW^{-1}$. Temperature in the aquarium did reach nearly 24 ºC in August, which is a dramatic increase from the temperatures found in the Solent wild (16.8 ºC, Figure 3.18). However, the acclimation temperature change for both Galway (-3 ºC) and Loch Ryan (- 1.9 ºC) for the summer experiments was greater than the Solent and they did not experience a similar variability in clearance rate. This could suggest that the Solent *O. edulis* population have a more diverse physiology than the other two locations. The seasonal fluctuations that occurred at local conditions were removed by the acclimation of all oysters to aquarium conditions. This experiment revealed that both clearance and respiration rates are a plastic phenotype of UK *O.*

edulis, which could be a result of the extensive and historically recent translocation of UK populations.

3.4.3 Conclusion

The aim of this work was to establish the importance of broodstock in physiological performance of *O. edulis* from three UK populations, and to determine if any effects seen were a result of local adaptation or simply phenotypic plasticity. Significant differences between populations were seen at local conditions, but these disappeared at common conditions. Although this suggests that UK *O. edulis* are likely to possess the same genetic profile and have not yet fully adapted to their local geographical regions, there was a significant decrease in body condition post translocation and acclimation to aquarium conditions. This study therefore highlights the importance of using local broodstock not only to restrict the spread of disease, but also to the reduce the stress of individuals as they are transplanted to a new environment for restoration of other restocking purposes. Of the three populations (Galway, Loch Ryan and the Solent), oysters from the Solent had the lowest body condition. These data were corroborated with evidence of increased pollutants and deficiencies of essential metabolites in the same oysters from the Solent. Although the Solent has a history of successful oyster production, these data raise serious concerns for oyster health/physiological function and advises restoration efforts to be implemented elsewhere.

Chapter 4 **An exploration of genetic and metabolomic markers in association with *Bonamia ostreae* infection in *Ostrea edulis***

Note: elements of this chapter have contributed to the publication Holbrook, Z., Bean, T. P., Lynch, S. A. and Hauton, C. (2021) ‘What do the terms resistance, tolerance, and resilience mean in the case of *Ostrea edulis* infected by the haplosporidian parasite *Bonamia ostreae*’, *Journal of Invertebrate Pathology*, 182, doi: 10.1016/j.jip.2021.107579.

4.1 Introduction

One key challenge for *Ostrea* spp. restoration lies in the potential impact of parasites such as the paramyxean *Marteilia refringens* (marteiliosis) and the haplosporidian *Bonamia* spp. (*B. ostreae*, *B. exitiosa*, *B. perspora*, and *B. roughleyi*) (bonamiosis) that have caused mass mortality of oysters worldwide (Culloty and Mulcahy, 2007). Whilst *Bonamia* spp. have not yet reached all countries hoping to restore flat oysters, their distribution across Europe alone within the last 40 years pose a serious and imminent threat to natural and commercial oyster beds. In the absence of any means to eradicate these pathogens, disease control in Europe is achieved by means of biosecurity, with European (EC Council Directive 2006/88/EC) underpinning national restrictions on the movement of diseased stocks. These restrictions attempt to control the spread of disease through the prohibition of movement of parasite-positive oysters from areas of existing disease into new areas with no oysters, or with oysters that are disease free. Some restoration practitioners have proposed benefits in translocating parasite-positive but otherwise healthy stocks, based on the premise that these stocks are likely to reflect the presence of ‘resistant’ or ‘tolerant’ oysters (Smaal *et al.*, 2015; Pogoda *et al.*, 2019). Although disease -resistance, -tolerance and -resilience are superficially familiar terms used to describe the various levels of response shown by an infected individual (Culloty *et al.*, 2004; Bonanno *et al.*, 2015; Gervais *et al.*, 2016; Pardo *et al.*, 2016), and have some formal definition in medical fields, absolute definitions of what these terms mean in individuals and populations are yet to be agreed (Holbrook *et al.*, 2021).

O. edulis previously exposed to *B. ostreae* have demonstrated a tolerance to the infection (Hervio *et al.*, 1995; Culloty and Mulcahy, 2007; Laing *et al.*, 2014), yet no *O. edulis* population has yet demonstrated full resistance (Naciri-Graven *et al.*, 1998; Woolmer *et al.*, 2011). da Silva *et al.* (2005) demonstrated that the level of disease ‘tolerance’ varied depending on family and origin of oyster population, finding higher tolerance from indigenous individuals, implying that local spat selection is important for restoration purposes. Programmes to develop *Bonamia*-‘resistant’ strains of *O. edulis* began in the mid-late 1980s in France (Baud *et al.*, 1997; Naciri-Graven *et al.*, 1998) and Ireland (Culloty *et al.*, 2004; Culloty and Mulcahy, 2007; Lallias *et al.*, 2010; Morga *et al.*, 2012). The Rossmore population of *O. edulis* located in Cork Harbour, Ireland were reported to demonstrate a certain *Bonamia*-'tolerance' as a result of continual exposure to the parasite over several generations (Laing *et al.*, 2005; Sobolewska and Beaumont, 2005). However, a higher prevalence of *B. ostreae* has more recently been observed in the Rossmore population in comparison to neighbouring bays (Cork and Lough Foyle) (Flannery *et al.*, 2016). Morga *et al.* (2017) characterised a reduced parasite burden in 'selected' oysters as a form of parasite resistance mediated through changes in immune performance. 'Selected' oysters demonstrated inhibited phagocytic activity that served to reduce the spread of parasites to wider tissues, whilst the expression of apoptosis-related genes was upregulated. Indeed, molecular responses are likely to be shaped by previous exposure to parasites. However, although often suggested as an essential intervention for the survival of *O. edulis* (Lynch *et al.*, 2014; Pardo *et al.*, 2016), selective breeding has not provided a definitive solution. Efforts of selective breeding in the USA to combat oyster parasite *Haplosporidian nelsoni* (MSX) were overtaken by natural resistance developed by MSX-infected oysters (Encomio *et al.*, 2005; Carnegie and Burreson, 2009; Ford and Bushek, 2012; Lynch *et al.*, 2014). Selective breeding is useful, yet protection is often against only one of many infectious diseases (Burreson and Ford, 2004). Laing *et al.* (2005) have reported that *Bonamia* is the "biggest biological factor" limiting oyster restoration for this species, despite much effort to develop *Bonamia*-resistant populations. It is clear that *Bonamia* resistance would benefit *O. edulis* populations and perhaps should be the focus of strategy for restoration projects (Laing *et al.*, 2006; Lallias *et al.*, 2010; Woolmer *et al.*, 2011). Many contemporary studies have started to explore the expression of proteins, genes, and micro-RNAs associated with phagocytosis, respiratory burst, and apoptosis and have compared *Bonamia*-naïve and *Bonamia*-exposed oysters to identify underlying mechanisms that might support a differential phenotype (Morga *et al.*, 2012, 2017; Martín-Gómez *et al.*, 2014; Gervais *et al.*, 2016; Pardo *et al.*, 2016; de la Ballina *et al.*, 2018; Ronza *et al.*, 2018; Vera *et al.*, 2019). In oysters, phagocytosis, respiratory burst, and apoptosis are three of several cellular mechanisms that have been associated with disease-resistance (Cheng, 1981; da Silva *et al.*, 2009; Comesaña *et al.*, 2012; Martín-Gómez *et al.*, 2012; Morga *et al.*, 2012), but phagocytosis also represents the mechanism by which the parasite *Bonamia* spp. gains entry to the haemocytes to facilitate the infection cycle. As such, oyster haemocytes are both the target tissue, and host defence against *B. ostreae* (Comesaña *et al.*, 2012; Gervais *et al.*, 2016). Past studies have concluded that *B. ostreae* can modulate the host immune response for its own benefit through the inhibition of the respiratory burst and phagocytosis (Morga *et al.*, 2009, 2011; Comesaña *et al.*, 2012), which might observationally be interpreted as tolerance, or even a mechanism of parasite virulence. Broad markers of resistance can be found in previously exposed populations by identifying significant selective sweeps in the genome of a population (Vera *et al.*, 2019). However, these same phenomena can make it difficult to characterise the specific biological mechanisms. In addition, a natural system is likely to experience the dilution of resistance genes as unexposed (susceptible) stock can often contribute to progeny of exposed areas, for example through the movement of gametes or larvae through the water column (Culloty *et al.*, 2001; Flannery *et al.*, 2014), and through the sale and consumption of resistant oysters before they contribute genes to future generations (Lauckner, 1983). Therefore, when analysing either the genetic architecture of disease resistance, or the biology of disease progression, it is often preferable to work with populations of oyster that have a highly structured population, have not been exposed to the parasite and can be challenged in a controlled manner (e.g. Hervio *et al.*, 1995). In doing so, variables associated with previous exposure (such as immune priming and selective sweeps) are accounted for (Vera *et al.*, 2019).

4.1.1 Molecular basis of oyster immunity

The history of extensive global translocation and consequential genetic interconnectivity of the species has added complexity to the genetic structure of the European *O. edulis* population. In addition, the use of different experiment techniques (allozyme markers v. microsatellite markers) has resulted in conflicting genetic profiles (Launey *et al.*, 2002). A genetic homogeneity of the entire European *O. edulis* population has been proposed (Launey *et al.*, 2002; Laing *et al.*, 2005; Sobolewska and Beaumont, 2005; Beaumont *et al.*, 2006; Lallias *et al.*, 2010; Bromley *et al.*, 2016a), but Vera *et al.* (2016) identified three distinct genetic profiles within the European *O. edulis* population and suggested they should remain independent of one another: (1) Denmark and The Netherlands, (2) England, Ireland and France, and (3) Spain. Similarly, genetic differences have been observed between Mediterranean and Atlantic *O. edulis* populations (Jaziri, 1990; Saavedra *et al.*, 1995).

A number of genes associated with oyster immunity have been identified as potential markers of *Bonamia*-resistance in oysters and have inspired debate into the possibility of pathogen recognition mechanisms in invertebrates (Morga *et al.*, 2012; Ronza *et al.*, 2018). Morga *et al.*, (2012) identified seven expressed sequence tags (ESTs) as potential markers of *Bonamia*-reistance: Inhibitor of apoptosis (*OeIAP*), fas-ligand (*OeFAS*), cathepsin B (*Cathep*), extracellular superoxide dismutase (*OeEcSOD*), ferritin (*Oefer*), C1q (*OeC1q*), and Cyclophylin B (*Oepepti*). Morga *et al.* (2012) observed higher expression of *OeIAP*, *OeFAS* and *OeEcSOD* in *Bonamia*-‘resistant’ oysters in comparison to wild oysters. Despite establishing the stability of five appropriate endogenous reference genes (ERGs) two years prior (elongation factor 1-α (*Ef1- α*), 60S ribosomal protein L5 (*L5*), glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*), polyubiquitin (*Ubiq*), and β-actin (*ACT*)) (Morga *et al.*, 2010), Morga *et al.* (2012) chose to use only one ERG in their analysis, which does not comply with current MIQE (Minimal Information for Publication of Quantitative Real-Time PCR Experiments) requirements and questions the stability/robustness of their argument in suggesting seven genes (*OeEcSOD*, *OeIAP*, *OeFAS*, *Cathep*, cyclophilin, ferritin and *Oe-C1q*) as reliable markers of *Bonamia*-resistance in oysters (Bustin *et al.*, 2009). In addition, Morga *et al.*'s (2012) analysis was conducted at only one time point, thus failing to acknowledge any seasonal effects such as the highly documented influence of temperature on pathogen prevalence and spread (Snieszko, 1974; Hine, 1991; Carnegie *et al.*, 2008). As such, Morga *et al.* (2012) only provides a snapshot into gene expression at one event, overlooking any seasonal variation. Therefore, the imperative was to test the findings of Morga *et al.* (2012) in different populations, during a seasonal cycle throughout the year 2018, and using multiple ERGs, which has not been attempted before.

4.1.2 Exploration of metabolomics to understand infection history

Metabolomics, the study of metabolites in an individual organism, is emerging as a useful tool in science and in medicine, and is already being widely used for application in pharmaceuticals and biomedical medical procedures through recognition of specific metabolic markers (Wishart, 2016). A biological marker, or biomarker, is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes” (Atkinson *et al.*, 2001; Montgomery and Brown, 2013). Metabolic markers are more frequently being used in precision medicine through development of personalised phenotyping and individual drug-response monitoring to detect early indicators of probable age-related conditions (Beger *et al.*, 2010; Wishart, 2016; Liu *et al.*, 2018). In fact, detection of abnormalities in specific biomarkers now help early diagnosis of Metabolic Syndrome in humans, which is associated with obesity and diabetes, and indicates a high risk of mortality as a result of cardiovascular disease, heart disease, stroke, and vascular dysfunction (Montgomery and Brown, 2013; Gao *et al.*, 2016; Srikanthan *et al.*, 2016). The growing information on metabolomics and an appreciation of a healthy human metabolomic profile means abnormal metabolites and cellular functions can be more rapidly identified and doctors can prescribe preventative measures (Atkinson *et al.*, 2001; Albert and Tang, 2018). Developing this same technique for early detection of diseases such as *B. ostreae* in *O. edulis* or metabolic markers of disease (bonamiosis) resistance would be extremely beneficial for restoration or culture of this species. As discussed in Chapter 3, a metabolic profile offers a physiological insight to the types of environmental conditions and pressures to which an individual organism has been exposed (Young *et al.*, 2015; Alfaro and Young, 2018). Metabolites arise from both external sources such as toxins and microbes, and from physiological processes generated by the host (Albert and Tang, 2018). The application of metabolomics to invertebrate, and specifically bivalve mollusc, studies is only beginning to be prosecuted (Kuehnbaum and Britz-Mckibbin, 2013; Kuhlisch and Pohnert, 2015; Alfaro and Young, 2018; Eymann *et al.*, 2020). However, this relatively new science has already shown promise within the aquaculture sector that relies upon rapid identification of stressors such as disease and nutrition deficiencies that the crop species might be experiencing (Alfaro and Young, 2018). Metabolite profiles help to establish and confirm change in organism phenotype that are suggested by gene expression (Kuehnbaum and Britz-Mckibbin, 2013; Young *et al.*, 2015). Irregular metabolic profiles have already been strongly linked with disease severity (Chace *et al.*, 1998; Kuehnbaum and Britz-Mckibbin, 2013), and metabolomics has also been used to identify stressed mollusc larvae (Young *et al.*, 2015, 2016). These findings suggest the use of metabolomics could be useful for understanding the metabolic demands placed on a bivalve host by an infectious microorganism.

Subsequent to the population scale study, a targeted approach was used to compare the expression of six genes associated with *Bonamia*-‘resistance’ between *Bonamia*-infected (n = 8) and non-infected (n = 40) *O. edulis* individuals to determine if they were robust markers of disease. This complementary work focussed on the Solent and Loch Ryan oysters from Chapter 3 (see Table 3.1). The metabolic profile of a larger group of oysters from the Solent, Loch Ryan and Galway (n = 114, extended from Chapter 3) was analysed to determine if metabolic profiles differed between groups (*Bonamia*-infected (n = 14) and non-infected (n = 100)) and to identify potential metabolic markers of infection status.

Aim

The aim was to confirm the use of genetic markers of resistance by comparing *Bonamia*-infected and non-infected oysters from Loch Ryan and the Solent using MIQE compliant relative quantitative PCR to establish changes in gene expression. Thereafter to explore the metabolic demands of infection by comparing the metabolite profiles of the same two groups (*Bonamia*-infected and non-infected) to identify potential markers of a disrupted metabolism associated with parasite infection.

Objectives

1. To quantify the expression of six genes (*OeIAP*, *OeEcSOD*, *OeC1q*, *OeFAS*, *Ubiq*, and *ACT*) previously identified by Morga *et al.* (2012) as an indication of ‘*Bonamia*-resistance’ in *Bonamia*-infected and non-infected oysters from Loch Ryan and the Solent using multiple (4) ERGs (*GAPDH*, *Ef1- α*, *L5*, and *Cathep*) by quantitative PCR (qPCR).
2. To explore the metabolic profile of oysters from the Solent, Loch Ryan, and Galway with and without a *B. ostreae* infection.

4.2 Methods

4.2.1 Oysters and parasite (Bonamia ostreae) burden

All Loch Ryan and Solent oysters from Chapter 3 were used for this study (see section 3.2.1). DNA was extracted from gill tissues using QIAGEN™ DNeasy Blood and Tissue Kit, and then parasite DNA was amplified using primers by Ramilo *et al.* (2013) in a PCR. Amplified DNA was size fractionated by gel electrophoresis and presence of *B. ostreae* was identified through amplicon size (208 bp) (Ramilo *et al.*, 2013). *B. ostreae* was found present in 8 of the 48 oysters (17%) (as seen in 3.3.2).

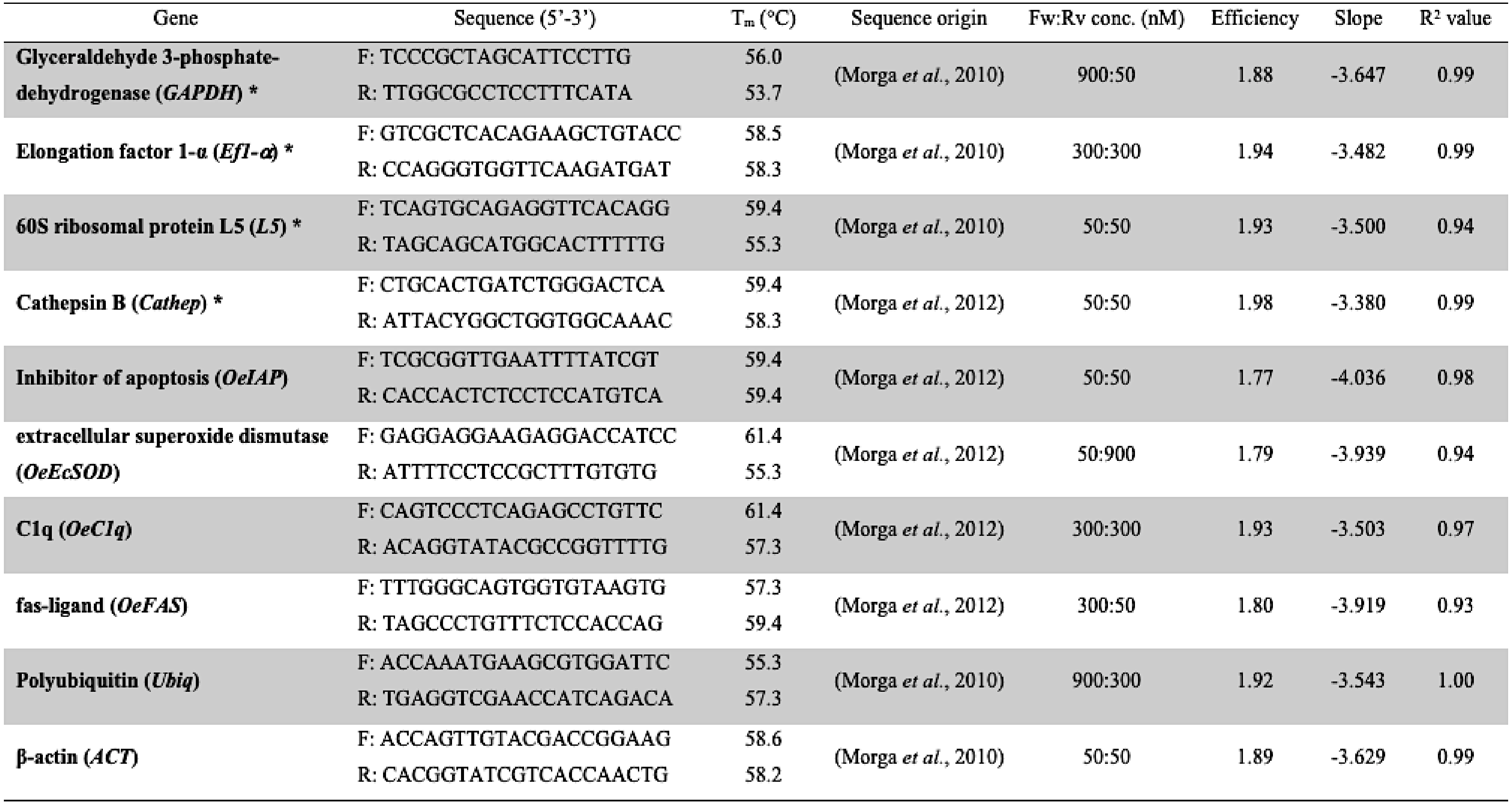
4.2.2 Expression of genes associated with Bonamia-resistance

Total RNA was extracted from gill tissue (acquired as in 3.2.2) using TriReagent (see Appendix D). Twelve samples were used to test RNA integrity using a BioRad Experion^{TM} automated electrophoresis assay, which returns an RNA quality indicator (RQI) (Bio-Rad Technical note 5761) of between 1 (most degraded RNA) and 10 (most intact RNA) (Riedmaier *et al.*, 2010). The 12 samples chosen all returned RQI values of 10, confirming that the method of tissue preservation and then total RNA extraction were appropriate to isolate high quality intact total RNA templates. Reverse transcription (RT) was achieved using SuperScript III™ reverse transcriptase (Invitrogen) as recommended by Udvardi, Czechowski and Scheible (2008), to produce complementary DNA (cDNA). To follow MIQE compliance as closely as possible, four reference genes specific to *O. edulis* were used for the quantitative polymerase chain reaction (qPCR) to quantify the expression of six genes associated with *Bonamia* resistance as described by (Morga *et al.*, 2012) (Table 4.1).

The qPCR assay was optimised by first manipulating the primer concentration to find the best combination for an early and clear quantification cycle (C_q) value. The C_q gives the number of PCR cycles before a clear signal can be detected in the samples, and an earlier signal is preferable as this improves assay sensitivity. The chosen primer concentration was then taken forward to create a standard curve with 10-fold dilution of cDNA to measure the efficiency of each gene assay (Table 4.1).

All qPCR analysis was achieved using qbase+ software (Biogazelle), which is based on geNorm (Vandesompele *et al.*, 2002) and qBase technology (Hellemans *et al.*, 2007). Four reference genes were used for the qPCR procedure to comply with MIQE standard requirements; glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*), Elongation factor 1 alpha (*Ef1-*α), 60S ribosomal protein L5 (*L5*), established for *Ostrea edulis* assays by (Morga *et al.*, 2010), and Cathepsin B (*Cathep*). Morga *et al.* (2012) found the expression of *Cathep* to be significantly increased in ‘resistant’ oysters, yet the expression of this gene remained stable across our samples and could be used as a reference gene.

Table 4.1. The 10 genes used for analysis, with respective sequences and melting temperature (T_m). Chosen concentrations for the forward and reverse primers (Fw:Rv conc, nM), and efficiency of qPCR assay, slope and R^2 value for each gene. Reference genes are marked with asterisk '*'.



Gene	Sequence (5'-3')	T_m (°C)	Sequence origin	Fw:Rv conc. (nM)	Efficiency	Slope	R^2 value
Glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*) *	F: TCCCGCTAGCATTCCTTG	56.0	(Morga *et al.*, 2010)	900:50	1.88	-3.647	0.99
	R: TTGGCGCCTCCTTTCATA	53.7					
Elongation factor 1-α (*Ef1-α*) *	F: GTCGCTCACAGAAGCTGTACC	58.5	(Morga *et al.*, 2010)	300:300	1.94	-3.482	0.99
	R: CCAGGGTGGTTCAAGATGAT	58.3					
60S ribosomal protein L5 (*L5*) *	F: TCAGTGCAGAGGTTCACAGG	59.4	(Morga *et al.*, 2010)	50:50	1.93	-3.500	0.94
	R: TAGCAGCATGGCACTTTTTG	55.3					
Cathepsin B (*Cathep*) *	F: CTGCACTGATCTGGGACTCA	59.4	(Morga *et al.*, 2012)	50:50	1.98	-3.380	0.99
	R: ATTACYGGCTGGTGGCAAAC	58.3					
Inhibitor of apoptosis (*OeIAP*)	F: TCGCGGTTGAATTTTATCGT	59.4	(Morga *et al.*, 2012)	50:50	1.77	-4.036	0.98
	R: CACCACTCTCCTCCATGTCA	59.4					
extracellular superoxide dismutase (*OeEcSOD*)	F: GAGGAGGAAGAGGACCATCC	61.4	(Morga *et al.*, 2012)	50:900	1.79	-3.939	0.94
	R: ATTTTCCTCCGCTTTGTGTG	55.3					
C1q (*OeC1q*)	F: CAGTCCCTCAGAGCCTGTTC	61.4	(Morga *et al.*, 2012)	300:300	1.93	-3.503	0.97
	R: ACAGGTATACGCCGGTTTTG	57.3					
fas-ligand (*OeFAS*)	F: TTTGGGCAGTGGTGTAAGTG	57.3	(Morga *et al.*, 2012)	300:50	1.80	-3.919	0.93
	R: TAGCCCTGTTTCTCCACCAG	59.4					
Polyubiquitin (*Ubiq*)	F: ACCAAATGAAGCGTGGATTC	55.3	(Morga *et al.*, 2010)	900:300	1.92	-3.543	1.00
	R: TGAGGTCGAACCATCAGACA	57.3					
β-actin (*ACT*)	F: ACCAGTTGTACGACCGGAAG	58.6	(Morga *et al.*, 2010)	50:50	1.89	-3.629	0.99
	R: CACGGTATCGTCACCAACTG	58.2					

4.2.3 Metabolic profiling

Tissue samples were taken from the hepatopancreas (HP) (see section 3.2.2, Figure 3.1), flash frozen in liquid nitrogen and placed in -80 ˚C. HP tissue samples of oysters from the Solent (n = 36), Loch Ryan (40), and Galway (n = 38) were sent to the McCullagh Metabolomics Laboratory for untargeted Metabolomics, Department of Chemistry at the University of Oxford for analysis. Each sample was analysed using three methods (ion exchange (IC-MS/MS), C18-reverse phase chromatography (C18-MS/MS), and derivatised C18-reverse phase chromatography (dC18-MS/MS)) as reported in section 3.2.2.

4.2.4 Statistical analysis

The qBase+ software first ranked the endogenous reference genes in order of expression stability, by comparing the ratio of each gene with the other reference genes and giving it a value of gene-stability (*M)*. A low *M* value reflects a more consistent ratio between the control genes across all samples regardless of experimental condition and a more desirable ERG (Vandesompele *et al.*, 2002) (the four genes with lowest *M* can be found in Appendix E, Figure E1). The qBase+ programme then determined the minimal number of genes required for use as ERGs, by comparing the change in expression of the genes of interest (GOIs) based on the geometric mean expression of the ERGs. This change in expression with the addition of each ERG is given as a value of *V* (see Appendix E, Figure E2). Vandesompele *et al.* (2002) suggested a cut off V value of 0.15, below which the addition of a further ERG is not required. For this study, four ERGs (*GAPDH*, *Ef1- α*, *L5*, and *Cathep*) were required.

All statistical analyses was conducted in the R environment (R Core Team, 2018) with packages ggbiplot, lsmeans, ggplot2 and devtools (Wickham, 2009; Vu, 2011; Lenth, 2016; Wickham *et al.*, 2018). Figures with the raw relative expression values for each gene can be found in Appendix E (Figures E3-E12). Genes were first analysed individually and a linear model (LM) or generalised linear model (GLM) was fitted to the data where appropriate. Principal components analysis (PCA) was then used to find any correlations between expression of all six genes, achieved through new variables (principal components (PCs)) derived by the dataset itself. PCA does this by dismantling the original data of its dimensions and forming new linear relationships (PCs) that maximise variance and remain unrelated to one another.

The metabolomics data were collected and analysed as seen in section 3.2.3. Four pieces of data were collected for each ion measured by each of the three methods (IC-MS/MS, C18-MS/MS and dC18-MS/MS): retention time, accurate mass, abundance and fragmentation patter. However, whilst identifying the structure of every ion fragment or compound detected is at least technically feasible, a complete metabolomics analysis is beyond the scope of this thesis. Individual ion data were clustered using a PCA, and a partial least squares discrimination analysis (PLS-DA) to maximise the separation between groups of samples by iteratively removing one type of data (one of the four above) to generate a less confounded model. Permutation analysis was used to test the validity of the PLS-DA for the three methods (IC-MS/MS, C18-MS/MS and dC18-MS/MS). Each ion was given a unique identification number based on retention time and accurate mass, and those that contributed the greatest ‘difference’ between the two groups (*Bonamia*-infected and non-infected oysters), and could be identified, were visualised in a variable importance plot. Finally, a receiver operating characteristic (ROC) curve was created for the top two ion features for each of the three methods (IC-MS/MS, C18-MS/MS and dC18-MS/MS) by plotting the true positive rate (TPR) against the false positive rate (FPR) at various threshold settings. This final analysis sought to determine the value of an ion feature as a predictor of *Bonamia*-infection.

4.3 Results

The expression of 6 genes (*OeIAP*, *OeEcSOD*, *OeC1q*, *OeFAS*, *Ubiq*, and *ACT*) was compared between *Bonamia*-infected and non-infected oysters irrespective of season or origin (Solent / Loch Ryan) to determine if they were significant markers of infection. Each gene was analysed individually, before correlations were made between the whole data using a principal components analysis (PCA).

4.3.1 The effect of Bonamia-infection on gene expression

The presence of *B. ostreae* had a significant effect on the variability in the calibrated normalised relative quantity (CNRQ) of expressed *OeC1q* (GLM, $F_{1,63} = 12.406$, $P < 0.001$) (Figure 4.1), *OeFAS* (GLM, $F_{1,63} = 17.713$, $P < 0.001$) (Figure 4.2), and *ACT* (GLM, $F_{1,63} = 7.859$, $P = 0.007$) (Figure 4.3). Genes *OeIAP*, *OeEcSOD*, and *Ubiq* did not consistently discriminate infection.

Adding the effect of season confounded the response to infection for both *OeC1q* (GLM, $F_{2,59} = 4.741$, $P = 0.012$) (Figure 4.4) and *OeFAS* (GLM, $F_{2,59} = 5.776$, $P = 0.005$) (Figure 4.5); Significantly higher expression of *OeC1q* and *OeFAS* was seen in *Bonamia*-positive individuals in comparison to non-infected individuals for each season apart from in the Autumn, when lower expression was seen in *Bonamia*-positive individuals. No *Bonamia*-infections were detected by traditional PCR in Winter.

Figure 4.1

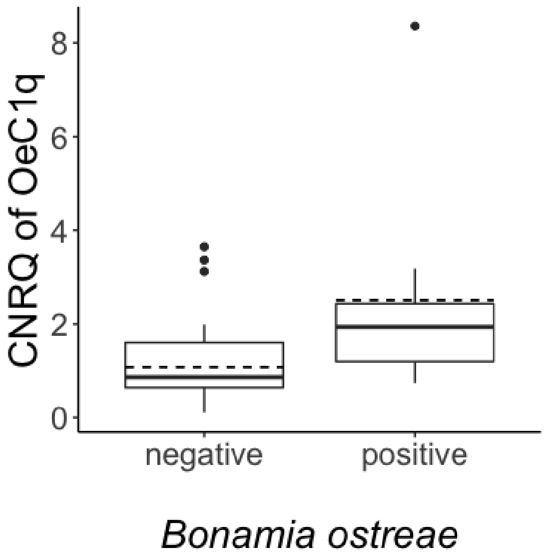


Figure 4.2

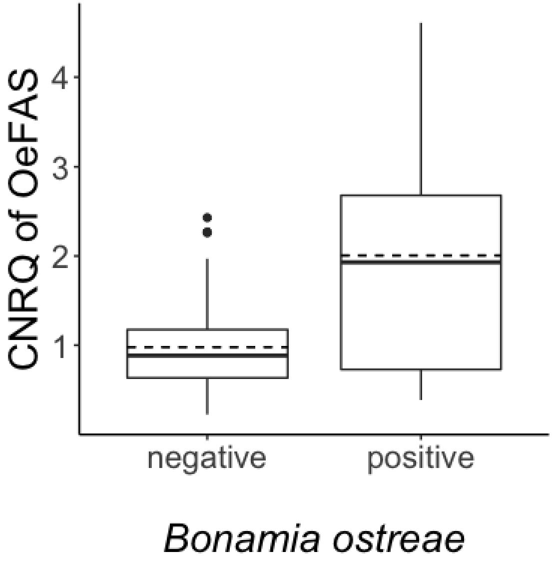


Figure 4.3

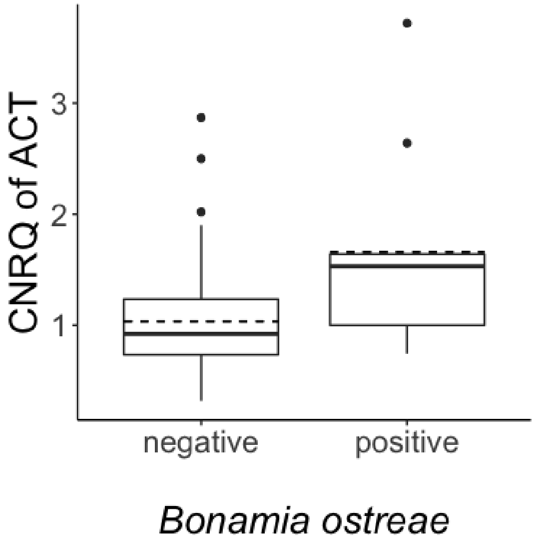


Figure 4.1, Figure 4.2, and Figure 4.3. Presence *of Bonamia ostreae* had an effect on the calibrated normalised relative quantity (CNRQ) of *OeC1q* (GLM, $F_{1,63} = 12.406$, $P < 0.001$) (Figure 4.1), *OeFAS* (GLM, $F_{1,63} = 17.713$, $P < 0.001$) (Figure 4.2), and *ACT* (GLM, $F_{1,63} = 7.859$, $P = 0.007$) (Figure 4.3). The solid and dashed lines represent the median and mean averages respectively (n = 8:40, *B. ostreae* +ve : *B. ostreae* -ve).

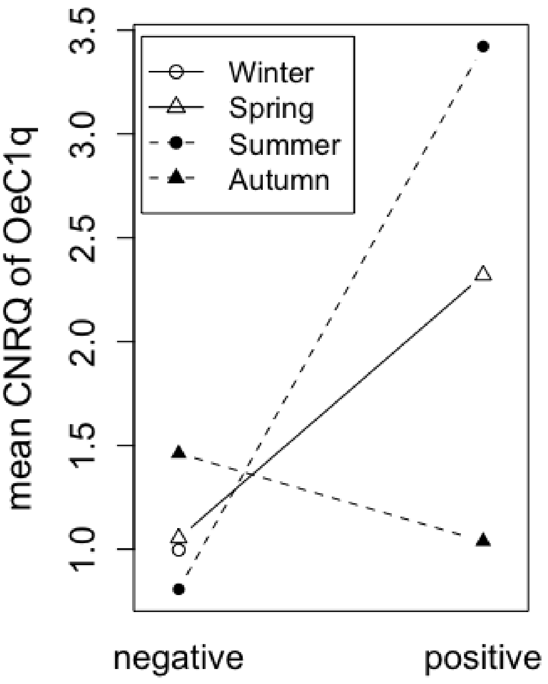


Figure 4.4. The effect of *Bonamia ostreae* infection on calibrated normalised relative quantity (CNRQ) of *OeC1q* depended on season. Symbols (circles and triangles) represent the mean, winter n = 16:0, spring n = 15:1, summer n = 11:5, autumn n = 13:3 (*B. ostreae* -ve : *B. ostreae* +ve, respectively)

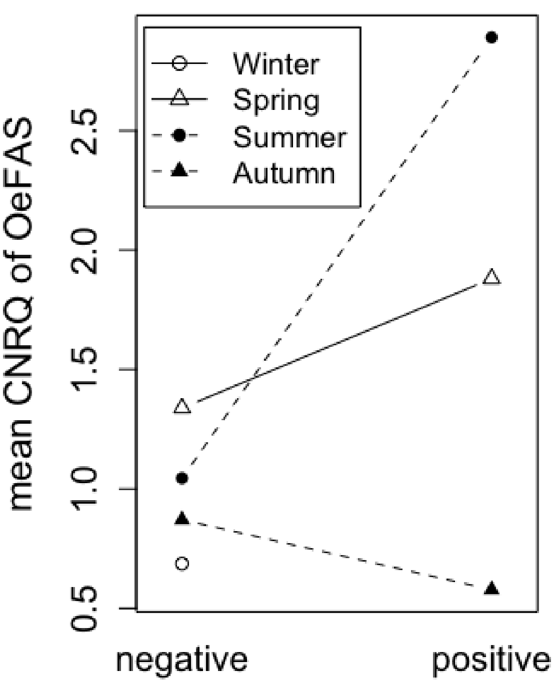


Figure 4.5. The effect of *Bonamia ostreae* infection on calibrated normalised relative quantity (CNRQ) of *OeFAS* depended on season. Symbols (circles and triangles) represent the mean, winter n = 16:0, spring n = 15:1, summer n = 11:5, autumn n = 13:3 (*B. ostreae* -ve : *B. ostreae* +ve, respectively)

4.3.2 Correlation in gene expression between oysters

A principal component analysis (PCA) was conducted to explore the correlation between expression of the six genes (*Ubiq*, *ACT*, *OeIAP*, *OeEcSOD*, *OeC1q* and *OeFAS*) in oysters with and without a *B. ostreae* infection. The data were normalised to account for the different variance within each gene, and then a PCA was performed using the prcomp command in R (R Core Team, 2018). The first two principal components (PCs) accounted for 35.9% and 27.0% of variation respectively, and this correlation matrix was visualised with a biplot (Figure 4.6) with points to represent oyster individuals and arrows to represent the variable markers (increasing values in the arrow direction). The most highly correlated genes were *OeIAP* and *Ubiq* (correlation = 0.71, *P* = 4.992e-11), which were also highly correlated with PC1 (represented by the horizontal (x) axis, Figure 4.6). *OeC1q* and *OeFAS* were correlated (correlation = 0.43, *P* = 0.0004) and also strongly correlated with PC2 (represented along the vertical (y) axis, Figure 4.6).

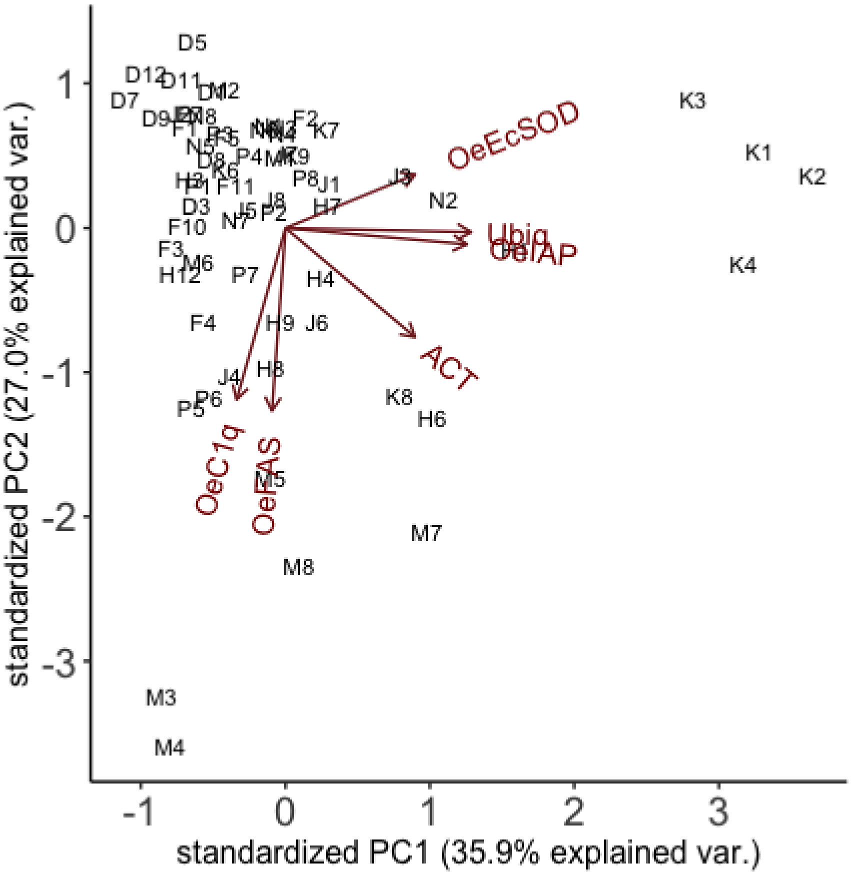


Figure 4.6. A correlation matrix derived from a principal components analysis (PCA) to observe the spread of (normalised) data (letter and number combinations represent individual oysters, see Table 3.1 in Chapter 3), characterised by expression of six genes (represented with red arrows). These data are all oysters irrespective of disease status. *Ubiq* and *OeIAP* are highly correlated with principal component (PC) 1 (horizontal axis) while *OeC1q* and *OeFAS* are highly correlated with PC2 (vertical axis).

Grouping the data on the biplot by disease status (Figure 4.7) showed the strength of these effects on the expression of the six genes in question. Interestingly, oysters infected with *B. ostreae* were characterised more by their expression of *OeC1q* and *OeFAS* than by *OeEcSOD*, *Ubiq* or *OeIAP* (Figure 4.7).

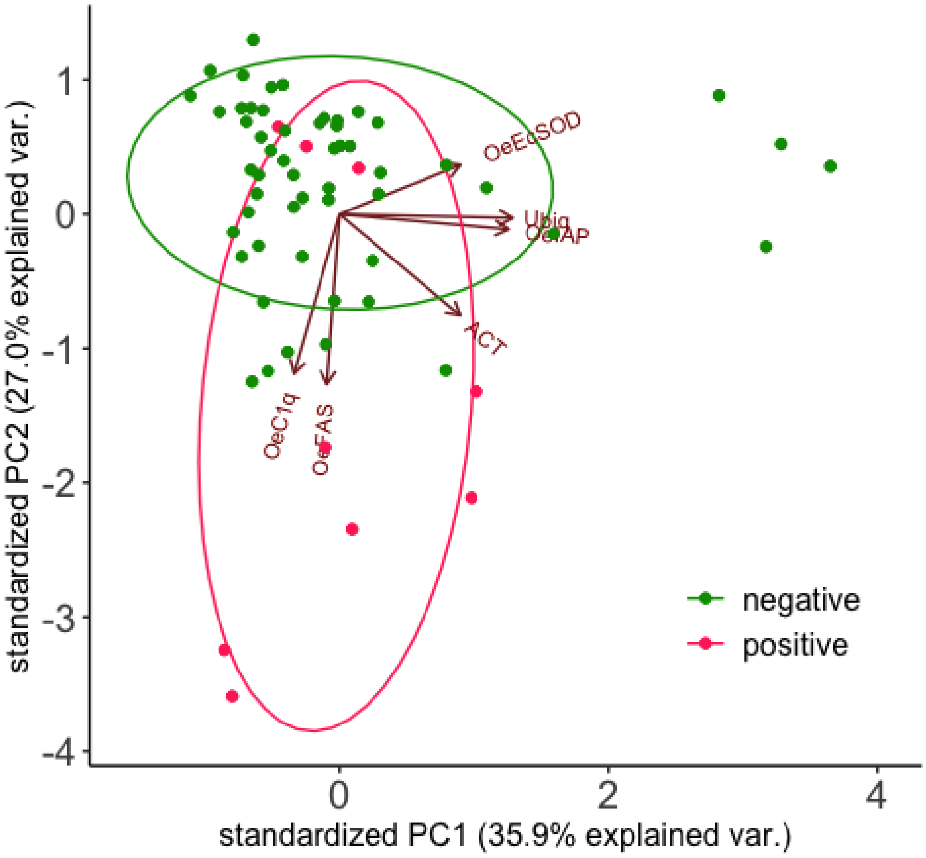


Figure 4.7. A correlation matrix derived from a principal components analysis (PCA) to observe the spread of (normalised) data, characterised by expression of six genes (represented with red arrows) and grouped by disease status (*Bonamia*-negative and -positive individuals in green and red respectively). The ellipses represent a 68% confidence level. Oysters infected with *B. ostreae* are more characterised by their expression of *OeC1q* and *OeFAS* than by *OeEcSOD*, *Ubiq* and *OeIAP*.

4.3.3 Exploring the effect of disease (Bonamia ostreae) infection status on metabolic profiles

Four pieces of data were collected for each ion measured by the three methods used to establish the metabolic profile for each oyster (see 3.2.2). A principal components analysis (PCA) was performed to combine these data for each individual, and a partial least squares discrimination analysis (PLS-DA) was used to maximise the separation between the *Bonamia*-infected and non-infected groups for each of these three methods (IC-MS/MS (Figures 4.8 and 4.9), C18-MS/MS (Figures 4.10 and 4.11) and dC18-MS/MS (Figures 4.12 and 4.13).

