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Basic and applied biology of the photosynthetic flatworm *Symsagittifera roscoffensis*

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Submitted to Swansea university in fulfilment of the requirements for the Degree of "Doctor of Philosophy".

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Summary

Photosymbiosis exists in both terrestrial and aquatic environments. Photosymbionts within a marine setting, utilising a photosynthetic partner, such as algae, provide photoassimilates to the host via photosynthesis. An example of this is photosymbiosis between *Symsagittifera roscoffensi*s and the alga *Tetraselmis convolutae*. The host (*S. roscoffensis*) is entirely dependent on the alga for its nutrition to the extent that the host becomes photoautotrophic. *Symsagittifera roscoffensis* inhabits the dynamic intertidal zone along the Atlantic coast, adapting to conditions and light availability while minimizing dispersal. As this organism is found along the Atlantic coast and has limited swimming ability, thus giving rise to an unclear intra-species genetic diversity.

To enhance understanding of how environmental conditions affect the worms, in Chapter 2, I examined the impact of changing abiotic factors on the symbiont's photosynthesis. Salinity (20, 30, 40) and nutrient (f/8, f/4, f/2), showed no significant effect, while increases in temperature, light intensity and photoperiod resulted in significantly lower photosynthesis. These data are crucial for understanding how the symbiont provides photoassimilates in an ever-changing environment and in vitro culture optimization.

Chapter 3; I explored *S. roscoffensis'* behaviour responses to stimuli. Aposymbiotic juveniles displayed positive chemotaxis towards algae. Adults were able to balance light exposure and dispersal risk by repeated up and down movements. Worms avoided excessive light intensity by burrowing into the substrate. Mechanical vibrations triggered a downward movement above a threshold, below the threshold worms did not respond, allowing them to remain at the surface to continue photosynthesising These behaviours enable the *S. roscoffensis* to optimise photosynthesis while maintains its position within the intertidal zone.

In Chapter 4; I analysed field conditions at East Aberthaw, Wales for 13 months and assessed genetic diversity among populations of *S. roscoffensis* and its symbiont *T. convolutae* from Wales, France, Guernsey, Spain and Portugal. Environmental changes, primarily temperature, influenced the size of the population at the local site. Limited gene flow between populations along the Atlantic coast was encountered; suggesting little migration, allowing populations to become locally adapted.

Chapter 5; I used qPCR to quantify algal cells in juvenile and adult worms. Juveniles showed little difference in the algae cell's numbers. While adult worms contained more algal cells than previously reported in the literature.

Chapter 6; I investigated *S. roscoffensis* as an ornamental fish feed. Nutritional analysis showed essential polyunsaturated fatty acids, and feeding trials indicated palatability for both freshwater and marine species. Aposymbiotic juveniles formed symbiosis with other *Tetraselmis* species, suggesting a high level of plasticity with regards to the algal symbiont.

This thesis represents a substantial knowledge gain regarding on*S. roscoffensis* and photosymbiosis, presenting insights into host-symbiont relationships and promoting its use as a model for further study in this field.

Declarations

Declarations

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

Signed. [redacted] .....

Date 15.9.2023 .....

This thesis is the result of my own investigations, except where otherwise stated. Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

Signed. [redacted] .....

Date 15.9.2023 .....

I hereby give consent for my thesis, if accepted, to be available for electronic sharing

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The University's ethical procedures have been followed and, where appropriate, that ethical approval has been granted.

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Date 15.9.2023 .....

Declaration:

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Manuscript 1: Environmental constraints on the photosynthetic rate of the marine flatworm *Symsagittifera roscoffensis*
Manuscript 2: To move or not to move: Taxis responses of the marine flatworm *Symsagittifera roscoffensis* to different stimuli
Manuscript 3: *In situ environmental drivers and molecular identification of the photosymbiotic marine flatworm Symsagittifera roscoffensis*
Manuscript 4: Prospecting the photosynthetic flatworm *Symsagittifera roscoffensis* as a novel fish-feed

Manuscript 1
Author 1: Nathan Thomas, Swansea University. Lead author, conceptualization, carried out experiments, methodology and data handling, writing original draft.
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Author 3: Professor Kam Tang, Swansea University. Primary supervisor, conceptualization, supervision, resources, original draft, writing and editing, project administration.

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Ethical statements

Ethical approval was given for the experiments not involving vertebrate SU-Ethics-Student-011019/1904. Experiments involving vertebrates were given approval by AWERB: SU-Ethics-Student-190321/3739.

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Abbreviations

ALA	Alpha linolenic acid
BLR	Binomial linear regression
Cox	Cytochrome oxidase
DNA	Deoxyribose nucleic acid
DHA	Docosahexaenoic acid
dNTP	Deoxynucleotide triphosphate
EPA	Eicosapentaenoic acid
mL	Milliliter
ng	Nanogram
nmol	Nanomole
PCR	Polymerase chain reaction
PUFA's	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
se	Standard error
snp	Singal nucleotide polymorphism
ug	Microgram
uL	Microlitres
µm	Micrometres
°C	Celsius

Glossary

Acoel	A member of the Acoela group of marine worms, which lack a true digestive cavity with definite walls.
Aposymbiotic	Two organisms that form symbiosis living apart. In this thesis it also refers to a host that is lacking its symbiont.
Bootstrap	A statistical method that resamples a single dataset to create many simulated samples.
Kleptoplasty	The removal of a chloroplast from a cell and using it for photosynthesis in a the host.
Nucleotides	The basic building blocks of DNA and RNA
Photoassimilates	A compound formed by light dependant reactions.
Photoautotrophic	The ability to use sunlight as a food source.
Photosymbiosis	Symbiosis between two organisms one of which is capable of photosynthesis.
Phylogeny	The depiction of the evolutionary decent of different spices.
Symbiosis	A close and prolonged interaction between two organisms who both benefit from the interaction (mutualism).
Thermocycling	The use of a thermocycler to change the temperature in order to amplify DNA.

Chapter 1：General introduction

1.1 General ecology and biology of photosymbiosis

Symbiosis has played a fundamental role in the evolution of life on Earth and is common across all domains of life (Margulis, 1970). Symbiosis was defined by Heinrich de Barry in 1887 as 'two separate organisms living together', and this relationship is often intimate and sophisticated (de Bary, 1878). The original definition of symbiosis broadly includes three types of interactions, mutualistic where both partners benefit from the relationship, parasitic where one partner is exploited, and commensal where one partner benefits with no effects on the other.

The formation of a new symbiotic relationship is considered one of the main driving forces in the evolutionary formation of new taxa (Bermudes and Margulis, 1987; Margulis and Fester, 1991), allowing these partners to occupy new habitats that neither partner would be able to do alone (Norris, 1996). In the many examples of symbiosis, the relationship develops gradually over time, with one member feeding or parasitizing upon the other and eventually developing strategies to become fully integrated (Bermudes and Back, 1991). Symbiotic relationships are powerful tool that can be utilized by a multitude of organisms; it allows the host to obtain resources that it would otherwise not have access to.

A particular type of symbiotic relationship is photosymbiosis. Photosymbiosis is the formation of a relationship between a photosynthetic symbiont and a heterotrophic host. Photosymbiosis is widespread within the marine habitats, and oligotrophy is considered to be the primary factor driving its formation (Table 1.1) (Taylor *et al.*, 1982; Decelle *et al.*, 2015).

Table 1.1. Examples of symbiosis with marine animals and algae (adapted from Venn *et al*., 2008) .

Host	Symbionts	References
Porifera	*Cyanobacteria* primarily in marine sponges, *Symbiodinium* in marine sponges	(Lee *et al.*, 2001)
Cnidaria	*Symbiodinium* in benthic organisms e.g., marine corals and sea anemones, *Scripsiella* spp. in pelagic taxa, e.g., *Velella*	(Banaszak, *et al.*, 1993; Stat, *et al.*, 2006)
Platyhelminthes	Multiple species of marine turbellarians	(Parke and Manton, 1967; Apelt, 1969)
Xenacoelomorpha	Acoels e.g., *Tetraselmis* and *Symsagittifera roscoffensis*	(Douglas, 1983a)
Mollusca	Dinoflagellates, most commonly *Symbiodinium* found in marine gastropods and bivalves.	(Belda-Baillie *et al.*, 2002)
Ascidia	Cyanobacteria, commonly found in *Prachloron* and other ascidians	(Lewin and Cheng, 1989)

Photosymbiosis allows the host organism to obtain nutrition directly from sun light. An example of this is the symbiotic relationship that occurs in coral reefs. The dinoflagellate *Symbiodinium* sp. (also known as zooxanthellae) is one of the most well-known photosymbionts that can from a relationship with a multitude of marine organisms, the most common of which is corals (Farmer *et al.*, 2001). The symbiont via photosynthesis then can provide 95% of the hosts' nutritional requirements, and the energy flow between the host and the symbiont is often complex and requires a high level of cooperation between both partners (Figure 1.1) (Muscatine, 1990). The benefits of the photosymbiotic relationship are that the

coral receives photosynthates from its symbiont, and in return the coral provides the symbiont with shelter and nitrogen (Stanley and Lipps, 2011).

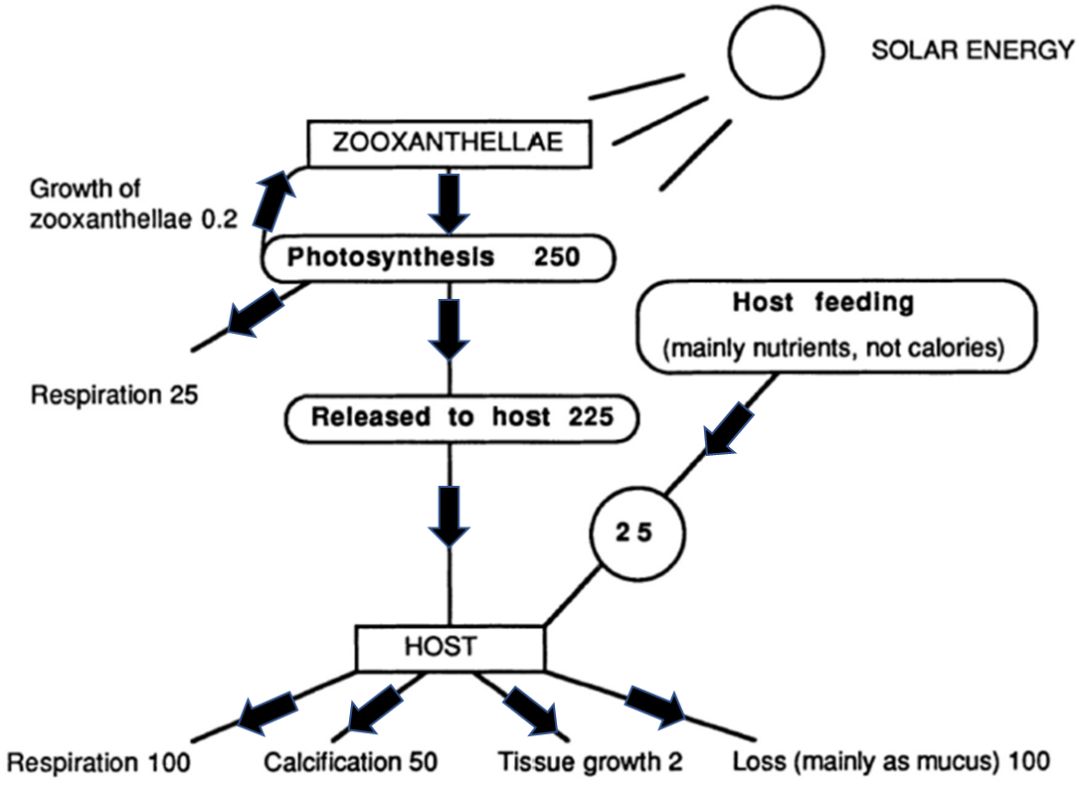

Figure 1.1. An energy budget for the photosymbiont zooxanthella in symbiosis with a marine coral. *Pocillopora*. 250 units of solar energy is harnessed by zooxanthellae via photosynthesis at the top; 25 units are lost to respiration; the remaining 225 units are released to the host. The host also acquires an additional 25 units from heterotrophic feeding. The host then uses the energy for respiration, calcification, tissue growth and mucus secretion. (From Cowen, 1988).

The word kleptoplasty translates to stolen plasmids. While kleptoplasty is not strictly a form of photosymbiosis, as the alga cell is destroyed and all that remains is the non-reproducing chloroplast organelles, there are some similarities between kleptoplasty and photosymbiosis, because the host can harness the sun's energy without the need to develop photosynthetic apparatus itself (Rumpho *et al.*, 2011). Functional kleptoplasty is known to occur in different organisms, mainly protists such as ciliates and foraminifera, in metazoans, kleptoplasty is restricted to the clade of *Sacoglossa* (Pillet and Pawlowski, 2011; Pillet and Pawlowski, 2013; Not *et al.*, 2016). Sacoglossans are a type of herbivorous sea slug; their diet is comprised of different algae in particularly green algae of the genera *Caulerpa* and *Codium.* They are also known to feed on red algal species such as *Griffithsia* spp. (Ansell *et al.*, 2002).

Once the host has acquired its kleptoplasts, the latter are provided with inorganic nutrients, CO_2 and protection (Pearse and Muscatine, 1971; Stat, Carter and Hoegh-Guldberg, 2006; Yellowlees, Rees and Leggat, 2008). The stolen kleptoplasts can remain photosynthetically active for a few weeks. There are, however, examples of the plasmids remain photosynthetically active in sea slugs for up to 9 months; this is remarkable considering that there is no input of genetic information from the alga nucleus to maintain the plasmids (Rumpho *et al.*, 2000; Ansell *et al.*, 2002). Previous studies indicated that when the sea slug *Elysia viridis* was deprived of food (algae) and light, individuals lost weight twice as quickly as individuals that were just deprived of food but still given access to light, indicating that individuals with algal organelles were able to photosynthesise. However, the contribution of kleptoplasty to the nutritional needs of sea slugs remains uncertain, with reports of as little as 1% (Rauch *et al.*, 2017), to as much as 60% (Raven *et al.*, 2001).

1.2 Phylogeny of Aceols

The phylogeny of acoels over the last 100 years has been fraught with uncertainties, and competing hypotheses have emerged on the phylogeny of acoels and its wider taxonomic grouping (von Graff, 1891; Hyman, 1951; Ehlers, 1986; Carranza, Baguna and Riutort, 1997; Ruiz-Trillo *et al.*, 1999; Ax, 2000). The first documented acoel was described in 1806, *Convoluta convoluta*, initially classified as a planarian (Müller and Abildgaard 1789). Often, early classifications were based on phenotypic similarities and due to the ciliated, gutless nature of *C. convoluta*, it was assigned to the taxon Turbellaria, within the phylum Platyhelminthes (Ehlers 1986). More recent nucleotide data place acoels with nemertodermatids to form the taxon Acoelmorphora; the addition of xenoturbellids forms the phylum Xenacoelomorpha, placed in the wider clade of bilaterians (Figure 1.2). Typically characterised by the bilateral symmetry during embryogenesis, Xenacoelomorpha is a sister group to Nephrozoa of Deuterostomia (Cannon *et al.*, 2016). Its placement at the base or the root of Bilateria is due to the shared morphology and similar characteristics with Cnidarians; for instance, they both share a single opening that can, in some cases, lead to a digestive tract (Hejnol and Martín-Durán, 2015). Other shared characteristics associated with bilaterians similar structures to the mesoderm and longitudinal musculature (Hejnol and Martindale, 2008; Haszprunar, 2016). The shared characteristics of Xenacoelomorpha enable it to be a model candidate to study the evolution of the bilaterians through body plan features, the acquisition of different cell types

and associated organs (Hejnol *et al.*, 2009), and ultimately the evolution of bilaterian traits (Hejnol and Pang, 2016).

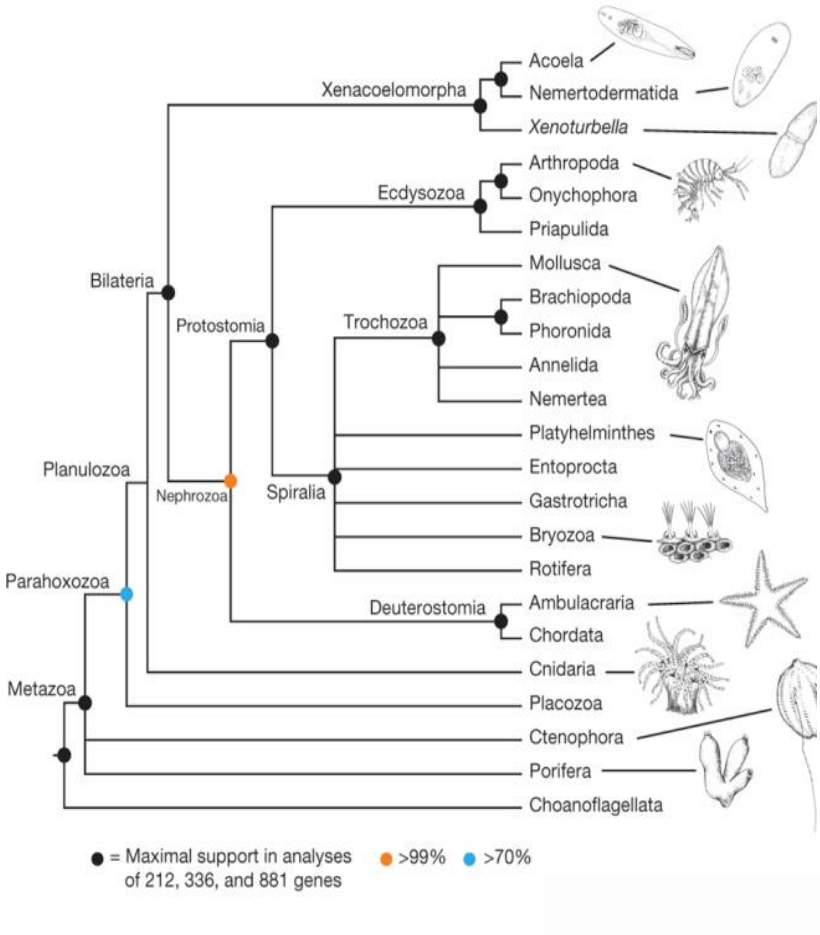

Figure 1.2. The phylogenetic position of Xenacoelomorpha within Bilateria (From Cannon *et al.*, 2016).

1.3 The biology and natural history of the Acoel flatworms

Acoelomorpha within the phylum Xenacoelomorpha contain roughly 400 members. Typically acoels are on average one millimetre in size, however, some acoels can be much larger; individuals are bilaterally symmetric, most of which are found in benthic marine habitats, although some can be found in fresh water (Faubel, 1978; Tyler *et al.*, 2005). Acoels do not possess a true gut. The space in which the gut would normally reside contains parenchyma cells and is typically a solid syncytium. The majority of acoels are free living, but some form photosymbiotic relationships with algae, some individuals are also parasites and found in the digestive system of echinoderms (Jennings, 1971). The diet of acoels can range from unicellular algae, bacteria, crustaceans, small bivalves, and even other acoels (Achatz *et al.*, 2013).

The body plan of acoels is dependent on their habitats. Individuals living within a sandy habitat tend to have a long and slender body shape, while those living within a muddy habitat tend to be compact and droplet-shape. The different body shapes reduce friction and allow them to move more freely through the respective substrates. Most acoels are translucent or milky, although some are coloured by their algal symbionts, pigments or the presence of rhabdoids, which are granular secretions (Smith *et al.*, 1982; Achatz *et al.*, 2013). Rhabdoid glands are often pigmented and are considered to secrete mucus to reduce friction during reproduction or to allow the worm to move more freely through its environment (Pedersen, 1965).

Acoels are ‘surprisingly muscular’ and move freely with the use of a multiciliate epidermis (Tyler and Rieger, 1999), namely ciliary gliding. The cilium each consists of a configuration of nine fringe microtubules in pairs of two, surrounded by two central microtubules (9x2+2). The shape of the cilia among acoels is distinctive, having a marked shelf at the tip. This shelf, when viewed using electron microscopy, can be seen as a distinctive narrowing at the tip of the cilia from 0.27 µm to 0.15 µm in length (Tyler, 1979; Rieger *et al.*, 1991).

The statocyst is another notable feature of acoels and it contains the lithocyte, within which is one statolith. The primary function of a statocyst is to detect gravity, allowing acoels to orient themselves (Lanfranchi, 1990). In some species, at the anterior of the body there are

paired eye spots containing pigmented granules, which allow for the detection of light (Yamasu, 1991) (Figure 1.3).

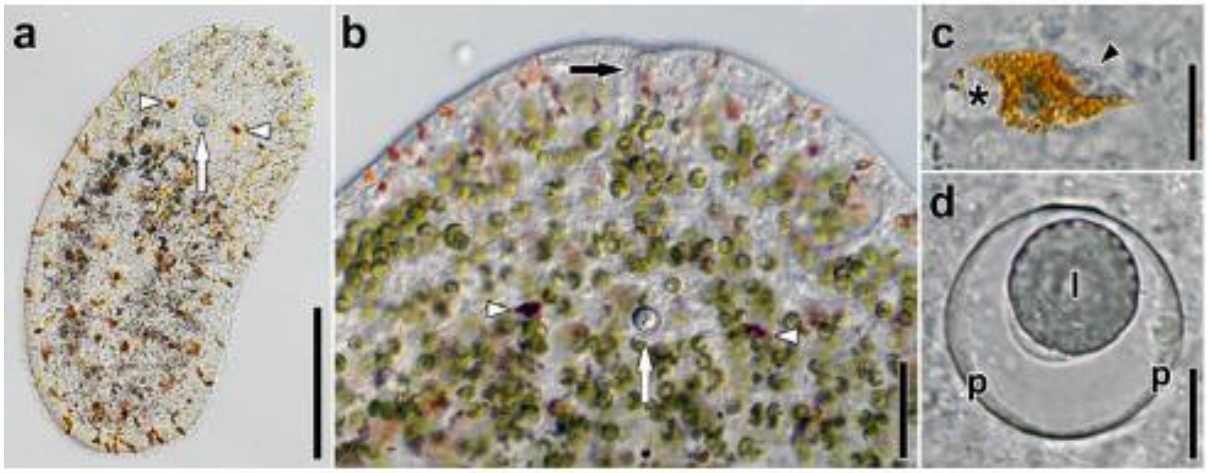

Figure 1.3. The sensory organs found in *Symsagittifera roscoffensis*. A) A newly hatched juvenile *S. roscoffensis*. Short arrows point to the eye spots; long arrow indicates the position of the statocyst. Due to the lack of symbiont in the juvenile the rhabdoids are clearly present (orange pigments). Scale bar = 100 µm. B) The anterior end of an adult *S. roscoffensis* with photosymbiont. Short arrows indicate the positions of the eye spots; long white arrow indicates the position of the statocyst; black arrow indicates the position of the frontal organ. Scale bar = 50 µm. C) An eye of an adult *S. roscoffensis*. Asterisk marks the position of the nucleus of the eye, while the arrow points to a pigment contained inside vacuole, the pigment contains reflective inclusions known as concernments. Scale bar = 10 µm. D) The statocyst of an adult *S. roscoffensis*: l = lithocyst, p = parent cell. Scale bar = 10 µm. (From Achatz *et al*., 2013).

The brain of an acoel is a circular ring shape, formed by a dense mass of neuronal cells surrounding a central neuropil. It then forms a complex mass of connectives and commissures (Bery *et al.*, 2010; Semmler *et al.*, 2010). The neuropil contains several types of neurites and these are classified based on the type of neurotransmitter they use. Nerve cords run from the neuropil through the length of the body at different dorso-ventral positions (Bery *et al.*, 2010). The slender body plan and well-developed neural system, allow the worms to move freely and respond quickly to stimuli. These features have resulted in a large family that have different body plans occupying a range of aquatic habitats.

1.4 Photosymbiosis within Aceolmophora

Within the phylum Xenacoelomorpha, symbiosis has been described in multiple species of the taxon Convolutidae, such as *Amphiscolops langerhansi* and the alga *Amphidinium klebsii* (Trench and Winsor, 1987). *A. langerhansi* is also able to utilise other members of *Amphidinium* for symbiosis if its preferred organism is not available (Trench and Winsor, 1987). The literature also reports that *Amphiscolops* sp. is able to form photosymbiosis with two algal species simultaneously: *Amphidinium* sp. and *Symbiodinium* sp. (Trench and Winsor, 1987). Coexistence of symbionts frequently occurs in Convolutidae, suggesting that species within the Convolutidae can utilise multiply symbionts, in some cases two symbionts at once. Such as the case of *Waminoa litus* and *Waminoa brickneri*, which can form symbiosis with *Amphidinium* sp. and *Symbiodinium* sp. (Ax and Apelt, 1965; Winsor, 1990; Barneah *et al.*, 2007). *Convoluta convoluta* forms a symbiotic relationship with the diatoms *Licmophora* (Apelt, 1969), while *Convolutriloba longifissura* forms symbiosis with the alga *Tetraselmis* sp. (Adam and Balzer, 2002). Another example is *Symsagittifera roscoffensis* forming symbiosis with the alga *Tetraselmis convolutae*. The range of photosymbiotic relationships within the different genus of the family Convolutidae suggest that there are multiple, convergent evolutionary events that have led to the formation of symbiosis across multiple genera (Douglas, 1983a) (Table 1.2).

Table 1.2. Photosymbionts found in members of Convolutidae. (Cases where there are two photosymbionts listed, indicates the coexistence of photosymbionts.)

Acoel	Photosymbiont	Reference
Amphiscolops langerhansi	*Amphidinium klebsii* or other member of this genus	(Trench and Winsor, 1987)
***Amphiscolops* spp.**	*Amphidinium* sp., *Symbiodinium* sp.	(Trench and Winsor, 1987)
Amphiscolops marinelliensis	*Symbiodinium* sp.	(Beltagi and Khafaji, 1984)
Waminoa litus	*Amphidinium* sp. *Symbiodinium* sp.	(Ax and Aplt 1965; Winsor 1900; Barneah et al., 2007)
Waminoa brickneri	*Amphidinium sp. Symbiodinium* sp.	(Ax and Aplt 1965; Winsor 1900; Barneah et al., 2007)
Convoluta convoluta	*Licmophora communis*	(Beltagi and Khafaji, 1984)
Convolutriloba longifissura	*Tetraselmis* sp.	(Adam and Balzer, 2002)
Symsagittifera roscoffensis	*Tetraselmis convolutae*	(Douglas, 1983a)

The symbiotic relationship within the Convolutidae family appears to be species specific, with host species having a preferred symbiont species, or two in some cases. After establishing symbiosis, the algal partner fulfils some or all nutrition needs, reducing or eliminating the reliance on heterotrophic feeding. This relationship creates a unique scenario where organisms have plant-like (autotrophic) feeding yet retain animal-like mobility. However, while the nutritional needs may be satisfied, photosymbiosis presents new challenges to the individuals. For instance, acoels that are dependent on photosymbiosis must now meet the requirements of the symbiont by providing sunlight for photosynthesis. Living within the intertidal zone in shallow pools of water, photosymbiotic acoels are subjected to frequent changes in ambient temperature, salinity, light and nutrient availability. Organisms living within these environments must develop strategies to cope with the changing environmental conditions, while still keeping their symbionts photosynthetically active. This suggests that these photosymbiotic individuals require environmental plasticity, to tolerate a range of

conditions within its habitat. Acoels and in particular photosymbiotic acoels have the ability to switch algal species required for photosymbiosis (Arboleda *et al.*, 2018), This ability may be a remnant of a previous heterotrophic lifestyle, or an adaptation to allow photosymbiosis to remain active under changing conditions (Boulotte *et al.*, 2016), i.e., a coping mechanism for when there is a lack of the preferred symbiont. The ability to utilise an entirely different species of algae for photosymbiosis is a feature of Convolutidae and is rarely documented among other photosymbiotic species. In other photosymbiotic relationships, a strict species and/or subspecies association usually occurs with the hosts (Muller-Parker *et al.*, 2015).

1.4 The biology, ecology, and biogeography of *Symsagittifera roscoffensis*

Symsagittifera roscoffensis – previously known as *Convoluta roscoffensis* (von Graff, 1891) is commonly referred to as the mint sauce worm or the Roscoff worm. *Symsagittifera roscoffensis* is an acoel in the family Convolutidae and forms a symbiotic relationship with the alga *Tetraselmis convolutae* (von Graff, 1891**)** (Douglas, 1983a; Jondelius *et al.*, 2011; ITIS, 2019). Adult *S. roscoffensis* are larger in size than the average acoel and are typically between 2-4 mm long and roughly 550 μm wide (Bailly *et al*., 2014). The shape of its body plan is described as longitudinal with a curve in the middle that lacks a defined coelom. Two main areas of the body are easily distinguishable: the anterior or the head, and the posterior end. Its anterior is defined by the presence of a central neuropil domain comprised of roughly 700 neurons, surrounded by a statocyst. The statocyst is opposed by two pigmented eyespots. Within the anterior end there is a mouth-like opening, which is used to take in algae to start a photosymbiotic relationship, allowing *S. roscoffensis* to become completely photoautotrophic (Mamkaev and Kostenko, 1991; Bailly *et al*., 2014; Arboleda *et al*., 2018). As with all acoels, *S. roscoffensis* does not possess a true hind gut that has a defined lumen. Instead, its mouth connects to a cavity filled with parenchyma cells (Achatz *et al.*, 2013).

First discovered in Roscoff in 1879 (Geddes, 1879), *S. roscoffensis* was described in 1910 as a "Plant-Animal" thanks to its ability to photosynthesise (Keebles, 1910). *S. roscoffensis* has been used as an alternative model for the study of the mammalian glial cell (Freeman and Doherty, 2006; Sprecher *et al.*, 2015). Individuals are capable of regeneration and tissue renewal (Bely and Sikes, 2010) due to the presence of totipotent stem cells referred to as neoblasts (Bailly *et al.*, 2014; Sprecher *et al.*, 2015). Neoblasts are capable of

differentiation into all cell types needed for regeneration, growth and development (Gschwentner *et al.*, 2001; Gehrke and Srivastava, 2016). *S. roscoffensis* has the ability to regenerate both the posterior and anterior portions of its body. Brain regeneration occurs within 20-25 days post amputation (Bailly *et al*., 2014)

S. roscoffensis has been documented in locations around the coast of Europe, on beaches within the intertidal zone, including the northwest coast of Roscoff, France (Keebles, 1910; Doonan and Gooday, 1982; Selosse, 2000), the southern tip of Portugal (Carvalho *et al*., 2013) and the Channel Isles (Keebles, 1910; Doonan and Gooday, 1982). *S. roscoffensis* can be found along the coast in East Aberthaw, South Wales (Mettam, 1979; Mcfarlane, 1982) (Figure 1.4), where it is exposed to generally cooler temperatures and more rainfall than its counterparts in continental Europe. In fact, when first reported by Mettam (1979) in Wales, the author stated that the worm was present during the coldest parts of the winter, when the beach was made inaccessible due to the presence of snow. *Symsagittifera roscoffensis* seemed to be unaffected by the cold weather, as it was still found immediately after the thaw (Mettam, 1979).

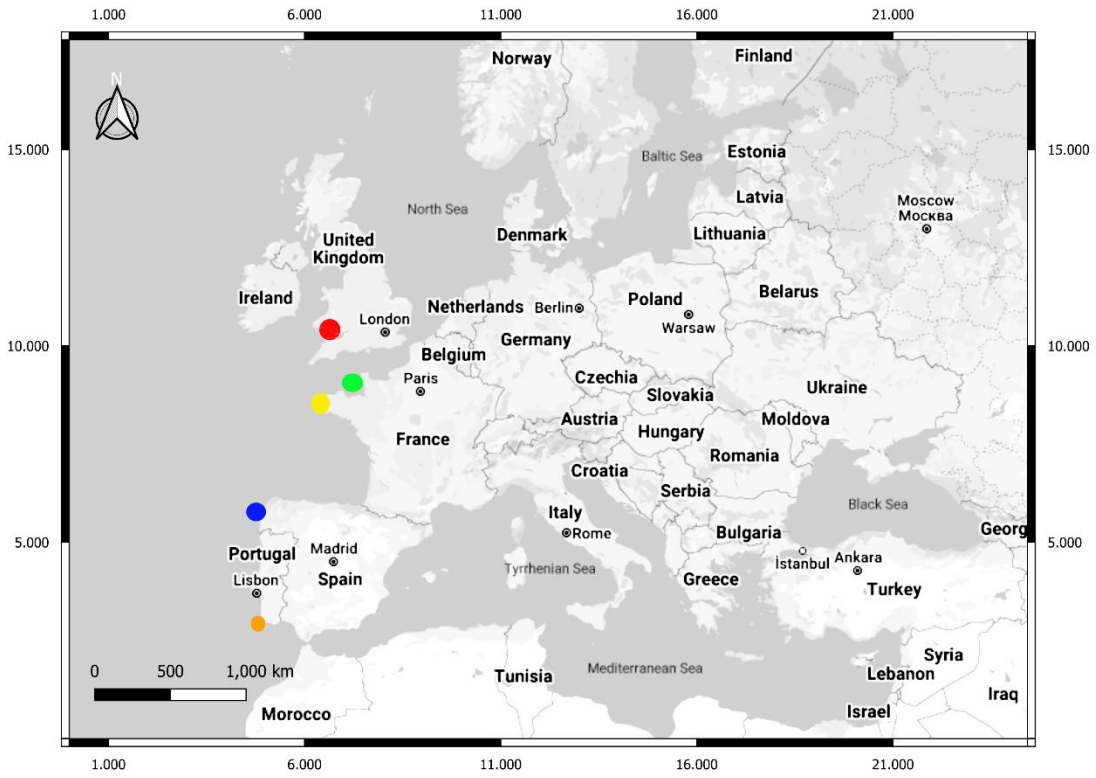

Figure 1.4. Distribution of the reported populations of *Symsagittifera roscoffensis.* Locations of confirmed populations are represented by the colours. (Red) Wales, (Green) Channel Isle, (Yellow) France, (Blue) Spain, (Orange) Portugal.

The breeding period of *S. roscoffensis* is reported to be from September to June (Douglas, 1983), based on individuals taken from the Channel Isles, and it may differ for other locations due to temperature differences. When the environmental conditions are favourable, the population size of *S. roscoffensis* can reach thousands of individuals appearing as large green mats along the coasts (Geddes, 1879; Bailly *et al.*, 2014) (Figure 1.5). Individuals are most noticeable during low tides. *S. roscoffensis* is hermaphroditic, each sexually mature adult possesses both the male and female reproductive organs. However, reproduction is through sexual means and a mate is required to produce viable embryos (Bailly *et al.*, 2014). During the breeding season, a sexually mature adult lays around 20-30 eggs in a translucent cocoon encased in a sticky mucilage (Douglas, 1983). As with most acoel species, embryos emerge through the side of the body wall. In order for this to happen, *S. roscoffensis* must first rotate end over end, while at the same time releasing an encapsulating mucilage over the embryos

and itself. The worm subsequently frees itself from the mucus cocoon structure leaving the embryos behind (Costello and Costello, 1939). *S. roscoffensis* is known to be an obligate phototroph with its algal symbiont providing all of its nutritional needs (Provasoli 1968; Bailly *et al.*, 2014; Arboleda *et al*., 2018). However, when the juveniles emerge from the cocoon they are aposymbiotic; that is, they completely lack a photosymbiont. The photosymbiont must then be acquired from the environment (horizontal transfer), in the absence of a symbiont the juveniles will die in 20 days (Douglas, 1983). Early experiments indicted that removing the mucilage layer surrounding the embryos keeps the juveniles aposymbiotic (Provasoli *et al.*, 1968; Douglas, 1985). This may indicate that the mucilage from the parental body and encapsulating the cocoons may contain algal cells, potentially providing a source of algae, In such an instance when the algae is inherited from the parents, the symbiont species would remain the same across multiple generations.

Figure 1.5. *Symsagittifera roscoffensis* at different field locations. Green colonies of *S. roscoffensis* are highlighted by the red arrows. A) *S. roscoffensis* in East Aberthaw, South Wales. B) *S. roscoffensis* on a beach in Roscoff, France. Picture credit: (Bailly, 2016).

Tetraselmis convolutae undergoes phenotypical changes once incorporated by *S. roscoffensis* such as the loss of the theca, its quad-flagella and the eye spot (Provasoli *et al.*, 1968). There are estimated 10,000-100,000 algal cells held within the upper epidermis in each adult worm (Douglas 1962; Bailly *et al*., 2014). Douglas (1983c) proposed that *T. convolutae* utilizes metabolic waste produced by *S. roscoffensis* as a nitrogen source. In return, *S.*

roscoffensis receives photosynthates such as mannitol, glucose, fructose, and lactate; *S. roscoffensis* then uses them to synthesise a range of compounds. Taylor, (1974) showed that when given lactate ex-vitro, *S. roscoffensis* used it to synthesise amino acids such as lysine, alanine, and phenylalanine-tyrosine. However, *S. roscoffensis* is unable to synthesise essential fatty acids and instead depends upon the algal symbiont to provide them (Meyer *et al*., 1979). The reliance on the symbiont for essential fatty acids could lead to host preferences for algal species that are known to produce large amounts of polyunsaturated fatty acids (PUFA). Provasoli *et al.* (1968) was the first to demonstrate that *S. roscoffensis* was able to incorporate algal species other than *T. convolutae*. However, once *T. convolutae* became available, *S. roscoffensis* would replace the already incorporated alga with its preferred *T. convolutae.* The ability of *S. roscoffensis* to switch its algal symbiont was further demonstrated by Arboleda *et al.* (2018) in the laboratory, showing that *S. roscoffensis* formed a symbiotic relationship with other algal species when its preferred *T. convolutae* was not available (Figure 1.6). Mcfarlane (1982) and Douglas (1985) both suggested that the frequency of symbiosis at the Welsh site varied between the eastern and western fringes of the population. Macfarlane, (1982) reported that at the eastern side of the beach some individuals had an alternative member of *Tetraselmis,* and *T. convolutae* accounted for only 20% of the symbionts. However, towards the western side *T. convolutae* accounted for 45-60% of the symbionts. Photosymbiont of the subgenus *Prasinocladia* was reported to occur in more than 70% of *S. roscoffensis* on the eastern side of the Welsh site (Mcfarlane, 1982). Taken together the earlier laboratory and field observations, it appears that the photosymbiotic relationship in *S. roscoffensis* is much more fluid than previously thought and that utilisation of alternative algal partner can occur.

