THE BURROWING BEHAVIOUR AND DIET OF THYASIRID BIVALVES FROM BONNE BAY, NL

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Abstract

Thyasirids are an interesting model group due to the presence of sulfur-oxidizing symbionts in some species but not all. Symbiotic thyasirids form numerous burrows (i.e. pedal tracts) to obtain sulfides. Asymbiotic thyasirids were not expected to produce similar burrows as they have no need for sulfides. Comparisons of the burrowing behaviour of symbiotic and asymbiotic thyasirids from Bonne Bay, Newfoundland were made. Unexpectedly, no differences in burrowing depth, number or length of pedal tracts were found between symbiotic and asymbiotic thyasirids. To explore whether pedal tracts of asymbiotic thyasirids might be used for "farming" chemoautotrophic bacteria, we performed stable isotopic analysis (carbon and nitrogen). As expected, the symbiotic taxon consumes mainly thiotrophic bacteria, and one of the asymbiotic taxa consumes mainly suspended material. Contrary to expectations, the other asymbiotic taxon consumed mainly thiotrophic bacteria, in a greater proportion than the symbiotic taxon, indicating that “microbial farming” is likely occurring for that taxon.

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List of Abbreviations and Symbols

SPOM – Suspended particulate organic matter

OM – Organic matter

OTU – Operational taxonomic unit

C - Carbon

N - Nitrogen

SPM – Suspended particulate matter

MODIS – Moderate resolution imaging spectroradiometer

Chl *a* – Chlorophyll A

HCl – Hydrochloric acid

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Introduction

Chemosymbiosis

Animals have evolved in environments dominated by bacteria, and have found many ways to take advantage of the vast metabolic repertoire of these prokaryotes. Bacteria play important roles in animal nutrition, either by serving as prey or by contributing digestive enzymes or nutritional molecules that can be used by the animal (McFall-Ngai *et al.* 2013). In some marine invertebrates, a highly specific association with chemoautotrophic (sulfur-oxidizing or methanotrophic) bacteria provides nutritional benefits to the host; this type of association, called chemosymbiosis, was first discovered in the l980s at hydrothermal vents (Cavanaugh *et al.* 1981). Chemosymbiotic bacteria use chemicals such as sulfide as an energy source to autotrophically produce organic carbon compounds that can be used by both the bacteria and the host species. Most chemoautotrophic hosts maintain their symbionts intracellularly and have molecular or behavioural adaptations for supplying their symbionts with the energy sources they require (Taylor & Glover 2010). Chemosymbioses typically occurs in what we consider harsh or high organic matter environments such as cold seeps, whale falls or sulfide rich muds, but can also be found in less organically-enriched sediments (Dubilier *et al.* 2008).

Bivalves are some of the first organisms in which chemosymbiosis was discovered outside of hydrothermal vents (e.g. in marine muds and salt marshes; Cavanaugh *et al.* 1983). Chemosymbiosis has been confirmed in five bivalve families living in diverse habitats: the Solemyidae, the Mytilidae, the Lucinidae, the Vesicomyidae, and the Thyasiridae (Taylor & Glover 2010), with putative

chemoautotrophic bacteria observed in three other families (Nucinellidae, Montacutidae and Basterotiidae; Oliver 2013, Oliver *et al.* 2013). The extent to which species within these families rely on their symbionts for their nutrition varies widely, with solemyids and vesicomyids being highly symbiont-dependent, while lucinids and thyasirids are mixotrophic and consume both chemoautotrophic bacteria-derived nutrients and other particulate food (Rodrigues *et al.* 2013, Rossi *et al.* 2013, van der Geest 2014). Mixotrophy is considered to be an adaptive strategy in unstable environments, allowing chemosymbiotic hosts to exploit multiple food sources according to nutrient availability or host metabolic demands (Dufour & Felbeck 2006, van der Geest 2014). For example, chemosymbiotic thyasirids in fjord sediments depend more on symbiont-derived carbon in years when sediment sulfide concentrations are high than when they are low (Dando & Spiro 1993). The mechanisms through which mixotrophic bivalves control the balance between chemosynthetic and particulate food sources, and the importance of mixotrophy in chemosymbiosis evolution and establishment are unknown.

The Bivalve Family Thyasiridae

The bivalve family Thyasiridae displays a range of chemosymbiotic characteristics, making it an interesting group for studying the evolution of such symbioses and of mixotrophy (see Fig. 1 for an image of a thyasirid). Thyasirids are globally distributed in cold waters and live infaunally in shallow to abyssal depths (Payne & Allen 1991); they are the most dominant lamellibranch family in the deep-sea (Allen 2008). Thyasirid species are either asymbiotic or symbiotic, and in the latter, bacteria are extracellular to the gill epithelial cells in all but one species (Southward 1986, Fujiwara *et*

al. 2001, Dufour 2005). In comparison to most other chemosymbiotic bivalves, the extracellular location of thyasirid symbionts is considered to be less derived (Taylor *et al.* 2007). Among bivalves, the Thyasiridae is unique in having chemosymbiotic species distributed in more than one clade, often within the same clades as asymbiotic species (Taylor *et al.* 2007). The evolutionary history of the Thyasiridae is not well known, but it is hypothesized here that there have either been multiple, independent symbiont acquisition events within the family or that some species have independently lost their chemoautotrophic partners through time.

Figure 1: Image of *Thyasira flexuosa* with foot extended. Shell diameter = 5 mm.

Most examined thyasirid symbionts are thiotrophs that likely use sedimentary sulfides as an energy source (Distel 1998). In homogenates of *Thyasira sarsi* and *T.*

flexuosa gill tissue, the activities of ribulosebisphosphate carboxylase (Rubisco) and adenylylsulfate reductase demonstrated the thiotrophic activity of symbionts in those species (Dando & Southward 1986). The transfer of chemoautotrophically derived carbon from symbionts to host tissue has been demonstrated in *T. sarsi* and *T. equalis* using carbon stable isotope analysis (Dando & Spiro 1993); this study also showed that the nutritional dependence of thyasirids upon their symbionts fluctuates according to the availability of sedimentary sulfides. Dufour & Felbeck (2006) have shown that differing sulfide levels in the sediment also affect thyasirid symbiont abundance. In mixotrophic thyasirids, the non-symbiont derived fraction of their nutritional demands is met by particulate feeding (Dufour & Felbeck 2006), with thyasirids generally considered to be suspension-feeders, obtaining food through the semi-permanent inhalant tube constructed using their foot (Allen 1958).

The Burrowing Behaviour of Thyasirids

All chemosymbiotic host species, particularly those found outside hydrothermal vents, need to ensure that their symbionts have access to the chemicals they require. In addition to forming an inhalant tube, through which oxygenated water and particulate food can circulate, thyasirids and lucinids use their foot to create extensive burrows (henceforth called pedal tracts) radiating down and outwards from their shell (Allen 1958). These pedal tracts are thought to be used to access patches of sulfides from deeper sediments (Dando & Southward 1986, Dufour & Felbeck 2003). The maximal depth of thyasirid pedal tracts is considered to be equivalent to the length of the foot while extended, and corresponds to the length of their inhalant tube (Allen 1958). Large species

or individuals are expected to be able to burrow more deeply than smaller ones, yet Anderson (2014) found no clear correlation between size and burial depth (i.e., where the shells are located within sediments, not the maximal depth reached by pedal tracts). Dufour and Felbeck (2003) discovered that when sediment sulfide concentrations were greater, the pedal tracts of symbiotic thyasirids were shorter and less numerous, suggesting a relationship between thyasirid pedal tracts and the provision of sulfides for their symbionts. The burrowing behaviour of asymbiotic thyasirids has been virtually unexplored, and it is unclear if asymbiotic species form pedal tracts in addition to an inhalant tube.

Thyasirids in Bonne Bay, Newfoundland

The natural diversity and abundance of thyasirids in Newfoundland creates an opportunity to investigate the burrowing and feeding behaviours of these bivalves. Thyasirids have been reported in Newfoundland waters (Ramey & Snelgrove 2003), including the fjord of Bonne Bay (Quijón & Snelgrove 2005; Batstone *et al.* 2014). Notably, a cryptic species complex was recently discovered in the latter site, consisting of three closely related taxa resembling *Thyasira gouldi* (hereafter *Thyasira* cf. *gouldi*) in which Operational Taxonomic Units (OTU) 1 and 2 have chemoautotrophic bacterial symbionts, OTU 3 is asymbiotic (Batstone *et al.* 2014). An additional, undescribed asymbiotic species, *Parathyasira* sp., also inhabits this fjord (Batstone 2012).

Bonne Bay is a large subarctic fjord with two arms, South Arm and East Arm (Fig. 2). Thyasirids have so far only been found in East Arm at three locations: South East Arm, Deer Arm and Neddy's Harbour (Batstone *et al.* 2014). All *Thyasira* cf. *gouldi*

OTUs and *Parathyasira* sp. co-occur at South East Arm and Deer Arm, whereas only symbiotic *T.* cf. *gouldi* (OTUs 1 and 2) have been reported from Neddy's Harbour (Batstone 2012, Batstone *et al.* 2014). The distribution of thyasirids at these sites is patchy. At Deer Arm, asymbiotic thyasirids (*T.* cf. *gouldi* OTU 3 and *Parathyasira* sp.) tend to be more abundant than symbiotic thyasirids, while symbiotic thyasirids are predominate at South East Arm, and reach higher densities than they do at Deer Arm (Batstone *et al.* 2014, Batstone & Dufour in press). Symbiotic thyasirid densities at Neddy's Harbour are spatially variable and comparable to those at the other two sites (Batstone & Dufour in press).

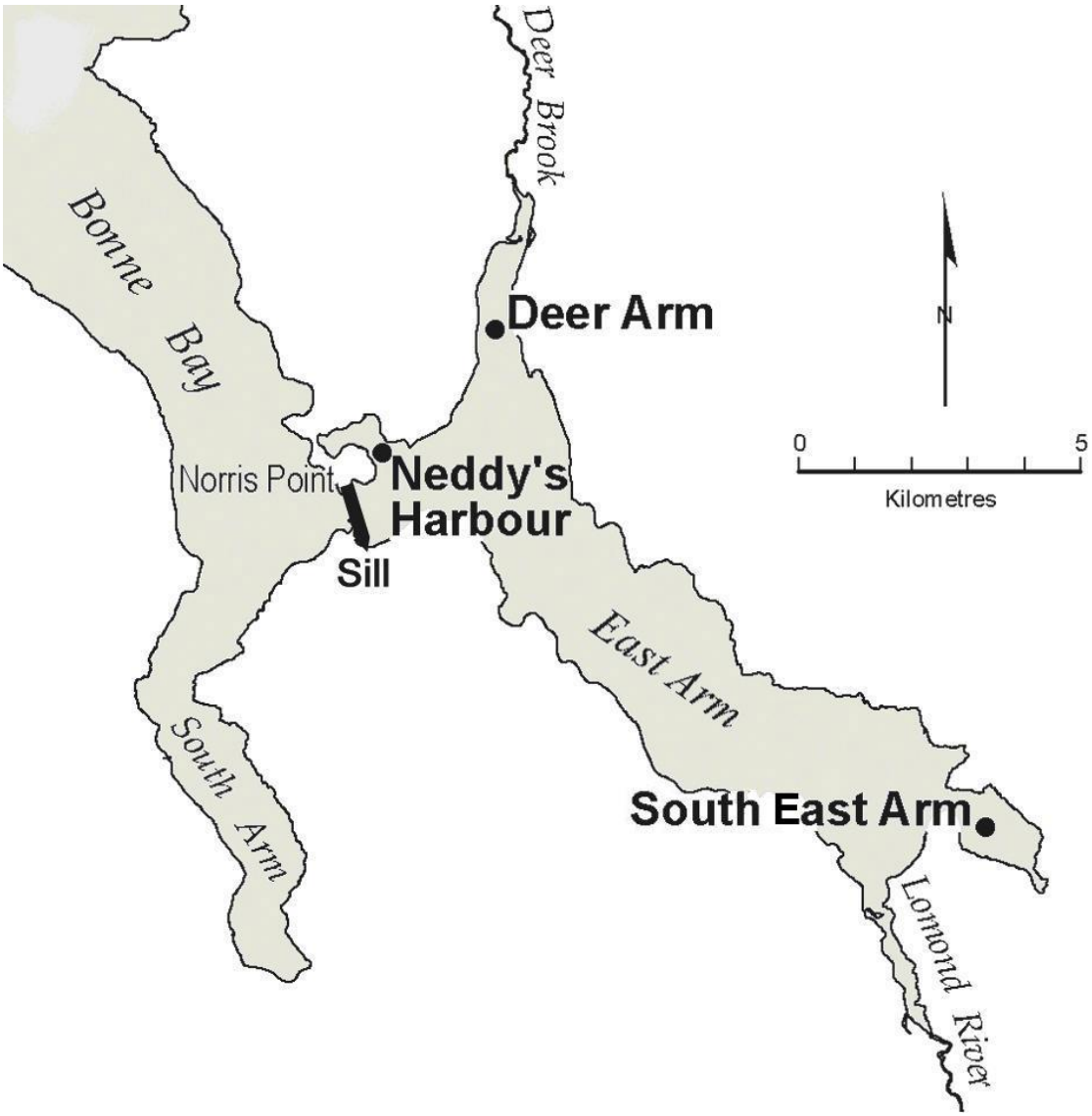

Figure 2: Map of Bonne Bay, NL. Sill and sample locations indicated. Bonne Bay opens to the Gulf of St Lawrence. Modified from Batstone *et al*. (2014)

Thesis Objectives

The availability of both symbiotic and asymbiotic thyasirids within Bonne Bay provides us with the opportunity to study burrowing behaviours and investigate possible

feeding modes in this group. This thesis has two main objectives: the first is to investigate the burrowing behaviour of the Bonne Bay thyasirids using mini aquaria (Chapter 1). Although burrowing behaviours have been studied in some thyasirids and lucinids (Dufour & Felbeck 2003; Stanley 1970), that of asymbiotic species remains largely unexplored. Comparing the behaviours of closely related *Thyasira* cf. *gouldi* OTUs that live either with or without symbionts is of particular interest, as it may indicate whether symbiont presence might be directly associated with burrowing behaviours, as suggested by Dufour & Felbeck (2003). The second objective of this thesis is to determine the relative importance of various food sources for the three thyasirid taxa from Bonne Bay (Chapter 2). Diet studies are often conducted by examining gut content (where direct dissection and investigation of gut content is possible) or by characterizing stable isotope composition. The latter approach considers the ratios between the stable isotopic forms of certain elements (typically C and N), which can help to determine the food sources of organisms and trophic relationships within ecosystems (Peterson & Fry 1987). Carbon isotope ratios in a consumer reflect the source of carbon originally fixed by a producer; given that different producers have differing degrees of selectivity for the stable isotopes of carbon, it is possible to differentiate between organic carbon fixed by different classes of organisms, such as various photoautotrophs and chemoautotrophs (Peterson & Fry 1987, Robinson & Cavanaugh 1995). Estimates of the relative contribution of carbon that is symbiont-derived versus photosynthetically-produced and then consumed by a mixotrophic bivalve can be made by characterizing $\delta^{13}C$ (Spiro *et al.* 1986, Dando & Spiro 1993, van der Geest *et al.* 2014). Nitrogen stable isotopes provide information on

the trophic level of the organism, as nitrogen fractionates in a predictable manner through food chains (Peterson & Fry 1987). Previous studies have used carbon isotope ratios of thyasirid tissues as indicators of the proportion of the diet that is derived from sulfur-oxidizing symbionts (Spiro *et al.* 1986, Dando & Spiro 1993). A recent study also documented the nitrogen isotopic composition of a thyasirid species from Japan (Kiyashko *et al.* 2014). In this project, the stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) of symbiotic and asymbiotic thyasirids from Bonne Bay were compared, and mixing models were used to evaluate the contribution of various food sources to the diet of those bivalves.

By considering together the results from those two thesis chapters, a more detailed perspective is gained on the diversity of feeding modes in thyasirids; in particular, a new feeding mode is proposed for some asymbiotic thyasirids. Results are incorporated in a new model of chemosymbiosis evolution in the bivalve family Thyasiridae.

Co-Authorship Statement

In regards to the *design and identification of the research proposal*, my supervisor assisted with the initial research idea and gave constant feedback on the written proposal that I wrote and submitted during BIOL 7000. Regarding the *practical aspects of the research*, I conducted all of the work except for preparation of some of the stable isotope samples, which were done by Dr. Flora Salvo who has been given Co-authorship of that chapter. Regarding the *data analysis*, I fully conducted this work, receiving and applying advice given by my supervisor and Dr. Flora Salvo. Finally, I contributed fully to the *manuscript preparation*, writing each draft and incorporating the feedback given by my supervisor and committee.