Figure 4.8

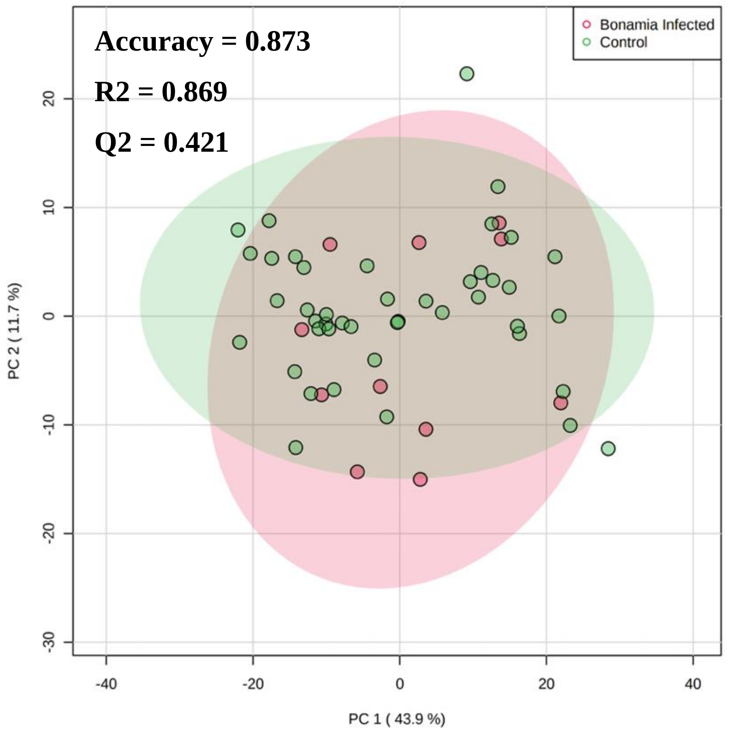


Figure 4.9

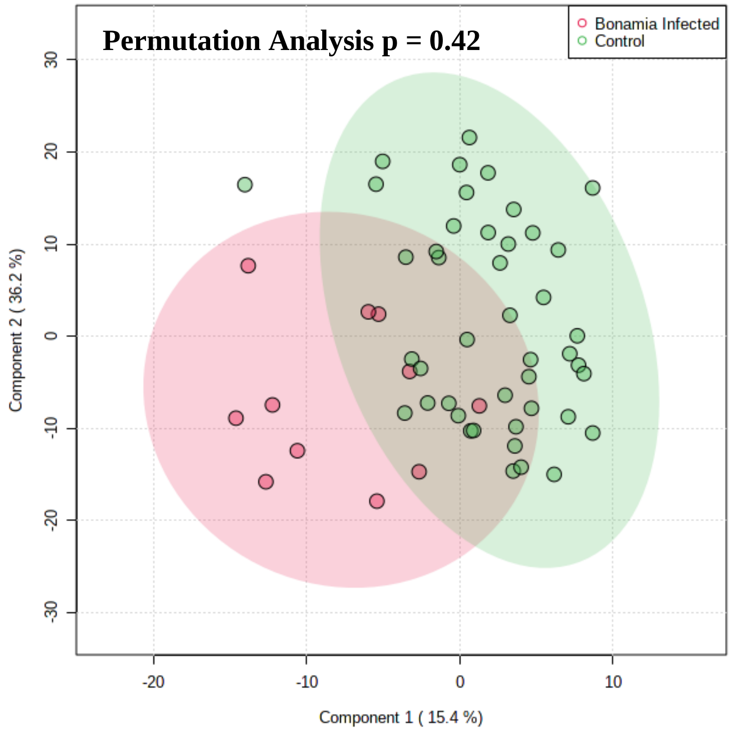


Figure 4.8 and Figure 4.9. Principal components analysis (PCA) (Figure 4.8) and partial least squares discrimination analysis (PLS-DA) (Figure 4.9) to show the contribution of individual ions to the magnitude of change noted between the 2 groups (*Bonamia*-infected and non-infected *Ostrea edulis* individuals). Statistical analysis for the top ion features for the ion exchange method (IC-MS/MS). Statistical analysis quantifies the potential strength of the mathematical model that can be constructed (R2) and compares to the actual data collected (Q2). Permutation analysis was used to determine whether PLS-DA was an appropriate analysis technique to determine the validity of this analysis method. Figures created by D. Hauton at the University of Oxford.

Figure 4.10

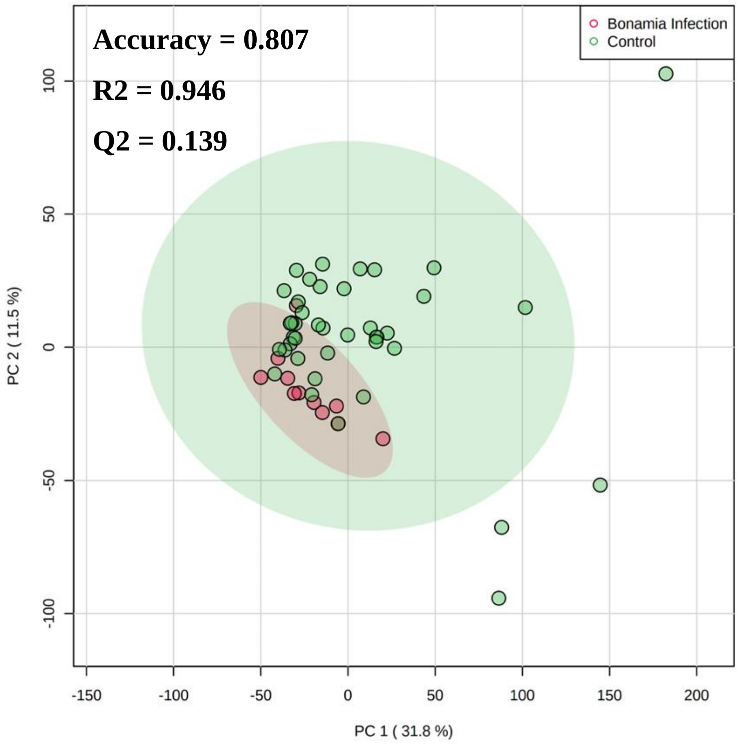


Figure 4.11

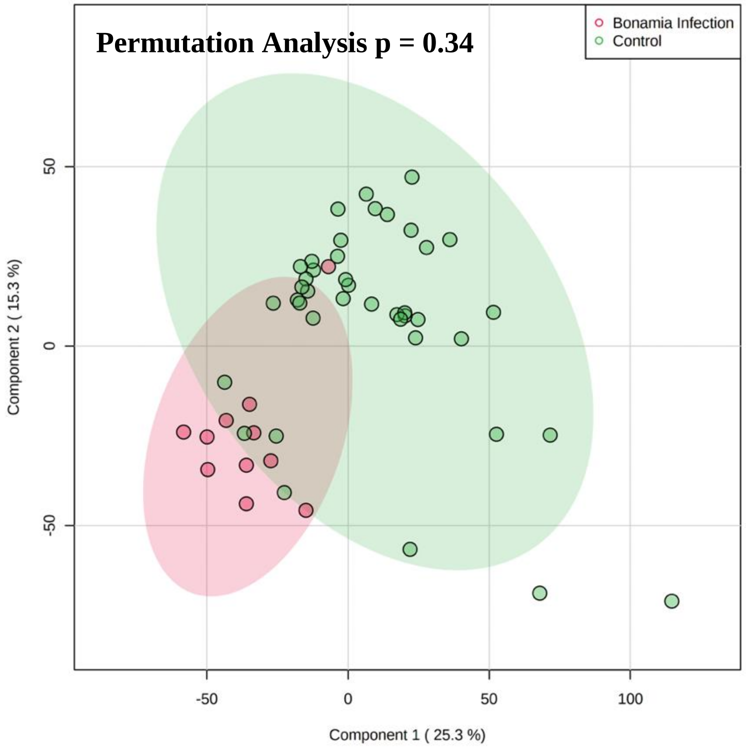


Figure 4.10 and Figure 4.11. Principal components analysis (PCA) (Figure 4.10) and partial least squares discrimination analysis (PLS-DA) (Figure 4.11) to show the contribution of individual ions to the magnitude of change noted between the 2 groups (*Bonamia*-infected and non-infected *Ostrea edulis* individuals). Statistical analysis for the top ion features for the C18-reverse phase chromatography method (C18-MS/MS). Statistical analysis quantifies the potential strength of the mathematical model that can be constructed (R2) and compares to the actual data collected (Q2). Permutation analysis was used to determine whether PLS-DA was an appropriate analysis technique to determine the validity of this analysis method. Figures created by D. Hauton at the University of Oxford.

Figure 4.12

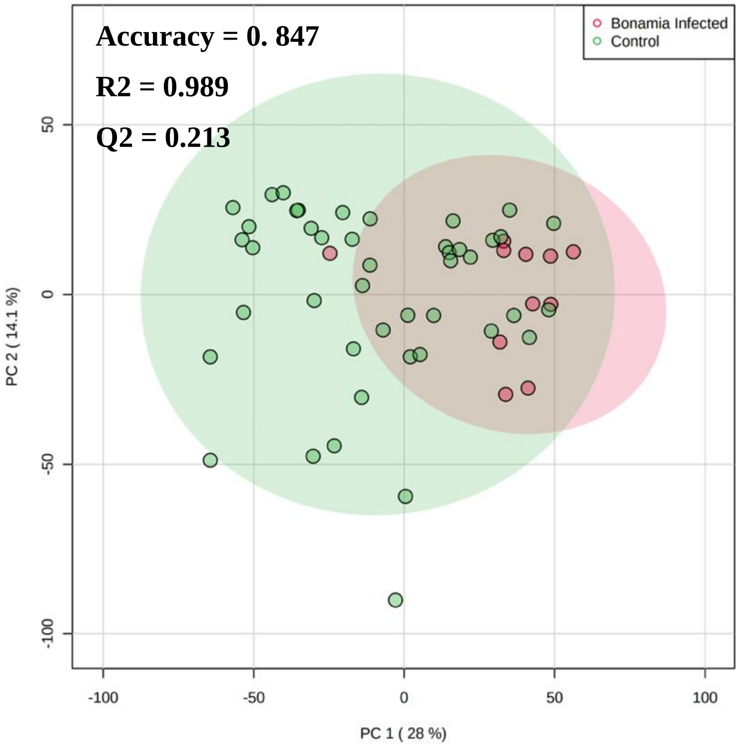


Figure 4.13

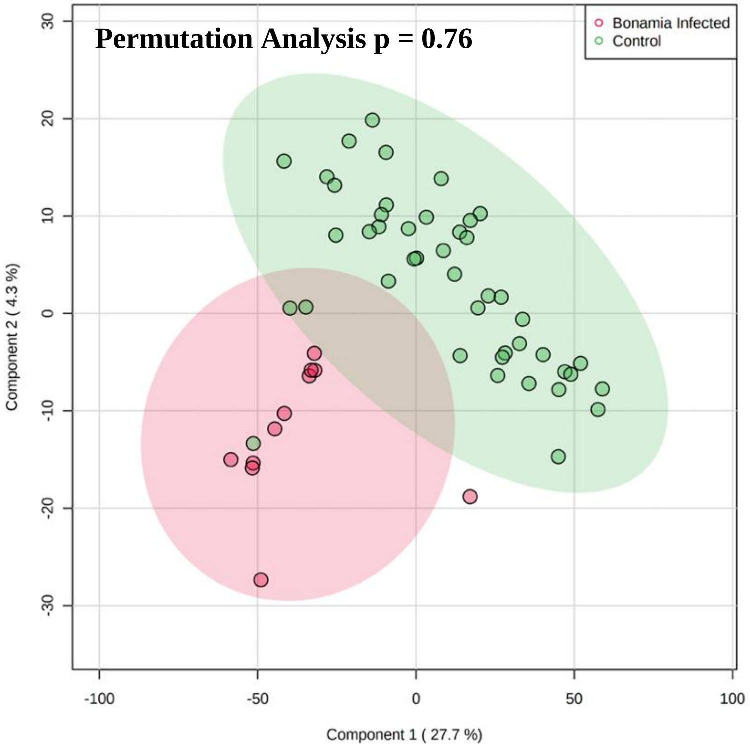


Figure 4.12 and Figure 4.13. Principal components analysis (PCA) (Figure 4.12) and partial least squares discrimination analysis (PLS-DA) (Figure 4.13) to show the contribution of individual ions to the magnitude of change noted between the 2 groups (*Bonamia*-infected and non-infected *Ostrea edulis* individuals). Statistical analysis for the top ion features for the derivatised C18-reverse phase chromatography method (dC18-MS/MS). Statistical analysis quantifies the potential strength of the mathematical model that can be constructed (R2) and compares to the actual data collected (Q2). Permutation analysis was used to determine whether PLS-DA was an appropriate analysis technique to determine the validity of this analysis method. Figures created by D. Hauton at the University of Oxford.

The ions that contributed the greatest difference between the two groups (*Bonamia*-infected and non-infected oysters) could be identified by their name or unique number, and were visualised in Variable Importance Plots; only methods that identified specific ion names are visualised below (IC-MS/MS, Figure 4.14) and dC18-MS/MS (Figure 4.15). Identified ions were Ciceritol, 1,4-Ipomeadiol, Phenyl vinyl s (sulphide), and Pyrimidine nuc (nucleotide) using the IC-MS/MS method (Figure 4.14), and Dehydrospermin using the dC18-MS/MS method (Figure 4.15).

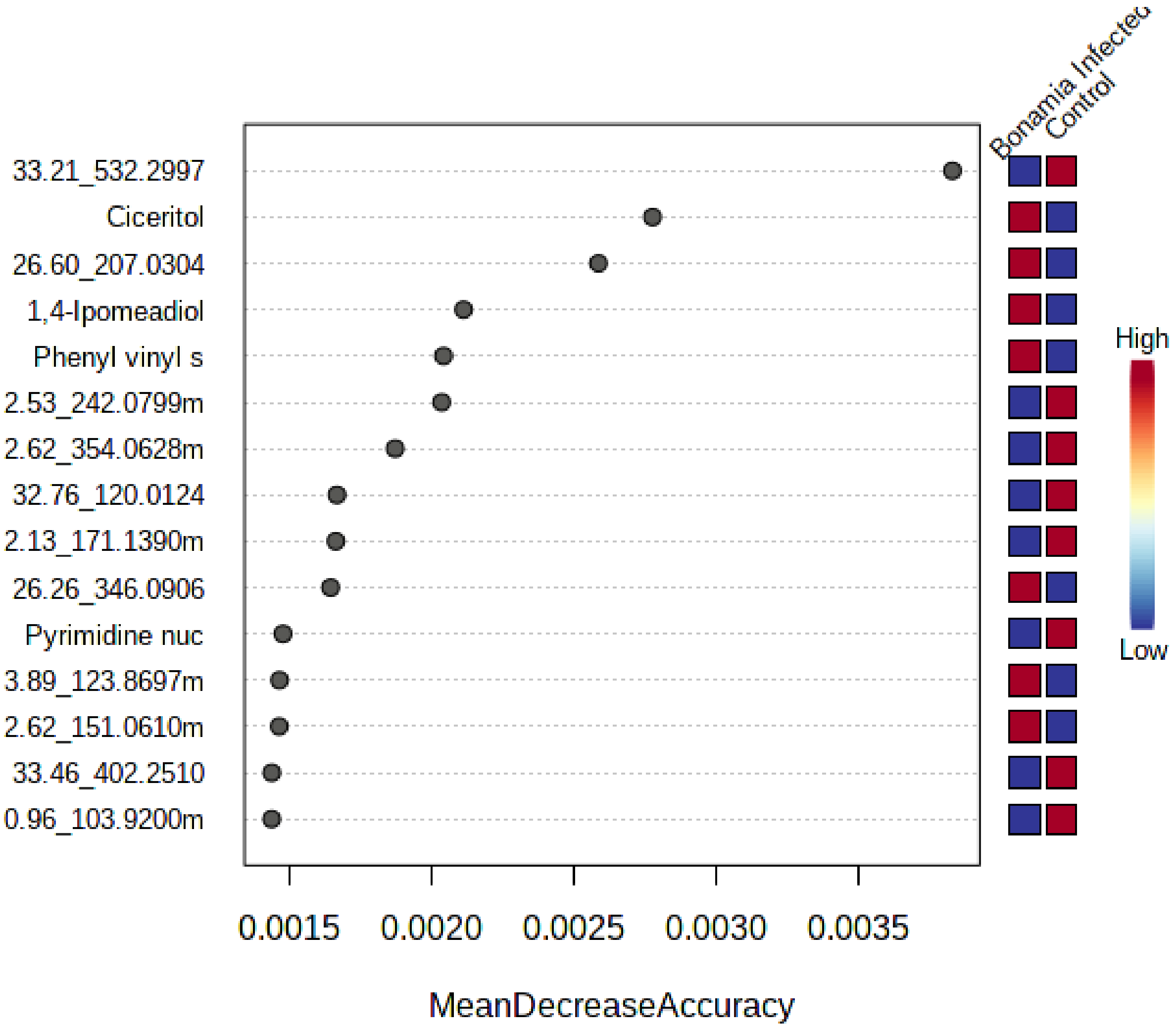


Figure 4.14. Variable Importance Curve to show the contribution of individual ions to the magnitude of change noted between the 2 groups (*Bonamia*-infected and non-infected oysters). Curve analysis for the top two ion features for ion exchange (IC-MS/MS). Each ion is given a unique identification number based on retention time and accurate mass. Those compounds with a positive ID based on an authenticated standard are named in the figure. Figure created by D. Hauton at the University of Oxford.

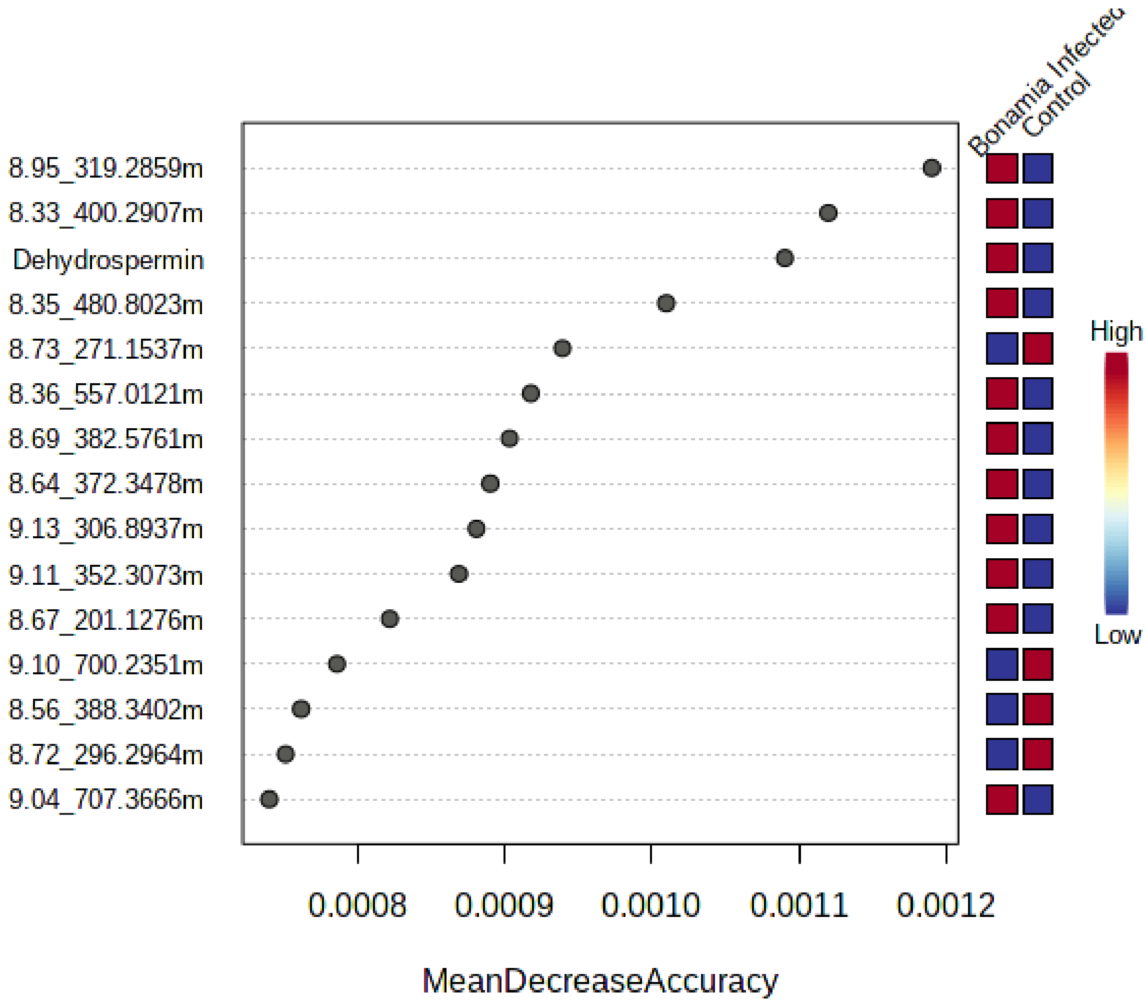
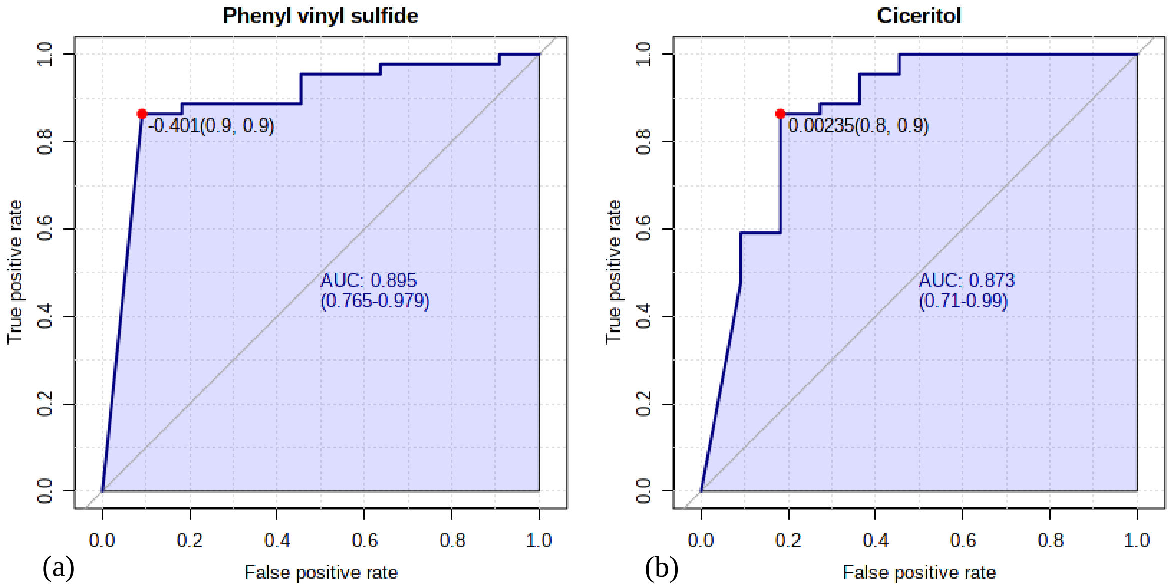


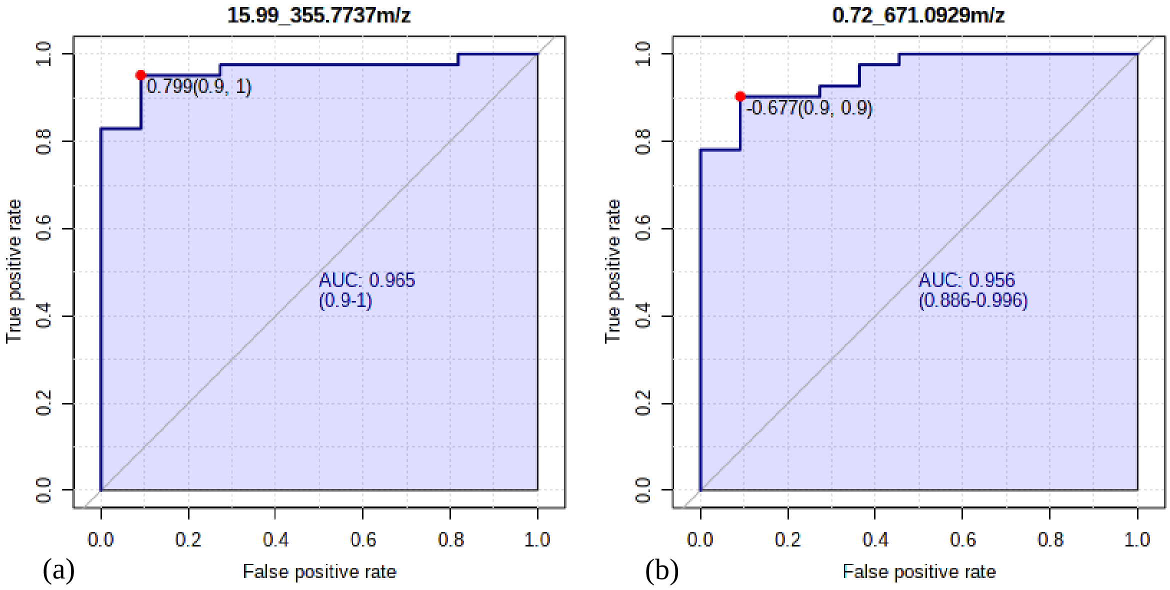
Figure 4.15. Variable Importance Curve to show the contribution of individual ions to the magnitude of change noted between the 2 groups (*Bonamia*-infected and non-infected oysters). Curve analysis for the top two ion features for derivatised C18-reverse phase chromatography (dC18-MS/MS). Each ion is given a unique identification number based on retention time and accurate mass. Those compounds with a positive ID based on an authenticated standard are named in the figure. Figure created by D. Hauton at the University of Oxford.

Finally, receiver operator characteristic (ROC) curve analysis for two of the top ion features was used to determine the value of an ion feature as a predictor of *Bonamia*-infection for IC-MS/MS (Figures 4.16a and 4.16b), C18-MS/MS (Figures 4.17a and 4.17b), and dC18-MS/MS (Figures 4.18a and 4.18b). Ion features were characterised by retention time and accurate mass to identify specific features, and where possible identified with reference to authenticated standards.



(a) (b)

Figures 4.16 (a and b). Receiver operator characteristic (ROC) curve analysis for the top two ion features for ion exchange (IC-MS/MS). Ion features were characterised by retention time and accurate mass to identify specific features. Where possible, features were identified with reference to authenticated standards. Figures created by D. Hauton at the University of Oxford.



(a) (b)

Figure 4.17 (a and b). Receiver operator characteristic (ROC) curve analysis for the top two ion features for C18-reverse phase chromatography (C18-MS/MS). Ion features were characterised by retention time and accurate mass to identify specific features. Where possible, features were identified with reference to authenticated standards. Figures created by D. Hauton at the University of Oxford.

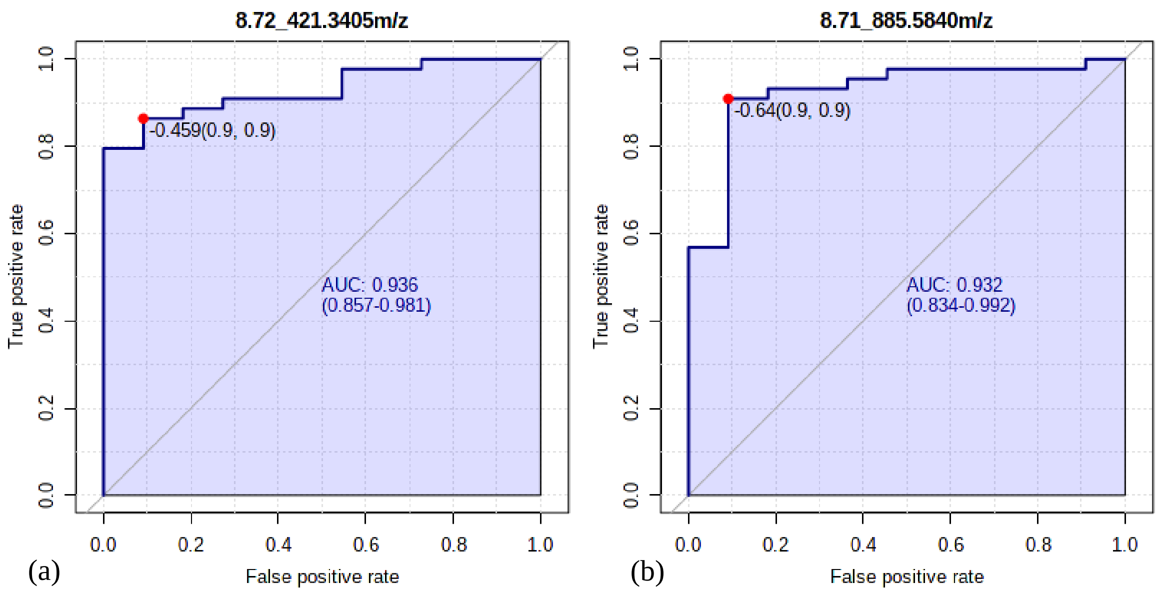


Figure 4.18 (a and b). Receiver operator characteristic (ROC) curve analysis for the top two ion features for derivatised C18-reverse phase chromatography (dC18-MS/MS). Ion features were characterised by retention time and accurate mass to identify specific features. Where possible, features were identified with reference to authenticated standards. Figures created by D. Hauton at the University of Oxford.

These ROC curve analyses plot diagnostic accuracy across the test values acquired, by plotting sensitivity along the y axis, with (1-sensitivity) along the x axis. The diagonal line that runs from (0,0) to (1,1) represents random chance that the ion in question will distinguish between oysters with *Bonamia*-infection and those without. Often shown as a curve, the ROC curve is the line (here shown in dark purple) that stretches from the diagonal line towards (0,1). The area below this line (AUC) is a summary measure of the accuracy in using this ion as a predictor of *Bonamia*-infection, with numbers closer to 1 representing higher test accuracy. For example, ions 15.99 _355.7737m/z and 0.72_671.0929m/z (Figures 4.17 a and b) could be used as markers of *Bonamia*-infection with high accuracy (AUC = 0.965, AUC = 0.956 respectively). Models (of individual ions) below a critical AUC value of 0.75 might not be considered statistically reliable.

4.4 Discussion

This chapter aimed to test the hypothesis that there are genetic and metabolic markers of disease resistance. The expression of six genes (inhibitor of apoptosis (*OeIAP*), extracellular superoxide dismutase (*OeEcSOD),* C1q (*OeC1q*), fas-ligand (*OeFAS)*, polyubiquitin (*Ubiq*), and β-actin (*ACT*)) have been identified to have an association with *Bonamia*-resistance (Morga *et al.*, 2012). These genes were analysed between *Bonamia*-infected (n= 8) and non-infected (n = 40) *Ostrea edulis* from Solent and Loch Ryan regardless of origin and season, to determine if they were robust markers clearly defined by disease status. The metabolic profiles of an extended number of *Bonamia*-infected (n = 14) and non-infected (n = 100) oysters from the Solent, Loch Ryan and Galway were then compared to determine the value of an ion feature as a predictor of *Bonamia*-infection.

4.4.1 The effect of Bonamia-infection on gene expression

Since its arrival to the south coast of England in 1982, *Bonamia ostreae* has contributed to the disproportionate decline in the Solent *O. edulis* population and is still present in many surviving individuals. The ability of these oysters to tolerate and live with, or actively resist *B. ostreae* infection has motivated further research into the *O. edulis* immune system and physiological pathogen defence mechanisms. A high variability in susceptibility to *B. ostreae* is now seen in *O. edulis* populations, with reduced susceptibility often seen in *Bonamia*-exposed oysters (Narcisi *et al.*, 2010). A greater understanding of *O. edulis* immune system could advise our use of these oysters in restoration and conservation efforts.

For this study, the expression of *OeIAP*, *OeEcSOD*, *OeC1q*, and *OeFAS* identified by Morga *et al.* (2017) to be associated with *Bonamia*-resistance, was compared between *Bonamia*-infected oysters (n = 8) and non-infected oysters (n = 40) from Loch Ryan and the Solent. Although *Ubiq* and *ACT* are occasionally identified as stable ERGs (Morga *et al.*, 2010; Wang *et al.*, 2013), enough variation was seen between our samples (by qbase+) to also require their comparison between treatments. Four reference genes, glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*), elongation factor 1-α (*Ef1- α*), 60S ribosomal protein L5 (*L5*), and cathepsin B (*Cathep*) were used to comply with MIQE requirements. Gill tissue from each oyster was tested for the presence of *B. ostreae* in order to compare gene expression between infected and non-infected oysters.

The infection status of *O. edulis* did explain significant differences in variation between the calibrated normalised relative quantity (CNRQ) of *OeC1q*, *OeFAS* and *ACT*. *OeC1q* is from the family of C1 proteins responsible for immunity against pathogens in vertebrates (Kishore and Reid, 2000) and invertebrates (Zhang *et al.*, 2008) that are considered reliable markers for resistance in other organisms such as clams (*Mercenaria mercenaria*) to diseases such as Quahog Parasite Unknown (QPX) (Perrigault *et al.*, 2009; Morga *et al.*, 2012; Ronza *et al.*, 2018). C1q proteins are involved in recognition of pathogens by the invertebrate innate immune system (Zhang *et al.*, 2008; Gerdol *et al.*, 2015; Wang *et al.*, 2018), and are an essential component of innate immunity as part of the complement system. The complement system coordinates the removal of non-self particles such as pathogens, dying or deceased cells, and abnormal molecular structures through a cascade system known as the classical pathway, which is initiated by the proteolytic enzyme called the C1 complex (Mortensen *et al.*, 2017). The C1 complex is activated with the binding of recognition molecule C1q, using its globular head, with a variety of activator molecules (Morga *et al.*, 2012; Mortensen *et al.*, 2017; Wang *et al.*, 2018), thus elevating the expression of this gene. *OeC1q* has previously demonstrated different expression in *O. edulis* owing to bonamiosis (Martín-Gómez *et al.*, 2014; de la Ballina *et al.*, 2018). Similarly, in this study, a significant increase in expression of *OeC1q* was seen in *Bonamia*-infected oysters in comparison to non-infected oysters. Further evidence is required to determine whether this is simply evidence of an innate immune response or whether it could represent a response specific to *Bonamia ostreae*. Fas ligands play a central role in the mediation of apoptosis in mammalian cells, but their purpose in invertebrates such as *O. edulis* are just beginning to be understood (de Zoysa *et al.*, 2009). However, upregulation of Fas ligand, *OeFAS*, has already been specifically linked to *O. edulis* either infected with, or previously exposed and potentially ‘resistant’ to, *B. ostreae* (Morga *et al.*, 2012; Cocci *et al.*, 2020). Cocci *et al.* (2020) suggested that the increased expression of *OeFAS* seen in oysters infected with *B. ostreae* indicates a modulation of the apoptotic pathway by the host, as the parasite manipulates the same apoptotic system in order to neutralise host defence mechanisms. If this is the case, then perhaps *OeFAS* could become a marker of *tolerance* rather than *resistance*. While resistant individuals may demonstrate the ability to actively reduce parasite burden and neutralise its virulence (Råberg *et al.*, 2007; Schneider and Ayres, 2008; Ferrandon, 2009; Lynch *et al.*, 2014; Louie *et al.*, 2016; Morga *et al.*, 2017), disease tolerance is the ability to survive whilst maintaining a parasite burden. Tolerance can be beneficial for both host and parasite, in that host fitness is not greatly affected by the presence of the parasite, regardless of its successful proliferation in host tissues (Schneider and Ayres, 2008; Råberg *et al.*, 2009). Cao *et al.* (2009) reported tolerance in their *O. edulis* population in Ría de Ortigueira, which they identified as low disease prevalence and low mortality. Tolerance may be the result of a parasitic virulence mechanism whereby the parasite is able to neutralise the host immune reaction to the infection (Mauel, 1984; Cheng, 1987; Ferrandon, 2009; Råberg *et al.*, 2009). This again highlights the complex interaction of physiological mechanisms used by the host and by the parasite that determine infection virulence (Snieszko, 1974). Phagocytosis is essential to innate immune response of bivalves and is a process dependent on the family of actin proteins (May and Machesky, 2001; Li and Xiang, 2013). Beta-actin (ß-actin or *ACT*) is a cytoskeletal protein involved in cell structure and motility, and embryonic development (Bunnell *et al.*, 2011). Although this conservative protein is often considered a stable reference gene, *ACT* has more recently been recognised as an essential component in innate antiviral immune response by regulating specific proteins (Xie *et al.*, 2019), and contributing to important protein complexes involved with invertebrate immune defence such as that found in shrimp against white spot syndrome virus (WSSV) (Li and Xiang, 2013). Xie *et al.*, (2019) found that the absence of mitochondria-targeted *ACT* was detrimental to the innate antiviral immune signalling that initiates an immune response, which suggests that *ACT* plays a much larger role in invertebrate immunity than previously thought. In this study, expression of *ACT* was significantly different between oysters with and without a *B. ostreae* infection, irrespective of season or origin. This variability demonstrates the unsuitability of the repeated use of *ACT* as an ERG, and suggests it is an influential component of *O. edulis* immunity encouraging further analysis into its specific role against a *B. ostreae* infection.

However, the effect of season confounded the infection response for *OeC1q* and *OeFAS*; Significantly higher expression of *OeC1q* and *OeFAS* was seen in *Bonamia*-positive individuals in comparison with non-infected individuals for each season apart from in the Autumn, when lower expression was seen in *Bonamia*-positive individuals. Previous reports that have found a correlation in temperature and *Bonamia* prevalence; lower temperature results in lower prevalence of *Bonamia* (Hine, 1991; Carnegie *et al.*, 2008). Temperature always plays an important role in the prevalence and spread of pathogens (Snieszko, 1974), and the effect is evident here in the form of seasonal fluctuations. If temperature has an influence on prevalence of *B. ostreae*, then perhaps this suggests that *OeC1q* and *OeFAS* are significant genes for immune defence specific to *B. ostreae*, as expression is elevated during the months when *B. ostreae* would be most prevalent. However, what we view at a single time point is part of a continuum of effect, which does not always have a precisely defined end point. Infection status is an interaction between host, parasite and environment, and whatever the status of a population or individual, environmental perturbation may in fact completely alter that status; something which is particularly important in sessile, aquatic animals. Although it is possible to assign terminology to the state of an infected individual, i.e. tolerant or resistant, it must be appreciated that an individual or population may change status depending on the temporal development of the host-pathogen interaction, and the environment in which they find themselves (Holbrook *et al.*, 2021). The observed gene expression may be part of an immune response, but it may also be part of the pathology of a dying animal. Although an appreciation for invertebrate immunity from individual observations of molecular features is limited, a comprehensive appreciation of the mechanisms controlling resistance, tolerance and resilience might lead to more efficient control measures and a heightened understanding of host-parasite interaction (Schneider and Ayres, 2008).

4.4.2 Correlations in gene expression

Principal components analysis (PCA) highlighted the correlation between *OeC1q* and *OeFAS* in their upregulation in *Bonamia*-infected oysters (correlation = 0.43). As discussed above, C1q proteins are pattern recognition receptor (PRR) molecules that can recognise molecules found in pathogens, and *OeFAS* is likely an essential component to the apoptotic pathway. The clustering of oysters expressing these two oyster genes (*OeC1q* and *OeFAS*) could suggest the genes play a combined immune role in defence against infection (Figure 4.6). Fas-ligand in the mollusc abalone (*Haliotis discus discus*) has been shown to activate both apoptotic and non-apoptotic pathways (de Zoysa *et al.*, 2009). This further indicates that, similar to C1q, Fas-ligand is crucial for early detection of pathogens and signalling pathways associated with the innate immune response to infection.

Expression of *OeIAP* and *Ubiq* were also highly correlated (correlation = 0.71), and regulation of *OeEcSOD* was closely clustered. The clustering of these three genes is highly comprehensible owing to the role of these genes in the control of cell turnover as a result of apoptosis (*OeIAP*), oxidative stress (*OeEcSOD*), and protein breakdown (*Ubiq*). Inhibitors of apoptosis (IAP's), found in both invertebrates and vertebrates, are responsible for regulating apoptosis, a common immune response to pathogen cell invasion. Superoxide dismutase (*SOD*) is a haemocyte-specific enzyme that is abundant during proliferation of hematopoietic stem cells in oysters (Jemaá *et al.*, 2014); upregulation of *SOD* could therefore potentially reflect an increase in the antioxidant pathways related to cell abundance and consequently immune defence. In addition, *SOD* plays a role in protection from naturally accumulating oxygen radicals, and is therefore not only an indicator of immune function, but also for the preservation of general tissues and cellular repair (Zelko *et al.*, 2002; Manduzio *et al.*, 2003; Monari *et al.*, 2005, 2007). In their study of *SOD* in bivalve clam *Chamelea gallina*, Monari *et al.* (2007) conceded that low levels of *SOD* would reduce efficiency of haemocytes and immune response against disease. The polypeptide Ubiquitin (*Ubiq*) has roles in both innate and specific immunity of organisms from plants to the most complex animal (Goldstein *et al.*, 1975). This conserved protein has been recognised in multiple immunity associated processes such as initiating the synthesis and degradation of other proteins involved with apoptosis of viral cells (Yan and Chen, 2012), differentiating between lymphocytes, and modulating signalling pathways or transcription factors (Liu, 2004). Ubiquitin is recognised as an important component in the coordination of signals responsible for immune mechanisms such as apoptosis and inflammation (Skaug *et al.*, 2009).

This study aimed to test the *Bonamia*-resistant genes observed by (Morga *et al.*, 2012) with a more robust MIQE compliant analysis by using different populations of *O. edulis*, sampling throughout the seasons, and using multiple ERGs. The results from this study show that use of genetic markers to identify resistance to *Bonamia ostreae* is more nuanced than that proposed by Morga *et al.* (2012). Whilst this study observed significant increases in expression of *OeC1q*, *OeFAS* and *ACT* in *Bonamia*-infected oysters in comparison to non-infected oysters, there was a pronounced seasonal effect that confounded the interpretation for *OeC1q* and *OeFAS*. The significant variance seen in expression of *ACT* between *Bonamia*-infected and non-infected oysters indicates that the common use of this gene as an ERG is inappropriate for *O. edulis*. This study highlights the importance of following MIQE compliance and generating robust analyses to further the understanding of *O. edulis* immunity associated with mechanisms of tolerance and resistance to *B. ostreae*.

4.4.3 Exploration of metabolomics to understand infection history

Hepatopancreatic tissue samples of oysters from the Solent, Loch Ryan and Galway (n = 114) were used to assemble a metabolome profile for each oyster individual. The variable importance plots (Figures 4.14 and 4.15) revealed potential metabolic markers; several ion features or compounds were significantly different between groups (*Bonamia*-infected and non-infected oysters). Two of the top ions demonstrating this difference were analysed for each of the methods using a receiver operator characteristic (ROC) curve analysis (Figures 4.16 - 4.18), which highlighted that these top ion features could either be detected in infected individuals or were absent in infected individuals. Most of these distinctive ions or compounds could only be referenced by their unique number, but a number of metabolites were recognised as ciceritol, 1,4-ipomeadiol (*DIOL*), phenyl vinyl sulphide (*PVS*), pyrimidine nucleotide, and dehydrospermin. Cysteine proteases from human parasites are known to play a role in haemoglobin degradation, blood cell invasion, and processing cellular surface proteins (Lecaille *et al.*, 2002; Rosenthal *et al.*, 2002; Verma *et al.*, 2016). Phenyl vinyl sulphide (*PVS*) is a cysteine protease inhibitor (CPI) that can block these processes and neutralise the active degradation of proteins (Verma *et al.*, 2016). In fact, *PVS* has been recommended as a treatment for Schistosomiasis, a human disease caused by parasitic flatworm *Schistosoma mansoni*, owing to its parasiticidal properties (Sajid and Mckerrow, 2002; Abaza *et al.*, 2013; Mahmoud *et al.*, 2017). High levels of *PVS* seen in *Bonamia*-infected oysters in comparison to non-infected oysters could indicate a natural host mechanism in defence against the *Bonamia*-infection. If so, this metabolite holds potential as a marker of disease or of resistance to this disease. Pyrimidine nucleotides are the energy sources for most molecular reactions including DNA, RNA and protein synthesis (Vogels and van der Drift, 1976; Kamatani *et al.*, 2014). The significantly lower concentration of pyrimidine nucleotides seen in *Bonamia*-infected oysters in comparison to non-infected oysters could indicate that presence of the parasite (*B. ostreae)* disrupts basic nucleic acid metabolism, essential for regenerating cellular material. Metabolomics is a relatively new field of science, and it is challenging to determine why the metabolites featured in this study (named and numbered) are related to infection. The use of metabolomic markers in human medicine to detect molecular abnormalities that could lead to disease, demonstrates the potential for their similar use for other species. Although restricted to mainly human metabolic pathways for reference, this study was able to demonstrate that there are potential metabolic markers of *Bonamia*-resistance and would urge further research to identify the specific roles of the named and numbered metabolites and their association with *Bonamia*-infection.