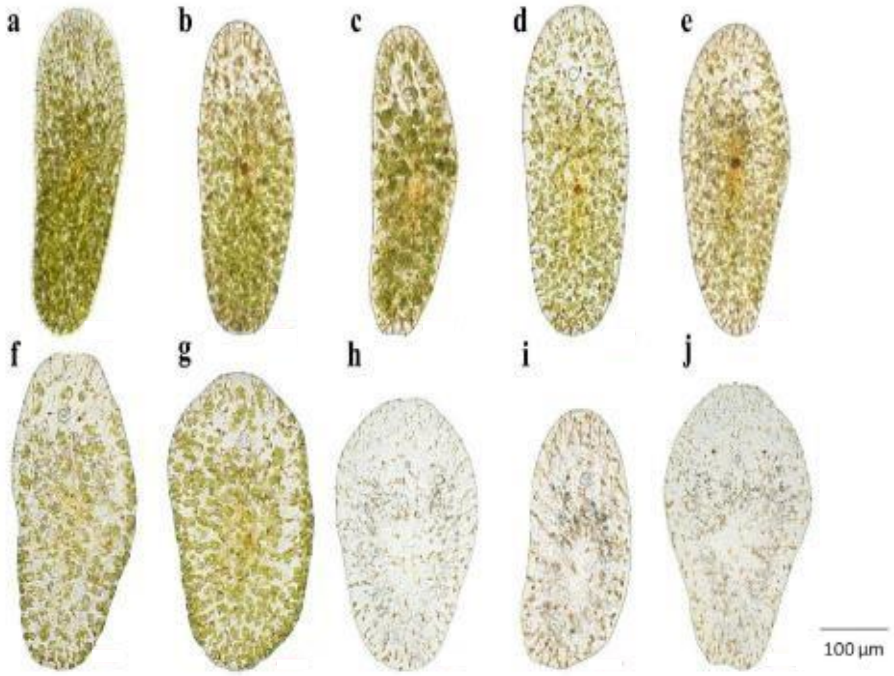

Figure 1.6. *Symsagittifera roscoffensis* forming symbiosis with alternative symbionts. (A*) Tetraselmis convolutae*, (B) *T. chuii* (C) *T. marina*, (D) *T. rubens*, (E) *T. striata*, (F) *T. subcordiformis*, (G) *T. suecica*, (H) *Dunaliella salina*, (I) *Nanochloropsis* sp., (J) aposymbiotic juveniles without any algae. (From Arboleda *et al*., 2018)

1.5 Objectives of this thesis

The overall objectives of this thesis were to understand how *Symsagittifera roscoffensis* responds to basic changes in abiotic factors; both in culture and in the wild. As well as its behavioural reposes and the genetic relatedness of populations and its potential applications in the biotechnology and aquaculture sectors.

Chapter 2: This chapter aimed to investigate the environmental constraints of *S. roscoffensis* and how changing abiotic factors, such as salinity, temperature, light intensity, photoperiod and nutrient concentrations, affect photosynthesis of the symbiont via oxygen production. A secondary objective was to determine the optimal conditions for establishing a long-term culture. To achieve this, I conducted a laboratory experiment in which I systematically varied one abiotic factor at a time and recorded the oxygen production rates of the worms at set time points.

(*Manuscript published*)

Chapter 3: This chapter aimed to investigate how *S. roscoffensis* responds to stimuli, including light stimulation (phototaxis), physical disturbance, and the presence of an algal source (chemotaxis). I conducted a series of laboratory experiments to study the behavioural responses of *S. roscoffensis* to these stimuli. In the phototaxis experiment, the aim was to understand how the worms balance their need for light against the risk of dispersal within the water column. In a series of lab experiments, I manipulated a light source within a water column and recorded the worm's response. A secondary aim of the phototaxis experiments was to understand how the worms use phototaxis when subjected to increasing light intensity. In this experiment, I manipulated the light intensity and recorded the number of worms present during photosynthetic hours. When investigating chemotaxis, I introduced aposymbiotic juveniles to an algal source and recorded their movements. In the mechanical vibration experiments, I exposed the worms to varying vibration intensities and then recorded the time taken for the worms to descend the water column.

(*Manuscript published*)

Chapter 4: The first aim of this chapter was to investigate the abiotic environmental conditions at the field location, including salinity, light intensity, and temperature and the resulting change in the worm's colony size. A secondary aim of this chapter was to examine

the genetic relatedness of populations and its symbionts. To understand the environmental conditions, I measured the colony sizes and the environmental conditions at the field location in Wales for a period of 13 months. The secondary aim was achieved by collecting samples from known locations along the Atlantic coast (Wales, Guernsey, France, and Portugal). Genetic markers for the worms (COX1) and algae (18s rRNA) where utilised, and a cladogram was constructed to assess the genetic relationships of populations from different locations and their algae symbionts.

(*Manuscript published*)

Chapter 5: The main aim of this chapter was to investigate the number of algal cells inside the worms during symbiosis and how the algal cell density changes from the initial incorporation and establishment to an adult. To achieve this, I developed a qPCR protocol for quantifying the number of algal cells within the worms. This protocol was subsequently applied to juvenile aposymbiotic worms introduced to an algae source. Additionally, I used the protocol on adult specimens obtained field locations in order to quantify the number of algae inside an adult.

Chapter 6: The main aim of this chapter was to explore the potential aquaculture applications of *S. roscoffensis.* This chapter covers three major aims. First, I assessed the palatability of the worms by observing the behaviour response of marine and freshwater fish. To accomplish this, I observed the fish's reaction when introduced to the worms and the number of worms eaten for each fish species. The second aim of this chapter was to determine the flexibility of *S. roscoffensis* in utilising an alternative symbiont. This was achieved through a laboratory-based experiment in which aposymbiotic juveniles were introduced to alternative symbionts. The third aim of this chapter was to analyse the biochemical composition of the worms grown in different media. I accomplished this by a laboratory-based experiment in which the worms were subjected to different media concentrations, and then the protein, carbohydrate and fatty acid content were quantified.

(*Manuscript published*)

Chapter 2 : Environmental constraints on the photosynthetic rate of the marine flatworm *Symsagittifera roscoffensis*

A version of this chapter has been published as "**Nathan J. Thomas**, Christopher J. Coates, Kam W. Tang (2023), Environmental constraints on the photosynthetic rate of the marine flatworm *Symsagittifera roscoffensis*, Journal of Experimental Marine Biology and Ecology, Volume 558, ISSN 0022-0981"

2.1. Abstract

Symsagittifera roscoffensis is an Acoel flatworm that lives within the intertidal zone, first discovered over a century ago as a "plant animal" due to its symbiotic relationship with the alga *Tetraselmis convolutae*. Although commonly used as a model organism in biomedical research, there is little information regarding its life history or how environmental factors affect this organism. In this study, the effects of a range of abiotic factors on *S. roscoffensis* was investigated by measuring its photosynthetic oxygen production rate under different salinities, temperatures, light intensities, photoperiods, and nutrient concentrations over 6 days. Salinity (20, 30 and 40) had little effect on photosynthetic rate, whereas a temperature of 14 °C yielded significantly higher final oxygen production than 0 and 30 °C. The lowest light intensity (21 µmol $m^{-2}s^{-1}$), the shortest photoperiod (8L:16D) and the intermediate nutrient concentration (f/4) tested resulted in the highest final oxygen production rates. The results show that *S. roscoffensis* had the ability to tolerate and remain photosynthetically active under a wide range of conditions that it is likely to experience within the intertidal zone. Using experimental data, it was estimated that *S. roscoffensis* was able to produce 174% body C d^{-1} via photosynthesis alone, which could explain the total absence of heterotrophic feeding in this organism. The organism appeared to be robust and easy to cultivate, which should open new opportunities for its wider applications.

2.2. Introduction

Symbiosis occurs within different marine phyla such as Porifera, Cnidaria (Banaszak *et al.*, 1993; Lee *et al.*, 2001; Belda-Baillie *et al.*, 2002). A particular type of symbiosis—photosymbiosis—is rather rare, and perhaps the most well-known example is between corals and zooxanthellae (*Symbiodinium* spp.). As a result of this symbiotic relationship, corals can harness solar energy and achieve large population densities with minimal feeding (Muscatine, 1990). By comparison, a lesser-known example of photosymbiosis is between the flatworm *Symsagittifera roscoffensis* and its algal symbiont. Unlike corals, which supplement photosynthates with heterotrophic feeding on suspended food particles, *S. roscoffensis* is completely reliant on its symbionts for nutrition. There are also other stark differences between corals and *S. roscoffensis*. For example, corals are fully submerged, whereas *S. roscoffensis* live in the intertidal zone where it is exposed to strong variations in environmental conditions. While adult corals are sessile organisms, *S. roscoffensis* remains freely mobile and can quickly relocate when disturbed. These characteristics make *S. roscoffensis* a unique organism that has been previously described as a 'plant animal' (Keebles, 1910).

Symsagittifera roscoffensis, previously known as *Convoluta roscoffensis* (von Graff, 1891), is an Acoel in the phylum Xenacoelomorpha (previously platyhelminths; (ITIS, 2019)). The adult is between 2 and 4 mm in length and lacks a defined coelom (Bailly *et al.*, 2014). It has a vivid green colour due to the presence of the symbiotic chlorophyte *Tetraselmis convolutae* (Douglas, 1983a; Arboleda *et al.*, 2018). *S. roscoffensis* is hermaphroditic but it reproduces by mating (Provasoli *et al.*, 1968; Douglas, 1983; Bailly *et al.*, 2014). Newly hatched individuals are aposymbiotic and must acquire the algal symbiont from the environment (Keebles, 1910; Provasoli *et al.*, 1968). The anterior of the body has a mouth-like opening that takes in algae to initiate the symbiotic relationship (Mamkaev and Kostenko, 1991; Arboleda *et al.*, 2018). After the initial acquisition by *S. roscoffensis*, phenotypical changes occur in the algae, such as loss of the cell wall, eyespot, and flagella; the algal cells are then incorporated into the upper epidermis of *S. roscoffensis* (Douglas, 1983). The symbiotic *T. convolutae* provides the host with photoassimilates such as mannitol, glutamic acid, and lactic acid (Boyle and Smith, 1975), which are then used as precursors to synthesise more complex molecules (Taylor, 1974). The host does not perform any heterotrophic feeding, and it relies entirely upon the symbiont to provide for its nutritional needs; consequently, a true

digestive tract is absent in the worm (Jennings, 1971; Mamkaev and Kostenko, 1991; Bailly*et al.*, 2014; Arboleda *et al.*, 2018).

Symsagittifera roscoffensis is capable of regeneration and tissue renewal thanks to the presence of totipotent stem cells (Bely and Sikes, 2010). Accordingly, *S. roscoffensis* has been used extensively as a model organism in biomedical research concerning tissue development and regeneration (Reuter *et al*, 1998; Bailly *et al.*, 2014; Dittmann *et al.*, 2018). By comparison, information on its natural history, ecophysiology and other potential applications is limited since its discovery at the Station Biologique de Roscoff, Roscoff, France (Geddes, 1879).

The known geographical range of *S. roscoffensis* covers the temperate zone from South Wales (51°23′00.8″N 3°22′27.9″W) to the southern tip of Portugal (37°05′22.2″N 8°11′18.4″W). Bailly *et al*., (2014) reported that the habitat temperature for the population in Roscoff, France is between 10 and 20 ºC, but populations found in the lower and higher latitudes likely experience very different temperatures. For instance, the water temperature at the South Wales site reaches as low as 6 ºC and remains <10 ºC for much of the winter. The Welsh population can also be exposed to ice and snow occasionally in the winter. Mettam (1979) reported that the South Wales site was inaccessible due to the presence of snow and that the population was still present after the snow thawed. In the summer months, water temperature at the South Wales site can reach up to 30 ºC, and *S. roscoffensis* are still present. It appears that the Welsh population of *S. roscoffensis* may have a different thermal preference or tolerance than the other populations.

The algal symbiont requires light for photosynthesis, but photoperiod and light intensity both vary temporally and spatially across the latitudinal range of *S. roscoffensis*. While photoperiod varies by ∼5 h seasonally at the lower latitudes such as in Portugal, the population in South Wales experiences a 10 h difference in daylength between summer and winter. The average light intensity also decreases toward the higher latitudes. Therefore, populations at different locations are expected to adapt to different light environments. Surprisingly, there is very limited information on how variations in light intensity and photoperiod affect the photosynthetic activity of *S. roscoffensis* (Androuin *et al.*, 2020).

Unlike the submerged environment, salinity along the intertidal zone will fluctuate due to inundation, evaporation, precipitation, and surface runoff, but information is lacking on how *S. roscoffensis* responds to different salinities. My own observations at the South Wales site suggest that the salinity can fluctuate between 10, and 30, within a day. Carvalho *et al*., (2013)

showed that *S. roscoffensis* can take up nutrients from the surrounding water to provide for the algal symbiont. Nutrient levels along the shore can vary due to discharge and runoff from land, but it remains unclear how the worm's photosynthetic activity may respond to different ambient nutrient concentrations.

To fill those knowledge gaps and improve the understanding of the basic biology of this organism, I conducted a series of experiments to determine how the different abiotic factors—salinity, temperature, light intensity, photoperiod, and nutrient concentration – affect the photosynthetic activity of *S. roscoffensis* collected from South Wales. The results not only enable a better understanding of the natural history and ecophysiology of the organism but may also open possibilities to customise culturing conditions for the organism for different research applications.

2.3. Methods

2.3.1 Sample collection and master culture

Symsagittifera roscoffensis was collected from a beach in East Aberthaw in South Wales, U.K. (N 51. 23′ 2.506″ W3. 22′ 28.004″), between late September and early October 2020. It is found in the upper limit of the intertidal zone at low tide as patches of green on the sand, in small rock pools and between pebbles (Figure 2.1). The worms were collected with a pipette into test tubes and returned to the laboratory within 2 h. In the laboratory, the worms were transferred into 300-mL glass containers to establish a master culture. The containers held autoclaved sand that was collected from the same location; the seawater was drawn from Swansea Bay and was sterilised by filtration, UV radiation and autoclaving before use (salinity 30, pH 8.1). Inorganic nutrients were added in the form of 0.22 μm-filtered Guillard (Guillard and Ryther, 1962) f/2 medium at 10 mL L^{-1} (f/4 final conc.) The master culture was placed inside an LMS incubator set to a temperature of 14.5 ºC; light was provided by a light panel inside the incubator at an intensity of 69 μmol m^{-2} s^{-1} and a photoperiod of 16 L:8 D. One quarter of the water was renewed every 3 days to replenish the nutrients, and the worms were transferred to new containers weekly to avoid build-up of waste and detritus.

Figure 2.1. *Symsagittifera roscoffensis*. Left: Dense patches of *Symsagittifera roscoffensis* in the intertidal zone at the South Wales field site. Right: An individual *S. roscoffensis* under a light microscope.

2.3.2. Photosynthetic activity

Photosynthetic activity of the worms was measured as oxygen production through time with a Pyroscience Firesting optical O_2 system (Model: FSO2-C4) inside the incubator. For that, the worms were placed inside a 2-mL transparent respiration vial that was sealed air-tight with a lid. The base of the vial had an O_2-sensitive patch attached and connected through optic fibre to a Firesting O_2 meter, which recorded the dissolved O_2 level inside the vial continuously. O_2 measurements were always performed during daytime, and each measurement lasted 40 min. A temperature probe connected to the Firesting was used to account for any temperature fluctuation during the measurements.

The Firesting system was calibrated using a 2-point calibration with 100% O_2-saturated water and a 0% O_2 solution (30 g L^{-1} Na_2SO_3), following the manufacturer's instructions.

2.3.3 Salinity experiment

In addition to the base seawater described earlier (salinity 30 with added nutrients), solutions of lower (20) and higher salinity (40) were made up by mixing the base seawater with the appropriate amount of deionised water or sea salt. The final salinities were verified by a DD True Seawater Refractometer calibrated following the manufacturer's instructions.

To begin the experiment, worms each *ca*. 2 mm in length were transferred from the master culture into three sets of five 100-mL containers (200 worms each) with seawater solution of a specific salinity (20, 30 or 40). Temperature, light intensity, and photoperiod remained at 14 °C, 69 µmol s^{-1} m^{-2} and 16 L:8 D, respectively throughout the experiment.

From each of the 15 treatment containers, a group of 50 worms was randomly selected and placed inside the respiration vial to measure photosynthetic activity. To account for any background O_2 changes, a blank measurement was included using water (without worms) from the same treatment container. On Day 3, another group of 50 worms was taken from each treatment container (plus the corresponding blank) to measure photosynthetic activity; the procedures were repeated on Day 6.

2.3.4. Temperature experiment

To test the effect of different temperatures, the incubator temperature was set to 0, 14 or 30 °C. Sets of five treatment containers were exposed to each of the experimental temperatures. The salinity was kept at 30 and illumination remained at 69 μmol s^{-1} m^{-2} and 16 L:8 D. Oxygen production by the worms (with the corresponding blank) was measured on Day 0, 3 and 6 as described above.

2.3.5. Light intensity experiment

The amount of light inside the incubator was adjusted to create different light intensity levels of 21, 69 and 475 μmol m^{-2} s^{-1}, as verified by an Apogee quantum PAR sensor. Temperature was kept at 14 °C, salinity at 30 and photoperiod at 16 L:8 D. Sets of five treatment containers were exposed to each of the light intensity levels and oxygen production by the worms (with the corresponding blank) was measured on Day 0, 3 and 6 as described above.

2.3.6. Photoperiod experiment

For this experiment, the light intensity level was kept at 69 μmol s^{-1} m^{-2} but the photoperiod was set to 8 L:16 D, 16 L:8 D or 24 L:0 D. Temperature and salinity remained at 14 °C and 30, respectively. Photosynthetic activity of the worms exposed to the different photoperiods was measured in the same manner as described earlier.

2.3.7. Nutrient experiment

To create different nutrient treatments, Guillard solution was added to autoclaved base seawater in the amount of 20-, 10- or 5-mL L^{-1} for the equivalent final nutrient level of f/2, f/4, and f/8, respectively. Sets of five treatment containers were set up for each nutrient concentration. Temperature (14 °C), salinity (30) and light (69 μmol s^{-1} m^{-2}; 16 L:8 D) conditions were kept constant for the experiment. Photosynthetic activity of the worms exposed to each nutrient concentration was measured in the same manner as described earlier.

2.3.8. Respiration rate

To determine the respiration rate of *S. roscoffensis* we randomly selected 50 worms and placed them inside the same respiration vial mentioned above. These were placed inside an incubator under the conditions for my master stock cultures at salinity 30, temperature 14 °C, light intensity 69 μmol m^{-2} s^{-1}, photoperiod 16 L:8 D and nutrient concentration f/4. Net oxygen production rate was obtained by recording data for 40 min with the light turned on and was repeated with the light turned off to obtain the respiration rate. This was replicated on three separate occasions.

2.3.9. Carbon budget

In order to calculate the daily carbon budget, a body water content of 89% was used, a carbon content of 25% and a wet body weight of 1.6 mg ind^{-1}; from this I calculated the dry weight carbon content. I then calculated the global average oxygen production rate for my master stock cultures at salinity 30, temperature 14 °C, light intensity 69 μmol m^{-2} s^{-1}, photoperiod 16 L:8 D and nutrient concentration f/4 from 0 to 6 days using the oxygen data presented below. The global average was converted to a daily value assuming 12 h of active photosynthesis; converting the daily global average oxygen production to carbon production using a 2.6:1 (g/g) O_2-to-C conversion ratio. I then used my carbon production per day and body carbon content to predict a % body C d^{-1} ind^{-1} ratio.

2.3.10. Data analysis and statistics

Data analysis was conducted in R studio version 1.41717. To avoid initial fluctuations in the oxygen readings due to opening and closing the incubator door, oxygen readings in the first 10 min were excluded from further analysis. The oxygen readings in the subsequent 30 min in both treatments (with worms) and controls (without worms) were fitted to a linear model (lm) to generate the respective slopes – the difference between the two slopes represents the O_2 production rate by the worms via photosynthesis (expressed in μg O_2 h^{-1} ind^{-1}). I calculated the mean and standard error among the five replicates. The same procedures were applied to data from Day 0, 3 and 6 of each of the experiments. ANOVA (aov) with a Tukey's post hoc test for pairwise comparisons were carried out (R package emmeans) to test for putative significant effects due to treatment, time, and their interactions. Graphs were generated using the package tidyvers that included ggplot 2 in R studio.

2.4.1. Results

2.4.2. Salinity

At salinity 20, the oxygen production rate (mean ± s.e.) on day 0 was at 11.0 ± 3.0 µg O_2 h^{-1} ind^{-1}, but it decreased to 8.7 ± 1.8 µg O_2 h^{-1} ind^{-1} on Day 6 (Figure 2.2). The oxygen production rate in salinity 30 decreased from 10.7 ± 2.5 to 7.8 ± 3.4 µg O_2 h^{-1} ind^{-1} over the course of the experiment (Figure 2.2). In salinity 40, the oxygen production rate was relatively stable, varying between 9.8 ± 2.8 µg O_2 h^{-1} ind^{-1} on Day 0 and 8.6 ± 2.5 µg O_2 h^{-1} ind^{-1} on Day 6 (Figure 2.2). There was no significant effect due to salinity (p = 0.977), time (p = 0.979) or their interaction (p = 0.842).

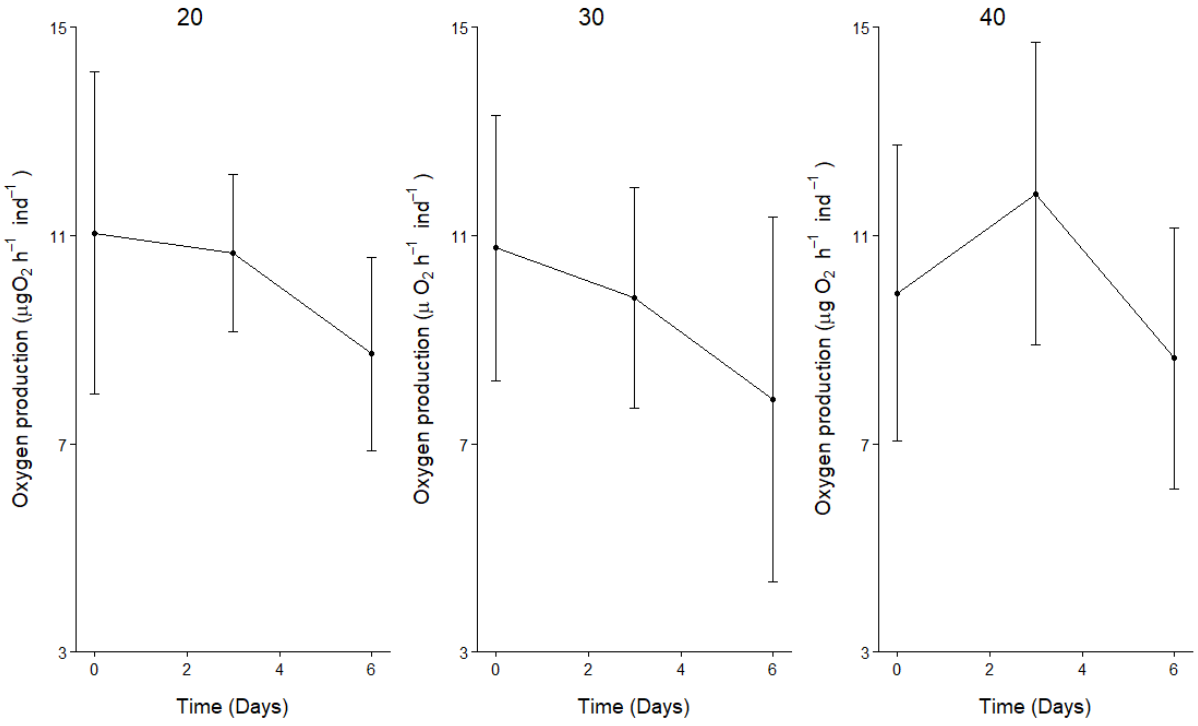

Figure 2.2. O_2 production rate of *Symsagittifera roscoffensis* in different salinities. Data are plotted as mean ± s.e. Based on ANOVA, there was no significant effect due to salinity, time or interaction between temperature and time ($p > 0.05$). Each data point represents the mean of five replicates, with N=50 individuals per replicate.

2.4.3. Temperature

Oxygen production rate at 0 °C started at a high level of 28.2 ± 5.3 µg O_2 h^{-1} ind^{-1} on Day 0 but then decreased rapidly to 5.8 ± 1.3 µg O_2 h^{-1} ind^{-1} by Day 6, resulting in an 80% drop over the course of the experiment (Figure 2.3). Oxygen production rate was relatively

stable but low at 30 °C, varying between 7.9 ± 1.4 and 11.8 ± 3.2 µg O_2 h^{-1} ind^{-1} (Figure 2.3). Oxygen production rate was also stable at 14 °C and remained higher than the other temperature treatments at the end of the experiment (23.1 ± 3.3 µg O_2 h^{-1}; Figure 2.3). There was a significant effect due to temperature ($p < 0.001$), time ($p = 0.045082$) and the interaction between temperature and time ($p < 0.001$).

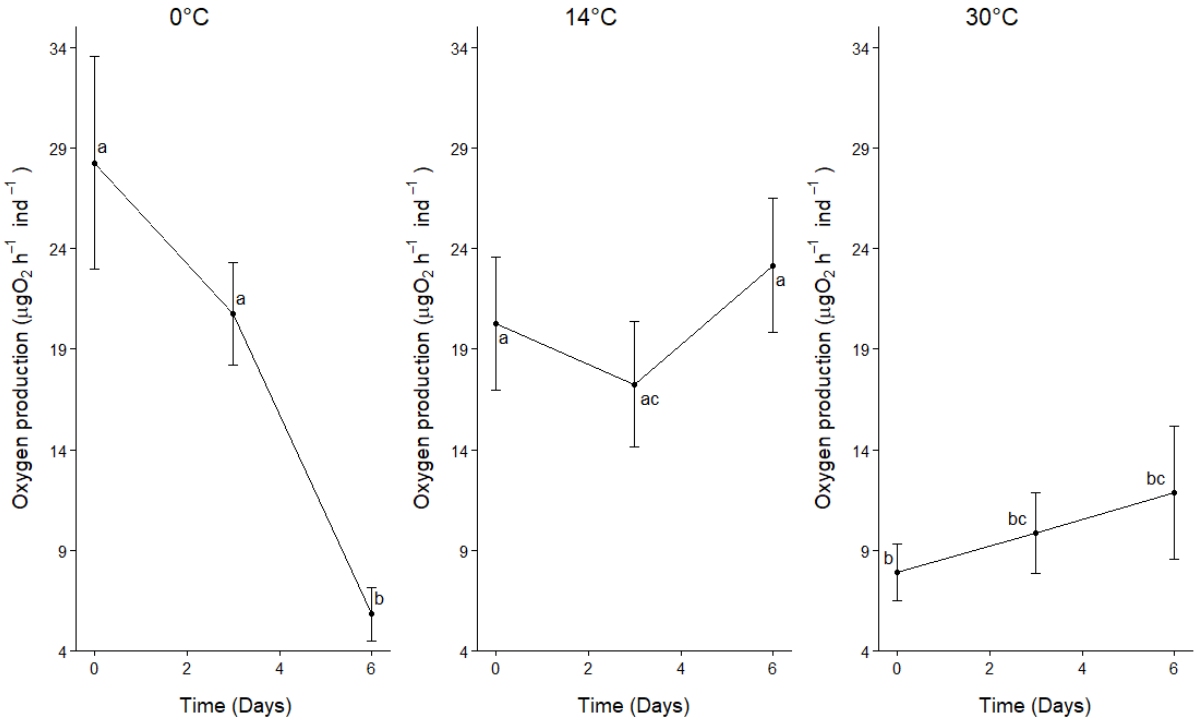

Figure 2.3. O_2 production rate of *Symsagittifera roscoffensis* in different temperatures. Data are plotted as mean ± s.e. Based on ANOVA there was a significant effect due to temperature, time and interaction between temperature and time (ANOVA; $p < 0.05$). Unshared letters indicate significant differences determined via ANOVA and Tukey's post-hoc tests (Tukey; $p < 0.05$). Each day represents five replicates with N=50 different individuals involved in each replicate.

2.4.5. Light intensity

The oxygen production rate was highest at 36.3 ± 3.0 µg O_2 h^{-1} ind^{-1} under light intensity 21 µmol m^{-2} s^{-1} on Day 0; despite a drop between Day 0 and Day 3, it rebounded to 31.9 ± 2.9 µg O_2 h^{-1} ind^{-1} on Day 6 (Figure 2.4). The oxygen production rate was considerably lower at 69 µmol m^{-2} s^{-1}, and it increased from 11.2 ± 4.0 µg O_2 h^{-1} ind^{-1} to 17.1 ± 5.8 µg O_2 h^{-1} ind^{-1} over the course of the experiment, an increase of 34% (Figure 2.4). At 475 µmol m^{-2} s^{-1}, the oxygen production rate varied between 20.3 ± 1.9 and 17.7 ± 2.3 µg O_2

h^{-1} ind^{-1} (Figure 2.4). There was a significant effect due to light intensity ($p < 0.001$), time ($p < 0.001$) and the interaction between light intensity and time ($p < 0.001$).

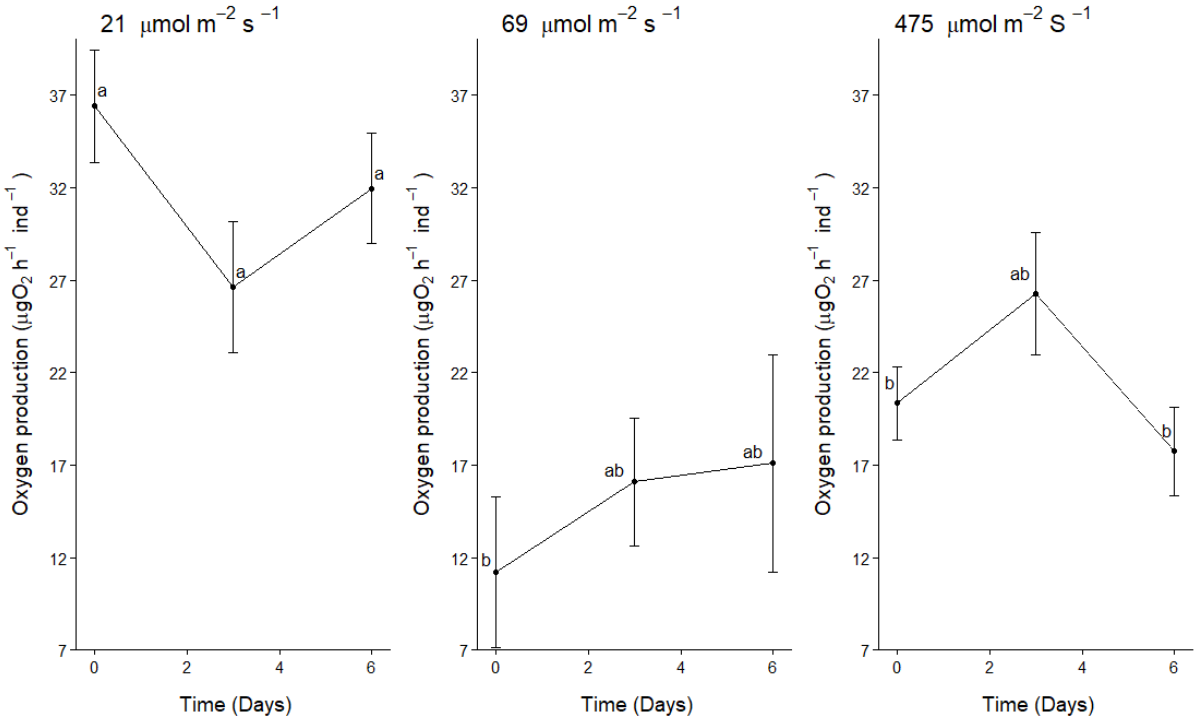

Figure 2.4. O_2 production rate of *Symsagittifera roscoffensis* in different light intensities. Data are plotted as mean ± s.e. Based on ANOVA there was a significant effect due to light intensity, time and interaction between light intensity and time (ANOVA; $p < 0.05$). Unshared letters indicate significant differences determined via ANOVA and Tukey's post-hoc tests (Tukey; $p < 0.05$). Each day represents five replicates with N=50 different individuals involved in each replicate.

2.4.6 Photoperiod

Under the 8 L:16 D photoperiod, the oxygen production rate doubled over the course of the experiment, increasing from 16.8 ± 3.4 µg O_2 h^{-1} ind^{-1} on Day 0 to 32.1 ± 2.4 µg O_2 h^{-1} ind^{-1} on Day 6. (Figure 2.5). In the 16 L:8 D photoperiod treatment, the oxygen production rate increased by 115%, from 9.7 ± 2.1 to 20.9 ± 2.8 µg O_2 h^{-1} ind^{-1} over the 6-day period (Figure 2.5). Under continuous light (24 L), the oxygen production rate began at 24.3 ± 0.4 µg O_2 h^{-1} ind^{-1} and after a slight increase to 31.1 ± 6.6 µg O_2 h^{-1} ind^{-1}, decreased sharply to 12.0 ± 3.3 µg O_2 h^{-1} ind^{-1} at the end of the experiment, representing a 61% drop (Figure 2.5). There was no statistically significant effect attributable to photoperiod alone ($p = 0.194$). However, there was a significant effect due to time ($p < 0.001$) and the interaction between photoperiod and time ($p = 0.00848$).

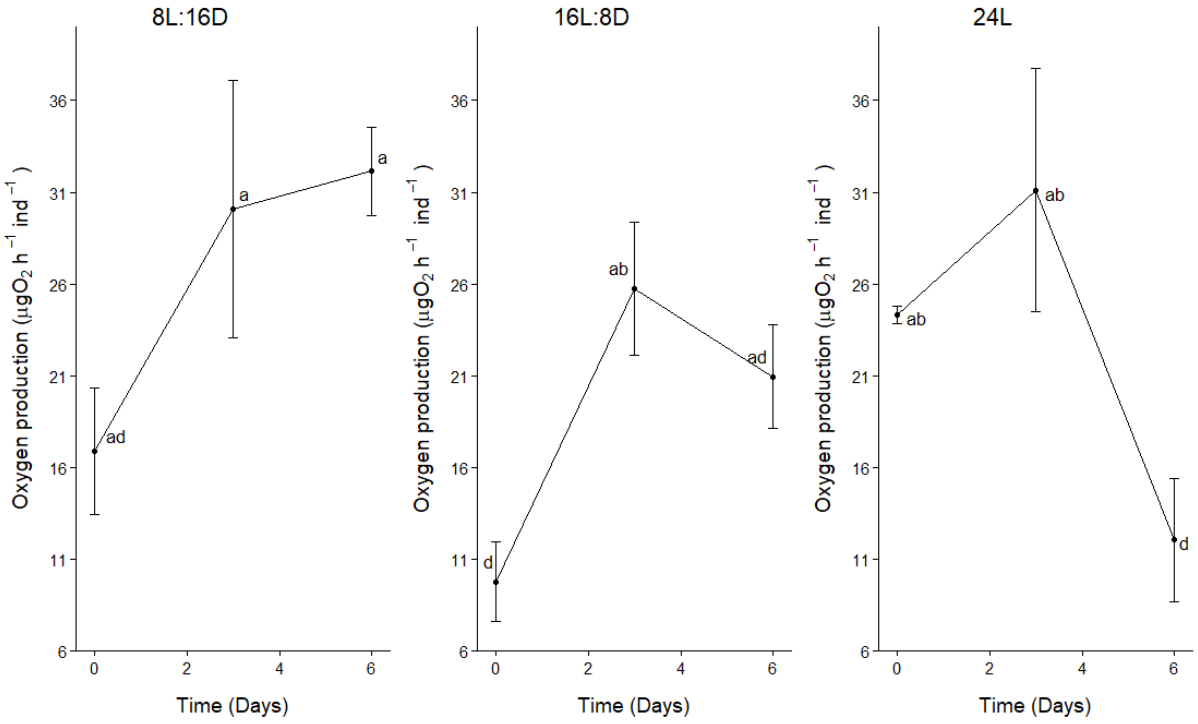

Figure 2.5. O_2 production rate of *Symsagittifera roscoffensis* in different photoperiods. Data are plotted as mean ± s.e. Based on ANOVA there was a no significant effect due to photoperiod (ANOVA; $p > 0.05$). However, there was a significant effect due to time as well as photoperiod and time (ANOVA; $p < 0.05$). Unshared letters indicate significant differences determined via ANOVA and Tukey's post-hoc tests. (Tukey; $p < 0.05$). Each day represents five replicates with N=50 different individuals involved in each replicate.

2.4.7. Nutrients

In the highest nutrient concentration (f/2), the oxygen production rate decreased by 53% from 18.3 ± 3.8 to 8.5 ± 3.7 µg O_2 h^{-1} ind^{-1}, over the 6-day period (Figure 2.6). The oxygen production rate in the intermediate nutrient concentration (f/4) remained high over the course of the experiment, varying between 19.9 ± 2.4 and 18.3 ± 2.1 µg O_2 h^{-1} ind^{-1}. In the lowest nutrient concentration (f/8), the oxygen production rate decreased from 20.4 ± 2.7 to 11.4 ± 1.3 µg O_2 h^{-1} ind^{-1}, a 44.1% decrease over the course of the experiment (Figure 2.6). There was no significant effect due to nutrient concentration ($p = 0.271$), time ($p = 0.0938$), or their interaction ($p = 0.2512$).

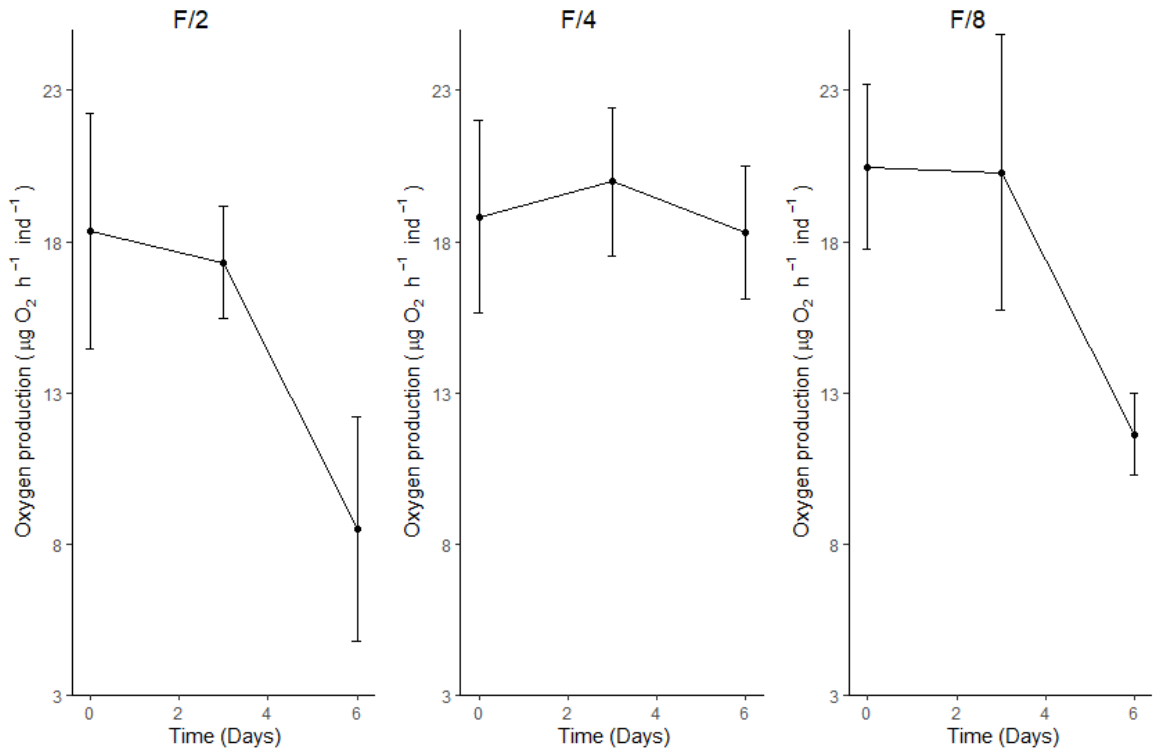

Figure 2.6. O_2 production rate of *Symsagittifera roscoffensis* in different nutrient concentrations. Data are plotted as mean ± s.e. Based on ANOVA nutrient treatments did not have a significant effect on oxygen production between days, neither did the interaction between nutrients and time (ANOVA; $p > 0.05$). Each day represents five replicates, with N=50 individuals per replicate.