Chapter 1: The Burrowing Behaviour of Symbiotic and Asymbiotic Thyasirids from Bonne Bay, Newfoundland

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Abstract

Within the bivalves family Thyasiridae there are species that are chemosymbiotic with sulfur-oxidizing bacteria and others that are asymbiotic. Chemosymbiotic species create extensive burrow structures called pedal tracts. The purpose of these pedal tracts is thought to gain access to sulfides in sediment. It is expected asymbiotic species would lack pedal tracts. Within Bonne Bay, NL, there are three closely related taxa of thyasirids, one symbiotic and two asymbiotic. Burrowing behaviour was investigated to determine if the asymbiotic create pedal tracts. Photographic images and X-radiographs of mini tank experiments were taken. All three taxa created pedal tracts with no difference in the depth, total length or number of pedal tracts. We hypothesized that the formation of pedal tracts in the asymbiotic taxa may be due to, 1) a previous symbiotic state, and/or 2) an adaptation for pedal feeding, which may also include `farming` of bacteria along burrow walls.

Introduction

Of all groups of invertebrates living within marine sediments, bivalves are among the most conspicuous and evolutionarily successful. The earliest bivalves are considered to have been shallow living organisms possibly oriented in a similar manner to the modern *Glycymeris glycymeris* (i.e. partially buried in surface sediments; Morton 1996). The bivalves can be subdivided into those that remained on, or very close to, the sediment-water interface, and others which began burrowing to deeper depths and developed a suspension feeding mode of life (Morton 1996; Bieler & Mikkelsen 2006). The colonization of the endobenthic realm by bivalves was facilitated by the evolution of the foot as an effective hydraulic burrowing organ (Seilacher 1984).

The generalized form of the bivalve foot is cylindrical with a central haemal space surrounded by circular and longitudinal muscles that allow for extension and contraction. The bivalve foot is variable in shape (e.g., vermiform or slipper-shape), may have specialised regions such as a heel, or be highly extensible dependent upon preferred substrates and mode of life of the bivalve. The variations in form of the foot are commonly related to the function. For example, a slipper-shaped foot is adapted for digging shallow burrows, and a heel can both improve anchoring and act as a plough in deep burrowing forms (Allen 1958).

Bivalve burrowing typically consists of a repetitive succession of behaviours: 1) expansion of the shells to form a proximal anchor; 2) probing of the foot causing shell contraction and liquefaction of the sediment; by 3) contraction of the foot's longitudinal muscles to create a bulbous distal anchor; 4) contraction of the pedal retractor muscles to

draw the shell towards the distal anchor made by the foot. The contraction of the longitudinal muscles may be accompanied by a rocking motion of the shell, and/or by valve closure and ejection of water that leads to sediment liquefaction around the shell. This succession usually continues until the bivalve has reached the length of its siphons or foot, depending on the group (Allen 1958; Trueman 1966, 1983). Due to the fact that burrowing bivalves need to maintain access to surface water for oxygen (and for suspension-feeders, particulate food), they either burrow only as deep as the maximum length of their siphons (typical of most bivalve species; Trueman 1983), or create a mucus-lined inhalant tube by extending their foot to the surface (as in the Lucinoidea and the Thyasiroidae; Allen 1958).

Among bivalves, one of the most accepted functions of burrowing is to gain protection from predators. The 'radiation of predators' seen in the Mesozoic is thought to have provided selection pressure driving the evolution of deeper and faster burrowing bivalves (Stanley 1973). Since most epi-benthic predators search the sediment-water interface for their prey, shallow-tier bivalves are at a greater risk of being preyed upon (Zwarts & Wanink 1989). In intertidal regions, burrowing can also protect bivalves from being washed out by waves and tides, as well as from desiccation and fluctuations in temperature and salinity (Ratcliffe *et al.*1981). Although suspension-feeding is the dominant feeding mode for most bivalves, other species deposit-feed: either from the sediment-water interface or at depth in the sediment (Stanley 1970). Living infaunally gives deposit-feeding bivalves greater access to sedimentary organic matter than shallow living taxa. Bivalves having a symbiotic relationship with chemoautotrophic sulfur-

oxidizing bacteria (Cavanaugh *et al.*1981; Cavanaugh 1983) create deep burrows to access sulfidic pore waters in the anoxic or suboxic zones of the host sediment.

Chemosymbiotic bivalves (especially vesicomyids) are commonly associated with hydrothermal vents. Vesicomyids lie partly buried in sediments and access sulfides, using the extensible foot, either from sediment pore-waters or directly from the effusive conduits of hydrothermal vents. In vesicomyid clams, the anterior end of the bivalve is either burrowed in sediments, or nestled in cracks at hydrothermal vents, with the remaining half to quarter of their body remaining above the surface in contact with oxygenated water (Arp *et al.* 1984; Hashimoto *et al.*1995). With their extendable foot in the chemically rich waters, vesicomyids gain access to sulfides in the subsurface or cracks; sulfides diffuse into the haemolymph of the foot, bind to proteins and travel to the gills (Arp *et al.* 1984; Childress et al 1991). In contrast, the shallow marine intertidal protobranch *Solemya* sp. creates a permanent U-shaped burrow, sometimes becoming y-shaped by the addition of a downwardly directed "sulfide well" from which *Solemya* sp. pumps sulfidic water for the symbionts hosted on the gills (Seilacher 1990; Stewart & Cavanaugh 2006). Another type of behaviour is seen in the lucinids and thyasirids that create mucus-lined inhalant tubes that may allow sulfide rich pore-water into the ventilation systems of those clams by creating currents (Dando & Southward 1986; Seilacher 1990, Dando *et al.* 2004). Additionally, these groups are known to create extensive, ramifying pedal tracts below their shells that are inferred to be sulfide wells designed to access deeper sulfidic zones (Stanley 1970; Dando & Southward 1986;

Dufour & Felbeck 2003). In some species of symbiotic thyasirids, the foot can extend up to 30 times the length of the shell (Dufour & Felbeck 2003).

Burrow ventilation by symbiotic thyasirids is considered to be related to sulfide mining behaviour. Some symbiotic thyasirids pump in oxygenated water from the overlying water-column through their inhalant tube, subsequently reversing the flow to pump out oxygen-depleted water, probably including sulfidic water from their pedal tracts (Batstone 2012). Burrow ventilation allows the bivalve and the bacterial symbionts to alternately gain access to the oxidant reductant they require (i.e. oxygen and reduced sulfur in the clam and symbionts, respectively). One result of burrow ventilation is the oxidation of the near burrow environment (Dando & Southward 1986), which facilitates colonization of the sediment by organisms that could not normally survive in deep low-oxygen environments, an example of ecosystem engineering (Dando *et al.* 2004).

Thyasirids are an interesting model group for studying relationships between burrowing and irrigation behaviours and the presence of sulfur-oxidizing symbionts, because both symbiotic and asymbiotic species are known (Dufour 2005). Research on burrowing and irrigation behaviours has focused on symbiotic thyasirids (Dando & Southward 1986; Dufour & Felbeck 2003), while asymbiotic species of thyasirids have received little attention. The presence of both symbiotic (*Thyasira* cf. *gouldi* Operational taxonomic unit (OTU)s 1 and 2) and asymbiotic (*T.* cf. *gouldi* OTU 3 and *Parathyasira* sp.) thyasirids in the fjord of Bonne Bay, Newfoundland (Batstone *et al.* 2014) provides a unique opportunity to investigate the effect of symbiont presence/absence on burrowing behaviour and burrow morphology formation. In order to test the hypothesis that pedal

tracts are a burrow feature designed for collection of sulfidic pore-water, symbiotic and asymbiotic thyasirids were introduced into mini aquaria that would facilitate study of burrow morphology using X-radiography imaging. It was predicted that asymbiotic thyasirid burrows would lack pedal tracts below the shell, as they do not need to access reduced sulfur for symbiotic bacteria.

Materials and Methods

Study site

Bonne Bay is a subarctic fjord on the western coast of Newfoundland separated from the Gulf of St. Lawrence by a sill. The fjord is composed of two arms, East Arm and South Arm, with South Arm further separated from the other arm by a medial sill (Fig. 2**Figure 2**). Thyasirids are found in three locations within East Arm: South East Arm (49°27’719 N, 57°42’909 W; depth of 30-33 m), Deer Arm (49°33’225 N, 57°50’395 W; depth of 29-32 m), and Neddy’s Harbour (49°31’66 N, 57°52’236 depth of 14.5-17 m).

Collection of Thyasirids

Sediment and clams were collected from Bonne Bay in April, August, and November of 2013 using a Peterson grab (length: 12 cm). Sediment from South East Arm was dry sieved through a 1 mm mesh to collect thyasirids, and the sediment that passed through the sieve was retained for use in the experiments. Sediment from the other locations was wet sieved through a 1 mm sieve to collect the thyasirids and the sediment discarded. Thyasirids from all the three sites were pooled and sorted into three groups based on external shell morphology (cf. Oliver *et al.* 2002; Batstone *et al.* 2014): 1) *Thyasira* cf. *gouldi* OTU 1 or 2 (grouped in this study, and hereafter referred to as

"symbiotic *T.* cf. *gouldi*"); 2) *Thyasira* cf. *gouldi* OTU 3 (hereafter referred to as "asymbiotic *T.* cf. *gouldi*"); or 3) *Parathyasira* sp.. Confirmation of identification was done after experiments were completed. All specimens were measured (to the nearest 0.5 mm) and retained in filtered seawater at 4˚C until experimentation began (maximum of 3 days).

Experimentation

Thin mini aquaria were set up using pieces of glass with ¼ inch tubing sandwiched between them, and filled with natural sediment from the collection site (Fig. 1-1). In April and August, sediments were deposited as layers, to mimic natural sedimentation processes. The thin aquaria used for experimentation in August had been set up in May (with sediments collected in April) and maintained in seawater prior to experimentation, to increase the likelihood of sulfur accumulation within the sediments. The thin aquaria used for experimentation in November were prepared in September (using sediment collected in August); however, no layering was used in the final experiment as it was found that more detail was obtained from X-radiography images when sediments were not layered.

Figure 1-1: Sediment filled mini aquaria mini. An adjacent tank (not seen in this image) is also sandwiched between the same glass plates, to the left of the one seen here

A single clam was introduced into each mini aquarium, which were placed into one large aquarium with flow-through seawater from Bonne Bay (between 4ºC - 7ºC). The number of specimens of each taxon, the date of introduction into the mini aquaria, and the size range are listed in Table 1-1. There were no differences in size between the three taxa based on Kruskal-Wallis tests (p-value = 0.1862).

Table 1-1: Number and size range of specimens introduced into mini aquaria at each experimentation period. Values represent only the specimens whose identity was confirmed via gill thin sectioning at the end of the experiment.

	April		August		November	
	Number of specimens	Size range (mm)	Number of specimens	Size range (mm)	Number of specimens	Size range (mm)
Symbiotic *Thyasira* cf. *gouldi*	6	3-5	5	3-5	4	3-5
Asymbiotic *Thyasira* cf. *gouldi*	0	-	3	2-3	4	2-3
Parathyasira sp.	6	3-4	6	2-5	6	4-5

Digital photographs were taken of the mini aquaria approximately every day over a 2-week period during the months of April, August, and November, to monitor the location and possible extent of burrows. After an initial period of burrow establishment (approx. 2 weeks), X-radiographs were taken using a MinXray HF 8015+ ultra-light machine at 50 kV for 0.24 seconds. A disposable aluminum baking sheet was placed in front of the mini aquaria during X-radiography to help reduce noise and to enhance the image (Van Geet *et al.* 2000).

Following X-radiography, the mini aquaria were dismantled and the clams retained. Because of external morphological similarities between symbiotic and asymbiotic *Thyasira* cf. *gouldi* all individuals of this species were dissected to confirm symbiont presence or absence (cf. Batstone *et al.* 2014). Gills were individually fixed in 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer for approximately 24 hours, and then placed in the same buffer prior to post-fixation and embedding. Post-fixation was conducted by immersing the gills in a 2% osmium tetroxide solution in 0.1 M sodium cacodylate buffer for 15 minutes followed by dehydration in an ascending ethanol series (50% to 100% ethanol). The tissues were then bathed in two changes of Epon resin, placed into moulds with the same resin and cured in the oven (80°C) overnight. The blocks were trimmed around the gill and 3 μm sections were made using an ultramicrotome, placed on glass slides and then stained with 1% toluidine blue to identify nucleic acids and polysaccharides. At this point, the determination of bacterial symbiont presence/absence was possible (cf. Batstone *et al.* 2014).

Analysis

X-radiographs were digitized using a scanner with images enhanced using Photoshop C5. These images were imported into ImageJ and depth (the linear distance between the bivalve and the surface); length of central channel (the distance traveled by the bivalve to reach its position in the sediment); and both the length and number of pedal tracts (root-like, located deeper in sediments, with ramifications originating from the presumed location of the shell) were measured (Fig. 1-2). No measurement of the inhalant tube was undertaken as it was not visible on most X-radiographs.

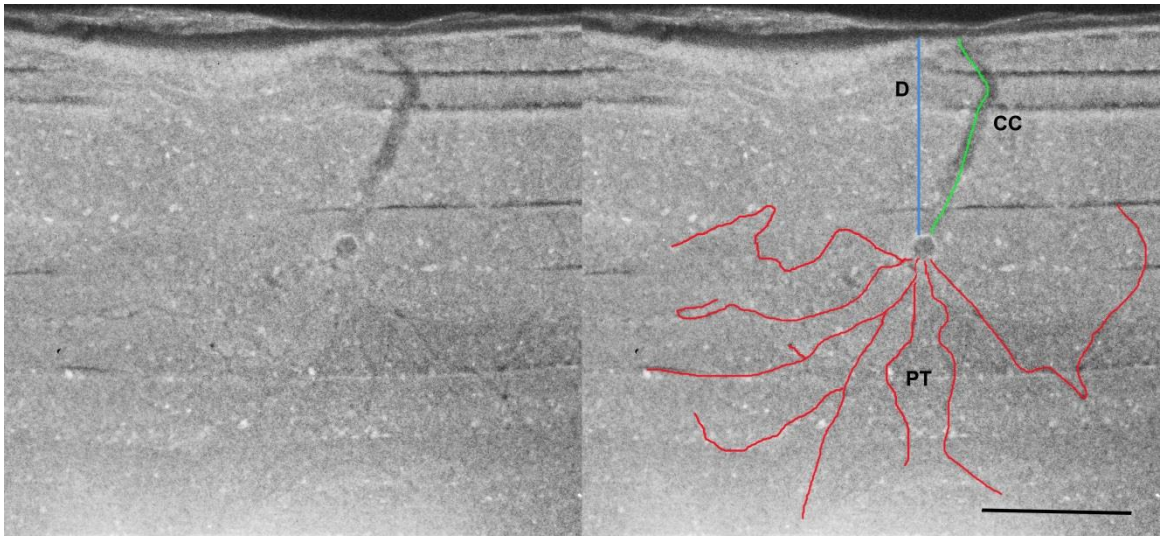

Figure 1-2: Example of X-radiograph image of a symbiotic *T.* cf. *gouldi.* The central channel (green), depth of burial (blue) and the pedal tracts (red) were traced for measurement in ImageJ. D: Depth, CC: central channel, PT: pedal tracts Black bar is 5cm.

Statistical analyses were performed to test three main questions: 1) whether there were differences in burrow depths between the three taxa; 2) whether the three taxa had different total pedal tract lengths; and 3) whether thyasirid size had an effect on the depth or pedal tract length observed during this study. Shapiro-Wilks and Breusch-Pagan tests

were used to test parametric assumptions, and ln transformations were done for any data that were not initially found to be normal or to have equal variances. ANOVAs were performed in R and for which parameter assumptions were met. Shell size data could not be normalized so nonparametric tests were done using Kruskal-Wallis tests in R (R Core Team 2013).

Results

Most (90%) of the specimens studied burrowed in the tank sediments within two days following their placement at the sediment surface. Thereafter, burrows became evident along the walls of most of the mini aquaria (generally 2-5 days later), and many burrows could be identified as either central channels, inhalant tubes, or pedal tracts. Examples of burrows visible from the tank exterior are shown in Figure 1-3, and examples of X-radiographs in Figure 1-4. Only individuals whose identity as symbiotic or asymbiotic state could be confirmed and only X-radiographs clear enough were considered. Pedal tracts visible along the tank wall were created by 13 of the symbiotic *Thyasira* cf. *gouldi*, 6 of the asymbiotic *T.* cf. *gouldi*, and 16 of the *Parathyasira* sp. X-radiographs, allowed data collection from 15 symbiotic *T.* cf. *gouldi*, 2 asymbiotic *T.* cf. *gouldi*, and 12 *Parathyasira* sp. Some individuals showed photographic and X-radiographic evidence of burrows, but not all individuals had both usable X-radiographs and photographs. X-radiographs of some symbiotic *T.* cf. *gouldi* and *Parathyasira* sp. show the shell, but no pedal tract, therefore no measurements were possible likely due to X-radiography quality.