4.4.4 Conclusion

Investigation into six genes associated with *O. edulis* immunity showed a significant difference in the expression of C1q (*OeC1q*), fas-ligand (*OeFAS*) and β-actin (*ACT*) between *Bonamia*-infected and non-infected oysters. However, seasonal effect on this variation in expression of *OeC1q* and *OeFAS* confounded the response to infection and suggests the use of genetic markers is more nuanced than previously considered. The significant difference in expression of the commonly used ERG, *ACT*, between *Bonamia*-infected and non-infected oysters demonstrates the importance of using multiple ERGs for stability when performing qPCR. We, the science community, are clearly a long way from understanding *O. edulis* disease status and resistance associated with *B. ostreae*, and the results from this study do not support the use of the Morga *et al.* (2012) genes as markers of resistance. We urge supplementary research to rigorously follow MIQE compliant qPCR methods in order to further this research with robust results that will best benefit restoration practice. This study demonstrated that metabolic markers do have the power to discriminate between *Bonamia*-infected and non-infected *O. edulis* individuals and highlights the potential for developing this for use in immune research into *Bonamia*-resistance. One metabolite (*PVC*) identified in this study as a potential marker of *Bonamia*-resistance has already been identified to benefit from parasiticidal properties, strengthening the need to further our understanding of metabolic pathways associated with a *B. ostreae* infection. Combined cellular and molecular approaches might, in time, provide mechanistic evidence to support our understanding of *O. edulis* immunity to *B. ostreae* and other pathogens. Unfortunately, we cannot rely solely upon host genotype alone to predict whether an individual will produce a defined immune response, be it disease–tolerant, –resistant or –resilient, as no host parasite interaction explanation is complete without an environmental context.

Chapter 5 Indicators of restoration success: a study to observe the effect of density and elevation on oysters from Loch Ryan, Scotland, deployed in the Beaulieu River, England

5.1 Introduction

Although restoration of European flat oyster *Ostrea edulis* is not a new concept (Cole, 1951; Van Banning, 1979; Elston *et al.*, 1987), the practice has not yet been standardised within Europe. The selection of re-laying density and elevation of oysters are two important considerations in restoration design that can influence population development and sustainability. For example, *O. edulis* is a broadcast spawner, and therefore reproduction requires oysters to be in close proximity, yet high density can increase prevalence of disease (see section 1.6.6). More evidence is required to support the benefits associated with re-laying density and elevation on the success of *O. edulis* restoration. To add complexity, measuring restoration ‘success’ is challenging. Different restoration goals, for different restoration organisms, require unique indicators for monitoring, and therefore restoration success is highly subjective. However, general monitoring of (a) the associated epibenthic community, (b) parasite prevalence amongst the restored population, and (c) the physiological performance of the target organism, would build important and essential information about the progression of any marine restoration subject.

5.1.1 The epifaunal community associated with an Ostrea edulis bed

Ostrea edulis restoration, whether introducing oysters to a new area or replenishing dwindling stocks, will alter the benthic community. As discussed in Chapter 1, oysters are a keystone species that provide shelter for benthic organisms through their 3D structure and contribute to the recycling of nutrients at the benthic water interface (Korringa, 1951; Grabowski *et al.*, 2005; Grabowski and Peterson, 2007; Kellogg *et al.*, 2013; Smaal *et al.*, 2015; Perry *et al.*, 2020; Lown *et al.*, 2021). The epifaunal organisms often associated with an oyster bed (e.g. barnacles, tunicates, sponges, polychaetes) also contribute to these supporting and regulating ecosystem services, by water and nutrient cycling through their suspension feeding (Brumbaugh *et al.*, 2005). The presence of epifaunal species is an attraction for mobile organisms such as gastropods and crustaceans that, in turn, attract higher trophic predators. This bottom-up control, by providing resources (such as an oyster bed and associated epifauna) to attract predators, can lead to diverse communities and influence the abundance of commercially important organisms such as fish (Lynam *et al.*, 2017). If left undisturbed, the oyster bed and development/growth of the associated community would have a synergistic effect on the aforementioned ecosystem services and improve biodiversity and species richness in the benthic environment. However, deploying high numbers of oysters simultaneously provides an abundance of prey for predators such as crabs and gastropods, such as the tingle. Evidence of increased predation as a result of restoration (Rindone and Eggleston, 2011), or as a result of a heavy natural spatfall (Key and Davidson, 1981), has raised concerns that restoration could disturb the natural balance between predator and prey. Just as restoring a predator would endanger a prey species, restoring a prey species to an area could simply create a feeding ground by rapidly increasing food availability for oyster predators (Rindone and Eggleston, 2011). However, both live and recently dead oysters have been shown to release chemical pheromone-type cues that attract epifaunal organisms, which would allow a benthic community to develop even in periods of high oyster predation (Brumbaugh *et al.*, 2005; Mesías-Gansbiller *et al.*, 2013). Ecosystem functionality and predator-prey interaction is a naturally fluid dynamic that responds to seasons and weather events (Bertram *et al.*, 2001; Trathan *et al.*, 2007; Tablado *et al.*, 2014; Kauppi *et al.*, 2017), but human interference, such as dredge fishing, have actively and irreversibly altered the environment on a local and global scale. Rapid changes to trophic structure can imbalance ecosystem functionality, which can increase the incidence of pollution, hypoxic environments, and toxic algal blooms called 'red tides' (Orth and Moore, 1983; Officer *et al.*, 1984; Cushing, 1988; Newell, 1988; Elmgren, 1989; Smayda, 1990; Burkholder *et al.*, 1992; Cooper and Brush, 1993; Rothschild *et al.*, 1994; Nixon, 1995; Jonas, 1997; Jackson *et al.*, 2001). Appreciating the complexity of prey survivorship in habitats threatened by anthropogenic and climate-induced stressors is essential for sustainable restoration (Humphries *et al.*, 2011). It is now generally accepted that a 'whole ecosystem' approach to restoration yields the greatest profit for humans. Aiming to restore oyster stocks to historical levels is over-ambitious, but stakeholders could benefit by re-establishing a healthy oyster habitat. Monitoring presence and activity of *O. edulis* predators (such as crabs and tingles) in association with a restoration site is essential to monitor the effect of the restoration on the community.

Contemporary observations of substrate type at proposed restoration sites prior to active deployment would also complement historical records of the environment by increasing knowledge about the ecosystem as a whole. The two recognised habitat classification schemes in the UK are the Marine Habitat Classification for Britain and Ireland established by The Joint Nature Conservation Committee (JNCC) (https://jncc.gov.uk) under the UK Government, and the European Nature Information System (EUNIS) (https://www.eea.europa.eu/data-and-maps/data/eunis-habitat-classification). Specific codes were established by the JNCC, building from the most basic substrate type (e.g. SS for Sublittoral sediment (SS)) to details including specific species present (e.g. *O. edulis* beds on shallow sublittoral muddy mixed sediment (SS.SMx.IMx.Ost)), that can be used as descriptors of a changing habitat. The European Environment Agency (EEA) initiated the EUNIS scheme in 2014, and uses a coding heavily based on the JNCC UK system (Parry, 2019). Both systems offer a succinct and standardised habitat description that can be monitored to determine the effect of restoration on the immediate environment.

5.1.2 The effect of density and elevation on physiological performance

As explained in Chapter 2, local hydrodynamics are an essential consideration for appropriate *O. edulis* restoration site selection. Similarly, decisions concerning restoration design and implementation (such as re-laying density and elevation of oysters from the seabed) are important to promote the highest physiological performance of the oysters and reduce threats such as disease to population sustainability.

Ecosystem stability depends on a balance of resources and an interaction of trophic relationships that ensure a synergistic network of organisms that support and sustain the habitat. The carrying capacity of a single species within an ecosystem is site specific and depends on factors such as food availability and pollution, and an imbalance of resources can influence the prevalence of disease (Héral, 1993). Oyster growth rate and survivability has been linked to population density; In their study on Pacific oyster *Crassostrea gigas* culture, Héral (1993) reported oyster growth rate halving as a result of an increase in oyster production (from 54000 tonnes to 110000 tonnes) in Hiroshima Bay in 1980. The high densities of oysters in Chesapeake Bay, USA (615000 tonnes in 1884, (Rothschild *et al.*, 1994)) and the Marennes-Oléron basin, France (170000 tonnes (1955-65), 140000 tonnes (1986-) (Héral *et al.*, 1986)) were considered important in the subsequent reduction in environmental conditions (such as an increase in sedimentation), resulting in an extension of the duration of their breeding cycle, a decrease in their growth rate, and increases in outbreaks of disease amongst the population (Héral *et al.*, 1986), all detrimental for restoration.

A positive correlation between *O. edulis* population density and *Bonamia ostreae* prevalence is often alluded to in the literature (Le Bec *et al.*, 1991; McArdle *et al.*, 1991; Kennedy and Roberts, 1999; Hawkins *et al.*, 2000; Berghahn and Ruth, 2005), yet there are few direct observations with statistical evidence to support this theory. Le Bec *et al.* (1991) theorised that density would impact prevalence of *B. ostreae*, yet although they found a significant increase in survival of *O. edulis* in densities of 200 oysters per bag in comparison to 400 oysters per bag, they found no significant effect of density on prevalence of disease and actually witnessed a decreased incidence of *B. ostreae* with density. These results were also confounded by the presence of *Crassostrea gigas* and by their exclusion of dead oysters in their consideration of *B. ostreae* prevalence. In addition to improved growth rates and shell size, Hawkins *et al.* (2000) did observe that oysters restocked at low density (10 oysters m^{-2}) coped with a *B. ostreae* disease challenge better than those stocked at high density (30 oysters m^{-2}). However, this evidence was based on histological quantification of the number of circulating haemocytes post disease challenge, thus not specifically a representation of parasite prevalence amongst the population. Observations have been made, but further analysis into the effect of density on *B. ostreae* prevalence is required to establish an economically viable density for restoration with the least potential for a disease outbreak.

Increased oyster density is, however, thought to have a positive effect on reproductive success owing to increased proximity of reproducing adults; a higher concentration of sperm in the water column would lead to increased egg fertilisation (Vogel *et al.*, 1982), and a larger substrate surface area for larval settlement is likely to improve recruitment (Smyth *et al.*, 2018; Rodriguez-Perez *et al.*, 2019). Additionally, in areas with less disturbance such as fishing, high density populations would increase brooding and spawning capacity, thus increasing recruitment potential. In 1974, during its peak performance, the Solent Oyster Fishery recorded a healthy population density of 32 oysters m^{-2} (Key and Davidson, 1981). This high density is unrealistic today owing to the presence of *B. ostreae*, and a new oyster re-laying density that will support both the oysters and a balanced ecosystem needs to be identified.

Increased siltation, turbidity, pollution, and smothering of natural communities caused by the busy shipping activity in the Solent has changed the benthic environment and induced physical loss to the ecosystem (Cefas, 2001). Higher turbidity has been shown to reduce feeding rates of benthic organisms such as *O. edulis* (Korringa, 1952; Grant *et al.*, 1990; Sawusdee *et al.*, 2015); Sawusdee *et al.* (2015) observed significantly increased clearance rates in oysters raised only 0.4 m off the seabed in comparison to those on the seabed in all months sampled (September, April, August and November). Oyster clearance rate is highly indicative of individual scope for growth and therefore a significant determinant of population development (Barber *et al.*, 1991). As seen in Chapter 2, local hydrodynamics and seabed topography play a role in the distribution of food particles to benthic organisms by vertical movement and resuspension of phytoplankton (Colden *et al.*, 2016), and food availability near the seabed can be low (Fréchette *et al.*, 1989). In addition, a significantly higher bacterial load can be found at the seabed in comparison to the immediate water column (Sawusdee *et al.*, 2015). In his study of the physiological performance of Pacific oyster *Crassostrea virginica* in association to reef height, Lenihan (1999) showed that elevated oysters (at the top of the reef) showed reduced mortality, improved growth, and were exposed to less hypoxia than those on the seabed at the base of the reef. Lenihan (1999) argued that these oysters raised off the seabed were in a generally less stressful environment that would render them less prone to disease, which has implications for restoration. These data have meant the use of structures such as reef balls or oyster castles to elevate oysters from the seabed is commonly applied for restoration of *Crassostrea* spp., especially in areas with a high proportion of silt in the sediment (zu Ermgassen *et al.*, 2016). However, the height at which *O. edulis* perform most effectively has yet to be defined. Although using elevated structures for restoration can add complexity to a restoration project, evidence of the physiological benefits of elevating *Crassostrea* spp. for restoration in the USA (Lenihan, 1999; zu Ermgassen *et al.*, 2016), and similar benefits shown in the elevation of *O. edulis* from the seabed in the UK (Sawusdee *et al.*, 2015), indicate that these parameters must be tested to ensure restoration of *O. edulis* is best designed to develop and support a self-sustaining population.

5.1.3 The Beaulieu River

Beaulieu is a village that lies at the south of the New Forest National Park, England, with a river running from the New Forest into the Solent (Figures 5.1a and 5.1b). The Beaulieu River is designated as a Site of Special Scientific Interest (SSSI) that “supports many characteristic, rare and endangered species, habitats, and natural features” (https://naturalengland-defra.opendata.arcgis.com/datasets/f10cbb4425154bfda349ccf493487a80_0), a Special Area for Conservation (SAC) of habitats and species owing to the “specific physiographic sub-type of estuary and hydrographic regime” (https://sac.jncc.gov.uk/site/UK0030059), a Special Protection Area (SPA) for the conservation of wild birds, a RAMSAR site for protection of wetlands (Ramsar Convention in 1975) and is opposite a Marine Conservation Zone (MCZ) protecting important marine habitat and species from Yarmouth to Cowes on the Isle of Wight. Designated by the UK Government body DEFRA (Department of Environment Food and Rural Affairs), these designations highlight the importance of this ecosystem in providing for a high variety and abundance of critical species and habitats (JNCC, 2005). The Beaulieu River has been privately owned since 1204, originally by the monks who founded the Beaulieu Abbey, and then by the current Montagu family resident on the Beaulieu Estate since 1538 (Holland, 1985). Management of any fishery efforts have therefore fallen under control of the Estate, allowing private regulations to keep disturbance to the seabed to a minimum. The Newtown and Beaulieu Oyster Fishery was established in 1880 by Sir John Stephen Barrington Simeon and Lord Henry Scott, and management of the oyster beds was taken over by the Beaulieu Estate, although these early efforts were cut short in 1886 due to low recruitment and low economic return (Isle of Wight Record Office, 1867-86). Regardless of this initial failure, the Beaulieu River has played a major role in the current Solent Oyster Fishery. Historically, the Beaulieu River has been a significant source of oyster spat in the Solent due to its abundant population of healthy and actively fertile broodstock (Key and Davidson, 1981), and an effective dispersive hydrodynamic environment as seen in Chapter 2 (Figure 2.33). The significant spatfall seen in the early 1970s that revived the Solent *O. edulis* population is thought to have originated from a batch of *O. edulis* imported from Brittany, France, to the Beaulieu River in 1958 (Davidson, 1976; Key and Davidson, 1981; Kamphausen *et al.*, 2011). In addition, Key and Davidson (1981) attributed the natural recruitment regularly seen at Stanswood Bay (c. 5.1 km from the Beaulieu River) as likely to be larval output from the Beaulieu River broodstock. However, subsequent *B. ostreae*-attributed mortalities in the Beaulieu River led to a mass removal of *O. edulis* from the Beaulieu River bed between 1986 and 1989, leading to an immediate halt of the *O. edulis* culture there (Hudson and Hill, 1991; Laing *et al.*, 2005; Kamphausen, 2012). Oyster fishing in the Beaulieu River has since become secondary to the busy yachting activity in the River, which is the Estate’s economic priority. The Beaulieu River still benefits from restrictions on fishing and is therefore an appropriate location to establish an *O. edulis* sanctuary area where a developing oyster bed protected from fishing pressure can be monitored. The history of *O. edulis* culture in the Beaulieu River suggests it has desirable abiotic and biotic conditions to support a self-sustaining population and could be a robust location for restoration.

A field study was conducted in association with Blue Marine Foundation to deploy *O. edulis* in the Beaulieu River, and monitor the physiological performance of individuals, parasite prevalence in the population and associated epifaunal community over a 2-year period (2017-2019). The deployment design incorporated treatments to test the effect of both re-laying density, and elevation from the seabed on the physiological performance of *O. edulis*. Due to previous experience with mass mortality events attributed to *B. ostreae*, the Beaulieu Estate requested the sole use of *Bonamia*-free oysters for this research. This provided an opportunity to test whether *Bonamia*-naïve oysters would survive in a *Bonamia*-exposed area.

Aims

The aim of this study was to determine whether restoration supports biodiversity in the Beaulieu River, and whether density and elevation had an effect on physiological performance of individuals, and whether density influenced population parasite (*Bonamia ostreae*) prevalence. Oysters from Loch Ryan were deployed into the Beaulieu River in November and December, 2017.

Objectives

1. Observations were made of the associated epifaunal community and environment in the Beaulieu River.
2. The effect of density and elevation on oyster physiological performance (clearance, respiration and growth rates and condition index) was tested each year post oyster deployment (in 2018 and 2019).
3. Prevalence of *Bonamia ostreae* was measured in oysters at high and low density after two years.

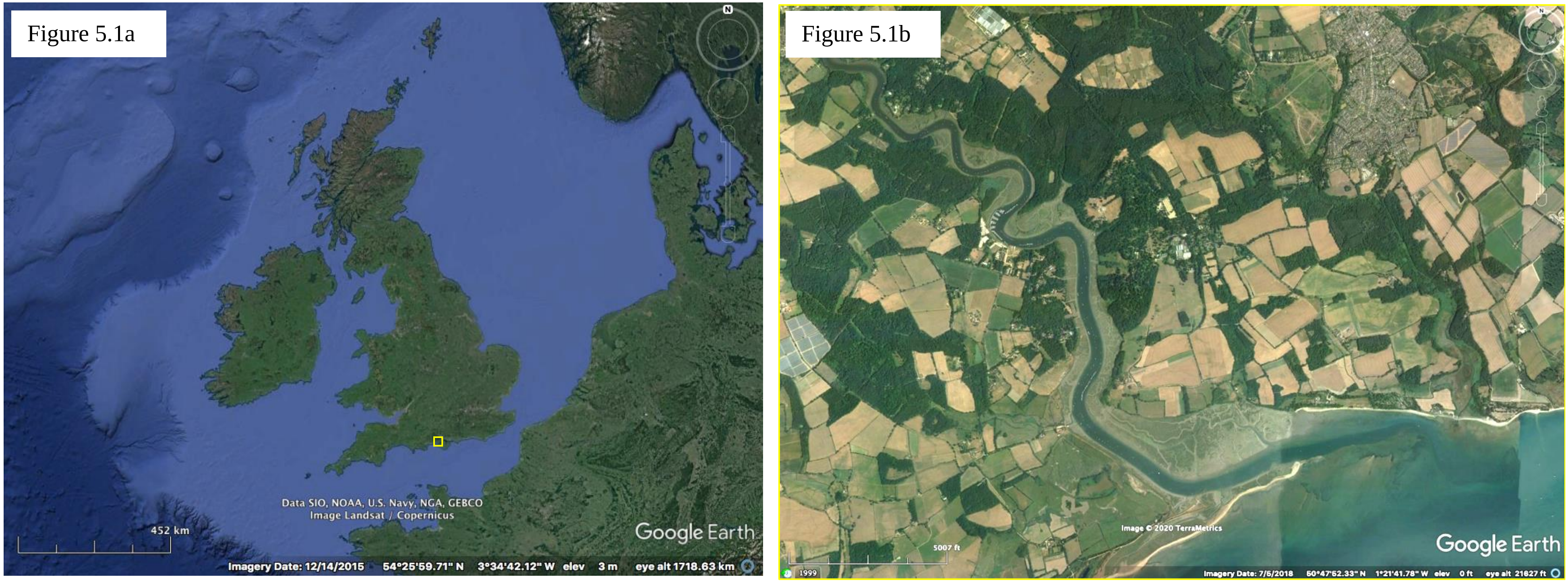


Figure 5.1 (a and b). Location of the Beaulieu River in the UK (marked by the yellow box in Figure 5.1a) and at a higher resolution (Figure 5.1b).

5.2 Methods

5.2.1 A baseline assessment of the Beaulieu River benthic habitat

A baseline study was conducted on the 21st June 2017 to provide a snapshot of the natural condition of the Beaulieu River bed before oyster deployment using established JNCC and EUNIS habitat classification codes. Previously published and established environmental conditions of the Beaulieu River are documented in Table 5.1 for reference. Sediment grabs and video footage of the seabed were taken at three haphazardly selected stations along the Beaulieu River (Figure 5.2). Sediment was collected at each station using a hand-held Van Veen grab, with two replicates (Figure 5.3). Video footage of the River bed was taken using a GoPro Hero 3+ attached to a metal scaffold frame measuring 0.35 x 0.45 x 0.35 m in height, length and width respectively (Figure 5.4), and suspended from the side of the vessel. These dimensions ensured the entire video captured by the GoPro Hero 3+ was framed and accounted for within the rectangular base (0.16 m^2) of the scaffold. At each site, the scaffold with GoPro 3+ was lowered onto the River bed and left there for 10 seconds to allow the silt to settle and capture a clear image. After this 10 seconds, the scaffold was lifted approximately 1-2 m above the River bed for 5 seconds to allow it to drift, and lowered again. This action was repeated until the frame had touched the seabed 10 times, therefore capturing 1.58 m^2 of the Beaulieu River bed in total. The vessel was drifting during this deployment, which ensured that each drop down captured a slightly different area of riverbed at each site. This was replicated two or three times for each station with the boat returning to the original location at the start of each replicate.

Table 5.1. Environmental conditions in the Beaulieu River.

Parameter	**Beaulieu River**	**Reference**
Depth of estuary	≤ ~ 1 m	(Goring-Harford *et al.*, 2020)
Tidal range	0.1 - 4 m	(Goring-Harford *et al.*, 2020)
Freshwater residence time	~ 7 days	(Fang, 1995)
Temperature (min-max)	6.9 – 23.6 °C	(Rodhouse, 1978)
Salinity	0 - > 30	(Goring-Harford *et al.*, 2020)
pH	6.5 – 7.8	(Hopwood *et al.*, 2014)
Dissolved oxygen	< 370 μmol L^{-1}	(Goring-Harford *et al.*, 2020)
Dissolved iron (dFe)	8-21 μM	(Goring-Harford *et al.*, 2020)
Dissolved organic carbon	250 – 1800 μM	(Hopwood *et al.*, 2014)

Figure 5.2. Haphazardly chosen sampling stations along the Beaulieu River for sediment grabs and video footage of the River bed (Stations 2-4).

Figure 5.3. A hand-held Van Veen Grab for sediment.

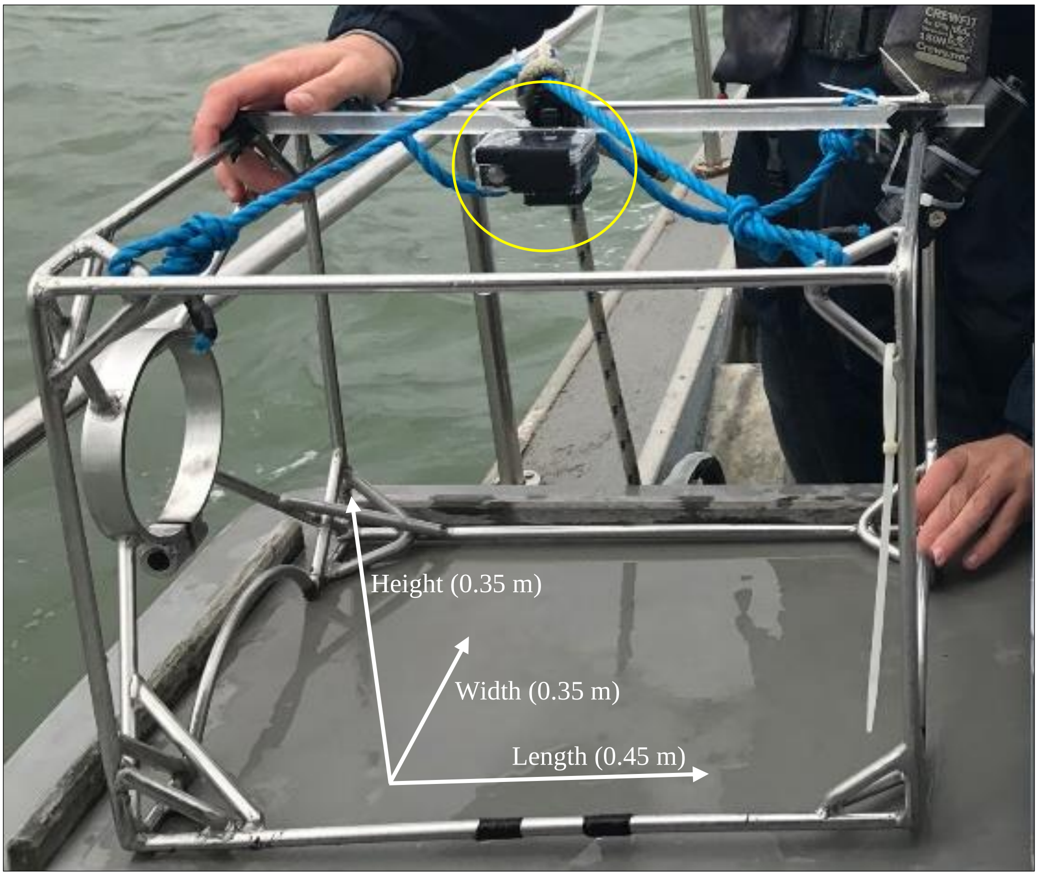


Figure 5.4. GoPro Hero 3+ camera (within the yellow circle) and scaffold with height, width and length dimensions, measured in metres (m), to video capture the riverbed.

Sediment was suspended in ultrapure water and Calgon (5%) to disaggregate the sample whilst maintaining colloidal stability (Walker *et al.*, 1974). A subsample (1 ml) of this sediment-Calgon solution was then processed using a Coulter counter with an aperture of 200 µm to determine particle grain size. The 200 µm aperture ensured all particles were accounted for, sizing particles within an analytical range of 125-1 µm. Three replicates of each sample were analysed in the Coulter counter, and sediment type was consequently named according to (Blott and Pye, 2001) using the Gradistat Excel macro (Table 5.2). The drop-down video data at stations 2 to 4 allowed for qualitative description of common species seen with each deployment of the camera and frame. Information of both sediment and species present enabled habitat biotope codes (as established by JNCC and EUNIS) to be allocated to the Beaulieu River (Parry, 2019).

Table 5.2. Grain size according to Gradistat excel macro used for sediment analysis (Blott and Pye, 2001).

Sediment type	**ϕ**	**Size (µm)**
Very fine sand	4 to 3	63 – 125
Very coarse silt	5 to 4	31 – 63
Coarse silt	6 to 5	16 – 31
Medium silt	7 to 6	8 – 16
Fine silt	8 to 7	4 – 8
Very fine silt	9 to 8	2 – 4
Clay	9 +	< 2

5.2.2 Gabions

Four 1 m^3 gabions were deployed in the Beaulieu River to test the two important restoration considerations: density and elevation. Gabions were manually assembled using materials acquired from Hy-Ten Ltd.; 1 m^2 sheets of PVC-zinc coated steel wire mesh were fixed into a cube shape using a combination of lacing wire and cable ties. Fourteen concrete breeze blocks were placed into the base of each gabion for stability, and shelves (using the same steel mesh) were added at a low and high elevation of either 0.2 m or 0.8 m from the base. Each gabion was then attached to a plastic pallet base with thick cable strops woven through for lifting purposes (Figure 5.5a and 5.5b). The lid of each gabion was attached with plastic cable ties to allow easy access to the oysters. Each gabion weighed < 400 kg.

5.2.3 Oysters

A batch of 840 *Bonamia*-naïve oysters were acquired from Loch Ryan, Scotland and maintained in Empress Dock at the National Oceanography Centre Southampton. Oysters were scrubbed clean of any epifauna and then divided into the four 1 m^3 gabions using a microreef (MR) system as designed by *O. edulis* fisherman Tony Legg (Figure 5.6). Recent fieldwork in the Solent using this MR system to deploy *O. edulis* in marinas had found a significantly higher larvae abundance being produced from oysters at lower density (20 oysters MR^{-1}) than at their high density (40 oysters MR^{-1}) (Luke Helmer, pers. comm). Herein, a density of 20 oysters MR^{-1} was used as the high-density treatment, and a new low-density of 12 oysters MR^{-1} was also included. Ten or twelve MRs (at the two respective densities) were placed onto the shelf of each gabion resulting with either a low density of 120 oysters m^{-3} (10 MRs), or with a high density of 240 oysters m^{-3} (12 MRs) (Table 5.3).


Figure 5.5a

Figure 5.5b

Figure 5.5 (a and b). A 1 m^3 gabion with high elevation shelf (0.8 m above the seabed) holding a high density of oysters (240 oysters m^{-3}) side view (Figure 5.5a) and plan view (Figure 5.5b). Fourteen concrete blocks sit at the bottom of the gabion for stability and the purple strops provide the lifting mechanism.

Figure 5.6. One microreef (MR) as designed by Jersey Sea Farms containing *Ostrea edulis*.

5.2.4 Gabion deployment

Gabions 1-4 were deployed between 24th November and 5th December 2017 at three navigation piles (15, 17 and 19, Table 5.3) on the northern side of the entrance to the Beaulieu River (Figure 5.7).

Table 5.3. Densities and elevation from the seabed of LR oysters in each gabion, and the navigation pile (Nav. pile) with Latitude (Lat.) and Longitude (Long.) in degree decimal minutes (DDM) at which they were deployed.

Gabion	***Oyster density***		***Elevation***		***Nav. pile***	***Lat. (DDM)***	***Long. (DDM)***
1	High	240 oysters m^{-3}	High	0.8 m	17	50 46.80 N	001 23.34 W
2	Low	120 oysters m^{-3}	Low	0.2 m	19	50 46.73 N	001 23.79 W
3	High	240 oysters m^{-3}	Low	0.2 m	15	50 46.88 N	001 23.06 W
4	Low	120 oysters m^{-3}	High	0.8 m	15	50 46.88 N	001 23.06 W

5.2.5 Sea water temperature

Seawater temperature in the Beaulieu River was collected using a YSI Exo3 Multiparameter sonde deployed at the Royal Southampton Yacht Club pontoon (50 47.05 N, 1 24.77 W), 1.5 km from the gabions (marked with a black circle in Figure 5.7). The EXOsonde3 was deployed within a plastic pipe alongside a beam on the Royal Southampton Yacht Club pontoon (Figure 5.8). Temperature data were also collected using a HOBO UTBI-001 Tidbit v2 Temperature Logger attached to the top of gabion 1 (less than 1 m from the oysters) on navigation pile 17 (marked with a black cross in Figure 5.7). Temperature data acquired from both the EXOsonde3 and the HOBO logger throughout 2017, 2018 and 2019 were pooled, and the monthly fluctuations were similar to temperatures seen in 1974 and 1975 by Rodhouse (1978) (Figure 5.9).

5.2.6 Sampling

Sampling occurred in August 2018 (1st) and April 2019 (9th and 11th). At each sampling event, oyster mortality and epifauna on the oysters and in the gabions was recorded. On both sampling dates, eight oysters were retrieved from each gabion and returned to the National Oceanography Centre Southampton for analysis of clearance, respiration and growth rates, and body condition index (as seen in section 3.2.2). During experiments, oysters were kept in an outside tank at the National Oceanography Centre Southampton with a flow through system of water directly from the Southampton Water at temperatures similar to those the oysters had been experiencing in the Beaulieu River upon retrieval. Temperatures in the Beaulieu River exceeded 25 °C in June and July, and therefore experiments were conducted at 24 °C in August 2018, and at 10.5 °C in April 2019.

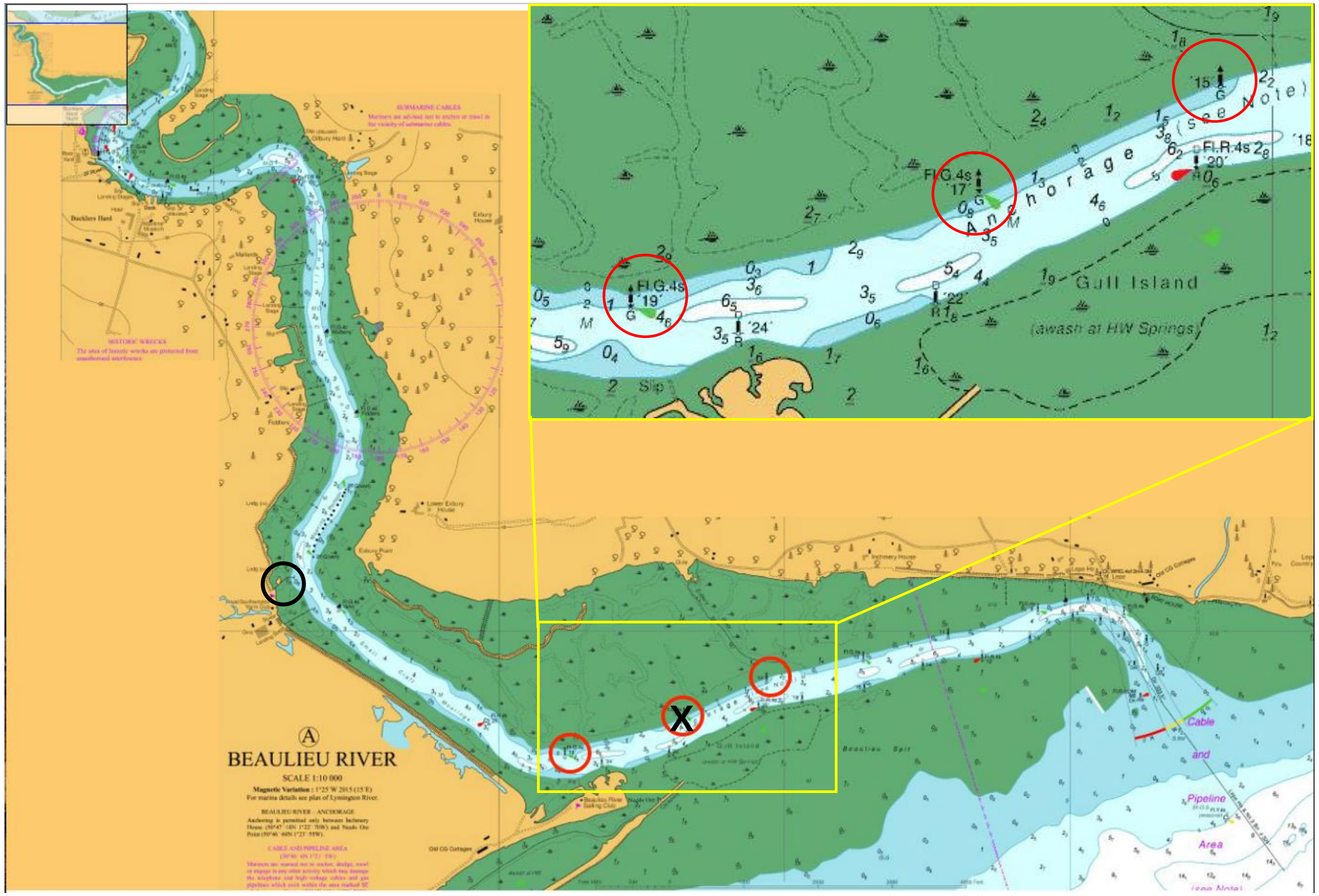


Figure 5.7. Location of navigation piles (15, 17 and 19) (marked with red circles) at which gabions were deployed. Locations of temperature loggers (a) YSI EXOsonde3 at the Royal Southampton Yacht Club (marked with a black circle) and (b) HOBO temp logger (marked with a black cross). Chart map (scale 1:10 000) acquired from www.visitmyharbour.com on 24th

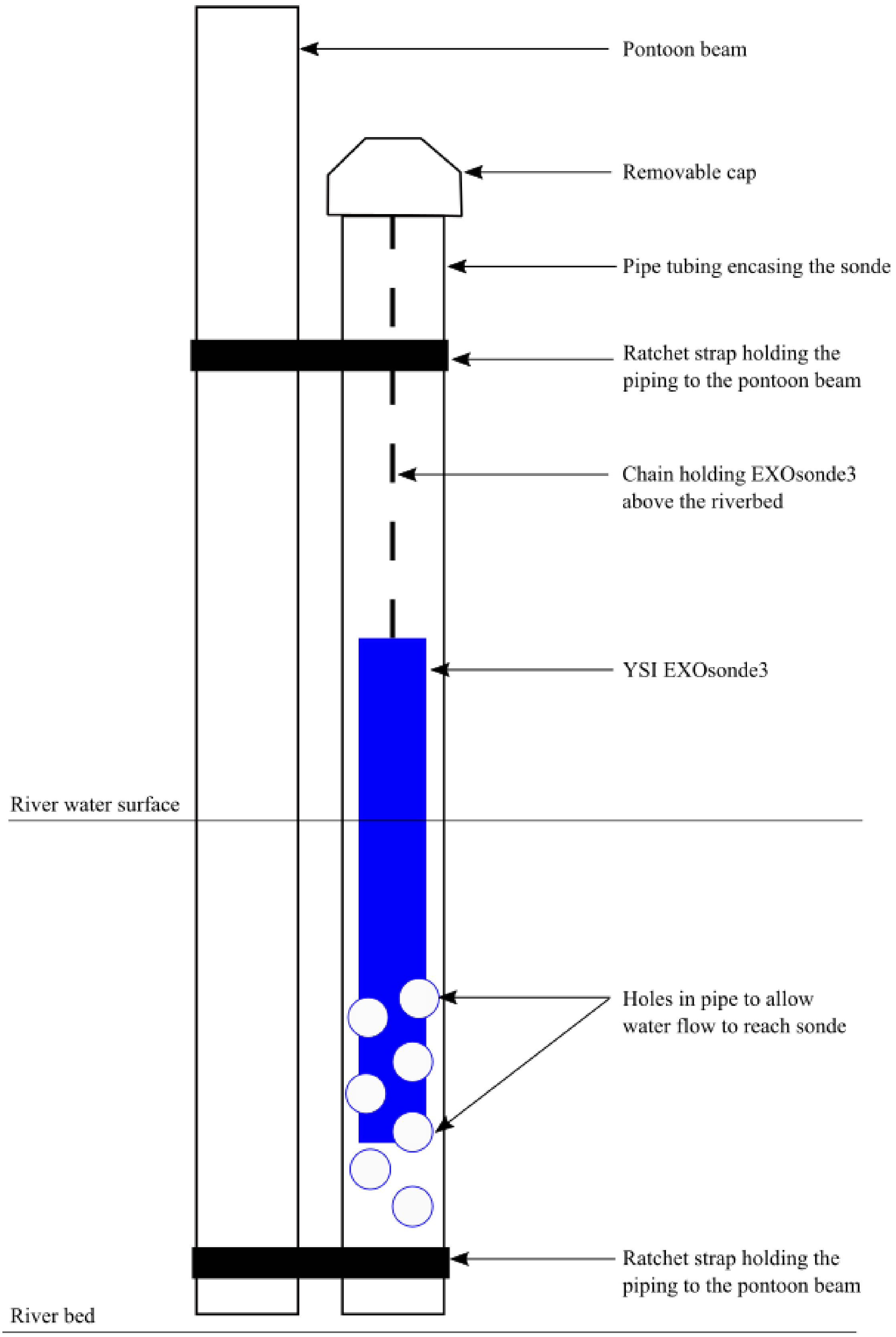


Figure 5.8. Deployment of the YSI EXOsonde3 with labelled components. Not to scale. Length of EXOsonde3 and piping was 0.59 m and 2.4 m respectively.

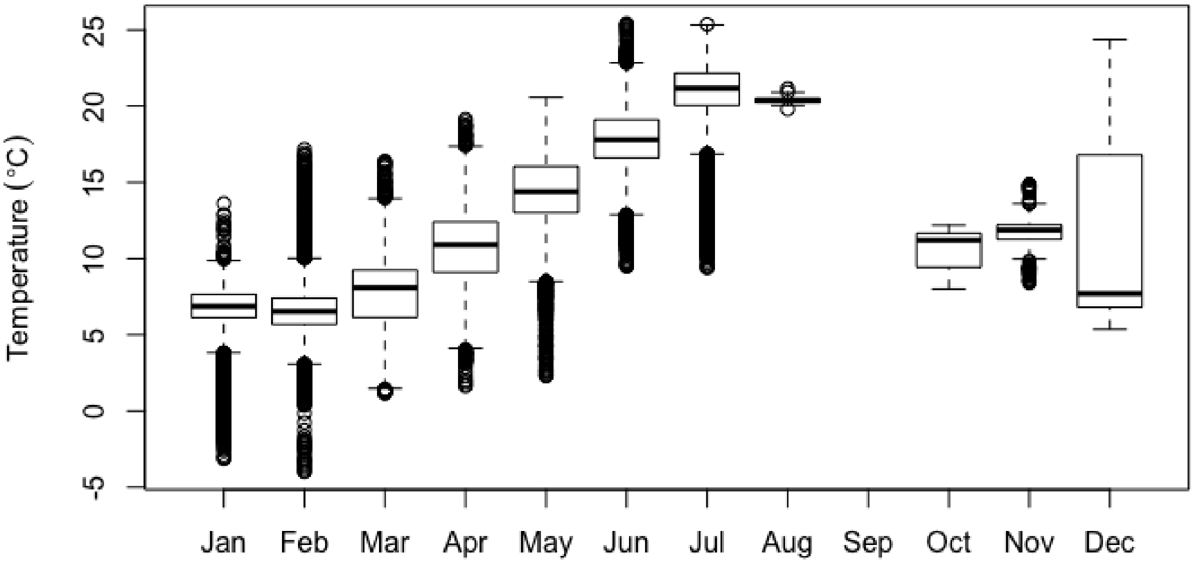


Figure 5.9. Water temperature (ºC) in the Beaulieu River in 2017 (n = 2544), 2018 (n = 20400), and 2019 (n = 28626) per month. No data were collected in September. Boxes hinge from the 1st to the 3rd quartiles (25th and 75th percentiles) and whiskers extend to the highest and lowest value within 1.5 * IQR of each hinge, where the IQR is the inter-quartile range, or distance between the 1st and 3rd quartile. Data beyond the whiskers are considered outliers and plotted as individual points.

5.2.7 Epifauna associated with an Ostrea edulis bed

At each sampling event, epifauna observed within the gabions and on the oysters were recorded. Particular note was made of instances of *O. edulis* predators such as tingle *Ocenebra erinacea*. Biotope codes from JNCC and EUNIS were used to describe the epifaunal community prior oyster deployment (in the baseline study) and post oyster deployment to determine the effect of *O. edulis* restoration in the Beaulieu River.

5.2.8 Oyster survival and mortality

Mortality was calculated by number of sampled oysters found gaping or with empty shells in April 2019 and given as a percentage of the sample total.

5.2.9 Physiological analysis

Clearance and respiration rates were acquired from eight Loch Ryan (LR) oysters from gabions 1 to 4 at two sampling events (August 2018 and April 2019) using methods as seen in section 3.2.2.

5.2.10 Oyster growth and body condition

Oyster growth measurements (height (mm), length (mm), and wet weight (g) (Galtsoff, 1964), see 2.2.1) were acquired through the comparison between 48 LR oysters at deployment in November 2017 and 48 LR oysters in August 2019. Oyster body condition was observed in 12 LR oysters in November 2017 upon arrival from Loch Ryan and compared with 32 LR oysters from the gabions in August 2018 and in April 2019. Body condition was acquired by weight index of dried soft tissue to dried shell (Lucas and Beninger, 1985) (see section 3.2.2).