2.4.8. Respiration rate

In the respiration experiment, under the conditions of salinity 30, temperature 14 C, light intensity 69 µmol m^{-2} s^{-1}, photoperiod 16 L:8 D and nutrient concentration f/4, an oxygen production rate of 7.9 ± 0.8 µg O_2 h^{-1} ind^{-1} was calculated proceeded to fall to -3.1 ± 1.3 µg O_2 h^{-1} ind^{-1} when placed into the dark (Figure 2.7). There was a significant difference between the oxygen production rate and the respiration rate (ANOVA; $p = 0.00228$).

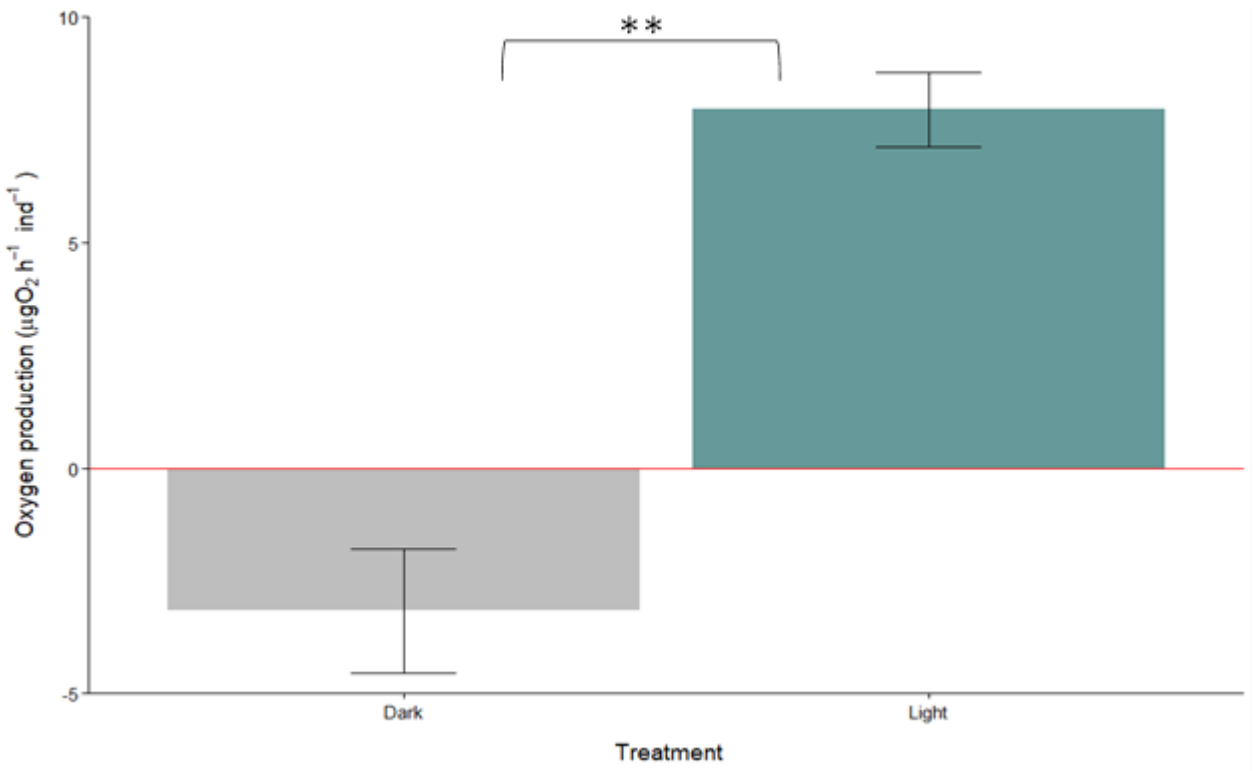

Figure 2.7. O_2 production and respiration rate of *Symsagittifera roscoffensis*, in the light and dark. Data are plotted as mean ± s.e. Based on ANOVA, there was a significant difference in oxygen production between the light and dark treatments (ANOVA; $p < 0.05$). Each treatment represents three replicates, with N=50 individuals per replicate.

2.4.9 Carbon budget

First, I calculated an oxygen production rate of 199.8 µg O_2 d^{-1} ind^{-1} and a wet weight of 1.6 mg ind^{-1}, of which 0.18 mg ind^{-1} is dry weight. With an estimated carbon content of 44 µg C ind^{-1}, and using the oxygen production rate, I determined the daily carbon production rate of 76.8C d^{-1} ind^{-1}. Finally, I calculated the body % carbon content, which gave me *S. roscoffensis'* s body weight to carbon ratio of 174% body C d^{-1} ind^{-1}.

2.5.1. Discussion

The intertidal acoel flatworm *Symsagittifera roscoffensis* is a rare example of photosymbiosis where the host is sustained entirely by the algal symbionts. South Wales is considered the northern limit of its geographical range (Bailly *et al*., 2014). Although the organism has been discovered for over 100 years (Geddes, 1879), much remains unknown about its natural history and ecology. The purpose of this study was to determine how the different abiotic factors—salinity, temperature, light intensity, photoperiod, and nutrient level, affect the photosynthetic activity of the South Wales' population of *S. roscoffensis.*

2.5.2. Effects of salinity

The oxygen production rate did not vary much with time and salinity, and it remained quite comparable among all treatments, at between 7 and 8 μg O_2 h^{-1} ind^{-1} at the end of the experiment. These results suggest that *S. roscoffensis* was able to cope with the salinity range of 20–40, not unexpected for an organism that lives within the intertidal zone (Bailly *et al.*, 2014). On the beach where I collected my samples, salinity can vary frequently due to evaporation, inundation, sea spray, precipitation and surface runoff, and *S. roscoffensis* must be able to withstand a wide salinity range (Geng *et al.*, 2016). While a salinity of 40 may be extreme, I occasionally saw some of the worms basking in the sun at low tide at the field site and its surrounding salinity could be much higher than 30. Overall, my results show the ability of *S. roscoffensis* to maintain its photosynthetic activity in a wide salinity range that they likely experience *in situ*.

2.5.3. Effects of temperature

While there is little literature information on the temperature preference of *S. roscoffensis,* Bailly *et al.* (2014) suggested an optimal temperature range of 10–20 ºC based on the environmental temperature in Roscoff. In my experiments, the oxygen production rate remained stable at 14 C and the final value was significantly higher than that of other temperature treatments (0 and 40 ºC), which seemed to be consistent with Bailly *et al.*'s suggestion. Interestingly, at 0 °C the oxygen production rate showed a sharp decrease over time whereas a slight increase was observed in 30 °C, suggesting that cold temperature was more stressful to *S. roscoffensis* than warm temperature. While it was not tested for a duration longer than 6 days, the results did suggest that *S. roscoffensis* from South Wales was able to cope with a temperature range of 0 to 30 °C. This is not unexpected as the habitat temperature along the South Wales's coast can vary widely depending on tides and seasons. For example, Mettam

(1979) reported the presence of the worm after the beach had been sealed off by snow. Observations at the field site also suggest that *S. roscoffensis* could experience close to 0 °C and 30 °C during an average year, and *S. roscoffensis* was found in both the coldest and warmest parts of the year, without any sign of ill effects.

2.5.4. Effects of light intensity

The oxygen production rate appeared steady at 69 μmol m^{-2} s^{-1}, which was the light intensity the worm is accustomed to in the master culture. Surprisingly, the highest oxygen production rate was observed in the lowest light intensity tested (21 μmol m^{-2} s^{-1}). This indicates that 69 μmol m^{-2} s^{-1} light intensity was already too high for the worm. This may be the case as it lives within dense colonies and among pebbles (Figure. 2.1) that may offer some shading. Franks *et al.* (2016) showed that within an *S. roscoffensis* colony, individuals rotate in a circular motion, which may allow them to photosynthesise in short bursts and avoid over exposure. In contrast, in the highest light level (475 μmol m^{-2} s^{-1}) the oxygen production rate decreased considerably after 3 days, suggesting a stress response. Androuin *et al*., (2020) found that for *S. roscoffensis* that had been acclimatised to low light – it showed a decrease in oxygen production rate when exposed to light above 200 μmol m^{-2} s^{-1}, akin to a photoinhibition effect. Nevertheless, the worm remained photosynthetically active after 6 days in my experiments, affirming that *S. roscoffensis* was able to cope with the seasonal range of irradiance that it is likely to encounter in situ.

2.5.6. Effects of photoperiod

Among the tested photoperiods, 8 L:16 D yielded the largest increase and highest final oxygen production rate. This photoperiod is comparable to the shortest winter day in South Wales. The results suggest that the South Wales population of *S. roscoffensis* preferred a shorter daylength, which seemed to agree with my own anecdotal field observations where the worm population appeared to be more abundant in the winter months. The 16 L:8 D photoperiod is close to the longest summer day in South Wales, and the measured oxygen production rate remained high toward the end of the experiment although there was a small decrease from Day 3. Data from these two photoperiod treatments support the field observations that *S. roscoffensis* was present in both summer and winter. In contrast, the oxygen production rate of the worm increased slightly first under 24 L, but then decreased sharply toward the end. These observations suggest that while the worm required light for photosynthesis, continuous exposure to light could have a negative effect like photoinhibition.

2.5.7. Effects of nutrients

By the end of the experiment, the oxygen production rate of *S. roscoffensis* was highest in the f/4 medium. The oxygen production rate was considerably lower in f/8 medium, perhaps reflecting nutrient limitation for the algal symbiont. Interestingly, despite being a standard medium for culturing microalgae including free-living *Tetraselmis* species, f/2 medium resulted in a substantial decrease in the oxygen production rate over the 6-day period. Indeed, when I attempted to use f/2 for the master culture, the worm did not do well and quickly perished. Carvalho *et al*. (2013) suggested that *S. roscoffensis* can be used to treat nutrient-rich wastewater stream. I would, however, caution against such suggestion because the worm appeared to be stressed when exposed to high nutrient level, perhaps reflecting the fact that *S. roscoffensis* and its algal symbiont from South Wales may have adapted to a lower nutrient concentration than the Portuguese population used in Carvalho *et al*.'s study.

2.5.8. Respiration rate

The oxygen production rate and the respiration rate for *S. roscoffensis* in the light and dark. Using these data, I determined the gross oxygen production rate to be 11.1 ± 2.1 μg O_2 h^{-1} ind^{-1}. The gross oxygen production rate is comparable to the net oxygen production obtained for day 1 for the other data sets i.e., salinity, photoperiod, and light intensity. The respiration rate that I detected would have been comprised of respiration from both the algae symbiont and the worm.

2.5.8. Daily carbon production

No information on the dry weight or carbon content of *S. roscoffensis* is present in the literature. Nevertheless, I measured the wet weight of an individual *S. roscoffensis* from the stock and found it to be 1.6 mg ind^{-1}. Assuming that, *S. roscoffensis* has the same body water content (89%) and carbon content (25% of dry weight) as algae (Beal *et al.*, 2018), was estimated to be a dry weight of 0.18 mg ind^{-1} and a carbon content of 44 μg C ind^{-1}.

Using the experimental results, the carbon budget of *S. roscoffensis* when exposed to the environmental conditions used in the stock culture, i.e., salinity 30, temperature 14 °C, light intensity 69 μmol m^{-2} s^{-1}, photoperiod 16 L:8 D and nutrient concentration f/4. For each of the environmental parameters, I used the average of the photosynthetic oxygen production rate across the experimental period (Day 0 to Day 6), then calculated a global average photosynthetic oxygen production rate of 16.4 μg O_2 h^{-1} ind^{-1}. I then extrapolated this to a daily value assuming 12 h of active photosynthesis per day, giving me a daily rate of 199.8 μg

O_2 d^{-1} ind^{-1}. Using a 2.6:1 (g/g) O_2-to-C conversion, this translates to a daily carbon production rate of 76.8 μg C d^{-1} ind^{-1} via photosynthesis, which is equivalent to 174% body C d^{-1} ind^{-1}. While this carbon budget estimation is rather crude and relying on several assumptions, it shows that *S. roscoffensis* has a highly efficient photosynthetic machinery thanks to its algal symbionts, which may explain the absence of the need for heterotrophic feeding in this organism. The high % body C production rate may also allow for the energy and resources it needs to cope with the dynamic and potentially stressful intertidal environment.

2.5.9. Cultivation method and potential applications

For this study, I established a master culture of *Symsagittifera roscoffensis* in the laboratory. I was, however, unable to find any detailed description of long-term culturing methods for this organism in the literature. Nevertheless, using my simple master culturing protocol (see Methods), I was able to keep a continuous stock of *S. roscoffensis* for over a year. During this time, there was no noticeable changes in the vivid green colour of the worm that may indicate poor physiological condition. To the contrary, I observed small photosynthetic juveniles appearing in the culture, indicating successful reproduction.

Because *S. roscoffensis* in the wild may experience different and variable conditions, I used a gradual acclimatisation of the worms to the culture condition. I initially recorded the *in-situ* temperature and salinity in the field, and then transported them in ambient seawater back to the laboratory. In the laboratory, I initially set the incubator to the *in-situ* temperature then adjusted it by ≤2 °C every 2 days until it reached the desired temperature. Likewise, I also adjusted the water salinity incrementally every 2 days until it reached the desired salinity. On one occasion, I accidentally left a container of *S. roscoffensis* in the dark for a month, and afterward I did not notice any mortality or visual change to the size, behaviour, or colour of the worm, suggesting that *S. roscoffensis* is a rather robust organism that can survive an extended period of unfavourable conditions, which should make it fairly easy to cultivate.

2.6.1. Conclusion

The results indicate that *S. roscoffensis* can withstand a range of conditions and still remains photosynthetically active. However, changes to temperature, light intensity, and photoperiod result in significant changes to the oxygen production rate and should be kept within the optimal range in order to maximise oxygen production rate. Moreover, while *S. roscoffensis* has been used as a model organism in biomedical research, its highly efficient photosynthetic ability and the relative simplicity of its cultivation and maintenance should open

other applications/possibilities. For example, the worm has a body size similar to certain life stages of common live-feed organisms (e.g., *Artemia* and copepods) and *Tetraselmis* is generally regarded as a beneficial algal genus for aquaculture (Tulli *et al.*, 2012; Pereira *et al.*, 2020); therefore, *S. roscoffensis* has the potential to be developed into a low-maintenance, high-yield feed for aquaculture. Further research into the biochemical profile of *S. roscoffensis* under different growth conditions and its palatability to aquaculture species should be considered.

Chapter 3 : To move or not to move: Taxis responses of the marine flatworm *Symsagittifera roscoffensis* to different stimuli

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3.1 Abstract

The acoel worm *Symsagittifera roscoffensis* lives in the intertidal zone forming a symbiotic relationship with the alga *Tetraselmis convolutae* and relying entirely on photosynthesis by the algal symbiont for its nutritional needs. Juveniles are born without algal symbionts (i.e., aposymbiotic) and must acquire the algae from the surrounding water. Once symbiosis is established, the worm positions itself to harness sunlight for photosynthesis, while also minimising the risk of being washed away in the physically dynamic environment. To gain insights into the worm's behavioural adaptations to living within the intertidal zone, I conducted a series of experiments on its movement when presented with an algal source (chemotaxis), varied light gradient and increasing light intensities (phototaxis), and mechanical vibrations. Aposymbiotic juvenile worms showed approximately three times larger positive displacement towards an algal cue than towards an artificial worm or plain seawater. Adult worms showed positive phototaxis but retreated into the substrate when exposed to a light intensity of ≥1400 μmol $m^{-2}s^{-1}$. The worms ascended at 0.11±0.01 cm s^{-1} and stayed near the surface of calm water, and only began to descend when the mechanical vibrations exceeded a threshold. All worms descended at 0.66±0.07 cm s^{-1} when the vibrations reached 1.764 $m^{-}s^{-2}$. Altogether, these data suggest that *Symsagittifera roscoffensis* has a chemotactic ability to acquire algae from its surroundings, a crucial step to complete its life cycle, and that it may maintain populations within the intertidal zone by seeking light for photosynthesis while minimising the risk of dispersal and photoinhibition.

3.2.1 Introduction

The marine acoel worm *Symsagittifera roscoffensis* (Graff 1891), formerly *Convoluta roscoffensis*) of the Family Convolutidae, was first discovered in Roscoff, France (Geddes 1879) and subsequently observed in Portugal (Carvalho *et al*., 2013), the Channel Isle's (Doonan and Gooday 1982) and South Wales, U.K. (Mettam 1979), with the latter considered the northern limit of its known distribution (Mettam 1979; Mcfarlane 1982). It is often found in shallow pools of water (< 10 cm deep) at the upper limit of the intertidal zone in dense patches of thousands of individuals (Doonan and Gooday 1982; Bailly *et al*., 2014). Individuals are easily identifiable by their vivid green colour, due to the presence of the symbiotic microalga *Tetraselmis convolutae* held within the upper epithelium (Bailly *et al*., 2014), earning it the nickname mint-sauce worm. The worm lacks a true digestive tract and relies entirely on the algal symbionts to provide nutrition through photosynthesis (Bailly *et al*., 2014; Chapter 2). While earlier studies focused on its geographical distribution (Mettam 1979; Stoecker *et al*., 1989), life cycle (Provasoli *et al*. 1968; Douglas 1985) and ecology (Parke and Manton 1967; Nozawa *et al*., 1972; Douglas 1983), recent research focus has shifted towards its use as a model organism to study developmental and neural biology (Semmler *et al*., 2010; Bailly *et al*., 2014; Sprecher *et al*., 2015). Nevertheless, the ability of *S. roscoffensis* to acquire an algal symbiont, harness light and maintain its position within the dynamic intertidal zone raises some interesting questions about its behavioural adaptations.

Symsagittifera roscoffensis is hermaphroditic but reproduces by mating. The embryos are encased in a cocoon and emerge as aposymbiotic juveniles, i.e., lacking algal symbionts (Provasoli *et al*., 1968; Bailly *et al*., 2014). The juvenile worms must find the algae in the vicinity and establish photosymbiosis within a few days to survive (Bailly *et al*., 2014; Chapter 6), but how the juveniles detect and acquire the algae *in situ* remains unclear. Although *S. roscoffensis* can perceive light with photoreceptors, it lacks eyesight. While no other sensory organs have been described, it is not uncommon for closely related species to have a range of sensory organs that enable it to respond to other stimuli (Pearl 1903; Inoue *et al*., 2004; Inoue *et al*., 2014). Its inability to swim freely and that it resides in shallow pools of water upon the sand also raises the question: Where may it find algal cells to establish photosymbiosis? In this chapter, I posit that aposymbiotic juveniles can seek out the "right" algae by sensing the chemicals (positive chemotaxis) from settled algal cells or nearby adult worms.

In the natural environment, the direction to seek light is to move upward. There is the suggestion that the worm's upward movement is linked to high tides (Arboleda *et al*., 2018), but because the tides shift daily, this would mean *S. roscoffensis* must continuously adjust its movement to align with the tidal cycle. It is also questionable what benefit the worm may gain by responding to high tides at night. Indeed, Arboleda *et al*., (2018) noted that the alleged tidal migration disappeared when *S. roscoffensis* was kept in the dark, implying that the movement was cued to the light-dark cycle rather than the tidal cycle, but the authors did not provide any further details. Movement in response to light requires the ability to sense light. Photosensitive cells have evolved many times (Burr 1984a; Plachetzki *et al*. 2005), giving rise to wide-spread phototaxis (positive or negative) in metazoans (Burr 1984b). *Symsagittifera roscoffensis* has photoreceptors, and its positive phototaxis has been reported in the literature (Serôdio *et al*., 2011; Nissen *et al*., 2015).

Light intensity at the intertidal zone can reach a very high level especially in a clear summer day, and it is well known that free-living microalgae can suffer photodamage when exposed to excessive light (Straka and Rittmann 2018). Serôdio *et al*., (2011) proposed that the worms would retreat to avoid excessive light and prevent photoinhibition of the algal symbionts. However, Nissen *et al*., (2015) disagreed and instead suggested the worms lack the ability to regulate photosynthesis or avoid photoinhibition. In the Channel Isles (Guernsey), population sizes of *S. roscoffensis* were lower in the summer months (Douglas *et al*., 1982). In Wales, I had anecdotal evidence that in the summer months, very few worms were present on the beach surface when the ambient light level reached ~2,000 µmol m^{-2} s^{-1}, implicating a negative response to excess light, although detailed data were lacking.

While there are examples of photosymbiosis in sessile invertebrates that are exposed to strong tidal and wave actions, most notably corals, sponges and ascidians, the detached body of *S. roscoffensis* creates a dilemma: The requirement of light for photosynthesis means the worms must expose themselves in the intertidal zone. At the same time, water motions due to waves, tides and water runoff place the worms in danger of being washed away. During collection in the field, the candidate observed that strong winds continuously agitated the water, but the worms remained positioned just below the water surface unfazed by the agitation. However, as soon as the water was disturbed with sampling gear, the worms immediately retreated into the sand, similar to that reported by Gamble and Keeble (1904). These observations suggest that *S. roscoffensis* is able to tolerate some level of physical

disturbance and continue to photosynthesise within the intertidal zone, but retreat when the physical disturbance crosses some threshold.

To better understand the behavioural adaptations of *S. roscoffensis* to the intertidal environment, I conducted a series of laboratory experiments focusing on chemotaxis, phototaxis and physical disturbance. I tested if *S. roscoffensis* exhibits positive chemotaxis towards an algal chemical signal, and its movements under different light gradients and light intensities. Lastly, physical vibration was simulated and tested if *S. roscoffensis* would respond only to a certain level of disturbance. The results would shed light on how the worm may acquire the essential algal symbionts and the sunlight it needs for photoautotrophy, while avoiding unwanted dispersal in a physically dynamic environment.

3.3.1 Materials and methods

3.3.2. *Symsagittifera roscoffensis* collection and master culture

Worms were collected and cultured as previously described in Chapter 2.

3.3.3. Chemotaxis experiments

I harvested cocoons taken from the adult worms and hatched them in autoclaved seawater to produce ten aposymbiotic juveniles per trial (Provasoli *et al*., 1968; Chapter 2 & 6). These were placed to one side of an 8.5 cm wide Petri dish filled to 0.7 cm in height with seawater. Using a camera (Olympus UC30) attached to a dissecting microscope (Olympus SZX16), the movement of the worms was recorded for 10 min with no stimuli to establish their 'background' movement patterns.

To test chemotaxis, freeze-dried adult worms were used to provide an algal chemical cue. To produce the freeze-dried worms, adult worms full of endosymbiotic algae were taken from the master culture and held at -80 °C for 24h and then placed into a freeze dryer (Model:Edwards Modulyo) 24h prior to the experiment. Freeze-drying was used as a preservation method to maintain intact algal cell walls (Min *et al*., 2022), protein, lipids (Aljabri *et al*., 2023), phenols (Badmus *et al.*, 2019) and carbohydrates (Badmus *et al.*, 2019), such that the end product resembled closely the original chemical characteristics of the algae. A set of 10 aposymbiotic juvenile worms were added to a Petri dish; directly opposite approximately 4 cm away I placed a freeze-dried worm. The movement of the juvenile worms was recorded for 10 min. For the negative control, the experiment was repeated replacing the freeze-dried worms with a plastic artificial worm which was blue in colour and made from

polyethylene terephthalate and was the approximate size of an adult worm. Each of the treatments (plain seawater, freeze-dried worm, artificial worm) were tested five times, each time with a new Petri dish and a new set of juvenile worms.

Once the video footage was obtained, the programme AnTracks v1018 was used to analyse the movement of the juvenile worms from their starting positions, in response to freeze-dried worm, artificial worm or plain seawater, and was measured as displacement positive (towards the cue) or negative (away from the cue). Displacement was used instead of distance due to the fact that the worm's movement was non-linear. The displacement values were compered between treatments using the Kruskal-Wallis and a pair-wise comparison using the Wilcoxon rank sum test with a Bonferroni correction.

3.3.4. Phototaxis experiments

In the first experiment, I used a glass cylinder (1.7–2.3 cm dia.) with 10 cm height of seawater (salinity 30). A single adult worm was placed into the cylinder and allowed to settle to the bottom. Once settled, an LED lamp (Barrian; 6500K) was turned on and focused to illuminate the water column evenly at 69 µmol m^{-2} s^{-1}, and the worm's vertical position was observed continuously for 30 min. The cylinder was left undisturbed throughout the observations, and 50 trials were conducted with a new worm and fresh seawater in each trial.

In the second experiment, the cylinder was placed under a dark cover without light. In the third experiment, the same LED light beam was focused on the surface to create a down-gradient of light. In the fourth experiment, the LED light beam was focused at the bottom to create a reverse light gradient. In each case, the worm's position was recorded either continuously or every 5 min for a total of 30 min. A total of 30 trials were conducted in these latter experiments.

To aid the analysis of the movements and comparison with data in existing literature, time each worm spent in the upper (7–10 cm) and lower (0–3 cm) sections of the water column were calculated. Time distributions between sections by individual worms were compared using a Mann-Whitney test.

3.3.5. Light intensity experiments

Experiments were conducted to test whether the adult worm would avoid excessive light. 50 worms were placed in a glass jar that contained 2 cm sand as a substrate and 5 cm deep (100 mL) seawater (enriched with f/4) of the same salinity as the candidate's master

culture. The glass jar was placed inside the LMS incubator at 14.5°C. A Kessil A360X Tuna Sun adjustable LED lamp was placed, with the colour turning knob set to white, at 12.5 cm from the water surface. When using the Tuna Sun, I did not use a spectral controller or the associated Wi-Fi dongle. Using an Apogee quantum light meter, I measured and adjusted the light intensity to 70 µmol m^{-2} s^{-1} at the start of the experiment (Day 0). The photoperiod was kept at 16L:8D throughout. The number of worms that were present above the sand was counted on Day 3, one hour after the light was turned on. After counting, the light intensity was increased to the next level: 150 µmol m^{-2} s^{-1} on Day 3, 280 µmol m^{-2} s^{-1} on Day 6, 525 µmol m^{-2} s^{-1} on Day 9, 1400 µmol m^{-2} s^{-1} on Day 12, and finally 2500 µmol m^{-2} s^{-1} on Day 15. The counting continued every third day until Day 18. The experiment was then repeated with a new jar and another 50 worms, for a total of three times. At the end of the final experiment, the light intensity was lowered back down to 70 µmol m^{-2} s^{-1} for 24 h and recorded the number of worms present above the sand.

For the phototaxis experiments and the light intensity experiments the candidate chose to use 69-70 µmol m^{-2} s^{-1} as this was the light intensity that was used for the master culture conditions; previous data also suggested that photosynthesis remained stable at this intensity (Chapter 2).

After confirming that the data was normally distributed by the Kolmogorov-Smirnov test (R studio package DHARMa V4.1.3), A non-linear generalised Poisson regression was used to test if there was a statistical difference in the number of worms that were presented above the sand between the different light intensities.

3.3.6. Behaviour observed under mechanical stimulation

Experiments were conducted to study the vertical movement of *S. roscoffensis* in response to physical disturbance in the form of vibrations. A single adult worm was placed inside a glass cylinder (2.3 cm dia.) with 10-cm deep seawater (salinity 30) and allowed to settle to the bottom. A smartphone pre-programmed (mobile application Vibrator strong) to create a vibration level of 1.764 m s^{-2}. The smartphone was placed (with vibration off) on top of the column. The cylinder was evenly illuminated by an LED lamp and the worm was observed continuously. When it began to move upwards, its vertical movement was tracked and timed. After the worm had reached the surface and stayed there for at least 30 s, the vibration function was turned on and the downward movement of the worm was observed and timed until it reached the bottom. Afterwards, the cylinder was cleaned and refilled with

seawater and the experiment was repeated with a new worm, for a total of thirty times. Upward and downward speeds were calculated as vertical distance travelled per second and compared using a Mann-Whitney test.

3.3.7. Vibration intensity experiments

After confirming that the worms responded negatively to vibrations (1.764 m s^{-2}) in the previous experiments, I tested whether there was a threshold level of disturbance before the adult worm would react, by gradually increasing the level of disturbance. The smartphone was pre-programmed to different vibration intensities (m s^{-2}) with different on/off (ms) cycles to create eight levels of disturbance, each lasting 1 min (Table 3.1). One adult was placed worm into the illuminated cylinder and waited for it to reach the top of the water column as mentioned earlier. The treatment started from disturbance level 1 and increased sequentially to level 8, and the disturbance level was noted that triggered the worm's decent and the descent velocity. Afterwards, the cylinder was cleaned, and the experiment was repeated with a new worm, for a total of ten times. The results were compared between the different disturbance levels using ANOVA; normality was confirmed by the Kolmogorov-Smirnov test (studio package DHARMa V4.1.3).

3.4.1. Results

3.4.2 Effects of chemical cues

In the chemotaxis experiment, the juveniles showed a significantly larger positive displacement towards the freeze-dried worm than towards the artificial worm or plain seawater (Kruskal-Wallis; Chi-squared = 157.29; $p < 0.001$; Figure. 3.1). The juveniles in plain seawater showed only small positive displacement from their starting positions, which increased to 35.9 ±5.6 mm (accumulative ± s.e.) at the end of the experiment. In the artificial worm treatment, the juveniles showed small but negative displacement (i.e. away from the cue) in the first 450s, then changed to a positive displacement of 30.1±13.4mm by 600 s. In the freeze-dried worm treatment, juveniles consistently showed positive displacement, which increased steadily from 19.4±2.9 mm at 75 s to 89.4±14.1 mm at the end of the experiment.

Wilcoxon rank sum test scores indicated significant differences between the artificial worm and plain seawater treatments ($p = 0.0075$), between the freeze-dried worm and plain seawater treatments ($p< 0.001$), and between the freeze-dried worm and artificial worm treatments ($p< 0.001$).

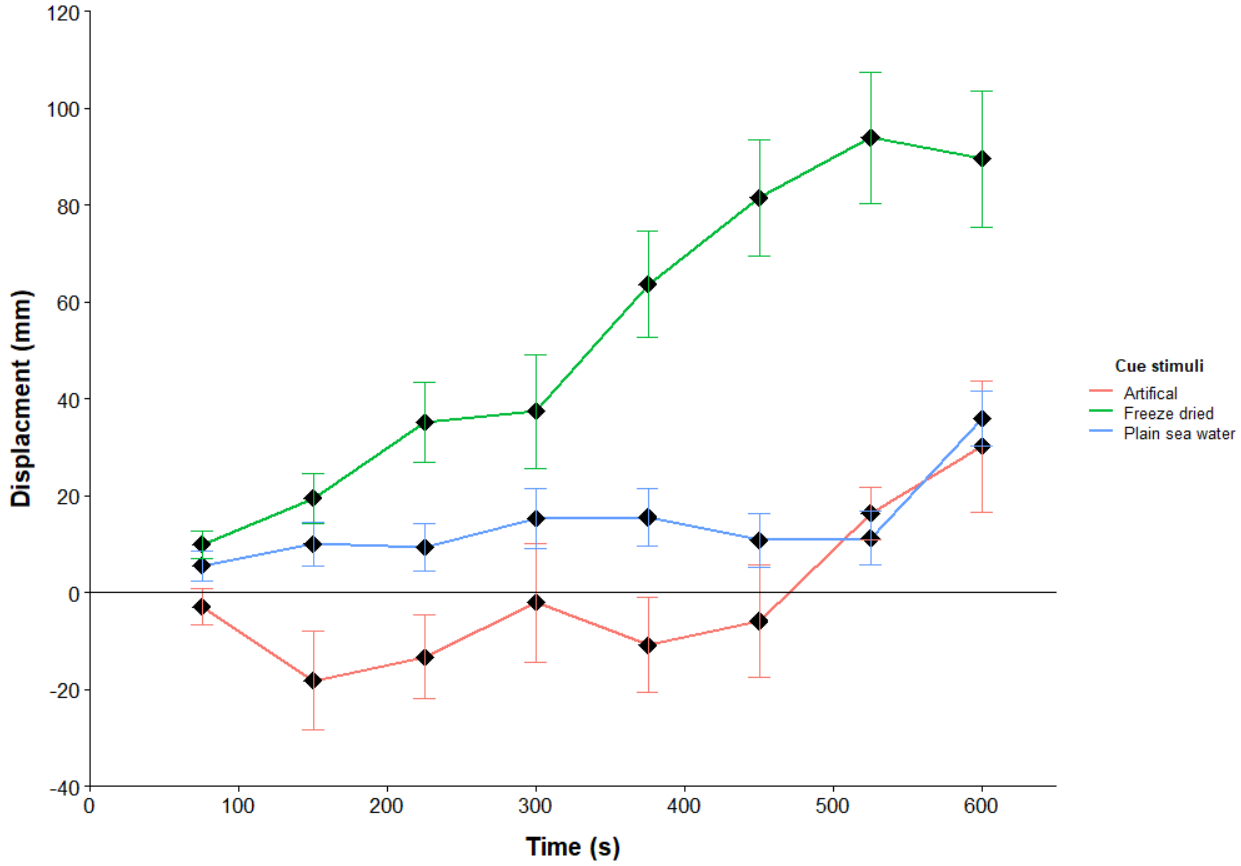

Figure 3.1. Chemotactic displacement (mm) of *Symsagittifera roscoffensis* when presented with plain seawater, a freeze-dried adult worm, and an artificial worm. The overall displacement direction (accumulative mean ± s.e) in each treatment is positive when towards the stimuli and negative when away from the stimuli. N=10 juvenile worms per treatment per replicate. There was a significant overall difference in the displacement values among the treatments (Kruskal-Wallis; Chi-squared = 157.29; $p < 0.001$).

3.4.3 Effects of light gradient and intensity

In the first experiment with even illumination, the timing of ascent varied between individuals, with some moving upwards after *ca.* 200 s, whereas others remained at the bottom for nearly 700 s before ascending. Some individuals reached and stayed at the surface, whereas others moved up and down repeatedly, and they did not stop for any noticeable amount of time when in transit. On average, the worm spent more time in the upper section (50 ± 0.04 %; mean ± s.e.) than the lower (28 ± 0.04 %) section of the water column (Mann-Whitney, w = 1752.5 $p = 0.0005$) (Figure. 3.2).

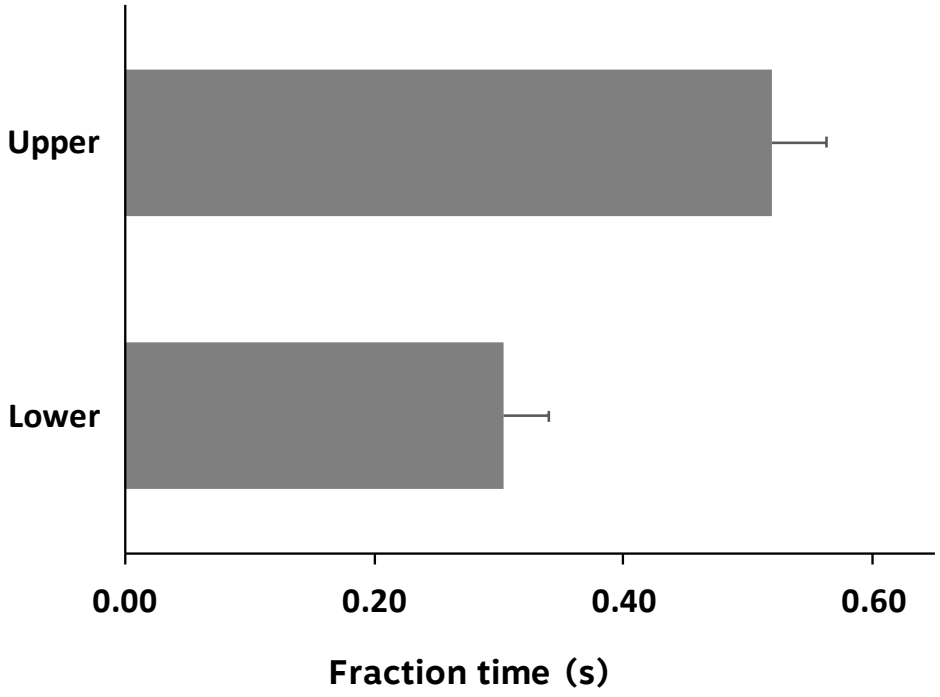

Figure 3.2. Fraction of time (mean + s.e.) spent by *S. roscoffensis* in the upper (7–10 cm) and the lower (0–3 cm) sections of the water column. Worms were monitored for 30 minutes at 69 µmol m^{-2} s^{-1} in even illumination, n=50 worms. All of the worms moved. A fraction time of 0.18 was spent in transition between the upper and lower sections and is not included in the graph. There was a significant difference in the time spent between the two sections (Mann-Whitney; w = 1752.5, p = 0.0005).

In the second experiment where the cylinder was in darkness, 17 of the 30 individuals stayed at the bottom and did not register any vertical movement. Of the other 13 individuals, nine of them travelled up and down multiple times. These 13 individuals spent 46 ± 26% of their time in the upper section of the water column (Figure 3.3). In the third experiment where the light beam was focused at the surface, only two individuals remained at or close to the bottom the entire time. The other 28 individuals travelled the entire length of the water column and spent on average 64 ± 27% (12 individuals spending >80%) of their time in the upper section (Figure 3.3). In the fourth experiment where the light beam was focused at the bottom, only three individuals showed any noticeable upward movement and only two of them reached the surface. These three individuals spent on 28 ± 31% of their time in the upper section. The other individuals did not register any upward movement and remained at the bottom the entire time (Figure 3.3).

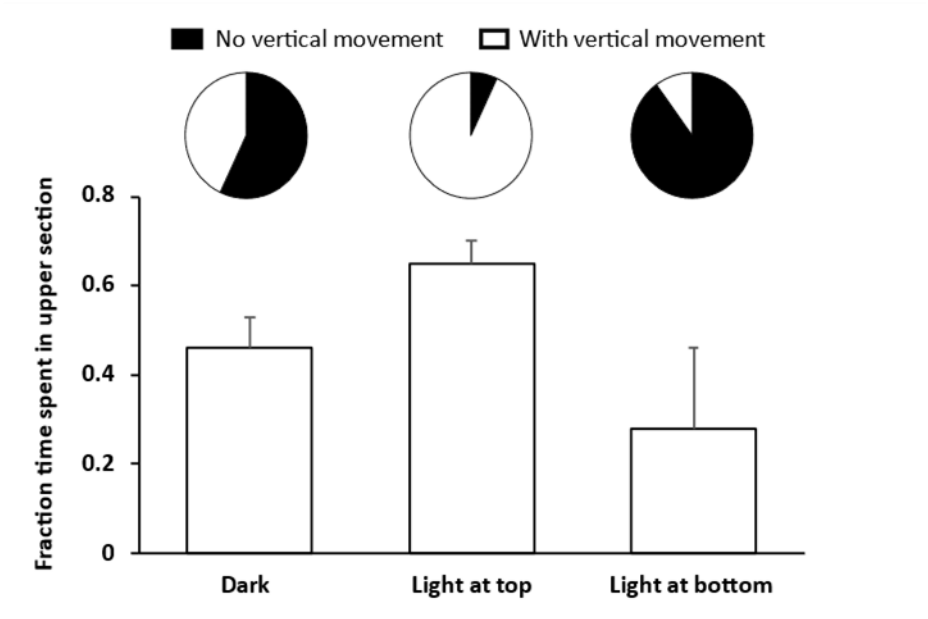

Figure 3.3. Movement of *S. roscoffensis* under different light conditions. Pie charts show the proportions of worms with or without vertical movement; n = 30 worms. Bar graph shows, for the ones that moved, the fraction of time spent in the upper section of the water column (mean + s.e.).