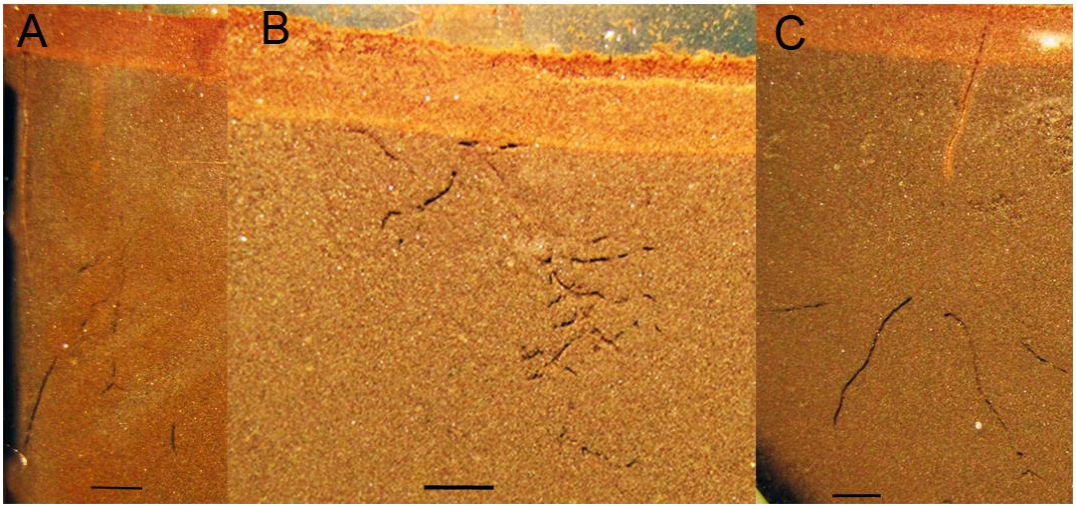

Figure 1-3: Examples of pedal tracts visible on tank walls throughout the experimentation period. A) symbiotic *T.*cf. *gouldi* B) asymbiotic *T.* cf. *gouldi* C) *Parathyasira* sp. Black bars represent 5 mm in each image.

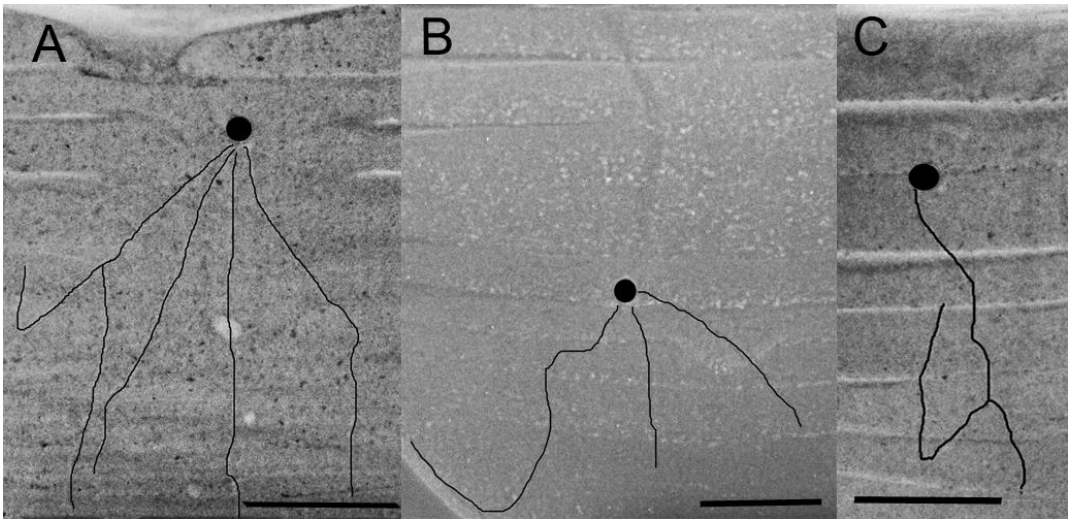

Figure 1-4: Examples of X-radiographs taken of each taxon examined following introduction of thyasirid 2 weeks prior. A black circle shows the location of the shell, and black lines show the pedal tracts. A) symbiotic *T* .cf. *gouldi* B) asymbiotic *T.* cf. *gouldi* C) *Parathyasira* sp.. Black bars represent 5 cm.

Depth of burrowing, and inhalant tube

The three thyasirid taxa were observed to burrow depths of between 12.2 and 64.2 mm (Fig. 1-5). Shell size did not influence the depth of burial (p-value = 0.4589), nor were there any significant differences among taxa (p-value = 0.1837). The shallowest burrower was a symbiotic *T.* cf. *gouldi* specimen (12.2 mm), while the deepest burrower was a *Parathyasira* sp. (64.3 mm). Colour changes in the near-burrow sediment, attributed to sediment oxygenation, was present in 44% of the symbiotic *T.* cf. *gouldi*, 11% of asymbiotic *T.* cf. *gouldi*, and 55% of t *Parathyasira* sp. aquaria (Fig. 1-6). X-radiographs show that a zone of inferred oxidation surrounds the inhalant tube that connects the clam to the surface for bringing in oxygen rather than the central channel created by the initial burrowing of the clam. In April, at least three *Parathyasira* sp. changed their position within the sediment, after having formed pedal tracts (Fig. 1-7). Similar lateral movement was not observed in other months or in association with the other taxa. As X-radiographs were only taken at the end of the experiment, it is not known when these individuals changed location.

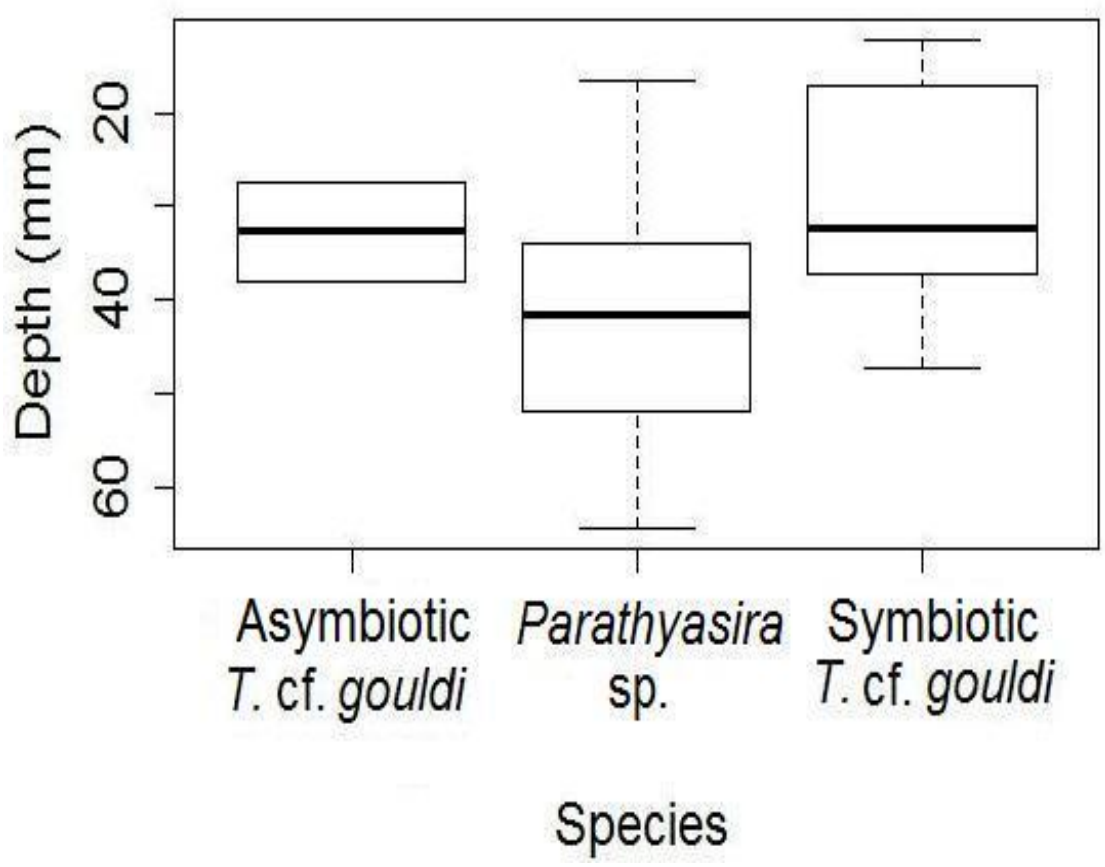

Figure 1-5: Depth of burrowing observed in thyasirids from Bonne Bay, NL, (symbiotic *T.* cf. *gouldi* n=15, asymbiotic *T.* cf. *gouldi* n=2, *Parathyasira* sp. n=12). This value represents the linear distance between the sediment surface and the location of the bivalve. Within the image the box represents the 25 to 75th percentiles with the whiskers representing the maximum and minimum values.

Figure 1-6: Digital image (Day 13) showing evidence of pedal tracts and oxidation of sediments around the inhalant tube of a *Parathyasira* sp. IT: inhalant tube, PT: Pedal tracts. Black scale bar represents 5cm.

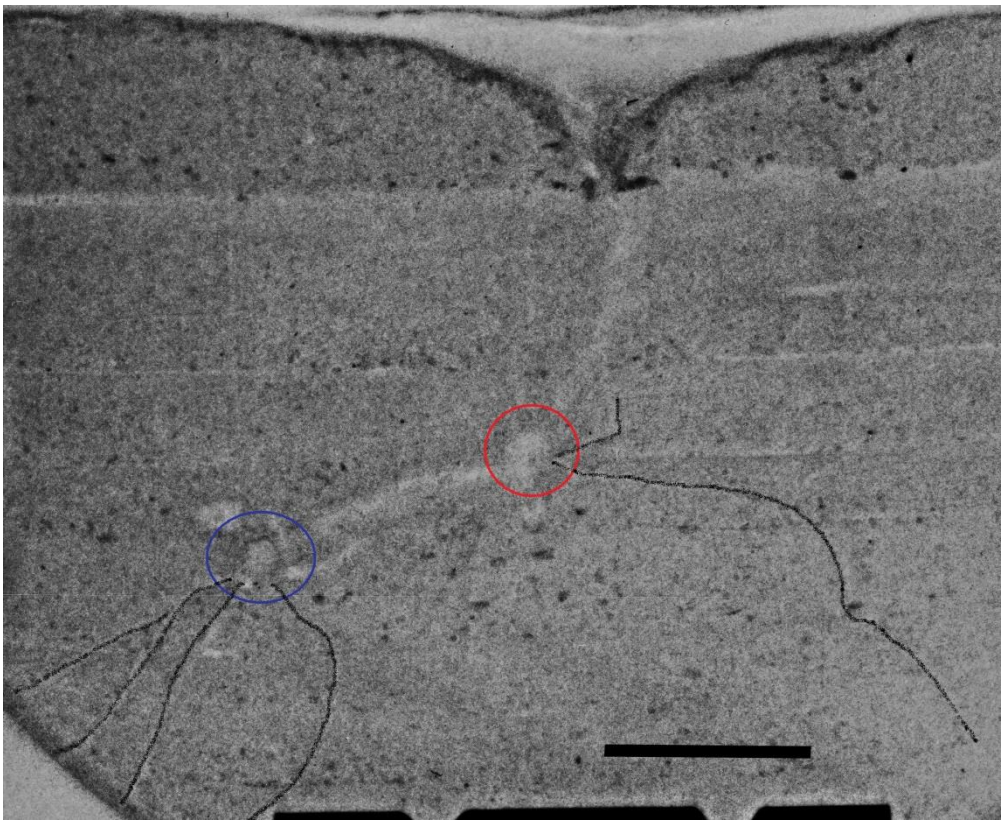

Figure 1-7: X-radiograph of *Parathyasira* sp. showing relocation after an initial phase of pedal tract generation. The area circled in red represents its initial position within the sediment, and the blue circle shows the final position at the end of the 2-week period. Black scale represents 5cm.

Pedal tracts

Pedal tracts were established by all three thyasirid taxa, during all experimentation periods (Figures 1-3, 1-4 & 1-8). A greater number of pedal tracts were observed in the mini aquaria set up in April, with some individuals of symbiotic *Thyasira* cf. *gouldi* and *Parathyasira* sp. having more than seven pedal tracts. The thyasirids in experiments started in August and November and all had less than five pedal tracts. In August and November, pedal tracts were more likely to be > 40 mm in length, with only a few measuring < 10 mm (range: 8.3 mm to 61.5 mm), whereas many of the pedal tracts were

< 20 mm in April. The longest pedal tract recorded (76.6 mm) was formed by a symbiotic *T.* cf. *gouldi*. There were no obvious morphometric differences between the pedal tracts established in layered sediments and in non-layered sediments.

Total pedal tract lengths ranged between 28.3-258.9 mm in symbiotic *Thyasira* cf. *gouldi*, between 54.4 -59.9 mm in asymbiotic *T.* cf. *gouldi* and between 46.4 - 157.2 mm in *Parathyasira* sp. (Fig. 1-8). The three taxa showed no significant differences in total pedal tract length in (p-value=0.1661) or shell size (p-value=0.438).

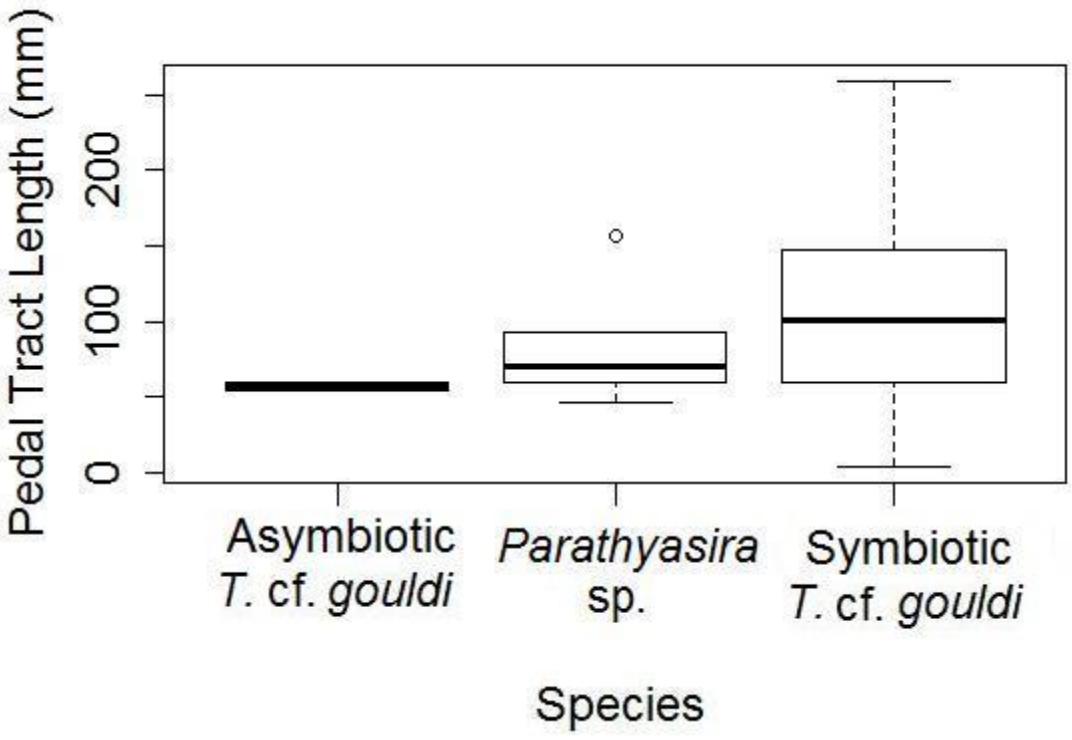

Figure 1-8: Total pedal tract lengths observed in thyasirids from Bonne Bay, NL, all experimental periods combined (symbiotic *T.* cf. *gouldi* n=11, asymbiotic *T.* cf. *gouldi* n=2, *Parathyasira* sp. n=6). Within the image the box represents the 25 to 75th percentiles with the whiskers representing the maximum and minimum values.