5.2.11 Parasite (Bonamia ostreae) burden

In August 2019, an additional 40 Loch Ryan (henceforth named 'LRinfection') oysters were taken from gabions 2 and 3 (low elevation) to determine the effect of re-laying density on parasite prevalence amongst the population. DNA was extracted from each individual and, where present, parasite DNA was amplified with the use of PCR using primers designed by Ramilo *et al.* (2013). Positive and negative control samples were run for each PCR reaction, and presence of *B. ostreae* was identified through amplicon size (208 bp) (Ramilo *et al.*, 2013) (see 3.2.2 for full method).

5.2.12 Statistical analysis

All statistical analysis was conducted in the R environment (R Core Team, 2018) with packages ggbiplot, lsmeans, ggplot2 and devtools (Wickham, 2009; Vu, 2011; Lenth, 2016; Wickham *et al.*, 2018). A linear model (LM) or general linear model (GLM) was fitted to the data where possible, for analysis of variance. Sediment was analysed using the Microsoft Excel Gradistat macro (Blott and Pye, 2001) to calculate grain size statistics (including mean, mode, sorting, and skewness) arithmetically, geometrically (in metric units), and logarithmically (in phi units). All other data (such as epifaunal observations) were qualitative and required no statistical model.

5.3 Results

5.3.1 A baseline assessment of the Beaulieu River

A baseline study was conducted along the Beaulieu River in 2017 prior to the deployment of oysters in gabions. At three stations along the Beaulieu River (see Figure 5.2), sediment grabs were taken to assess the substrate type, and video footage of the riverbed was collected for qualitative data on benthic community already present.

Two sediment grabs were taken at each of the three stations. The collected sediment from the Beaulieu River was predominantly silt with a small proportion of fine sand (Figure 5.10). The percentage of sand particles increased (from 5.2% to 8.6%) towards the mouth of the River (Table 5.4).

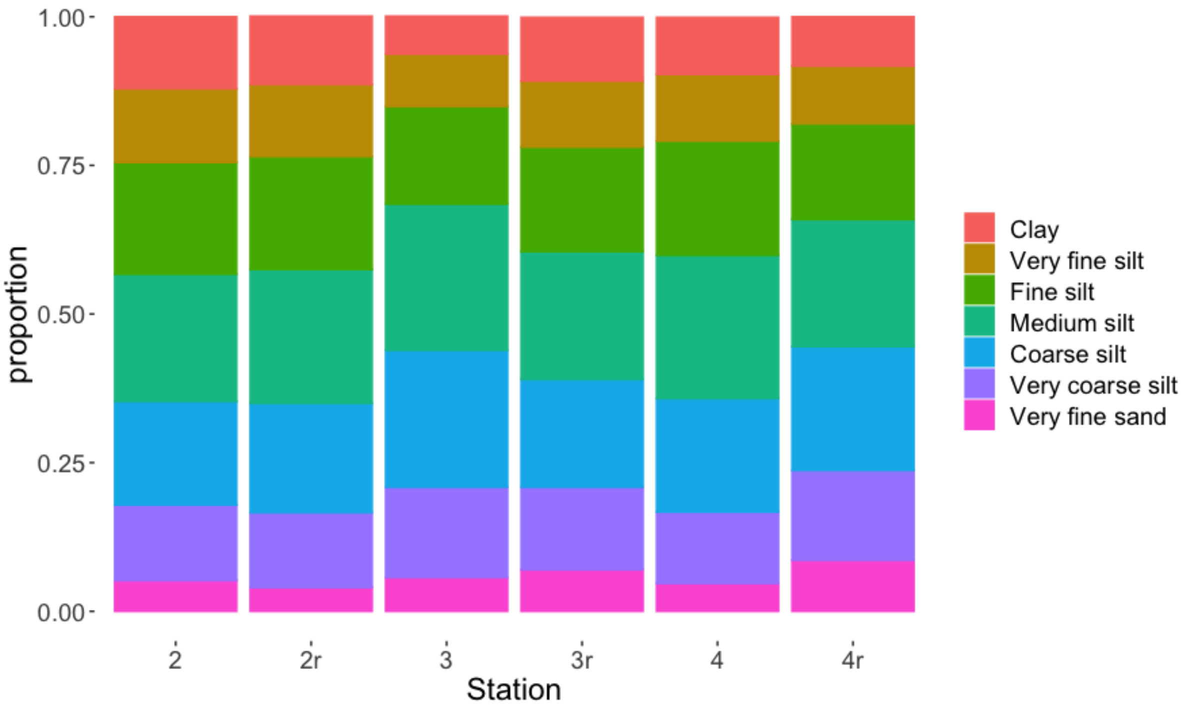


Figure 5.10. The proportion of sediment type at stations 2, 3 and 4 along the Beaulieu River (see Figure 5.7) with repeats (2r, 3r, and 4r). Sediment type was assessed using a Coulter Counter and classed according to the Gradistat Excel macro (Blott and Pye, 2001)

Table 5.4. Grain size analysis of sediment collected at stations 2, 3 and 4 (with replicates referenced as 2r, 3r and 4r) in the Beaulieu River using the Gradistat Excel macro (Blott and Pye, 2001). Each sediment type is represented as a percentage of the entire sample.

Sediment Type	**Overall Average**	**2**	**2r**	**3**	**3r**	**4**	**4r**
Very fine sand	5.9%	5.2%	4.0%	5.7%	7.0%	4.7%	8.6%
Very coarse silt	13.6%	12.7%	12.6%	15.2%	13.9%	12.0%	15.1%
Coarse silt	19.4%	17.4%	18.4%	23.0%	18.1%	19.1%	20.8%
Medium silt	22.6%	21.4%	22.5%	24.6%	21.5%	24.1%	21.4%
Fine silt	17.9%	18.8%	19.0%	16.4%	17.6%	19.2%	16.1%
Very fine silt	10.9%	12.4%	12.1%	8.8%	11.1%	11.2%	9.7%
Clay	9.8%	12.1%	11.5%	6.4%	10.7%	9.6%	8.3%

Video footage revealed evidence of a diverse epifaunal community (detail in Appendix G, Tables G1, G2, and G3), with several species of interest including fan worms *Sabella* spp., but only two *O. edulis* individuals (or shells) were recorded on camera (both captured at station 2, Figure 5.11)

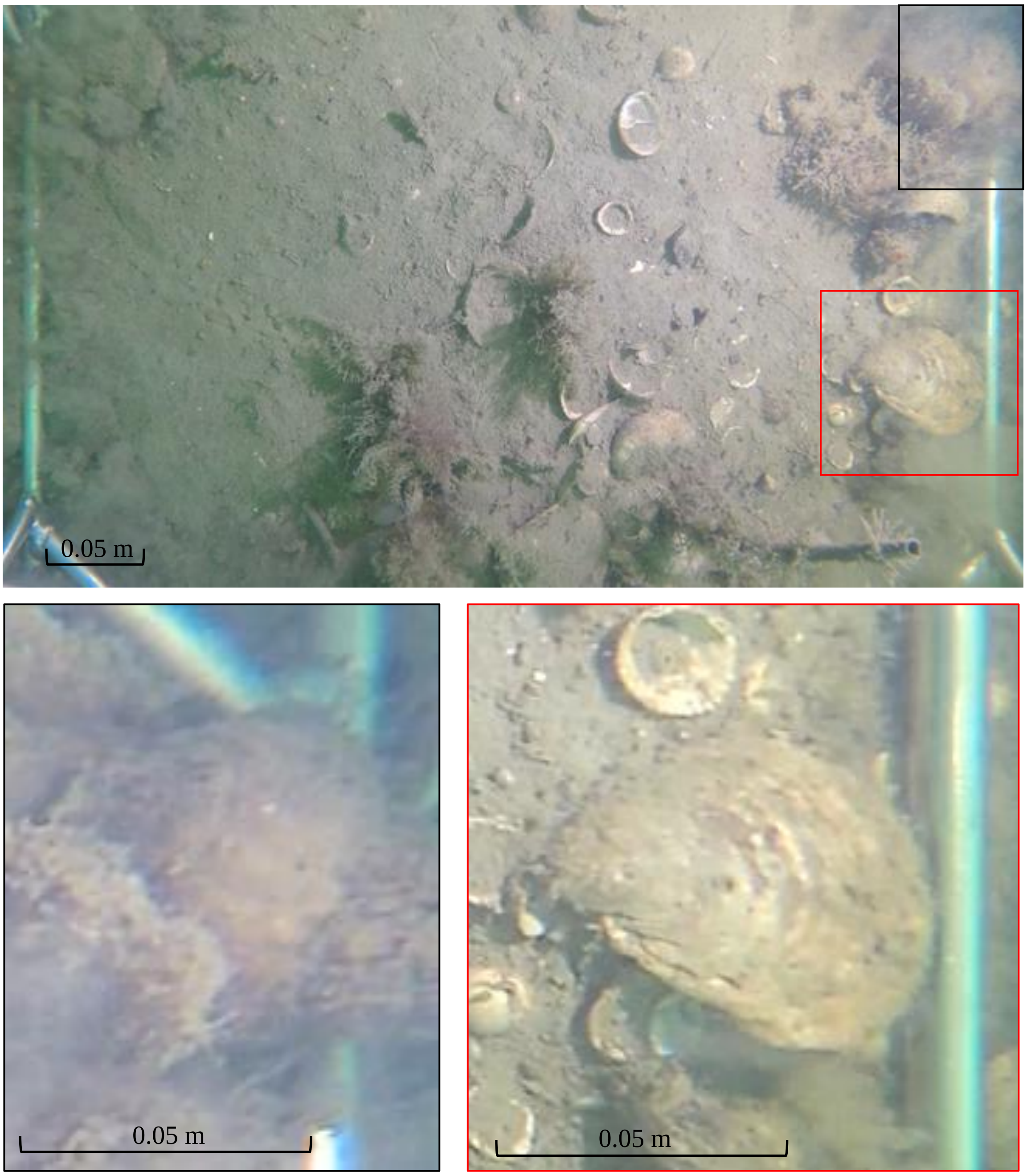


Figure 5.11. Two *Ostrea edulis* oysters were seen in video footage taken at station 2 (see Figure 5.2 for location) with the GoPro Hero 3+ camera.

Typical fauna along the Beaulieu River were fan worms (Figure 5.12), exposed mud with evidence of infauna (Figure 5.13), layered broken clam and cockle shells (Figure 5.14), and silt covered red and green algae (Figure 5.15).

Figure 5.12

Figure 5.13

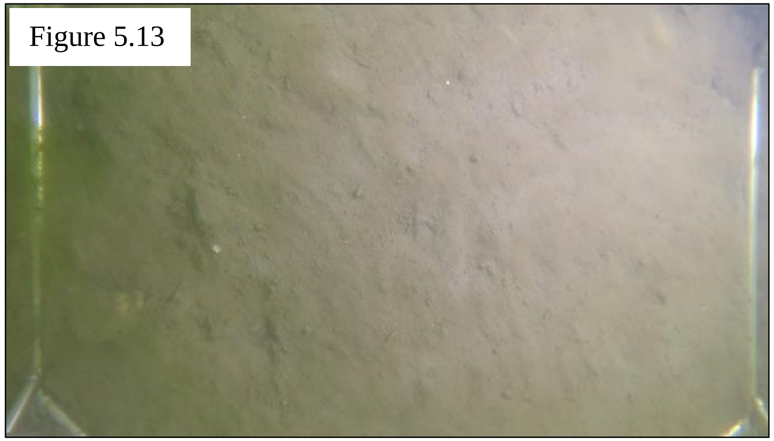


Figure 5.12 and Figure 5.13. Fan worms (*Sabella* spp.) (Figure 5.12) and exposed mud with evidence of infauna (Figure 5.13).

Figure 5.14

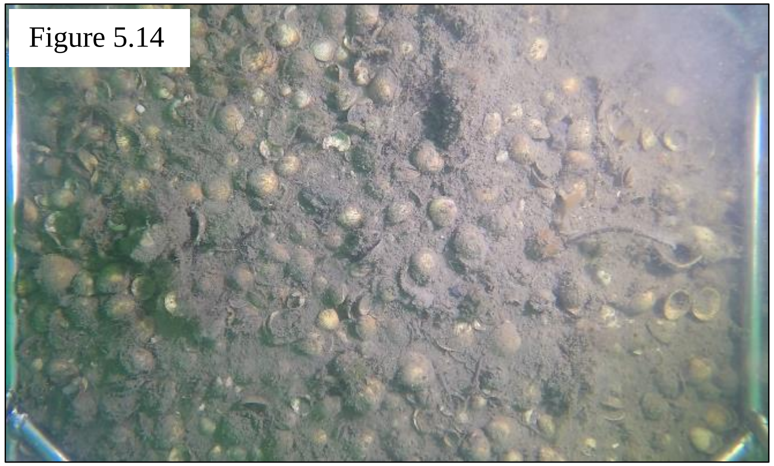


Figure 5.15

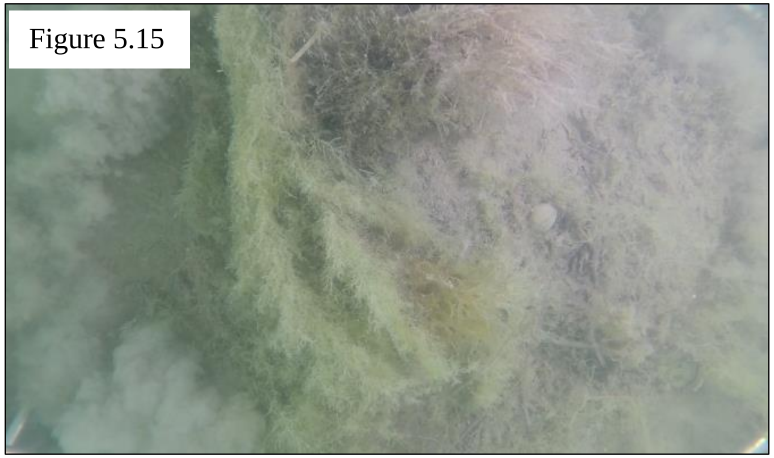


Figure 5.14 and Figure 5.15. Layered broken cockle and clam shells (Figure 5.14) and silt covered red and green algae (Figure 5.15).

5.3.2 Epifauna associated with the oyster gabions

Upon observation in August 2018 and April 2019, the gabions were heavily fouled with algae and benthic colonial species such as tunicates (images for this section can be found in Appendix G, Figures G1 and G2 respectively). Common organisms seen to be inhabiting amongst the oyster microreefs were fish (*Taurulus* spp., Figure G3), squat lobster (*Galathea squamifera*, Figure G4), crabs (*Carcinus* spp. Figures G5 and G6), and sea spiders (*Pantopoda* spp., Figure G7). Worms such as the sand mason, *Lanice conchilega*, terebellid polychaete, *Amphitrite* spp., and annelid worm, *Polydora spp.* (Figures G8, G9 and G10 respectively) were observed amongst the oyster shells, as were juveniles of species such as the sea mouse, *Aphrodita aculeata* (Figure G11). Oysters were encrusted with organisms such as keel worms, *Pomatoceros triqueter*, and tunicates (particularly *Ascidia conchilega*, *Ascidiella aspera*, *Ciona intestinalis* and *Distomus variolosus*) (Figures G12, G13, and G14a and G14b). Barnacles, *Semibalanus balanoides*, and *Balanus* spp., were common encrusting species, evident from either empty outer shells/plates (Figure G15), or living specimens (Figures G16a and G16b) on the LR oysters. The *O. edulis* predator, tingle *Ocenebra erinacea*, was seen at each sampling event (Figure G17). Although abundance was low (only four observed individuals per sampling date), the size of the individuals (shell length measured > 4 mm in August 2018, see Figure G18) were in the upper range of sizes recorded (Hancock, 1960; Anon, 1985; Hawkins and Hutchinson, 1990; Gibbs, 1996); average shell length of *O. erinacea* in the Solent was recorded at 34.6 ± 6 mm (Mardones *et al.*, 2020). Oysters within each gabion were being used as an egg depository for gastropods including *O. erinacea* (Figures G19 and G20) and in August 2018, four spider crabs (*Maja squinado*) with a carapace of over 12 cm were found within the gabions (Figures G21 and G22). There was evidence of attachment by boring sponge *Cliona* spp., from abandoned holes in the oyster shell (Figure G23), live specimens found on individuals (Figure G24), or inside the shell with layers of sponge causing the valves to crumble upon opening (Figures G25 and G26). Oyster spat were seen attached to LR oyster shells in both sampling events (2018 and 2019) (Figures G27a and G27b), but this study did not have the resources to discriminate between morphologically similar spat of molluscs present in the south coast of England, such as *O. edulis*, *Crassostrea* spp., and saddle oyster, *Anomia ephippium* (Figures G28, G29, G30 and G31). Upon sacrifice of the LR oysters for further analysis, fertilised eggs within the mantle known as ‘white sick’ was observed in several (uncounted) oysters, indicating successful reproduction (Figures G32, G33 and G34).

The observed epifauna post deployment of *O. edulis* gabions in the Beaulieu River was higher in diversity than that observed prior to oyster deployment (Table 5.5). Official biotopes as set out by the Joint Nature Conservation Committee (JNCC) (JNCC, 2015), and by the European Environmental Agency (EEA) EUNIS scheme could be used to further identify the habitat before and after the oyster deployment (Table 5.6 and 5.7 respectively).

Table 5.5. Qualitative data of common species seen in the Beaulieu River prior- and post-deployment of gabions containing *Ostrea edulis* (see Table 5.3, Figure 5.7 for specific location). Observations prior to deployment was collected from video footage taken with the GoPro Hero 3+ camera, and post deployment were acquired by direct observation during sampling. Three replication samples were taken at station 2, each with 10 video drop-down events. Asterisks (*) represents presence of the given species or description for each replication sample.

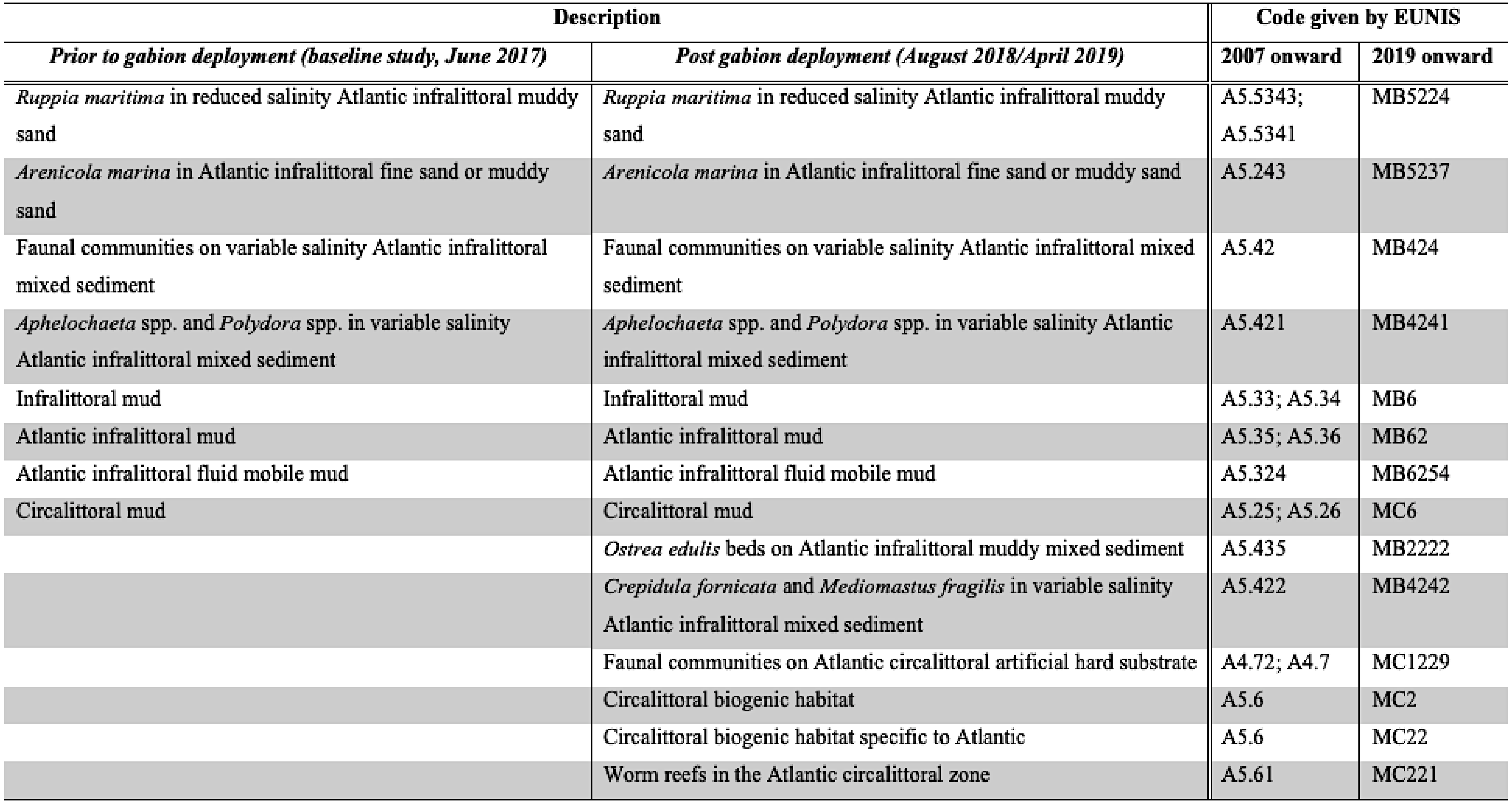
Prior to gabion deployment (baseline study, June 2017)	***Post gabion deployment (August 2018/April 2019)***
Soft muddy sediment – silt cloud suggesting very soft sediment	Soft muddy sediment – silt cloud suggesting very soft sediment
Broken (dead) cockle / clam shells	Broken (dead) cockle / clam shells
Evidence of infauna	Evidence of infauna
Evidence of epifauna (e.g. crabs)	Evidence of epifauna (e.g. crabs)
Coral weed (*Corallina offinalis*)/wire weed (*Sargassum* spp.)/harpoon weed (*Asparagopsis armata*/*phycodrys* spp.)	Coral weed (*Corallina offinalis*)/wire weed (*Sargassum* spp.)/harpoon weed (*Asparagopsis armata*/*phycodrys* spp.)
Gut weed (*Ulva intestinalis*)	Gut weed (*Ulva intestinalis*)
Green and red algae (*Cladophora rupestris, Ulva, Plocamium cartilagineum, Anotrichum barbatum*?)	Green and red algae (*Cladophora rupestris, Ulva, Plocamium cartilagineum, Anotrichum barbatum*?)
High abundance of fan/tube worms (*Sabella* spp.?)	High abundance of fan/tube worms (*Sabella* spp.?)
Porifera (*Cliona* spp., *Axinella* spp., *Homaxinella* spp.?)	Porifera (*Cliona* spp., *Axinella* spp., *Homaxinella* spp.?)
High abundance of colonial ascidians (*Botryllus* spp., *Botrylloides* spp.)	High abundance of colonial ascidians (*Botryllus* spp., *Botrylloides* spp.)
Crepidula fornicata	*Crepidula fornicata*
Tunicates (*Ascidia*/*Ascidiella* spp.)	Tunicates (*Ascidia*/*Ascidiella* spp.)
	Tunicates (*Ciona intestinalis*, *Distomus variolosus*)
	Sting winkle (*Ocenebra erinacea*)
	Crabs (Spider crab, *Maja squinado*, and *Carcinus* spp.)
	Squat lobster (*Galathea squamifera*)
	Fish (*Taurulus* spp.)
	Sea spiders (*Pantopoda* spp
	Worms (sand mason, *Lanice conchilega*, terebellid polychaete, *Amphitrite* spp., and annelid worm, *Polydora spp.)*
	Sea mouse (*Aphrodita aculeate*)
	Keel worms (*Pomatoceros triqueter*)
	Barnacles (*Semibalanus balanoides*, *Balanus* spp.)

Table 5.6. Relevant JNCC recommended marine habitat classification biotopes* for Britain and Ireland associated with the substrate type found in the Beaulieu River. Full descriptions of each JNCC biotope code can be found in Appendix G in Table G4.

Description		**Code given by JNCC**
Prior to gabion deployment (baseline study, June 2017)	***Post gabion deployment (August 2018/April 2019)***	
Sublittoral sediment	Sublittoral sediment	SS
Sublittoral cohesive mud and sandy mud communities	Sublittoral cohesive mud and sandy mud communities	SS.Smu
Sublittoral mud in variable salinity (estuaries)	Sublittoral mud in variable salinity (estuaries)	SS.Smu.SmuVS
Circalittoral sandy mud	Circalittoral sandy mud	SS.Smu.CsaMu
Sublittoral mixed sediment	Sublittoral mixed sediment	SS.SMx
Sublittoral mixed sediment in variable sediment (estuaries)	Sublittoral mixed sediment in variable sediment (estuaries)	SS.SMx.SMxVS
Sabella pavonina with sponges and anemones on infralittoral mixed sediment	*Sabella pavonina* with sponges and anemones on infralittoral mixed sediment	SS.SMx.Imx.SpavSpAn
Infralittoral mobile sand in variable salinity (estuaries)	Infralittoral mobile sand in variable salinity (estuaries)	SS.SSa.SSaVS.MoSaVS
	Sublittoral biogenic reefs on sediment	SS.SBR
	Ostrea edulis beds on shallow sublittoral muddy mixed sediment	SS.SMx.Imx.Ost
	Crepidula fornicata and *Mediomastus fragilis* in variable salinity infralittoral mixed sediment	SS.SMx.SMxVS.CreMed
	Venerupis senegalensis, *Amphipholis squamata* and *Apseudes latreilli* in infralittoral mixed sediment	SS.SMX.IMx.VsenAsquAps
	Ampelisca spp., *Photis longicaudata* and other tube-building amphipods and polychaetes in infralittoral sandy mud	SS.SMU.ISaMu.AmpPlon
	Circalittoral fouling faunal communities	CR.FCR.FouFa

*JNCC habitat biotopes accessed on 05.09.2017 at http://jncc.defra.gov.uk/marine/biotopes/hierarchy.aspx.

Table 5.7. Relevant EUNIS recommended marine habitat classification biotopes for Britain and Ireland associated with the substrate type found in the Beaulieu River. Full descriptions of each EUNIS biotope code can be found in Appendix G in Table G5.



Description		Code given by EUNIS	
Prior to gabion deployment (baseline study, June 2017)	***Post gabion deployment (August 2018/April 2019)***	**2007 onward**	**2019 onward**
Ruppia maritima in reduced salinity Atlantic infralittoral muddy sand	*Ruppia maritima* in reduced salinity Atlantic infralittoral muddy sand	A5.5343; A5.5341	MB5224
Arenicola marina in Atlantic infralittoral fine sand or muddy sand	*Arenicola marina* in Atlantic infralittoral fine sand or muddy sand	A5.243	MB5237
Faunal communities on variable salinity Atlantic infralittoral mixed sediment	Faunal communities on variable salinity Atlantic infralittoral mixed sediment	A5.42	MB424
Aphelochaeta spp. and *Polydora* spp. in variable salinity Atlantic infralittoral mixed sediment	*Aphelochaeta* spp. and *Polydora* spp. in variable salinity Atlantic infralittoral mixed sediment	A5.421	MB4241
Infralittoral mud	Infralittoral mud	A5.33; A5.34	MB6
Atlantic infralittoral mud	Atlantic infralittoral mud	A5.35; A5.36	MB62
Atlantic infralittoral fluid mobile mud	Atlantic infralittoral fluid mobile mud	A5.324	MB6254
Circalittoral mud	Circalittoral mud	A5.25; A5.26	MC6
	Ostrea edulis beds on Atlantic infralittoral muddy mixed sediment	A5.435	MB2222
	Crepidula fornicata and *Mediomastus fragilis* in variable salinity Atlantic infralittoral mixed sediment	A5.422	MB4242
	Faunal communities on Atlantic circalittoral artificial hard substrate	A4.72; A4.7	MC1229
	Circalittoral biogenic habitat	A5.6	MC2
	Circalittoral biogenic habitat specific to Atlantic	A5.6	MC22
	Worm reefs in the Atlantic circalittoral zone	A5.61	MC221

5.3.3 Oyster survival and mortality rates

Across all gabions, there was a survival rate of 67.19% (n = 64), between deployment in November 2017 and recovery in April 2019. Mortality rates were between 30 and 35% in each gabion and were not affected by density (mortality in high density = 32.5%, n = 40, low density = 33.33%, n = 24) or elevation (mortality high elevation = 34.4%, n = 32, low elevation = 31.3%, n = 32).

5.3.4 Physical metrics, growth and mortality

The dimensions of LR oysters from all four gabions were taken at deployment in November 2017 and in April 2019. The Loch Ryan oysters were deployed at an average height, length and wet weight of 69.2 mm ± 3.9, 63.8 mm ± 7. 4 and 69.9 g ± 7.3 (mean ± sd, n = 32) respectively in November 2017, and were retrieved with a mean height, length and wet weight of 73.1 mm ± 5.6, 67.5 mm ± 6.4, and 81.0 g ± 10.0 (mean ± sd, n = 32) respectively in April 2019. This produced an estimated growth rate of 5.6 g y^{-1}. Many individuals were observed with a clear/translucent frill along the edge of the shell, demonstrating new growth (examples shown in Appendix G, Figures G35 and G36). Significant variation in LR oyster wet weight (WW) (g) could be statistically explained by the interaction between re-laying density and elevation from the seabed (LM, $F_{1,60}$ = 8.29, P = 0.006, Figure 5.16), and between years (LM, $F_{1,60}$ = 27.90, P = 1.934e^{-06}, Figure 5.17). There was an increase in oyster WW after 2 years, and the heaviest oysters were seen at either high elevation and high density, or at low elevation and low density.

5.3.5 Clearance rate

Clearance rates could be compared between the samples from August 2018 and those from April 2019 as temperature did not have a significant effect (LM, $F_{1,63}$ = 1.372, P = 0.246). Clearance rates were between 0.01 and 8.69 L h^{-1} g $AFDW^{-1}$ with an average of 1.73 ± 2.20 L h^{-1} g $AFDW^{-1}$ (mean ± standard deviation) in August 2018, and between 0.09 and 7.41 L h^{-1} g $AFDW^{-1}$ in April 2019 with an average of 2.39 ± 2.30 L h^{-1} g $AFDW^{-1}$. Variance in clearance rate could not be statistically explained by differences between years (GLM, $F_{1,61}$ = 1.34, P = 0.252), oyster re-laying density (GLM, $F_{1,61}$ = 0.34, P = 0.564), or elevation from the seabed (GLM, $F_{1,61}$ = 0.96, P = 0.332).

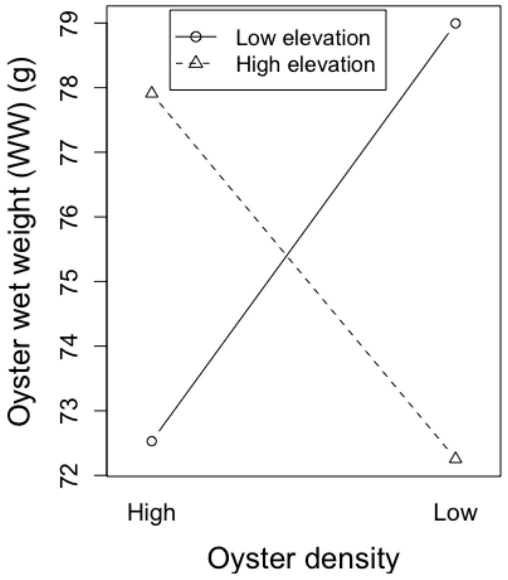


Figure 5.16. Difference in wet weight (WW) could be explained by the interactive effect of density and elevation (LM, $F_{1,60}$ = 8.29, P = 0.006), suggesting the effect of density depended on elevation, and vice versa (high:low density, n = 40:24 , elevation, n = 32).

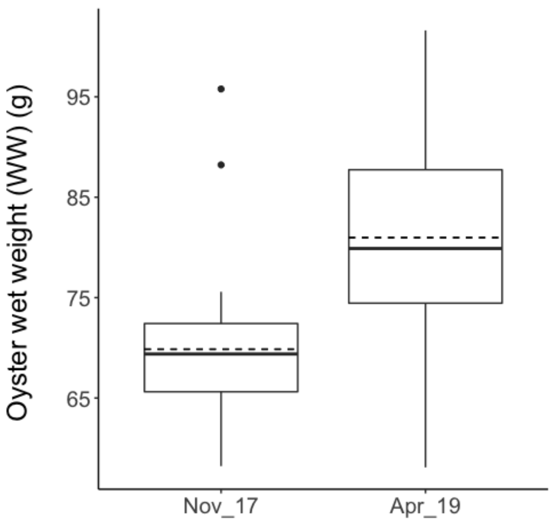


Figure 5.17. Regardless of density or elevation, the wet weight (WW) of LR oysters was significantly different between sampling events in November 2017 (Nov_17) and April 2019 (Apr_2019) (LM, $F_{1,60}$ = 27.90, P = 1.934e^{-06}) demonstrating considerable growth (n = 32).

5.3.6 Metabolic rate acquired from respiration rate

The difference in experiment water temperature (24 °C in August 2018 and 10.5 °C in April 2019) did not have a significant effect on respiration rate (LM, $F_{1,63}$ = 1.269, P = 0.264), which meant the effects of density and elevation could be robustly compared between the two events. Respiration rates were between 0.02 and 2.66 mg O_2 L^{-1} g $AFDW^{-1}$. Variation in respiration rate was statistically explained by the interaction between elevation and density (GLM, $F_{1,58}$ = 12.38, P < 0.001, Figure 5.18), and the effect of both elevation and density was different each year (GLM, $F_{1,58}$ =10.27, P = 0.002, Figure 5.19), and (GLM, $F_{1,58}$ = 7.28, P = 0.009, Figure 5.20).

Figure 5.18

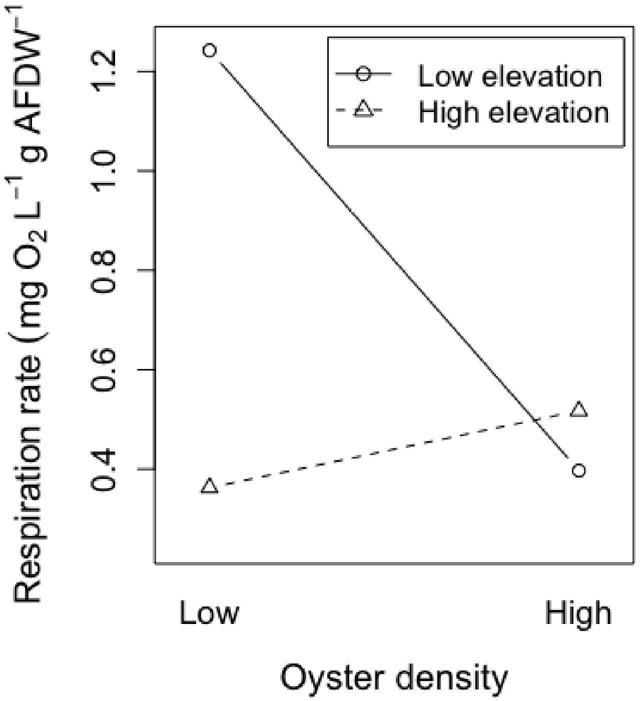


Figure 5.19

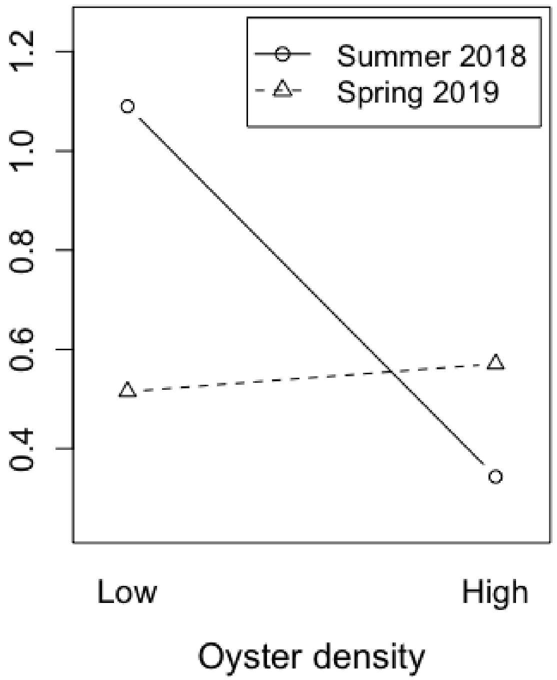


Figure 5.20

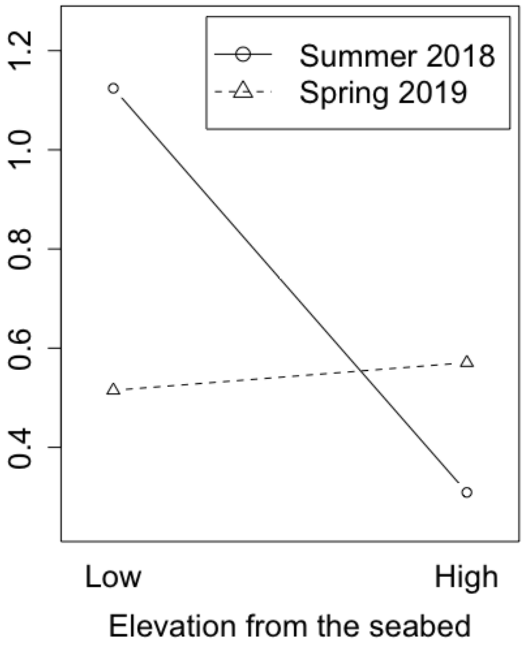


Figure 5.18, Figure 5.19, and Figure 5.20. The variation seen in respiration rate between high and low densities depended on elevation (Figure 5.18), and sampling event (summer 2018 and spring 2019) (Figure 5.19). The variation seen in respiration rates between high and low elevations was also dependent on sampling event (Figure 5.20). Each marker represents the mean for 8 oysters.

5.3.7 Condition index

There was a significant difference in oyster body condition between sampling events (GLM, $F_{1,60}$ = 21.76, $P < 0.0001$, Figure 5.21). There was an interactive effect of both effects (density and elevation) in summer 2018 (GLM, $F_{1,29}$ = 9.442, P = 0.005, Figure 5.22), and in spring 2019 (GLM, $F_{1,29}$ = 7.8951, P = 0.009, Figure 5.23), suggesting condition of oysters at each elevation depended on the density at which the oysters were laid; the best LR oyster body condition was seen at either high density and low elevation, or low density and high elevation.

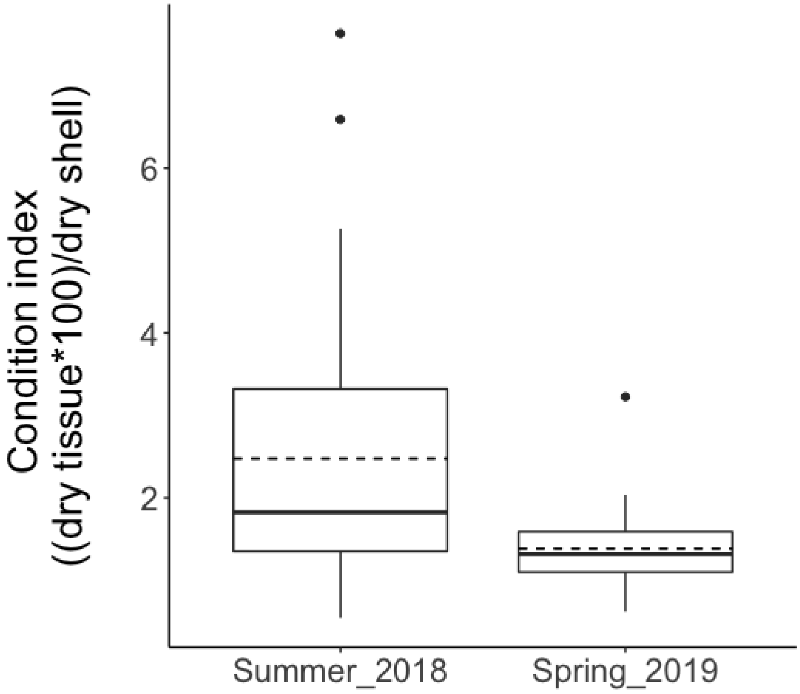


Figure 5.21. The condition of oysters decreased between the years 2018 and 2019 (n = 32), irrespective of density or elevation.

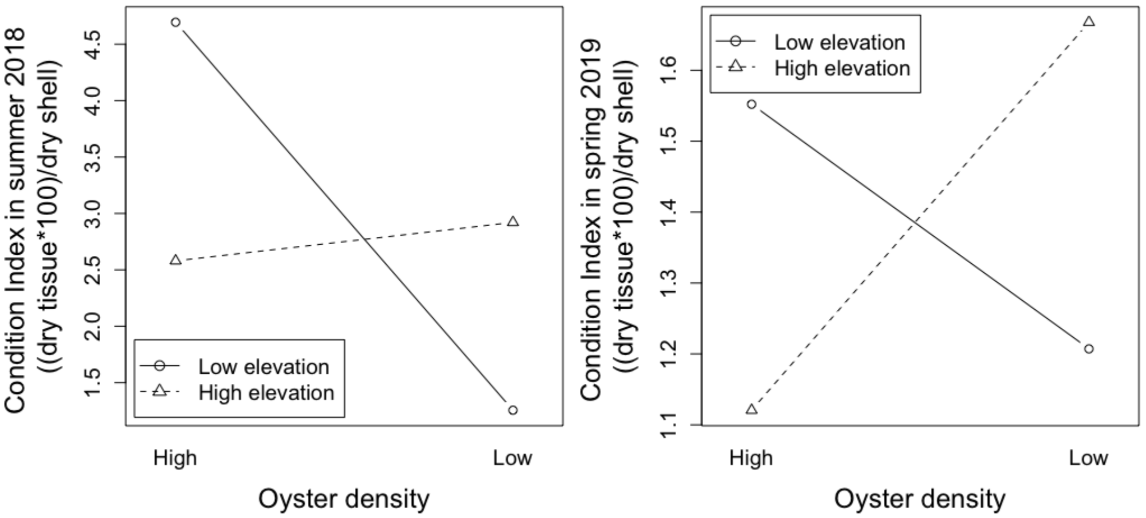


Figure 5.22 and Figure 5.23. The interaction between re-laying density and elevation from the seabed had a significant effect on the variation in condition index in summer 2018 (Figure 5.22), and in spring 2019 (Figure 5.23). Each marker represents the mean for 8 oysters.

The condition of the LR oysters in November 2017 before deployment in the Beaulieu River was 3.29 ± 1.35 (mean average ± sd, n = 12), which then decreased to 2.48 ± 1.68 (n = 32) in August 2018 and 1.39 ± 0.50 (n = 32) in April 2019. Although a small number of oysters were measured in 2017, these values shows a general decrease in the body condition of the LR oysters post deployment in the Beaulieu River.

5.3.8 Effect of Bonamia ostreae infection

To quantify the prevalence of *B. ostreae* amongst the (previously *Bonamia*-free) Loch Ryan population after two years in the *Bonamia*-exposed Beaulieu River, gill tissue from a larger group of (‘LRinfection’) oysters from gabions 2 and 3 (both with low elevation at only 0.2 m from the seabed) was taken in August 2019 for molecular analysis. Only four of the forty-eight LRinfection oysters (8.33%) had contracted a *B. ostreae* infection by 2019. There was a relationship between infection status, density and oyster wet weight (WW) (GLM, $F_{1,91}$ = 9.93, P = 0.002, Figure 5.24); *B. ostreae* infected oysters were significantly lighter at high oyster densities than those at low densities.