The next experiment tested the effect of light intensity. At 70 µmol $m^{-2}s^{-1}$, 46.6 ± 1.2 (mean ± s.e.) worms (out of 50) were present above the sand (Figure. 3.4). With the light intensity increasing every 3 days, the number of worms present decreased accordingly: 28.6±4.0 worms at 150 µmol $m^{-2}s^{-1}$, 20.3 ± 3.1 worms at 280 µmol $m^{-2}s^{-1}$, 12.3 ± 4.4 worms at 552 µmol $m^{-2}s^{-1}$, and no worms were visible at 1400 µmol $m^{-2}s^{-1}$ and 2500 µmol $m^{-2}s^{-1}$. The results can be described by a non-linear generalised Poisson regression: Number of worms = exp(4.0 – 0.00353 × light intensity) (Z-value = -9.553, $R^2 = 0.92$, $p < 0.0001$). At the end of the final trial, the light intensity was decreased back to 70 µmol $m^{-2}s^{-1}$ and 23.6 ± 1.4 worms re-emerged after 24 h.

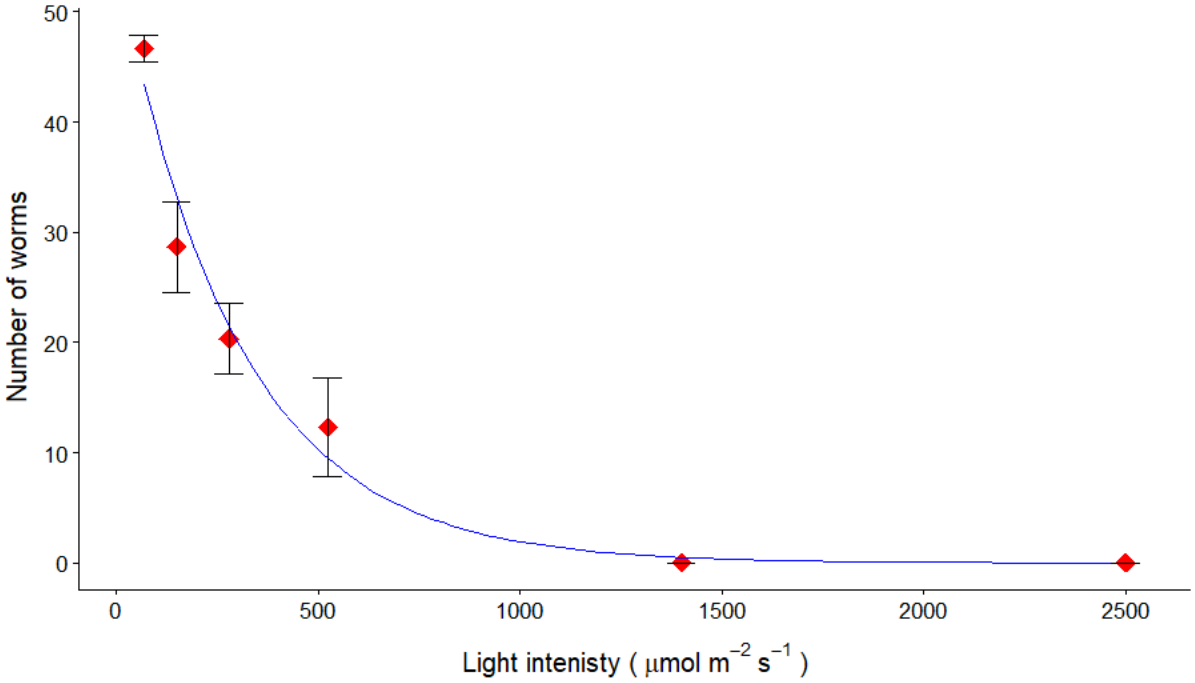

Figure 3.4. Number of worms present above the sand when exposed to increasing light intensity (mean ± s.e.). n=50 worms per replicate. The results can be described by a non-linear generalised Poisson regression; Number of worms = exp(4.0 – 0.00353 × light intensity), represented by the blue line (R^2 = 0.92, Z-value = -9.553, p <0.0001).

3.4.4 Effects of physical disturbance and vibration intensity

When testing the effect of vibration, the worms initially ascended at a speed of 0.11±0.01 cm s^{-1} (mean ± s.e.), equivalent to ca. 0.6 body lengths per second (Figure. 3.5). If no disturbance was applied, the worms maintained the position at the top of the water column similar to that presented in Figure 3.3. At the onset of a vibration of 1.764 m s^{-2}, all of them moved downwards almost immediately in a freefall-like manner, at a speed of 0.66 ± 0.07 cm s^{-1} (ca. 3.4 body lengths per second), significantly faster than the ascent speed (Mann-Whitney, W = 37, p =< 0.001) (Figure. 3.5).

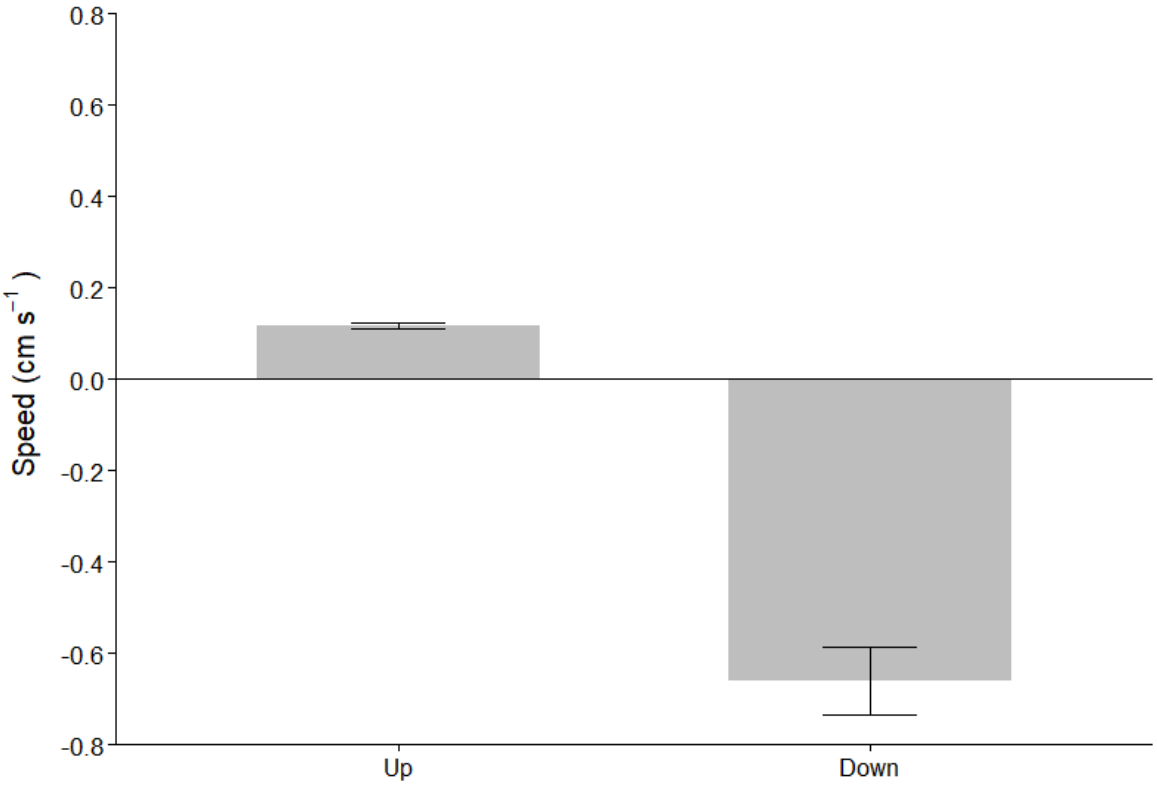

Figure 3.5. Vertical movement speed (mean ± s.e.) of undisturbed *S. roscoffensis* (upwards; positive values) and after disturbance (downwards; negative values) (Mann-Whitney, W = 37, $p < 0.001$). N=30 worms.

In the experiment where increasing levels of disturbance was tested, the worms did not descend under disturbance levels 1-4 (Table 3.1). Disturbance levels 5-6 only triggered descent in one out of 10 trials, at a speed of 0.16–0.18 cm s^{-1} (mean ± s.e.). Disturbance levels 7-8 triggered more responses: Level 7 caused descent in three trials at a speed of 0.5±0.05 cm s^{-1}, whereas level 8 resulted in descent in 5 trials at 1±0.5 cm s^{-1}. There was a significant difference in the number of worms that descended between the different disturbance levels (ANOVA; F-value =16.95, $p = 0.006$).

Table 3.3. Vibration intensities and durations used to create different levels of disturbance. The durations that the vibration was turned on and off are given in milliseconds. Total number of worms tested (out of 10 total) showing downward movement and their corresponding speed (s.e. in parenthesis where applicable) are presented.

Setting	Intensity ($m\ s^{-2}$)	On duration time (ms)	Off duration time (ms)	Number of worms descending	Downward speed ($cm\ s^{-1}$)
1	0.21	120	1000	0	0
2	0.43	240	875	0	0
3	0.63	360	750	0	0
4	0.84	480	625	0	0
5	1.05	600	500	1	0.16
6	1.27	720	375	1	0.18
7	1.481	840	250	3	0.5 (0.05)
8	1.693	960	125	5	1 (0.5)

3.5.1. Discussion

The acoel *S. roscoffensis* must find the optimum algae to establish photosymbiosis. It must also expose and orient itself towards the light, while avoiding unwanted dispersal in the physically dynamic intertidal zone. Using a series of experiments, I aimed to gain further understanding of the worm's behavioural adaptations to chemical, light, and mechanical stimulations in such an environment.

After birth, the aposymbiotic juvenile must acquire algal symbionts within days to survive. Because the juvenile worm does not display any swimming or filter feeding capabilities that may allow it to capture freely suspended algal cells, I suspect that it likely acquires algal cells that have settled or algae from nearby adult worms (Bailly *et al*., 2014). In the chemotaxis experiments, juvenile worms showed a larger displacement towards a freeze-dried adult worm than either plain seawater or an artificial worm, suggesting an attraction towards the algal source via chemotaxis. I also repeated the experiment using adult worms full of algal symbionts, but the adult worms showed no movement and remained stationary (data not shown), suggesting that the chemotactic response was only present in aposymbiotic juveniles in search of algae, but it is no longer needed once photosymbiosis has been established in the adult worms.

Chemotactic ability is not uncommon in soft bodied marine meiofauna; for instance, planarians use chemotaxis to detect food in the surrounding environment (Inoue *et al*., 2015). For the aposymbiotic juvenile of *S. roscoffensis,* the most readily available source of algae *in situ* would be the adult worms, each containing over 100,000 alga cells (Bailly *et al.,* 2014; Arboleda *et al.,* 2018). Adults are covered in mucus and during reproduction, eggs rupture from the side wall of the adult's body. It is conceivable that some algal cells get lodged into the mucus as the adults lay eggs (Costello and Costello 1939). As the adults then move around, they shed the mucus, which may act as an algal source for the juveniles. Chemotactic ability would allow the juveniles to locate the 'right' algae in the vicinity (Provasoli *et al*., 1968; Bailly *et al*., 2014). Although the net displacement seems rather modest—less than 90 mm over 10 min in my experiment, this may be sufficient within a dense congregation of *S. roscoffensis* in the field. While the data in this chapter shows that juveniles were attracted to the algae contained within the adults, it may be possible that they were attracted to other chemicals from the adults also. Either way, this behaviour would allow the aposymbiotic juveniles to locate a

suitable algal source. Further work should consider comparing algal isolates and algae-free adults to determine the actual source of chemical cue that the juveniles are attracted to.

Previously, Nissen *et al*., (2015) indicated that adult worms exhibited a clear positive phototaxis, spending 69% or more time in the illuminated side of a Petri dish than in the shade. Notably, their experiment involved the worms moving along a horizontal surface without exposure to potential danger of water motion. In the wild, the light gradient is more vertical than horizontal, and any vertical movement along a water column has to balance the need for light against the risk of unwanted dispersal. In this chapter where I used even illumination, *S. roscoffensis* spent more time in the upper part of the vertical water column. However, by tracking the individual movements, I discovered a considerable amount of variability between individuals, suggesting asynchronous movements within a population. Interestingly, many of them did not stay at the top continuously, but rather they moved up and down repeatedly. Although the worm needs light for photosynthesis, a prolonged stay at the surface may increase the risk of unwanted dispersal by sudden water motion. This may explain why they returned to the bottom repeatedly, perhaps as a safety precaution.

The anterior of *S. roscoffensis* has, in addition to photoreceptors, a statocyst that senses gravity (Bailly *et al.*, 2014). It was observed that the worm lied horizontally when at the bottom or just underneath the water surface, but it assumed a vertical posture when in transit, suggesting that it could determine its direction (upwards or downwards) based on the statocyst's orientation. This might explain why the worm didn't pause during transit, but only halted when it encountered a boundary such as the water surface or the bottom substrate, and adopted a horizontal posture, even in the absence of light.

Between the two light gradient experiments, the results reinforced one another in showing that the worm's movement was positively phototactic, individuals were attracted to the light, regardless of its position at either the top or bottom (Figure.3.3). It was not believed that any vertical movement in darkness would be observed, but some individuals still moved upwards. *S. roscoffensis* requires light to survive and in nature, the most logical direction to seek light is upwards. Whether an individual should move or not in darkness may depend on its internal state and how strong is the need to search for light. I randomly selected the individuals for the experiments, and prior differences in their photosynthetic history and physiological conditions may contribute to the variations in their vertical movement in the dark. During the course of the experiments and in my own culture maintenance, I observed no vertical movement of *S. roscoffensis* that could be linked to tidal cycle, which also has been

confirmed by others (Arboleda *et al.,* 2018). Instead, I postulate that the cyclical vertical movement reported in the literature (Keebles 1910; Arboleda *et al*., 2018) may have been linked to a circadian rhythm induced by a strong phototactic response (Stanton *et al.,* 2022).

Excessive light can harm the algal photosynthetic system, which in turn could be detrimental to the host worm (Androuin *et al*., 2020). In a recent study, it was observed that at a light intensity of 475 µmol µmol m^{-2} s^{-1}, the worm's photosynthetic oxygen production began to decline after four days, indicating photoinhibition (Chapter 2). In this current Chapter, I also observed that as light intensity increased, more worms burrowed into the sand, and no worms were visible at the surface at ≥ 1400 µmol µmol m^{-2} s^{-1}. Some worms re-emerged when the light intensity was decreased. These findings aligned with Doonan and Gooday (1982) who noted a lower number of worms in situ during the summer months when light intensity was highest, and the idea proposed by Serôdio *et al*., (2011) that *S. roscoffensis* burrows into substrate to avoid excessive light.

In the mechanical stimulation experiment, the worm ascended at an average speed of 0.11 cm s^{-1}, identical to the horizontal speed reported earlier (Nissen *et al.,* 2015). In comparison, all individuals descended ~6 times faster in response to physical disturbance. *Symsagittifera roscoffensis* lives in an environment where water movement poses the risk of unwanted dispersal. The worm requires a mate to reproduce despite being hermaphroditic (Bourlat and Hejnol 2009). Therefore, one may posit that the danger of being removed from the habitat and the population outweighs the need for light, and it is necessary for *S. roscoffensis* to descend and secure itself to the bottom as quickly as possible when it senses strong disturbance. However, in the intertidal zone where there can be frequent water movement, overly sensitive reaction to any disturbance could be counter-productive because the worm would be spending energy unnecessarily and moving away from the light. My observations in the wild found that the worms do not respond to minor agitation in the water. This suggests that they would tolerate background disturbances up to a certain threshold. This was confirmed in the current experiment where the worms did not respond to weak vibrations, which allowed the worm to remain near the surface for photosynthesis and avoid unnecessary exertion.

Combining the results from the phototaxis experiments and mechanical stimulation experiments, I propose a “decision scheme” to describe the response of *S. roscoffensis* to external stimuli such as disturbance and light, moderated by its internal state and a built-in “safety measure” (Figure.3.6). This scheme does not imply any conscious thinking by the

worm; rather, it illustrates how the different external and internal factors work together to influence the worm's behaviour.

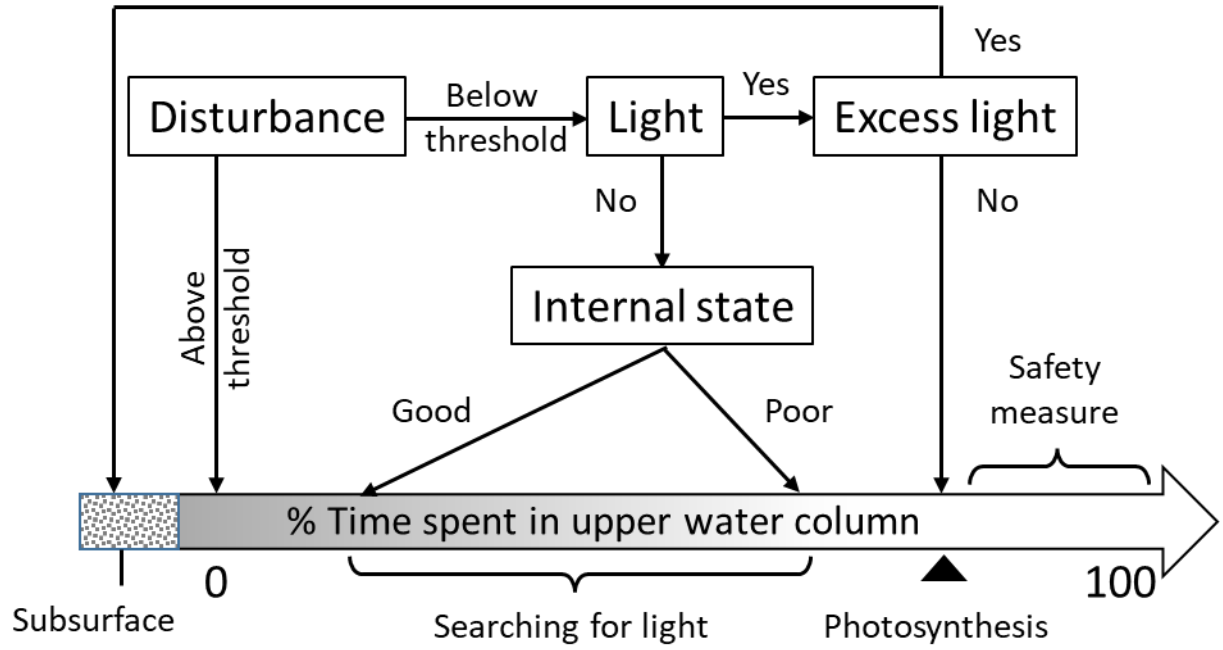

Figure 3.6. "**Behavioural response scheme**" depicting the influence of external factors (disturbance, light) and internal factors (internal state, built-in safety measure) in determining the vertical movement of *S. roscoffensis.* Good internal state refers to an individual with sufficient or excess photoassimilates and therefore having little need for photosynthesis; poor internal state refers to the opposite condition.

Further work on the chemotactic ability of *S. roscoffensis* should consider identifying the compound(s) responsible for attracting juveniles. Determining the attractant would not only enhance the understanding of what the worms are drawn to but could also facilitate additional experiments involving the detection threshold and associated concentration to which the worms respond. Identifying the attractant could also help us determine the sensory organs involved.

Additional research on how the worms avoid higher light intensity should explore how they achieve photosynthesis during the summer months, or perhaps, photo repair mechanisms. For instance, assessing whether the worms shift their photosynthesis to the dawn and dusk periods of the day when light intensity is lower. Understanding how the worms survive during the summer months will contribute to determining population dynamics in these periods.

3.6.1 Conclusions

The experimental results describe the behavioural adaptions of *S. roscoffensis* as a photosynthetic acoel living in the dynamic intertidal zone. The aposymbiotic juvenile exhibited chemosensing ability to seek out the algal source (found in adults) for establishing photosymbiosis. The worm showed an intricate balance between positive phototaxis to acquire light by ascending through the water column, and quick descent —at a certain threshold of disturbance—to avoid unwanted dispersal by water movement. The worm also burrowed itself to avoid excessive (harmful) light. Collectively, these behaviours would allow *S. roscoffensis* populations to establish and persist in the intertidal zones, such as those found in South Wales and along the Atlantic coast of continental Europe.

Chapter 4 :*In situ* environmental drivers and molecular identification of the photosymbiotic marine flatworm *Symsagittifera roscoffensis*

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4.1 Abstract

Known as the Roscoff worm or mint-sauce worm, *Symsagittifera roscoffensis* is a marine flatworm distinguishable due to the presence of symbiotic alga *Tetraselmis convolutae*, held beneath the epidermis of the worm. Isolated populations of *S. roscoffensis* span a broad geographical range along the north-eastern Atlantic coast, from Wales to Portugal. The only known population of the worm in the United Kingdom was discovered in Wales decades ago, but very little research has been done on it since. For 13 months, I measured how environmental conditions such as temperature, salinity and light intensity influenced the population size at the Welsh field site. To establish phylogenetic relationships among the different populations and their algal symbionts, new polymerase chain reaction (PCR) oligonucleotides were designed to assess the nucleotide diversity of the mitochondrial cytochrome c oxidase I subunit (COI) gene in gDNA extracted from worms across their known range (Wales, France, Portugal, Spain, and Guernsey). I also targeted the 18S rRNA gene of their algal symbiont, *Tetraselmis convolutae*. Temporal shifts in environmental factors coinciding with fluctuating worm colony size were observed, notably temperature. Based on the molecular data, the worm exhibited different ecotypes across locations, while the algal symbiont showed little genetic variation.

4.2.1 Introduction

Symsagittifera roscoffensis (previously *Convoluta roscoffensis*) is an Acoel in the phylum Xenacoelomorpha (previously Platyhelminthes; ITIS, 2019), and it lives within the intertidal zone. First taxonomically described over a century ago, it was termed a "plant animal" due to its symbiotic relationship with the chlorophyte alga *Tetraselmis convolutae* (von Graff, 1891). An adult is 2-4 mm in length, lacks a defined coelom (Bailly *et al*., 2014), and is characterised by a vivid green colour due to its algal symbiont (Douglas, 1983; Arboleda *et al.*, 2018). The relationship between *T. convolutae* and *S. roscoffensis* provides the host with all of its nutritional needs and no heterotrophic feeding is known to take place (Bailly *et al*., 2014; Arboleda *et al*., 2018; Chapter 6). While symbiosis can occur with other members of the Genus *Tetraselmis* in the laboratory, suboptimal algal species can result in increased mortality (Arboleda *et al*., 2018;Chapter 6). Horizontal transfer of algae between adults and juveniles occurs after birth and not vertically via the parental line (Bailly *et al*., 2014; Provasoli *et al*., 1968).

Since its first discovered in Roscoff in 1879 (Geddes, 1879), populations of *S. roscoffensis* have been reported in Wales, France, the Channel Isles, Spain and Portugal (Jondelius *et al*., 2011; Carvalho *et al*., 2013; Bailly *et al*., 2014; Franks *et al*., 2016; Mettam 1979). The intertidal zone, where *S. roscoffensis* resides, is a dynamic environment. Inhabitants are often exposed to prevailing weather conditions during low tide, and surrounding conditions can change rapidly as a result. The only sighting of the worm in the UK was in Limpet Bay in East Aberthaw, Wales (Mettam 1979). The beach is rocky with limited amounts of sand; the rocks are primarily limestone arising from the falling cliff-face of the Jurassic coastline (Figure.4.1A and 4.1B). Open patches of sand are rare on the beach, instead clear spaces are dominated by thick clay and no sand (in some places the rocks are upon the clay with no visible sand underneath). Rocks in the area where worms are found are also small and have been eroded by the sea. Larger rocks are in place behind *S. roscoffensis*' habitat closer to the cliff face. *Symsagittifera roscoffensis* is found in the small pools of water, between these rocks where the underlaying substrate is sand. Upon discovery of a population at this Welsh site, Mettam (1979) speculated that S. *roscoffensis* had made its way up the Bristol Channel from the nearest population in the Channel Islands; however, there has been no genetic data on the intra-specific diversity of *S. roscoffensis* to confirm or refute this hypothesis.

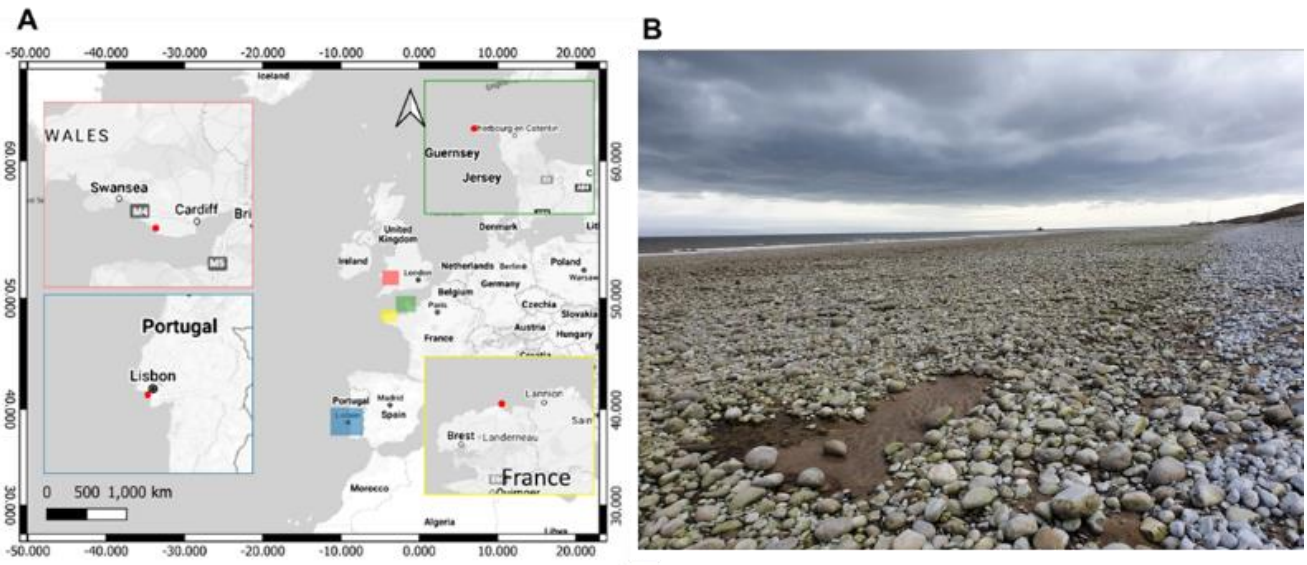

Figure 4.1. Locations of *Symsagittifera roscoffensis* populations used in the present study. *Symsagittifera roscoffensis* populations exist along the Atlantic cost (**A**). Limpet Bay (red), East Aberthaw (Wales, UK) represents the study site; green, Alderney, Guernsey; blue, Costa da Caparica, Portugal; yellow, Roscoff, France. (**B**) Image of Limpet Bay (Wales, UK). Worms are located along the tide mark where there is a distinctive colour change in the rocks (from intertidal to supratidal).

Newly emerged worms are aposymbiotic, i.e. lacking the *T. convolutae* symbiont. Survival and successful establishment of a new colony requires the worms to acquire the algal symbionts post-hatching (Oschman, 1966; Douglas and Gooday, 1982). Symbiotic *T. convolutae* may travel with the founder worm population and be released upon the worm's death and establish a local supply of algal cells or may already be present in the substrate upon the worm's arrival. The ability of the worms to acquire the algae externally from the environment may lead to distinct clades of *T. convolutae* being associated with different populations of *S. roscoffensis* (Riewluang and Wakeman, 2023), it may also be the case that alternative symbionts are associated with distinct populations. The first to suggest this was Mettam (1979) and Macfarlane (1982), stating that individuals of the Welsh site indeed formed a symbiotic relationship with a different local species of *Tetraselmis*, with early results suggesting that the alternative symbiont could be found in up to 55% of individuals. The variation between the preferred symbiont *T. convolutae* and an alternative symbiont reported by Mettam (1979) and Mcfarlane (1982) was dependent upon its location along the intertidal zone. However, there were no reports of mixed symbionts within a single worm. The authors distinguished the different algal symbionts by microscopy, based on differences in the shape

of the pyrenoid between species. Macfarlane (1982) suggested the reason for differences in symbiont profiles was due to the preferred *Tetraselmis* being less abundant at the Welsh field site.

The site in Wales is the most northerly location of *S. roscoffensis'* known distribution (Mcfarlane, 1982), but the literature provides scant information regarding this colony. To address this knowledge gap, I measured environmental parameters and population characteristics of the worm at the Welsh site for a period of 13 months. Secondly, I isolated worm DNA from geographically distinct populations (Wales, Guernsey, France, Portugal) and used the cytochrome c oxidase I gene to determine population relatedness. Lastly, using the same worm extracts, I probed DNA from the algal symbionts of *S. roscoffensis* collected from Wales, Guernsey, France and Portugal using the 18S rRNA gene to confirm the identity of the algal symbiont.

4.3.1 Materials and Methods

4.3.2 Field data

Data collection started in August 2020 and ended in August 2021. Twice per month I collected measurements at the field location in East Aberthaw, Wales (GIS:51.38158, -3.36363). I selected six colonies along a transect at the habitat range of *S. roscoffensis*; each colony was assigned a permanent marker point. I then returned to the same point each time to measure colony size, temperature, salinity, and light intensity. During the entire study period (13 months), and subsequent visits for sample collections and observations, during this period I noted that low or high tides did not seem to cover the colonies at any point.

About 50 ml of sea water was collected as close as possible to each marker, and the water temperature was measured immediately with a thermometer (Silverline digital). Salinity was measured with a refractometer (D-D True Seawater). Light intensity was measured using an Apogee MQ-500 Quantum meter placed directly above the worm colony. Environmental data are presented as mean ± s.e for each month (at least 6 technical measures were taken across the site on a given sampling day).

Photographs of each colony were taken alongside a reference object for scale, images were processed using Image J software. I then set the scale in Image J based on the reference object in the picture, then measured the size of each colony as surface area. The individual colony size measurements were summed to calculate the cumulative colony size, yielding two

cumulative colony size measurements per month. Population size data are expressed as cumulative colony size ± s.e for each month.

4.3.2 Worm collection and DNA extraction

Samples of *S. roscoffensis* collected from France, Guernsey, and Portugal (Figure. 4.1A) were preserved in 70% ethanol and stored at -80 °C upon arrival and prior to gDNA extractions. Samples from Wales were collected at East Aberthaw (see Sect. 4.3.3), maintained temporarily using culture conditions described by Chapter 2, salinity of 30 enriched with 10 ml per L of Guillards solution (Guillard and Ryther, 1962) (f/4), 14.5 ºC, 16 L/8D, and 69 µmol m^{-2} s^{-1}. DNA was extracted from live worms collected from the Welsh field site only.

Worms suspended (approx. 50 individuals) in 1.5 ml of f/4 medium, seawater or ethanol were centrifuged at 10 x *g* for 10 min at room temperature (~ 22 °C); the pellet was retained, and the supernatant was discarded. Each preparation was probe for both the worm (*cox1*) and algal (18S rRNA) gene targets. Genomic DNA was extracted using a Qiagen blood and tissue kit (https://www.qiagen.com). The manufacturer's protocol was followed with a minor amendment to the lysis time: Fresh samples were incubated at 56 °C for 10 min with 15s vortexing every 5 min. For each of the samples preserved in 70% EtOH and frozen at -80 °C, the incubation time was increased to 1 h with intermittent vortexing for a maximum duration of 15s. Post gDNA purification, elutants were assessed for potential contaminants (salt, protein) using the Nanodrop Spectrophotometer.

4.3.3 Targeting of the cytochrome oxidase I and 18S rRNA genes

Amplification of both genes was achieved using end-point PCR. For the host *S. roscoffensis,* I targeted the cytochrome oxidase I gene (*cox1*), whereas for the algal symbiont, I targeted the 18S ribosomal RNA gene (18S rRNA). *Cox 1* amplification was performed using newly designed oligonucleotide primers (synthesised by Eurofins, Ebersberg, Germany): Forward, 5′-GCTTATAATGTGGTRATTACTGCTC–3′, and Reverse, 5′-CAGTAAGAAGTATTGTAATACCTCCTGC-3′. These primers were selected following multiple alignments (Clustal Omega; https://www.ebi.ac.uk/Tools/msa/clustalo/), and scrutiny, of available sequences for *S. roscoffensis* (HM233750, FR837904) and *Convolutriloba retrogemma* (EU710942, EU710925) in GenBank. Each PCR reaction was carried out in a total of 25 µL using 2X Master Mix (New England Biolabs), containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 25 units/ml *Taq* DNA Polymerase, 1 µl of each primer at 10 mM working stock and ~ 190 ng template DNA per reaction. Thermocycling conditions consisted of an initial

denaturation step of 94 °C for 2 min, followed by 34 cycles of 94 °C for 30 s, 57 °C for 1 min and 72 °C for 1 min, prior to a final extension step of 72 °C for 5 min (post PCR, samples were stored at 4 °C).The algal 18S rRNA gene was amplified using published primers and thermocycling conditions; Forward: 5′-GCGGTAATTCCAGCTCCAATAGC–3′ and Reverse: 5′-GACCATACTCCCCCCGGAACC-3′ (Lim *et al.*, 2012). PCR reactions were carried out in a total of 25 µL reaction volume using 2X Master Mix (New England Biolabs), containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 25 units/ml Taq DNA Polymerase, 1 µl of each primer at 10 mM working stock and ~ 152 ng template DNA per reaction. An initial denaturation step of 94 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 1 min, prior to a final extension step of 72 °C for 10 min (post PCR, samples were stored at 4 °C).

PCR-derived amplicons of the expected size for *cox1* (478 bp) and 18S (549 bp) were confirmed using 2% (w/v) agarose gel electrophoresis and a NZYDNA ladder V (www.nzytech.com) ranging from 100 to 1000 bp. Both the *cox1* and 18S rRNA gene targets were cleaned-up prior to sequencing using a Thermo-Scientific EXOSAP kit, post amplification DNA yields were confirmed using a Qubit fluorometer (Thermo Fisher Scientific). Samples were sent to Eurofins genomics (Ebersberg, Germany) for Sanger sequencing using both forward and reverse reactions.

4.3.5. Sequence identity and phylogenetic analyses

Eight *cox1* sequences (two per worm) were used to construct consensus sequences. For the algae, 14 samples were sent for sequencing (18S rRNA), but two failed to produce reliable data. All resolved DNA sequences were inspected and trimmed manually of their primer regions. BLASTn for the 18S rRNA gene and BLASTX for the *cox1* gene search algorithms (Altschul *et al.*, 1990) were used to confirm sequence identities (top three matches for each are listed in Tables 4.1 and 4.2). For the *cox1* target, I gathered a broader set of 24 reference sequences from the Convolutidae (GenBank), which were added to the newly generated sequences herein (GenBank: OQ536360 to OQ536363), yielding 28 in total, each spanning ~ 424 nucleotides (or 140 amino acids) (See Table 4.3). For the 18S rRNA target, 51 reference sequences retrieved from GenBank covering multiple algal genera (e.g., *Tetraselmis*, *Dunaliella*, *Chlorella*) were added to the new sequences (GenBank: OQ538146 to OQ538151), yielding 57 in total (See Table 4.3).

Table 4.1. The top 3 BLAST results for *cox 1* for *Symsagittifera roscoffensis*.

Obtained sequence (query length)	E-value	Coverage	Identity	GenBank accession number	Sequence match
Symsagittifera roscoffensis from south Wales (431bp)	2e-68	98%	90.14%	FR837904.1	*S. roscoffensis*
	7e-68	98%	88.03%	LC515766.1	*Symsagittifera* sp.
	7e-68	98%	88.03%	LC515768.1	*Symsagittifera* sp.
Symsagittifera roscoffensis from Portugal (431 bp)	3e-64	99%	86.86%	FR837904.1	*S. roscoffensis*
	5e-64	99%	86.43%	LC515766.1	*Symsagittifera* sp.
	8e-64	99%	86.43%	LC515768.1	*Symsagittifera* sp.
Symsagittifera roscoffensis from Guernsey (426)	9e-68	99%	89.36%	FR837904.1	*S. roscoffensis*
	2e-67	99%	87.23%	LC515766.1	*Symsagittifera* sp.
	3e-67	99%	87.23%	LC515768.1	*Symsagittifera* sp.
Symsagittifera roscoffensis from Roscoff (426)	8e-79	99%	90.07%	FR837904.1	*S. roscoffensis*
	2e-76	99%	87.23%	MZ519776.1	*Symsagittifera schultzei*
	1e-75	99%	89.36%	NC014578.1	*S. roscoffensis*

Table 4.2. BLAST results for 18S rRNA gene from the algal symbionts of *Symsagittifera roscoffensis*.

Obtained sequence (query length)	E-value	Coverage	Identity	Accession number (GenBank)	Sequence match
Tetraselmis convolute from Welsh population	0.0	100%	100%	MK542679.1	*Tetraselmis* sp.
	0.0	100%	99.29%	KY054995.1	*T. marina*
	0.0	100%	99.29%	KX904704.1	*T. rubens*
Tetraselmis convolute from Portugal	0.0	99%	100%	KT860914.1	*T. convolute*
	0.0	99%	100%	KT860913.1	*T. convolute*
	0.0	99%	99.79%	EF526921.1	Uncultured marine organism
Tetraselmis convolute from Roscoff	0.0	99%	99.80%	KT860914.1	*T. convolute*
	0.0	99%	99.80%	KT860913.1	
	0.0	99%	99.60%	EF526921.1	
Tetraselmis convolute CCAP?	0.0	100%	100%	MT489380.1	*Tetraselmis* sp.
	0.0	100%	100%	MT489359.1	*Tetraselmis* sp.
	0.0	100%	100%	MN721295.1	*T. tetrathele*

Table 4.3. The convolutidae and algae sequences used to reconstruct phylogenies.