Discussion

This study provides insight into the burrowing behaviour of thyasirids both asymbiotic and symbiotic. The X-radiography of mini aquaria used in this research was similar to that used by Stanley (1970) to investigate burrowing in 63 species of bivalves, including chemosymbiotic species such as *Lucina pensylvanica* and *Solemya* sp. There have, however, been few observations of burrowing behaviour in thyasirids, with most studies focusing on symbiotic species (Dando & Southward 1986, Dufour & Felbeck 2003; Dando *et al.* 2004). Previous work found that pedal tracts were formed by thyasirid species that were symbiotic (*Thyasira. equalis*, *T. flexuosa* and *T. sarsi*), with none observed in association with the asymbiotic species investigated (*T. obsoleta* and *T. ferruginea*; Dufour and Felbeck 2003). This study is the first evidence of pedal tracts in asymbiotic species of thyasirids.

Depth of burrowing

Our experiments showed that there was no significant difference in depth of burrowing among the three taxa (Fig. 1-5). The similarity in burrow depth is possibly due to the comparable shell sizes (and likely the length of the foot) among the specimens from which data was obtained. It should be noted that symbiotic *Thyasira* cf. *gouldi* can attain larger sizes than asymbiotic *T.* cf. *gouldi*, and that there are some differences in shell shape between these taxa (Batstone *et al.* 2014). However, Anderson (2014) found that the size and shell shape of individuals in lucinids and thyasirids was unrelated to the depth of burrowing and the larger species often burrow to similar depths as those of the smaller species such as *T. flexuosa*, although this work only pertained to symbiotic

species. Our data showed that asymbiotic thyasirids are able to burrow to at least 6 cm depth. Previous work on asymbiotic thyasirids has documented a maximum depth of burrowing of 1.5 cm (Dufour & Felbeck 2003), much shallower than in the current study. This difference in depths between the two studies could be due to shell size differences, as the asymbiotic species in Dufour & Felbeck (2003) were smaller than those studied here, but further work relating shell size to burrow depth is required.

Inhalant tube

Formation of the semi-permanent inhalant tube is characteristic of thyasirids, which lack an inhalant siphon and continually probe the tube to keep it open and intact (Allen 1958). Where the burrow was located away from the glass margin of the mini aquaria, the location of the inhalant tube was commonly recognised by colour differences in the sediment inferred to be due to oxidation (Fig. 1-6). Sediment oxidation around the inhalant tube was more prevalent around the inhalant tubes of *Parathyasira* sp. and the symbiotic *Thyasira* cf. *gouldi* than those of asymbiotic *T.* cf. *gouldi*. Differences in the ventilation cycle including the duration or periodicity of the inhalation and exhalation events could influence sediment oxidation around the inhalant tube (Batstone 2012). A longer period of experimentation may have led to a greater proportion of oxidized inhalant tubes, as well as possible oxidation along the pedal tracts.

Pedal tracts

The three thyasirid taxa created long, branching pedal tracts (Fig. 1-8) in both layered and non-layered sediments. Previous studies of burrowing in thyasirids did not document pedal tracts in association with asymbiotic species (e.g. Dufour & Felbeck

2006). A possible reason for the lack of pedal tracts in asymbiotic species in previous studies may be a result of the species used in the investigation. The shape of the foot in *Thyasira obsoleta* is rather short yet bulbous (Oliver *et al.* 2002), while the foot of *T. equalis* appears to be much longer (Oliver *et al.* 2002), similar to that seen in the taxa found in Bonne Bay. Although found in other species, the presence of pedal tracts made by asymbiotic thyasirids is unexpected, given that the pedal tract in thyasirids has been inferred to be exclusively for the collection of dissolved sulfides to provide an energy source for thyasirid symbionts (Dufour & Felbeck 2003).

Given that pedal tracts occur in association with demonstrably asymbiotic *Thyasira*. cf. *gouldi*, two alternative explanations for pedal tract formation are posited: 1) pedal tracts form no useful function and their formation is a vestige of a previously symbiotic state; and 2) the pedal tracts are used for pedal feeding by collecting particles on their foot and bringing to their mouth.

Pedal track explanations

Symbiosis appears to have evolved multiple times in different thyasirid lineages (Taylor *et al.* 2007; Duperron *et al.* 2013). In some symbiotic lineages, the association with chemoautotrophic bacteria may have broken down in some species, possibly as a secondary loss due to habitat expansion into areas with fewer free-living bacteria within the sediment; as thyasirids acquire their symbionts from their sedimentary environment (Dufour *et al.* 2014). Asymbiotic *Thyasira* cf. *gouldi* OTU 3, therefore, may be more derived than the symbiotic OTUs 1 and 2 in Bonne Bay (Batstone *et al.* 2014) with pedal tract-forming behaviour retained despite the loss of symbionts. Similarly, *Parathyasira*

sp. may be derived from symbiotic ancestors that formed pedal tracts as a sulfur mining mechanism. The phylogenetic relationships between thyasirids of this family remain unresolved; it is difficult therefore to further evaluate the likelihood that pedal tracts are a vestigial behaviour in the asymbiotic thyasirids studied here.

It is proposed here that the formation of pedal tracts in some asymbiotic thyasirids may be a feeding adaptation. Pedal feeding is a common method of particulate feeding in juvenile bivalves that is retained into maturity in some small bivalve species (Reid *et al.*1992, Morton 1996). Pedal feeders collect organic material on the muco-ciliary surface of the foot, which transports particles into the mantle cavity and deposit it into the mouth (Reid *et al.*1992). Thyasirids from Bonne Bay may use this approach to acquire organic matter and sediment-dwelling bacteria, and pedal tracts could be evidence of this mode of feeding. The chemical conditions along the lining of pedal tracts are likely to be particularly suitable for microbial growth. The burrow linings of several infaunal organisms are hotspots of bacterial activity, particularly when the burrows are irrigated (Papaspyrou *et al.* 2006). Large pH gradients have been reported around both the inhalant tube and the pedal tracts of *Thyasira sarsi*, suggesting that there is active inhalant and exhalent pumping of the water throughout all burrow structures that creates fluctuating redox boundaries in the near-burrow environment (Hakonen *et al.* 2010). One type of bacteria that would likely be attracted to pedal tracts is magnetotatic bacteria, which orient along magnetic field lines to locate oxic/anoxic interfaces in sediments (Lefèvre & Bazylinski, 2013), and have recently been found in association with *Thyasira* cf. *gouldi* in Bonne Bay (Dufour *et al*., 2014). These bacteria are often chemoautotrophic sulfur

oxidizers that seek redox boundaries in sediments because of the availability of both of the nutrients they require for energy production (reduced sulfur and oxygen) in those particular areas (Lefèvre & Bazylinski 2013). Therefore, thyasirid burrow irrigation could effectively promote "microbial gardening" (Lopez & Levinton 1987) with pedal feeding being a means of harvesting these microbes. Although no bacteria were found to colonize the gills of asymbiotic *T.* cf. *gouldi* or *Parathyasira* sp, such that they have no obvious need for reduced sulfur, these taxa may still create ideal environments for sulfur-oxidizing and sulfate reducing bacteria along their pedal tracts and attract bacteria in these regions. Investigations of the diet of asymbiotic thyasirids (e.g. through isotopic analysis to identify chemoautotrophic signatures) are needed to test the likelihood and relative importance of pedal feeding in those bivalves.

Although accessing sulfides may be the main purpose of pedal tracts in symbiotic thyasirids, these bivalves may also use pedal feeding to gain nutrients. *Thyasira flexuosa* and other chemosymbiotic bivalve species are mixotrophs, nutritionally relying on more than just their symbionts, as evidenced by differences in stable isotope composition among conspecifics (Spiro et al 1986; Dando & Spiro 1993) and variability in symbiont abundance (Dufour & Felbeck 2006). In populations of the symbiotic *T.* cf. *gouldi* from Bonne Bay, symbiont abundance varies cyclical, annual and periodicity (Laurich *et al.* submitted), suggesting that behaviours such as pedal feeding may be utilized during periods of low symbiont abundance.

The length of individual pedal tracts was highly variable in this study, possibly due to heterogeneity in sediment sulfide concentrations within the mini aquaria, resulting

in the thyasirids having to search more intensively in some conditions compared to others. Dufour & Felbeck (2003) discovered that in low sulfide conditions, the length and number of pedal tracts created by symbiotic thyasirids was greater than in high sulfide conditions. The increase in the number of pedal tracts observed in April may be due to a seasonal depletion of nutrients in the sediments due to low rates of degradation over the winter months (Rozan *et al.* 2002), resulting in lower sulfide concentrations at this time. It has been observed that in Bonne Bay, sulfides are the highest in August and lowest in March and April (Batstone 2012). Additionally, given that sediments used in April were freshly sieved and oxygenated and, unlike in the August and November experiments which had not experienced a prolonged settling time prior to experimentation, overall sulfide concentrations were likely comparatively lower in the April mini aquaria. Dufour and Felbeck (2003) used a short settlement time (4 days) to be considered their low sulfide treatment, with longer settlement time for their high sulfide treatment and found a difference in burrowing between the two, indicating that the differences seen in pedal tracts for our study may be a result of settlement time as it relates to sulfide accumulation. Lower sulfide content in April would explain the greater number of pedal tracts observed at that experimental period, but the relatively shorter length of pedal tracts in April remains enigmatic. The change in location of some of the *Parathyasira* sp. (Fig. 1-7), only observed in April may also be related to a greater search effort for nutrients.

Maintenance of pedal tracts

In most cases, pedal tracts were visible on the exterior of the mini aquaria within a few days after bivalves were introduced. Over the course of experimental periods, some

of the pedal tracts appeared to collapse; similarly, Dufour & Felbeck (2003) reported that some pedal tracts began to collapse after one week, likely from disuse. It remains unclear whether thyasirids maintain their pedal tracts, or consistently form new ones, particularly in natural environments where burrowing endobenthos may disrupt such structures. Further, pedal tracts did not show any discernible spatial pattern within the sediment and did not appear to follow the redox layers that were visible within the mini aquaria, or the boundaries of sediment layers. It is not yet known if the foot is able to sense sulfides, or how the directionality of thyasirid pedal tracts is determined. Additional work on burrow formation, maintenance, and irrigation, coupled with fine-scale microbial distribution and sedimentary redox oscillation patterns are needed to better understand the controls on pedal tract formation and their interactions with surrounding sediments.

Conclusion

Thyasirid burrow irrigation/microbial gardening behaviour might be a precursor to the establishment of chemosymbiosis in this group. It is conceivable that sediment-dwelling bacteria accidentally trapped on the gill epithelium during transfer of food particles from the foot to the pallial organs might colonize the thyasirid gill, starting the symbiotic relationship common in many modern thyasirids. The bacteria found associated with symbiotic *Thyasira* cf. *gouldi* in Bonne Bay contain magnetosomes and were probably acquired via the pedal tracts or inhalant tubes (Dufour *et al.* 2014).

This study demonstrates for the first time that at least some asymbiotic thyasirids burrow and create pedal tracts in a similar manner to symbiotic thyasirids. The fact that two different taxa of asymbiotic species show similar burrowing behaviours suggests that

other asymbiotic thyasirids might share this behaviour; the form of the foot may be a good predictor of the likelihood of pedal tract formation in thyasirids.

Additional research on feeding mechanisms and ventilation patterns in various thyasirid species would help us better understand the role of pedal tracts in both asymbiotic and symbiotic thyasirids, and the evolution of these behaviours in relation to chemosymbiosis. In particular, the possibility that pedal tracts might be associated with microbial gardening and pedal feeding activities should be investigated.

Chapter 2: Dietary Analysis of the thyasirids of Bonne Bay, NL using Stable Isotope Techniques

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Abstract

The bivalve family Thyasiridae has both chemosymbiotic species as well as asymbiotic species. Chemosymbiotic species derive part of their nutrition from the sulfur-oxidizing symbionts whereas asymbiotic species are presumed to be suspension feeders. Within Bonne Bay, NL, there are three species of thyasirids, one symbiotic, two asymbiotic. To investigate the feeding mechanisms of these taxa, stable isotope analyses with carbon and nitrogen were conducted. As expected, the symbiotic taxon consumes mainly bacteria, and one of the asymbiotic taxon consumed mainly suspended material. However, contrary to expectations, the other asymbiotic taxon consumed mainly thiotrophic bacteria, in an even greater proportion than the symbiotic taxon. Although we currently do not know why, it may indicate that "microbial gardening" is likely occurring for that taxon, which may be a possible explanation about how chemosymbiosis evolved within some species of the Thyasiridae.

Introduction

Chemosymbiosis refers to a long-term relationship between a multicellular organism host including marine invertebrates, and chemolithoautotrophic bacteria, mainly sulfur-oxidizing gammaproteobacteria, where the invertebrate host receives nutrients from its symbionts (Dubilier *et al.* 2008). Chemosymbioses have evolved in a variety of marine habitats where the proximity of oxic and anoxic conditions produces an optimal setting for chemolithotrophic bacterial production. By maintaining such bacteria either at the surface of, or within their cells, host invertebrates ensure an efficient transfer of nutrients between symbiotic species and their bacteria. The nutritional advantage gained by forming chemosymbioses has allowed many groups of invertebrates to thrive in sulfide or methane-rich environments, such as hydrothermal vents, cold seeps, organically polluted sites, and in marine sediments experiencing varying degrees of organic enrichment (Dubilier *et al.* 2008).

Whereas some chemosymbiotic invertebrates (e.g. giant tubeworms and solemyid bivalves) obtain all, or nearly all their nutrients from their symbionts, others are mixotrophic, having retained the ability to capture and digest food particles (Rodrigues *et al.* 2013, Rossi *et al.* 2013, van der Geest 2014). The importance of particulate food can be significant, and several mixotrophic bivalves show a fluctuating reliance on symbionts and particulate matter. For example, lucinid bivalves inhabiting seagrass beds exhibit a seasonal pattern in the relative importance of nutritional input from suspended particulate organic matter (SPOM) and bacterial symbionts (van der Geest *et al.* 2014). The nutritional and energetic value of SPOM is considered to be greater than that of

symbionts (Le Pennec *et al.* 1995, Pile & Young 1999), and many shallow water and deep-sea chemosymbiotic bivalves may adjust their trophic strategies as a response to environmental or seasonal factors such as phytoplankton blooms (Le Pennec *et al.* 1995, van der Geest *et al.* 2014).

Mixotrophy is also present in the Thyasiridae (SF Thyasiroidea), a bivalve family in which there are both chemosymbiotic and asymbiotic species. Thyasirids exhibit a high degree of plasticity in nutrient utilization from sulfur-oxidizing symbionts (Dando & Spiro 1993, Le Pennec *et al.* 1995); the balance of their nutritional requirements is considered as being derived from SPOM (Rozemarijn *et al.* 2011). The contribution of symbiont-derived carbon to the diet of thyasirids appears to be influenced by the availability of dissolved sulfide in sediments (Dando & Spiro 1993). Seasonal studies of the ultrastructure of the digestive tract of thyasirids indicate a reduction of particulate feeding over winter months (Donval *et al.* 1989). The abundance of symbionts in thyasirids from subarctic fjords also varies temporally, most likely as a response to seasonal changes (Laurich *et al.* submitted). The potential importance of pulses of SPOM to the diet of thyasirids has not yet been examined.

Most research on thyasirids has focused on symbiotic species (e.g. Southward 1986, Dando & Spiro 1993, Dufour & Felbeck 2003, 2006, Dando *et al.* 2004, Dufour 2005), with little known on the biology and feeding mode of asymbiotic thyasirids. The latter are smaller than symbiotic thyasirids (Dufour 2005, Batstone *et al.* 2014), common in the deep-sea (Payne & Allen 2001), expected to be less tolerant of reducing conditions than symbiotic thyasirids due to lack of symbionts to regulate sulfide concentrations, and

considered to be suspension-feeders (Allen 1958). Given their anterior-posteriorly elongated shell shape, many asymbiotic thyasirids are interpreted as being motile, shallow burrowers (Payne & Allen 2001). According to Dufour & Felbeck (2003), no asymbiotic thyasirids were observed to form elongated, ramified pedal tracts beneath their shells, a behaviour interpreted as sulfide-mining in chemosymbiotic thyasirids. However, a recent study of burrowing behaviour has shown that asymbiotic thyasirids from Bonne Bay, Newfoundland form extensive pedal tracts and burrow as deeply as co-occurring symbiotic thyasirids (Chapter 1). These findings raise questions on the potential benefit of such behaviours in asymbiotic species that presumably do not require sulfide. Thyasirid burrowing behaviours may be associated with a microbial farming strategy: burrow irrigation creates steep pH gradient along the burrow lining (Hakonen *et al.* 2010) where the production of microbes (e.g. sulfur-oxidizing bacteria) is likely enhanced (Lopez & Levinton 1987). Thyasirids may collect microbes on the muco-ciliary surface of their foot (i.e., pedal feeding; Reid *et al.* 1992, Morton 1996) and subsequently ingest them (Chapter 1). The existence of pedal feeding, a form of subsurface deposit-feeding, has not yet been investigated in thyasirids. Such a feeding mode could be important in asymbiotic thyasirids, and could be employed by mixotrophic species in addition to, or instead of, suspension-feeding.