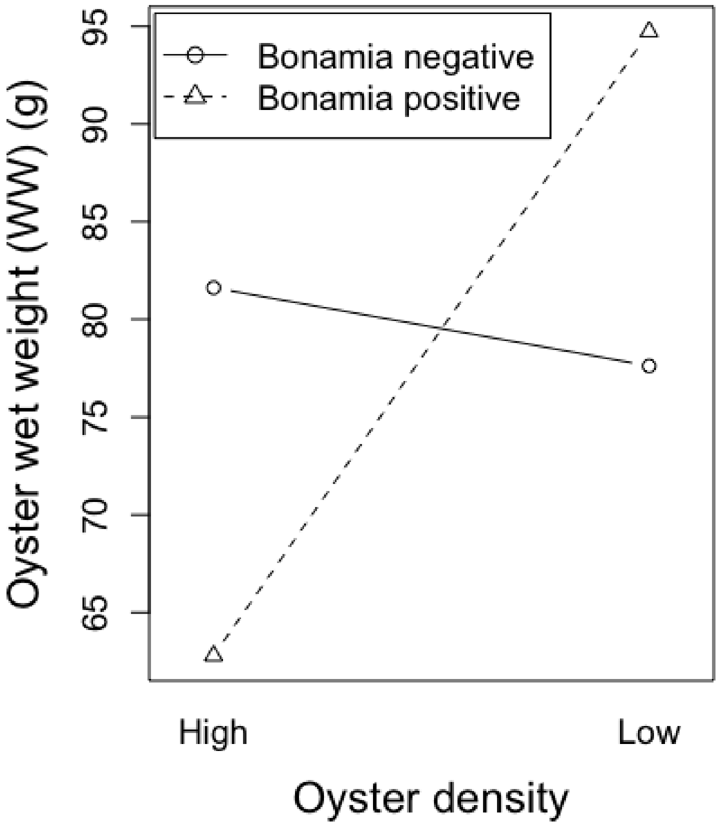


Figure 5.24. The effect of re-laying density on LRinfection oyster wet weight (WW) (g) was dependent on whether the oyster was infected with *B. ostreae*.

5.4 Discussion

The aim of this work was to determine (a) whether restoration supports biodiversity in the Beaulieu River, (b) if density and elevation had an effect on physiological performance of individuals, and (c) whether density influenced population parasite prevalence two-years after deployment in the Beaulieu River, England. Oysters from Loch Ryan, Scotland (‘LR’ oysters) were placed into four gabions at one of two densities (120 or 240 oysters m^{-3}) on shelves at one of two elevations (0.2 or 0.8 m) from the seabed and deployed into the Beaulieu River in November 2017. The epifaunal community was observed with a baseline study prior to this oyster deployment and recorded at each subsequent sampling event in August 2018 and April 2019. At these two sampling events, oyster mortality was recorded, and a sample of oysters were returned to the National Oceanography Centre Southampton to determine the effect of density and elevation on growth, condition, and physiological performance in the form of clearance and metabolic (respiration) rates. Forty oysters (‘LRinfection’ oysters) were then acquired from both gabions 2 (low elevation, low density) and 3 (low elevation, high density) to determine the effect of density on parasite prevalence amongst the (previously *Bonamia*-free) population after two years in the *Bonamia*-exposed Beaulieu River.

5.4.1 The Beaulieu River benthic environment and epifaunal community

The removal of oyster habitat from the Beaulieu River in the 1980s meant any epifauna associated with the oyster bed was also removed, thus shifting the ecosystem structure and functionality (Posey and Hines, 1991). The baseline study in the Beaulieu River on the 21st June 2017 offered a snapshot insight into the natural benthic environment prior to oyster deployment. The high proportion of silt in the sediment suggested any organisms deployed onto the riverbed would likely be at risk of smothering or sedimentation. Video imagery prior to the deployment of oysters for this study revealed expansive areas of exposed silt with evidence of infauna, with only 2 oysters observed in an area covering 12.64 m^2 indicating an abundance of 1 oyster every 6.32 m^2. The high incidence of broken clam and cockle shell seen in the video footage was likely a result of recent clam fishing in the Beaulieu River, either from the active replacement of clam shell cultch in the river to support further recruitment or from natural mortality of a dense population. The baseline study revealed a low relief, two dimensional and heavily-silted benthic environment in the Beaulieu River prior to oyster deployment.

Deployment of the gabions containing *O. edulis* for this study added a three-dimensional structure to the Beaulieu River benthic environment, and an increase in associated epifauna was observed. At both sampling events (August 2018 and April 2019), the *O. edulis* gabions supported a diverse epifaunal community, which was indicative of the extensive ecosystem habitat support offered by an oyster bed and suggested rapid succession of the benthic community associated with *O. edulis*. Three layers of succession were evident, with species colonising (1) the oysters themselves, (2) the microreefs walls and space within, and (3) the gabion mesh walls and space within. Oyster shells were encrusted with tunicates (*Ascidia conchilega*, *Ascidiella aspera*, *Ciona intestinalis* and *Distomus variolosus*), keel worms (*Pomatoceros triqueter*), barnacles (*Semibalanus balanoides*, and *Balanus* spp.), sponges (*Cliona* spp., *Axinella* spp., *Homaxinella* spp.), algae (*Cladophora rupestris*, *Ulva*, *Plocamium cartilagineum*, *Anotrichum barbatum*, *Corallina offinalis*, *Sargassum* spp., *Asparagopsis armata*, *phycodrys* spp., and *Ulva intestinalis*), egg capsules and spat, with more mobile invertebrates such as polychaetes (*Lanice conchilega*, *Amphitrite* spp., and *Polydora spp.*), and sea spiders (*Pantopoda* spp.) moving within shell layers. The hard plastic of the microreefs and the mesh walls of each gabion similarly supported colonies of algae, tunicates, and bryozoans. The presence of predator (adult) tingle *Ocenebra erinacea* amongst the oysters was low at each sampling event for this study, although the observed specimens were large and there were multiple clumps of egg capsules suggesting successful reproduction. The spider crabs, *Maja squinado*, seen in the gabions in August 2018 were too large to exit through the mesh, suggesting they had entered post moult and were thriving on the organisms within the gabion (Figures G21 and G22 in Appendix G). There is no evidence to suggest that *M. squinado* is a predator of *O. edulis* or that their claws would be strong enough to crush an *O. edulis* shell. However, *M. squinado* is an opportunistic omnivore known to consume other bivalve molluscs such as *Mytilus* spp., and fragments of unknown molluscs have been found in the gut of *M. squinado* (Bernárdez *et al.*, 2000). The diet of *M. squinado* also reportedly includes polychaetes (such as *Nereis* spp), gastropods (*Bittium* spp.), echinoderms (de Kergariou, 1974), crabs (*Carcinus* spp.) (Brosnan, 1981), algae (such as Phaeophyta, Rhodophyta, and Laminaiaceae) (Bernárdez *et al.*, 2000), and other invertebrates commonly associated with an oyster bed. If *M. squinado* were not able to prey on *O. edulis* owing to shell strength, then their presence could represent a synergistic relationship, as clearing algae and other fouling organisms would benefit the oysters by ensuring the mesh was clear to allow water to freely pass through and provide associated oxygen and food (macroalgae) to the oysters. The presence of *M. squinado* is evidence of a complex trophic community establishing, as mobile predators (attracted by the developing epifaunal community) begin to colonise the site. Attachment by boring sponge *Cliona* spp. was evident from external holes in the LR oyster shells, live specimens active on the LR oyster shell, or found layered within the internal LR oyster shell structure upon separation of the valves (Figures G23-G25 in Appendix G). *Ostrea edulis* can repair puncture wounds in their shell as a result of predation by tingle *O. erinacea* or boring sponge *Cliona* spp. (Quayle and Newkirk, 1989), but repeated repair patches within the shell structure often result in mud blisters, where sediment is caught between the shell layers. Trapped sediment causes a discolouration of the inner shell and renders the oyster unsuitable for consumer markets. This has implications for fisheries, as oysters are often served on the half shell, which means the shell quality and appearance is an important selling point. Further research would be required to determine the effect of *Cliona* spp. presence on sustainability of an *O. edulis* population.

The provision of hard substrate in the form of oyster shells (and the gabion structure itself) attracted a diverse community, which changed the JNCC and EUNIS biotope habitat codes from infralittoral/circalittoral sandy mud environment (SS.Smu.Csa.Mu (JNCC) / MB6, MB62 and MC6 (EUNIS)) with occasional tube worms such as *Sabella* spp. (SS.SMx.Imx.SpavSpAn (JNCC) / MB5237 (EUNIS)), to an *O. edulis* bed (SS.SMx.Imx.Ost and SS.SBR (JNCC) / MB2222, MC2, and MC22 (EUNIS)) supporting large numbers of tube building amphipods and polychaetes (SS.SMU.ISaMu.AmpPlon and SS.SMX.IMx.VsenAsquAps (JNCC) / MC221 (EUNIS)), tunicates (SS.SMx.SMxVS.CreMed (JNCC) / MB4242 (EUNIS)), and larger mobile organisms such as *Maja squinado* (CR.FCR.FouFa (JNCC) / MC1229 (EUNIS)). This study demonstrated how *O. edulis* restoration supports biodiversity; the complexity of the three-dimensional gabion structure with *O. edulis* shifted the habitat classification and increased the epifaunal community in the Beaulieu River. The gabion scaffold alone provided hard mesh substrate for organisms to attach, provided security from larger predators such as harbour seals with the size-constrained mesh walls (7 mm diameter holes), and promoted turbulence of the water passing through the structure, which would have reduced siltation and increased the amount of organic particles available for consumption by the species within (Colden *et al.*, 2016). A complementary study comparing the epifaunal community in gabions with and without *O. edulis* is now recommended in order to determine whether the observed improvement in habitat biodiversity is a function of the oysters, or whether it is simply a function of the gabion structure itself.

For this study, the gabion structure was designed to test two contested parameters of restoration (re-laying density and elevation from the seabed, see Figures 5.5a and 5.5b) on oyster mortality, growth and body condition (index) and physiological performance such as clearance and respiration rates.

5.4.2 Oyster mortality

Natural mortality of oyster spat has been well documented, and high cumulative mortalities of between 66% and 92% are generally accepted (Korringa, 1940; Cole, 1951; Walne, 1961, 1979). However, mortality of adult *O. edulis* is often reported in relation to environmental stressors such as climate fluctuations (Cole, 1940; Waugh, 1954; Carnegie and Barber, 2001), pollution (Kamphausen, 2012; Green, 2016), siltation and associated disruption to feeding (Korringa, 1952; Walne, 1979; Laing *et al.*, 2005), increased predation (Walne, 1961; Key and Davidson, 1981) or increased disease prevalence (Alderman *et al.*, 1977; Figueras, 1991; Hudson and Hill, 1991; Robert *et al.*, 1991; Cigarría *et al.*, 1995; Baud *et al.*, 1997; Culloty *et al.*, 2001; Montes *et al.*, 2003). The oysters used for this study came from a healthy wild population in *Bonamia*-free Loch Ryan in Scotland at the request of the Beaulieu Estate and in accordance with the approval of the Cefas Fish Health Inspectorate. These oysters were translocated c. 450 miles to the *Bonamia*-exposed Solent, experiencing a seawater temperature increase of approximately 2 °C, and left for 2 years to adjust to new water quality and food resources. The main predators found in the Beaulieu River (tingles, crabs and starfish) are present in Loch Ryan and therefore did not pose an unfamiliar challenge for the oysters, although levels of predation and abundance of these predators was not measured. *Bonamia ostreae* has reportedly caused mortalities of nearly 40% in Galicia (NW Spain) (Figueras, 1991), 52-87% in Quiberon Bay (France) (Baud *et al.*, 1997), 60-80% in the Bay of Archaeon, France (Robert *et al.*, 1991), 74-91% in the Eo Estuary (N Spain) (Cigarría *et al.*, 1995), 90% in Cork Harbour, Ireland (Culloty *et al.*, 2001), 90% in the Fal Estuary (UK), and 95% in Poole Harbour (UK) (Hudson and Hill, 1991). Establishing an acceptable proportion of population mortality is challenging as *O. edulis* survival is site-specific and dependent on a complex balance of conditions (Orton, 1923). If the risk of *Bonamia*-exposure in the Beaulieu River is considered as a potential influence on population mortality, then the 32.9% mortality observed in this study over a 2-year period is a relatively small proportion of the population. However, although none of the oysters included in the mortality count were tested for the presence of *B. ostreae*, only 8.33% (n = 48) of the live oysters were found to be infected with *B. ostreae*. This low prevalence of the parasite suggests that something other than *B. ostreae* was killing the oysters. In healthy, uninfected *O. edulis* in Galicia (NW Spain), Montes *et al.* (2003) observed natural cumulative mortality of 22.8% within 1 year, while Cáceres-Martínez *et al.* (1995) associated *Bonamia*-free oysters with only 5.6% mortality (although a specific time frame was not given). Others have observed a natural mortality rate of between 5 and 15% (Marteil, 1979; Paquotte and Moriceau, 1987; Robert *et al.*, 1991), and therefore the accumulative mortality of 32.9% in this study over the 2 year period could be considered quite high.

5.4.3 Seasonality and the influence of temperature

The mean average AFDW of the oysters used in this study was 0.85 g, and they had a mean average clearance rate of 2.06 L h^{-1} g $AFDW^{-1}$. Therefore (on average) each oyster was filtering 42.02 L water per day, which means our low-density gabions were filtering 5042.88 L water per day, and our high-density gabions were filtering 10085.76 L water per day. A clearance calculator tool developed for *C. virginica* has proven useful in the USA to quantify the environmental and economic value of oyster restoration (zu Ermgassen *et al.*, 2016). Although *O. edulis* has a much lower clearance rate than *C. virginica*, evidence that *O. edulis* clearance rate remains constant over time suggests that a similar calculator tool could be created for this oyster species. Rodhouse (1978) observed clearance rates of c 1.0 L h^{-1} g $AFDW^{-1}$ at 10 °C, and 2.5 L h^{-1} g $AFDW^{-1}$ at 24 °C. Nielsen et al (2017) observed higher *O. edulis* clearance rates of 7.3 ± 1.7 L h^{-1} g^{-1} at a temperature of 22 ± 1.0 °C, but only tested four oysters.

As Nielsen et al (2017) highlighted, it is difficult to make direct comparisons to the literature with such different environmental conditions, but it is generally accepted that physiological rates are usually stimulated by an increase in temperature (Walne, 1972; Newell *et al.*, 1977; Rodhouse, 1978; Haure *et al.*, 1998; Eymann *et al.*, 2020). That typical behaviour was not seen in this study, as the difference in temperature (14.5 °C) between experiments in August 2018 (25 °C) and April 2019 (10.5 °C) did not have a significant effect on the clearance or respiration rates of the LR oysters (n = 32). As seen in Chapter 3, physiological performance is influenced by season (see section 3.3.3 and 3.3.4), and the difference in sampling date each year for this study (August (summer) 2018 and April (spring) 2019) may have confounded the data. Pre spawning oysters invest energy into gametes rather than somatic body condition and somatic growth. The observed decrease in body condition in April 2019 (see section 5.3.7, Figure 5.21) may therefore have been a result of this transfer of resources to reproductive organs.

An average growth rate of 5.6 g wet weight y^{-1} was seen between 2017 when the Loch Ryan oysters were deployed into the Beaulieu River and 2019 when they were recovered. The technique applied to track the growth of individual oysters (using nail varnish applied to their shells) unfortunately did not work, and therefore these averages were acquired from random groups of oysters in both years. This observed ‘growth’ of the LR oysters was significantly lower than natural growth rates previously observed in the UK; Walne (1958) reported annual growth rates of 17-26 g in oysters in Anglesey (Northern Wales), and (Kamphausen, 2012) saw an annual growth rate of 29 g in 2009 in the Solent. In addition, a significant reduction in the Loch Ryan oyster body condition index was seen over the two years in the Beaulieu River (from 3.29 ± 1.35 (mean average ± sd, n = 12) in 2017 to 1.39 ± 0.50 (n = 32) in 2019), which suggests that these oysters were directing resources to increase their shell content rather than soft body tissues over the two observed years. A lower soft tissue to shell ratio could be the result of nutritional deficiency (e.g. post investment in gametogenesis) (Nelson, 1947; Gabbott and Walker, 1971; Askew, 1972; Ruiz *et al.*, 1992; Pogoda *et al.*, 2011), a high energy environment (e.g. water currents) (Pogoda *et al.*, 2011), or predation pressure (Lemasson and Knights, 2019), all in reaction to the immediate environment. Due to boat availabilities, sampling of the LR oysters took place first in August (2018) and then in April (2019), which fall either side of the main spawning period; spawning and brooding oysters in April would have been directing less resources into their own somatic body tissue in order to reproduce than spent oysters in August that would be building up their somatic body resources again. In addition, food availability between August and April may have been different; phytoplankton and zooplankton may have been more abundant during the summer month of August in comparison to April, which may have impacted oyster condition. Growth is likely to fluctuate naturally, and a decrease in oyster growth has been attributed to spent spawning energy (Orton, 1928). However, as seen in Chapter 3, oysters from the Solent area were in a generally lower condition than the Loch Ryan oysters regardless of season, classified by a thicker shell and lower soft tissue content. This could be a result of nutrient deficiencies in the diet available to the oysters in the Solent. *O. edulis* are known to be selective feeders, and clearance rates change with different available algae species (Haure *et al.*, 1998; Nielsen *et al.*, 2017), and algae concentration (Hutchinson and Hawkins, 1992; Haure *et al.*, 1998); Haure *et al.* (1998) identified from published literature a reduction in clearance rate (-70%) with increasing algae concentration (1-7 mg L^{-1}). The composition of algal species and associated concentrations in the Beaulieu River was not measured in this study. However, the general decline in oyster body condition and slow growth rates could indicate a nutrient deficit in the diet available to the oysters in the Beaulieu River in comparison to that available in Loch Ryan from where the oysters originated. As discussed in Chapter 3, the lower condition of the Solent oysters in comparison to the Loch Ryan oysters could also be a result of exposure to pollutants. Increased pollution such as antifouling agents and biocides leached from shipping activity and the human population, both of which are greater in the Solent than in Loch Ryan, has been shown to increase oyster shell thickness (Axiak *et al.*, 1995; Newkirk *et al.*, 1995), and negatively influence the condition index of the Solent oysters.

5.4.4 Effect of re-laying density

The 'OSPAR Convention' (https://www.ospar.org/convention) ratified by the UK in 2009 agreed to recognise an oyster bed habitat at a density of 5 oysters m^{-2}, although the current distribution of *O. edulis* in most historical or natural wild oyster beds are much reduced (Haelters and Kerckhof, 2009; Helmer *et al.*, 2019). In order to be the most effective, restoration densities are likely to be higher than current natural beds, but lower than aquaculture efforts. It is essential to differentiate between studies aiming to enhance fishery or aquaculture practice by testing higher densities, with those aiming to restore a natural self-sustaining population at lower densities. Regardless of research intent, there is a general trend for higher survival of *O. edulis* populations at lower oyster densities due to presence of disease (Le Bec *et al.*, 1991; Kennedy and Roberts, 1999; Carlucci *et al.*, 2010; Engelsma *et al.*, 2010). *B. ostreae* prevalence has been intrinsically linked to oyster population density, and so careful consideration for restoration oyster density would likely have a positive impact on both disease prevalence and physiological performance of the re-laid population. Mortality of the LR oysters in this study was not affected by density, and only 8.33% of the previously *Bonamia*-naïve Loch Ryan oysters had contracted *B. ostreae* after 2 years in the gabions in the *Bonamia*-exposed Beaulieu River, in a high-density environment (120 and 240 oysters m^{-3}). However, a direct comparison to the above studies referenced (Le Bec *et al.*, 1991; Kennedy and Roberts, 1999; Carlucci *et al.*, 2010; Engelsma *et al.*, 2010) could be considered inappropriate, as the LRinfection disease oysters in this study were stacked in three dimensional microreef structures and elevated ≥ 0.2 m from the riverbed offering a different solution to restoration. Grizel and Tigé (1973) saw a decline in growth rate of oysters with a *B. ostreae* infection, but Robert *et al.* (1991) saw no significant effect between infected and uninfected oysters.

Although the effect on growth rate is contested, oyster condition has been shown to decline with increasing intensity of *B. ostreae* infection (Rogan *et al.*, 1991). A difference in wet weight between *B. ostreae* infected and uninfected LRinfection oysters in this study was dependent on re-laying density; *B. ostreae* infected oysters from the high-density gabions were significantly lighter (g) than *B. ostreae* infected oysters in the low-density gabions. Increased oyster density has been shown to reduce growth rate due to crowding (Carnegie and Barber, 2001) and similar results have been shown in *O. edulis* mixed with Pacific oyster *Crassostrea gigas* (Le Bec *et al.*, 1991; Robert *et al.*, 1991). Re-laying density did not have a significant effect on the clearance rates of *O. edulis* in this study, which suggests that re-laying adult oysters at a higher density would not necessarily improve water quality within this area. Water clarity as a product of *O. edulis* restoration will therefore not be at a disadvantage by precautionary and conservative oyster re-laying abundance.

5.4.5 Effect of elevation from the seabed

The effect of elevation on LR oyster respiration rates changed between years; In August 2018, higher respiration rates were seen in oysters at low elevation (1.12 ± 0.90 mg O_2 L^{-1} g AFDW $^{-1}$, mean ± sd) in comparison to those at high elevation (0.31 ± 0.26 mg O_2 L^{-1} g AFDW $^{-1}$), but this effect was not seen in April 2019 when elevation did not have a significant effect. *O. edulis* has shown signs of anaerobic metabolism above temperatures of 25 °C (Newell *et al.*, 1977; Eymann *et al.*, 2020), and therefore the temperature seen in the Beaulieu River (and therefore the experimental temperature) of 24 °C in August 2018 was reaching the upper thermal limits for effective *O. edulis* physiological functioning. It is therefore not surprising that the oyster's metabolic response at this time was more variable. These results suggest that when oysters are under additional pressures such as an increase in temperature, the physiological effect of elevating the *O. edulis* (above excess turbidity) was more pronounced.

The expressed LR *O. edulis* respiration rates of between 0.02 and 2.66 mg O_2 L^{-1} g AFDW $^{-1}$ fall within respiration rates seen by others (Beiras *et al.*, 1995; Haure *et al.*, 1998; Sytnik and Zolotnitskiy, 2014; Sawusdee *et al.*, 2015). The fact that no significant difference in respiration rate was seen in spring 2019 could be evidence that the Loch Ryan oysters were acclimating to their new environment. Significant variation in wet weight could be explained by the interaction between re-laying density and elevation (Figure 5.16); the greatest wet weight was observed at either low elevation and low density, or at high elevation and high density. This suggests elevation from the seabed might be beneficial for high density stocks. Sawusdee *et al.* (2015) found elevated oysters (0.4 m from the seabed, in a water column of 10-12 m) to have higher clearance rates, which would logically improve growth rates (Barber *et al.*, 1991; Hutchinson and Hawkins, 1992). A direct comparison is not appropriate, as the oysters in this study were deployed in a different location, in shallower and probably more turbulent water. However, the absence of an effect of elevation (0.2 or 0.8 m from the seabed) on oyster clearance rate in this study suggests that efficient oyster growth and water clearance will occur with any restoration design in the Beaulieu River. This information will relieve restoration efforts and budgets, as growth rates are not necessarily enhanced from more intricate (and potentially more expensive) elevated structures. In general, elevating the oysters did show small improvements in physiological performance, but further research into the balance between these physiological benefits and the associated cost implications of elevated restoration structures is required.

The interaction between treatments (density and elevation) had an effect on some physiological performance: the effect of density on LR oyster condition depended on the elevation from the seabed; oyster condition was better either at low density and high elevation, or at high density and low elevation. One reason for this could perhaps be food availability, as the topography of the seabed has been shown to influence the number of large particles in suspension (Colden *et al.*, 2016), and therefore there could be a higher abundance of particles nearer the seabed than at 0.8 m elevation.

5.4.6 Conclusion

This study established that restoration does support biodiversity and can enhance a habitat from a two-dimensional system into a three-dimensional species-rich ecosystem. Although further research is required to fully appreciate the predator-prey relationship in this oyster bed ecosystem, the abundance and diversity of the epifaunal community associated with the gabions in this study is demonstrative of the diverse ecosystem offered by an oyster bed within the rapid succession of only 2 years. However, the Loch Ryan oysters declined in condition during these two years in the Beaulieu River. This would not necessarily affect habitat or epifaunal community development as the shell substrate and three-dimensional structure remain, but it could imply that this is an environment unsuitable for most productive oyster development and impact efforts associated with a fishery. Only a small proportion of the oysters acquired *Bonamia ostreae* infections, which is good considering mass mortality from *B. ostreae* was the reason for removal of *O. edulis* from the Beaulieu River in the 1980s, and considering our understanding that *B. ostreae* remains in a location long after oysters have been removed. These *B. ostreae* infected oysters were significantly lighter in mass (wet weight) at high oyster density than those at low density, which has implications for large scale oyster production in *Bonamia*-exposed areas. The effect of elevation on respiration rate (metabolic rate) was significant, which corroborates literature that suggests metabolic rates decrease in highly turbid areas. However, these results were confounded by sampling event, which supports previous studies that have shown seasonal or annual fluctuations in effects on *O. edulis* physiology. In conclusion, re-laying Loch Ryan oysters in the Beaulieu River provided a stable environment for the development of a diverse benthic community, but their decrease in body condition suggests they would not support large-scale oyster production for commercial purposes.

Chapter 6 Conclusions and recommendations for *Ostrea edulis* restoration

In the mid-2000s, as part of the International Convention on Biodiversity (JNCC, 1994, 2007), *Ostrea edulis* was identified as a high priority species for conservation in the UK and in Europe due to the ecosystem services offered by individuals of this species and the associated three-dimensional habitat formed by its shells. As a result, a significant increase in *O. edulis* restoration projects in the UK and in Europe has emanated. Owing to signficiantly low population abundance (a reduction of ~90% in the last 100 years), *O. edulis* restoration efforts must include the re-laying of high numbers of oyster individuals to support the development of a self-sustaining population. There is a wealth of knowledge about *O. edulis* physiology, but information on how to apply this knowledge to large-scale restoration is lacking. The literature offers little information on the role of hydrodynamics in physical and physiological interference to oyster development, which should significantly influence choice of restoration site selection. Similarly, choice of broodstock is currently restricted by efforts to control the spread of parasite *Bonamia ostreae*, which has masked discussions about the differences in physiological performance of different populations and the consequential effect on oyster population development. Molecular biomarkers are being explored to improve our understanding about *O. edulis* immune mechanisms against *B. ostreae*, but the science is not yet robust enough to support restoration practice. Establishing an appropriate restoration design (e.g. a re-laying density that will support growth and development without increasing disease prevalence) is essential to progress this field on an international level.

As detailed in the general introduction (Chapter 1), the aim of this thesis was to address the gaps in our knowledge of how to restore *O. edulis* to a self-sustaining population using the Solent as a model system with which to develop and test ideas. A critique of the literature identified these gaps, and laboratory and field experiments were used to achieve the following objectives:

a) Understanding the importance of hydrodynamics for restoration site selection by analysing both small- and large-scale effects of water flow on *O. edulis* with implications for re-laying adults, juveniles and cultch (Chapter 2)
b) Understanding the effect of broodstock origin on physiological performance with a comparison of physiological efficiency between three UK *O. edulis* populations (Chapter 3)
c) Exploring the potential for molecular and metabolic markers of resistance to pathogenic agent *Bonamia ostreae* (Chapter 4)

d) Testing the effects of re-laying density and elevated restoration (as an exemplar of one method) on habitat and the physiological efficiency of a population of *O. eduli*s with a field study (Chapter 5).

The success of the thesis in addressing these gaps in the knowledge are discussed below, with specific recommendations for further research and application in *O. edulis* restoration.

6.1 The importance of site selection with specific reference to hydrodynamics (Chapter 2)

The hydrodynamic environment associated with an oyster bed can contribute to the resuspension of silt and sediment with the potential to smother benthic organisms (Colden *et al.*, 2016). However, turbulence has been shown to aid oyster larval settlement and increase the abundance of organic particles available to an oyster bed for consumption, by slowing the water flow immediately above the seabed (Wethey, 1986; Mullineaux and Butman, 1991; Fuchs *et al.*, 2007; Whitman and Reidenbach, 2012; Fuchs and Reidenbach, 2013; Colden *et al.*, 2016). The naturally rough surface of oyster shells increases the turbulence of water flow, thus improving the immediate environment with respect to both recruitment and feeding (Eckman, 1990; Mullineaux and Garland, 1993; Soniat *et al.*, 2004; Koehl and Hadfield, 2010; Whitman and Reidenbach, 2012). American slipper limpet *Crepidula fornicata* was first reported as an accidental introduction to Europe in 1893 with imports of Eastern oyster *Crassostrea virginica* (Crouch, 1893), and high numbers of the smooth chains constructed as multiple *C. fornicata* individuals attach to one another have replaced *O. edulis* beds in the south of England (Barnes *et al.*, 1973; Blanchard, 1997, 2009). Over the last 19 years, a 96% decrease in *O. edulis* has occurred concurrently to a 441% increase in *C. fornicata* in Chichester Harbour alone (Helmer *et al.*, 2019). The flume work achieved in Chapter 2 of this project demonstrated that an *O. edulis* bed created three times the shear stress than a *C. fornicata* bed (see section 2.3.1, Figures 2.29 and 2.30). At present, the Solent seabed is dominated by *C. fornicata*, and benthic boundary layer (BBL) thus characterised by high velocities and low turbulence in association with this low shear stress created by the chains made by this species. This indicates that the present benthic environment on the seabed in the south of England is not the most accessible for oyster larvae, and could be negatively influencing annual recruitment. This study also demonstrated that high water velocity (≥ 0.19 m s^{-1}) causes oyster valves to close (see section 2.3.1, Table 2.3), which could influence oyster feeding and respiration rates. Presence of *C. fornicata* in areas proposed for *O. edulis* restoration should therefore pose concern for the recruitment of future generations of *O. edulis* and self-sustainability of the population. Restoration efforts can overcome this by laying some form of cultch (e.g. oyster shell, mussel shell, spat-on-shell) for substrate preparation to create rugosity and turbulence. However, as shown in this study, cultch is similarly dependent on local hydrodynamics and restoration must ensure efforts are not wasted by cultch being washed away.

At a larger scale, contemporary restoration with a goal to restore a self-sustaining population requires re-laying broodstock oysters to produce a high concentration of larvae offspring (Korringa, 1946; Key and Davidson, 1981; Smyth *et al.*, 2018).
Although somewhat influenced by individual larval swimming (or milling) activity, the trajectory of bivalve larvae is highly dependent on local hydrodynamics, both vertically and horizontally (Cole and Knight-Jones, 1939; Cranfield, 1973; Cragg and Gruffydd, 1975; Hidu and Haskin, 1978; Willis *et al.*, 2019). The three-dimensional model used in this study identified that deploying broodstock in Lymington, Newtown Creek and the Beaulieu River on the western side of the Solent has a higher probability of dispersing larvae to the wider Solent, than re-laying broodstock at Saxon Wharf, River Hamble, and at Land Rover Ben Ainslie Racing (LRBAR) in the central and eastern side of the Solent that would more likely retain larvae (see section 2.3.2). Both dispersive and retentive environments are beneficial for restoration depending on the project goals; a dispersive environment may benefit restoration aiming to naturally repopulate a wider area over a longer time frame, and a retentive environment may more rapidly form reef-like structures as recruitment occurs locally. Models offer a comprehensive and cost-effective method of analysis for large scale restoration (Bergström *et al.*, 2021). This thesis recommends the use of hydrodynamic models for restoration site selection to determine where larval offspring of any re-laid broodstock will settle, so that these broodstock locations can be situated in the most effective place with the most appropriate hydrodynamics, sediment type and food availability for a healthy adult self-sustaining population.

This project has substantiated the importance of site selection with respect to hydrodynamics by demonstrating the effectiveness of water flow in controlling oyster valve activity, by revealing the natural turbulence created by oyster shells at the benthic boundary layer, and by forecasting the large-scale dispersal of *O. edulis* larvae. The modelled larvae trajectory used in this study would benefit from further *in situ* sampling to determine if natural settlement actually occurs in the predicted settlement locations. Additional experiments to observe the vertical activity and behaviour of oyster larvae in the Lab Carousel flume would benefit this work by testing the assumptions of the model.

6.2 The importance of broodstock origin (Chapter 3)

This study identified a significant gap in the knowledge of the effect of broodstock origin on the physiological performance of *O. edulis* individuals and consequently the self-sustaining potential of a population.

Restoration of *O. edulis* requires the re-laying of adult or juvenile oysters, but there are limitations as to the sourcing of those oysters. The low population abundance of *O. edulis* in the UK and in Europe means there is only a handful of *O. edulis* hatcheries and fisheries still active and able to provide oysters for restoration (Lynch and O'Hely, 2001; OSPAR, 2015; Adamson *et al.*, 2018). Hatcheries are more likely to be able to support the high number of individuals required for restoration than wild fisheries (Bayraktarov *et al.*, 2016), but the genetic diversity of hatchery produced oysters is greatly reduced to that seen in the wild due to a reduced number of active parents (Gosling, 1982; Hedgecock and Sly, 1990; Gaffney *et al.*, 1992; Saavedra and Guerra, 1996; Saavedra, 1997; Launey and Hedgecock, 2001; Boudry *et al.*, 2002; Sobolewska and Beaumont, 2005; Laing *et al.*, 2005; Appleyard and Ward, 2006; Taris *et al.*, 2007; Lind *et al.*, 2009; Lallias *et al.*, 2010). Further limiting the availability of oyster adults and spat are the Government biosecurity regulations (EC Council Directive 2006/88/EC) restricting the movement of *O. edulis* in attempt to reduce the spread of parasite *Bonamia ostreae*.

Genetic variability of *O. edulis* individuals and populations in the UK and in Europe is uncertain. Although the frequent and extensive translocation of *O. edulis* for culture during the mid 1800s to late 1900s might lead to the assumption of genetic homogeneity amongst UK and European populations, distinct regional genetic and physiological traits have been observed (Johannesson *et al.*, 1989; Saavedra *et al.*, 1993; Maršić-Lučić, 1999; Launey *et al.*, 2002; Sobolewska and Beaumont, 2005; Beaumont *et al.*, 2006; Donnan, 2007; Bromley *et al.*, 2016a). Vera *et al.* (2016) identified three distinct genetic European populations ((i) Denmark and The Netherlands, (ii) UK and France, and (iii) Spain), and Sawusdee (2015) observed differences in physiological traits in populations within 50 miles of one another. Therefore, it was important to determine if acquiring stocks from any available source would be sufficient for successful restoration, or if restoration efforts need to be more selective.
This project revealed that the physiological performance of individual oysters was highly distinctive between the three UK populations studied (Loch Ryan (Scotland), Galway (Ireland), and Solent (England)) (see section 3.4.1). However, these differences disappeared when the populations were brought to common conditions, suggesting the differences were the result of phenotypic plasticity and not long-term genetic adaptation. Although these results suggest a genetic homogenisation of *O. edulis* populations in the UK, there was a significant decrease in body condition of the Loch Ryan and Galway oysters acclimated to aquarium conditions using water from the Solent (see section 3.3.8, Figure 3.22). This study therefore promotes the concept of using local adult oysters for broodstock, not only to restrict the movement of pathogens (*B. ostreae*), but also to reduce the negative results of the stress associated with acclimating to a new environment.

The Solent oysters were in a worse condition to those from Loch Ryan or Galway (see section 3.3.6, Figure 3.12), indicating either dietary or other environmental pressures/stress. A decrease in condition of

Loch Ryan oysters was also observed post deployment in the Beaulieu River (Solent water) for 2 years (see section 5.3.7, Figure 5.21), which suggests this location is not ideal for effective oyster development. This theory was supported by metabolic data that highlighted potential environmental pressures such as increased pollution and reduced diet availability in the Solent in comparison to Galway and Loch Ryan (see section 3.4.1 and Figure 3.17). This should concern restoration efforts in this region, as there are ethical issues with the removal of healthy disease-free oysters from more appropriate oyster bed sites (such as the *Bonamia*-free Loch Ryan) for relocation to inappropriate locations (such as the *Bonamia*-exposed Beaulieu River) only to reduce in soft tissue weight and potentially contract *Bonamia ostreae*. It is therefore recommended that *O. edulis* restoration supports the use of locally sourced *Bonamia*-free broodstock with similar physiology in order to reduce stress and increase likelihood of a self-sustaining population.

6.3 Finding markers of resistance to *Bonamia ostreae* (Chapter 4)

Bonamia ostreae has been considered the most challenging aspect of restoration to overcome (Laing *et al.*, 2005; Lallias *et al.*, 2010; Woolmer *et al.*, 2011). Reports of individual and population survival in *Bonamia*-exposed areas are driving researchers to identify markers of *Bonamia*-resistance, yet discussions as to what *resistance* actually means are still underway (Holbrook *et al.*, 2021). Morga *et al.* (2012) identified six potential genetic markers of *Bonamia*-resistance in *O. edulis*, but used only one endogenous reference gene (ERG), which does not comply with the current minimum information for publication of real-time quantitative PCR experiments (MIQE) regulations (Bustin *et al.*, 2009, 2013). This provided an opportunity to test in a more robust way whether these genetic markers of resistance were conservative and whether they could be recognised in *Bonamia*-infected or non-infected oysters across different populations. The results established that the concept is more nuanced than originally expressed by Morga *et al.* (2012) as only three of these markers (*OeC1q*, *OeFAS* and *ACT*) were distinguishable between infected and non-infected individuals from Loch Ryan and The Solent, and the variation in expression of *OeC1q* and *OeFAS* was dependent on season (Chapter 3). This thesis has exposed the complexity in finding conservative genetic markers of *Bonamia*-resistance and challenges further research to explore these genes using multiple ERGs and multiple populations before confirming their power of discrimination.

Although the field of metabolomics to support shellfish research is in its infancy, this thesis has demonstrated its power to discriminate between *Bonamia*-infected and non-infected individuals (Kuehnbaum and Britz-Mckibbin, 2013; Kuhlisch and Pohnert, 2015; Alfaro and Young, 2018; Eymann *et al.*, 2020). Several metabolic compounds were either detected in infected individuals, or were absent in infected individuals, which offers potential for their use as markers of *B. ostreae* infection.

Environmental conditions and pressures to which an organism has been exposed is revealed through a metabolic profile, which can provide information as to how that organism might deal with further stress (Kuehnbaum and Britz-Mckibbin, 2013; Young *et al.*, 2015; Alfaro and Young, 2018). Metabolomics are successfully used in medical science for early detection of disease and risk of mortality, which offers promise for restoration projects that currently have no early detection methods of disease outbreaks (Beger *et al.*, 2010; Montgomery and Brown, 2013; Gao *et al.*, 2016; Srikanthan *et al.*, 2016; Wishart, 2016; Liu *et al.*, 2018). It is highly recommended that the specific metabolites identified in this study should be analysed further and considered for use in both helping find robust broodstock populations for restoration, and detecting early outbreaks of disease so that preventative measures can be taken.

6.4 Applied restoration in the Beaulieu River, and the importance of restoration design (Chapter 5)

The objective of this study was to apply our knowledge of *O. edulis* physiology to determine if restoration was effective in supporting biodiversity in the Beaulieu River and if density and elevation had an effect on physiological performance of individuals. Although the (*Bonamia*-free) Loch Ryan oysters declined in condition during their two-year deployment in the (*Bonamia*-exposed) Beaulieu River, the significant development of a diverse epifaunal community was evident (see section 5.3.2). Several trophic levels were observed in the oyster gabions, from primary producers (e.g. macroalgae, see Appendix G, Figure G1 and G2) and filter feeding sessile organisms (e.g. barnacles, see Appendix G, Figures G15 and G16) to motile scavenger species (e.g. crabs, see Appendix G, Figures G4-G6, G21 and G22) and fish (see Appendix G, Figure G3). Evidence of reproduction of both oysters and other epifauna (see Appendix G, Figures G19, G20, and G27-G34) was a good indication that the conditions were appropriate for supporting ecosystem growth and development. While benefits such as an increased fishing catch can be given a direct value (i.e. landing effort and commercial oyster price), others such as habitat stability are harder to measure (Stagl, 2004). This study used the recognised habitat classification methods developed by The Joint Nature Conservation Committee (JNCC) and the European Environmental Agency (EEA) (EUNIS scheme) to show that oyster restoration in the Beaulieu River changed the habitat description and increased the abundance and diversity of species recorded (Tables 5.4, 5.5 and 5.6) (JNCC, 2015; Parry, 2019). Although indicators such as endangered species abundance and habitat richness might resonate louder with environment-concerned local government bodies than with fishermen, restoration outcomes can benefit most stakeholders if requirements are prioritised during the early restoration planning stage. The acknowledgement of realistic timescales for restoration in advance of work will benefit long term management of marine environments (Sheehan *et al.*, 2013). *O. edulis* restoration is thought to require at least 20-25 years of support to ensure a self-sufficient population or ‘reef’ has been established, which has economic implications (Laing *et al.*, 2005; Donnan, 2007; Gercken and Schmidt, 2014; England and Wilkes, 2018). However, the oyster gabions in this study showed a diverse and rich epifaunal community after only 2 years, which is promising for the habitat ecosystem services from an oyster bed. Other studies have similarly found an increase in biodiversity and epifaunal community in association with structures containing *O. edulis*, including species of conservation interest such as eels and seahorses (https://www.bbc.com/news/uk-england-hampshire-53961417). The next step is to test the significance of the cage itself in the development of this observed three-dimensional ecosystem by comparing the development of epifaunal community in cages with and without oysters.

High levels of turbidity at the benthic boundary layer can slow feeding and negatively affect the metabolic rate, which can subsequently affect oyster growth and condition (Korringa, 1952; Grant *et al.*, 1990; Sawusdee *et al.*, 2015). The results from this study support evidence that elevating oysters from the seabed significantly improves metabolic rate (see section 5.3.6), but indicated potential seasonal fluctuations in the effect on oyster physiology. Further research with more frequent sampling is required to determine the seasonal effect on *O. edulis* physiology at elevation. Elevation with the use of structures such as cages and reef building tools (such as castles, (Baggett *et al.*, 2014; zu Ermgassen *et al.*, 2016)) has been highly recommended for restoration of *Crassostrea* spp. in the United States, and our research has similarly established the physiological benefits for *O. edulis*. The deployment of heavy structures would also deter unwanted fishing activities such as dredging should the restoration goal be to support recovery of ecosystem and not for fishery purposes (Schulte *et al.*, 2009). Elevation has been proven to benefit the physiology of *O. edulis*, and therefore any opportunity to raise an oyster bed should be taken.

The density at which to re-lay oysters for restoration is contested owing to the implications for reproduction and disease prevalence; proximate distance between oyster individuals reduces sperm dilution and improves successful egg fertilisation, but high densities can lead to increased disease prevalence (Vogel *et al.*, 1982; Denny and Shibata, 1989; Dame *et al.*, 2002). There is a knowledge gap between recognised definitions of healthy wild *O. edulis* beds (OSPAR, 5 oysters m^{-2}) and applicable densities for restoration, likely to be much greater. Although the physiological performance of *O. edulis* was not significantly influenced by the two densities tested (120 and 240 oysters m^{-3}), this study observed that *Bonamia*-infected oysters were significantly lighter in mass (wet weight, g) at high oyster density than at low density (see section 5.3.8, Figure 5.23). This again accentuates the physiological issues associated with applying *O. edulis* restoration in a *Bonamia*-exposed area. Although re-laying *O. edulis* at high density was linked to reduced wet weight in oysters infected with *B. ostreae* in this study, it is recommended that adult oysters should be placed in close proximity to stimulate successful reproduction, enhance recruitment by providing appropriate substrate, and to build a three-dimensional habitat for other species.

The Solent and The Beaulieu River as a location for restoration (Chapter 5)

Bonamia ostreae has been and still is the most effective disease agent in the Beaulieu River, and is likely to have further effects on survival of future populations. The host-parasite interaction is impacted by abiotic and biotic factors, which allow each to dominate different niches (Snieszko, 1974; Laing *et al.*, 2014). Current intelligence on these interactions is likely to change as the climate changes as a result of global warming. Extreme weather conditions are already evident in the increased frequency of ‘cold snaps’ that cause frost damage to marine organisms found in intertidal and coastal waters (Pinnegar *et al.*, 2020), and heatwaves that can reduce available phytoplankton and dissolved oxygen to benthic organisms (Joos *et al.*, 2003; Oliver *et al.*, 2018). *O. edulis* has shown signs of anaerobic metabolism above temperatures of 25 °C (Newell *et al.*, 1977; Eymann *et al.*, 2020), and therefore the temperature seen in the Beaulieu River (and consequently the experimental temperature) of 24 °C in August 2018 was toward the upper thermal limits for effective *O. edulis* physiological functioning. *B. ostreae* is known to be limited by low temperatures, yet seawater temperature is rising and therefore less places will be limiting for the parasite (Rowley et al. 2014). Warmer seawater may increase dissemination and prevalence of diseases but may also provide a platform for a stronger immunity for host individuals. This study only found *B. ostreae* infections in 8.33% (n = 48) of the previously *Bonamia*-naïve Loch Ryan oysters post a two-year deployment in the *Bonamia*-exposed Beaulieu River (see section 5.3.8), but longer monitoring is essential to determine the long-term effects of the parasite on the population.