Convolutidae species	GenBank id	Gene target	Algae species	GenBank id	Gene target
Symsagittifera roscoffensis	OQ536360 OQ536361 OQ536362	COI	*Tetraselmis convolutae*	OQ538146 OQ538147 OQ538148 OQ538149	18S rRNA

	OQ536363 FR837904.1 NC014578.1			OQ538150 OQ538151 KT860913.1 MT982710.1	
Symsagittifera schultzei	MZ519776.1	COI	Tetraselmis chuii	JQ423150.1 DQ207405.1	18S rRNA
Symsagittifera psammophila	FR837903.1	COI	Tetraselmis suecica	FJ559381.1 JQ423151.1	18S rRNA
Symsagittifera sp.	LC515767.1, LC515766.1, LC515768.1	COI	*Tetraselmis* sp.	FJ559406.1 JQ423158.1 FR744761.1 MH055453.1 AJ431370.2 MH055456.1 KT860916.1 MH055454.1 KT860627.1 KT860876.1 AB058392.1 MH071711.1 MH055448.1 MH055449.1 MH055452.1 MH055444.1 KX998797.1	18S rRNA
Convolutriloba macropyga	EU710922.1	COI	*Tetraselmis striata*	GQ917220.1	18S rRNA
Convolutriloba hastifera	EU710926.1	COI	*Tetraselmis gracilis*	KP662695.1	18S rRNA
Convolutriloba retrogemma	EU710924.1	COI	*Tetraselmis astigmatica*	JN376804.1	18S rRNA
Convolutriloba longifissura	FR837853.1	COI	*Dunaliella primolecta*	DQ009764.1	18S rRNA
Convoluta convoluta	FR837852.1	COI	*Dunaliella salina*	EU589199.1 JQ423154.1	18S rRNA
Praesagittifera naikaiensis	LC515740	COI	*Dunaliella* sp.	FJ164062.1	18S rRNA
Stomatricha hochbergi	FR837902.1	COI	*Chlorella vulgaris*	FR865683.1	18S rRNA
Amphiscolops bermudensis	FR837839.1	COI	*Chlorell*a sp.	JQ423156.1 JF950558.1	18S rRNA
Heterochaerus blumi	FR837864.1	COI	*Chaetoceros muellerii*	AY485453.1 AY625896.1 JQ423153.1	18S rRNA
Anaperus gardineri	FR837840.1	COI	*Chaetoceros calcitrans*	DQ887756.1	18S rRNA
Neochildia fusca	FR837876.1	COI	*Chaetoceros* sp.	FR865488.1	18S rRNA

Anaperus rubellus	FR837841.1	COI	*Nannochloropsis oceanica*	FJ896231.1 HQ710567.1	18S rRNA
Anaperus singularis	FR837842.1	COI	*Nannochloropsis* sp.	HQ710568.1 JQ423160.1	18S rRNA
Archaphanostoma sublittoralis	KM527303.1	COI	*Nannochloropsis oculata*	GU220364.1	18S rRNA
Parahaploposthia cerebroepitheliata	MZ519768.1	COI	*Porphyridium purpureum*	KR904907.1	18S rRNA
Daku woorimensis	FR837854.1	COI	*Pavlova lutheri*	JQ423159.1 JF714238.1	18S rRNA
Diopisthoporus sp.	FR837856.1	COI	*Pavlova salina*	AF106059.1 JQ423155.1	18S rRNA
			Pavlova sp.	JF714245.1	18S rRNA
			Isochrysis galbana	HM149543.1 HQ877903.1 JQ423157.1	18S rRNA

Multiple sequence alignments for the *cox1* and 18S rRNA datasets were performed in MEGA11 using the MUSCLE function (Tamura *et al.*, 2021). Evolutionary reconstructions were performed using the maximum likelihood method (1,000 bootstrap re-samplings) based on either the Tamura 3-perameter model (Tamura, 1992) for *cox1* or the Kimura 2-parameter model for 18S rRNA (Kimura, 1980). DNA substitution models were selected based on the ranked Bayesian information criteria via ModelFinder in MEGA11.

Additionally, the same multiple sequence alignments for *cox1* and 18S rRNA were used to reconstruct Bayesian trees in BEAST (v2.6.7); based on a yule model and MCMC chain length of 10,000,000. I used a burn in rate of 10% to summarise the posterior sample of my trees to produce the maximum clade credibility tree in tree annotator v2.6.7. FigTree software (v 1.4.4 http://tree.bio.ed.ac.uk/software/figtree/) was used to visualise the initial outputs.

Final trees were formatted in iTOL (Letunic and Bork, 2019) using the Bayesian tree topologies (outputs from both maximum likelihood and Bayesian inference were in good agreement).

4.3.6 Statistical analysis

For the environmental data (colony size, temperature, light intensity, and salinity), statistical analysis was performed using a binomial generalised linear model. Normality was confirmed using the R package DHARMa version 4.1.3 (R studio version 1.41717) that utilises the Kolmogorov-Smirnov test.

4.4.1 Results

4.4.2. Population size and environmental conditions

The population of *Symsagittifera roscoffensis* at the Welsh site was at its largest during the spring months, peaking in May with 489.7 ± 27.8 cm^2 (accumulative $\pm$ s.e; Figure 4. 2). During May, water temperature was 19.7 ± 0.2 °C (mean $\pm$ s.e) while salinity was 22 ± 0.7 and light intensity was 1007.4 ± 228.7 µmol m^{-2} s^{-1}. The smallest colony sizes were observed during summer, 28.05 ± 3.1 cm^2 in July, when water temperature was at its highest (27.2 ± 0.8 °C), while salinity was 24 ± 1.6 and light intensity was 1338 ± 169 µmol m^{-2} s^{-1}. Throughout the winter, colony sizes decreased gradually: 206.15 ± 21.6 cm^2 in December, 140.05 ± 22.4 cm^2 in January and 125 ± 23.6 cm^2 in February. Temperature in the winter months ranged from 13.3 to 5.3 °C, salinity was between 23 and 14, while light intensity was between 96.19 and 26.54 µmol m^{-2} s^{-1}. Using binomial generalized linear models (GLMs), the variation in colony sizes between months was deemed significant, and temperature was ranked as a significant predictor variable associated with the colony size of *S. roscoffensis* (Table 4.4).

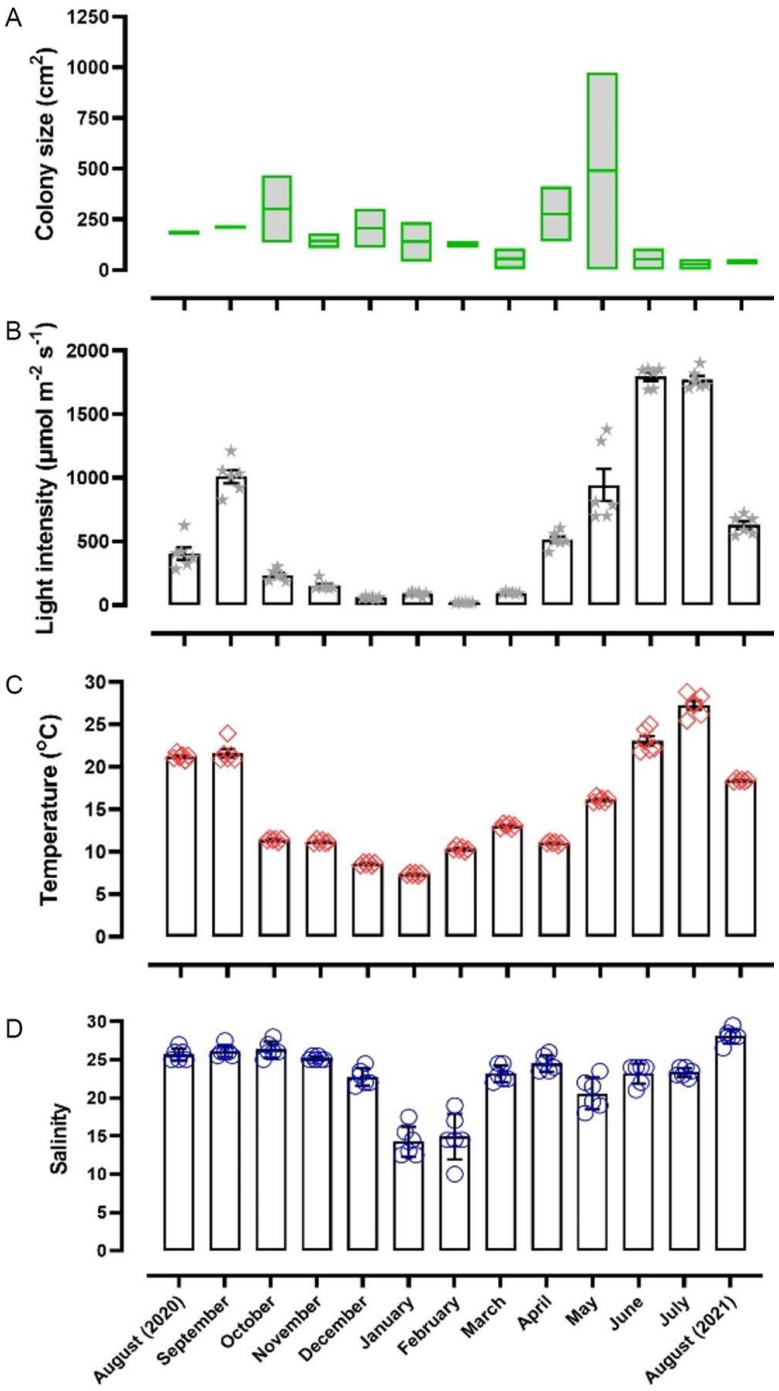

Figure 4.2. Data from the field site in East Aberthaw, Wales for A) cumulative colony size (accumulative ± s.e.) of *Symsagittifera roscoffensis*, B) salinity (mean ± s.e), C) water temperature (mean ± s.e.) and D) ambient light intensity (mean ± s.e), from August 2020-August 2021. There were significant differences between months and concerning the effect of temperature on colony sizes (Binomial, Generalized linear model; $p < 0.05$). Salinity and light intensity did not have a significant effect on colony sizes (GLM; $p > 0.05$).

Table 4.5. Outputs from the binomial generalised linear model for the environmental data.

	Coefficient Estimate	Std. error	Z-value	P-value
Intercept	4.2801590	1.3370863	3.201	**0.001**
Months	0.2908985	0.1249699	2.328	**0.01**
Salinity	-0.0882170	0.0635780	-1.215	0.224548
Temperature	-0.2223250	0.0648652	-3.427	**0.0006**
Light intensity	0.0007326	0.0005339	1.372	0.170023

4.4.3 Phylogenetic analyses of worm ecotypes and their algal symbionts

4.4.3.1. *S. roscoffensis*

BLASTX searches of the *cox1* sequence amplified from the Welsh worms shared ~ 90% identity to S. *roscoffensis* collected from Spain in 2010 (GenBank acc. No. FR837904.1). Worms from Portugal, Guernsey and those re-sequenced from France were ~ 88 to > 90% similar to the same sequence from Spain (FR837904.1). In a recent publication of the genome of *S. roscoffensis*, Martinez *et al.* (2023) stated that the population of the worms have a high level of heterozygosity; however, they did not reassess the mitochondrial genome. Independent evolutionary analyses of the *cox1* gene from the Convolutidae – using both maximum likelihood (ML) and Bayesian approaches – yielded trees of near identical topology (Figure. 4.3). All worm sequences I generated from this study, and existing sequences for France (Roscoff) and Spain (Galiza), formed a highly supported clade with 99% ML bootstrap support and a Bayesian posterior probability (BPP) of 1 (Figure. 4.3). This clade bifurcated (BBP = 0.99) between the sequence from Portugal (OQ536361) and all the other sampling locations (Figure. 4.3), and further separated the sequence from Spain (FR837904; BBP = 0.99) to those from Wales, France, and Guernsey.

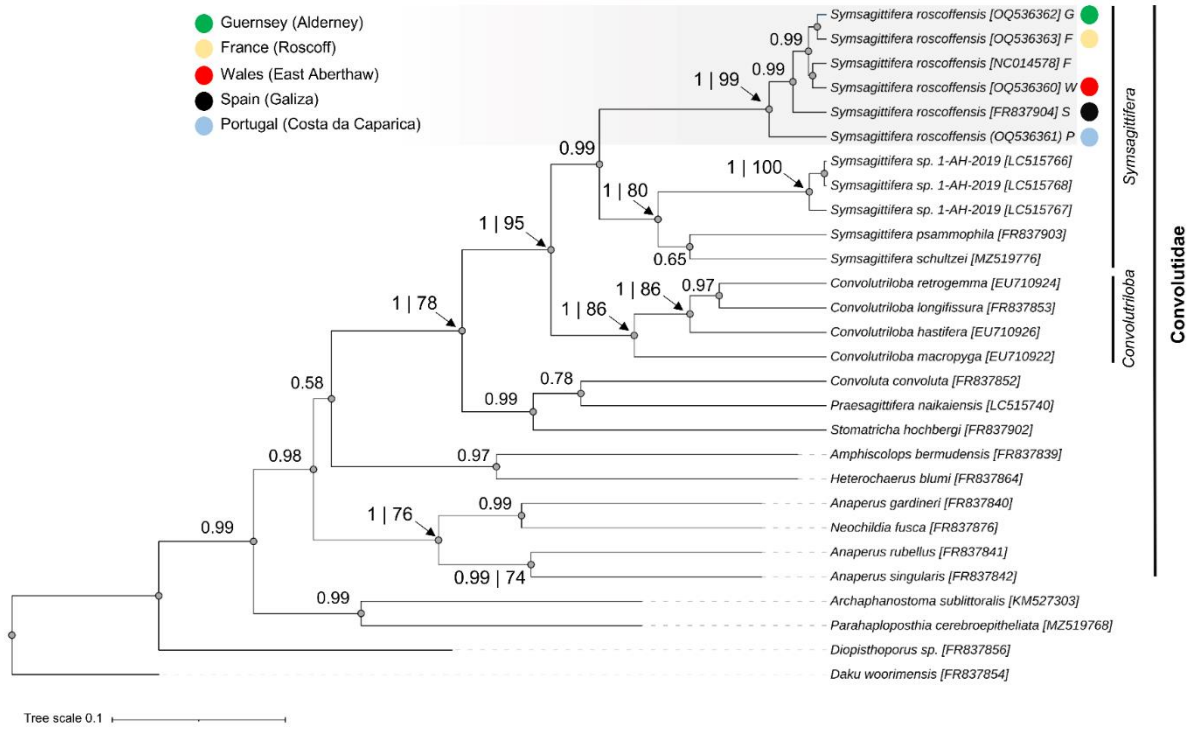

Figure 4.3. Bayesian phylogenetic tree of the partial cytochrome c oxidase subunit 1 gene from marine flatworms (Convolutidae). Bayesian posterior probability (BPP) >0.5 and maximum likelihood (ML) bootstrap support >70 (from 1000 resamplings) are placed beside the respective node. The tree is rooted with a COI sequence from *Daku woorimensis* (FR837854). In total 28 nucleotide sequences were used for reconstructions, with four of those generated from the present study (GenBank: OQ536360 to OQ536363). Coloured circles indicate sample locations for *Symsagittifera roscoffensis*. The scale bar represents nucleotide substitutions per site.

Notably, the two sequences from Roscoff were not identical, one is 10 years old (NC014578), and the other I generated for this study (OQ536363). Closer inspection of the nucleotide sequences revealed three (A to G) substitutions, i.e., transitions (Supp. Figure 1A), which coincided with two hydrophobic amino acid substitutions (i.e., methionine to isoleucine), and one lysine to serine substitution (Supp. Figure. 1B). Looking at all the *cox1*, transitions were the most frequent single point mutations, as expected (Supp. Figure 2).

4.4.3.2 Algal symbionts (*Tetraselmis* spp.)

The 18S rRNA sequences retrieved from algal symbionts in the Welsh worms had 100% similarity to *Tetraselmis sp.* (MK542679.1) from Roscoff, France (2019 sample) (Figure 4.5.). Resident algae from worms located in Portugal, Roscoff and Guernsey shared 99.6-100%

sequence identity to *T. convolutae* (KT860914.1). Algae initially extracted from living *S. roscoffensis* and subsequently grown in culture at Swansea University for ~ 6 months had a similarly high identity (99.8%) to *T. convolutae* (KT860914.1) – again, from Roscoff, France (2015). Interestingly, the partial 18S rRNA gene from *T. convolutae* – an archived sample from CCAP66/36 – shared 100% similarity to *Tetraselmis* sp. SMS19 (MT489380.1).

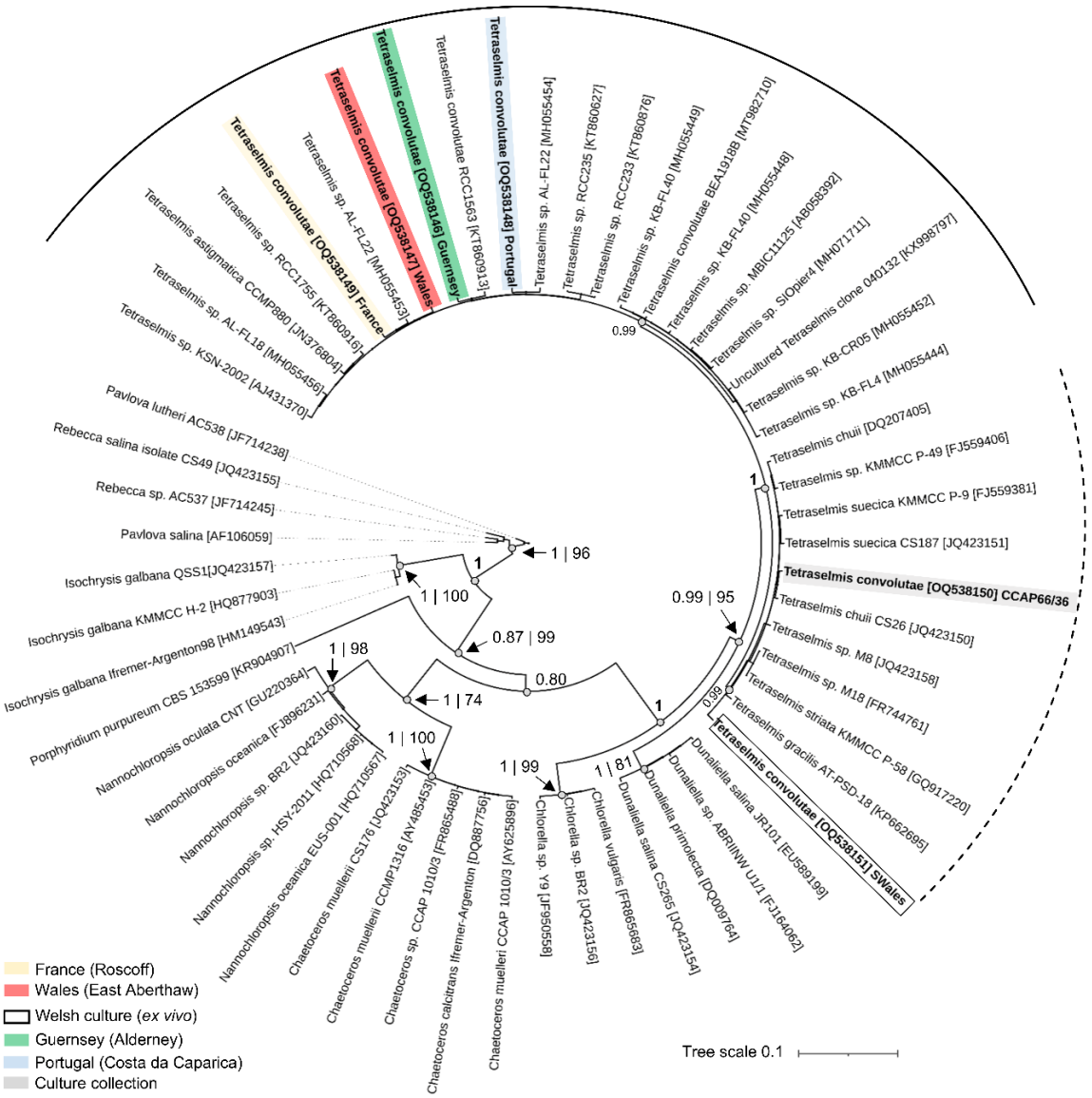

Figure 4.5. Bayesian phylogenetic tree of the partial 18S rRNA gene from algae. Bayesian posterior probability (BPP) >0.80 and maximum likelihood (ML) bootstrap support >70 (from 1000 resampling's) are placed beside the respective node. The tree is rooted with the 18S rRNA sequence from *Pavlova lutheri* AC538 (JF714238). In total 57 nucleotide sequences were used for reconstructions, with six of those generated from the present study (GenBank: OQ538146 to OQ538151). Coloured circles indicate sample locations for *Symsagittifera roscoffensis* (containing algal symbionts). The scale bar represents nucleotide substitutions per site. The black lines, continuous and broken, represent two putative subclades within the *Tetraselmis* genus.

Independent evolutionary analyses of 18S rRNAs gathered from diverse algal genera, using both maximum likelihood (ML) and Bayesian approaches, produced trees with consistent topology (Figure 4.5). The genus *Tetraselmis* was highly supported, distinct to *Dunaliella* (ML = 81%; BPP = 1), and both of which formed an independent clade to *Chlorella* (ML = 99%; BPP = 1). The sequences isolated from France, Portugal, Guernsey, and Wales formed a large, highly supported clade (BPP = 0.99) with *T. convolutae*, some uncultured species, and one *T. astigmatica* sequence (JN376804.1). These data clearly indicated the algal symbionts of worms from all locations represent *Tetraselmis convolutae*. Interestingly, both 18S rRNA sequences from the culture collection (*T. convolutae* CCAP66/36) and Swansea University short-term culture formed a diverse clade (BPP = 0.99) with all the other *Tetraselmi*s species; *T. striata*, *T. gracilis*, *T. chuii* and *T. suecica* (Figure 4.5), indicating some potential contamination from sub-culturing, or the presence of an algal consortium associated with *S. roscoffensis*.

4.5.1 Discussion

A 13-month field campaign monitoring the environmental conditions of *S. roscoffensis* colonies in the least studied population of its known distribution, i.e., Wales, UK. Additionally, I gathered DNA from Welsh worms as well as those from other populations and assessed the genetic relatedness between populations (using *cox1*) and its algal symbiont (using 18S rRNA).

4.5.2 Environmental influences on *S. roscoffensis* colonies

Originally, I expected to see the largest colony size during the summer months (Arboleda *et al.*, 2018; Bailly *et al.*, 2014; Douglas, 1985), as this would be the period of maximum photosynthetic activity, hence growth of the worms. I also expected that during the winter months, there would be few to no worms because of the harsh environmental conditions. Findings showed that during the winter months when both water temperature and light intensity dropped precipitously (below 10°C and below 100 µmol m^{-2} s^{-1}, respectively), colony sizes decreased but remained at sizeable numbers at the Welsh site (Figure 4.2), suggesting that the worms were able to survive the winter condition and remain active.

Contrary to my expectation, during the summer months, population sizes reduced to lower levels than those observed in the winter months. During this period, the worms were exposed to ambient water temperature reaching >27°C, for comparison the average ocean temperature for the same month was 17.7°C (Accessed: 26-10-2023. World Sea Temperatures 2023). The worms also experienced very high light intensities (> 1000 µmol m^{-2} s^{-1}) that could have resulted in photoinhibition (Androuin *et al.*, 2020). At low tides, the worms were often

trapped in small pools of water only 2-10 cm deep and 1-40 cm wide; these small water bodies offered little buffering capacity against environmental stresses. Multiple worm colonies were present along the edge of the supratidal zone at the Welsh Site (Figure 4.1B). During field sampling, it was noted that the average incoming tide at this location did not reach the supratidal zone; therefore, these colonies would be exposed to extended periods of temperature or osmotic stresses and photoinhibition during photosynthetically active hours. While colonies of the worm would not normally survive without additional water from the incoming tide (i.e., desiccation), the unique location of the Welsh population means that it is situated in front of a saltmarsh. This could act as a potential saltwater source keeping the worms' substrate wet in the absence of tidal water. I speculate that this could be the reason why the Welsh population is limited to one area of the beach.

The field data of the current study indicated that colony sizes increased steadily during the spring months (March-May; Figure 4.2). During this period, salinity varied little and the temperature was between 15-20 °C, which was comparable to the reported optimal temperature for the worm in the laboratory study (10-20°C;Chapter 2). Salinity did not seem to have an overall effect on colony sizes in situ, which complements my previous work on worm photosynthetic output in vitro (salinity variation from 20 to 40 had little effect on photosynthetic rates; Chapter 2). It is unsurprising that organisms living within the intertidal zone are adapted to deal with fluctuating salinity. Light intensity steadily increased from < 100 to ~ 1000 µmol m^{-2} s^{-1}, which would have allowed the worm to increase photosynthetic activity and growth, thereby increasing their abundance. However, once temperatures exceeded 20 °C, light intensity exceeded 1200 µmol m^{-2} s^{-1} in the summer months (June-August), colony sizes decreased sharply (Figure. 4.2), suggesting that the environmental conditions became too stressful for the worm.

From my BLR model, temperature was ranked as the most likely predictor of colony size. A decrease in temperature during the wintertime could coincide with a reduction in worm populations. Temperature flux beyond the thermal (optimal) range can adversely affect the photosynthetic output of *S. roscoffensis*. Under laboratory conditions, oxygen production decreased by > 50% when temperature was raised from 14°C to 30°C (Chapter 2). Data from the field site suggests that populations of *S. roscoffensis* survive the seasonal variation in conditions at the site, reflecting broad environmental plasticity (Chapter 2). Intraspecific variation in temperature tolerance between the different geographical populations of *S. roscoffensis* is likely.

4.5.3 Molecular and phylogenetic assessments of *S. roscoffensis* and their algal symbionts

Using the *cox1* mitochondrial gene, both maximum likelihood and Bayesian phylogenetic reconstructions placed all the *S. roscoffensis* samples together. There were some subtle differences between the worm populations tested here (Figure 4.3). My data goes some way to addressing the limited molecular and biogeographical information available for *S. roscoffensis*. First, the worms from Portugal were the most distantly related to those from France and Guernsey, followed by Spain, although they shared a common ancestor. Second, if I speculate that the worms from France were the founder population (first discovered in Roscoff), the species has developed distinct ecotypes as it spread north (Guernsey and Wales), and south (Spain and Portugal). Interestingly this gradient complements the ecological conditions that populations would experience at their respective locations. For instance, worm populations in Spain and Portugal would experience on average higher mean temperatures and longer day lengths in comparison to populations further north. While the sequences retrieved from Wales, France and Guernsey formed a subclade within the species (Figure 4.3), they further placed the Welsh worms and a 10-year-old sequence from Roscoff together, whereas the two sequences I retrieved from Roscoff and Guernsey in this study were clustered. The sequences that were generated from worms taken from the Roscoff site did not show an identical match to those already in GenBank. Given the fact that the sequences in the data base are >10 years apart, I suspect that these differences were due to single nucleotide polymorphisms (SNPs) within my sequences. Transitions between A/Gs and C/Ts occur regularly in such populations. The substitution of methionine to isoleucine is considered a "safe" substitution and does not result in a conformational change in protein structure (Bordo and Argos, 1994; Ohmura *et al.*, 2001); therefore, it is likely to persist in the population. Non-deleterious SNPs are also known to accumulate in populations that have little to no gene flow between populations, acting as a driver for natural selection, such as the case of the worm populations (Ferchaud *et al.*, 2020). Considering the disparate geographical areas of the worm's known distribution, it is probable that some populations exist but have yet to be discovered. The worms have very limited mobility and are unlikely to swim across large distances. Isolation by distance also occurs to even larger, more mobile marine species such as reef fish and invertebrates due to restriction by physical barriers such as ocean currents (Planes and Fauvelot, 2002; Johannesson *et al.*, 2010). In other marine invertebrates that are isolated by distance, the ecotypes that form become locally adapted to conditions, for instance, temperature

or salinity (Johannesson and André, 2006; Barrett and Schluter, 2008). This may also be the case for *S. roscoffensis* given the fact that different populations span large geographical and environmental gradients and as such, each *S. roscoffensis* population would be adapted to local conditions and vary in their tolerances to environmental conditions. Of course, further field data is needed to attribute local environmental conditions to local adaptations for known populations of *S. roscoffensis*.

Concerning the algal symbiont, the results are more straightforward. *T. convolutae* is specific to all worms across all locations tested (Figure.4.6). While *S. roscoffensis* in a laboratory setting can be manipulated to expel and switch its algae symbiont (Dupont *et al*., 2012; Arboleda *et al*., 2018;Chapter 6), in the field I did not find any evidence that supports a more diverse symbiont profile other than *T. convolutae*. The current findings contradict Mettam (1979) and Macfarlane (1982), who both claimed that populations at the Welsh field site differed in their resident algae. It should be noted that Mettam (1979) and Macfarlane (1982) relied on microscopy, whereas my 18S rRNA data provided arguably more reliable algal species identity. While the worms do not acquire the algae directly from the parents, I have data to suggest that aposymbiotic worms can detect algae within their surrounding environment and move towards it (Chapter 3). Given the fact that the worms will reject any alga in the presence of *T. convolutae,* populations are likely to maintain the same symbiont species across multiple generations (Provasoli *et al*., 1968).

Tetraselmis convolutae CCAP66/36 and my own short-term culture maintained at Swansea University clustered together with diverse *Tetraselmi*s spp. This difference may be due to the fact that algae, when in symbiosis, have slower growth than its free-living counterpart. For instance, algae in symbiosis has a doubling time of between 70-100 days, while free-living algae can double every 3 days (Wooldridge, 2010). Therefore, *T. convolutae* may have a lower growth rate when residing inside the worm and differences seen within my trees may be due to non-deleterious SNPs. Over time, it is also possible that the cultures became contaminated.

4.6.1 Conclusion

Environmental conditions at the Welsh field site coincided with fluctuating *S. roscoffensis* population size, with temperature identified as the main predictor. Representative worms from the disparate populations studied here are distinct ecotypes (or species subtype). Future experiments should look to examine whether the location-specific ecotypes of *S.*

roscoffensis differ from each other in their physiology, behaviour and other traits (local adaptations). The algal symbiont, *T. convolutae*, showed little genetic diversity between the worms sampled, illustrating the intimate relationship between the worms and its symbiont across many generations and locations.

4.7 Supplementary

All DNA sequences generated for Chapter 4 have been deposited in GenBank under the following accession numbers: OQ536360-OQ536363 (worm *cox1*) and OQ538146-OQ538151 (algal 18S rRNA)

1A) DNA sequences:

```
OQ536360        -------------TTTTTTTTTTTGTAATGCCTGTTTTAATAGGGGGTTTTGGTAATTGA  47
NC_014578       GGACTGATTATAATTTTTTTTTTTGTAATGCCTGTTTTAATGGGGGGTTTTGGTAATTGA  240
                              *************************** ******************

OQ536360        TTACTACCCATTATGTTGGGGTGTGCCGATATGGCTTTTCCTCGATTAAACAATTTATCT  107
NC_014578       TTACTACCCATTATGTTGGGGTGTGCCGATATGGCTTTTCCTCGATTAAACAATTTATCT  300
                ************************************************************

OQ536360        TTCTGGCTTCTTCCTCCTAGAATTAGATTATTAATTTTTAGAAAACTTATTGAAACGGGA  167
NC_014578       TTCTGGCTTCTTCCTCCTAGAATTAGATTATTAATTTTTAGAAGACTTATTGAAACGGGA  360
                ****************************************** ***************

OQ536360        GTGGGAACTGGGTGAACATTATACCCGCCTTTATCTAGATTAATTGGACATCAAAGAATA  227
NC_014578       GTGGGAACTGGGTGAACATTATACCCGCCTTTATCTAGATTAATTGGACATCAAAGAATA  420
                ************************************************************

OQ536360        GGTGTAGATTTAGGTATTTTTAGAATACACATTGCAGGAGCTTCCTCTATTGGTGGGTCT  287
NC_014578       GGTGTGGATTTAGGTATTTTTAGAATACACATTGCAGGAGCTTCCTCTATTGGTGGGTCT  480
                ***** ********************************************************

OQ536360        ATTAATTTCTTATGTACAATTAGAAATTTACGATCTCCCGAAATCACTTGAGAAAATCTT  347
NC_014578       ATTAATTTCTTATGTACAATTAGAAATTTACGATCTCCCGAAATCACTTGAGAAAATCTT  540
                ************************************************************

OQ536360        ACCCTTTTTGTATGAGGGGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCTGTA  407
NC_014578       ACCCTTTTTGTATGAGGGGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCTGTA  600
                ************************************************************

OQ536360        TTTGCAGGAGGTATTAC----------------------------------------------  424
NC_014578       TTTGCAGGAGGTATTACAATACTTCTTACTGATCGTAATTTCAATACATCCTTCTTTGAT  660
                *****************
```

1B) Protein sequences:

```
OQ536360         FFFVMPVLMGGFGNWLLPIMLGCADMAFPRLNNLSFWLLPPSISLLIFSKLIETGVGTGW        60
France_NC        FFFVMPVLMGGFGNWLLPIMLGCADMAFPRLNNLSFWLLPPSISLLIFSSLIETGVGTGW        60
                 ************************************************.**********

OQ536360         TLYPPLSSLIGHQSMGVDLGIFSMHIAGASSIGGSINFLCTISNLRSPEITWENLTLFVW        120
France_NC        TLYPPLSSLIGHQSIGVDLGIFSIHIAGASSIGGSINFLCTISNLRSPEITWENLTLFVW        120
                 **************:********:************************************

OQ536360         GVFFTAILLVLSLPVFAGGI140
France_NC        GVFFTAILLVLSLPVFAGGI140
                 ********************
```

Supplementary Figure 1 (Chapter 4). (A) Nucleotide and (B) protein sequence alignments for two worm samples from Roscoff (using Clustal Omega: https://www.ebi.ac.uk/Tools/msa/clustalo/). Single point differences are highlighted yellow.

```
OQ536361_Portugal       TTTTTTTTTTTTGTGATGCCTGTTTTAATAGGGGGTTTTGGTAATTGATTACTACCCATCA   60
FR837904_Spain          TTTTTTTTTTTTGTAATGCCTGTTTTAATGGGAAGGTTTTGGTAATTGATTACTACCCATCA   60
OQ536362_Guernsey       TTTTTTTTTTTTGTAATGCCTGTTTTAATGGGGGGTTTTGGTAATTGATTACTACCCATTA   60
OQ536363_France         TTTTTTTTTTTTGTAATGCCTGTTTTAATGGGGGGTTTTGGTAATTGATTACTACCCATTA   60
OQ536360_Wales          TTTTTTTTTTTTGTAATGCCTGTTTTAATAGGGGGTTTTGGTAATTGATTACTACCCATTA   60
NC014587_France         TTTTTTTTTTTTGTAATGCCTGTTTTAATGGGGGGTTTTGGTAATTGATTACTACCCATTA   60
                        ************* ************** ** ************************** *

OQ536361_Portugal       TGTTGGGGTGTGCTGATATAGCTTTTCCTCGATTAAACAATTTATCTTTCTGACTTCTTC   120
FR837904_Spain          TGTTGGGGTGTGCCGATATGGCTTTTCCTCGATTAAACAATTTATCTTTTTGACTTCTTC   120
OQ536362_Guernsey       TGTTGGGGTGTGCCGATATGGCTTTTCCTCGATTAAACAATTTATCTTTCTGGCTTCTTC   120
OQ536363_France         TGTTGGGGTGTGCCGATATGGCTTTTCCTCGATTAAACAATTTATCTTTCTGGCTTCTTC   120
OQ536360_Wales          TGTTGGGGTGTGCCGATATGGCTTTTCCTCGATTAAACAATTTATCTTTCTGGCTTCTTC   120
NC014587_France         TGTTGGGGTGTGCCGATATGGCTTTTCCTCGATTAAACAATTTATCTTTCTGGCTTCTTC   120
                        ************* ***** ***************************** ** *******

OQ536361_Portugal       CGCCTAGGATTAGATTATTAATTTTTAGAAGACTTATTGAGACAGGAGTAGGAACTGGGT   180
FR837904_Spain          CTCCTAGAATTAGATTATTAATTTTTAGAAAACTTATTGAAACGGGAGTGGGAACTGGGT   180
OQ536362_Guernsey       CTCCTAAAATTAAATTATTAATTTTTAAAAAACTTATTGAAACGGGAGTGGGAACTGGGT   180
OQ536363_France         CTCCTAGAATTAGATTATTAATTTTTAAAAAACTTATTGAAACGGGAGTGGGAACTGGGT   180
OQ536360_Wales          CTCCTAGAATTAGATTATTAATTTTTAGAAAACTTATTGAAACGGGAGTGGGAACTGGGT   180
NC014587_France         CTCCTAGAATTAGATTATTAATTTTTAGAAGACTTATTGAAACGGGAGTGGGAACTGGGT   180
                        * ****  **** ************** ** ********* ** ***** **********

OQ536361_Portugal       GAACACTATACCCACCTTTATCTAGATTAATTGGACATCAAAGAATAGGTGTTGATTTAG   240
FR837904_Spain          GAACATTATACCCACCTTTATCTAGATTAATTGGACATCAAAGAATGGGTGTAGATTTAG   240
OQ536362_Guernsey       GAACATTATACCCGCCTTTATCTAGATTAATTGGACATCAAAAAATAGGTGTGGATTTAG   240
OQ536363_France         GAACATTATACCCCCCTTTATCTAGATTAATTGGACATCAAAAAATAGGTGTGGATTTAG   240
OQ536360_Wales          GAACATTATACCCGCCTTTATCTAGATTAATTGGACATCAAAGAATAGGTGTAGATTTAG   240
NC014587_France         GAACATTATACCCGCCTTTATCTAGATTAATTGGACATCAAAGAATAGGTGTGGATTTAG   240
                        ***** ******* *************************** *** ***** *******

OQ536361_Portugal       GAATTTTTAGAATACACATTGCAGGAGCTTCCTCTATTGGTGGGTCTATTAATTTCTTGT   300
FR837904_Spain          GAATTTTTAGAATACACATTGCAGGAGCTTCCTCCATTGGTGGGTCTATTAATTTCTTAT   300
OQ536362_Guernsey       GTATTTTTAAAATACACATTGCAGGAGCTTCCTCTATTGGTGGGTCTATTAATTTCTTAT   300
OQ536363_France         GTATTTTTAGAATACACATTGCAGGAGCTTCCTCTATTGGTGGGTCTATTAATTTCTTAT   300
OQ536360_Wales          GTATTTTTAGAATACACATTGCAGGAGCTTCCTCTATTGGTGGGTCTATTAATTTCTTAT   300
NC014587_France         GTATTTTTAGAATACACATTGCAGGAGCTTCCTCTATTGGTGGGTCTATTAATTTCTTAT   300
                        * ******* ********************** ********************** *

OQ536361_Portugal       GTACAATTAGAAATTTACGATCTCCTGAAATTACTTGAGAAAATCTTACCCTTTTTGTGT   360
FR837904_Spain          GTACAATTAGAAATTTACGATCTCCCGAAATCACTTGAGAAAATCTTACCCTTTTTGTAT   360
OQ536362_Guernsey       GTACAATTAAAAATTTACAATCTCCCGAAATCACTTGAAAAAATCTTACCCTTTTTGTAT   360
OQ536363_France         GTACAATTAGAAATTTACAATCTCCCGAAATCACTTGAGAAAATCTTACCCTTTTTGTAT   360
OQ536360_Wales          GTACAATTAGAAATTTACGATCTCCCGAAATCACTTGAGAAAATCTTACCCTTTTTGTAT   360
NC014587_France         GTACAATTAGAAATTTACGATCTCCCGAAATCACTTGAGAAAATCTTACCCTTTTTGTAT   360
                        ********* ******** ****** ***** ****** ****************** *

OQ536361_Portugal       GAGGAGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCTGTATTTGCAGGAGGTA   420
FR837904_Spain          GAGGGGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCGGTATTTGCAGGAGGTA   420
OQ536362_Guernsey       GAGGGGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCTGTATTTGCAGGAGGTA   420
OQ536363_France         GAGGGGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCTGTATTTGCAGGAGGTA   420
OQ536360_Wales          GAGGGGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCTGTATTTGCAGGAGGTA   420
NC014587_France         GAGGGGTTTTTTTTACAGCTATTTTATTAGTTCTTTCTTTGCCTGTATTTGCAGGAGGTA   420
                        **** ************************************ ****************

OQ536361_Portugal       TTAC--          424
FR837904_Spain          TTACAA          426
OQ536362_Guernsey       TTACAA          426
OQ536363_France         TTACAA          426
OQ536360_Wales          TTAC--          424
NC014587_France         TTAC--          424
                        ****
```

Supplementary Figure 2 (Chapter 4). Nucleotide sequence alignments for worms from all five locations studied (using Clustal Omega: https://www.ebi.ac.uk/Tools/msa/clustalo/). Single point differences are highlighted yellow.