Here, we use stable isotope analysis (carbon and nitrogen) and mixing models to assess the importance of various food sources (SPOM, sedimentary organic matter (OM)), and sulfur-oxidizing bacteria) to the diet of symbiotic and asymbiotic thyasirids from a fjord in Newfoundland, Canada. Stable isotope analysis is widely used in ecology

to study trophic pathways, and has been particularly useful in discriminating among carbon sources in environments such as estuaries and fjords, where terrestrial organic matter can make up a significant proportion of the energy budget (McCallister *et al.* 2004, McLeod & Wing 2007). Carbon isotope ratios ($\delta^{13}C$ values) can help identify the origin of fixed carbon assimilated by a consumer, as different modes of primary producers are more or less selective towards the lighter stable isotope of carbon which leads to different producer $\delta^{13}C$ values. In particular, terrestrial C3 plants (and detritus derived from them) typically have a range of $\delta^{13}C$ values between -28 and -25‰ (Fry & Sherr 1984 in Hedges *et al.* 1997), where marine phytoplankton in temperate environments show $\delta^{13}C$ values of -28 to -18‰ (Spiro *et al.* 1986, Peterson 1999, Fry & Sherr 1984 in Hedges *et al.* 1997). Chemoautotrophic organisms with sulfur-oxidizing bacteria have $\delta^{13}C$ values depending on the form of Rubisco they contain: -35‰ to -27‰ for Rubisco form I, or -15‰ to -9‰ for Rubisco form II; Childress & Fisher 1992; Robinson & Cavanaugh 1995). Methanotrophs have even lower $\delta^{13}C$ signatures due to the very low carbon isotope values of methane (-70‰ to -50‰; Wahlen 1993). In chemosymbiotic bivalves, carbon isotope ratios have been used to estimate the proportion of the host's carbon obtained from symbiont-derived chemoautotrophy versus that assimilated through particulate feeding. Typically, the $\delta^{13}C$ signature of non-gill tissues is compared to that of the gills (where symbionts are maintained), and often the gill signature is used as an approximation of the isotopic signature of the symbionts (Dando *et al.* 1994).

Nitrogen isotope ratios ($\delta^{15}N$ values) provide information on the trophic position of organisms given that nitrogen fractionates as it moves through food webs due to losses

of the lighter isotope during excretion (Peterson & Fry 1987). As dependence upon sulfur-oxidizing bacteria leads to depleted nitrogen signatures due to low trophic level consumption (Conway *et al.* 1991).

Several thyasirid taxa co-occur in Bonne Bay, Newfoundland, of which *Thyasira* cf. *gouldi* OTUs 1 and 2 are symbiotic and *T.* cf. *gouldi* OTU 3 and *Parathyasira* sp. are asymbiotic (Batstone *et al.* 2014). By comparing the isotopic signature of potential food sources (SPOM, sedimentary OM and symbionts) to those of thyasirids, we attempt to identify whether these thyasirids are suspension-feeders or deposit-feeders; if pedal feeding is indeed occurring, then the signature of asymbiotic thyasirids should resemble that of sedimentary OM or of free-living chemoautotrophic bacteria instead of SPOM. We also aim to determine the importance of chemoautotrophic symbionts to the diet of the symbiotic taxa at different times of the year when symbiont abundances differ; (Laurich *et al.* submitted) and at different sampling sites. In particular, the SPOM availability and quality is expected to change over time: following spring and fall phytoplankton blooms, deeper water masses (where thyasirids occur) experience influxes of SPOM (Tian *et al.* 2003). Terrestrial OM is also brought into the fjord via riverine input and accumulates in sediments; seasonal changes in this terrestrial influx, coupled with fluctuations in the activity and abundance of benthic communities, likely influence the quality and quantity of sediment organic matter.

Materials and Methods

Study site

Bonne Bay is a subarctic fjord on the western coast of Newfoundland separated from the Gulf of St. Lawrence by a sill. The fjord is composed of two arms, East Arm and South Arm, with East Arm being further separated from the other arm by another sill near Norris Point (Fig. 2). Sampling took place in three sites within East Arm, where thyasirids have been collected previously (Batstone *et al.* 2014): South East Arm (S; 49°27'719 N, 57°42'909 W; depth range: 30 – 33 m), Deer Arm (D;49°33'225 N, 57°50'395 W; depth range: 29 – 32 m) and Neddy's Harbour (N; 49°31'66 N, 57°52'236; depth range: 14.5 – 17 m).

Site Characteristics

The South East Arm site (30 m depth) is the furthest from the Gulf of St. Lawrence, being located at the very end of the East Arm of Bonne Bay. This site receives freshwater input from South East Brook. Based on visual observations during sampling, the sediments are very fine-grained and are mainly composed of dark-black muds with some occasional cobble. Previous studies of the area have found that the OM content of South East Arm sediments is approximately 12%, with average porewater dissolved sulfide concentrations determined to be 5.4 ± 4.7 µM (N = 17 samples) in April 2011, and 8.7 ± 4.9 µM in August 2011 (N = 20 samples) (Batstone, unpublished data).

The Deer Arm site (30 m depth) receives freshwater from Deer Brook (Fig. 2). The freshwater influence appears greater at Deer Arm than at South East Arm as

indicated by organic material in the sediment: Deer Arm sediment contains large (cm scale) particles of wood debris and other organic material, and is otherwise less muddy than South East Arm sediments. The OM content of Deer Arm sediment is similar to that of South East Arm sediment, at approximately 11%, and measured dissolved porewater sulfides at Deer Arm (4.2 ± 2.2 µM, N = 15 samples in April 2011, and 9.4 ± 4.3 µM, N = 22 samples in August 2011) are also similar to values from South East Arm (Batstone, unpublished data).

Sediments from the Neddy's Harbour site (15 m depth) are considerably different from other sites as there is little nearby freshwater input, and anthropogenic effects may be more important. The sediment here is largely sandy, although silt is present due to dredging of the harbour every few years (Hooper pers. com.). Neddy's Harbour sediment has the lowest OM content of the three sites at 3.5%, yet porewater sulfide levels are similar to those at Deer Arm and South East Arm (4.6 ± 4.0 µM, N = 18 samples in April 2011, and 9.6 ± 5.0 µM, N = 21 samples in August 2011; Batstone, unpublished data).

Over the past few years, occasional measurements at the three sampling sites have shown that salinity and temperature measurements near the benthic environment are similar among sites within a sampling period (salinity: approximately 30.0, S. Dufour, unpublished data; see below for temperatures). Differences in sediment composition and depth likely contribute to the differences in thyasirid taxonomic composition among these three sites.

Seasonal Differences

As in most coastal waters in temperate regions, phytoplankton blooms occur at certain times of the year, and constitute a large influx of food for many organisms, including benthic species. The suspended particulate matter (SPM) present during a phytoplankton bloom is of much higher quality and quantity than at other times of the year. In Bonne Bay, the spring phytoplankton bloom typically occurs at the end of April or May (Tian *et al.* 2001) and phytodetritus settlement occurs throughout the spring and summer. A second, smaller phytoplankton bloom can also occur in the autumn (Laurich *et al.*, submitted).

Along with increases in organic material through the end of spring and summer, there is an increase in temperature that continues into the autumn (periodic temperature measurements near the seafloor at the sampling sites have ranged from 0.7˚C to 14˚C between April - August; unpublished data). Changes in sediment temperature have been found to influence porewater sulfides (Goldhaber & Kaplan 1975, Westrich & Berner 1984). At the Bonne Bay sampling sites, a significant increase in porewater sulfides was observed between April and August (Laurich *et al.* submitted). Such an increase in the concentration of chemicals required by chemoautotrophic bacteria might be expected to influence the abundance of symbionts within the thyasirids. Indeed, Laurich *et al.* (submitted) found that symbiont abundance in *Thyasira* cf *gouldi* increased over the course of the summer and autumn, as temperatures got warmer (and the sulfide concentration increased), and then decreased over winter months when the temperatures dropped. If symbiont abundance is a measure of the amount of food available to

thyasirids through phagocytosis, then thyasirids might be expected to rely more heavily on their symbionts rather than on other food sources when symbionts are most abundant. However, Laurich *et al.* (submitted) found that the density of membrane whorls (which represents the extent of symbiont consumption in host gill epithelial cells) does not appear to fluctuate seasonally and remains constant throughout the year. As noted previously, sediment sulfide concentrations have been related to the extent of symbiont-derived nutrients in some mixotrophic thyasirid species, albeit over larger timescales (Dando & Spiro 1993). Also, sediment porewater sulfide content influences the pedal mining behaviour of symbiotic thyasirids (Dufour & Felbeck 2003), with unknown consequences to host nutrition. Additional work is required to better characterize the importance of symbiont-derived and other food sources to symbiotic thyasirids, particularly on seasonal timescales.

Sample Collection

Sediment, seawater and thyasirids were collected from the three sampling sites in April, August and November of 2013. Seawater was collected at approximately 1 m above the bottom using a 3 L Niskin bottle prior to sediment sampling (i.e., to avoid collecting sediments re-suspended during grab sampling) until a 20 L Nalgene bottle was full (previously cleaned with 10% HCl) and brought to the lab for immediate processing.

Peterson grabs (diameter and depth of penetration: 12 cm) were used to collect sediment and bivalves. As soon as grabs were brought into the boat, sediment samples from different depths were collected: 1) a surface sample (upper 1 cm); 2) a deep/anoxic sample (dark colouration, approximately 8 cm from the sediment surface); and 3) an

intermediate sample (paler than the deep/anoxic sample, at approximately 4 cm from the sediment surface). Sediment samples were transferred to plastic centrifuge tubes, and kept in a cooler while onboard. Sediment samples were collected from 3 sediment grabs from each site and in each month.

Thyasirids were obtained from the remainder of the sediments by wet or dry sieving on a 1 mm mesh. The bivalves were sorted into three taxa: 1) *Thyasira* cf. *gouldi* OTU 1 or 2 (grouped in this study, and hereafter referred to as "symbiotic *T.* cf. *gouldi*"); 2) *Thyasira* cf. *gouldi* OTU 3 (hereafter referred to as "asymbiotic *T.* cf. *gouldi*"); or 3) *Parathyasira* sp. The *T.* cf. *gouldi* specimens were sorted into symbiotic or asymbiotic groups based on shell shape and the location of the ferrugineous patch on the dorsal end of the shell (Batstone *et al.* 2014); no further confirmation of symbiont presence or absence was done on these specimens. Although identifications were done with the greatest care, we cannot guarantee that all were correctly identified. All thyasirids were measured (to the nearest 0.5 mm) and held in 0.7 µm-filtered seawater from Bonne Bay at 4˚C for a minimum of 24 hours to allow for gut clearance prior to further processing.

Sample Processing

Seawater samples

Upon returning to the lab (within a few hours from sampling), Nalgene bottles containing seawater samples were gently inverted to re-suspend particulate matter. For each site and date, SPM was collected onto four separate GF/F Whatman filters (47 mm diameter, 0.7 µm porosity) to determine the concentration of SPM and chlorophyll *a* (chl

a), and the stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) within SPM, as described below.

To determine the concentration of SPM (mg/l of seawater), between 2 – 3 L of seawater was run through a combusted (450°C, 4h) and pre-weighed filter (GF/F Whatman filters, 47 mm diameter, 0.7 μm porosity), which was then dried at 60°C for 48 hours and re-weighted. For chl *a* determinations, 0.5 – 1.5 L of seawater was run through a filter (GF/F Whatman filters, 47 mm diameter, 0.7 μm porosity), which was then wrapped in aluminum foil and frozen (-20˚C). Care was taken to perform this filtration in subdued light. For stable isotope ratio determinations, 2.5 – 5.5 L of seawater was run through previously combusted (450°C, 4h) filters (one each for $\delta^{13}C$ and $\delta^{15}N$ quantifications), which were then placed into cleaned (10% HCl) and pre-combusted (450°C, 4h) glass scintillation vials and dried at 60°C for 48 hours. These vials were kept in a dry environment until further processing could be done.

Prior to stable isotope analysis, one SPM sample from each site and time was fumigated with hydrochloric acid for 24 hours as similarly described in Lorrain *et al.* (2003). The acidification step removes inorganic carbon that may be present on the filter, so that the $\delta^{13}C$ of SPOM could be determined, while a non-acidified sample is required to obtain an unaffected $\delta^{15}N$ signature (Lorrain *et al.* 2003). Particulate matter was scraped off the filters and placed in tin (for $\delta^{15}N$) or silver (for $\delta^{13}C$) caps for stable isotope analysis.

Sediment samples

Tubes containing sediment samples were covered in aluminum foil and frozen (-20 ˚C). The sediments were later freeze-dried and ground using a mortar and pestle for chl *a*, organic matter and stable isotope analysis. Sediment for the grain size analysis was also freeze dried though no grinding was done to prevent reductions in grain size. For stable isotope and elemental analyses, two subsamples from each combination of depth (surface, intermediate, bottom/anoxic), site and time were prepared: one was placed in a pre-weighted tin cap and a second in a pre-weighted silver cap. To remove carbonates, 2 mL of 0.3N HCl was added to each sediment sample within silver caps. These samples were then dried at 60°C overnight and the process repeated again until there was no more visible effervescence, as in Nieuwenhuize *et al.* (1994). The silver and tin caps were then weighed and ready for stable isotope analysis.

Bivalve Samples

Following a minimum of 24 hours in filtered seawater, the bivalves were dissected and two sets of samples were prepared: one set containing gill tissue (for symbiotic thyasirids, this includes bacterial symbionts) and one containing non-gill and non-digestive gland tissue (i.e., mantle, muscle and foot; hereafter referred to as "non-gill"). The non-gill tissue was targeted for use in mixing models because it does not include the signature of symbionts and represents organic matter assimilated over approximately 1 month (Dubois *et al.* 2007). Because of the small size of these bivalves, tissues from multiple individuals were pooled to ensure a sufficient mass of dried tissue for isotopic analysis. Due to our identification method, the pooling may have resulted in samples

containing some of both symbiotic and asymbiotic *Thyasira* cf. *gouldi* tissue, though we believe the majority of each sample were correctly identified. For each taxon, the gills of 5-10 individuals were pooled and placed into pre-weighed tin caps. The non-gill tissues of those same individuals were pooled and placed into other pre-weighted tin caps. For each site and date, between 1 and 3 tissue samples were obtained for each taxa and location, depending on the number of specimens that were collected. All tissue samples were dried overnight (70°C).

None of the bivalve tissue samples were acidified due to concerns about sample size and the resulting sample loss that can occur during acidification. We considered that the mass of carbonates within tissue samples were negligible and would not significantly impact our results; similarly, other stable isotopic studies of thyasirids present results of samples analyzed without prior acidification (Spiro *et al.* 1986; Dando *et al.* 1994) and van der Geest (2014) determined that there was no significant difference in lucinid signatures whether acidification was done or not.

Sample Analysis

Stable Isotope and Elemental Analyses

Samples were weighed and analyzed at the CREAIT Stable Isotope Laboratory at Memorial University of Newfoundland using a Finnigan MAT252 Gas Source IRMS coupled with a Carlo Erba NA1500 Series II Elemental Analyser. Data is expressed in the standard δ notation:

$$\delta^{13}C_{sample} \text{ or } \delta^{15}N_{sample} = [(R_{sample}/R_{reference})-1]*1000$$

where $R = {}^{13}C/{}^{12}C$, ${}^{15}N/{}^{14}N$.

Isotopic compositions were calibrated against internal standards and reported relative to Vienna Pee Dee Belemnite for $\delta^{13}C$ and atmospheric air for $\delta^{15}N$, analysis uncertainly based upon standards was less than 0.1‰.

Chlorophyll a *Analysis*

Chl *a* was extracted from filtered seawater and sediment samples following the method of Aminot & Rey (2000) & Danovaro (2010) with some modifications as described below. All steps were done in the dark. Filters containing SPM were placed into glass centrifuge tubes along with 5 mL of 90% acetone. Filters were mechanically ground using a glass rod and an additional 5 mL of 90% acetone was added to each tube. Samples were gently agitated and stored at 4°C for a maximum of 18 hours. Processing of sediment samples was as follows: 2 g of freeze-dried, homogenized sediments were placed into glass centrifuge tubes along with 8 mL of 90% acetone; samples were agitated and stored at 4°C for up to 18 hours.