Although clearance rate and respiration rates were stable across both years, the condition of the Loch Ryan oysters was significantly reduced after the two years. Oyster growth was slow over the two years in comparison to previous observations (Walne, 1958; Kamphausen, 2012), potentially with the development of a thicker shell than desired for fishery purposes. As of yet, there is no evidence to suggest that these thick-shelled oysters would have a reduced reproductive output, which is essential for supporting a self-sustaining population and keeping recruitment high. The information acquired throughout this study suggests that the Beaulieu River would not necessarily profit as a fishery, but could continue to be a suitable sanctuary restoration site, left to enhance the habitat with a reef-type structure, and population of adult broodstock that would disperse larvae to boost the wider Solent *O. edulis* populations.

6.5 Further recommendations

This thesis does not address the anxiety surrounding present *O. edulis* harvesting methods such as dredging (Kennedy and Roberts, 1999; Hugh-Jones, 2004; Long *et al.*, 2017), nor does it discuss the benefits of sanctuary areas. Fishermen play an important role in successful management of a restoration project; according to Smyth *et al.* (2009), the Spanish *O. edulis* restoration efforts in Galicia in 1960 were not successful owing to minimal interest and effort from local fishermen. There are obvious flaws in the UK fishing industry that contributed to the decline of the European flat oyster and therefore must be addressed head-on at these early stages of the *O. edulis* restoration revolution. For example, although there are restrictions on oyster dredge size (the front edge or blade must not exceed 1.5 m in width, https://www.southern-ifca.gov.uk/byelaws#Oyster-Dredges), there is currently no maximum oyster landing size, which would ensure mature oysters are returned to the seabed to continue as effective brooders within the population. However, there is an argument that "fishery restoration and native oyster restoration cannot be achieved at the same time" (Schulte *et al.*, 2009). Setting up 'sanctuary sites' as part of restoration is imperative to allow the population to recover without disturbance and for us to achieve the maximum benefits (ecosystem services) of the oyster bed such as the three-dimensional habitat for the benthic community. Although the provisional and cultural services provided by oysters are significant, the pressures of historical and current exploitation of *O. edulis* must be acknowledged and the devastated state of wild populations requires focus to be placed on environmental recovery rather than production for consumption.

6.6 Conclusion

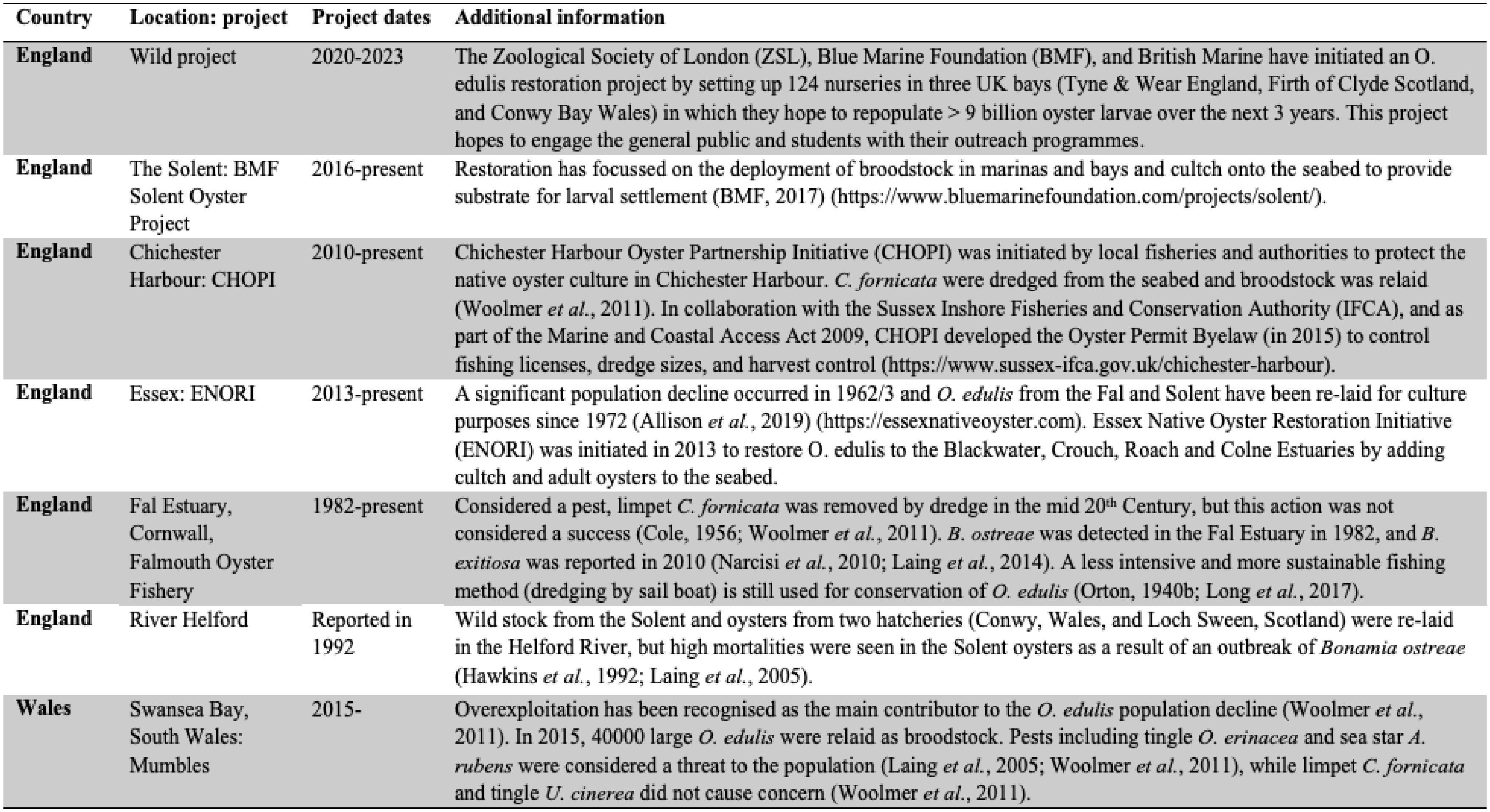
Restoration of *Ostrea edulis* is threatened with continued fishing pressure, an altered hydrodynamic benthic environment dominated by *Crepidula fornicata*, and parasite *Bonamia ostreae*. Restoration efforts in the Solent and surrounding area should prioritise the goal of ecosystem regeneration and development over commercial oyster production. This recommendation is owing to indications that oyster condition is depleted in this marine environment, potentially as a result of heavy pollution and an absence of specific dietary compounds that are essential for oyster physiology. Where possible, restoration practitioners should source local *Bonamia*-free *O. edulis* for use as broodstock and elevate them from the seabed. Evidence of metabolic markers of *Bonamia*-resistance in this study should inspire further research to use molecular and metabolic techniques to find broodstock populations robust to this pathogen to support a self-sustaining population. In order to restore a substantial amount of *O. edulis* habitat to coastal areas of the UK and Europe, efforts should be concentrated in supporting existing beds by restricting fishing activity, and further investigating *Bonamia*-resistance as a restoration strategy.

Appendix A. *Ostrea edulis* restoration in the UK and in Europe

Table A1. *Ostrea edulis* restoration in Europe (excluding the UK).

Country	Location: project	Project dates	Additional information
Ireland	**Cork: Rossmore population**	1988-present	Three to four year old *Ostrea edulis* 'survivors' post exposure to *Bonamia ostreae* were used as broodstock in controlled spawning events in attempt to produce *Bonamia*-resistant offspring (Lynch *et al.*, 2014). A certain level of *Bonamia*-resistance was observed amongst the Rossmore population (Laing *et al.*, 2005; Sobolewska and Beaumont, 2005) .
Ireland	**Galway, Lough Swilly, Clew Bay, and Kilkieran Bay**	2017-present	Funded by the Marine Institute Galway, the European Maritime and Fisheries Fund, and various Non-Governmental Organisations, oyster restoration is active in 4 bays in Ireland. Cultch has been deployed in most locations. Community based organisation, Cuan Beo, aims to restore the *O. edulis* population in Galway as part of its pledge to improve the quality of the environment and community (*CuanBeo*, 2020). Adult oysters have been relocated to Lough Swilly with the hope to increase recruitment. Monitoring for presence of *Bonamia ostreae* is occurring in Galway and Clew Bay.
Spain	**Galacia: SETTLE**	2008-2010	Collapse of the *O. edulis* population began in 1778 (Smyth et al 2009). SETTLE was initiated with 10 partners from 4 countries (Spain, France, Ireland and Norway) to address the issues surrounding broodstock conditioning and recruitment (https://cordis.europa.eu/project/id/222043). Their aim was to develop optimum hatchery methods during autumn and winter to extend the natural reproduction season and increase oyster production to satisfy consumer demand.
Germany	**North Sea: RESTORE**	2016-2022	The German Federal Agency for Nature Conservation (BfN) partnered with the Alfred-Wegener-Institue (AWI) to establish RESTORE (https://www.awi.de/en/science/biosciences/shelf-sea-system-ecology/main-research-focus/european-oyster/restore.html#c64212).
The Netherlands	**Lake Grevelingen/ Oosterschelde**	1930s-1964	The Netherlands have attempted to restore *O. edulis* in the Oosterschelde (Smaal et al 2015). Repeated importations of 'foreign spat' to replenish overexploited wild *O. edulis* stock in Grevelingen Lake in the Netherlands was successful, but this stopped in 1964 and no significant fishery has since been re- established in this region (Korringa, 1946; Drinkwaard, 1999b; Lallias *et al.*, 2010).
France		1960s-	In response to the negative effects of *Martelia refringens* (1960s, Balouet & Chastel 1979; Kennedy & Roberts 1999) and *Bonamia ostreae* (late 1970s, McArdle et al 1991; Kennedy & Roberts 1999) to their *O. edulis*, France attempted to replace stocks with related species; *Ostrea chilensis* (Grizel et al 1983), *O. denselamellosa* (Le Borgne and Le Pennec 1983), *O. angasi* (Bourgrier et al 1986), and *O. puelchana* (Pascual et al 1991) were all unsuccessful introductions (Robert et al 1991).

Table A2. Existing and obsolete *Ostrea edulis* restoration in the UK.



Country	**Location: project**	**Project dates**	**Additional information**
England	Wild project	2020-2023	The Zoological Society of London (ZSL), Blue Marine Foundation (BMF), and British Marine have initiated an O. edulis restoration project by setting up 124 nurseries in three UK bays (Tyne & Wear England, Firth of Clyde Scotland, and Conwy Bay Wales) in which they hope to repopulate > 9 billion oyster larvae over the next 3 years. This project hopes to engage the general public and students with their outreach programmes.
England	The Solent: BMF Solent Oyster Project	2016-present	Restoration has focussed on the deployment of broodstock in marinas and bays and cultch onto the seabed to provide substrate for larval settlement (BMF, 2017) (https://www.bluemarinefoundation.com/projects/solent/).
England	Chichester Harbour: CHOPI	2010-present	Chichester Harbour Oyster Partnership Initiative (CHOPI) was initiated by local fisheries and authorities to protect the native oyster culture in Chichester Harbour. *C. fornicata* were dredged from the seabed and broodstock was relaid (Woolmer *et al.*, 2011). In collaboration with the Sussex Inshore Fisheries and Conservation Authority (IFCA), and as part of the Marine and Coastal Access Act 2009, CHOPI developed the Oyster Permit Byelaw (in 2015) to control fishing licenses, dredge sizes, and harvest control (https://www.sussex-ifca.gov.uk/chichester-harbour).
England	Essex: ENORI	2013-present	A significant population decline occurred in 1962/3 and *O. edulis* from the Fal and Solent have been re-laid for culture purposes since 1972 (Allison *et al.*, 2019) (https://essexnativeoyster.com). Essex Native Oyster Restoration Initiative (ENORI) was initiated in 2013 to restore O. edulis to the Blackwater, Crouch, Roach and Colne Estuaries by adding cultch and adult oysters to the seabed.
England	Fal Estuary, Cornwall, Falmouth Oyster Fishery	1982-present	Considered a pest, limpet *C. fornicata* was removed by dredge in the mid 20th Century, but this action was not considered a success (Cole, 1956; Woolmer *et al.*, 2011). *B. ostreae* was detected in the Fal Estuary in 1982, and *B. exitiosa* was reported in 2010 (Narcisi *et al.*, 2010; Laing *et al.*, 2014). A less intensive and more sustainable fishing method (dredging by sail boat) is still used for conservation of *O. edulis* (Orton, 1940b; Long *et al.*, 2017).
England	River Helford	Reported in 1992	Wild stock from the Solent and oysters from two hatcheries (Conwy, Wales, and Loch Sween, Scotland) were re-laid in the Helford River, but high mortalities were seen in the Solent oysters as a result of an outbreak of *Bonamia ostreae* (Hawkins *et al.*, 1992; Laing *et al.*, 2005).
Wales	Swansea Bay, South Wales: Mumbles	2015-	Overexploitation has been recognised as the main contributor to the *O. edulis* population decline (Woolmer *et al.*, 2011). In 2015, 40000 large *O. edulis* were relaid as broodstock. Pests including tingle *O. erinacea* and sea star *A. rubens* were considered a threat to the population (Laing *et al.*, 2005; Woolmer *et al.*, 2011), while limpet *C. fornicata* and tingle *U. cinerea* did not cause concern (Woolmer *et al.*, 2011).

Table A2 continued.

Wales	Milford Haven, South Wales	Surveyed: 2016-2017, restoration: 2019-2023	*Bonamia ostreae* (first reported by CEFAS in 2006), high densities of *C. fornicata*, cultch availability or quality and eutrophic water likely contributed to the decline of the *O. edulis* population in Milford Haven (Woolmer *et al.*, 2011; zu Ermgassen, 2017).
Scotland	Loch Ryan	1880s-present	In the 1880s and 1960s, *O. edulis* from France, Holland or Essex were re-laid in Loch Ryan, which increased spatfall and replenished stocks (Beaumont et al 2006; Lallias et al 2010). Continued private management of the wild fishery has helped sustain the *O. edulis* population (Lallias et al, 2010).
Scotland	Dornoch Firth: DEEP (Glenmorangie)	2014-present	The Dornoch Environmental Enhancement Project (DEEP) (https://www.glenmorangie.com/en-int/partnerships), was initiated as a partnership project between Herriott-Watt University, the Marine Conservation Society, and Glenmorangie to restore *O. edulis* to Dornoch Firth, Scotland. This is being achieved through monitoring larval settlement environmental cues for settlement and researching the most appropriate cultch type. In 2017, DEEP re-laid 300 oysters into the Dornoch Firth.
Ireland	Strangford Lough	1998-	The fishery in Strangford Lough significantly collapsed in the 19th Century, and although a fishery was regained in the 1970s, it subsequently crashed in 1998, and again in 2003 due to overexploitation and unsuitable substrate (Smyth et al 2009). Occasional pockets of oyster beds are thought to have been naturally enhanced by commercial aquaculture efforts in the surrounding area (Kennedy & Roberts 1999; Smyth et al 2009). In 1998, 75 tonnes of cultch were deployed, and 250000 oyster spat laid (Allison, 2017).
Ireland	Lough Foyle, Ireland	1970-	In 1970, 250000 oysters were re-laid on natural beds (Kennedy and Roberts, 1999). *B. ostreae* was first reported amongst the *O. edulis* population in 2005 (Flannery *et al.*, 2014; Laing *et al.*, 2014).

Appendix B. Flume specifics

There was no obvious difference between the drag coefficient (C_D) over the *O. edulis* bed and that over the *C. fornicata* bed, but the Reynolds numbers (*Re*) were higher in association with the *O. edulis* bed (Figure B1, Table B1). The C_D was calculated for each height above the seabed for each profile and experimental flume velocity (Figure B2), and occasional differences in the value along the vertical plane for each profile suggests that using the quadratic stress law to acquire a constant C_D for a water body is limited.

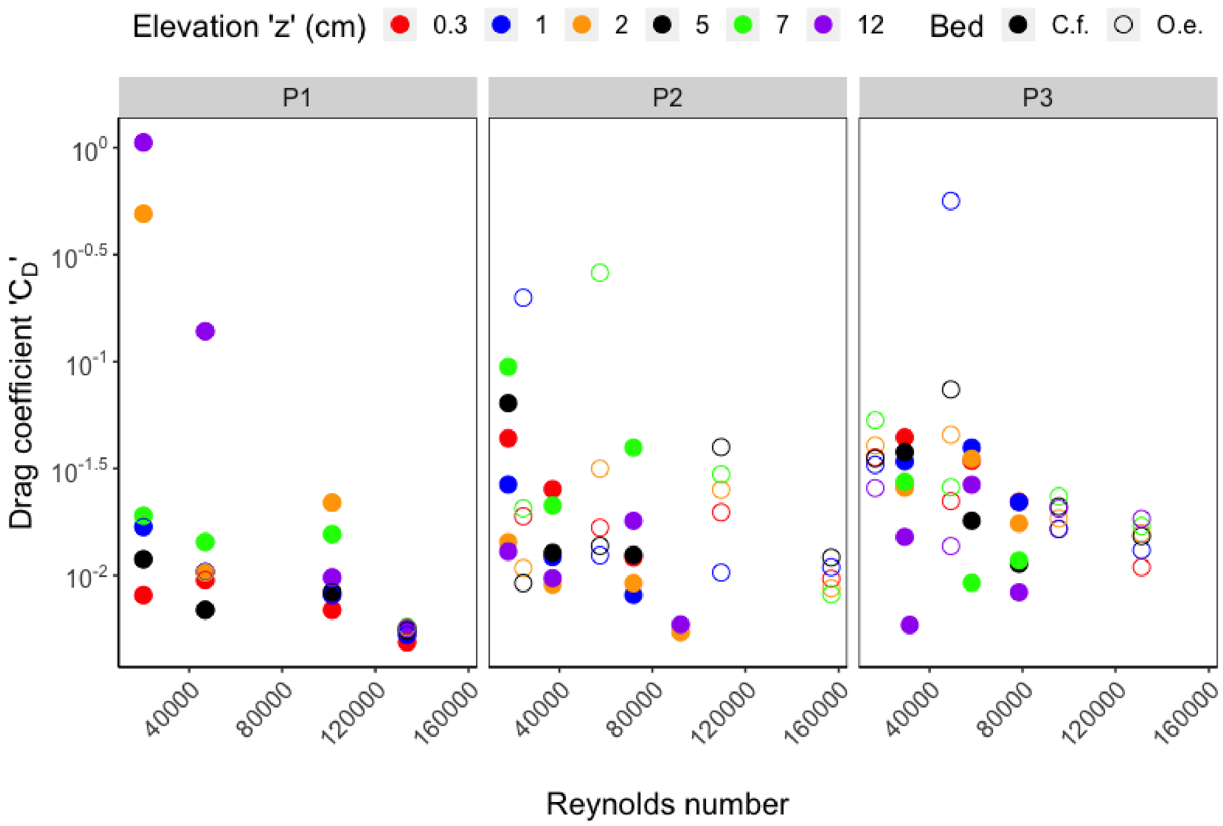


Figure B1. Drag Coefficient ‘C_D’ prior to (P1), above (P2), and after (P3) an *Ostrea edulis* (O.e.) bed (empty circle) and a *Crepidula fornicata* (C.f.) bed (filled circle) in relation to Reynolds number ‘Re’. Only experimental velocities at which both *O. edulis* and *C. fornicata* were both tested were included (0.06, 0.13, 0.31 and 0.42 m s^{-1}).

Table B1. Depth averaged velocity (U_d) was calculated between elevation z = 0 and z = 0.17 m. The coefficient of drag (C_D) was calculated from the Quadratic Stress Law (equation 4). Roughness length-scale (z_0) and shear velocity (u*) were estimated using the Log Law of the Wall

Exp. flume velocity (m s^{-1})	**Profile**	**$\bar{U}_d$**	***Ostrea edulis***					***Crepidula fornicata***				
			Z_0	u* (eq. 5)	**C_d (z =1)**	**C_d (z=17)**	**Re**	**Z_0**	u* (eq. 5)	**C_d (z =1)**	**C_d (z=17)**	**Re**
0.06	P1	0.057	5.832E-03	0.004	0.017	0.019	20388	5.832E-03	0.004	0.017	0.019	20388
	P2	0.069	4.374E-02	0.007	0.199	0.021	24494	1.037E-14	0.001	0.027	0.095	18027
	P3	0.047	8.863E-51	0.000	0.033	0.053	16638	-	-0.020	-	-	31509
0.13	P1	0.131	1.461E-09	0.003	0.010	0.014	46913	1.461E-09	0.003	0.010	0.014	46913
	P2	0.161	1.871E-03	0.009	0.012	0.260	57494	-	-0.002	0.012	0.021	37095
	P3	0.138	-	-0.002	0.564	0.026	49163	-	-0.002	0.034	0.027	29337
0.22	P1	0.204	-	-	-	-	72719	2.674E-20	0.002	0.009	0.011	72719
	P2	0.156	-	-	-	-	-	3.017E-21	0.001	0.012	-	55604
	P3	0.126	-	-	-	-	-	-	-0.002	0.033	0.017	45101
0.31	P1	0.284	2.674E-20	0.002	0.008	0.016	101464	1.412E-10	0.005	0.008	0.016	101464
	P2	0.307	3.987E-02	0.030	0.010	0.030	109571	-	-0.003	0.008	0.040	71911
	P3	0.268	6.205E-06	0.008	0.016	0.023	95566	-	-0.002	0.040	0.009	58168
0.42	P1	0.374	1.412E-10	0.005	0.005	0.006	133668	9.189E-09	0.008	0.005	0.006	133668
	P2	0.439	1.547E-02	0.035	0.011	0.008	156815	-	-0.006	-	-	92119
	P3	0.367	1.579E-04	0.015	0.013	0.017	131107	-	-0.003	0.022	0.012	78523
0.52	P1	0.465	1.617E-07	0.011	0.004	0.004	165913	-	-	-	-	-
	P2	0.521	1.914E-03	0.030	0.039	0.004	186077	-	-	-	-	-
	P3	0.426	2.041E-03	0.024	0.014	0.017	152270	-	-	-	-	-

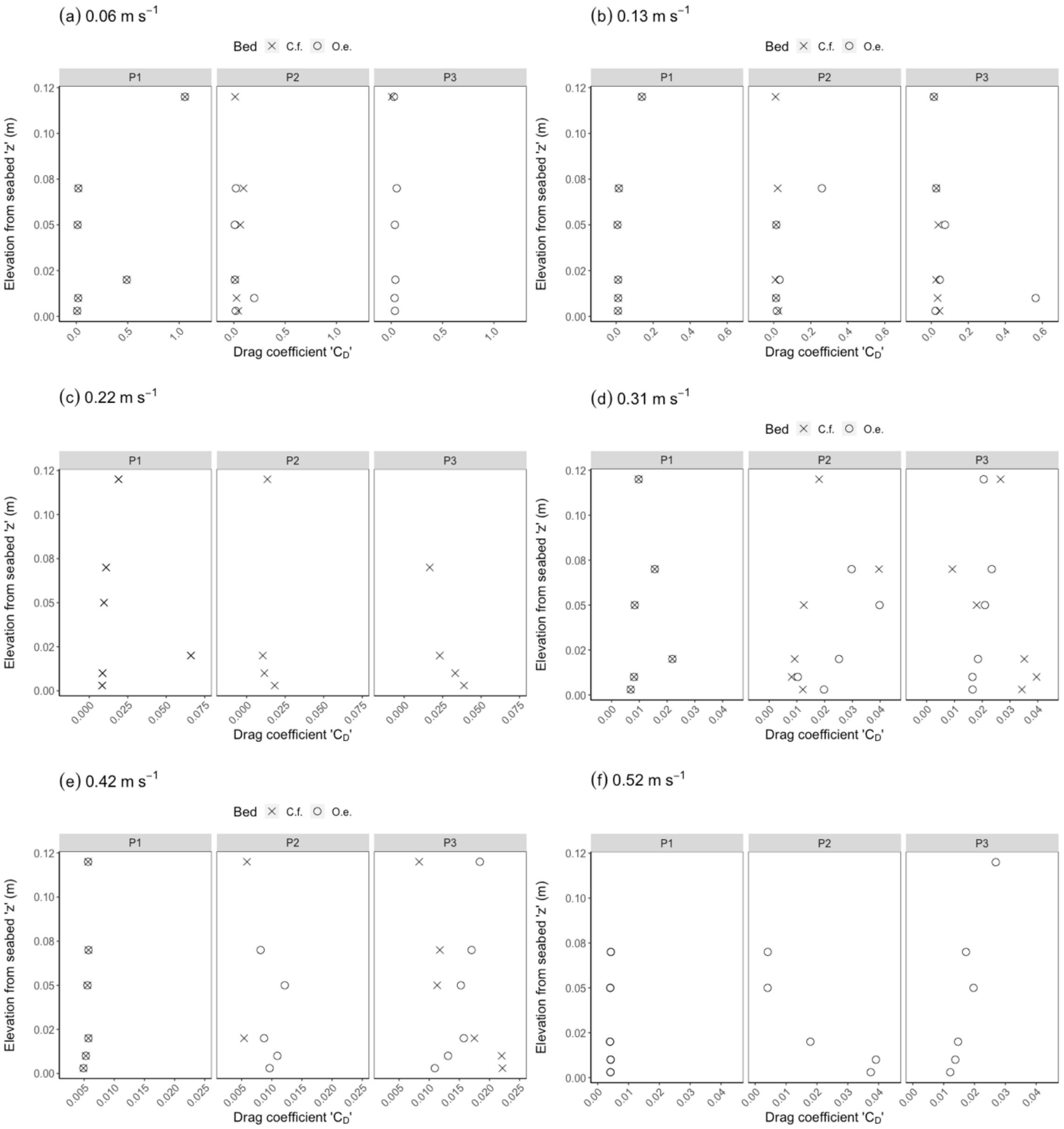


Figure B2 (a-f). Drag coefficient 'C$_D$' as a factor of elevation from the seabed 'z' (m) at the three measured positions prior to (P1), above (P2) and after (P3) an *Ostrea edulis* (O.e.) bed (circles), and *Crepidula fornicata* (C.f.) bed (crosses) at each experimental flume speed: (a) 0.06 m s^{-1}, (b) 0.13 m s^{-1}, (c) 0.22 m s^{-1}, (d) 0.31 m s^{-1}, (e) 0.42 m s^{-1}, and (f) 0.52 m s^{-1}.

Appendix C. Metabolic markers of resistance

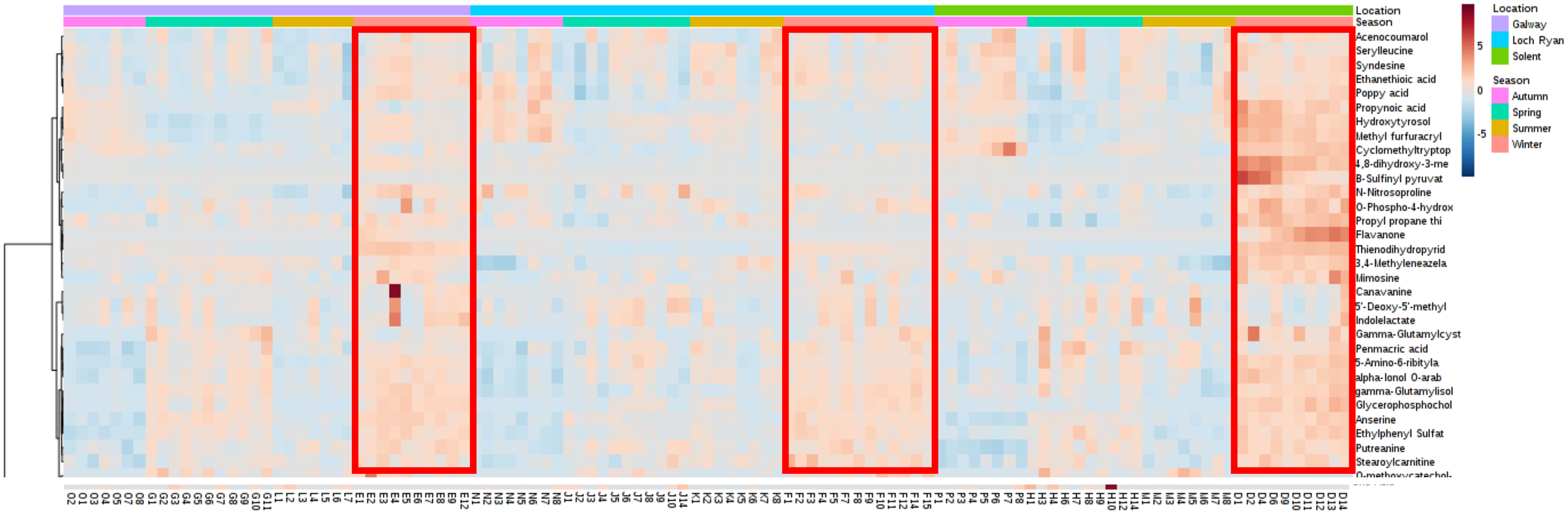


Figure C1. Close up heatmap of the ion compounds showing significantly higher concentration in the Solent population compared to the Galway and Loch Ryan populations in winter.

Figure C2. Close up heatmap of the ion compounds showing significantly higher concentration in the Galway and Loch Ryan populations compared to the Solent population in the autumn.

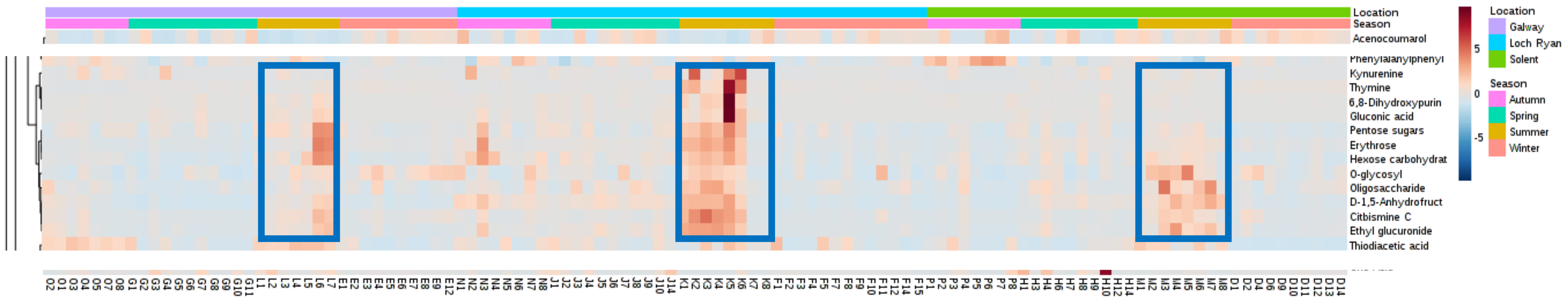


Figure C3. Close up heatmap of the ion compounds showing significantly higher concentration in the Loch Ryan population compared to the Solent and Galway populations in the summer.

Appendix D. RNA Extraction

Extraction of total RNA from biological tissues using TRI REAGENT (Hauton 001)

- ***Work in the fume cupboard in room 454-07 or 784/04***
- ***All waste solutions, eppendorfs, paper towels, gloves and pipette tips should be disposed of in the designated 'halogenated organics' special waste container in 454-07***
- **This standard protocol can be followed outside of normal working hours**

TRI REAGENT is a mixture of guanidine thiocyanate and phenol in a mono-phase solution. This reagent effectively dissolves DNA, RNA, and protein on homogenization or lysis of tissue sample. After adding chloroform and centrifuging, the mixture separates into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA and an organic phase containing proteins.

Hazardous chemicals used in this protocol (AS IDENTIFIED ON THE ATTACHED COSHH FORMS):

TRI reagent: EUH032, H314-H341-H412-H311-H301-H330-H373, contact with acids liberates very toxic gas, causes severe skin burns and eye damage, suspected of causing genetic defects, harmful to aquatic life with long lasting effects, toxic in contact with skin, toxic if swallowed, fatal if inhaled, may cause damage to organs through prolonged or repeated exposure.

Chloroform: H319-H335-H315-H334, causes serious eye irritation, may cause respiratory irritation, causes skin irritation, may cause allergy or asthma symptoms or breathing difficulties if inhaled.

Procedure:

1) Homogenize tissue samples in TRI REAGENT (1 ml per 50-100 mg of tissue) in an eppendorf tube (or similar). The volume of the tissue should not exceed 10% of the volume of the TRI REAGENT. *Note: After the cells have been homogenized or lysed in TRI REAGENT, samples can be stored at -70 °C for up to 1 month.* Contaminated tips and tubes to special waste.

2) To ensure complete dissociation of nucleoprotein complexes, allow samples to stand for 5 minutes at room temperature.

3) Add 0.2 ml of chloroform (see note below) per ml of TRI REAGENT used. Cover the sample tightly, shake vigorously for 15 seconds and allow to stand for 15 minutes at room temperature. *Note: The chloroform used for phase separation should not contain isoamyl alcohol or other additives.* Contaminated tips and tubes to special waste.

4) Centrifuge the resulting mixture at 12,000*g* for 15 minutes at 4 °C. Centrifugation separates the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA).

5) Transfer the aqueous phase to a fresh tube and add 0.5 ml of isopropanol per ml of TRI REAGENT used in step 1 and mix. Allow the sample to stand for 5-10 minutes at room temperature.

Centrifuge at 12,000*g* for 10 minutes at 4 °C. The RNA precipitate will form a pellet on the side and bottom of the tube. Contaminated tubes and tips to special waste.

6) Remove the supernatant and wash the RNA pellet by adding 1 ml (minimum) of 75% ethanol per 1 ml of TRI REAGENT used in step 1. Vortex the sample and then centrifuge at 7,500*g* for 5 minutes at 4 °C.

7) Briefly dry the RNA pellet for 5-10 minutes by airdrying. Do not let the RNA pellet dry completely, as this will greatly decrease its solubility. Add an appropriate volume of DEPC-treated Miili-Q water. To facilitate dissolution, mix by repeated pipetting with a micropipette at 55-60 °C for 10-15 minutes.

Appendix E. Gene expression and choice of ERGs

Qbase+ was used to determine the number and choice of reference genes depending on expression stability across samples.

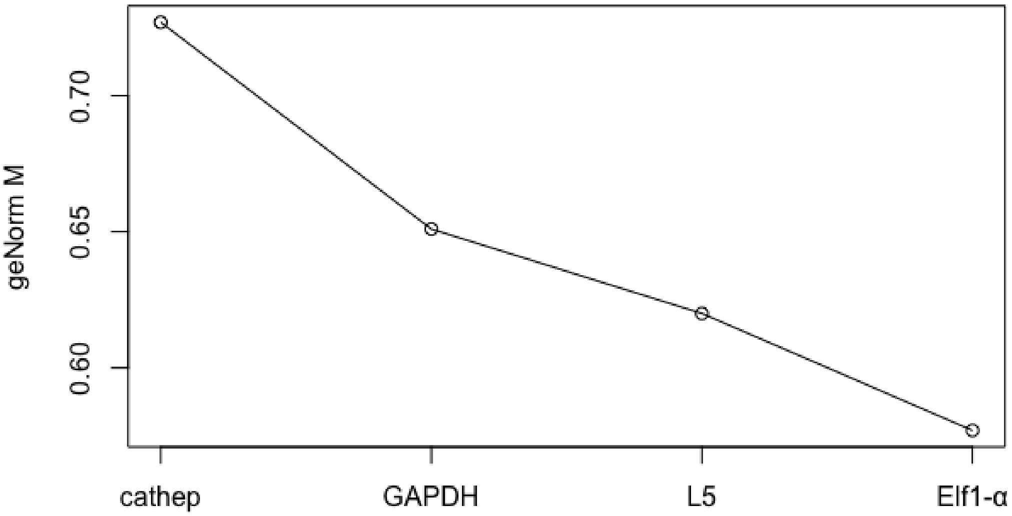


Figure E1. The four endogenous reference genes with lowest *M* value. A low *M* value reflects a more consistent ratio between the control genes across all samples regardless of experimental condition and a more desirable ERG.

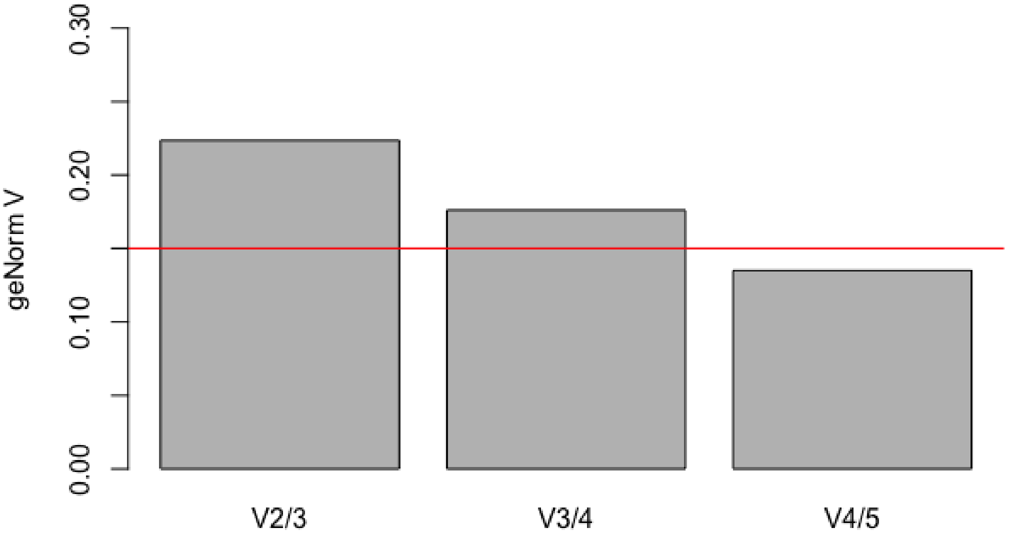


Figure E2. Determination of optimal number of reference targets (geNorm *V*). The *V* value determines the minimal number of genes required for use as ERGs, by comparing the change in expression of the genes of interest (GOIs) based on the geometric mean expression of the ERGs. Vandesompele *et al.* (2002) suggested a cut off *V* value of 0.15, below which the addition of a further ERG is not required.

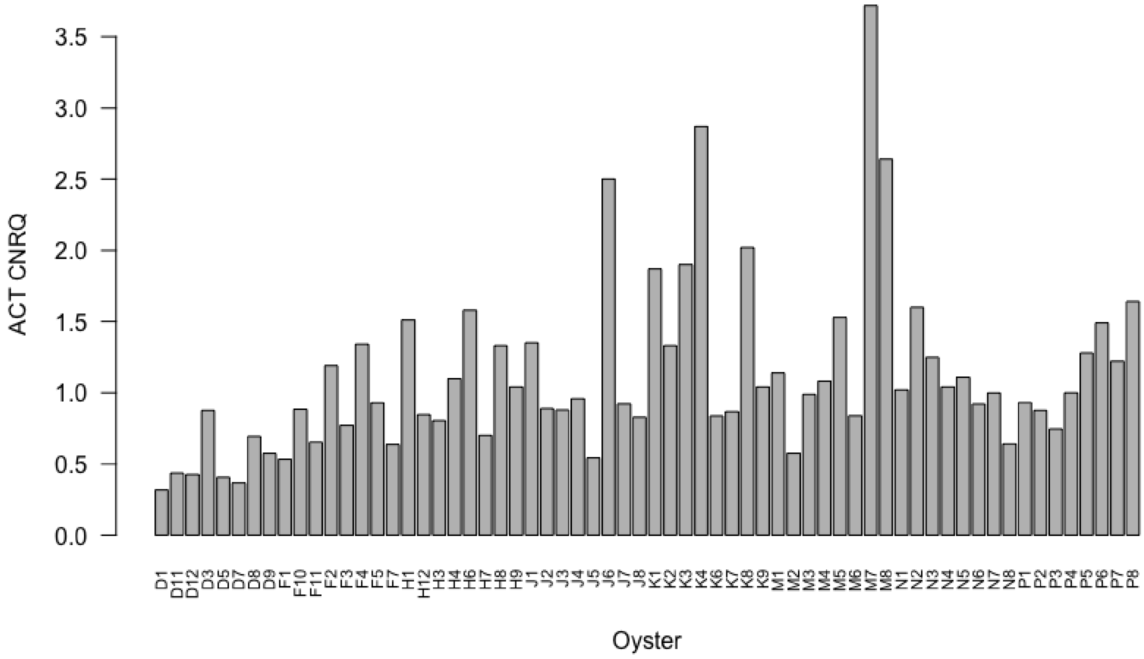


Figure E3. Calibrated normalised relative quantity (CNRQ) of β-actin (*ACT*) per oyster.

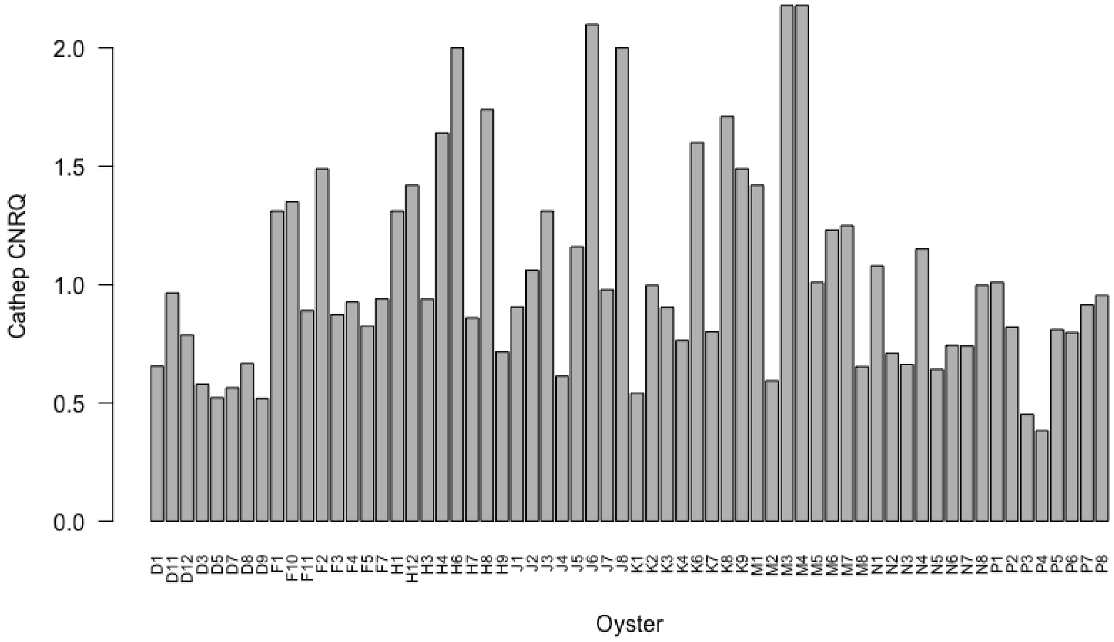


Figure E4. Calibrated normalised relative quantity (CNRQ) of Cathepsin (*Cathep*) per oyster.

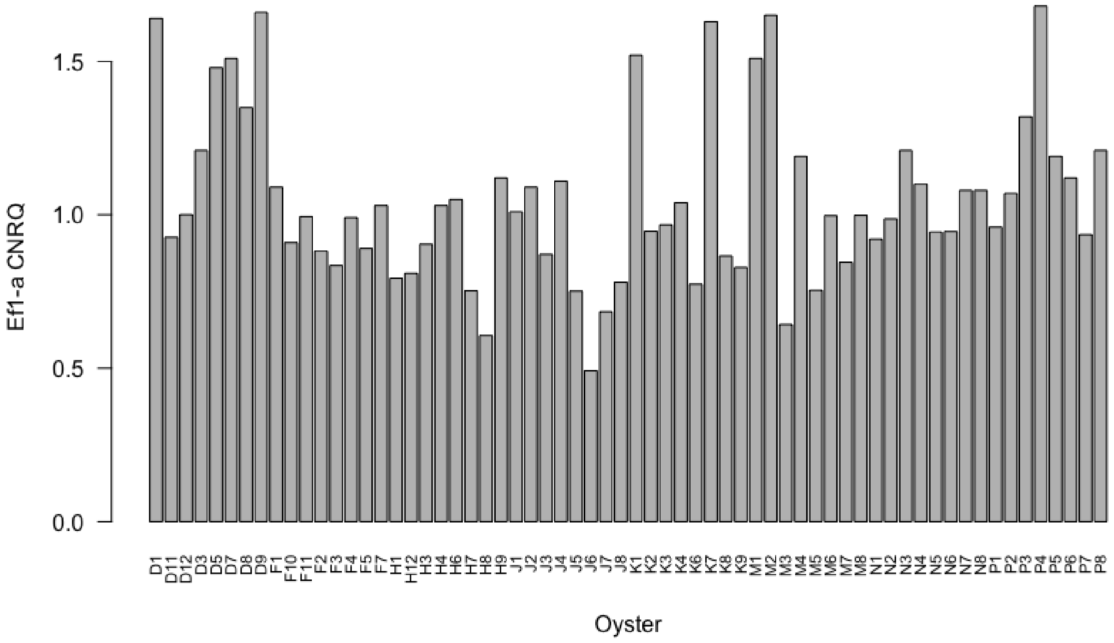


Figure E5. Calibrated normalised relative quantity (CNRQ) of elongation factor 1-α (*Ef1- α*) per oyster.