Chapter 5 : Quantification of algal symbionts in *Symsagittifera roscoffensis* using quantitative PCR (qPCR)

5.1. Abstract

Symsagittifera roscoffensis in an acoel that forms a symbiotic relationship with the alga *Tetraselmis convolutae*. Juvenile worms are born without the algal partner (aposymbiotic) and must acquire the algal partner from the environment. Upon the acquisition of the algal symbiont photosymbiosis is established, as juveniles age then it is assumed that the number of algal cells inside the worms' increases as more algal cells would be required to support the worms' nutritional needs. However, there are conflicting reports on the number of algal cells that are be found inside the worms. I developed a quantitative PCR (qPCR) to determine the number of algae cells inside the worms. I then utilised primers to target the 18s rRNA gene of the algae, while I was able to determine the number of algal cells per individual. Results suggest there was little difference in the number of algal cells between samples. I suspect that the copy number of the 18S rRNA gene varied between samples depending on the growth stage of the algae. As well as this low gDNA yields from the algae resulted in inconsistent amplification between samples. Further work is needed to assess the number of 18S rRNA copy number and optimising the protocol to get consistent gDNA yields in order to accurately use qPCR on the *Symsagittifera roscoffensis-Tetraselmis convolutae* relationship.

5.2.1 Introduction

Photosymbiosis occurs across multiple phyla within the marine environment, the most well-known and well-studied are those in the phyla Cnidaria (corals) and its dinoflagellate symbiont (zooxanthellae), which enables the corals to harness the solar energy to produce food. This relationship is highly effective and provides corals will a large percentage of its nutritional needs (Komyakova *et al.*, 2013). Upon the establishment of photosymbiosis algae cells are incorporated into the coral's own tissues. Determining the number of algal cells inside the hosts remains difficult due to the close relationship of the two (Fabricius *et al.*, 2004; Guillard and Sieracki, 2005). Algal cells in symbiosis are also manipulated by the host so that they can appear phenotypically different as well as have slower growth rates when compared to -living forms (Wooldridge, 2010) The ability of the host to increase or decreases the amount of algal cells in symbiosis also allows the host to adapt to changing environmental conditions such as temperature changes or changes in light intensity and photoperiods (Buddemeier and Fautin, 1993; Mieog *et al.*, 2007). Quantification of these changes could be used as an early indicator to determine the hosts response to changing environmental parameters.

Symsagittifera roscoffensis is a model organism for studying symbiosis (Bailly *et al*., 2014); as an obligated symbiont that forms a symbiotic relationship with the alga *Tetraselmis convolutae*. As is the case with many other photosymbiotic relationships, the algae fulfil the worms' nutritional needs via photosynthesis, however unlike some relationships (corals) no heterotrophic feeding is known to occur in the worms (Bailly *et al*., 2014; Arboleda *et al.*, 2018). Juveniles are born aposymbiotic; therefore, *T. convolutae* must be acquired from the environment (Provasoli *et al*., 1968). During the formation of the symbiotic relationship the alga is incorporated into the upper epidermis of the worms and phenotypic changes occur to the algal cell, such as loss of the cell wall, flagella, and eye spot (Provasoli *et al.*, 1968; Douglas, 1983a). Juveniles are approximately 100 µm (Chapter 6) in size while adults are between 3-4mm in size (Bailly *et al.*, 2014). As the worms increase in age so does the worm's requirements from the algae, the visible difference in size between the juveniles and the adults suggest that the worms increase the number of algal cells. Although no data in the literature can be found to support this suggestion.

Limited information can be found on the number of algal cells that reside in *Symsagittifera roscoffensis*. An early report by Douglas (1983a) indicated that the number of algae cells inside an adult was as little as 10,000 algae cells per adult (Douglas, 1983a), while the more recent study (Bailly *et* al., 2014) suggest that the number of algae cells per individual is in excess of 100,000. As the algae are essential to life cycle of *S. roscoffensis*, quantifying the alga cells during the growth phase of the worms would aid in understanding the symbiotic interaction between the two organisms, and the relative change in algal density during the life cycle of the worms. Such a tool could then be further utilised in exploring the fitness of individuals within a population in response to changing environmental conditions.

Here I developed a qPCR protocol to quantify the number of algal cells that are found inside of the worms. I tested this qPCR protocol on juvenile worms at set time points after the establishment of symbiosis to determine the quantify the number of algal cells. I also used this protocol on adults obtained from different field locations in order to quantify the number of algae cells inside adult worms.

5.3.1 Methods

5.3.2. Maintain master cultures of *symsagittifera roscoffensis*

The same culture methods used for *S. roscoffensis* were the same that have been described in Chapter 2. *S. roscoffensis* were harvested with a pipette and counted to the required number prior to extraction.

5.3.3. Generating aposymbiotic juveniles

In order to generate aposymbiotic juveniles, I used the method proposed by Provasoli *et al*., (1968) and used in Chapter 6. In summary, this involved placing adult worms a petri dish with f/2 (10ml L^{-1}) and sea water at a salinity of 30 and every three days they were checked for the presence of cocoons. The outer membrane of the cocoon was removed with the use of forceps and a scalpel. The embryos were then washed in autoclaved sea water (salinity 30) and transferred to a clean petri dish, where they were incubated in the dark for approximately 1-2 weeks at 14.5°C. Once hatched the worms where then placed into 80 mL of autoclaved sea water enriched with f/4 and 20 mL of algal cells at 275 per µL of *T. convolute* CCAP 66/36 was added and incubated in the same incubator as my master cultures (see Chapter 2 for conditions). At each sampling point, juveniles were harvested, washed with autoclaved sea water and incorporation of the algal cells was confirmed by using an epifluorescence

microscope (Olympus BX43), a lack of red fluorescence (from chlorophyll) under blue light excitation (480 nm) would indicate the absence of symbiotic algae. After washing, worms were centrifuged at 5 g for 5 min, the supernatant was removed, and I stored the worms in a -80 °C freezer, before beginning the extraction process.

5.3.4. Collection of adult worms

A total of 60 worms were collected from the Welsh field site and separated then into 3 Eppendorf tubes each containing 20 worms in 0.5ml of 70% ethanol at -80°C. The same number of adults were obtained from the field site in, Roscoff, France; these were preserved in RNA later. The worms obtained from France were removed from the RNA later, and extraction proceeded for both samples using the Qiagen plant kit.

5.3.5. Calibration curve of *Tetraselmis convolutae*

Tetraselmis convolutae (CCAP 66/36) was grown in a continuous growth phase, in f/2 (20 ml L^{-1}) filtered using autoclaved sea water salinity 30, pH 8.1 serially diluted from the master stock. 1 mL of each dilution was then placed into the flow cytometer (model: BD Accuri C6) to assess the number of algal cells per µL. After which, 10 mL of each serial dilution was transferred to 50 mL Flacon tube and centrifuged at RT at 4600 rpm and for 10 min, the remaining supernatant was removed. The algae cells were resuspended in 1ml of autoclaved sea water and centrifuged at 10 g for 10 min. The supernatant removed, and extraction proceeded with a Qiagen mini plant extraction kit with no deviations from the standard protocol. After extraction, the DNA yield was quantified using a Thermo Scientific, Nanodrop.

5.3.6 qPCR cycling conditions

I used a Qiagen SYBR PCR kit, to perform a qPCR using the following primers of my own design: Forward: *5′- CTTCGATTTCATTGTCAGAGGTG –3′* and Reverse: *5′- GTTTATGGTTGAGACTAGGACGG -3′* with an expected amplicon size of 150bp. The master mix final volume 20 µL consisted of 10 µL of SYBR Green PCR master mix, forward primer 1 µL final concentration of 0.7µM, reverse primer 1 µL at a final concentration 0.7 µM, 3 µL of DNA free water, 5 µL of template DNA at 8 ng/mL.

I used the following cycling conditions 2 mins at 95 °C, 5s at 95 °C, 10s at 60 °C for 35 cycles. After which I performed a melt curve analysis starting at 65 °C and 5 °C incremental increases every 5 s until 95 °C was achieved.

5.3.7. Data processing

Data processing was done in R studio, for the calibration curve I generated a linear model to determine the efficiency of the qPCR amplification for the relationship between the *Ct* values (cycle time) and the serial dilutions. For the juvenile worms I used ANOVA for the number of algal cells between days after confirming the data was normally distributed using the package DHARMa.

5.4.1 Results

The results presented in the calibration curve represent the *Ct* values from a serial dilution of algae cells of 3409, 988, 310, 95, 29, 30, 13 algal cells per µL. When adjusted to count for the starting concentration and volume used in the qPCR reaction the number of algae cells are 17.0, 4.95, 1.55, 0.47, 0.14, 0.15, 0.06 algae cells per µL (Figure 5.1). The relationship between the *Ct* values and the serial dilution can be explained by $Y= 17.0 + 2.2 \times log^{-10}$ (F-value: 5.276, $p=0.07$, R^2: 0.51).

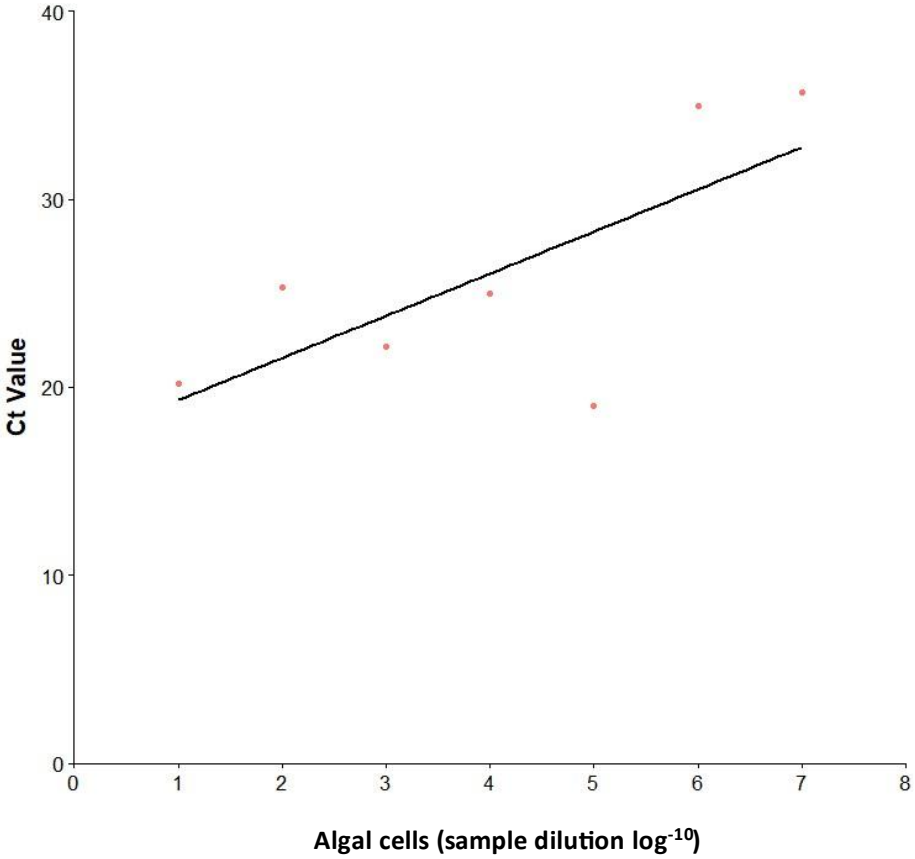

Figure 5.1. **The calibration curve for *Tetraselmis convolute***. Algal cells were diluted using a serial dilution, red dots indicate the respective *Ct* values while the black line indicates a liner model. The relationship between the *Ct* values and the serial dilution can be explained by Y= 17.0 + 2.2 × $\log^{-10}$, F- value: 5.276, p =0.07, R^2: 0.51.

Using qPCR, the number of algae cells at day 2 was 5800 algae cells ind^{-1}. At day 21 this had decreased to 2600 algae cells ind^{-1}, while at day 23 this had increased to 6000 algae cells ind^{-1} (Figure 5.2). There was no significant difference between the number of cells between days (F-value 0.741 p= 0.48).

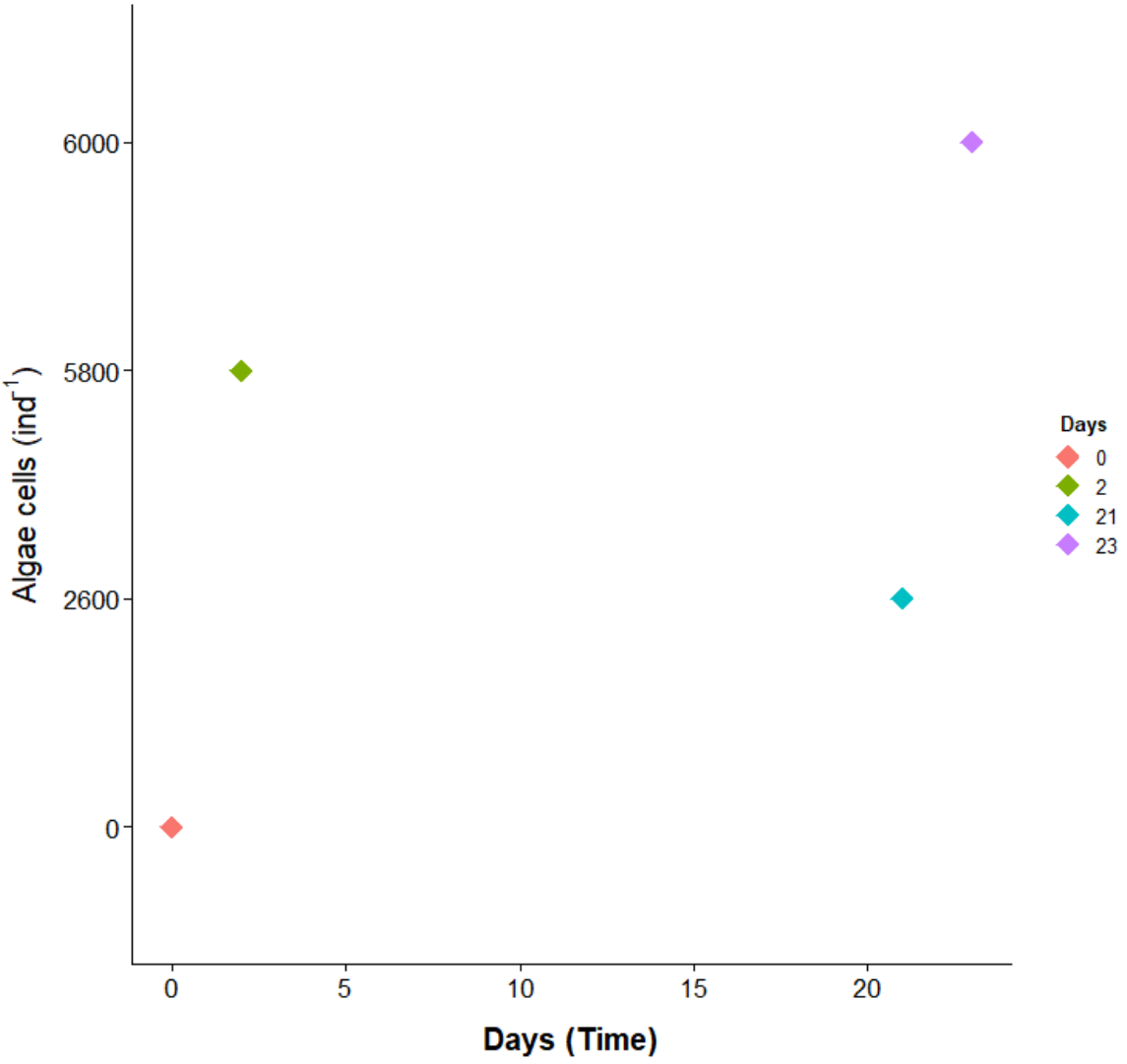

Figure 5.2.The number of algal cells for juvenile *Symsagittifera roscoffensis*. The number of algae cells found inside juvenile worms at day 0, 2, 21, 23. There was no significant difference in the number of alga cells between days (F-value 0.741 *p* =>0.05).

When assessing the algae content of the adults I found that both the adults collected from Wales and France had the same amount of algae cells at 170450 algae cells ind^{-1}.

5.5.1 Discussion

This chapter aimed to use qPCR to quantify the number of algal cells inside of *Symsagittifera roscoffensis.* The results suggest that the number of cells per adult is 170,550 algal cells per ind^{-1}. While the lowest number of alga cells in the juveniles was found to be at 2,600 algae cells ind^{-1} at 21 days, this had increased to 6,000 algae cells ind^{-1} at day 23. However, the low efficiency for the calibration curve suggests there is not an exponential increase in amplification for the number of algal cells between dilutions. When considering the juvenile worm samples, I also found inconsistencies, For instance, at Day 2, I recorded 5800 algae cells ind^{-1} while at day 21 the number of algae cells had decreased to 2600 algal cells ind^{-1}. However, at day 23 it had increased to 6000. This suggests that there is only a 200 algae cell difference between the day 2 and day 21 juveniles. Interestingly as there was no algae cells detected in day 0 after 1 hour of exposure, and I was able to detect algae at day 2, indicating that symbiosis is established within 48h of exposure to the algae. My estimate is that adult worms contain 170,450 algae cells ind^{-1}. My estimation, while larger adults is comparable to that has been previously reported 100,000 algae cells (Bailly *et al*., 2014).

When conducting the qPCR, some difficulties were encountered in obtaining the data. For instance, the qPCR efficiency is low calculated at 22%. This could be due to the fact that obtaining gDNA from algae remains difficult as there are limited extraction kits specifically designed for algae. One possibility as to why qPCR efficiency was low could be due to the presence of proteins and salts not removed fully by the extraction kits. These contaminates can lead to inhibition of the qPCR during DNA amplification. Using the worms for a qPCR reaction is further complicated by the fact that the algae is inside of another organism, and the worms could have been the source of the additional contamination, such as proteins. I also encountered low yields of gDNA when extracting from the algal cells, this could also suggest that the kit was not optimised for algae. For instance, the number of algal cells simply overwhelmed the extraction kit. This resulted in the spin columns becoming blocked and thus reducing the overall gDNA yield. However, lowering the number of algal cells so that the spin columns did not become blocked did not result in a higher yield of gDNA. I also designed my own primers from sequences generated from a previous study (Chapter 4). These primers could have had an off-target binding or low binding efficacy to the target sequence. This would result in low efficiency amplification of the target gene. It is worth nothing that the melt curve showed that the products of the amplification melted at the same temperature 82 °C showing that the target fragment size from the amplification was roughly the same size.

During this experiment I also encountered some technical difficulties, which were out of my control. Ongoing refurbishments to the departments existing infrastructure meant that there were repeated power outages with little to no notice. These power outages resulted in the incubators compressor failing and the temperature quickly increasing. Increasing the temperature rapidly over a short period resulted in death of a large proportion of the juveniles that would have been additional time points for the qPCR. This meant that I was left with a relatively small sample of time points. To further complicate matters the juveniles that I had remaining appeared to very in green colour, this suggests that some individuals are able to establish symbiosis quickly and rapidly increase the number of alga cells quicker than others. With more remaining samples I would have ideally excluded these individuals and instead chosen a sample that was more representative of the population as a whole or increased the number of samples to average out the variation However, this was not possible. This variation between individuals could account for the reason as to why juveniles at day 2 appear to have as much algae as the samples from day 21 (Figure 5.2). From the available literature, there is limited information on the establishment of symbiosis and how individuals effectively initiate and enable symbiosis. However, these changes that I observed in the juveniles may suggest that there is a large individual-individual variation in the worm's ability to effectively find and enable symbiosis between the of the algae.

Prior to initiating the qPCR protocol I also assumed that there was one copy of the 18S rRNA, however based on my own results this seems unlikely. While it is not known how many copies of the target gene *T. convolutae* has, having more copies will result in differences in the number of assumed cells. For instance, if I assumed one copy per cell then I could calculate the number of cells based on my *Ct* values. However, if that number changes, then the number of cells per reaction changes and as I did not know the number of gene copies then we are unable to calculate the number of cells using that method. To address this issue, I intended to compare the juvenile samples against a standard calibration curve. However, unknown to me prior to the experiment, the number of copies of the 18s rRNA gene per alga cell can change depending on the growth stage of the algae. Therefore, this makes it difficult to determine the number of alga cells in the samples without prior knowledge of for how this gene changes in different growth phases from the calibration curve.

Further work trying to quantify the number of alga cells in the *Symsagittifera roscoffensis-Tetraselmis convolutae* relationship should consider optimising the extraction protocol by utilising an additional lysis step or by preprocessing the samples in a bead shaker prior to extraction this would further disrupt the cells allowing for greater gDNA yield. Additional clean up steps should also be considered so that the maximum amount of protein can remove while achieving the highest gDNA yields. With regards to the copy number sequencing of the genome of *T. convolutae* is one of the best ways to determine the number of copies of the 18S rRNA gene. However, this is not always possible, therefore the number can be determined by producing a calibration curve using an alga that has been sequenced. In such as case the copy number can be compared to the reference algae. Once the copy number is known for the gene I could quantifying the amount of gDNA and I could make comparisons of algae in different growth stages to calculate how the 18S rRNA gene changes at different growth stages.

5.6 Conclusion

While I made progressing in utilising qPCR to determine the number of algae cells, I did however, encounter problems in developing this method. Algae genetics is complex due to the differences in the gene copy numbers not only between individuals in different growth phases, but also between difference species. While direct comparisons between closely related species is further complicated by genome organization, and gene duplication events further work is needed in this area to accurately calculate the copy number of rRNA18s genes inside the algae partner to enable the use of qPCR on the *Symsagittifera roscoffensis-Tetraselmis convolutae* relationship.

Chapter 6 : Prospecting the photosynthetic flatworm *Symsagittifera roscoffensis* as a novel fish-feed

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6.1 Abstract

Symsagittifera roscoffensis is an intertidal Acoel flatworm that forms a symbiotic relationship with the alga *Tetraselmis convolutae*. Members of the genus *Tetraselmis* are known to have a high nutritional value and have been widely used to enrich intermediate prey for fish within the aquaculture industry; therefore, *S. roscoffensis* could be a good candidate as a trophic shortcut to deliver algal nutrition to fish. In this Chapter, I investigated the likelihood of five ornamental tropical freshwater and six ornamental marine fishes to consume this worm, either as live feed or in freeze-dried form. I also tested the ability of *S. roscoffensis* to form a symbiotic relationship with alternative algal species, analysing the nutritional profile of *S. roscoffensis* when grown in different media. All the experimental fish consumed live worms to some degree, with the exception of one species (*Meiacanthus grammistes*); the response time to the worms ranged from 1.1–68.6 s for freshwater ornamental species to 1–24 s for marine ornamental species, and in most cases, this was comparable to or shorter than their response time to the reference diet *Artemia*. The fish showed no negative effects after consuming the worms. Similar results were obtained with freeze-dried worms in terms of the number of worms eaten, response time, and feeding time. *Symsagittifera roscoffensis* was able to form a symbiotic relationship with all the tested algal species of the genus *Tetraselmis*, but not with members of other genera. Worms grown in nutrient media (f/2 and f/4) had a significantly higher content of protein, pigments, and total and polyunsaturated fatty acids, including eicosapentaenoic acid (20:5n − 3) and α-linolenic acid (18:3n − 3), than those grown in seawater. These results show that *S. roscoffensis* was acceptable to many ornamental fish species, delivering key algal ingredients that are beneficial to fish health. Hence, it is a promising alternative to conventional fish feeds for the ornamental pet trade.

6.2.1 Introduction

Globally, it is expected that the pet trade industry will be worth $232.14 B by 2030, while the sale of live ornamental fish will be worth $330 M (Fortune business Insights, 2021;OEC, 2022). Within the UK, there are roughly 12,000 people employed across the 3000 pet shops that sell tropical fish and other ornamental aquatic species (OATA, 2019). It is estimated that 12 million UK households have pets, including ornamental aquatic species, spending £3 billion a year on pet food (OATA, 2019). Aquatic pet food can be categorised into dry feed and live feed. Most hobbyists feed their fish exclusively on dry feed in the form of flakes or pellets (Riehl and Baensch, 1997). However, dry feed is not suitable for all fish species, such as those that forage away from the surface, while pellets may sink and be buried in the substrate, thereby contributing to substrate and water fouling over time (Riehl and Baensch, 1997). Some fish species, especially wild-caught marine fish, will not readily accept dry feeds, due to the fact that in the wild, the only food they would have encountered would be live food (Andrews, 1990). Dry feeds that are left uneaten in the aquarium tend to dissolve quickly, thereby losing their nutritional contents. Dry feeds contain key ingredients, including polyunsaturated fatty acids (PUFAs) and proteins that are often derived from wild-caught fish and shellfish. A suitable alternative to harvesting wild fish and shellfish for dry feeds would help to alleviate the pressure on wild stocks and conserve the marine ecosystem (Benemann, 1992; Hemaiswarya *et al.*, 2011).

The other type of food for ornamental fish is live feed. Many microalgal species contain polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA, 20:5n − 3) and docosahexaenoic acid (DHA, 22:6n − 3), which are critical for fish health and nutrition (Brown *et al.*, 1997; Tredici *et al.*, 2015; Pratiwy and Pratiwi, 2020). While a limited number of fish, mainly freshwater species, have the ability to synthesise DHA via a distinct metabolic pathway (Δ4, Δ6), provided that the required precursors are available in the diet. Synthesis via these pathways usually results in a low conversion efficiency (Oboh *et al.*, 2017). However, microalgae can be good sources of DHA, EPA, and ALA, depending on the farming conditions (Brenna, 2002; Colombo *et al.*, 2006; Kumari *et al.*, 2010); they are also rich in key nutrients such as proteins and carotenoids, as well as those potentially beneficial to fish, such as micro-nutrients, carbohydrates, and bioactive compounds (e.g., polyphenols and sterols) (Michalak and Chojnacka, 2015; Daniel *et al.*, 2016).

One algal genus that has been widely used in fish aquaculture is *Tetraselmis* (Prasinophyceae, Chlorophyta), as it is rich in PUFAs (Brown *et al.*, 1997; Tredici *et al.*, 2015). Microalgal cells are too small for ornamental fish to consume directly, and the presence of the algal cell wall may also hinder digestion (Burr *et al.*, 2011; Tibaldi *et al.*, 2015). To circumvent this problem, an intermediate animal is usually given as live feed, most commonly zooplankton such as *Artemia* sp. and cladocerans, which are needed to deliver the desired algal constituents to the fish (Coutteau *et al.*, 1990; Abatzopoulos *et al.*, 2002). The production of animal live feed can be labour intensive and requires a cultivation system separate from the microalgae, or purchased as a pre-made enrichment paste or powder (Treece, 2000; Van Stappen *et al.*, 2020). This increases the likelihood of contamination and operational costs and results in a significant loss of production efficiency. Based on Lindeman's principle of ecological efficiency, on average, 90% of the energy is lost between the trophic levels, i.e., for every 100% microalgal production, only 10% is incorporated by an intermediate organism, which, in turn, transfers only 1% to the target fish species (Lindeman, 1942; Brown *et al.*, 2004). Finding an alternative live feed organism that is easy to cultivate and can deliver algal nutrients more efficiently to the higher trophic level will be hugely beneficial to the industry.

Symsagittifera roscoffensis is an Acoel flatworm within Xenacoelomorpha, which is found in intertidal areas in South Wales, UK, and the southern tip of Portugal, as well as Roscoff, France, and the Channel Isles (Chapter 4). It often occurs in dense patches of millions of individuals along the shore (Bailly *et al.*, 2014). In the wild, *S. roscoffensis* contains the algal symbiont *Tetraselmis convolutae* and relies on it entirely to meet its nutritional requirements (Bailly *et al.*, 2014). There has been suggestions that the worms can form an early symbiotic relationship with other *Tetraselmis* spp. when the preferred *T. convolutae* is unavailable (Mcfarlane, 1982; Arboleda *et al.*, 2018). The worm is a hermaphrodite but reproduces by mating. After successful mating, the worm produces a cocoon with 5–10 embryos inside. Newborns emerge from the cocoon after about 10 days as aposymbiotic individuals and must acquire the algal symbiont from the environment (Provasoli *et al.*, 1968; Douglas, 1983; Arboleda *et al.*, 2018). After the worm's initial acquisition, the *T. convolutae* cells undergo phenotypical changes, such as a loss of eye spot, flagella, and cell wall (Douglas, 1983a). It has been estimated that each individual adult worm contains more than 100,000 algal cells within its upper epidermis (Bailly *et al.*, 2014) and using photoassimilates such as lactic acid from the algal symbiont to synthesise other compounds (Jennings, 1971; Boyle and Smith,

1975; Arboleda *et al.*, 2018b). Interestingly, *S. roscoffensis* is unable to make any of its own PUFAs, which are instead synthesised by the algal symbiont (Meyer *et al.*, 1979).

There are several characteristics that make *S. roscoffensis* a good candidate as a novel fish feed for the aquaculture industry and, in particular, as a live feed within the ornamental fish trade: (1) *S. roscoffensis* is 3–4 mm in length, similar to that of common live feeds such as *Artemia* and cladocerans; (2) its symbiotic algae are from the genus *Tetraselmis,* which is known to have a high nutritional value for fish; (3) as the worm relies solely on its symbiotic algae for its nutrition, its cultivation does not require a separate food source and can be performed similarly to cultivating microalgae (Chapter 2); therefore, it may serve as a 'trophic shortcut' to delivering microalgal nutrition to the fish more efficiently; and (4) lastly, Douglas (1985a) and Mcfarlane (1982) reported that the Welsh population of *S. roscoffensis* contained different types of algae between the western and eastern fringes of their field site, which suggests that *S. roscoffensis* can incorporate different algal species if *T. convolutae* is not available. In the context of a novel feed development, this means the possibility of changing the algal symbiont and customising the biochemical characteristics of *S. roscoffensis* based on the needs of a given aquaculture industry.

To date, there is no information available on fish predation behaviour toward *S. roscoffensis,* its use as a fish feed in aquaculture, or its biochemical composition. To explore the suitability of *S. roscoffensis* as a feed for the marine and freshwater ornamental fish trade, I conducted experiments to (1) observe the fish's behavioural reactions to the worm; (2) quantify the fish's consumption of the worm; (3) test the ability of the worm to incorporate different microalgal species; and (4) analyse the biochemical compositions of the worm grown in different media.

6.3.1 Materials and Methods

6.3.2. Symsagittifera roscoffensis Collection and Master Culture

I used the same culturing methods to maintain the mater stocks as previously described in Chapter 2. *Symsagittifera roscoffensis* was collected from a beach in East Aberthaw in South Wales, U.K. (N 51° 23′ 2.506″ W 3° 22′ 28.004″), in early October 2021.

6.3.3. Feeding Trials with Freshwater Ornamental Fish

The freshwater ornamental fish were acquired from a local aquarium store. Each individual species was approximately the same age (sub adult) and had similar body sizes. I

obtained 10 individuals from each freshwater species: *Paracheirodon innesi* (common name Neon tetras), *Xiphophorus maculatus* (assorted Platy), *Trigonostigma heteromorpha* (Harlequin rasbora), *Danio margaritatus* (Galaxy rasbora), and *Danio albolineatus* (Pearl danio). I also obtained 30 individuals of *Pethia conchonius* (Rosy barb) and *Devario aequipinnatus* (Giant danio), which facilitated repeated trials (see below).

The fish were housed in 10 × 25 L in L37-W27-H35 cm holding tanks connected to a recirculation aquaculture system (RAS). Prior to the feeding trials, the fish were starved for 18 h to standardise their hunger levels. Because the selected fish species are known to live in groups in the wild, to mimic this, I placed ten individuals of each species in a clear 15 L experimental tank. The fish were allowed to acclimate to the experimental tank for 10 min; afterwards, 100 live worms were introduced into the tank at approximately half the tank's length from the fish. The behavioural responses of the fish were recorded for 10 min using a high-speed camera (Sony RX100 7, Tokyo, Japan). At the end of the 10 min trial, the remaining worms were counted, and the fish were returned to the holding tank, where they were monitored for adverse effects such as a loss of condition and changes in their behaviour, as well as an increased morbidity or mortality. Afterwards, I repeated the trials, but with *Artemia sp.* that were 72 h old and enriched with SELCO (INVE Aquaculture, Salt Lake City, USA) as the reference diet for comparison. *Artemia* sp. were cultivated at Swansea University in a salinity of 32 at circa 21°C and harvested on the day of the experiment. I used *Artemia* as a reference diet because they are easily accessible, and widely used as a live feed for many ornamental fish species within the aquatic trade. *Artemia* was also a part of the diet used at the aquarium store, prior to purchase and when housed in the holding tanks. The experiment was repeated two more times with Rosy barbs and Giant danio.

6.3.4. Feeding Trials with Marine Ornamental Fish

From a local aquarium store, I sourced five individuals of each of the following marine ornamental fish species. Each species was of a similar age (sub adult) and had similar body sizes: *Chrysiptera parasema* (Yellow tail), *Meiacanthus grammistes* (Striped blenny), *Gramma loreto* (Royal grammar), *Elacatinus oceanops* (Neon goby), *Amphiprion ocellaris* (Clown fish), and *Nemateleotris magnifica* (Firefish). The marine fish were housed in holding tanks of 6 × 50 L of sea water at 26 °C, with a salinity of 32. The marine fish experiments used the same set up as that for the freshwater fish experiments. Because the selected marine species can show aggression towards each other, I used one individual at a time per experiment. It

should be noted that one Clown fish and one Neon goby were removed from the experiment for ethical reasons due to the fish becoming visibly stressed in the experimental tank.

Each marine fish was allowed to acclimate to the experimental tank for 10 min; afterwards, 10 live worms were added, and the fish's behavioural response was video recorded for 10 min. The remaining worms were counted, and each fish was monitored for any signs of adverse effects. The same procedures were repeated with *Artemia* sp. as the reference diet.

6.3.5. Freeze-Dried Worms Feeding Trials

To test the suitability of *S. roscoffensis* as a dry feed for ornamental fish, I repeated the feeding trials using freeze-dried worms. To produce the dry feed, I first froze the worms in <0.5 mL of sea water at −20 °C overnight, then placed them in a freeze drier (Model:Edwards Super modulyo freeze dryer) at −20 °C under constant vacuum for 24 h. The freeze-dried *Artemia* were prepared as the reference diet in the same manner.

Only the freshwater ornamental fish species that showed a willingness to eat the live worms in the previous experiments were used for these experiments. Because of the rather low numbers of live worms eaten by the marine ornamental fish, they were not used for these experiments. The experimental set up and procedures were the same as before (see Section 6.3.3), with the exception that, instead of live worms, 100 freeze-dried worms (or *Artemia*) were given to the fish.

6.3.6. Ethical Approval

Prior to the start of any experiments involving either the freshwater fish or the marine species mentioned, the experimental design was approved via Swansea University's Animal Welfare and Ethics Review Body (AWERB), with the ethics approval number: 190321/3739. This included an acclimation period of two weeks to ensure the fish were healthy prior to the start of the experiments. All the experiments involving fish conformed to the UK Animals (Scientific Procedures) Act 1986.

6.3.7. Video Processing and Data Analysis

The videos from all the feeding trials (Sections 6.3.2–6.3.5) were processed in VLC media player (formerly VideoLAN Client) to determine the response time and feeding time of the individual fish. The response time was the time taken (in seconds) for each fish to start eating the worms or *Artemia* (live or freeze-dried) once they were placed into the experimental tank. The feeding time was the total time (in seconds) each fish spent eating the feeds once

they had been introduced. The number of animals (live or freeze-dried) eaten was determined by counting the remaining animals at the end of each experiment and with the use of the video data.

6.3.8. Algae Uptake Experiments

The ability of *S. roscoffensis* to establish symbiosis with different algal species: *Chlorella minutissima*, *Dunaliella primolecta*, *Isochrysis galbana*, *Nannochloropsis oculate*, *Porphyridium purpureum*, *Tetraselmis chuii*, *T. suecica*, *T. apiculata*, *T. gracilis*, *T. convolutae* (culture collection), and *T. convolutae* extracted from the Welsh worms (hereafter referred to as TC_W) was tested. Except for the TC_W, all the algal species were taken from Swansea University's algal culture collection or procured from the Culture Collection of Algae and Protozoa (CCAP). All the algal stock cultures were maintained in continuous growth in seawater enriched with f/2 medium, inside an LMS^{TM} (Kent, UK) incubator (14 °C, 69 µmol m^{-2} s^{-1}, and 16/8 light/dark cycle).

The method from Provasoli *et al.*, (1968) was used to obtain aposymbiotic juvenile worms for these experiments. Multiple adult worms were placed together in a small dish for about two weeks to allow reproduction to occur. The worms were checked every three days under a microscope; any cocoons produced were transferred into another Petri dish. Afterwards, the outer shell of each cocoon was carefully removed with a scalpel and a needle, freeing the embryos inside. Once freed, the embryos were rinsed in sterilised seawater (salinity 30 and pH 8.1) to remove any trace of algae. Ten embryos were then transferred into a small Petri dish and incubated at 14.5 °C for about ten days in the dark until they hatched.

Once hatched, the juvenile worms were checked to confirm their aposymbiotic status under an epifluorescence microscope (Olympus BX43, Tokyo, Japan), a lack of red fluorescence (from chlorophyll) under blue light excitation (480 nm) indicating an absence of symbiotic algae (Figure 6.1). Ten aposymbiotic individuals were then placed into a test tube with 100 mL of sterilised seawater enriched with 0.22 µm filtered Guillard f/2 medium at 10 mLL^{-1} (f/4 final conc.) and 10 mL inoculum of each test algal species; a total of 4 replicates were set up per algal species. The worms were checked every 3 days for 36 days and the number of surviving individuals was counted. Epifluorescence images were taken after 15 days; red fluorescence in the upper epidermis under blue light excitation would indicate that algal symbiosis had been established inside the worm.

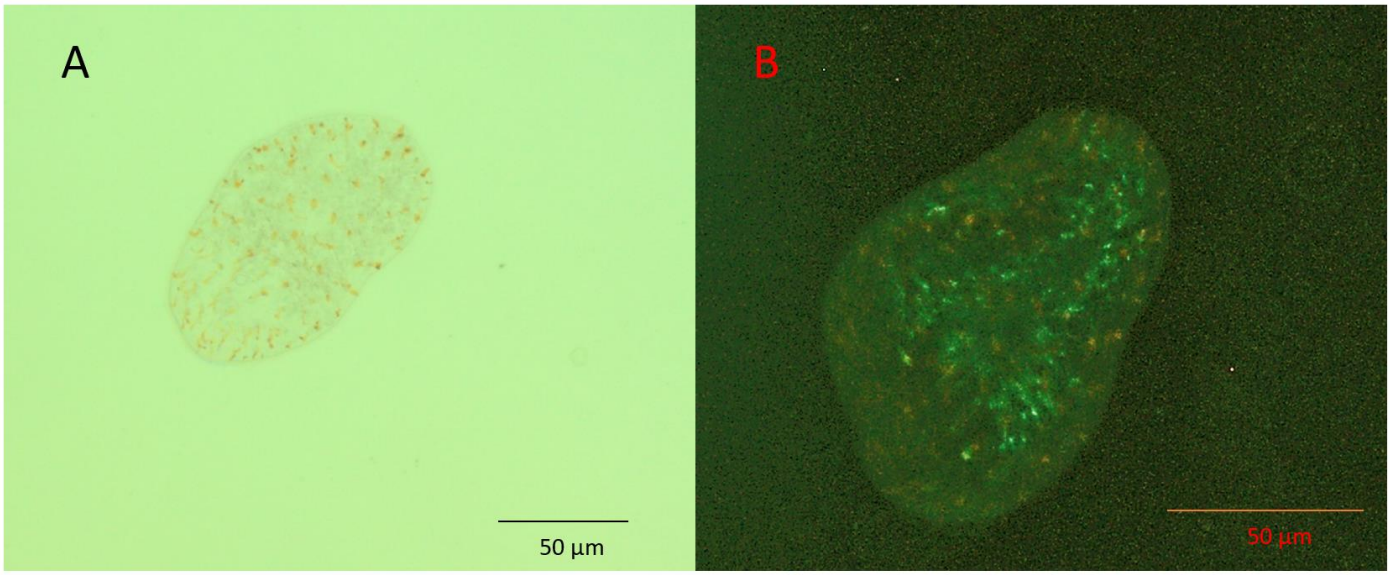

Figure 6.1. Aposymbiotic juvenile *Symsagittifera roscoffensis* visualised using (A) light microscopy, and (B) epifluorescence microscopy. The rhabdoid cells present on the upper surface give off a faint yellow-brown colour under blue light excitation; the lack of red fluorescence indicates the absence of symbiotic algae.