Samples were then transferred into plastic tubes for centrifugation. The filter samples were centrifuged at 3500 rpm for 20 min at 4°C while the sediment samples were centrifuged at 3000 rpm for 10 min at 4°C. Supernatants were then transferred back into clean glass tubes, and 2 mL of each sample was pipetted into a cuvette and the absorbance at wavelengths of 665 and 750 nm was measured on a Thermoscience Genesys 10S UV-Vis spectrophotometer with 90% acetone as a blank. A second measure of absorbance was read at the same wavelengths 2 min after adding 200 µL of 0.1M HCl to the cuvette containing the sample. The concentration of chl *a* and phaeophytin (the

product of chl *a* degradation) per L of seawater or per g of dry sediment were determined using the equations below (modified from Danovaro (2010) and Aminot & Rey (2000):):

Chl *a* sediment (µg/g dry sediment) = $26.7*(A665_o - A665_a)*V/P_s$

Phaeophytin sediment (µg/g dry sediment) = $26.7*((1.7*A665_a) - A665_o)*V/P_s$

Chl *a* filter (µg/L seawater) = $26.7*(A665_o - A665_a)*V/P_f$

Phaeophytin filter (µg/L seawater) = $26.7*((1.7*A665_a) - A665_o)*V/P_f$

Where:

$A665_o$ = [A665-A750]

$A665_a$ = [A665a-A750a]

V = volume of acetone (8 mL for sediment, 10 mL for filter)

P_s = weight of dry sediment

P_f = volume of water filtered

Organic matter analysis

Samples of freeze-dried sediment (between 1.5 and 2.5 g) were placed into dry, pre-weighed ceramic crucibles. These samples were then placed in an 80°C oven for a minimum of 1 hour to ensure that all the water had been removed. Once the weight no longer fluctuated, the crucibles were placed in a muffle oven at 550°C for 4 hours. After a cooling period the samples were reweighed and the % OM determined from the following equation:

% OM = ((dry weight – ash weight)/dry weight)*100

Grain size analysis

For grain size analysis the three depths samples were combined and samples of approximately 55g for each site were poured onto a stack of 7 sieves and shaken for 10 minutes to separate them into their component sediment size grades.

Data Analysis

Statistical analysis

Statistical analyses of stable isotope results were done using the software package R version 3.0.2 (R Core Team, 2013). Tests were run independently for all sample types to determine if any differences existed between samples according to site (Deer Arm, Neddy's Harbour, South East Arm) or sampling date (April, August, November). Each thyasirid taxon was considered a different sample type, thus each considered independently. For thyasirid, tests were also run to determine differences between tissue type (gill or non-gill), and for sediment additional tests were run to determine if there was a difference in depth (surface, intermediate or bottom/anoxic). Normality was tested using Shapiro-Wilks and homoscedasticity with Breusch-Pagan tests. For all sample types, non-parametric tests were run (Kruskal-Wallis and Mann-Whitney) to determine if differences existed between parameters and then between which they did exist. Significant levels of 0.05 were used.

Mixing Models

Since the stable isotopic signatures of sediment and SPM samples were found to be significantly different between sites, yet there were no significant differences

according to sampling date, all data from individual sites were pooled together and each site was processed independently with Bayesian mixing models in R using the SIAR package (Parnell *et al.* 2008). For the mixing models, the non-gill tissues were considered to be comparable among taxa and representative of the isotopic composition of the animal. Gills could not be considered due to chemoautotrophic bacteria with distinct isotopic ratios making up a considerable portion of the tissue mass in gills of symbiotic *Thyasira* cf. *gouldi*. The three sources used in the mixing model were SPM, sediments and the gills of the symbiotic *T.* cf. *gouldi* (the latter being a proxy for either symbiotic or free-living sulfur-oxidizing, chemoautotrophic bacteria with Rubisco Type II). Therefore, the model should assess the relative importance of suspension feeding, bulk deposit feeding, or selective deposit feeding and chemosymbiosis (on chemoautotrophic bacteria with Rubisco Type II) in the diet of those bivalves.

Since the three thyasirid taxa are closely related, they are assumed to have similar fractionation rates. For SPOM and sediments, we used a fractionation of 3 ± 1‰ for nitrogen and 1 ± 0.5‰ for carbon (McCutchan *et al.* 2003; Yokoyama *et al.* 2005). However, we used a lower fractionation (2 ± 1‰ for nitrogen and 0 ± 0.5‰ for carbon) for the chemoautotrophic thyasirid gill "source" because these tissues are an isotopic composite of both bacterial organic matter and animal tissue having already experienced fractionation. Models were run with 500,000 iterations, with the first 50,000 iterations discarded.

Results

Sources

Suspended particulate material

There was variability in characteristics of the SPM when comparing the three time periods and sample locations (Table 2-1). The total amount of SPM ranged from 6.2 to 14.61 mg/l, and with the exception of South East Arm, decreased over the sampling period. Chl *a* concentrations for all sites were greater in August than in November, with phaeophytin undetectable in the SPM at all sites and months (no data available for April). The SPM chl *a* concentrations were low compared to those of the sediment (Tables 2-1, 2-2, 2-3 & 2-4). At Neddy's Harbour and South East Arm, C:chl *a* increased from August to November. The C:N ratios were lowest at all sites in April, and increased in August (more markedly at Neddy's Harbour), with ratios either further increasing (Deer Arm and South East Arm), or slightly decreasing (Neddy's Harbour) in November.

Sediment

Organic matter content in sediments ranged overall from 3.9 to 13.8% (Tables 2-2, 2-3 & 2-4), with Neddy's Harbour having the lowest %OM and the other sites fluctuating by about 1% throughout the sampling period. Within a site, %OM values were fairly consistent regardless of depth in the sediment or sampling date, with the exception of samples from Deer Arm having a much higher %OM at the surface in August compared to the intermediate and deeper sediments.

Concentrations of chl *a* were highest near the surface and decreased with depth in the sediment, with South East Arm having the lowest chl *a* concentration of all the sites. There was an increase in chl *a* at all sites from April to August, then a decrease in November. Phaeophytin concentrations showed a similar pattern to that of chl *a* with the exception of surface sediment at South East Arm and deeper sediment at Neddy's Harbour, where phaeophytin increased from August to November. Chl *a*: phaeophytin ratios were generally greater near the surface than in deeper sediments. The largest chl *a*:phaeophytin ratios were observed in samples from Neddy's Harbour, where ratios in surface and intermediate sediments increased over time to a maximal value of nearly 5. In contrast, most sediment samples from South East Arm and Deer Arm had chl *a*: phaeophytin ratios between 1 and 2. The C:chl *a* ratios for sediment samples ranged between 403 and 7146, with lowest and highest values recorded from Neddy's Harbour and South East Arm, respectively. The C:N ratios for the different sites, sediment types and dates were variable and ranged from 8 to 22, with Deer Arm intermediate sediments having the largest ratio for all time periods.

Grain size analysis indicated that South East Arm is composed primarily of silt-clay (43.6% of total sample; see appendix A for grain size data), followed by very fine sand (25.4%). Deer Arm and Neddy's Harbour were similar with both having the largest proportions of sediment being very fine-grained sands (40% & 46.6% respectively). However, they differ with Neddy's Harbour in having 29% fine-grained sand, whereas Deer Arm is mostly silt and clay (28.5% vs the 13.7% for Neddy's Harbour) with 20.2% of the sediment being fine-grained sand.

Within each site carbon and nitrogen stable isotope ratios were similar among depths and dates considered (p-values ≥ 0.37 (df=2) for depths, p-values ≥ 0.71 (df=2) for dates). Average sedimentary $\delta^{13}C$ values were -25.95‰ at Deer Arm, -25.31‰ at South East Arm, and -23.75‰ at Neddy's Harbour, while average sedimentary $\delta^{15}N$ values were 2.62‰, 3.01‰ and 4.42‰ at those respective sites. Significant differences among sites were found for all sediment characteristics investigated (Table 2-5).

Table 2-1: Characteristics of suspended particulate material in seawater samples, collected near the bottom at the three Bonne Bay sites at each sampling date. A single sample was analyzed from each site and time. NA: not available; -: undetectable, *: infinite value

Characteristic	Deer Arm		
	April	August	November
Chl *a* (µg/l seawater)	NA	0.53	-
Phaeophytin (µg/l seawater)	NA	-	-
SPM weight (mg/l)	14.61	10.57	7.50
C:chl *a*	NA	1186.91	*
C:N	6.33	8.33	13.73
$\delta^{13}C$ ‰	-24.63	-24.65	-25.11
$\delta^{15}N$ ‰	6.04	4.95	6.54
Characteristic	**Neddy's Harbour**		
	April	**August**	**November**
Chl *a* (µg/l seawater)	NA	0.8	0.27
Phaeophytin (µg/l seawater)	NA	-	-
SPM weight (mg/l)	12.15	6.20	7.70
C:chl *a*	NS	1384.59	2743.58
C:N	8.00	14.42	13.19
$\delta^{13}C$ ‰	-23.73	-26.62	-24.36
$\delta^{15}N$ ‰	6.76	6.27	4.59
Characteristic	**South East Arm**		
	April	**August**	**November**
Chl *a* (µg/l seawater)	NA	0.71	0.27
Phaeophytin (µg/l seawater)	NA	-	-
SPM weight (mg/l)	NA	9.28	11.45
C:chl *a*	NA	1097.39	3823.60
C:N	7.92	9.59	11.87
$\delta^{13}C$ ‰	-23.07	-27.28	-25.52
$\delta^{15}N$ ‰	6.38	7.59	7.05

Table 2-2: Characteristics of sediments obtained from Deer Arm at the three sampling dates. Values are means ± standard deviations, for subsamples from different depths within sediments. The number of samples is indicated in parentheses when greater than one. NA: not available

Characteristic	April		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	8.40 ± 1.95 (2)	6.77	6.65 ± 0.95 (3)
Phaeophytin (µg/g)	5.22 ± 1.20 (2)	4.48	4.82 ± 0.15 (3)
Chl *a*: Phaeophytin	1.61 ± 0.00 (2)	1.51	1.38 ± 0.22 (2)
% OM	9.31 ± 0.64 (3)	10.6 ± 1.56 (2)	10.00 ± 1.01 (3)
C:chl *a*	2797.62	5140.32	NA
C:N	12.37	16.11 ± 4.19 (2)	NA
δ^{13}C ‰	-26.14	-26.0 ± 0.25 (2)	NA
δ^{15}N ‰	2.56	1.93 ± 0.78 (2)	NA
Characteristic	**August**		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	32.00 ± 7.67 (2)	8.21 ± 4.4 (2)	10.20 ± 2.79 (2)
Phaeophytin (µg/g)	9.38 ± 1.78 (2)	5.78 ± 2.24 (2)	4.49 ± 0.20 (2)
Chl *a*: Phaeophytin	3.55 ± 1.49 (2)	1.37 ± 0.23 (2)	2.29 ± 0.73 (2)
% OM	12.67 ± 2.76 (3)	9.47 ± 2.52 (3)	10.40 ± 2.92 (3)
C:chl *a*	765.63	3556.64	2275.39
C:N	15.31	17.18	8.03
δ^{13}C ‰	-26.00	-25.67	-25.76
δ^{15}N ‰	3.00	2.86	2.35
Characteristic	**November**		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	12.00 ± 4.66 (2)	6.22 ± 2.93 (2)	4.32 ± 0.24 (2)
Phaeophytin (µg/g)	6.14 ± 1.44 (2)	3.67 ± 0.54 (2)	2.81 ± 0.15 (2)
Chl *a*: Phaeophytin	1.92 ± 0.31 (2)	1.65 ± 0.55 (2)	1.53 ± 0.00 (2)
% OM	9.11 ± 1.42 (3)	8.48 ± 0.79 (3)	9.33 ± 0.96 (3)
C:chl *a*	1666.67	4533.76	5671.30
C:N	11.11	21.69	15.31
δ^{13}C ‰	25.76	-26.15	-26.03
δ^{15}N ‰	2.77	3.16	3.00

Table 2-3: Characteristics of sediments obtained from Neddy's Harbour at the three sampling dates. Values are means ± standard deviations, for subsamples from different depths within sediments. The number of samples is indicated in parentheses when greater than one. NA: not available.

Characteristic	April		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	6.87	NA	NA
Phaeophytin (µg/g)	2.74	NA	NA
Chl *a*: Phaeophytin	2.51	NA	NA
% OM	4.51 ± 0.07 (2)	3.95	4.13
C:chl *a*	1091.70	NA	NA
C:N	10.71	13.43	9.38
δ^{13}C ‰	-23.87	-24.37	-23.78
δ^{15}N ‰	4.26	3.95	4.54
Characteristic	**August**		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	19.6 ± 4.44 (2)	10.28 ± 1.84 (2)	9.85 ± 5.45 (2)
Phaeophytin (µg/g)	4.89 ± 0.29 (2)	3.00 ± 2.14 (2)	3.19 ± 0.23 (2)
Chl *a*: Phaeophytin	4.04 ± 1.15 (2)	3.46 ± 0.86 (2)	3.16 ± 1.94 (2)
% OM	4.53 ± 0.42 (2)	5.14 ± 0.38 (2)	4.02 ± 0.36 (2)
C:chl *a*	403.06	NA	791.88
C:N	13.17	NA	11.14
δ^{13}C ‰	-23.85	NA	-23.57
δ^{15}N ‰	4.02	NA	4.36
Characteristic	**November**		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	15.60 ± 9.20 (2)	8.37 ± 5.53 (2)	11.25 ± 4.03 (2)
Phaeophytin (µg/g)	3.23 ± 2.15 (2)	2.30 ± 0.43 (2)	5.99 ± 3.62 (2)
Chl *a*: Phaeophytin	4.98 ± 0.46 (2)	3.93 ± 3.14 (2)	2.04 ± 0.56 (2)
% OM	5.25 ± 1.44 (3)	4.05 ± 0.27 (3)	4.53 ± 0.53 (3)
C:chl *a*	705.13	1003.58	746.67
C:N	8.46	12.00	12.00
δ^{13}C ‰	-23.18	-23.19	-23.91
δ^{15}N ‰	4.57	5.60	5.60

Table 2-4: Characteristics of sediments obtained from South East Arm at the three sampling dates. Values are means ± standard deviations for subsamples from different depths within sediments. The number of samples is indicated in parentheses when greater than one.

Characteristic	April		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	6.44 ± 3.82 (2)	4.30 ± 3.53 (2)	3.76 ± 0.09 (2)
Phaeophytin (µg/g)	4.18 ± 2.31 (2)	3.06 ± 1.52 (2)	2.92 ± 0.04 (2)
Chl *a*: Phaeophytin	1.52 ± 0.07 (2)	1.28 ± 0.52 (2)	1.29 ± 0.01 (2)
% OM	10.64 ± 3.83 (3)	10.66 ± 2.83 (3)	9.85 ± 2.45 (3)
C:chl *a*	5326.09	8162.79	9760.64
C:N	11.06	13.50	14.12
δ^{13}C ‰	-25.09	-25.15	-25.00
δ^{15}N ‰	2.89	3.29	3.46
Characteristic	**August**		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	13.84 ± 7.76 (2)	4.76	4.38 ± 3.29 (3)
Phaeophytin (µg/g)	5.80 ± 4.84 (2)	5.31	3.99 ± 2.72 (3)
Chl *a*: Phaeophytin	2.80 ± 1.00 (2)	0.90	1.10 ± 0.29 (3)
% OM	12.74 ± 0.41 (3)	12.90 ± 0.56 (2)	9.95 ± 5.60 (4)
C:chl *a*	3041.91	11239.50	10114.16
C:N	13.58	13.38	11.97
δ^{13}C ‰	-25.11	-25.41	-24.08
δ^{15}N ‰	3.25	2.74	2.96
Characteristic	**November**		
	Surface	**Middle**	**Bottom/ Anoxic**
Chl *a* (µg/g)	11.77 ± 0.64 (2)	6.27 ± 0.75 (2)	5.01 ± 0.32 (2)
Phaeophytin (µg/g)	7.32 ± 2.18 (2)	3.77 ± 0.44 (2)	4.28 ± 0.08 (2)
Chl *a*: Phaeophytin	1.70 ± 0.59 (2)	1.68 ± 0.39 (2)	1.17 ± 0.05 (2)
% OM	13.50 ± 1.60 (3)	13.88 ± 0.73 (3)	12.34 ± 0.73 (3)
C:chl *a*	4231.10	6969.70	7145.71
C:N	14.65	13.66	10.85
δ^{13}C ‰	-25.88	-25.60	-25.71
δ^{15}N ‰	2.74	3.08	2.64

Table 2-5: Results of Kruskal-Wallis analysis of characteristics of sediment collected from Bonne Bay NL in April, August and November of 2013. (* = <0.05; ** <0.001). -: not significant; NA: not analyzed due to insufficient

	Sampling Date	Site	Depth in sediment
% OM	-	**	-
chl *a*	*	*	*
Phaeophytin	-	*	-
δ^{13}C ‰	-	**	NA
δ^{15}N ‰	-	**	NA

Bivalve tissues

Because thyasirid diversity differs among sites (Batstone *et al.* 2014) and their distribution is quite patchy, a different set of taxa was obtained from each site. In this study, we analyzed symbiotic *Thyasira* cf. *gouldi*, asymbiotic *T.* cf. *gouldi* and *Parathyasira* sp. from South East Arm, symbiotic and asymbiotic *T.* cf. *gouldi* from Deer Arm, and symbiotic *T.* cf. *gouldi* from Neddy's Harbour. Although *Parathyasira* sp. were also sampled from Deer Arm, we did not obtain enough specimens to perform isotopic analyses.