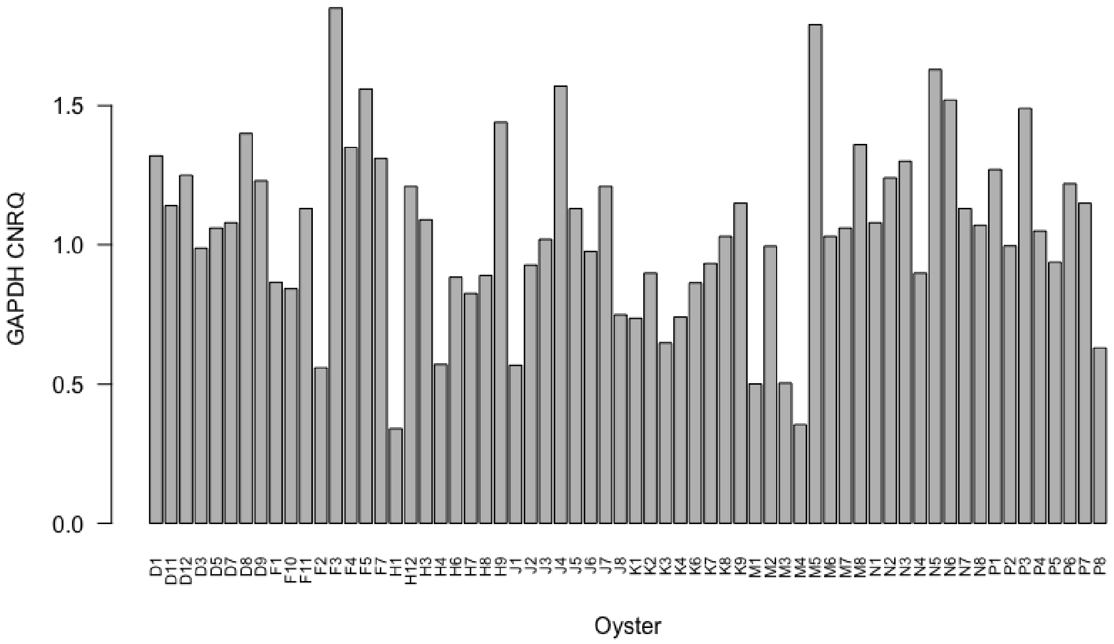


Figure E6. Calibrated normalised relative quantity (CNRQ) of glyceraldehyde 3-phosphate-dehydrogenase (*GAPDH*) per oyster.

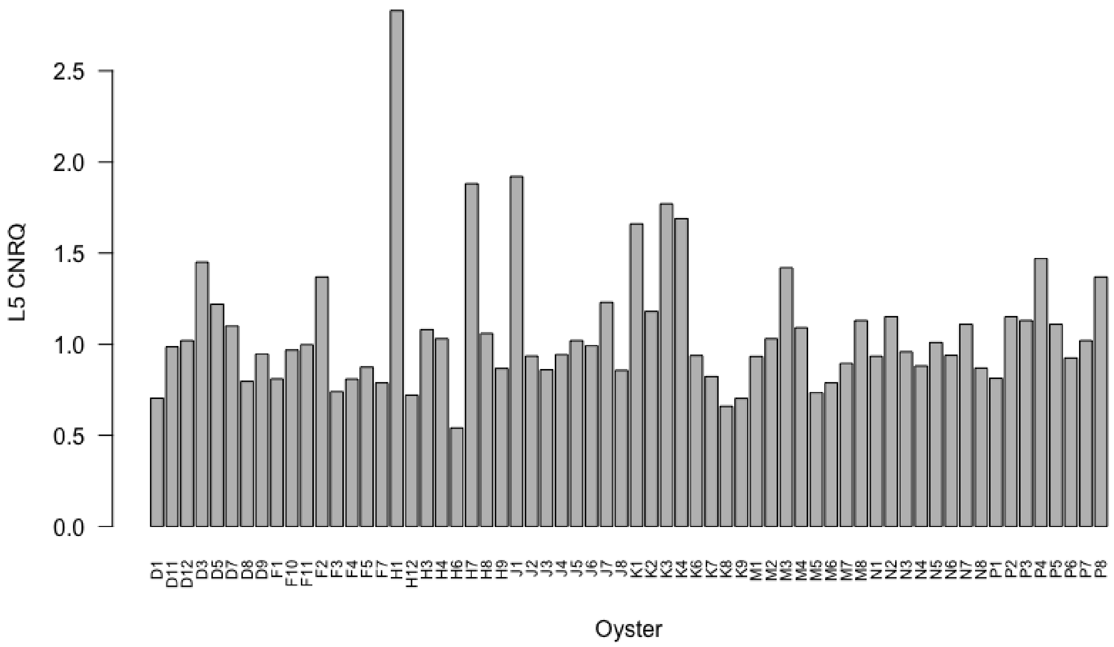


Figure E7. Calibrated normalised relative quantity (CNRQ) of 60S ribosomal protein L5 (*L5*) per oyster.

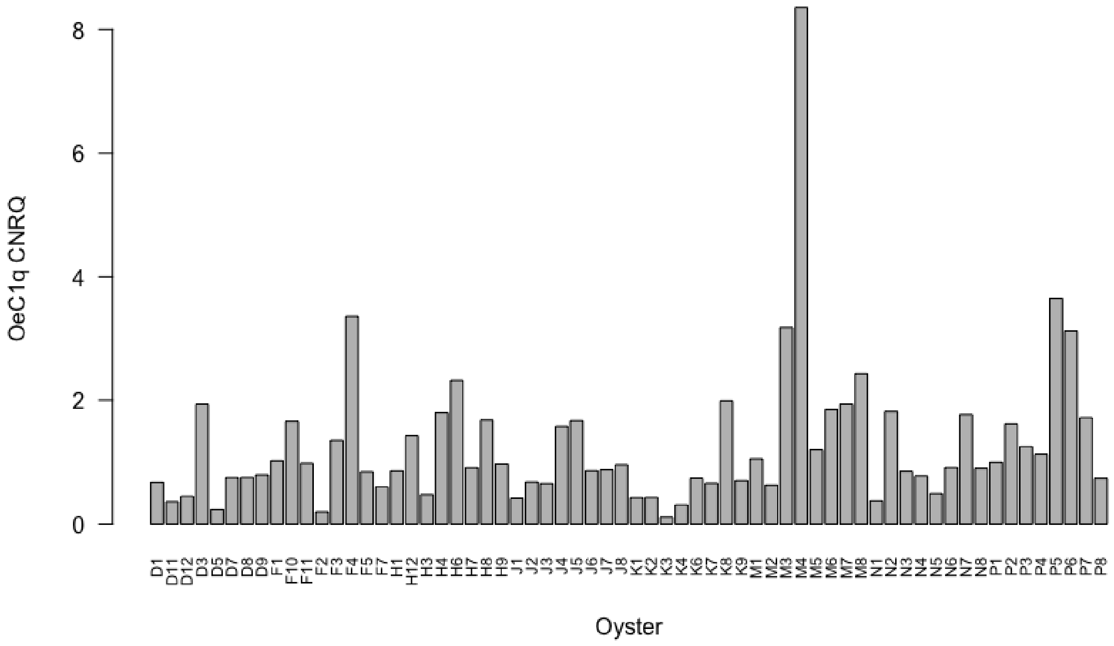


Figure E8. Calibrated normalised relative quantity (CNRQ) of C1q (*OeC1q*) per oyster.

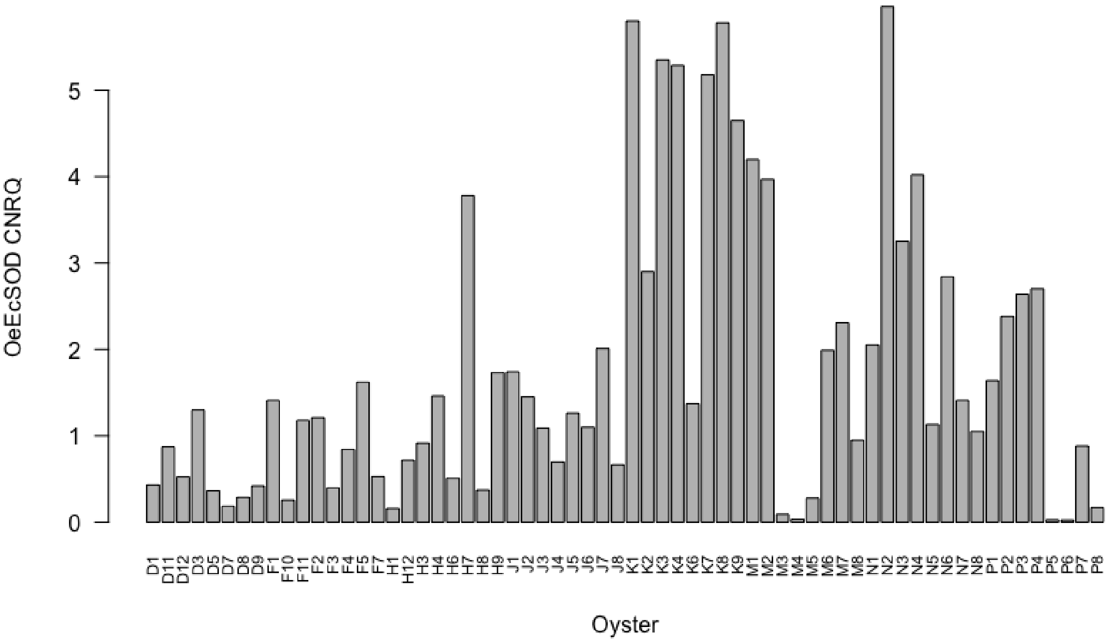


Figure E9. Calibrated normalised relative quantity (CNRQ) of extracellular superoxide dismutase (*OeEcSOD*) per oyster.

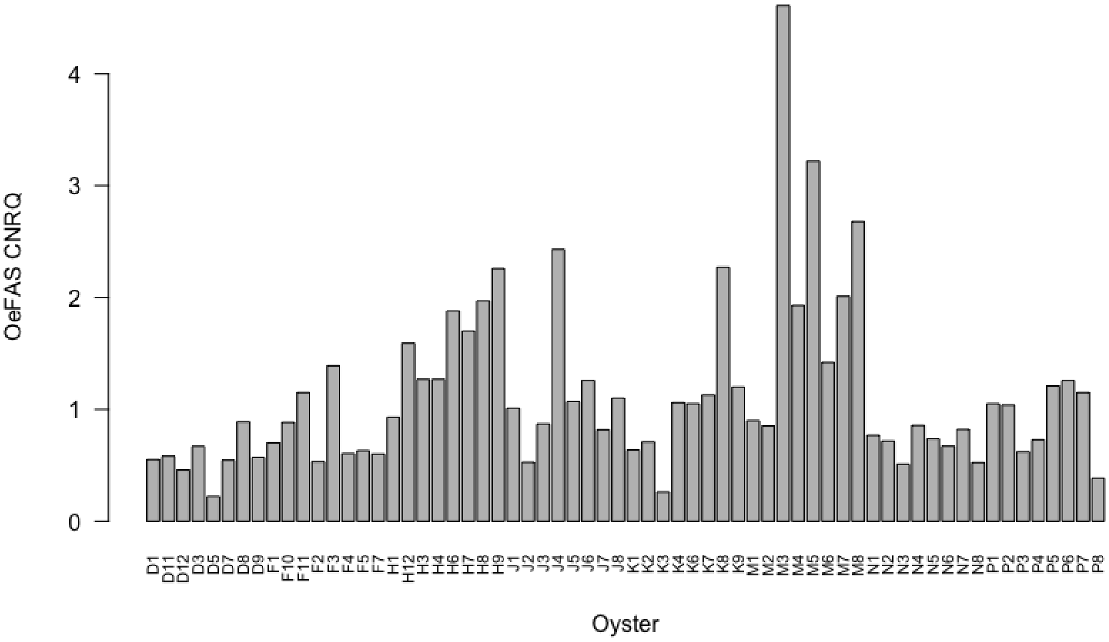


Figure E10. Calibrated normalised relative quantity (CNRQ) of Fas-ligand (*OeFAS*) per oyster.

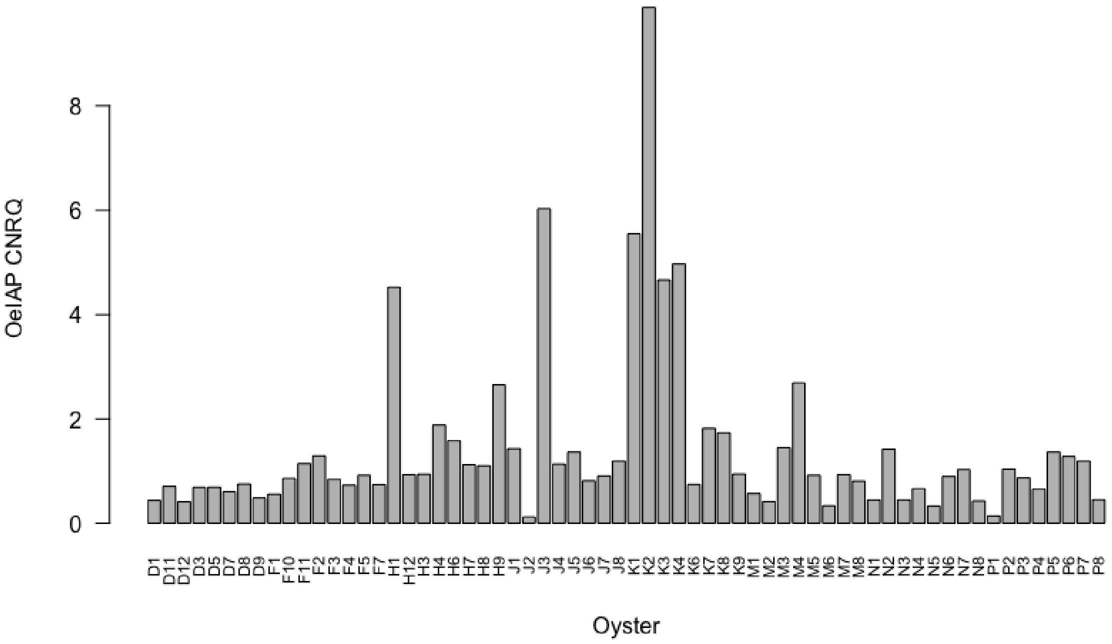


Figure E11. Calibrated normalised relative quantity (CNRQ) of Inhibitor of apoptosis (*OeIAP*) per oyster.

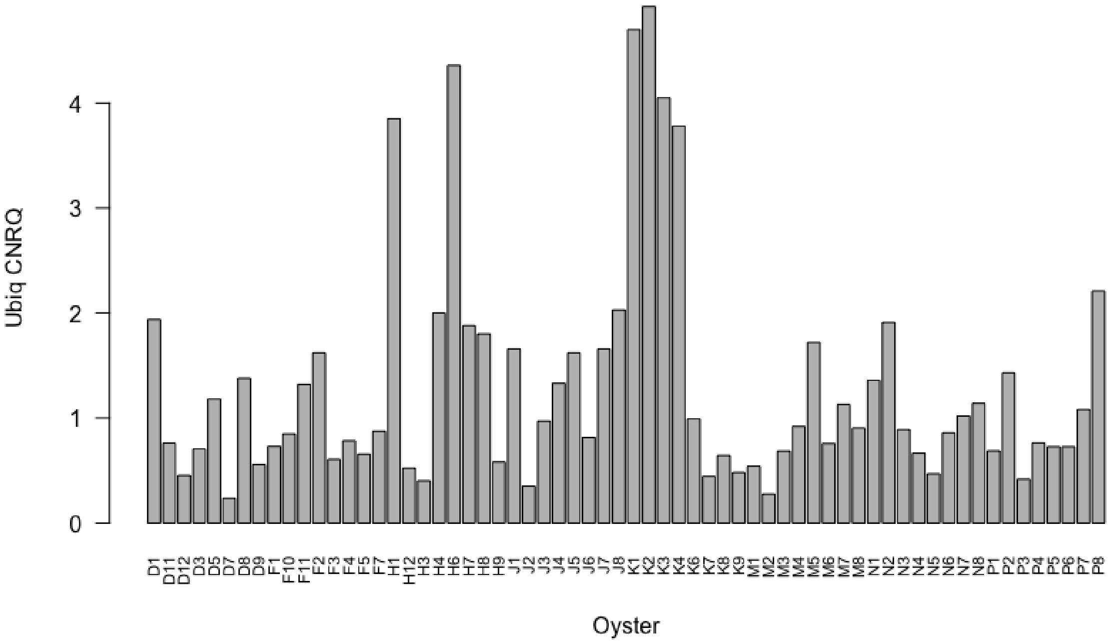


Figure E12. Calibrated normalised relative quantity (CNRQ) of polyubiquitin (*Ubiq*) per oyster.

Appendix F. Additional history of fishing in the Beaulieu River

In 1962, Lord Edward Montagu (3rd Baron Montagu of Beaulieu (1926-2015) was keen to restore *O. edulis* to the Beaulieu River and with help from cousin Maldwin Drummond, approached Cyril Lucas who was re-establishing oyster beds in Newtown Creek (Pers. Comm. Cyril Lucas). Cyril Lucas dredged the Beaulieu riverbed and found a natural settlement of approximately 2000 *O. edulis*, which he re-laid into Newtown Creek for closer management (Pers. Comm. Cyril Lucas) (Figure F1). More recently (until 2018), only one fisherman (David Mitchell 1941-2018) was responsible for the maintenance of the two established fisheries for *O. edulis* and Manila clams, *Ruditapes philippinarum*, (Beaulieu Estate, pers. Comm.).

Figures F1 and F2. Cyril Lucas (F1) harvesting and (F2) re-laying oysters into Newtown Creek c. 1962 (Photo courtesy of Bonnie Robinson, shown with permission from Cyril Lucas). Oytser dredge visible at the top of figure F1.

Appendix G. Epifauna

Table G1. Qualitative data of common species seen at station 2 (see Figure 5.7 for specific location) from video footage taken with the GoPro Hero 3+ camera. Three replication samples were taken at station 2, each with 10 video drop-down events. Asterisks (*) represents presence of the given species or description for each replication sample.

	Station 2		
Replications	1	2	3
Broken (dead) cockle / clam shells	*	*	*
Minimal algae	*	*	
Coral weed (Corallina offinalis)/wire weed (Sargassum spp.)/harpoon weed (Asparagopsis armata)/phycodrys spp.			*
Evidence of infauna	*	*	*
Evidence of epifauna (crabs etc)		*	*
High abundance of fan/tube worms (*Sabella* spp.?)			*
Porifera (*Cliona* spp., *Axinella* spp., *Homaxinella* spp.?)			*
Soft muddy sediment – silt cloud with frame movement suggesting very soft sediment	*	*	
High abundance of colonial ascidians (*Botryllus* spp., *Botrylloides* spp.)			*

Table G2. Qualitative data of common species seen at station 3 (see Figure 5.7 for specific location) from video footage taken with the GoPro Hero 3+ camera. Three replication (Rep.) samples were taken at station 3, each with 10 video drop-down events. Asterisks (*) represents presence of the given species or description for each replication sample.

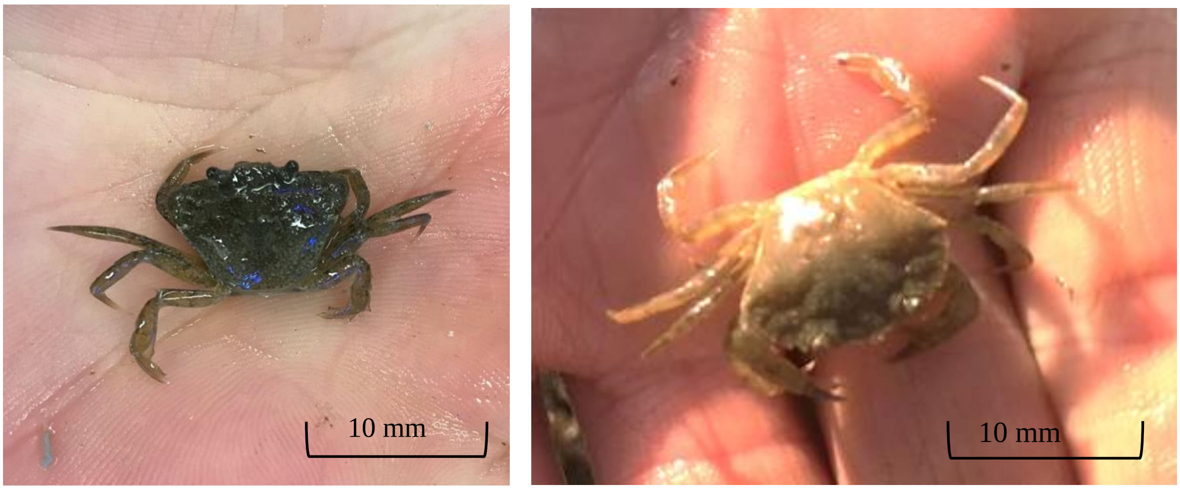
	Station 3		
Replications	1	2	3
broken (dead) cockle / clam shells		*	
evidence of infauna (Sea mouse – *Aphrodita* spp. in Rep. 2)	*	*	*
Evidence of epifauna (e.g. crabs)			*
Minimal epifauna	*		
Sea squirts (*Ascidia*/*Ascidiella* spp.)			*
Coverage with green & red algae (Cladophora rupestris, Ulva, Plocamium cartilagineum, Anotrichum barbatum?)	*		
Soft muddy sediment – silt cloud with frame movement suggesting very soft sediment	*	*	*
Gut weed (Ulva intestinalis)		*	
Harpoon weed (*Asparagopsis* spp.), *Ceramium* spp.? *Carpomitra* spp.?			*
porifera		*	

Table G3. Qualitative data of common species seen at station 4 (see Figure 5.7 for specific location) from video footage taken with the GoPro Hero 3+ camera. Two replication (Rep.) samples were taken at station 4, each with 10 video drop-down events. Asterisks (*) represents presence of the given species or description for each replication sample.

	Station 4	
Replications	1	2
Soft muddy sediment – silt cloud with frame movement suggesting very soft sediment	*	
Very soft sediment. Imprints left when frame removed form area.		*
High algae percentage, (*Corallina* spp., *Cryptopleura* spp., *Osmundea* spp., *Ulva*) but often covered in mud demonstrating high siltation	*	
High algae percentage, (*Sargassum* spp., *Carpomitra* spp., *Ulva*, *Cryptopleura* spp., *Plocamium* spp., *Calliblepharis* spp.) but often covered in mud demonstrating high siltation		*
Busy with epifauna	*	
Sea squirts (*Ascidia*/*Ascidiella* spp).		*
Fan/tube worms (*Sabella* spp.)	*	*
Higher concentration of *Crepidula fornicata* than previous drop downs	*	
Porifera present (Axinella spp., *Cliona* spp.)	*	*

Figure G1 and G2. Upon retrieval in both 2018 and 2019, gabions were fouled with red and green algae (Figure G1), and tunicates (Figure G2).

Figures G3 and G4. Scorpion fish, *Taurulus* spp., retrieved from the gabions in August 2019 (Figure G3), and squat lobster, *Galathea squamifera*, retrieved in August 2018 (Figure G4) (Hayward and Ryland, 2017).



Figures G5 and G6. Shore crabs, *Carcinus* spp., retrieved from the gabions in August 2018 (Figure G5) and August 2019 (Figure G6) (Hayward and Ryland, 2017).

Figure G7. Two sea spiders, *Pantopoda* spp., found on an oyster recovered from gabion 2 on 15th April 2019 (Hayward and Ryland, 2017).

Figures G8 and G9. A sand mason, *Lanice conchilega*, retrieved from within a microreef in gabion 4 on 15th April 2019 (Figure G8). A terebellid polychaete worm, *Amphitrite* spp., retrieved from gabion 1 on 15th April 2019 (Figure G9) (Hayward and Ryland, 2017).

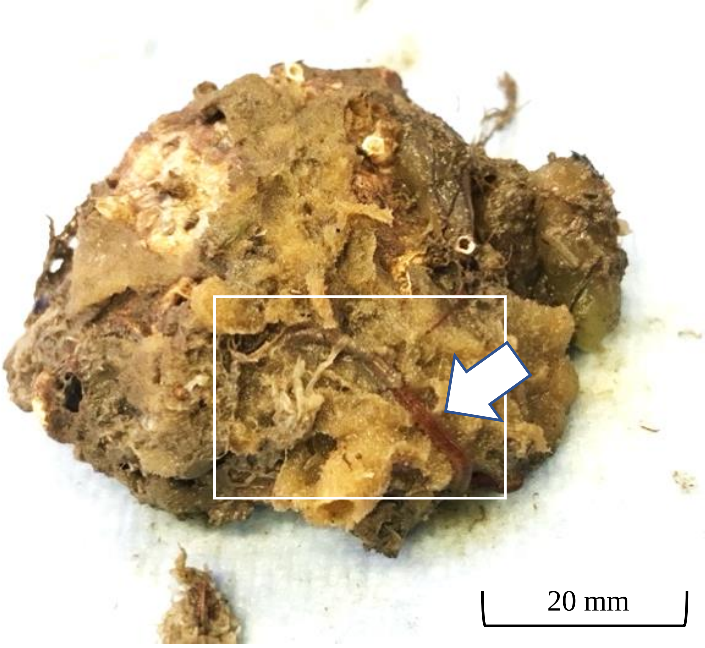


Figure G10. A polychaete worm (visible within the white box) living within an encrusting sponge on the left valve of an oyster recovered on 15th April 2019 from gabion 2.

Figure G11. A juvenile sea mouse, *Aphrodita aculeata*, retrieved from gabion 1 on 15th April 2019 (Hayward and Ryland, 2017).

Figures G12 and G13. Oysters (that had been scrubbed clean prior to deployment) were encrusted with keel worms, *Pomatoceros triqueter*, and tunicates (particularly *Ascidia conchilega*, *Ascidiella aspera*, and *Ciona intestinalis*). Both Figures show LR oysters retrieved form gabion 1 in August 2018 (Hayward and Ryland, 2017).

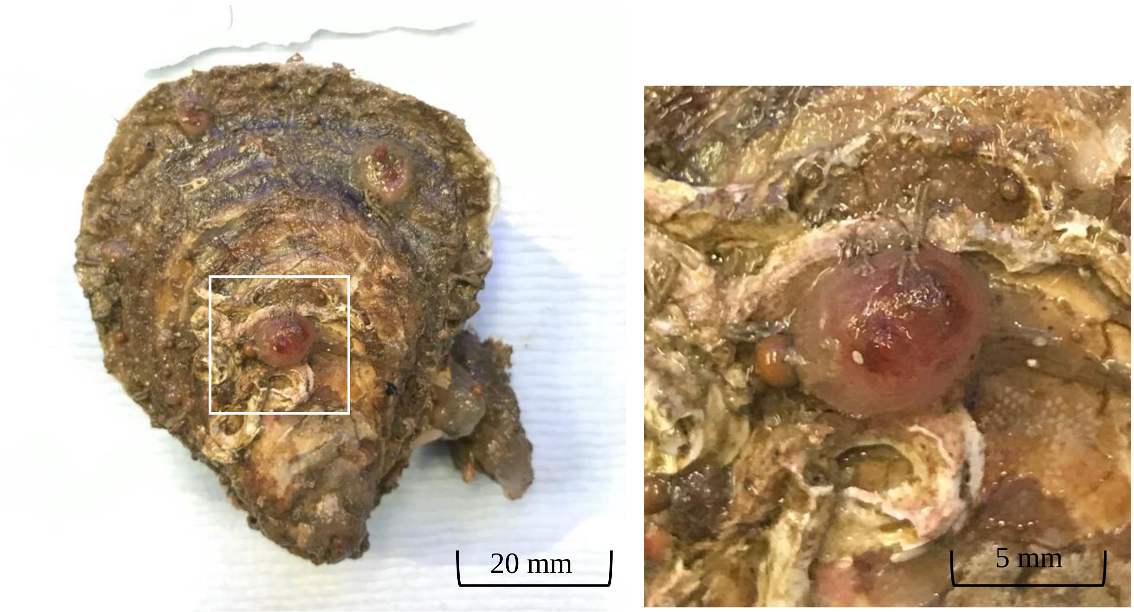


Figure G14a and G14b. Tunicates, *Distomus variolosus*, were commonly found on oyster shells. This specimen was found on the right valve of an LR oyster from gabion 3 retrieved on 15th April 2019 (Figure G14a) and is seen in close up in Figure G14b (Hayward and Ryland, 2017).

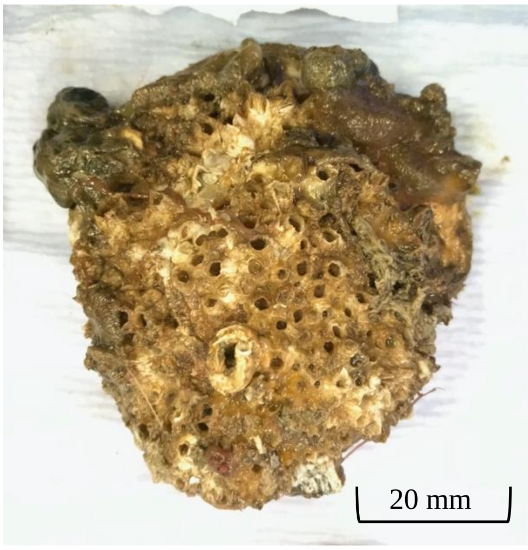


Figure G15. Evidence of a barnacle colony from empty plates on the left valve of a LR oyster recovered from gabion 4, 15th April 2019. Oysters were scrubbed clean of epifauna prior to deployment and therefore this represents new colonisation.

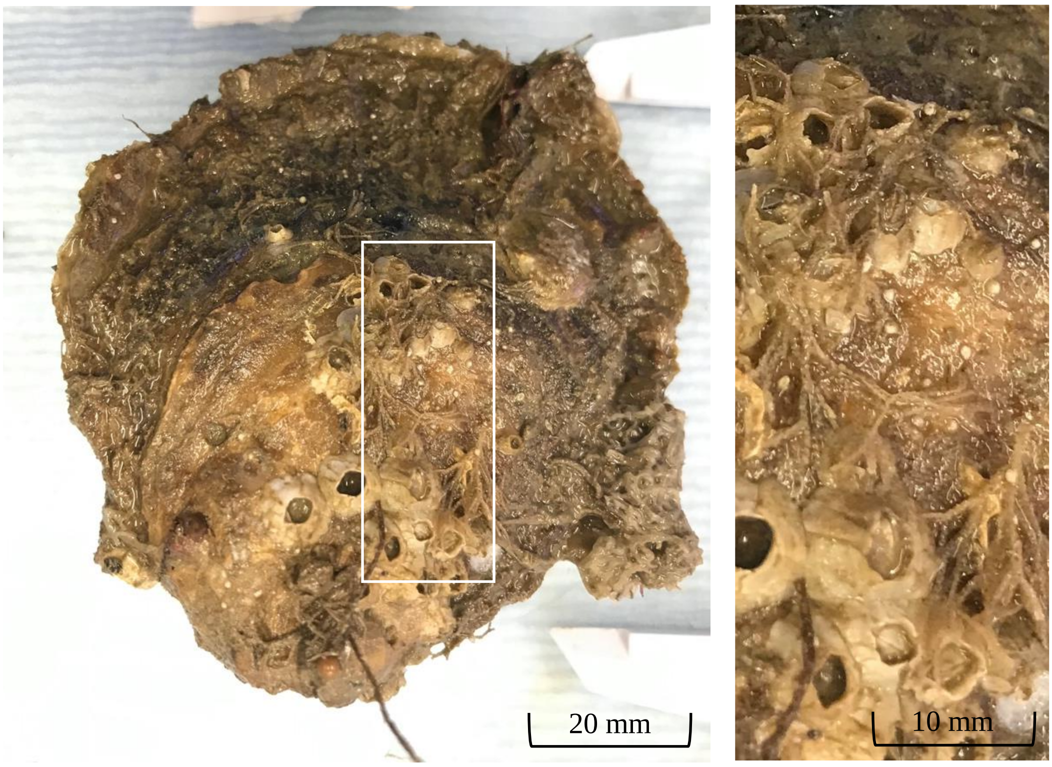


Figure G16a and G16b. Living barnacles observed encrusting on the right valve of a Loch Ryan oyster (within the white box in Figure G16a), and the white box at high resolution (Figure G16b).

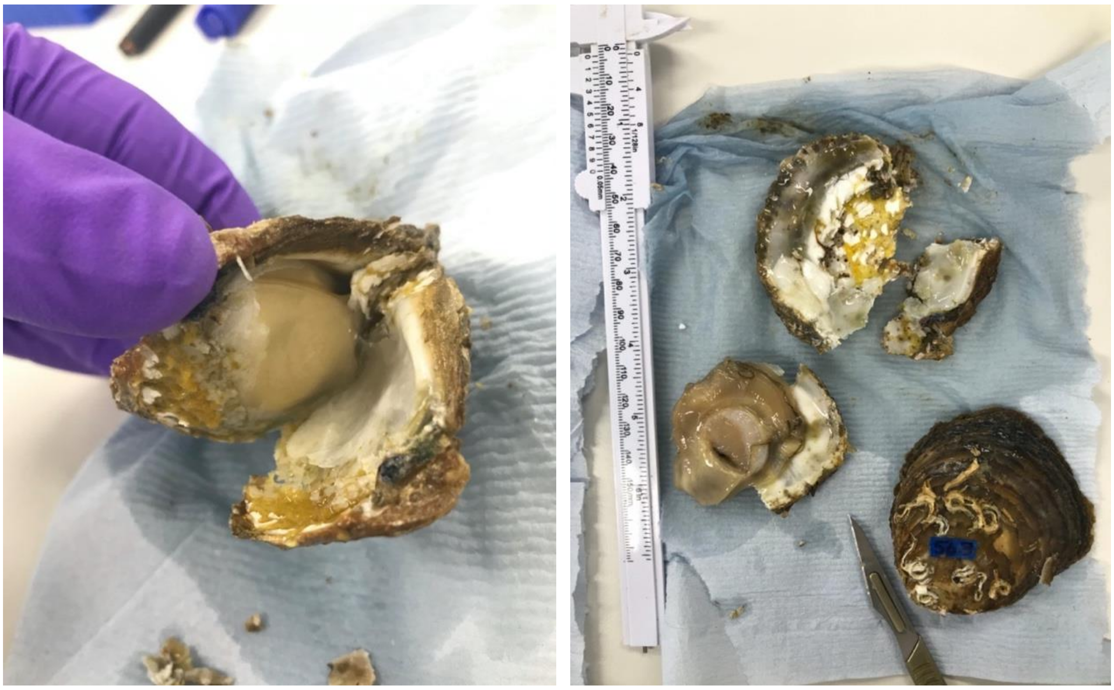
Figures G17 and G18. Four sponge covered *Ocenebra erinacea* were observed within the gabions in all sampling events (2018 and 2019) (Figure G17) (Hayward and Ryland, 2017). Each of the *O. erinacea* were large specimens that measured over 0.04 m in shell length (Figure G18).

Figure G19. An oyster recovered in August 2018 from gabion 3 with *Crepidula fornicata* egg capsules deposited onto the shell, visible as yellow egg capsules.

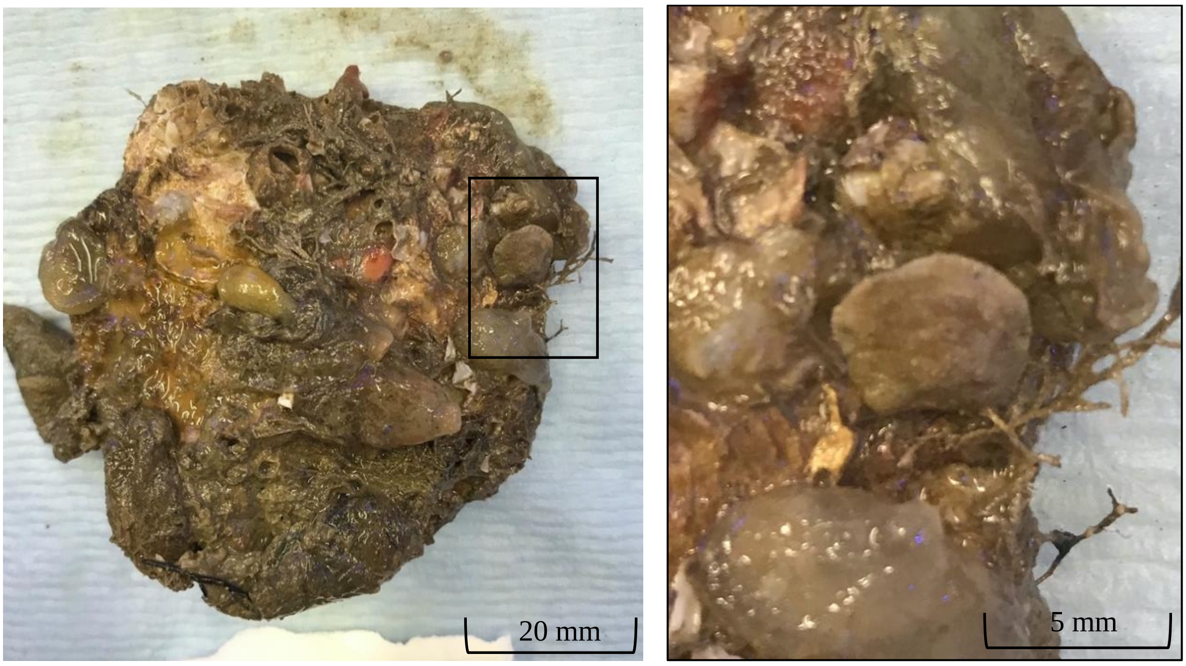
Figure G20. An oyster recovered in August 2018 from gabion 4 with *Crepidula fornicata* egg capsules deposited onto the shell, visible as yellow egg capsules

Figures G21 and G22. Spiny spider crab, *Maja squinado*, (Figure G21) were observed within the gabions in August 2018. Each of the four specimens recorded had a carapace of > 12 cm in length (Figure G22).

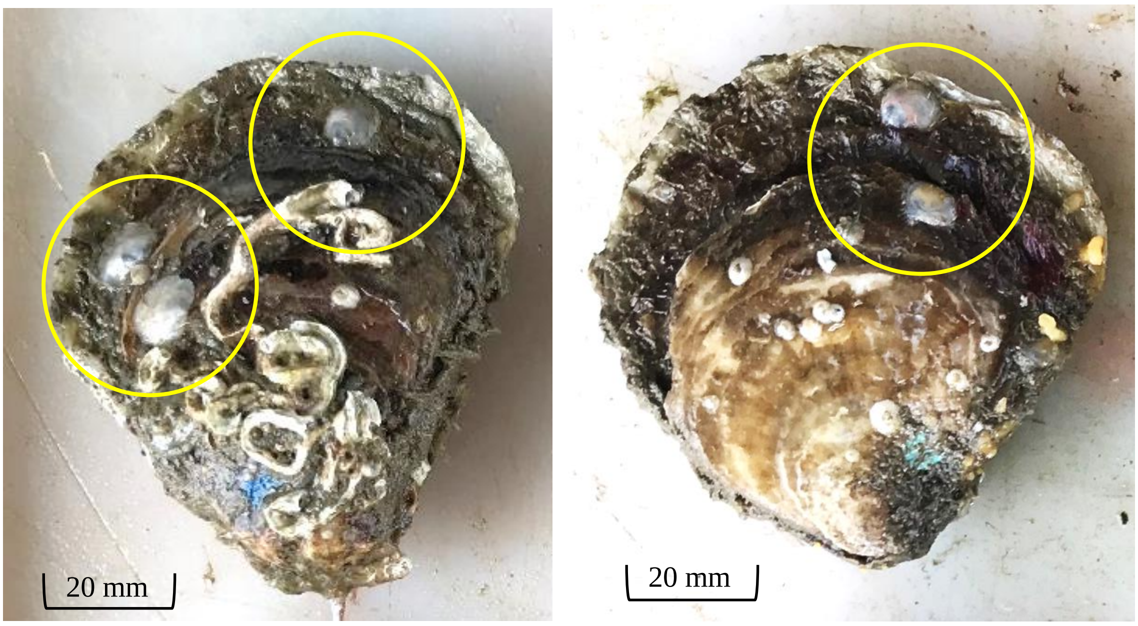
Figures G23 and G24. Evidence of boring sponge *Cliona* spp. from either absent holes from prior predation (Figure G23), or from live specimens on the shell (Figure G24). Both oysters were retrieved from gabions in April 2019.



Figures G25 and G26. Boring sponge *Cliona* spp. evident inside the shell of LRinfection oysters retrieved for disease study in August 2019 (Figure G25), causing the crumbling of the shell upon opening (Figure G26).

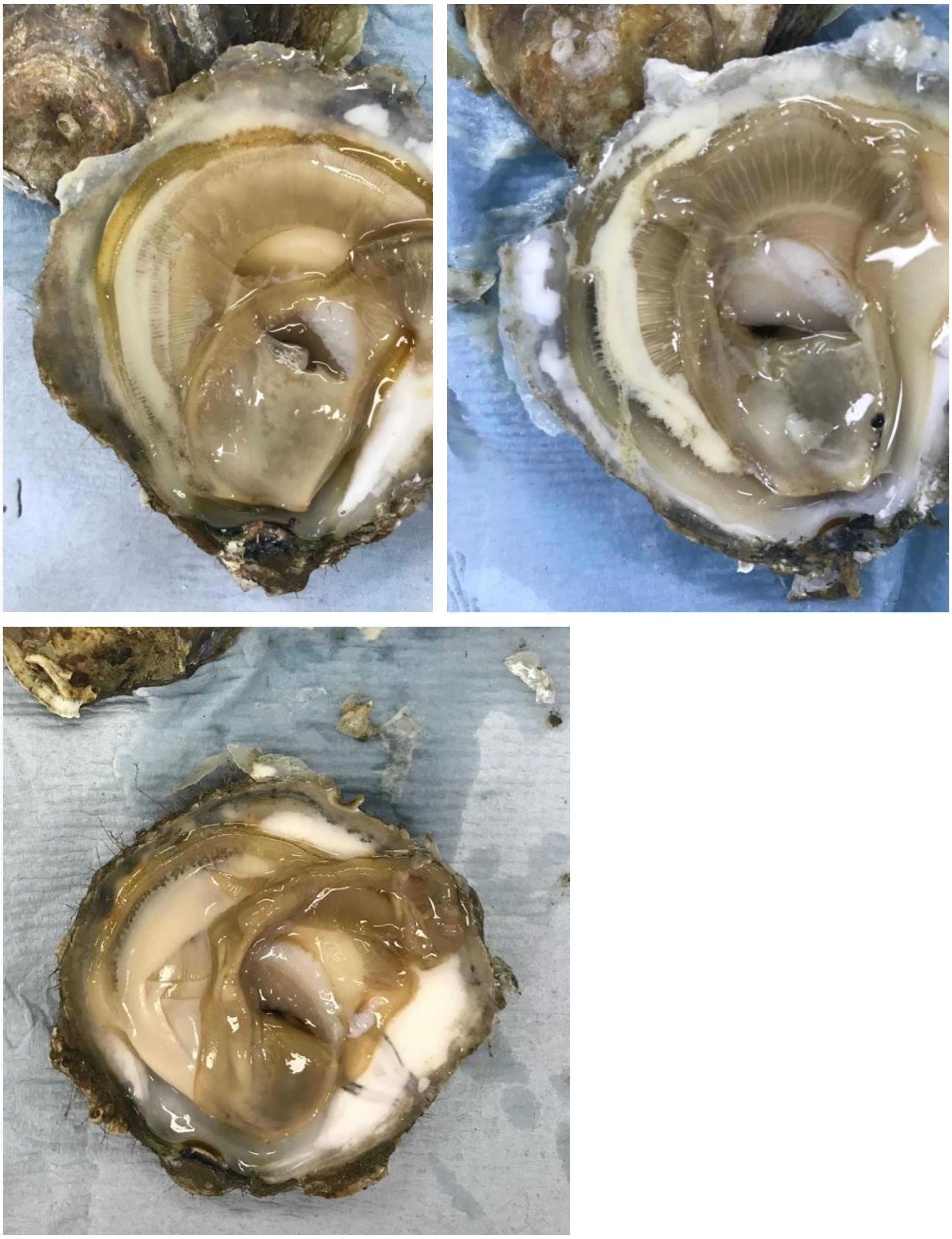


Figures G27a and G27b. Evidence of spat settlement on the left valve of a LR oyster from gabion 1, observed in April 2019 (within black box in Figure G27a), and at high resolution (Figure G27b).