6.3.9. Nutritional Profiling of *Symsagittifera roscoffensis*

To evaluate the nutritional value of *S. roscoffensis* as a potential feed for aquaculture, I analysed the biochemical compositions of *S. roscoffensis* grown in different media: filtered seawater, f/4, and f/2. The worms were kept in the corresponding medium (approximately 200 worms in 150 mL), which was renewed twice a week. After a 2-week period, the worms were collected and freeze-dried (Model:Edwards super modulyo freeze dryer); −20 °C under vacuum for 24 h), after which, the worms were separated from the remaining salt with tweezers. The biochemical characterisation of the freeze-dried samples followed standard methods, which are briefly described below.

6.3.9.1 Transesterification, Identification and Quantification of Fatty Acid Methyl Esters

All the chemicals and analytical reagents were of a high-performance liquid chromatography grade (Sigma-Aldrich, Dorset, UK), unless stated otherwise. The worm samples were weighed (~1 to 2 mg), followed by direct transesterification and fatty acid methyl esters (FAMEs) profiling (Griffiths *et al.*, 2010). The dried FAMEs were reconstituted in 300 µL of hexane prior to their identification and quantification on a GC-ToF-MS (Waters Corporation, Milford, MA, USA) using a TR-FAME capillary column (Mahanty *et al.*, 2015).

A six-point calibration curve was generated using one internal (C13:0) and two external standards (C17:0 and C19:0) to generate a C17:0/C13:0 calibration curve. The quantification of the FAMEs was performed by comparing the experimentally derived component peak areas with the calibration curve. In total, n = 3 replicates were run, and only the FAMEs identified in at least two replicates were considered to be true hits. The data were reported on a percent dry weight basis.

6.3.9.2. Analysis of Proteins, Pigments, and Carbohydrates

A combined colorimetric assay was used to measure the proteins, pigments, and carbohydrates according to Chen and Vaidyanathan, (2013). Briefly, the freeze-dried worm samples were treated with BCA reagents; the mixture was then centrifuged at 700× *g* for 3 min. Afterward, the different chemical fractions were removed to measure the total protein (absorbance at 562 nm), total carbohydrate (578 nm), chlorophyll a + b (416, 453, and 750 nm), and carotenoids (430, 450, 480, and 750 nm).

3.2.10. Statistical Analysis

Different statistical tests were applied to different data sets. In the freshwater and marine fish trials, I tested for differences between the feed treatments (worm vs. *Artemia*) for each fish species and for the number of worms eaten, response time, and total feeding time, using the Kruskal–Wallis test in R studio version 1.41717. Graphs were created using the R package ggplot2.

In the experiments in which *S. roscoffensis* was exposed to different algae, I tested the worm survival in the presence of different algal symbionts. This was analysed using the Log rank (Mantel–Cox) test for curve comparisons in GraphPad PRISM v9. When characterising the *S. roscoffensis* lipids, proteins, and carbohydrates, I tested for differences in the total fatty acids, proteins, pigments, and carbohydrates within the *S. roscoffensis* when they were kept in different media (seawater, f/4, and f/2). This was performed with a 1-way ANOVA after satisfying the requirement for normality, using the R package DHARMa version 4.1.3 that utilises the Kolmogorov–Smirnov test (R studio version 1.41717).

6.4.1 Results

6.4.2. Algae Uptake Experiments

The aposymbiotic juveniles of *S. roscoffensis* were incubated in the presence of ten different algal species to test whether the worm would incorporate alternative algal symbionts

in the absence of the native *T. convolutae*. Epifluorescence microscopy revealed that *S. roscoffensis* incorporated cells of all the tested *Tetraselmis* species throughout the upper layer of its body, suggesting that successful symbiosis had occurred (Figure 6.2.). On the contrary, the worms incubated with *C. minutissima*, *D. primolecta*, *I. galbana*, *N. oculata*, and *P. purpureum* had no algae inside, indicating that there was no uptake of those algal species.

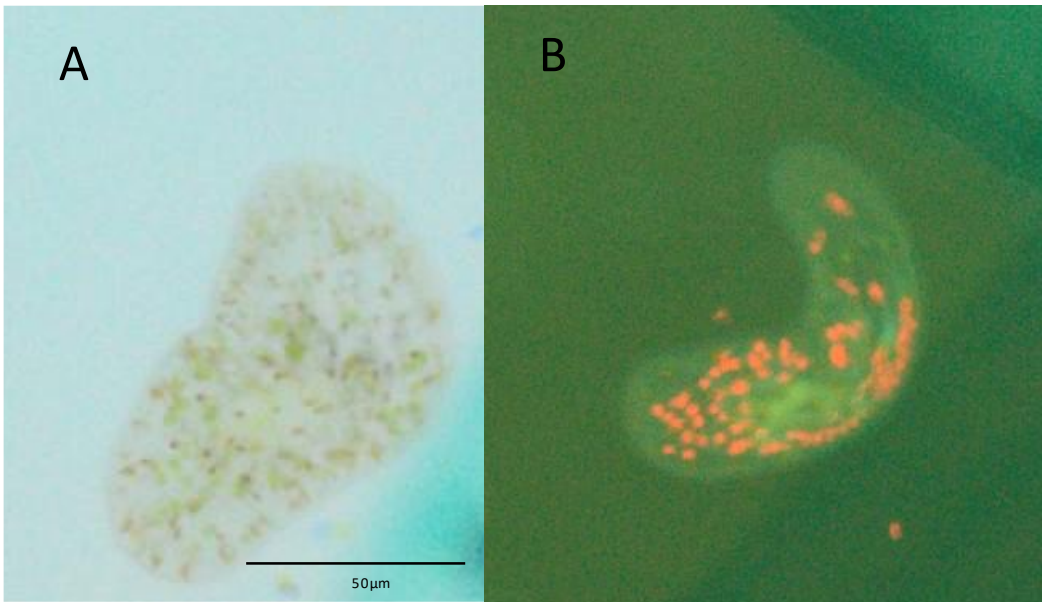

Figure 6.2. *Symsagittifera roscoffensis* visualised using epifluorescence microscopy when cultured in medium containing *Tetraselmis suecica* for 15 days. (**A**) Light green patches, i.e., chlorophyll, indicate the presence of algal cells within the worm when viewed under the light microscope. (**B**) The same worm displays punctate red patches under blue epifluorescent light, which confirms the presence of *T. suecica*.

Overall, the presence of different *Tetraselmis* species had a significant impact on the probability of the *S. roscoffensis* surviving ($X^2_{(5)}$ = 33.88, p < 0.001; Figure 6.3.). The differences in overall survival ranged from 5% to 23% over the duration of the experiment (36 days) and the median survival times ranged from 3 to 15 days (Table 6.1). Compared to *T. convolutae*, which is naturally found within the worms in situ, *T. apiculata* led to a significant reduction in survival by the experiment's end, from 20% to 10%, (day 36) with the shortest median survival time of 3 days. The worms with *T. chuii* and *T. suecica* demonstrated median survival times of 15 days each, with survival peaking at 23% for the former.

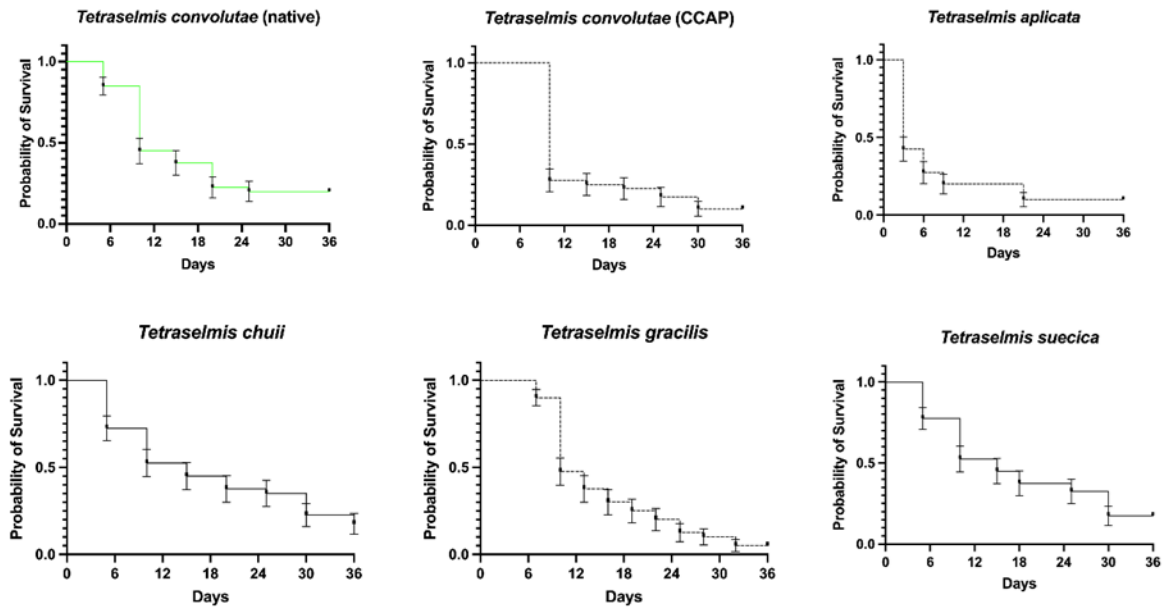

Figure 6.3. Survival of juvenile aposymbiotic *Symsagittifera roscoffensis* when offered *Tetraselmis* spp. as potential symbionts. Data are plotted as mean ± s.e. N = 40 per alga, with 240 in total. CCAP, Culture Collection of Algae and Protozoa.

Table 6.1. Survival analysis of *Symsagittifera roscoffensis* when offered a range of potential *Tetraselmis* symbionts. Log rank (Mantel–Cox) tests were used to compare curves.

	Survival (%) at Day 36 (Median Survival Time *)	**Curve Comparison with *T. convolutae* (Native)**
T. convolutae (native)	20% (10 days)	N/A
T. convolutae (CCAP)	10% (10 days)	$X^2_{(1)} = 0.23$, $p = 0.632$
T. apiculata	10% (3 days)	$X^2_{(1)} = 12.23$, $p < 0.001$
T. chuii	23% (15 days)	$X^2_{(1)} = 0.003$, $p = 0.959$
T. gracilis	5% (10 days)	$X^2_{(1)} = 1.069$, $p = 0.301$
T. suecica	18% (15 days)	$X^2_{(1)} = 0.011$, $p = 0.918$

CCAP, Culture Collection of Algae and Protozoa; N/A, not applicable. *, probability of survival equalling 50%.

6.4.3. Nutritional Profiles of S. roscoffensis

The nutritional profiles of the *S. roscoffensis* grown in different media (f/2, f/4, and filtered seawater) are presented in Figures 6.4 and 6.5 and Table 6.2. The worms had between 7.5 and 8.4 % DW of carbohydrates among all the treatments, and there were no significant differences among the treatments (ANOVA; $p = 0.2$) (Figure 6.4.). The protein contents were significantly different in the *S. roscoffensis* between the tested media (ANOVA; $p > 0.001$): it

was highest in f/2 (41.0 ± 0.0 %DW; mean ± s.e.) and lowest in the seawater (17.4 ± 0.0 %DW). The carotenoid contents varied between 0.4 ± 0.0 % DW in f/2, 0.3 ± 0.0 % DW in f/4, and 0.1 ± 0.5 % DW in seawater; there were significant differences among the tested media types (ANOVA; p = 0.02). The chlorophyll a + b contents of *S. roscoffensis* varied significantly (ANOVA; p = 0.002), from 0.7 ± 0.0 % DW in f/2 to 0.5 ± 0.0 % DW in f/4, while it was undetectable in the seawater (Figure 6.4).

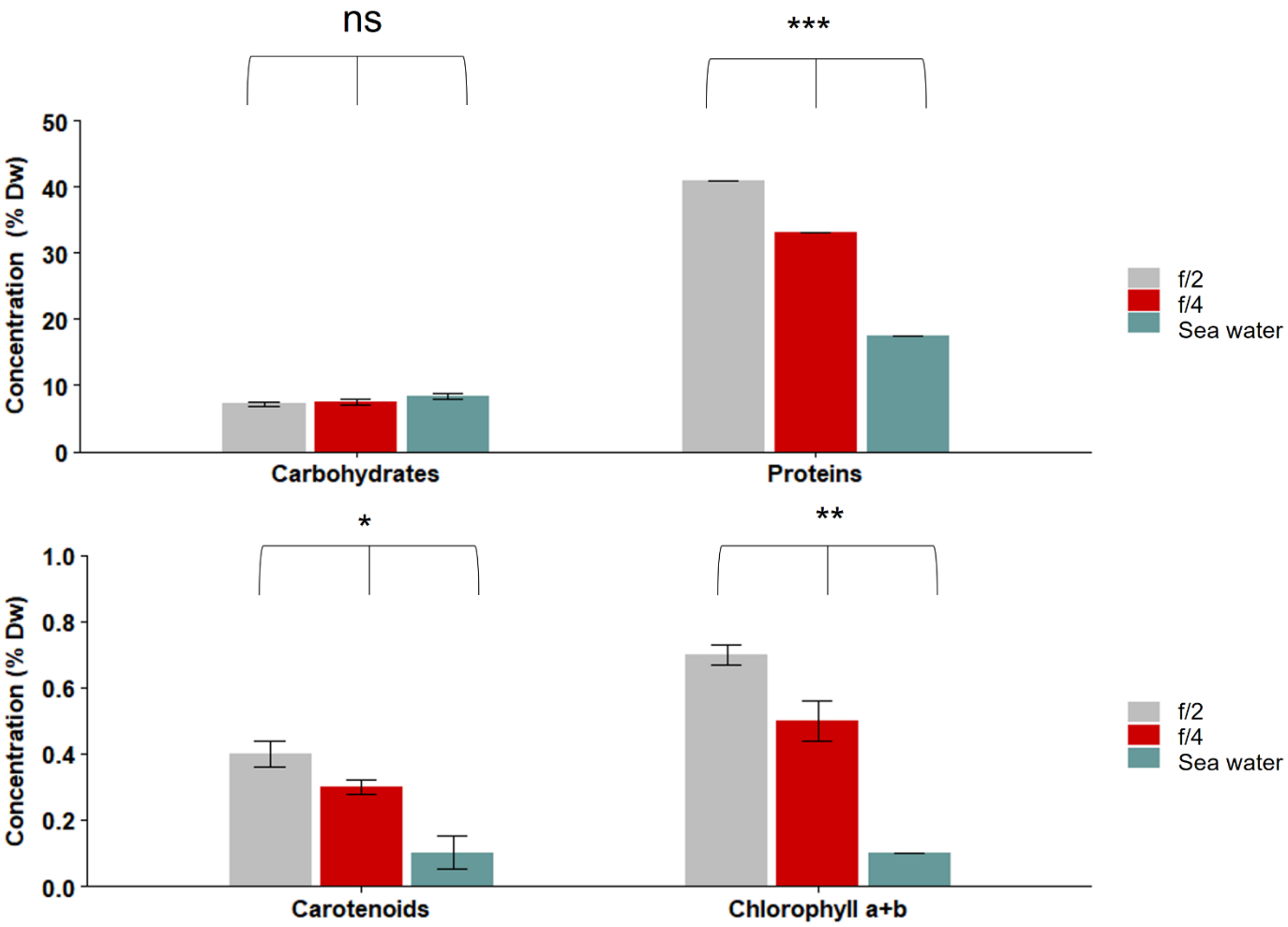

Figure 6.4. Carbohydrate, protein, carotenoid, and chlorophyll a + b contents of *Symsagittifera roscoffensis* cultured in different media (filtered sea water, f/4, and f/2). Carbohydrate concentrations did not differ significantly. Protein, carotenoids, and chlorophyll a + b were significantly different in *S. roscoffensis* depending on the medium used. Asterisks indicate significant differences (ANOVA: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ^{ns} not significant). n = 200 worms per experiment. Data are presented as % dry weight (DW; mean ± s.e.).

The total fatty acid contents were significantly different among the treatments (ANOVA; p = 0.01): the worms kept in f/2 had the highest total fatty acid content at 7.5 ± 0.52% DW (mean ± s.e.), followed by those kept in f/4 (5.86 ± 0.41% DW) and seawater (3.69 ± 0.24% DW) (Figure 6.5.). The PUFA contents of *S. roscoffensis* were also significantly different among the treatments (ANOVA; $p < 0.001$): it was higher in the f/2 treatment (2.77 ± 0.21% DW) than the f/4 treatment (2.2 ± 0.04% DW) and nearly absent in the seawater treatment (0.07 ± 0.01% DW) (Figure 6.5).

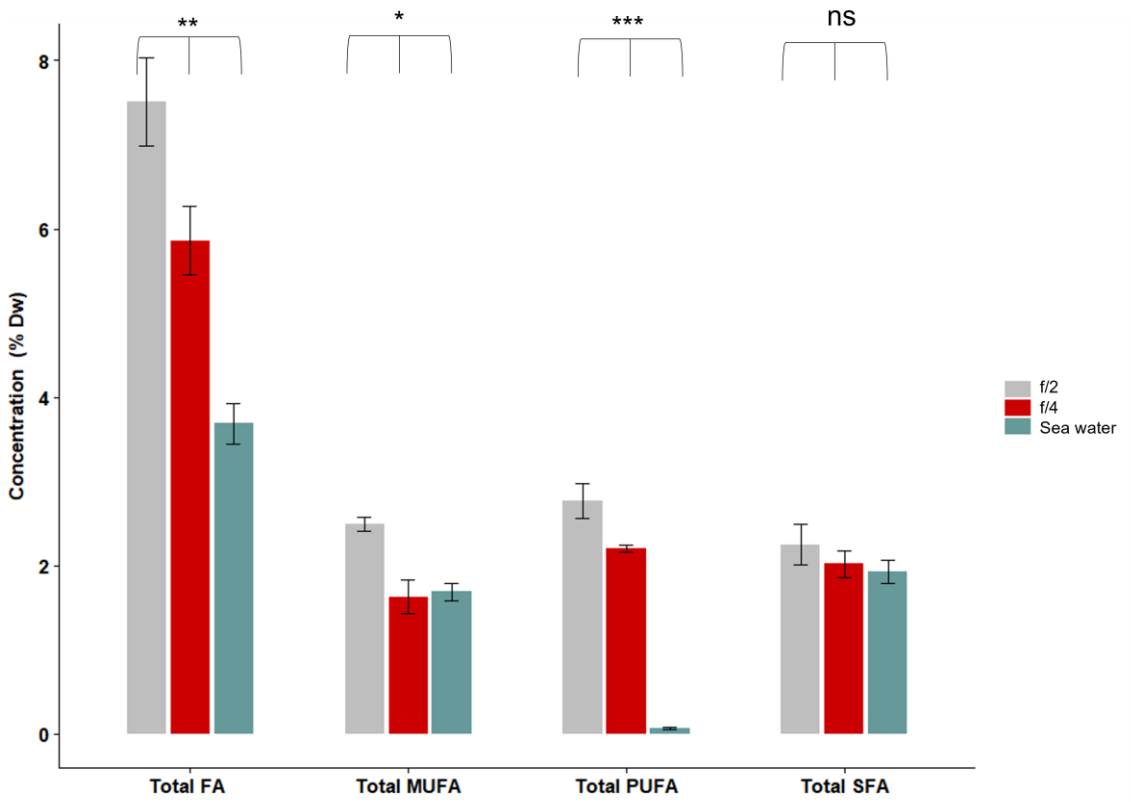

Figure 6.5. Total fatty acid content (FA), monounsaturated fatty acid (MUFA), polyunsaturated fatty acid (PUFA) and the saturated fatty acid (SFA) of *Symsagittifera roscoffensis* cultured in different media (f/2, f/4, or plain seawater). Total fatty acid content, monounsaturated fatty acid, polyunsaturated fatty acid was significantly different when *S. roscoffensis* was grown in different media. Saturated fatty acid was not significantly different when *S. roscoffensis* was grown in different media. Asterisks indicate significant differences (ANOVA: *p<0.05; **p<0.01; ***p<0.001; ^{ns} not significant). N=200 worms per experiment. Data are presented as % dry weight (DW; mean ± s.e.).

The fatty acid EPA (20:5n − 3) was present in the worms that were kept in the f/2 (1.75 ± 0.0% DW) and f/4 media (1.36 ± 0.0% DW), but it was nearly absent in the seawater treatment (0.01 ± 0.0% DW) (Table 6.2). DHA (22:6n − 3) was present, but only at 0.01% DW across all the treatments. ALA (18:3n − 3) was highest in the f/2 treatment (0.55 ± 1.7% DW), followed by the f/4 (0.45± 0.0% DW) and seawater treatments (0.02 ± 0.0% DW) (Table 6.2.).

Table 6.2. Fatty acid composition of *Symsagittifera roscoffensis* when grown in different media (f/2, f/4, or plain seawater) n = 3 per treatment.

	f/2 Medium		**f/4 Medium**		**Sea Water**
Fatty Acid	**%DW**	**SE**	**%DW**	**%DW**	**SE**
Caproic acid (C6:0)	0.01	0.0011	0.02	0	0
Caprylic acid (C8:0)	0.03	0.0012	0.05	0.01	0.00132
Capric acid (C10:0)	0.02	0.0034	0.03	0	0
Undecanoic acid (C11:0)	0	0	0.01	0	0
Lauric acid (C12:0)	0.02	0.0005	0.01	0.01	0.00057
Tridecanoic acid (C13:0)	0	0	0	0	0
Myristic acid (C14:0)	0.01	0.0001	0.08	0.03	0.0007
Pentadecanoic acid (C15:0)	0	0	0.01	0.01	0.00203
Palmitic acid (C16:0)	1.12	0.1625	1.09	1.47	0.03302
Heptadecanoic acid (C17:0)	0.02	0.0006	0.01	0	0
Stearic acid (C18:0)	0.23	0.0156	0.19	0.2	0
Arachidic acid (C20:0)	0.43	0.0604	0.3	0.16	0.10386
Heneicosanoic acid (C21:0)	0.31	0.0191	0.2	0.03	0.0016
Behenic acid (C22:0)	0.02	0.0002	0.02	0.01	0.00131
Tricosanoic acid (C23:0)	0.03	0.001	0.02	0	0
Lignoceric acid (C24:0)	0	0	0	0	0
Myristoleic acid (C14:1)	0	0	0	0.01	0.00055
Cis-10-Pentadecenoic acid methyl ester (C15:1)	0	0	0	0	0
Palmitoleic acid (C16:1)	0.06	0.0068	0.17	0.04	0.0037
Cis-10-Heptadecenoic acid methyl ester (C17:1)	0	0	0	0.01	0.00239
Elaidic acid (C18:1n9t)	0	0	0.01	0.01	0.00365
Oleic acid (C18:1n9c)	0.91	0.0387	0.65	0.97	0.03979
Cis-11-eicosenoic acid (C20:1n9)	0.93	0.001	0.47	0.44	0.00239

Erucic acid (C22:1n9)	0.38	0.0256	0.23	0.13	0.04778
Nervonic acid (C24:1n9)	0.2	0.0083	0.11	0.1	0.0067
Linoleic acid (C18:2n6c)	0	0	0	0	0
Linolelaidic acid methyl ester (C18:2n6t)	0.06	0.0066	0.05	0.01	0.00332
γ-Linolenic acid (C18:3n6)	0.01	0.0023	0.01	0	0
α-Linolenic acid (C18:3n3)	0.55	0.1793	0.45	0.02	0.00062
cis-11, 14-eicosadienoic acid (C20:2)	0.05	0.0011	0.05	0	0
cis-8, 11, 14-eicosatrienoic acid (C20:3n6)	0	0	0	0	0
Arachidonic acid (C20:4n6)	0.01	0.0039	0.01	0	0
cis-11, 14, 17-eicosatrienoic acid (C20:3n3)	0.35	0.007	0.26	0.01	0.00054
Eicosapentaenoic acid (C20:5n3)	1.73	0.0005	1.36	0.01	0.00045
Eicosadienoic acid	0.02	0.0001	0.02	0	0
Docosahexaenoic acid (C22:6n3)	0.01	0.0001	0.01	0.01	0.0015

6.4.4. Feeding Trials with Freshwater Ornamental Fish

In the first set of freshwater fish feeding trials with live feeds (Figure 6.6), all the tested fish species consumed significantly more *Artemia* than the worms. Platy consumed the most worms, at 4.8 ± 1.2 (mean ± s.e.) worms ind^{-1}, while Pearl danio consumed the least, at 1.0 ± 0.7 worms ind^{-1}. The other fish species ate between one and three worms ind^{-1}. When offered *Artemia*, Platy also consumed the most, at 10 ± 0 (mean ± s.e.) *Artemia* ind^{-1}, Pearl danio consumed 9.5 ± 0.5 *Artemia* ind^{-1}, and the other fish species consumed, on average, 8.6–8.8 *Artemia* ind^{-1}.

Platy had a response time of 68.6 ± 34.1 s to the worm, which was not significantly different from its response time to *Artemia* (42.6 ± 1.7 s) (Kruskal–Wallis chi-squared = 19; p = 0.08). Pearl danio reacted much quicker at 1.1 ± 0.7 s, which was a significantly shorter

response time (Kruskal–Wallis chi-squared = 19; $p = 0.01$) than that towards *Artemia* (32.1 ± 2.5 s). Harlequin rasbora apparently also responded faster to the worm (16.8 ± 9.1 s) than to *Artemia* (41.8 ± 6.6 s), but this difference was not statistically significant (Kruskal–Wallis chi-squared = 12.667; $p = 0.1$). Galaxy rasbora had a longer response time to *Artemia* (61 ± 10.3 s) than to the worms (28.9 ± 14.7 s), but this difference was also not statistically significant (Kruskal–Wallis chi-squared = 17.1; $p = 0.07$).

All the tested fish species spent significantly more time eating the *Artemia* than the worm (Figure 6.6). Platy spent the longest time eating the worm (16.8 ± 7.4 s) and Pearl danio spent the shortest time (0.6 ± 0.4 s); the remaining fish species spent between 4.9 s and 9.8 s eating the worm. Platy, when offered *Artemia*, had a total feeding time of between 108.6 s and 186.8 s.

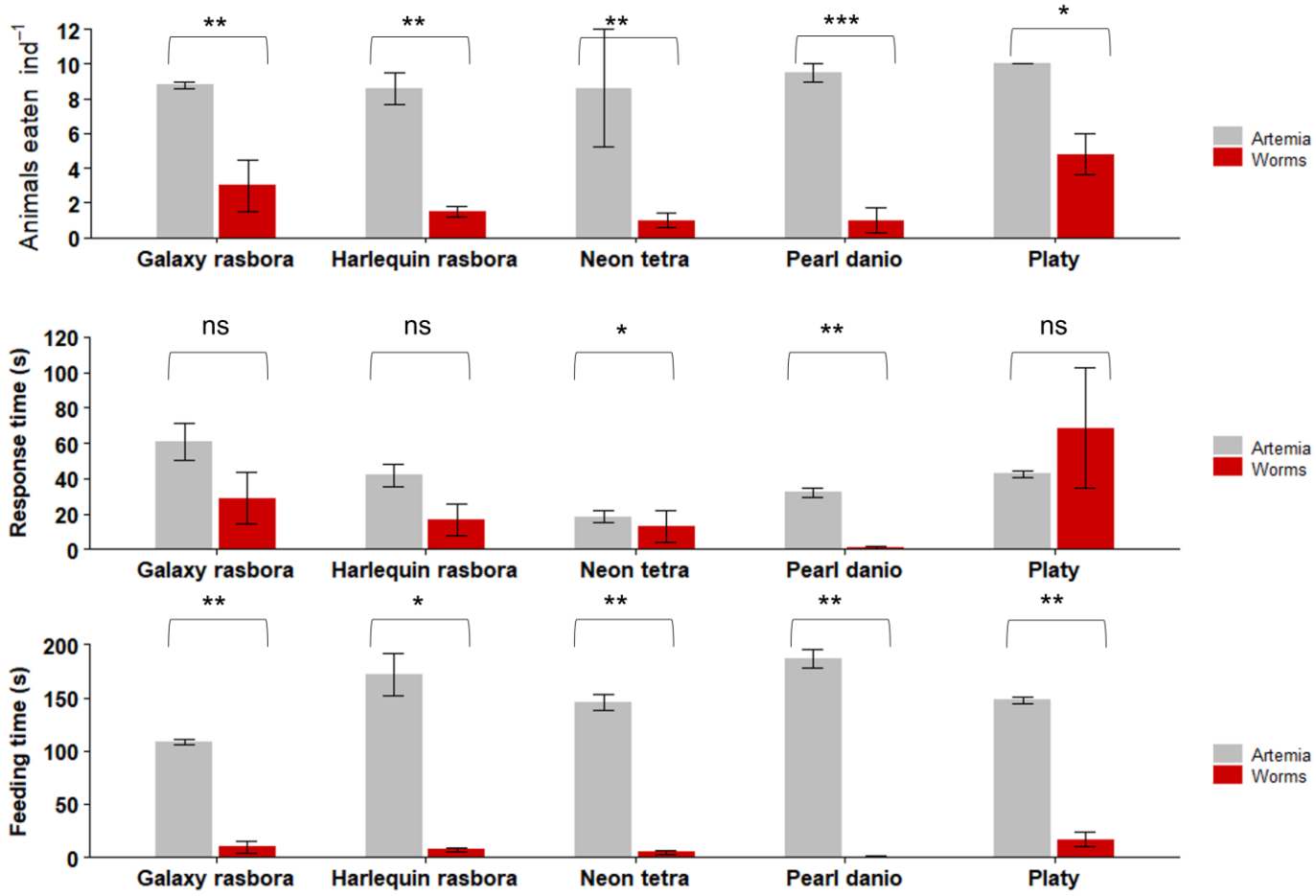

Figure 6.6. Feeding trials with freshwater ornamental fishes. Data are plotted as mean ± s.e. Asterisks indicate significant differences (Kruskal–Wallis: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ^{ns} not significant). n = 10 fish per experiment.

In the second set of experiments with repeated freshwater feeding trials (Figure 6.7), the Giant danio did not show a significant difference (Kruskal–Wallis chi-squared = 10.666; $p = 0.09$) in the number of live worms they consumed (9.5 ± 0.1 worms ind^{-1}; mean ± s.e.) vs. live *Artemia* (7.5 ± 1.2 ind^{-1}). Rosy barb also did not show a significant difference in the number of live animals consumed: 9.5 ± 0.1 worms ind^{-1} vs. 10 ± 0 *Artemia* ind^{-1} (Kruskal–Wallis chi-squared = 2.3824; $p = 0.4$). When offered freeze-dried feeds, Giant danio consumed 8 ± 0.1 dried worms ind^{-1} and 8.1 ± 0.1 dried *Artemia* ind^{-1}. Rosy barb, on the other hand, consumed slightly more dried *Artemia* (10.0 ± 0.0 dried *Artemia* ind^{-1}) than dried worms (9.6 ± 0.1 dried worms ind^{-1}). However, the differences between the freeze-dried worms and freeze-

dried *Artemia* were not statistically significant (Giant danio: Kruskal–Wallis chi-squared = 4.1943, $p = 0.1$; Rosy barbs: Kruskal–Wallis chi-squared = 4.3333, $p = 0.1$).

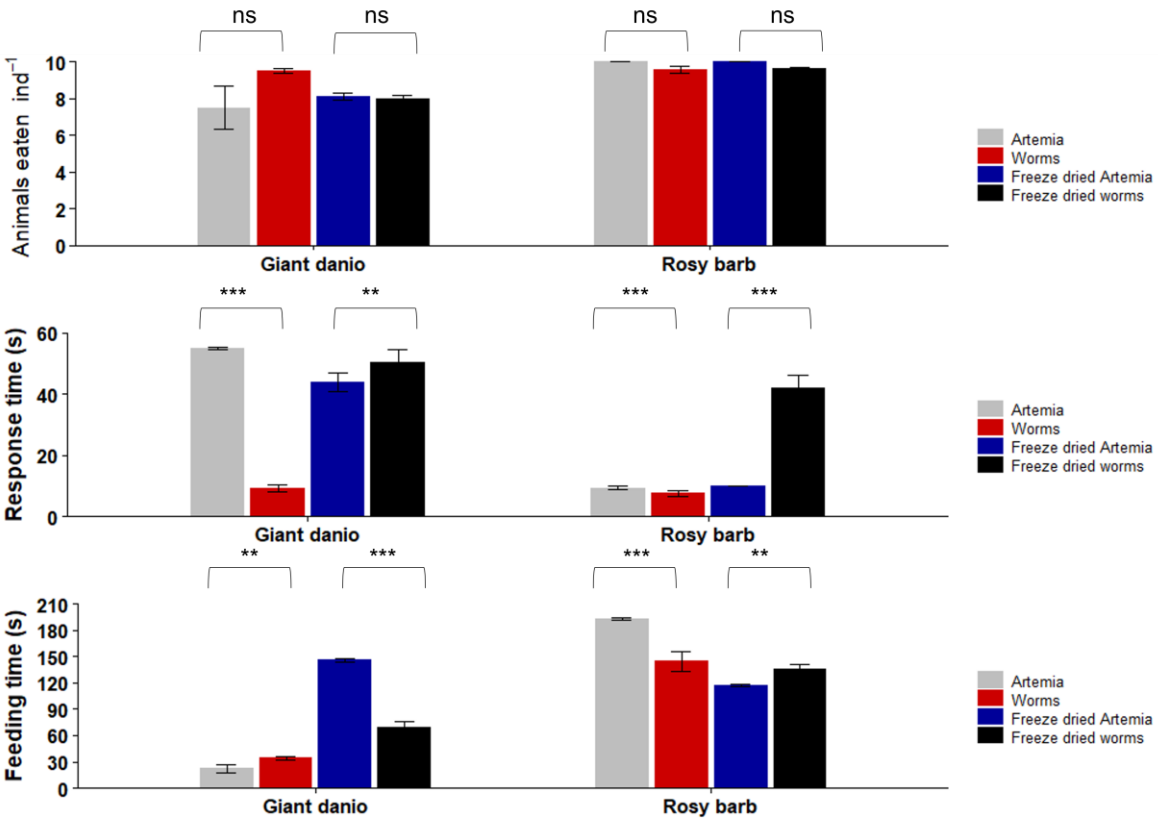

Figure 6.7. Repeated feeding trials with Giant danio (*Devario aequipinnatus*) and Rosy barb (*Pethia conchonius*) with both live and freeze-dried feeds. Data are plotted as mean ± s.e. Asterisks indicate significant differences (Kruskal–Wallis: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ^{ns} not significant). N = 10 fish per experiment.

The response time of Giant danio to the live worms (9.3 ± 1.1 s) was significantly shorter than that to the live *Artemia* (54.9 ± 0.4 s) (Kruskal–Wallis chi-squared = 39; $p > 0.001$). The Giant danio response time to the dried *Artemia* (43.9 ± 2.9 s) was significantly shorter than its response time to the dried worms (50.3 ± 4.0 s) (Kruskal–Wallis chi-squared = 35.533; $p = 0.01$). Rosy barb responded to the live worms (7.5 ± 0.9 s) significantly faster than it did to the live *Artemia* (9.4 ± 0.6 s) (Kruskal–Wallis chi-squared = 26.331; $p = 0.001$), but it was the opposite when the dried feeds were compared (10.0 ± 0.0 s to dried *Artemia*; 42.1 ± 4.1 s to dried worms; Kruskal–Wallis chi-squared = 30.333; $p = 0.004$).

When comparing the total feeding times, Giant danio spent significantly more time eating live worms (34.3 ± 2.2 s) than live *Artemia* (22.6 ± 4.6 s) (Kruskal–Wallis chi-squared

= 24.44; p = 0.006); but this was the opposite when dried feeds were used: 146.0 ± 2.2 s eating *Artemia* vs. 69.0 ± 6.6 s eating dried worms (Kruskal–Wallis chi-squared = 39; p < 0.001). Rosy barb spent significantly more time eating the live *Artemia* (193.5 ± 1.5 s) than the live worms (144.9 ± 11.2 s) (Kruskal–Wallis chi-squared = 39; p < 0.001), but the opposite was true when dried feeds were used (135.5 ± 5.4 s eating dried worms vs. 117.4 ± 1.6 s eating dried *Artemia*; Kruskal–Wallis chi-squared = 16.467; p = 0.05).

6.4.5. Feeding Trials with Marine Ornamental Fish

In the marine fish feeding trials (Figure 6.8.), Striped blenny did not eat any of the worms, whereas the other tested fish species ate between 0.6 (Royal Gramma) and 3 (Firefish) worms ind^{-1}. *Artemia* was consumed at 0.6–5 ind^{-1}. There were no significant differences between the two live feeds for any of the tested marine fish (Kruskal–Wallis; p = 0.37 to 0.57), excluding Striped blenny.

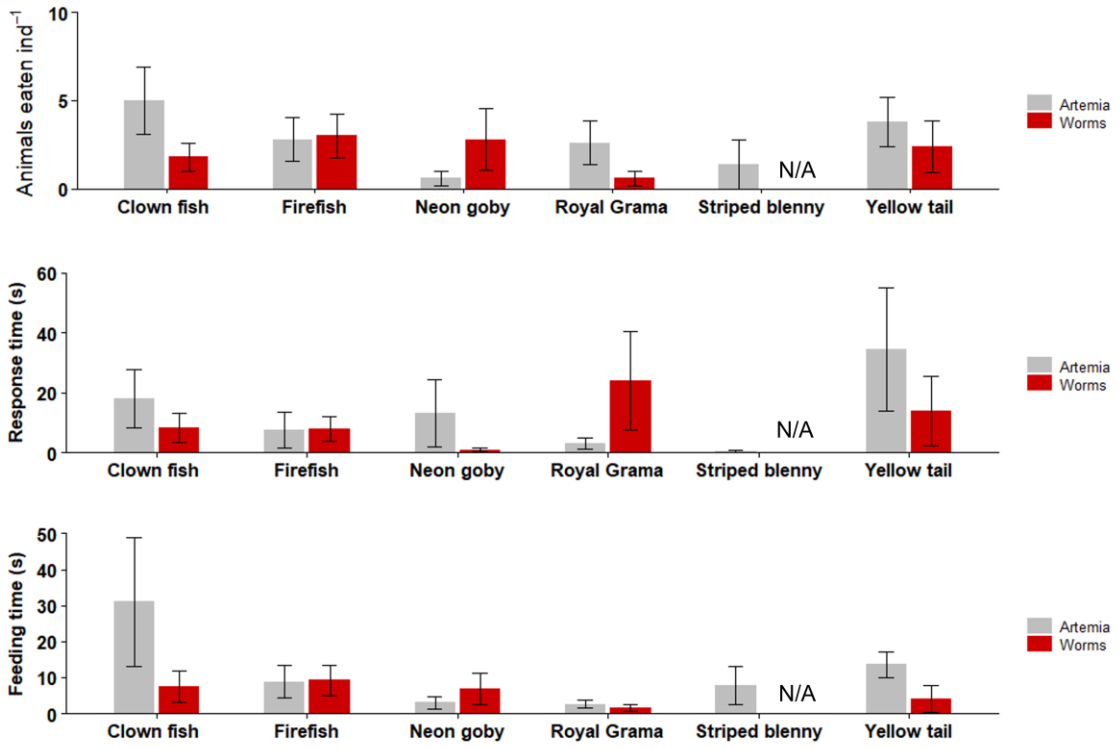

Figure 6.8. Feeding trials with marine ornamental fishes. Data are plotted as mean ± s.e. There were no significant differences between worm and *Artemia* treatments in terms of numbers of targets eaten, response time, and feeding time (Kruskal–Wallis: p > 0.05). n = 10 fish per experiment.