Considering all tissues, sites and dates, thyasirid taxa had overlapping ranges of δ^{13}C values (Tables 2-6, 2-7 & 2-8). The greatest variation in δ^{13}C values was observed in symbiotic *Thyasira* cf. *gouldi* (-24.0 to -18.56‰), while asymbiotic *T.* cf. *gouldi* and *Parathyasira* sp. had more restricted ranges of carbon isotope ratios (-21.24 to -19.20‰, and -21.40 to -20.02‰, respectively). For all taxa, gill tissues had heavier C signatures

than non-gill tissues, and the gills of symbiotic *T.* cf. *gouldi* were on average 1.61‰ heavier than asymbiotic gills.

Considering non-gill tissues only, average carbon isotopic signatures were most depleted in $\delta^{13}C$ (-20.87‰, SD=0.67) at South East Arm, with *Parathyasira* sp. being lightest (average: -21.28‰, SD=0.12), followed by the symbiotic *Thyasira* cf. *gouldi* (average: -20.80‰, SD=0.71) and asymbiotic *T.* cf. *gouldi* (average: -20.53‰, SD=0.84). In contrast, average $\delta^{13}C$ values were enriched at Neddy's Harbour (-19.36‰, SD=0.34), where only symbiotic *T.* cf. *gouldi* was collected. In Deer Arm, an average $\delta^{13}C$ signature of -20.56‰ (SD= 0.30) was found, with average values of -20.58‰ (SD=1.81) for asymbiotic *T.* cf. *gouldi* and -20.54‰ (SD=0.39) for symbiotic *T.* cf. *gouldi*.

Differences in the $\delta^{15}N$ signature of symbiotic and asymbiotic taxa were evident, with the former being isotopically lighter (Tables 2-6, 2-7 & 2-8). In the three taxa, gill tissues had lighter $\delta^{15}N$ values than non-gill tissues, with this difference being more pronounced (on average, 0.34‰ lighter) in symbiotic *Thyasira* cf. *gouldi* (Tables 2-6, 2-7 & 2-8) The gills of symbiotic T cf. *gouldi* often had negative $\delta^{15}N$ values (as low as -5.88‰), and were on average 0.28 ‰ lighter than asymbiotic gills.

At Deer Arm (Table 2-6), asymbiotic *Thyasira* cf. *gouldi* had average $\delta^{15}N$ values of 6.19‰ (SD=0.88) for gill and 7.17‰ (SD=0.39) for non-gill tissues, whereas symbiotic *T.*cf. *gouldi* had average $\delta^{15}N$ values of -3.09‰ (SD=3.19) for gill and 2.08‰ (SD=1.75) for non-gill tissues. At Neddy's Harbour (Table 2-7), gill and non-gill tissues of symbiotic *T.*cf. *gouldi* had average $\delta^{15}N$ values of -0.93‰ (SD=1.09) and 4.36‰ (SD=0.77), respectively. At South East Arm (Table 2-8), respective average gill and non-

gill tissue $\delta^{15}N$ values were 6.93‰ (SD=0.52) and 7.6‰ (SD=0.51) for asymbiotic *T*.cf. *gouldi*, 0.95‰ (SD=1.55) and 4.09‰ (SD=0.91) for symbiotic *T*.cf. *gouldi*, and 3.34‰ (SD=1.90) and 6.1‰ (SD=0.80) for *Parathyasira* sp.

At all sites, tissue C:N ratios were higher in symbiotic than in asymbiotic taxa, with *Parathyasira* sp. having the lowest ratio. The C:N ratios for non-gill tissues were generally lower in August at all sites.

Significant differences in $\delta^{13}C$ and $\delta^{15}N$ values were found among the different taxa, with all tissues and sampling dates considered (P-value = 0.024 and <0.0001, respectively, df=2). The gills differed significantly from those non-gill tissues for the asymbiotic taxa in terms of $\delta^{13}C$ (p-values $\leq$ 0.04, df=1) and for all taxa in terms of $\delta^{15}N$ (p-values, $\leq$ 0.02, df=1). Site-specific differences in isotopic composition were only found in symbiotic *Thyasira*. cf. *gouldi* (Table 2-9).

Table 2-6: Isotopic and elemental composition of the tissues from Deer Arm of symbiotic and asymbiotic *Thyasira* cf. *gouldi* sampled at each sampling date. Data are means ± standard deviations, N = 2 samples (pooled individuals) per combination of taxon, tissue type and sample date.

Characteristic	April					
	Symbiotic *T.* cf. *gouldi*			Asymbiotic *T.* cf. *gouldi*		
	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference
C: N	4.26 ± 0.12	3.83 ± 0.05		3.38 ± 0.04	4.42 ± 0.50	
δ^{13}C ‰	-19.48 ± 0.08	-20.33 ± 0.04	0.85 ± 0.04	-19.67 ± 0.22	-18.92 ± 2.72	1.77 ± 1.05
δ^{15}N ‰	-4.49 ± 0.60	2.26 ± 0.22	6.74 ± 0.38	5.79 ± 1.61	7.60 ± 0.06	1.82 ± 1.55
Characteristic	**August**					
	Symbiotic *T.* cf. *gouldi*			Asymbiotic *T.* cf. *gouldi*		
	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference
C: N	4.32 ± 0.35	3.90 ± 0.02		3.30 ± 0.08	3.69 ± 0.35	
δ^{13}C ‰	-22.45 ± 2.30	-21.04 ± 0.06	1.67 ± 1.99	-19.60 ± 0.04	-20.39 ± 0.03	0.80 ± 0.06
δ^{15}N ‰	0.52 ± 3.20	3.94 ± 0.25	3.43 ± 2.95	6.69 ± 0.04	7.32 ± 0.17	0.63 ± 0.13
Characteristic	**November**					
	Symbiotic *T.* cf. *gouldi*			Asymbiotic *T.* cf. *gouldi*		
	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference
C: N	4.07 ± 0.14	3.90 ± 0.07		3.73 ± 0.03	4.28 ± 0.16	
δ^{13}C ‰	-19.81 ± 0.75	-20.24 ± 0.06	2.79 ± 2.64	-19.43 ± 0.32	-20.65 ± 0.07	1.23 ± 0.25
δ^{15}N ‰	-5.29 ± 0.84	0.05 ± 0.21	7.11 ± 3.15	6.09 ± 0.67	6.78 ± 0.23	0.70 ± 0.45

Table 2-7: Isotopic and elemental composition of the tissues from Neddy`s Harbour of symbiotic *Thyasira* cf. *gouldi* sampled in at each sampling date. Data are means ± standard deviations, N = 2 samples (pooled individuals) per combination of tissue type in April and August. In November, only one sample was available for each tissue type.

Characteristic	April		
	Gill	Non-gill	Tissue Difference
C: N	3.97 ± 0.10	3.84 ± 0.10	
$\delta^{13}C$ ‰	-19.16 ± 0.81	-19.56 ± 0.31	0.41 ± 0.50
$\delta^{15}N$ ‰	-1.32 ± 0.37	3.98 ± 0.83	5.30 ± 0.53
Characteristic	**August**		
	Gill	**Non-gill**	**Tissue Difference**
C: N	3.94 ± 0.01	3.80 ± 0.08	
$\delta^{13}C$ ‰	-18.65 ± 0.12	-19.07 ± 0.29	0.42 ± 0.17
$\delta^{15}N$ ‰	-0.89 ± 1.95	4.44 ± 1.03	5.33 ± 0.93
Characteristic	**November**		
	Gill	**Non-gill**	**Tissue Difference**
C: N	4.13	3.88	
$\delta^{13}C$ ‰	-19.27	-19.53	0.26
$\delta^{15}N$ ‰	-0.22	4.95	5.17

Table 2-8: Isotopic and elemental composition of tissues from South East Arm of symbiotic and asymbiotic *Thyasira* cf. *gouldi* and *Parathyasira* sp. sampled at each sampling date. Data are means ± standard deviations, N = 2 samples (pooled individuals) per combination of taxon and tissue type in August and November, except for April and *Parathyasira* from November, when only sample per tissue type was available. NA: Not available.

Characteristic	April								
	Symbiotic *T.* cf. *gouldi*			Asymbiotic *T.* cf. *gouldi*			*Parathyasira* sp.		
	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference
C: N	3.74	4.18		NA	NA		3.35	3.97	
$\delta^{13}C$ ‰	-19.50	-19.70	0.20	NA	NA		-20.98	-21.40	0.42
$\delta^{15}N$ ‰	1.16	4.40	3.25	NA	NA		5.30	7.24	1.94
Characteristic	August								
	Symbiotic *T.* cf. *gouldi*			Asymbiotic *T.* cf. *gouldi*			*Parathyasira* sp.		
	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference
C: N	3.97 ± 0.05	3.84 ± 0.08		3.19 ± 0.28	4.20 ± 0.42		2.96 ± 0.97	3.56 ± 0.08	
$\delta^{13}C$ ‰	-19.97 ± 0.01	-20.77 ± 0.27	0.80 ± 0.25	-19.97 ± 0.65	-20.02 ± 1.02	1.18 ± 0.07	-20.45 ± 0.60	-21.28 ± 0.13	0.83 ± 0.74
$\delta^{15}N$ ‰	-0.61 ± 0.63	3.27 ± 0.95	3.88 ± 0.32	6.90 ± 0.35	7.68 ± 0.81	0.82 ± 1.11	2.40 ± 2.28	5.55 ± 0.04	3.15 ± 2.25
Characteristic	November								
	Symbiotic *T.* cf. *gouldi*			Asymbiotic *T.* cf. *gouldi*			*Parathyasira* sp.		
	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference	Gill	Non-gill	Tissue Difference
C: N	3.84 ± 0.08	4.05 ± 0.03		3.47 ± 0.08	3.97 ± 0.04		2.95	3.81	
$\delta^{13}C$ ‰	-20.24 ± 0.06	-21.39 ± 0.22	1.15 ± 0.28	-20.27 ± 0.32	-21.04 ± 0.28	0.79 ± 0.01	-20.26	-21.17	0.91
$\delta^{15}N$ ‰	2.41 ± 0.31	4.76 ± 0.32	2.35 ± 0.63	6.69 ± 0.83	7.53 ± 0.35	0.43 ± 0.29	3.26	6.07	2.81

Table 2-9: Results of Kruskal-Wallis analysis of stable isotope ratios run to detect differences according to sampling date or site or tissue on non-gill in the three thyasirid taxa collected in Bonne Bay NL. (* = p < 0.05, df=2).

Species		Sampling date	Site
Symbiotic *T.* cf. *gouldi*	$\delta^{13}C$ ‰	-	*
	$\delta^{15}N$ ‰	-	*
Asymbiotic *T.* cf. *gouldi*	$\delta^{13}C$ ‰	-	-
	$\delta^{15}N$ ‰	-	-
Parathyasira sp.	$\delta^{13}C$ ‰	-	-
	$\delta^{15}N$ ‰	-	-

Mixing models

Because of site differences in the isotopic signature of sources (Table 2-5) and of one of the thyasirid taxa (Table 2-9), sites are considered separately in mixing models. Source isotopic signatures were distinct at each site, with the exception of South East Arm where SPM and sediment have a similar carbon isotope signature (Fig. 2-1). Graphs of $\delta^{13}C$ vs $\delta^{15}N$ reveal that the signatures of all thyasirid non-gill samples are closer to the proxy signal for chemoautotrophic bacteria than to other sources (SPM and sediment organic carbon) (Fig 2-1). Therefore, chemoautotrophic bacteria appear to make a greater contribution to thyasirid isotopic signatures than do other sources, even asymbiotic taxa.

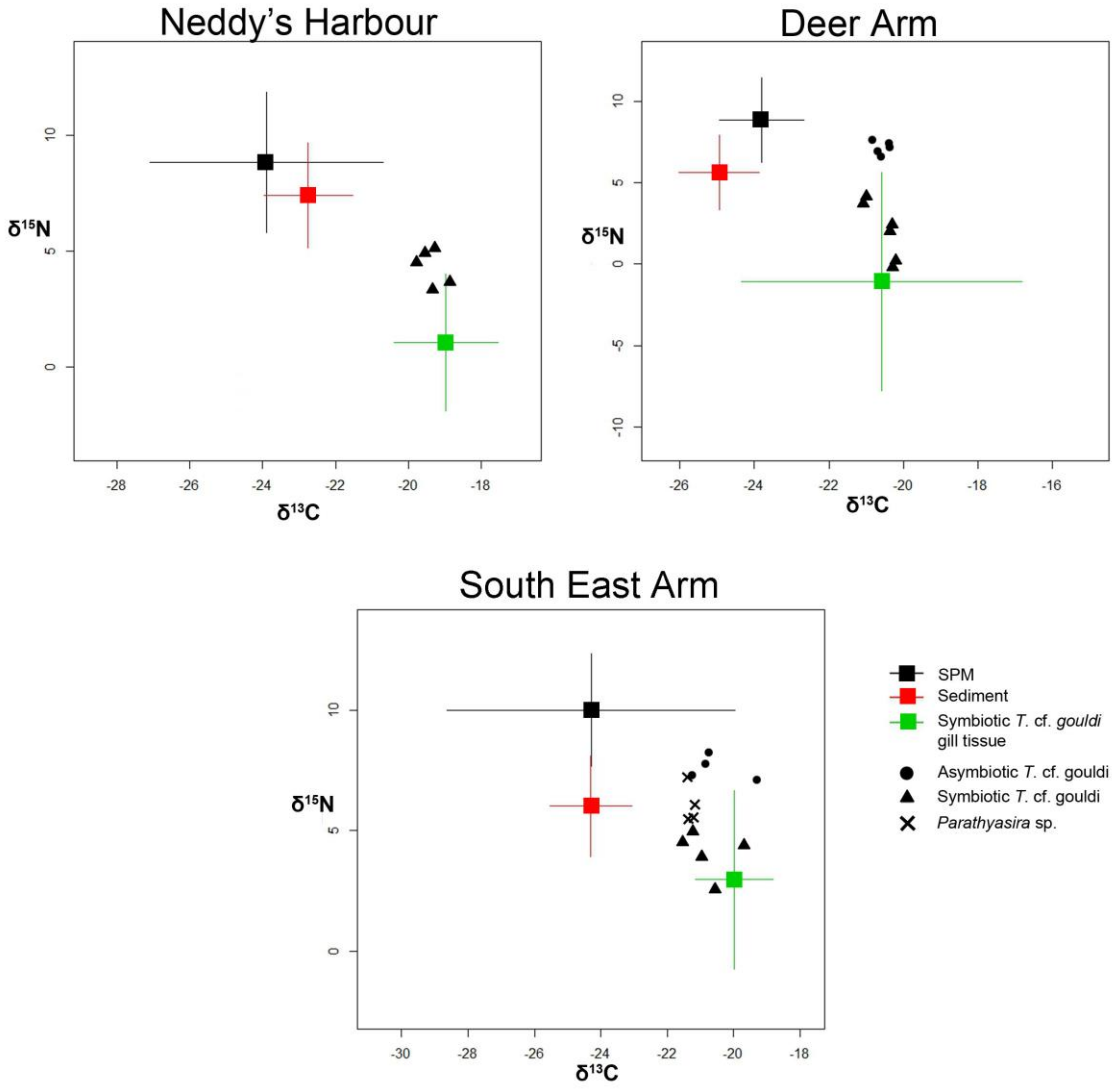

Figure 2-1: Stable isotopic signatures of thyasirid non-gill tissues and of potential food sources (SPM, sediment and gills of symbiotic *Thyasira* cf. *gouldi*, a proxy for chemoautotrophic bacteria) at the three sampling sites in Bonne Bay, NL. Sources have been fractionated and the standard deviations of the samples.