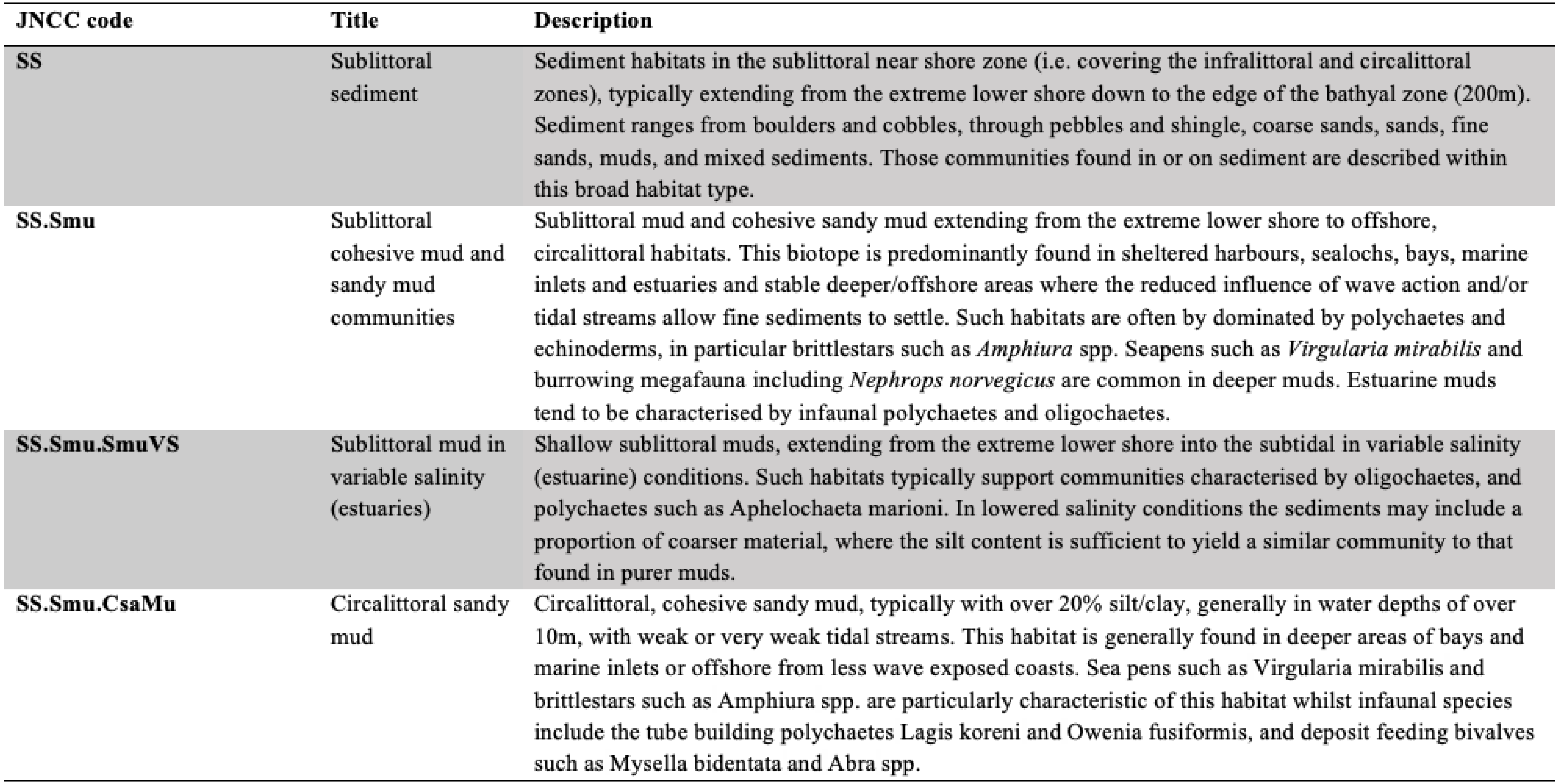
Figures G28 and G29. Evidence of spat settlement (within the yellow circles), potentially *O. edulis*. Observed in 2018.

Figures G30 and G31. Evidence of spat settlement (within the yellow circles), potentially *O. edulis*. Observed in 2018.



Figures G32, G33 and G34. White sick (fertilised eggs) observed within LR oysters indicating successful reproduction.

Table G4. JNCC biotope codes and their full description (Parry, 2019).



JNCC code	Title	Description
SS	Sublittoral sediment	Sediment habitats in the sublittoral near shore zone (i.e. covering the infralittoral and circalittoral zones), typically extending from the extreme lower shore down to the edge of the bathyal zone (200m). Sediment ranges from boulders and cobbles, through pebbles and shingle, coarse sands, sands, fine sands, muds, and mixed sediments. Those communities found in or on sediment are described within this broad habitat type.
SS.Smu	Sublittoral cohesive mud and sandy mud communities	Sublittoral mud and cohesive sandy mud extending from the extreme lower shore to offshore, circalittoral habitats. This biotope is predominantly found in sheltered harbours, sealochs, bays, marine inlets and estuaries and stable deeper/offshore areas where the reduced influence of wave action and/or tidal streams allow fine sediments to settle. Such habitats are often by dominated by polychaetes and echinoderms, in particular brittlestars such as *Amphiura* spp. Seapens such as *Virgularia mirabilis* and burrowing megafauna including *Nephrops norvegicus* are common in deeper muds. Estuarine muds tend to be characterised by infaunal polychaetes and oligochaetes.
SS.Smu.SmuVS	Sublittoral mud in variable salinity (estuaries)	Shallow sublittoral muds, extending from the extreme lower shore into the subtidal in variable salinity (estuarine) conditions. Such habitats typically support communities characterised by oligochaetes, and polychaetes such as Aphelochaeta marioni. In lowered salinity conditions the sediments may include a proportion of coarser material, where the silt content is sufficient to yield a similar community to that found in purer muds.
SS.Smu.CsaMu	Circalittoral sandy mud	Circalittoral, cohesive sandy mud, typically with over 20% silt/clay, generally in water depths of over 10m, with weak or very weak tidal streams. This habitat is generally found in deeper areas of bays and marine inlets or offshore from less wave exposed coasts. Sea pens such as Virgularia mirabilis and brittlestars such as Amphiura spp. are particularly characteristic of this habitat whilst infaunal species include the tube building polychaetes Lagis koreni and Owenia fusiformis, and deposit feeding bivalves such as Mysella bidentata and Abra spp.

SS.SMx	Sublittoral mixed sediment	Sublittoral mixed (heterogeneous) sediments found from the extreme low water mark to deep offshore circalittoral habitats. These habitats incorporate a range of sediments including heterogeneous muddy gravelly sands and also mosaics of cobbles and pebbles embedded in or lying upon sand, gravel or mud. There is a degree of confusion with regard nomenclature within this complex as many habitats could be defined as containing mixed sediments, in part depending on the scale of the survey and the sampling method employed. The BGS trigon can be used to define truly mixed or heterogeneous sites with surficial sediments which are a mixture of mud, gravel and sand. However, another 'form' of mixed sediment includes mosaic habitats such as superficial waves or ribbons of sand on a gravel bed or areas of lag deposits with cobbles/pebbles embedded in sand or mud and these are less well defined and may overlap into other habitat or biotope complexes. These habitats may support a wide range of infauna and epibiota including polychaetes, bivalves, echinoderms, anemones, hydroids and Bryozoa. Mixed sediments with biogenic reefs or macrophyte dominated communities are classified separately in the SBR and SMP habitat complexes respectively.
SS.SMx.SMxVS	Sublittoral mixed sediment in variable sediment (estuaries)	Shallow sublittoral mixed sediments in estuarine conditions, often with surface shells or stones, enabling the development of diverse epifaunal communities, e.g. Crepidula fornicata (IMX.CreAph), as well as infaunal communities. This biotope complex is therefore often quite species rich, compared with purer sediments.
SS.SMx.SMxVS.CreMed	*Crepidula fornicata* and *Mediomastus fragilis* in variable salinity infralittoral mixed sediment	Variable salinity mixed sediment characterised by the slipper limpet Crepidula fornicata and the polychaetes Mediomastus fragilis and Aphelochaeta marioni. Other numerically important taxa include the oligochaetes Tubificoides benedii, syllids such as Exogonenaidina and Sphaerosyllis, and Nephtys hombergii. Lepidonotus squamatus and Scoloplos armiger may also be common. Shell debris and cobbles are colonised by the ascidians Ascidiella aspersa, Ascidiella scabra, Molgula sp. and Dendrodoa grossularia (the ascidians may not be recorded adequately by remote infaunal survey techniques).
SS.SMx.Imx	Infralittoral mixed sediment	Shallow mixed (heterogeneous) sediments in fully marine or near fully marine conditions, supporting various animal-dominated communities, with relatively low proportions of seaweeds. This habitat may include well mixed muddy gravelly sands or very poorly sorted mosaics of shell, cobbles and pebbles embedded in mud, sand or gravel. Due to the quite variable nature of the sediment type, a widely variable array of communities may be found, including those characterised by bivalves (SMX.VsenAsquAps, SMX.CreAsAn, and SMX.Ost), polychaetes (SMX.SpavSpAn) and file shells (SMX.Lim). This has resulted in many species being described as characteristic of this biotope complex

		all contributing only a small percentage to the overall similarity (see below). This biotope complex may also include a newly proposed Chaetopterus biotope (Rees pers com.) recently found in the eastern English Channel. This biotope is characterised by an undescribed Chaetopterus sp. and small Lanice conchilega. Further sampling is need in order to assess and fully characterise this potential biotope. As a result, the Chaetopterus biotope has not been included in this revision. Infaunal data for this biotope complex are limited to that described in the biotope SMX.VsenAsquAps and so are not representative of the infaunal component of the whole biotope complex
SS.SMx.Imx.SpavSpAn	*Sabella pavonina* with sponges and anemones on infralittoral mixed sediment	Muddy gravelly sand with pebbles off shallow, sheltered or moderately exposed coasts or embayments may support dense populations of the peacock worm Sabella pavonina. This community may also support populations of sponges such as Esperiopsis fucorum, Haliclona oculata and Halichondria panicea and anemones such as Sagartia elegans, Cerianthus lloydii and Urticina felina. Hydroids such as Hydrallmania falcata and the encrusting polychaete Pomatoceros triqueter are also important. This biotope may have an extremely diverse epifaunal community. Less is known about its infaunal component, although it is likely to include polychaetes such as Nephtys spp., Harmothoe spp., Glycera spp., syllid and cirratulid polychaetes, bivalves such as Abra spp., Aoridae amphipods and brittlestars such as Amphipholis squamata.
SS.SSa.SSaVS.MoSaVS	Infralittoral mobile sand in variable salinity (estuaries)	Very mobile sand in areas of strong tidal currents and variable salinity. No stable community is able to develop within this extremely mobile and abrasive habitat. The fauna encountered in this habitat consists of epifaunal crustaceans or relatively low numbers of robust species, such as the isopod Eurydice pulchra or Mesopodopsis slabberi. The polychaete Capitella capitata may occur frequently in some areas. Other taxa such as the polychaetes Eteone spp. and Arenicola marina, the mysid Neomysis integer and the amphipods Bathyporeia spp. and Haustorius arenarius may also be washed in from adjacent communities. This biotope is found in tidal channels of estuaries and areas where water movement keeps silt and mud in suspension, and excludes even the more robust infauna. If oligochaetes, polychaetes and bivalves are present in any numbers within this habitat type then care must be taken to avoid the inclusion of juvenile or spat recruitment counts which may mask the presence of this biotope. This is particularly relevant as sampling usually occurs at slack water periods when settlement takes place.
SS.SMx.Imx.Ost	*Ostrea edulis* beds on shallow	Dense beds of the oyster Ostrea edulis can occur on muddy fine sand or sandy mud mixed sediments. There may be considerable quantities of dead oyster shell making up a substantial portion of the substratum. The clumps of dead shells and oysters can support large numbers of Ascidiella

	sublittoral muddy mixed sediment	aspersa and Ascidiella scabra. Sponges such as Halichondria bowerbanki may also be present. Several conspicuously large polychaetes, such as Chaetopterus variopedatus and terebellids, as well as additional suspension-feeding polychaetes such as Myxicola infundibulum and Sabella pavonina may be important in distinguishing this biotope, whilst the Opisthobranch Philine aperta may also be frequent in some areas. A turf of seaweeds such as Plocamium cartilagineum, Nitophyllum punctatum and Spyridia filamentosa may also be present. This biotope description may need expansion to account for oyster beds in England.
SS.SBR	Sublittoral biogenic reefs on sediment	Sublittoral biogenic reef communities. This complex includes polychaete reefs, bivalve reefs (e.g. mussel beds) and cold water coral reefs. These communities develop in a range of habitats from exposed open coasts to estuaries, marine inlets and deeper offshore habitats and may be found in a variety of sediment types and salinity regimes.
SS.SMX.IMx.VsenAsquAps	Venerupis senegalensis, Amphipholis squamata and Apseudes latreilli in infralittoral mixed sediment	Sheltered muddy sandy gravel and pebbles in marine inlets, estuaries or embayments with variable salinity or fully marine conditions, support large populations of the pullet carpet shell Venerupis senegalensis with the brittlestar Amphipholis squamata and the tanaid Apseudes latreilli. This biotope may be found at a range of depths from 5m to 30m although populations of V. senegalensis may also be found on the low shore. Other common species within this biotope include the gastropod Calyptraea chinensis, a range of amphipod crustacea such as Corophium sextonae and Maera grossimana and polychaetes such as Mediomastus fragilis, Melinna palmata, Aphelochaeta marioni, Syllids and tubificid oligochaetes. Many of the available records for this biotope are from southern inlets and estuaries such as Plymouth Sound and Milford Haven but V. senegalensis has a much wider distribution and it should be noted that northern versions of this biotope may a have a much lower species diversity than reported here.
SS.SMU.ISaMu.AmpPlon	*Ampelisca* spp., *Photis longicaudata* and other tube-building amphipods and polychaetes in infralittoral sandy mud	Sublittoral stable cohesive sandy muds occurring over a wide depth range may support large populations of semi-permanent tube-building amphipods and polychaetes. In particular large numbers of the amphipods Ampelisca spp. and Photis longicaudata may be present along with polychaetes such as Lagis koreni. Other important taxa may include bivalves such as Nucula nitidosa, Chamelea gallina, Abra alba and Mysella bidentata and the echinoderms Echinocardium cordatum and Amphiura brachiata. In some areas polychaetes such as Spiophanes bombyx and Polydora ciliata may also be conspicuously numerous. This community is poorly known, appearing to occur in restricted patches. In some areas it is possible that AmpPlon may develop as a result of moderate organic enrichment. A similar community in mud has also been reported in the Baltic which is characterised by large

		populations of amphipods such as Ampelisca spp., Corophium spp. and Haploops tubicola (see Petersen 1918; Thorson 1957) and it is not known if SMU.AmpPlon is a UK variant of this biotope.
CR.FCR.FouFa	Circalittoral fouling faunal communities	This biotope complex contains two biotopes which, although have different physical habitat characteristics, share the fact that they colonise new areas of artificial substrata relatively quickly. The *Ascidiella aspersa* fouling biotope (Aasp) is found on wave-sheltered artificial substrata such as discarded fishing nets/mooring lines. A separate fouling biotope (AdigMsen) is described for open coast wrecks. This biotope has a characteristic faunal community of *Alcyonium digitatum* and the anemone *Metridium senile*. Other species recorded in this complex (primarily under the AdigMsen biotope) include the hydroid *Nemertesia antennina*, the anemones *Actinothoe sphyrodeta* and *Sagartia elegans*, the cup coral *Caryophyllia smithii*, the bryozoans *Flustra foliacea* and *Bugula plumosa*, the crabs *Necora puber*, *Cancer pagurus* and *Maja squinado* and the lobster *Homarus gammarus*.

Table G5. EUNIS biotope codes and their full description (Parry, 2019).

EUNIS code	Title	Description
MB2222	*Ostrea edulis* beds on Atlantic infralittoral muddy mixed sediment	Dense beds of the oyster *Ostrea edulis* can occur on muddy fine sand or sandy mud mixed sediments. There may be considerable quantities of dead oyster shell making up a substantial portion of the substratum. The clumps of dead shells and oysters can support large numbers of *Ascidiella aspersa* and *Ascidiella scabra*. Sponges such as *Halichondria bowerbanki* may also be present. Several conspicuously large polychaetes, such as *Chaetopterus variopedatus* and terebellids, as well as additional suspension-feeding polychaetes such as *Myxicola infundibulum* and Sabella pavonina may be important in distinguishing this biotope, whilst the Opisthobranch *Philine aperta* may also be frequent in some areas. A turf of seaweeds such as *Plocamium cartilagineum*, *Nitophyllum punctatum* and *Spyridia filamentosa* may also be present. This biotope description may need expansion to account for oyster beds in England.
MB4241	*Aphelochaeta* spp. and *Polydora* spp. in variable salinity Atlantic infralittoral mixed sediment	In sheltered muddy mixed sediments in estuaries or marine inlets with variable or reduced/low salinity communities characterised by Aphelochaeta marioni and Polydora ciliata may be present. Other important taxa may include the polychaetes Nephtys hombergii, Caulleriella zetlandica and Melinna palmata, tubificid oligochaetes and bivalves such as Abra nitida. Conspicuous epifauna may include members of the bivalve family Cardiidae (cockles) and the slipper limpet Crepidula fornicata. This biotope is often found in polyhaline waters.
MB4242	*Crepidula fornicata* and *Mediomastus fragilis* in variable salinity Atlantic infralittoral mixed sediment	Variable salinity mixed sediment characterised by the slipper limpet *Crepidula fornicata* and the polychaetes *Mediomastus fragilis* and *Aphelochaeta marioni*. Other numerically important taxa include the oligochaetes *Tubificoides benedii*, syllids such as *Exogone naidina* and *Sphaerosyllis*, and *Nephtys hombergii*. *Lepidonotus squamatus* and *Scoloplos armiger* may also be common. Shell debris and cobbles are colonised by the ascidians *Ascidiella aspersa*, *Ascidiella scabra*, *Molgula* sp. and *Dendrodoa grossularia* (the ascidians may not be recorded adequately by remote infaunal survey techniques). Situation: This biotope occurs in the lower estuary where currents allow a stable environment to develop. It is associated with oyster beds and relict oyster beds, (MB2-222) in southern England and Wales. It may be found adjacent to or in conjunction with MB6-252 and MB4-241. It may be associated with MB4-233 and possibly form a component of MB6-242.
MB5224	*Ruppia maritima* in reduced salinity Atlantic infralittoral muddy sand	In sheltered brackish muddy sand and mud, beds of *Ruppia maritima* and more rarely *Ruppia spiralis* may occur. These beds may be populated by fish such as *Gasterosteus aculeatus* which is less common on filamentous algal-dominated sediments. Seaweeds such as *Chaetomorpha* spp., *Enteromorpha* spp., *Cladophora* spp., and *Chorda filum* are also often present in addition to occasional fucoids. In some cases the stoneworts *Lamprothamnium papulosum* and *Chara aspera* occur. Infaunal and epifaunal species may include mysid crustacea, the polychaete *Arenicola marina*, the gastropod *Hydrobia ulvae*, the

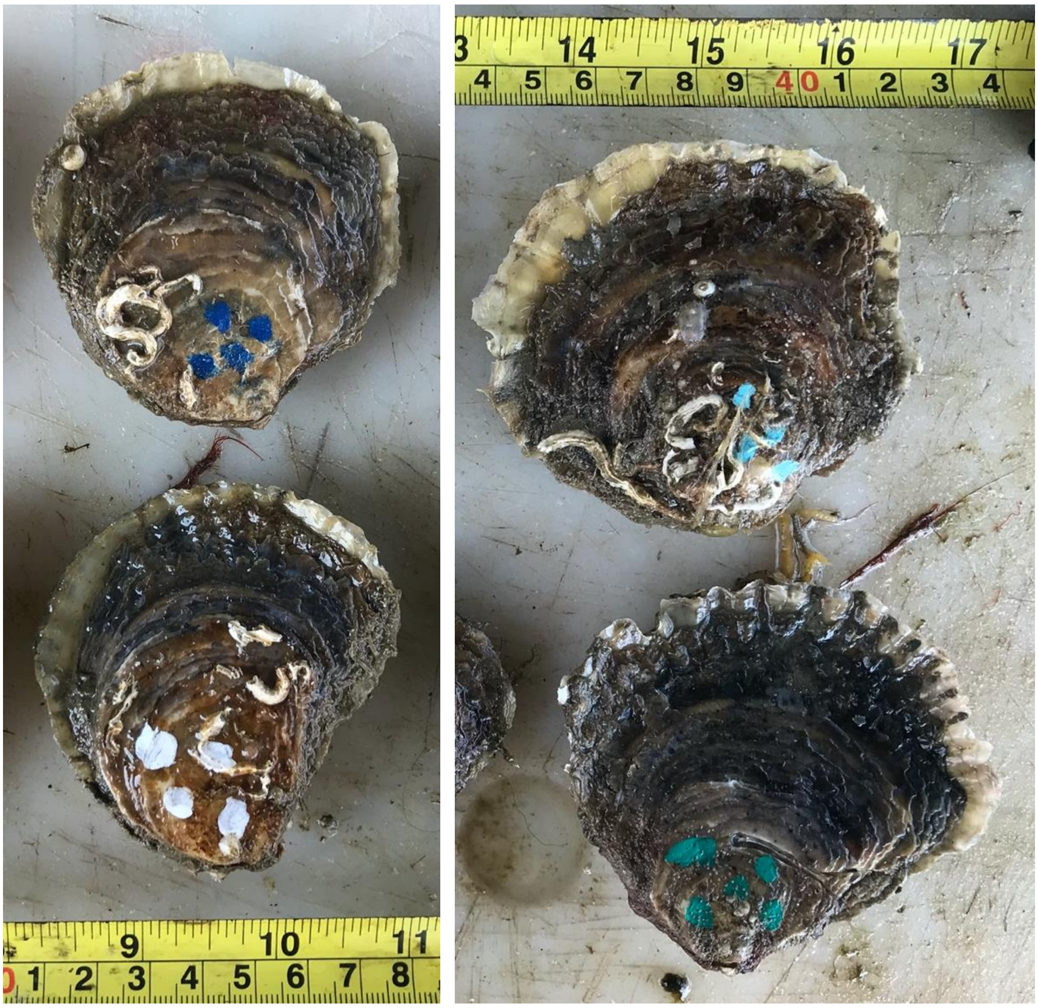
		amphipod *Corophium volutator* and oligochaetes such as *Heterochaeta costata*. In some areas *Zostera marina* may also be interspersed with the *Ruppia* beds.
MB5237	*Arenicola marina* in Atlantic infralittoral fine sand or muddy sand	In shallow fine sand or non-cohesive muddy sand in fully marine conditions (or occasionally in variable salinity) a community characterised by the polychaete *Arenicola marina* may occur. This biotope appears quite faunally sparse. Those other taxa present however, include scavenging crustacea such as *Pagurus bernhardus* and *Liocarcinus depurator*, terebellid polychaetes such as *Lanice conchilega* and the burrowing anemone *Cerianthus lloydii*. Occasional *Sabella pavonina* and frequent *Ensis* spp. may also be observed in some areas. The majority of records for this biotope are derived from epifaunal surveys and consequently there is little information available for the associated infaunal species. It is possible that this biotope, like unit MB5-235 (to which it is broadly similar) is an epibiotic overlay on other sublittoral sand biotopes. Temporal variation: At certain times of the year a diatom film may be present on the sediment surface.
MB6	Infralittoral mud	Sublittoral mud and cohesive sandy mud extending from the extreme lower shore to the lower limit of vascular plants. This biotope is predominantly found in sheltered harbours, sealochs, bays, marine inlets and estuaries and stable deeper/offshore areas where the reduced influence of wave action and/or tidal streams allow fine sediments to settle. Such habitats are often by dominated by polychaetes and echinoderms, in particular brittlestars such as *Amphiura* spp. Estuarine muds tend to be characterised by infaunal polychaetes and oligochaetes.
MB62	Atlantic infralittoral mud	Shallow sublittoral muds, extending from the extreme lower shore to about 15-20 m depth in fully marine or near marine conditions, predominantly in extremely sheltered areas with very weak tidal currents. Such habitats are found in sealochs and some rias and harbours. Infralittoral, cohesive sandy mud, typically with over 20% silt/clay, have a rich variety of polychaetes including *Melinna palmate*, tube building amphipods (*Ampelisca* spp.) and deposit feeding bivalves such as *Limecola balthica* (*Macoma balthica*) and *Mysella bidentata*. Sea pens such as *Virgularia mirabilis* and brittlestars such as *Amphiura* spp. may be present but not in the same abundances as found in deeper circalittoral waters. Mud with minimal sand content have populations of the lugworm *Arenicola marina* may be dense, with anemones, the opisthobranch *Philine aperta* and synaptid holothurians also characteristic in some areas. The extent of the oxidised layer may be shallow with some areas being periodically or permanently anoxic. In these areas bacterial mats may develop on the sediment surface. Infaunal records for this biotope complex are limited encompassing only one biotope. They are therefore not representative of the full suit of infaunal species found in this biotope.
MB6254	Atlantic infralittoral fluid mobile mud	Fluid mobile mud suspended and deposited on each tide. In areas with very high quantities of suspended particulate material in the water column it may become deposited around slack water when tidal currents fall. This can form fluid mud layers up to several metres thick (Warwick & Uncles 1980) becoming a transient habitat in its own right. Species present within this biotope will be those washed in from other communities such as *Nephtys hombergii* or *Capitella capitata*. This biotope may

		be under-recorded due to sampling problems, and also where sediment descriptions are absent from field data. Situation: It may be found adjacent to; MB6-256, MB6-253 and to some extent MB6-252.
MC1229	Faunal communities on Atlantic circalittoral artificial hard substrate	This habitat type contains two biotopes which, although have different physical habitat characteristics, share the fact that they colonise new areas of artificial substrata relatively quickly. The *Ascidiella aspersa* fouling biotope (MC1-2282) is found on wave-sheltered artificial substrata such as discarded fishing nets/mooring lines. A separate fouling biotope (A4.721) is described for open coast wrecks. This biotope has a characteristic faunal community of *Alcyonium digitatum* and the anemone *Metridium senile*. Other species recorded in this complex (primarily under subunit A4.721) include the hydroid *Nemertesia antennina*, the anemones *Actinothoe sphyrodeta* and *Sagartia elegans*, the cup coral *Caryophyllia smithii*, the bryozoans *Flustra foliacea* and *Bugula plumosa*, the crabs *Necora puber*, *Cancer pagurus* and *Maja squinado* and the lobster *Homarus gammarus*.
MC2	Circalittoral biogenic habitat	Biogenic reefs in the circalittoral zone formed by a variety of organisms, includes polychaete reefs, bivalve reefs (e.g. mussel beds) and cold water coral reefs in the circalittoral zone. These communities develop in a range of habitats from exposed open coasts to estuaries, marine inlets and deeper offshore habitats and may be found in a variety of sediment types and salinity regimes.
MC22	Atlantic circalittoral biogenic habitat	Atlantic circalittoral biogenic reef communities. This complex includes bivalve reefs (e.g. mussel beds) and cold water coral reefs. These communities develop in a range of habitats from exposed open coasts to deeper offshore habitats and may be found in a variety of sediment types.
MC221	Worm reefs in the Atlantic circalittoral zone	Sublittoral reefs of polychaete worms in mixed sediments found in a variety of hydrographic conditions. Such habitats may range from extensive structures of considerable size to loose agglomerations of tubes. Such communities often play an important role in the structural composition or stability of the seabed and provide a wide range of niches for other species to inhabit. Consequently polychaete worm reefs often support a diverse flora and fauna.
MC6	Circalittoral mud	Circalittoral mud and sandy mud typically with over 20% silt/clay, generally in water depths of over 10 m. Sea pens such as *Virgularia mirabilis* and brittlestars such as *Amphiura* spp. are particularly characteristic of this habitat.
EUNIS code	Title	Description
MB2222	*Ostrea edulis* beds on Atlantic infralittoral muddy mixed sediment	Dense beds of the oyster *Ostrea edulis* can occur on muddy fine sand or sandy mud mixed sediments. There may be considerable quantities of dead oyster shell making up a substantial portion of the substratum. The clumps of dead shells and oysters can support large numbers of *Ascidiella aspersa* and *Ascidiella scabra*. Sponges such as *Halichondria bowerbanki* may also be present. Several conspicuously large polychaetes, such as *Chaetopterus variopedatus* and terebellids, as well as additional suspension-feeding polychaetes such as *Myxicola infundibulum* and Sabella pavonina may be important in distinguishing this biotope, whilst the Opisthobranch *Philine aperta* may also be frequent in some areas. A turf of seaweeds

		such as *Plocamium cartilagineum*, *Nitophyllum punctatum* and *Spyridia filamentosa* may also be present. This biotope description may need expansion to account for oyster beds in England.
MB4241	*Aphelochaeta* spp. and *Polydora* spp. in variable salinity Atlantic infralittoral mixed sediment	In sheltered muddy mixed sediments in estuaries or marine inlets with variable or reduced/low salinity communities characterised by Aphelochaeta marioni and Polydora ciliata may be present. Other important taxa may include the polychaetes Nephtys hombergii, Caulleriella zetlandica and Melinna palmata, tubificid oligochaetes and bivalves such as Abra nitida. Conspicuous epifauna may include members of the bivalve family Cardiidae (cockles) and the slipper limpet Crepidula fornicata. This biotope is often found in polyhaline waters.
MB4242	*Crepidula fornicata* and *Mediomastus fragilis* in variable salinity Atlantic infralittoral mixed sediment	Variable salinity mixed sediment characterised by the slipper limpet *Crepidula fornicata* and the polychaetes *Mediomastus fragilis* and *Aphelochaeta marioni*. Other numerically important taxa include the oligochaetes *Tubificoides benedii*, syllids such as *Exogone naidina* and *Sphaerosyllis*, and *Nephtys hombergii*. *Lepidonotus squamatus* and *Scoloplos armiger* may also be common. Shell debris and cobbles are colonised by the ascidians *Ascidiella aspersa*, *Ascidiella scabra*, *Molgula* sp. and *Dendrodoa grossularia* (the ascidians may not be recorded adequately by remote infaunal survey techniques). Situation: This biotope occurs in the lower estuary where currents allow a stable environment to develop. It is associated with oyster beds and relict oyster beds, (MB2-222) in southern England and Wales. It may be found adjacent to or in conjunction with MB6-252 and MB4-241. It may be associated with MB4-233 and possibly form a component of MB6-242.
MB5224	*Ruppia maritima* in reduced salinity Atlantic infralittoral muddy sand	In sheltered brackish muddy sand and mud, beds of *Ruppia maritima* and more rarely *Ruppia spiralis* may occur. These beds may be populated by fish such as *Gasterosteus aculeatus* which is less common on filamentous algal-dominated sediments. Seaweeds such as *Chaetomorpha* spp., *Enteromorpha* spp., *Cladophora* spp., and *Chorda filum* are also often present in addition to occasional fucoids. In some cases the stoneworts *Lamprothamnium papulosum* and *Chara aspera* occur. Infaunal and epifaunal species may include mysid crustacea, the polychaete *Arenicola marina*, the gastropod *Hydrobia ulvae*, the amphipod *Corophium volutator* and oligochaetes such as *Heterochaeta costata*. In some areas *Zostera marina* may also be interspersed with the *Ruppia* beds.
MB5237	*Arenicola marina* in Atlantic infralittoral fine sand or muddy sand	In shallow fine sand or non-cohesive muddy sand in fully marine conditions (or occasionally in variable salinity) a community characterised by the polychaete *Arenicola marina* may occur. This biotope appears quite faunally sparse. Those other taxa present however, include scavenging crustacea such as *Pagurus bernhardus* and *Liocarcinus depurator*, terebellid polychaetes such as *Lanice conchilega* and the burrowing anemone *Cerianthus lloydii*. Occasional *Sabella pavonina* and frequent *Ensis* spp. may also be observed in some areas. The majority of records for this biotope are derived from epifaunal surveys and consequently there is little information available for the associated infaunal species. It is possible that this biotope, like unit MB5-235 (to which it is broadly similar) is an epibiotic overlay on other sublittoral sand biotopes. Temporal variation: At certain times of the year a diatom film may be present on the sediment surface.

MB6	Infralittoral mud	Sublittoral mud and cohesive sandy mud extending from the extreme lower shore to the lower limit of vascular plants. This biotope is predominantly found in sheltered harbours, sealochs, bays, marine inlets and estuaries and stable deeper/offshore areas where the reduced influence of wave action and/or tidal streams allow fine sediments to settle. Such habitats are often by dominated by polychaetes and echinoderms, in particular brittlestars such as *Amphiura* spp. Estuarine muds tend to be characterised by infaunal polychaetes and oligochaetes.
MB62	Atlantic infralittoral mud	Shallow sublittoral muds, extending from the extreme lower shore to about 15-20 m depth in fully marine or near marine conditions, predominantly in extremely sheltered areas with very weak tidal currents. Such habitats are found in sealochs and some rias and harbours. Infralittoral, cohesive sandy mud, typically with over 20% silt/clay, have a rich variety of polychaetes including *Melinna palmate*, tube building amphipods (*Ampelisca* spp.) and deposit feeding bivalves such as *Limecola balthica* (*Macoma balthica*) and *Mysella bidentata*. Sea pens such as *Virgularia mirabilis* and brittlestars such as *Amphiura* spp. may be present but not in the same abundances as found in deeper circalittoral waters. Mud with minimal sand content have populations of the lugworm *Arenicola marina* may be dense, with anemones, the opisthobranch *Philine aperta* and synaptid holothurians also characteristic in some areas. The extent of the oxidised layer may be shallow with some areas being periodically or permanently anoxic. In these areas bacterial mats may develop on the sediment surface. Infaunal records for this biotope complex are limited encompassing only one biotope. They are therefore not representative of the full suit of infaunal species found in this biotope.
MB6254	Atlantic infralittoral fluid mobile mud	Fluid mobile mud suspended and deposited on each tide. In areas with very high quantities of suspended particulate material in the water column it may become deposited around slack water when tidal currents fall. This can form fluid mud layers up to several metres thick (Warwick & Uncles 1980) becoming a transient habitat in its own right. Species present within this biotope will be those washed in from other communities such as *Nephtys hombergii* or *Capitella capitata*. This biotope may be under-recorded due to sampling problems, and also where sediment descriptions are absent from field data. Situation: It may be found adjacent to; MB6-256, MB6-253 and to some extent MB6-252.
MC1229	Faunal communities on Atlantic circalittoral artificial hard substrate	This habitat type contains two biotopes which, although have different physical habitat characteristics, share the fact that they colonise new areas of artificial substrata relatively quickly. The *Ascidiella aspersa* fouling biotope (MC1-2282) is found on wave-sheltered artificial substrata such as discarded fishing nets/mooring lines. A separate fouling biotope (A4.721) is described for open coast wrecks. This biotope has a characteristic faunal community of *Alcyonium digitatum* and the anemone *Metridium senile*. Other species recorded in this complex (primarily under subunit A4.721) include the hydroid *Nemertesia antennina*, the anemones *Actinothoe sphyrodeta* and *Sagartia elegans*, the cup coral *Caryophyllia smithii*, the bryozoans *Flustra foliacea* and *Bugula plumosa*, the crabs *Necora puber*, *Cancer pagurus* and *Maja squinado* and the lobster *Homarus gammarus*.

MC2	Circalittoral biogenic habitat	Biogenic reefs in the circalittoral zone formed by a variety of organisms, includes polychaete reefs, bivalve reefs (e.g. mussel beds) and cold water coral reefs in the circalittoral zone. These communities develop in a range of habitats from exposed open coasts to estuaries, marine inlets and deeper offshore habitats and may be found in a variety of sediment types and salinity regimes.
MC22	Atlantic circalittoral biogenic habitat	Atlantic circalittoral biogenic reef communities. This complex includes bivalve reefs (e.g. mussel beds) and cold water coral reefs. These communities develop in a range of habitats from exposed open coasts to deeper offshore habitats and may be found in a variety of sediment types.
MC221	Worm reefs in the Atlantic circalittoral zone	Sublittoral reefs of polychaete worms in mixed sediments found in a variety of hydrographic conditions. Such habitats may range from extensive structures of considerable size to loose agglomerations of tubes. Such communities often play an important role in the structural composition or stability of the seabed and provide a wide range of niches for other species to inhabit. Consequently polychaete worm reefs often support a diverse flora and fauna.
MC6	Circalittoral mud	Circalittoral mud and sandy mud typically with over 20% silt/clay, generally in water depths of over 10 m. Sea pens such as *Virgularia mirabilis* and brittlestars such as *Amphiura* spp. are particularly characteristic of this habitat.



Figures G35 and G36. Examples of new growth in the form of a frill along the edge of the shell of four LR oysters observed in April 2019.

Glossary of Terms

Amplicon: a length/piece of DNA or RNA that has been amplified using polymerase chain reactions (PCR).

Anthropogenic: originating from human activity.

Aquaculture: farming of marine organisms.

Benthic: the ecological region at the lowest level of water (at the seabed).

Biodiversity: variability and variety of living organisms.

Biofilm: surface layer created by a colony of microorganisms.

Biosecurity: referring to measures taken to prevent the introduction and spread of harmful organisms (bacteria/viruses) and minimise transmission of disease.

Bivalve: class of molluscs with a hard calcareous shell made of two parts.

Broodstock: a group of adult individuals with a sole purpose to reproduce offspring.

Coulter counter: the Coulter counter sizes and counts particles by measuring changes in electrical state produced by the nonconductive grain particles within an electrolyte by passing the particles through this small aperture (Coulter, 1953). The volume of electrolyte that is displaced by each particle can be measured as a voltage pulse that is proportional to the volume of the particle (Coulter, 1953).

Cryptic species: organisms that are morphologically identical, but are genetically distinct.

Cultivation: active farming/production.

Ecosystem: a biological interactive community of organisms and their physical environment.

Ecto-thermo-conformer: an organism that changes its temperature depending on their immediate environment.

Enzyme: a biological catalyst, often a protein.

Epifauna: organisms living on the surface of other organisms or substrates.

Euryhaline: (of an aquatic organism) that can tolerate a wide range of salinities.

Fitness: the ability of a genotype to reproduce.

Gabion: a cage or container filled with earth, stones, or other material for structural support.

Gametes: sexual organs.

Genotype: an organism’s set of heritable genes that can be passed from parent to offspring.

Geometric: relating to geometry, which is the mathematics that studies the sizes, shapes angles and dimensions of things (measured in metric units).

Haemocytes: cells that play a role in immunity (akin to blood cells in mammals).

Haplosporidian: a protozoan of the sporozoan subclass Haplosporea (internal parasites of lower vertebrates and invertebrates).

Hereditary: (of a gene or characteristic) determined by genetics that can be passed from parents to offspring.

Hermaphrodite: an organism that has both male and female reproductive organs.

Heterozygosity: the condition of having two different alleles at one (gene) locus (different forms of a particular gene). High heterozygosity means high genetic variation.

Hull fouling: the result of marine organisms attaching to the hull of a boat over time.

Hydrodynamics: ‘The physics that deal with the motion of fluids and the forces acting on solid bodies immersed in fluids and in motion relative to them’ (Merriam-Webster, no date).

Hypoxic: characterised by reduced/inadequate levels of oxygen.

Invertebrate: organism with no backbone.

Isotonic: relating to a solution that has the same osmotic pressure as another solution (eg. across a cell membrane).

Keystone species: an organism that defines an ecosystem (essential to ecosystem functionality).

Larviparous: an organism that produces its offspring in the form of larvae.

Logarithmic: the inverse function to exponentiation. Measured in phi units.

Mollusc: a phylum of invertebrate characterised by its soft body.

Morphology: a branch of biology studying the form, size, and structure of organisms and their specific features.

Omnivore: an organism that has a diet of both animal and plant. Often scavengers.

Organotin: chemical compound based on tin.

Osmoconformer: a marine organism that can maintain an internal environment that is isotonic to their external environment.

Osmoregulation: active regulatin of the osmotic pressure of an organism’s fluids.

Oyster dredge: a metal fishing apparatus deployed from the back of a boat. A weighted metal bar is dragged along the seabed to scoop oysters into the attached net (see Appendix F, Figure F1).

Pathogen: a biological agent that causes harm (disease or illness) to its host.

Poikilothermic: an organism whos internal temperature varies considerably.

Protandrous: a hermaphrodite that has male sexual organs reach maturity before female.

Protozoan: a single-celled microscopic organism from the family *Protista*, such as a flagellate, ciliate, amoeba, or sporozoan.

Recruitment: the process whereby individuals are added to a population. In relation to oysters, recruitment refers to the post-metamorphosis (spat) stage when settlement has taken place.

Rugosity: a measurement of surface roughness.

Salinity: saltiness, or amount of salt dissolved in a water body.

Sedimentation: the process of settling of being deposited as sediment.

Sessile: (of an organism) being fixed in one place (eg. an oyster or mussel).

Settlement: (in relation to oysters) the process of fixation to substrate in preparation for metamorphosis.

Siltation: the process by which water becomes dirty as a result of fine particles in suspension.

Somatic: in relation to cells of the body rather than reproductive cells.

Spatfall: the settling and attachment of young bivalves (such as oysters) to the substrate.

Spermatozoa: the male sex cells that fertilise the female egg.

Synergistic: the interaction or cooperation of two or more individuals, substances or agents to produce a combined effect greater than the sum of their separate parts.

Trajectory: the path followed by a propagule or object under the motion of forces.

Trophic: related to feeding and nutrition.

Turbidity: a measure of the presence of suspended solids/particles in water. Water loses its transparency as turbidity increases.

Turbulence: fluid motion characterised by chaotic changes in pressure and flow. Turbulence is in contrast to laminar flow, which occurs when fluid moves in parallel layers.

Veliger: planktonic larva.

Virulence: the severity or harmfullnes of a pathogen or disease.

Generalised linear model (GLM): For use when variance of data is not constant (heteroscedastic) and residuals are not normally distributed. If the distribution is one from the exponential family (poisson, negative binomial, or gamma), then there exists some functions of mean of Y which has linear relationship with model parameters (called a link function). GLMs impose a link function that allows us to express the transformed conditional mean of the dependent variable (y) as a linear combination of the regression variables (x) (e.g. a binomial residual can use a logit, and a poisson uses a log link function). A GLM is composed of three parts: a random component (specifying the conditional distribution of the response variable), a linear predictor (a linear function of regressors), and a link function (that transforms the expectation of the response variable to the linear predictor).

Linear model (LM): an equation that explains a relationship between two quantities that has a constant rate of change. Linear models assume that variance remains constant (homoscedastic). Requires normal distribution of data.

Partial least squares discrimination analysis (PLS-DA): a technique to maximise separation between groups (such as oyster origin) in the data by forming a linear regression. Similar to principal components analysis (PCA) (see below) in that it reduces dimensionality, but with full awareness of the class labels.

Principal components analysis (PCA): a technique to reduce the dimensionality of a data without minimising information loss. A PCA creates new uncorrelated variables (principal components) that maximise variance.

Tukey's post hoc test: compares all possible pairs of means after a multivariate test to learn if the specific groups are significantly different or similar with one another.

Permutation analysis: a randomisation test to compute the sampling distribution for any test statistic.

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