Except for the Striped blenny, which did not eat any worms, the response time to the worm was as short as 1 s (Neon goby) and ranged up to 24 s (Royal Grama). The response time to *Artemia* was between 3.2 (Royal gramma) and 34.6 s (Yellow tail), there were no significant differences between the two live feeds in terms of the response time for all of the tested marine fish (Kruskal–Wallis; $p = 0.4$ to 0.6), excluding Striped blenny.

The total time spent eating the worm ranged from 1.6 (Royal Gramma) to 9.2 s (Firefish) (excluding Striped blenny), and the total time spent eating the *Artemia* ranged from 3 (Neon gobies) to 31 s (Clown fish). There were no significant differences between the two live feeds for any of the marine fish tested (Kruskal–Wallis; $p = 0.25$ to 0.57), excluding the Striped blenny.

6.5.1 Discussion

The purpose of this chapter was to determine the palatability of *S. roscoffensis* for ornamental fish and its nutritional profile. The acoel flatworm *S. roscoffensis* forms a photosymbiotic relationship with the alga *T. convolutae*, providing the worm with all its required nutrition via photosynthesis. The *Tetraselmis* genus is known to be of a good nutritional value for aquaculture applications, and an adult *S. roscoffensis* is of a comparable size to common live feed for fish. These attributes make *S. roscoffensis* a good candidate as a novel feed for fish, but until now, there has been no information on its use as a fish feed or its nutritional contents. The worm's ability to establish photosymbiosis with other algal species was also tested, which may open up new opportunities to customise the worm for different applications in aquaculture and biotechnology.

The freshwater fish feeding trials showed that all the tested fish species consumed the worm to some degree, and several species appeared to be more attracted to the worm than the *Artemia*, as indicated by the shorter response times. The fish species that ate the highest number of worms were Giant danio and Rosy barb. Giant danio is an omnivore that lives within fast-flowing water, where it feeds on a variety of insects and plant material (Dey *et al.*, 2014). Interestingly, in the experiment, Giant danio consumed 19% more live worms than *Artemia* per individual; it also consumed as many freeze-dried worms, doing so as quickly as it consumed the freeze-dried *Artemia*. Rosy barb is also omnivorous and it consumes a varied diet (de Magalhães and Jacobi, 2013). However, the Rosy barb took more time to consume the same number of live worms as the Giant danio, which may reflect the more passive feeding behaviour of Rosy barb. It also consumed nearly the same number of dried worms as dried *Artemia*. Taken

together, the results suggest that *S. roscoffensis* was equally, if not more, accepted as a live or dried feed as *Artemia* by Giant danio and Rosy barb.

Harlequin rasbora consumed four times more *Artemia* than the worm in the experiment, reflective of its selective feeding behaviour (Robert Woods, 2019). The worm size (3–4 mm) being larger than the size of the *Artemia* (1–2 mm) may have imposed some limitation to its relatively small mouth. Galaxy rasbora forages for freshwater invertebrates in its natural habitat (Roberts, 2007), which may explain its significantly higher consumption of *Artemia*, which resembles its natural prey, relative to the worm.

Neon tetra and Pearl danio consumed low numbers of worms but continued to eat *Artemia* in comparable amounts to the other freshwater fish species. This was unexpected, since the other danio species in this experiment consumed the largest number of worms. I initially expected that the Pearl danio would also consume the worms in large amounts, as the diets of danio species tend to overlap, with the consumption of a range of insects and plant material (McClure *et al.*, 2006). Neon tetras are not known to be selective feeders and they regularly consume a variety of food when kept in aquaria; they are often recommended as ornamental fish for beginner hobbyists, as they have few special requirements (Michalak and Chojnacka, 2015). However, the fish may not have recognised the worms as a food source, resulting in a lower number being eaten. It may also be the case that a repeated encounter could improve their familiarity and increase the number of worms consumed. Moreover, in an aquarium, there is usually a mix of species that would all have different feeding habits, which would ultimately affect their feeding behaviour.

In the marine fish feeding trials, Firefish did not behave any differently to the worm and *Artemia*, in terms of the animals eaten, response times, and feeding times. Firefish are omnivorous and their natural diet consists of small zooplankton and algae (Kuiter and Tonozuka, 2001; Estelita, Emily, 2009); its non-selective feeding habits may have allowed it to consume the worm and *Artemia* equally well. Among the tested marine fish species, Neon goby had the quickest response to the worm, and it ate more worms than *Artemia* (albeit this was not statistically significant). In the wild, Neon gobies acts as a cleaner fish, removing parasites from other fish. *Symsagittifera roscoffensis* may resemble the common nematode parasites in marine fish (Moravec, 2007), due to a similarly shaped body size, thus triggering a feeding response from the Neon goby. Overall, all the tested marine fish species consumed small amounts of the worm, except for Striped blenny, although the Striped blenny did eat *Artemia*. *Artemia* is a common feed for many ornamentals within the aquaculture industry,

including the pet store from which the fish were purchased and in the in-house holding tanks. Long-term feeding with *Artemia* may result in the habituation of the fish to a particular feed, even though *Artemia* is not a natural prey for the fish in the wild. Habituation allows the fish to recognise *Artemia* as food and eat it more readily (Malison and Held, 1996; Macaulay *et al.*, 2021). It is thought that, if the same fish were offered the worms repeatedly over a longer time period, they too would become habituated, resulting in a higher number of worms eaten. This habituation effect could also be responsible for the larger number of *Artemia* eaten by the freshwater fish, such as Pearl danios and Neon tetras.

My data showed that the tested ornamental fish species ate the worm with no obvious ill effects. The differences in the numbers of worms consumed between the fish species could be due to the differences in the morphoanatomical mouth features of different fish species. As some fish found the worms palatable after processing, i.e., freeze dried. Processing the worm could be a method for improving the palatability for fish species with different morphoanatomical mouth features, thus increasing this palatability for a wider range of fish species. However, the results suggest that *S. roscoffensis* can be used as a suitable alternative feed in the ornamental fish trade. Because the worm is pre-enriched with *Tetraselmis* as algal symbionts, it can act as a "trophic short-cut" to delivering algal nutrition to the fish more efficiently and cost effectively.

The algae uptake experiments showed that *S. roscoffensis* successfully incorporated all the *Tetraselmis* species that I tested. On the other hand, the lack of uptake or ingestion of the non-*Tetraselmis* species further confirmed that *S. roscoffensis* lacked a functional digestive tract (Bailly *et al.*, 2014) and did not perform heterotrophic feeding, even in the absence of algal symbionts (Jennings, 1971; Mamkaev and Kostenko, 1991; Arboleda *et al.*, 2018). The observations support some of the literature reports, in that different *S. roscoffensis* populations in the wild may contain different algal species (Mcfarlane, 1982; Arboleda *et al.*, 2018), which indicates some flexibility for *S. roscoffensis* to establish symbiosis with different members of the *Tetraselmis* genus. However, I did notice that, while symbiosis did occur, the algae were not as abundant throughout the worm's body as seen in the field-collected samples. Free-living *Tetraselmis* cells have to undergo drastic structural and physiological changes after being incorporated into *S. roscoffensis*, such as a loss of cell wall, eyespot, and flagella (Douglas, 1983a; Douglas, 1985), which may require longer than the experimental time to proliferate throughout the worm's upper epidermis and provide the host with its full nutritional needs. This may also have contributed to the low survival of the worms in the experiment. Longer

experiments will be needed to test the stability and long-term physiological benefits of the different *S. roscoffensis*–*Tetraselmis* symbiotic pairings.

The nutritional profile of *S. roscoffensis* in the f/2 medium had similar protein and lipid contents to a closely related algal species, *T. suecica* grown on f/2 (Sharawy *et al.*, 2020): 38.7% DW of proteins and 12.4% DW of lipids. However, the worms had a much lower carbohydrate content (7.2 %DW) than *T. suecica* (44.3 %DW). The carbohydrates associated with the algal cell wall would have been lost as the cell wall was removed, along with other cell features when the cell was incorporated into the worm (Douglas, 1983a). Furthermore, the carbohydrates in free-living algae can be used for storage as starch grains (Raven and Beardall, 2003; Cheng *et al.*, 2017), but for symbiotic algae, excess carbohydrates may be metabolised by the host instead (Boyle and Smith, 1975).

Without the addition of inorganic nutrients (seawater treatment), the chlorophyll content of *S. roscoffensis* became nil; its total fatty acid and PUFA concentrations also decreased significantly. The fatty acid compositions of *S. roscoffensis*, when grown in f/2 and f/4, both contained EPA and DHA, as well as ALA, which can be a precursor to both EPA and DHA (Brenna, 2002; Oboh *et al.*, 2017). Notably, EPA and DHA were close to nil and ALA was absent when *S. roscoffensis* was grown in seawater. This supports the notion that the host worm relies on algal symbionts for PUFAs via the energy captured from photosynthesis, but this process requires chlorophyll and inorganic nutrients (Taylor, 1974). Therefore, by absorbing inorganic nutrients from its surroundings, the host worm could support its algal symbionts for mutual benefits (Carvalho *et al.*, 2013). The algal symbionts also increased the carotenoid content of the host in the presence of nutrients, which could provide important photoprotection to the worm when it is exposed to strong sunlight in the intertidal environment (Christaki *et al.*, 2013; Nissen *et al.*, 2015).

6.6.1 Conclusion

The result demonstrates the potential of *S. roscoffensis* as a novel feed—at least for the ornamental aquaculture industry—both in live and freeze-dried forms. The use of *S. roscoffensis* does not need enrichment and can deliver algae directly to fish species. All the freshwater and marine ornamental fish tested (except Striped blenny) consumed *S. roscoffensis* and displayed no adverse effects. Additionally, the first nutritional analysis of *S. roscoffensis* was performed, and in doing so, revealed the presence of ALA, EPA, and DHA, which are essential for fish health (Benemann, 1992; Hemaiswarya *et al.*, 2011). Microalgal cells are too

small for ornamental fish to directly consume, and the presence of the algal cell wall also reduces digestibility (Niccolai *et al.*, 2019; Machado, Carvalho and Pereira, 2022). The lack of cell walls in the symbiotic algae within *S. roscoffensis* theoretically improves its digestibility for fish.

Cultures from the laboratory collection have been maintained for over a year, with only one inoculation of worms collected from the wild and no addition of algae was required. Due to the way the algae are acquired by juvenile *S. roscoffensis*, no additional algal input would be required for the juveniles to maintain symbiosis in long-term cultures. Therefore, a continuous culture could be maintained with limited inputs. This simplifies the feed production process and reduces waste and the associated costs.

Chapter 7 General Discussion

The main aim of this thesis was to investigate the basic biology and behaviours of *Symsagittifera roscoffensis* and its algal symbiont *Tetraselmis convolutae* and provide insights into the environmental drivers at the field location and intra-species genetic diversity. I also in this thesis identified the potential aquaculture applications of this unique organism. To accomplish this, I focused on determining the environmental constraints of the worms, studying the worm's chemotaxis, geotaxis, phototaxis, and mechanical vibrations responses. I also conducted a field study of the populations and the environmental conditions as a genetic analysis of the intra-species relatedness from populations at known locations, potential aquaculture applications where assed by investigating the palatability of the worms to marine and freshwater fish and the ability to utilise a alternative symbiont and development of a qPCR protocol to assess the number of alga cells inside the worms.

7.1 Constraints of a photosymbiotic system

Symbiosis encompasses a wide range of interactions in a biological system, such as mutualism, parasitism and commensalism. Mutualistic symbiotic interactions between the host-and symbiont are often complex requiring a degree of cooperation between the two organisms. In a classical terrestrial symbiotic system, the benefits of symbiosis between the participants are clearly distinguishable. For instance, in the mycorrhizal system between plants and fungi, the symbiont or in this case the fungi receive additional nutrition in the form of photoassimilates from the plant, while the plant receives nitrates and phosphates from the fungi, these nitrates and phosphates are often unavailable for the plant to directly acquire. In other terrestrial examples, organisms use symbiosis to enhance fitness by conferring a stress tolerance to their hosts, allowing the host to survive in conditions in which it would usually not be able to (Bénard *et al*., 2020; Dunbar *et al.*, 2007).

Marine symbiosis such as photosymbiosis have historically been considered mutualistic, with each partner benefiting. In the *Symsagittifera roscoffensis* and *Tetraselmis convolutae* photosymbiotic relationship, the benefit to the host is apparent, the incorporated algal cells provide the host worm with photosynthetically derived nutrition (Yellowlees, Rees and Leggat, 2008). However, the benefits that the symbionts gain from the relationship is not well defined. Some authors speculate that the benefit to the algae is

protection and additional nutrients (Keeble, 1912; Provasoli *et al.*, 1968; Bailly *et al.*, 2014). However, these benefits seem marginal at best, especially considering that most other *Tetraselmis* species grow freely within the marine environment (He *et al.*, 2012), without the need for additional nutrients or protection that symbiosis provides. Moreover, the cost of symbiosis to the algae outweighs any potential benefits that they receive from symbiosis: During symbiosis the algae are subjected to a reduced growth rate (Wooldridge, 2010) and phenotypic changes (Provasoli *et al.*, 1968; Bailly *et al.*, 2014). Manipulation of the algae for the hosts own needs is observed in other marine photosymbiotic relationships in particular reef ecosystems, the most well studied being corals. In corals, the relationship is well-defined with a plethora of recent research (Weis, 2019; Gao *et al.*, 2024; Grupstra *et al.*, 2024). While the alga is manipulated in these systems it must also meet the demands of the coral for photoassimilates and even a small increase in ambient temperature, 1-2°C above the mean results in lower photosynthetic output, causing the alga to shift more of its resources into its own metabolic needs (Rowan, 2004; Carballo-Bolaños *et al.*, 2019). The shift in resources to the alga's own metabolic rate means that there is less photoassimilates available to the host. As a result, the coral expels the alga in an attempt to acquire another algal partner that can meet its demands; this breakdown in the relationship results in coral bleaching (Glynn, 1996; Hughes *et al.*, 2017).

While there are stark biological differences between corals and *Symsagittifera*, the similarities in marine photosymbiosis is evident. As such, the entire nature of the relationship seems to be defined by the host's requirement for photoassimilates rather than a mutually beneficial relationship. Such a relationship could be considered more akin to enslavement by the host or reverse parasitism (Decelle, 2013). In such a relationship the host manipulates the alga to sustain its own nutritional needs, with the algae receiving little to no benefit. Ultimately, when the host is no longer being provided with the high photosynthetic output that it requires of the algae, it discards the algae in hope of finding another more suitable.

Further evidence for the exploitation of algae by the host comes from the fact that *S. roscoffensis* is able to utilise alternative symbionts. The very nature of symbiotic relationships is defined as being highly specialised; however, contrary to this, Chapter 6 shows there is a large degree of plasticity in the choice of algal species used by the host. The plasticity that I reported on indicates that the host can utilise a range of species in the

absence of its preferred algae. It is possible that the only requirement of the host is to overcome the algae's defences and manipulate the algae to enable photosymbiosis. Symbionts of terrestrial symbiotic systems are elusive outside of the host and can be considered to be part of a rare biosphere (Nyholm and McFall-Ngai, 2004). In contrast, marine algae symbionts appear to thrive when not in symbiosis and can reach millions of individuals per L of seawater (Schoemann *et al.*, 2005), a vastly greater density than during its symbiotic life cycle (Decelle *et al.*, 2012).

Parasitism has to achieve the same outcome in order for the parasite to be successful. The parasite overcomes the hosts defence and then alters the molecular metabolism in favour of its own needs (Siddique and Grundler, 2018). However, in a parasitic system, the host is harmed by the parasite, resulting in a reduction in fitness. This, in turn, leads to a selection pressure where the host evolves new strategies to evade the parasite, resulting in an evolutionary arms race between the host and the parasite. In a traditional symbiotic system, both partners evolve to sustain the relationship in a co-evolving system (Moran and Wernegreen, 2000). However, in photosymbiotic systems such as corals, the main selection pressure experienced by the symbiont would be from within the host and would play an influential role in the selection of the algae (Decelle *et al.*, 2012). This, in turn, makes the symbiont more specialised towards the needs of the host and ultimately more susceptible to the hosts needs and manipulation, maintaining the symbiotic relationship.

In Chapter 6, the lack of selective evolution on alternative symbionts could be the reason why I did not see as much of the algae proliferating in the hosts tissues, as the algae were poorly adapted to the host and were able to resist the hosts exploitation to some extent. Evolutionary host selection also occurs in other photosymbiotic marine systems, such as corals, resulting in a reduction of the genome of *Symbiodinium*. Its genome is relatively small compared to other dinoflagellates (LaJeunesse *et al.*, 2005); resulting in a loss of non-essential genes; The *Symbiodinium* genome represents more of a host-dependent endosymbiotic bacterium than a dinoflagellate (McCutcheon and Moran, 2012).

While little genomic data is available for the *S. roscoffensis*-*T. convolutae* relationship, I found in Chapter 4 that there was little genetic variation between *T. convolutae* from the different locations. The low genetic variations between algae from different locations may indicate that the algae forming symbiosis with *S. roscoffensis* have

been subjected to an evolutionary selection by the host, enabling the host to manipulate the algal phenotype and maintain symbiosis, further supporting the reverse parasitism theory. If the photosymbiosis was more akin to terrestrial symbiosis, then it is possible that random individuals would find each other and form symbiosis via vertical transmission. Thus, it is possible that the genetic data in this case would detect small genetic differences between individuals.

Chapter 4 indicated that there are genetic differences between the *S. roscoffensis* populations. While my data provided more information, there are still unanswered questions regarding *S. roscoffensis,* such as how long have populations been separated and how did they arrive at the respected locations, as there is limited mobility between populations. Understanding the relatedness of the populations at different locations could provide additional details into the ecological history of populations and how they came to arrive at their respected locations. Additionally, reconstructing the ancestral position of the population could also lead to the discovery of new locations of populations.

In Chapter 6, I showed that there are potential aquaculture applications of the worms as a novel feed for fish. While I primarily tested ornamental fish in this chapter, it is important to note that there are numerous fish and invertebrate species that are farmed in aquaculture systems. The use of these worms in such systems could offer broader benefits to these species, including enhancing PUFA content of the fish or as live food that fish fry require.

7.2 Further work

Symsagittifera roscoffensis can be considered a model organism for studying photosymbiosis, primarily because of its ease if cultivation (Bailly *et al.,* 2014). Furthermore, in other symbiotic relationships it is extremely difficult to manipulate the host-symbiont relationship (Wang *et al.*, 2012). However, in *S. roscoffensis* the juveniles are born aposymbiotic (Provasoli *et al.*, 1968; Douglas, 1983a), a phenomenon not usually encountered in photosymbiotic systems except for coral bleaching induced by stress (Byler *et al.*, 2013).

The unique opportunity offered by *S. roscoffensis* allows us to conduct experiments aimed at gaining insights into host-symbiont interactions. Expanding upon the work carried out in Chapter 6, it would be interesting to explore further experiments on host-symbiont interactions. Early literature suggest that the algal cells undergo

changes, including the loss of flagella, eyespot and cell wall (Oschman, 1966; Provasoli *et al.*, 1968; Douglas, 1983a). However, it remains unclear how the worms facilitate these changes. It is possible that the worms can achieve this through genetic mechanisms such as downregulation or silencing off the genes associated with production of these structures (Bailly *et al*., 2014) Alternatively, the worms may employ other processes to remove them, wither through enzymatic or chemical degradation (Bailly *et al*., 2014). Moreover, the ability to induce morphological changes to the symbiont within the host is not well defined a natural setting. It also remains unclear if the morphological alterations induced in the algae are reversable.

Understanding how these morphological changes are induced could represent a strategy by the host to exploit photosynthates more efficiently. It may even redefine the very nature of photosymbiosis to a relationship more akin to 'enslavement' or 'reverse parasitism' of the algae by *S. roscoffensis* (Decelle, 2013).

To explore this further, a method similar to the one described in Chapter 6, involving microdissection to separate the embryos from the cocoons, could be used to produce aposymbiotic juveniles. These juveniles can then be introduced in free living *T. convolutae*; by monitoring the establishment of symbiosis over a set time frame, we can quantify the point at which the worms induce changes in the cell wall of the algae. Given that the cell wall of the algae is relatively small, additional microscopy stains such as Nan Orange (Grossart *et al.*, 2000) for the flagella and calcofluor white (Bidhendi *et al.*, 2020) could aid in visualisation.

Additionally, it is also worth exploring whether tissue homogenate from the worms may induce morphological changes in the free-living cells *in vitro*. If positive results are obtained, this work will guide future research to isolate and characterize the active compound in the homogenate.

In Chapter 6, I also demonstrated remarkable flexibility of *S. roscoffensis* in establishing symbiosis with other members of *Tetraselmis* not typically associated with symbiosis. This suggests a high degree of plasticity in *S. roscoffensis* to form photosymbiotic relationships. To expand on this work, I propose conducting a more rigorous experiments to investigate how symbiosis is induced in *S. roscoffensis,* using *Tetraselmis* and species from other genera, such as *Asteromonas* sp, and *Symbiodinium* sp.

Interestingly, other members of Convolutidae can also symbiosis with these species (Ax and Apelt, 1965; Winsor, 1990; Barneah *et al.*, 2007).

Upon establishment of symbiosis, one can measure various parameters, including the worm's photosynthetic performance, growth, reproduction and trans-generational transmission of the symbionts. Additionally, while there are reports of the worms switching back symbionts to its preferred symbiont, modern assays have not been used to test this hypothesis. To address this, I could reintroduce the wild-type *T. convolutae* to worms the worms that have previously been exposed to an alternative symbiont. After which I could perform a PCR using 18s rRNA primers on the *S. roscoffensis* to determine if the worms are able to swap the algae cells. This approach would identify the algae symbiont inside of the worms down to the species level. These experiments will produce valuable insights into the extent of plasticity in photosymbiosis of *S. roscoffensis* and the relative fitness of the different host-alga pairings.

This additional research on *S. roscoffensis* will advance theoretical and practical understanding about *S. roscoffensis* and more broadly photosymbiosis in ways that are not feasible with other symbiosis systems. The knowledge we gain from this research may also lay the foundation for novel applications, such as customising host-alga symbiosis for specific aquacultural and biotechnological uses (Chapter 6), considering the high productivity and nutritional value of *Tetraselmis* sp., as well as for developing oxygenic symbiont in human tissue engineering (Chávez *et al.*, 2020).

8.1 Concluding remarks

This thesis contributes to the understanding of photosymbiosis, with a particular focus *Symsagittifera roscoffensis*. It offers fresh insights into how environmental conditions influence its symbiont, the dynamics of gene flow between populations, and the role of behavioural responses in this relationship.

9 Appendices

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Swansea University
Prifysgol Abertawe

HEALTH & SAFETY
IECHYD A DIOGELWCH

Health and safety documents

Risk Assessment			
College/PSU	Bioscience	Assessment Date	26/07/21
Location	Wallace	Assessor	Nathan Thomas
Activity	DNA extraction and amplification	Review Date (if applicable)	
Associated documents	•	•	

Part 1: Risk Assessment

What are the hazards?	Who might be harmed?	How could they be harmed?	What are you already doing?	Do you need to do anything else to manage this risk?	Action by whom?
Handling of biological samples	Staff and other lab users	Biological hazards	Use Personal protective equipment when handling samples (lab coat, and disposable gloves). Clean up spills with 70% ethanol. All wase should be autoclaved before disposal.	No	Nathan Thomas

What are the hazards?	Who might be harmed?	How could they be harmed?	What are you already doing?	Do you need to do anything else to manage this risk?	Action by whom?	Action by when?	Do Yes
Handling DNA extraction chemicals	Staff and lab users	Chemical burnes	Use PPE and conduce a COSHH assessment for the chemicals that are being used (no hazards associated with chemicals)		Nathan Thomas	Pripr to using the lab	
Manual handling	Nathan Thomas	Physical injury	Use lab equipment according specific details provided in the introduction		Nathan Thomas	Prior to using equipment	Yes

Part 2: Actions arising from risk assessment

Actions	Lead	Target Date	Do Yes

COS Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol #	**Title:** Palatability study of *S. roscoffensis* on cleaner wrasse
Associated Protocols #.....	**Description:** determine the pliability of the roscofs worm when fed to cleaner wrasse. As this work will take place during the COVID-19 pandemic extra measures are in place to ensure safe working conditions and reduce chances of spreading the virus.

Location: CESAR

Identify here risks and control measures for work in this environment: general health risks (intruders, slips, fire, no help in emergency etc.)
Biosecurity risks: Prevent the introduction of an infectious agent into CESAR
Biosecurity control measures: footbaths to be used when entering and exiting the building. Hand washing after working and handling equipment in each area. All husbandry equipment sould be cleaned and disinfected after each use, and should not be transferred between facility's. use disposable aprons when working with animals and wet feeds.
PPE: White boots or shoe covers must be worm upon arrival, Lab coats, latex clothes and safety glasses must be worn, whilst working in the laboratory.
***Control measures*:** have access to a charged phone for emergencies, know emergency exit routes, keep work areas and paths clear to prevent tripping/slips, be familiar with fire safety procedures, do not move heavy loads which may cause injury.
COVID19 risks: infection & spread of virus
COVID19 control measures: self quarantine when any symptoms arise in myself or housemates & alert university + NHS Wales and self-isolate, avoid physical contact with others, wash hands regularly or use sanitiser at the designated points, avoid touching face, use the one way system that is in place. A maximum of two people are allowed in the C labs, always comply with social distancing, only come in when work is absolutely necessary. Face marks are to be worn at all times and all surfaces are to be cleaned down with 70% ethanol.

Chemicals	**Quantity**	**Hazards**	**Category (A,B,C,D)***	**Exp. Score**
Guillard's F/2	O.5L	N/A	D	1
Ethanol	<0.05L	Irritant, inhalation, flammable	C	2

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :-

Storage conditions and maximum duration :-flammable cupboard, F/2 in the Wallace 120 freezer

Secondary containment (of protocol) open bench, fume hood when diluting ethanol

Disposal e.g. autoclaving of biohazard, chemical disposal for large quantity of ethanol

COS Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name Nathan J Thomas ..... **Signature** [redacted] **date 10-3-21** .....

Supervisor* Kam Tang [redacted] ..... **Signature** [redacted] **date** 11-3-2021

Activity title Teaching ..... **Base location (room no.)** CESAR

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOS)

School Activity Serial # (enter Employee No. or STUREC No .....

Start date of activity (cannot predate signature dates) .....

End date of activity (or 'on going') .....

Level of worker (delete as applicable) .....

PG,

Approval obtained for Gene Manipulation Safety Assessment by UWS ? Yes/not applicable

Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? Yes/not applicable

Approval obtained for use of radioisotopes by COS ? Yes/not applicable

Record of specialist training undertaken

Course	date

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
1						11					
2						12					
3						13					
4						14					
5						15					
6						16					
7						17					
8						18					
9						19					
10						20					

See notes in handbook for help in filling in form (Continue on another sheet if necessary)

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol #	**Title:** DNA and PCR amplification for *S. roscoffensis*
Associated Protocols #**.....**	**Description:** DNA extraction and PCR amplification of S.*roscoffensis* samples. As this work will take place during the COVID-19 pandemic extra measures are in place to ensure safe working conditions, and reduce chances of spreading the virus.

Location: Wallace 123

circle which Bioscience and Geography Local Rules apply –

Boat Field Genetic-Manipulation Laboratory Office/Facility Radioisotope

Identify here risks and control measures for work in this environment, additional to Local Rules:
General lone working risks: general health risks (intruders, slips, fire, no help in emergency etc.)
***Lone working control measures*:** have access to a charged phone for emergencies, know emergency exit routes, keep work areas and paths clear to prevent tripping/slips, alert housemates when I will be back, be familiar with fire safety procedures, do not move heavy loads which may cause injury.
COVID19 risks: infection & spread of virus
COVID19 control measures: self quarantine when any symptoms arise in myself or housemates & alert university + NHS Wales, avoid physical contact with others, wash hands often and when coming into & out of the lab, avoid touching face, only do necessary lab work at university that cannot be postponed or done from home, avoid use of high-touch surfaces where possible (if not possible wash hands ASAP), keep 2m distance from others, travel to university by walking alone. Please see page 1 for additional information.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Qiagen DNA blood and tissue kit	<0.5L	N/A	D	1
DNA primers	<0.5L	N/A	D	1
Taq master mix	<0.5L	N/A	D	1
qPCR kit	<0.5L	N/A	D	1
DNA clean up kit	<0.5L	N/A	D	1
Agarose gel	<100g	N/A	D	2
Green safe premium	<1 ml	N/A	D	1
NYZ ladder V	<1ml	N/A	D	1
1X TBE	<100 ml	N/a	C	2

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the highest Exposure Score above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low ~~Medium~~ ~~High~~

Primary containment (of product) ***sealed flask***/~~bottle~~/***glass***/~~plastic/other (state)~~ :-

Storage conditions and maximum duration :- In the Wallace 123 freezer

Secondary containment (of protocol) open bench/~~fume hood~~/~~special (state)~~ :-

Disposal e.g. **autoclaving of biohazard**, ~~*SU chemical disposal*~~

Identify other control measures (circle or delete) – *nitrile gloves; safety glasses; lab coat*

Fire extinguisher available on site. Fire service to be called in case of fire. CESAR building alarm to be sounded for evacuation outside Fulton house.
Supervision/training for worker (circle) None required Already trained Training required Supervised always
Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures. *Name & signature of worker* Walter Thomas [redacted] 6-3-21 *Name & counter-signature of supervisor*..... [redacted] ..... *Date*..... 11-3-2021
Date of first reassessment Frequency of reassessments

Guidance for Completion of COS Protocol Risk Assessment Form

Note – you are strongly advised to complete electronic versions of this form, enabling you to readily expand and contract sections as required to ensure clarity and adequate documentation. Do **not** delete any sections! Instead, mark inappropriate sections with NA (not applicable) and contract the section to save space on the final printed form.

Protocol - any self-contained procedure. This could be any activities undertaken, be they lab-work, use of equipment, fieldwork or office work. Your complete research/teaching/administration **activity** (e.g. undergraduate project, PhD study, research grant, other) is therefore made up from separate **protocols**. If the protocol is mainly of low hazard, but with one or more hazardous components, consider making the manipulation of the latter a separate protocol and tie them together by completing the "*Associated Protocol*" box. This is because the entire protocol must be conducted under conditions required for the handling of the most hazardous component.

Title/Description - give sufficient detail to make it obvious what the protocol involves.

Location – identify which local rules apply. More than one rule may apply. Then add any additional risks and control measures peculiar to this protocol (e.g. site-specific fieldwork information; use of autoclaves, sonicators; mechanical, electrical hazards). You may also wish to stress any particularly important risks and controls even if indicated in local rules.

Chemicals etc. - give name, maximum quantity used, list hazards, hazard category (see Table 1) and calculate the **Exposure Score** (see Table 2) for **every** chemical used. Expand the area in the table as required.

Exposure Potential (see Table 3) - complete this section for the chemical which has the **highest** exposure score in your chemical list as this defines the highest risk factor.

Primary containment/Storage - detail how and where, and for how long, the resultant product from the protocol will be stored. The product must be labelled with the date of synthesis, and disposed of (see below) before the maximum duration time has elapsed.

Secondary containment - detail where the protocol will be performed (refer to Table 4).

Disposal - detail how you will dispose of surplus reagents and the product of the protocol. Final disposal must be undertaken within the period noted in the 'maximum duration' under 'Storage' (above).

Identify other control measures – typically these refer to special protective clothing etc.

Justification and controls for any work outside normal hours – out of hours working is only allowed under special conditions (e.g. 24h sampling, sampling related to tides etc.); convenience is

COS Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name Nelly Iberra ..... **Signature** [redacted] ..... **date** 7-10-19

Supervisor* Kam W. Tang ..... **Signature** [redacted] ..... **date** 2-10-2019

Activity title ..... **Base location (room no.)** Wallace 120

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOS)

School Activity Serial # (enter Employee No. or STUREC No......

Start date of activity (cannot predate signature dates) 3-10-19

End date of activity (or 'on going') on going

Level of worker (delete as applicable) PGR

UG,PG, research assistant, technician, administration, academic staff, other (state)

Approval obtained for Gene Manipulation Safety Assessment by UWS ? ~~Yes~~/not applicable

Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? ~~Yes~~/not applicable

Approval obtained for use of radioisotopes by COS ? ~~Yes~~/not applicable

Record of specialist training undertaken

Course	date
lab induction	7-10-19

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
1						11					
2						12					
3						13					
4						14					
5						15					
6						16					
7						17					
8						18					
9						19					
10						20					

See notes in handbook for help in filling in form (Continue on another sheet if necessary)

COS Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol #	**Title:**
Associated Protocols **#.....**	**Description:**

Location: lab

Identify here risks and control measures for work in this environment:

Use PPE dedicated work coat

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Sodium thiosulfate	15g	SOC please read MSDS sheet	D	0

Hazard Category (known or potential)

- **A** (e.g. carcinogen/teratogen/mutagen)
- **B** (e.g. v.toxic/toxic/explosive/pyrophoric)
- **C** (e.g. harmful/irritant/corrosive/high flammable/oxidising)
- **D** (e.g. non classified)

Exposure Potential Circle the **highest Exposure Score** above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low **Medium** **High**

Primary containment (of product) sealed flask/bottle/glass/plastic/other (state) :-

Storage conditions and maximum duration :-

Secondary containment (of protocol) open bench/fume hood/special (state) :-

Disposal e.g. autoclaving of biohazard, UWS chemical disposal

Identify other control measures (circle or delete) - latex/nitrile/heavy gloves; screens; full face mask; dust mask; protective shoes; spillage tray; ear-defenders; other (state)

Justification and controls for any work outside normal hours

N/A

Emergency procedures (e.g. spillage clearance; communication methods)

Fire extinguisher available on site. Fire service to be called in case of fire. Wallace building alarm to be sounded for evacuation.

Supervision/training for worker (circle)

None required Already trained Training required Supervised always

Declaration I declare that I have assessed the hazards and risks associated with my work and will take appropriate measures to decrease these risks, as far as possible eliminating them, and will monitor the effectiveness of these risk control measures.

Name & signature of worker .....

Name & counter-signature of supervisor..... *Date*.....

Date of first reassessment Frequency of reassessments

Bioscience and Geography Protocol Risk Assessment Form

(Expand or contract fields, or append additional sheets as required; insert NA if not applicable)

Protocol #	**Title:** DNA and PCR amplification for *S. roscoffensis*
Associated Protocols #**.....**	**Description:** DNA extraction and PCR amplification of S.*roscoffensis* samples. As this work will take place during the COVID-19 pandemic extra measures are in place to ensure safe working conditions, and reduce chances of spreading the virus.

Location: Wallace 123

circle which Bioscience and Geography Local Rules apply –

Boat Field Genetic-Manipulation Laboratory Office/Facility Radioisotope

Identify here risks and control measures for work in this environment, additional to Local Rules:
General lone working risks: general health risks (intruders, slips, fire, no help in emergency etc.)
***Lone working control measures*:** have access to a charged phone for emergencies, know emergency exit routes, keep work areas and paths clear to prevent tripping/slips, alert housemates when I will be back, be familiar with fire safety procedures, do not move heavy loads which may cause injury.
COVID19 risks: infection & spread of virus
COVID19 control measures: self quarantine when any symptoms arise in myself or housemates & alert university + NHS Wales, avoid physical contact with others, wash hands often and when coming into & out of the lab, avoid touching face, only do necessary lab work at university that cannot be postponed or done from home, avoid use of high-touch surfaces where possible (if not possible wash hands ASAP), keep 2m distance from others, travel to university by walking alone. Please see page 1 for additional information.

Chemicals	Quantity	Hazards	Category (A,B,C,D)*	Exp. Score
Qiagen DNA blood and tissue kit	<0.5L	N/A	D	1
DNA primers	<0.5L	N/A	D	1
Taq master mix	<0.5L	N/A	D	1
qPCR kit	<0.5L	N/A	D	1
DNA clean up kit	<0.5L	N/A	D	1

Hazard Category (known or potential)
A (e.g. carcinogen/teratogen/mutagen)
B (e.g. v.toxic/toxic/explosive/pyrophoric)
C (e.g. harmful/irritant/corrosive/high flammable/oxidising)
D (e.g. non classified)

Exposure Potential Circle the highest Exposure Score above. Use this to calculate the exposure potential for the entire protocol (see handbook). Indicate this value below.

Low ~~Medium~~ ~~High~~

Primary containment (of product) ***sealed flask***/~~bottle~~/***glass***/~~plastic/other (state)~~ :-

Storage conditions and maximum duration :- In the Wallace 123 freezer

Secondary containment (of protocol) open bench/~~fume hood~~/~~special (state)~~ :-

Disposal e.g. **autoclaving of biohazard**~~,~~ ~~*SU chemical disposal*~~

Identify other control measures (circle or delete) – *nitrile gloves; safety glasses; lab coat*

Justification and controls for any work outside normal hours NA

Bioscience and Geography Risk Assessment for Teaching, Administration and Research Activities

Swansea University; College of Science

Name Nathan J Thomas ..... **Signature** [redacted] **date** 3.8.21

Supervisor*Kam Tang ..... **Signature** [redacted] **date** 3.8.21

Activity title: PCR and DNA extraction of *S. roscoffensis* ..... **Base location (room no.)** **Wallace 123**

(* the supervisor for all HEFCW funded academic and non-academic staff is the HOC)

University Activity Serial # (enter Employee No. or STUREC No. .....
Start date of activity (cannot predate signature dates): ***August 2020*** .....
End date of activity (or 'on going') ***No later than December 2020*** .....

Level of worker (delete as applicable) PhD student.....

~~UG~~, PG, ~~research assistant, technician, administration, academic staff, other (state)~~

Approval obtained for Gene Manipulation Safety Assessment by SU ? **Not applicable**
Licence(s) obtained under "Animals (Scientific Procedures) Act (1986)" ? **Not applicable**
Approval obtained for use of radioisotopes by COS ? **Not applicable**

Record of specialist training undertaken

Course	date

Summary of protocols used; protocol sheets to be appended plus COSHH details for chemicals of category A or B with high or medium exposure

Protocol Details						Protocol Details					
#	Assessment					#	Assessment				
	1st date	Frequency of re-assessment	Hazard category	Secondary containment level	Exposure potential		1st date	Frequency of re- assessment	Hazard category	Secondary containment level	Exposure potential
1						11					
2						12					
3						13					
4						14					
5						15					
6						16					
7						17					
8						18					
9						19					
10						20					