Mixing model outputs reveal the proportion of each food source contributing to the isotopic composition of non-gill tissues (Fig. 2-2). For all sites the trends are similar. At Neddy's Harbour, symbiotic *Thyasira* cf. *gouldi* derive the greatest proportion of their

diet from chemoautotrophic bacteria (40.7 – 67.5%) and the least amount from the SPOM (7.8 - 29.2%). A similar trend follows at Deer Arm where symbiotic *T.* cf. *gouldi* derives the most from the bacteria (60.2 – 72.3%) and the least from SPOM (11.7 – 27.5%). At Deer Arm the opposite is true for the asymbiotic *T.* cf. *gouldi*, which consumes more SPOM (49 – 67%) and the least derived from chemoautotrophic bacteria (4.6 – 14.3%). At South East Arm symbiotic *T.* cf. *gouldi* derive a greater proportion (52.3 - 66.8%) of their diet from chemoautotrophic bacteria yet differs from the other sites because it consumes the least of sediment OM (10.9 – 24.5%). Asymbiotic *T.* cf. *gouldi* at South East Arm show a similar trend to Deer Arm with the greatest proportion from SPM (40.6 – 54.8%) and the least from chemoautotrophic bacteria (16 – 35.5%). *Parathyasira* sp. appears to derive a greater proportion of their diet from chemoautotrophic bacteria (66.6 – 75.8%) than do symbiotic *T.* cf. *gouldi* yet seems to also have a higher proportion from sediment OM(10.9 - 24.5%) than SPOM (4.8 – 15.7%).

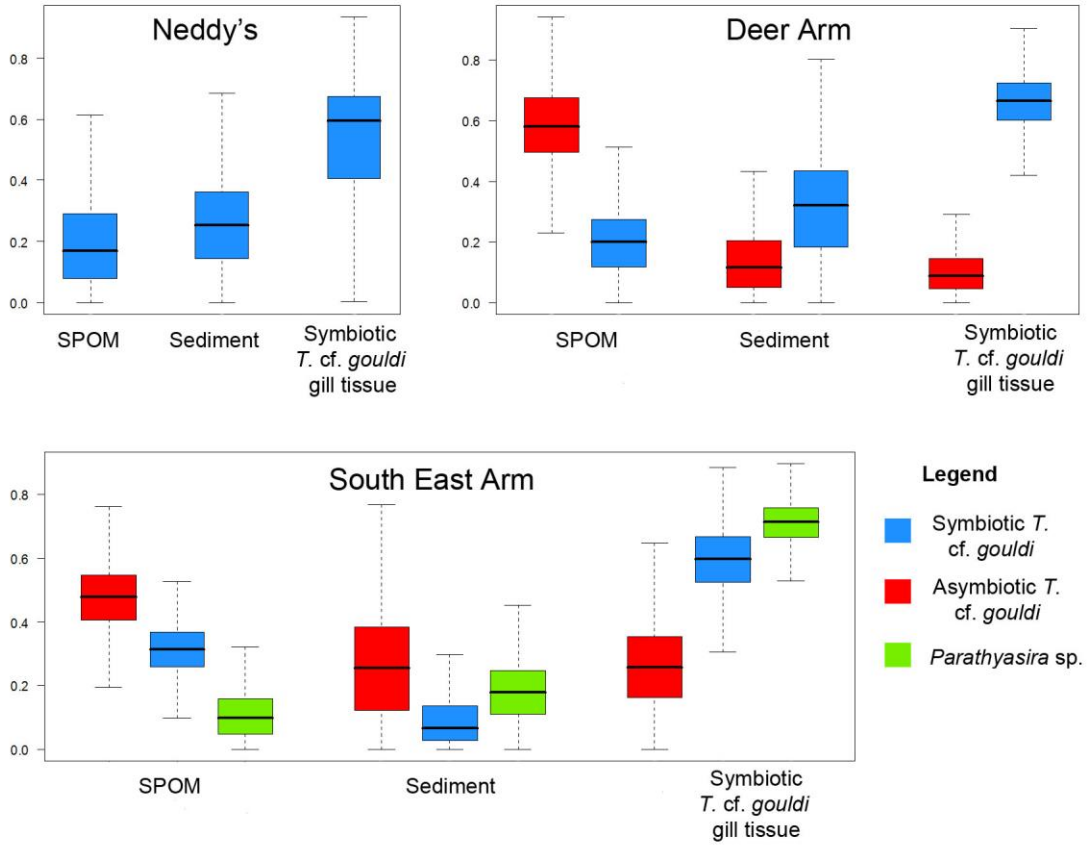

Figure 2-2: Stable isotope mixing model output for the three sampling sites in Bonne Bay, NL. The model is based upon three potential food sources: SPOM, sediment organic matter, and symbiotic *Thyasira* cf. *gouldi* gill tissue (a proxy for chemoautotrophic bacteria). The thyasirid taxa are represented by different colours. Box-and-whisker plots show the median, 25th and 75th percentiles, and maximum and minimum possible dietary ranges.

Discussion

The analysis of suspended particulate matter near the benthos (SPM, SPOM, Chl *a*) and sediment organic matter characteristics at the three Bonne Bay sampling sites revealed the extent of variability that exists both among those sites, and at different times of the year, as discussed below. In combination with the stable isotope analysis of

thyasirid tissues, the characterization of SPOM and sedimentary organic matter isotopic signatures at each sampling site helped us assess the importance of various food sources to the diet of symbiotic and asymbiotic thyasirids in this fjord.

Natural variation in potential food sources

Suspended particulate matter

The quality and quantity of SPM near the benthos differed slightly between sites, and more obviously among sampling dates in this study; however, due to the low number of samples, we could not determine if either temporal or spatial differences in SPM parameters were statistically significant. Overall, the concentration of SPM near the benthos was low compared to other estuarine regions (8.2-73.8 mg/L; Devlin *et al.* 2008), and, as in other fjords, was likely a mixture of inorganic and organic particles from both terrestrial and marine sources (McCallister *et al.* 2004). At the three sites, the relative importance of phytoplankton within SPM was probably greater in April and August than in November, for the following reasons: 1) the first two sampling periods coincided with phytoplankton blooms (late April and August) according to MODIS satellite color data from the adjacent Gulf of St Lawrence (Bedford Institute of Oceanography 2015); 2) Chl *a* concentrations were lower in November than in August (no data were available from the April sampling); 3) C:Chl *a* ratios were higher in November than in August (no data were available from the April sampling); 4) C:N ratios were lowest, and closest to the Redfield ratio (6.625) in April (Redfield *et al.* 1963). A previous study examining export fluxes between the surface and deep waters in Bonne Bay reported a bimodal pattern, with maximal sinking fluxes of similar magnitude occurring in the spring and late

summer (Tian *et al.* 2001). If a similar export pattern existed in 2013, then marine surface primary production likely formed a greater contribution to SPM near the benthos in April and August of this study.

Based on stable carbon isotope signatures (Table 2-1), much of the SPOM appears to be terrestrially derived (i.e., within the previously reported range of -28 to -18 ‰; (Fry & Sherr 1984 in Hedges *et al.* 1997) and was likely transported into the fjord by nearby brooks (Fig. 2). A predominant terrestrial origin for SPOM is also supported by high C:N values (> 12, Thornton & McManus 1994); this material appears to be detrital, given the relatively high C:Chl *a* ratios (> 200, Cifuentes *et al.* 1988). Detrital material constituted a greater proportion of the SPOM in November (when C:Chl *a* ratios were largest) than in April and August; as such, SPOM likely had a greater nutritional content in the earlier months than in November. Site-specific differences in the relative proportion of detritus within SPOM were not apparent which was unexpected, more research examining site differences at a more refined scale would likely to help clarify if this was a sampling method issue or if indeed there are no site differences.

Sediment organic matter

In Bonne Bay sediments, OM is abundant and may be directly consumed by thyasirids. While sediment organic matter characteristics varied significantly among sampling sites in this study, only Chl *a* concentration differed significantly according to sampling date or depth in sediments (Table 2-5). The higher Chl *a* concentrations observed in surface sediments compared to deeper layers (Tables 2-2, 2-3 & 2-4) are not surprising as surface layers contain a greater quantity of recently settled SPOM that has

not yet undergone much degradation as well as benthic algae and bacterial films. The ratios of Chl *a*: phaeophytin are also higher near the surface than in deeper layers (Tables 2-2, 2-3 & 2-4), indicating an increasing proportion of Chl *a* transformation into phaeophytin as this material gets buried and degrades (Michaud *et al.* 2009). The concentration of Chl *a* in sediments also differed significantly between sampling dates: values were lowest in April and highest in August (Tables 2-2, 2-3 & 2-4). Sediments collected in April had likely not yet received much input from the spring phytoplankton bloom or increased freshwater flow due to snowmelt (both of which likely contributed to the higher sedimentary Chl *a* values in August), but rather had experienced Chl *a* depletion over winter months. Based on differences in Chl *a* content according to depth in sediments, material in surface layers can be considered to be of greater nutritional quality than that in deeper layers, and might be targeted by thyasirids. However, we found only slight differences in the isotopic composition of sediments sampled at different depths (possibly due to high quantities of detrital matter), we did not consider these depth fractions separately in our mixing models.

Sampling sites differed in their sedimentary organic matter content and quality. Notably, OM content was lowest at Neddy's Harbour and highest at South East Arm. Sediment grain size also differed among sites, most likely in association with the amount and type of terrestrial sedimentary inputs from freshwater sources: sediments from Neddy's Harbour (further from land or streams) were predominantly sandy, while those from Deer Arm and South East Arm (receiving direct terrestrial inputs) were finer-grained, with abundant detrital plant material, particularly at Deer Arm. The relative

importance of terrestrial inputs was also reflected in C:N ratios, which were higher at Deer Arm and South East Arm than at Neddy's Harbour (C:N ratios >12 indicate a predominantly terrestrial origin; Thornton & McManus 1994). In high latitude fjords, Aracena *et al.* (2011) found that C:N ratios were higher for areas closer to rivers and glaciers indicating that fluvial inputs are important in these areas with South East Arm having more degraded organic matter based on C:Chl *a* values (Cifuentes *et al.* 1988).

The sediment stable isotope signatures showed site-specific differences in $\delta^{13}C$ and $\delta^{15}N$. $\delta^{13}C$ values were lighter in South East Arm and Deer Arm than in Neddy's Harbour, and $\delta^{15}N$ values were heaviest in Neddy's Harbour, and lightest in South East Arm, possibly reflecting a greater proportion of terrestrial organic matter at the former two sites. However, the isotopic signature of sedimentary organic matter was not observed to vary according to sampling date, despite the temporal variability in SPM quality and quantity, and other expected changes (e.g. sediment dissolved sulfide concentrations were previously observed to be higher in August than in April at the same Bonne Bay sites; Laurich *et al.*, submitted).

Considering solely SPOM and bulk sedimentary organic matter as potential thyasirid food sources according to typical stable isotope fractionation pathways suggests a missing food source, since the thyasirids are often lighter in nitrogen than both SPOM and sedimentary organic matter (Fig. 2-1). Most likely, the SPOM and sedimentary organic matter analyzed in this study consist of a complex mixture of various types of organic matter having unique signatures (represented herein by their average stable isotope composition), with thyasirids selectively feeding on some fractions of that organic

matter (e.g. chemoautotrophic bacteria). Including the gill tissues of symbiotic *Thyasira* cf. *gouldi* as a proxy for sulfur-oxidizing, chemoautotrophic bacteria that live either as symbionts or freely within sediments provides a better indication of the relative contribution of such bacteria to the diet of these bivalves.

Symbiotic Thyasira *cf.* gouldi *gills (proxy for sulfur-oxidizing, chemoautotrophic bacteria)*

Although symbionts can be isolated from gills using centrifugation (Distel *et al.* 1994; van der Geest 2014), we were unable to do so in this study given the small size and restricted number of specimens available. Instead, we used the isotopic signature of symbiotic *Thyasira* cf. *gouldi* gill tissue as a proxy for both free-living and symbiotic sulfur-oxidizing bacteria. Although isolated bacteria with Rubisco II would likely have a carbon signature closer to -11‰ (Robinson & Cavanaugh 1995), two factors likely lead to the more negative values observed here. First, the dissolved inorganic carbon pool available to *T.* cf. *gouldi* symbionts was likely isotopically depleted due to the decomposition of organic matter in sediments (McCorkle & Emerson 1988), resulting in a lighter chemoautotrophic $\delta^{13}C$ signature, as observed for *Solemya velum* (Scott *et al.* 2004). Second, the gill tissue analyzed here should contain a larger fraction of animal carbon, resulting in a lighter signature. Further, *T.* cf. *gouldi* likely has a mixotrophic diet (as do other symbiotic thyasirids, Dufour & Felbeck 2006), assimilating organic carbon from other sources besides their symbionts, and influencing the isotopic ratio of gills (and other tissues). Most other thyasirids (collected from fjords, cold seeps, reducing sediments) have gill tissue $\delta^{13}C$ values ranging between -40 and -30‰ (Dando & Spiro

1993), indicating that their symbionts use Rubisco type I (Robinson & Cavanaugh 1995); these thyasirids may also derive a greater proportion of their organic carbon from their symbionts than *T.* cf. *gouldi*. Only two other thyasirids were reported to have similar gill $\delta^{13}C$ values to those of symbiotic *T.* cf. *gouldi*: 1) *Parathyasira equalis* from the Swedish Gullmar fjord (-28.7 to -18.7‰, Dando & Spiro 1993), a species with a low abundance of chemoautotrophic symbionts (Southward 1986, Dufour 2005); and 2) *T.* (*Parathyasira*) sp. from abyssal stations in the Sea of Japan (-20.8 ± 0.7‰), a species appearing to be asymbiotic (Kiyashko *et al.* 2014). These two species are therefore more comparable to the asymbiotic *T.* cf. *gouldi* and *Parathyasira* sp. in this study than to symbiotic *T.* cf. *gouldi*.

The isotopic nitrogen signature of symbiotic *Thyasira* cf. *gouldi* gill tissues varied widely (Fig. 2-1); again, this may be due to the effect of variations in symbiont abundance and of a mixotrophic diet on host tissue signatures. Other chemosymbiotic bivalve species such as *Solemya velum* and bathymodiolin mussels have $\delta^{15}N$ values ranging from -9.8 to +4.4‰ for gill tissue (Conway *et al.* 1989) and from -12.9 to +3.0‰ for whole specimens (Brooks *et al.* 1987), respectively, indicating that a large $\delta^{15}N$ range is not uncommon. The $\delta^{15}N$ values of symbiotic *T.* cf. *gouldi* gill tissues were low (<0‰), indicating a low trophic level (i.e. chemoautotophic primary production), and possibly bacterial nitrogen fixation (Minagawa & Wada 1986). The only other available thyasirid $\delta^{15}N$ values are for the potentially asymbiotic *T.* (*Parathyasira*) sp. from the abyssal Sea of Japan: the heavy signature (9.9±1.0‰) (Kiyashko *et al.* 2014) suggests a higher trophic

level than in symbiotic *T.* cf. *gouldi* and is unlikely to reflect the signature of chemoautotrophic bacteria.

Isotopic composition of symbiotic and asymbiotic thyasirids

Comparisons of non-gill tissues revealed differences both among taxa within a site and among individuals of a given taxon at different sites, indicating diverse feeding modes and adaptations to local conditions in thyasirids. As expected, we found differences in the isotopic signature of symbiotic and asymbiotic thyasirids (especially in $\delta^{15}N$ values), reflecting a more important contribution of chemoautotrophic symbiont-derived nutrients in symbiotic thyasirids. While the presence of Rubisco type II in *Thyasira* cf. *gouldi* symbionts (Dufour *et al*. 2014) likely led to the absence of higher $\delta^{13}C$ signature than in other chemosymbiotic thyasirids (and closer to the $\delta^{13}C$ composition of asymbiotic benthic organisms, which typically range from -20 to -16‰; Spiro *et al.* 1986), the $\delta^{15}N$ composition of symbiotic thyasirid tissues helped in the discrimination of potential food sources.

The isotopic composition of non-gill tissues of symbiotic *Thyasira* cf. *gouldi* suggested a mixotrophic diet, given that $\delta^{13}C$ values were slightly lower, and $\delta^{15}N$ often higher than would expected if these organisms obtained all their nutrients from chemoautotrophic symbionts. A mixotrophic diet has previously been suggested for other thyasirids, on the basis of $\delta^{13}C$ values that differed widely between sampling sites and dates (Spiro *et al.* 1986; Dando & Spiro 1993), and because the abundance of symbionts varies according to SPM availability (Dufour & Felbeck 2006) and temporally (Laurich *et al.* submitted). However, a clear relationship between symbiont abundance and the degree

of nutritional input from symbionts may not exist: although symbiont abundance in Bonne Bay *T.* cf. *gouldi* populations varied in a cyclical fashion over two years, with highest and lowest abundances in the fall and spring, respectively, a similar trend in membrane whorl density, an indicator of the quantity of digested symbionts, was not observed (Laurich *et al.* submitted). Alternatively, the nutritional input from symbionts may remain relatively constant over short periods of time (e.g. over a year), while the input from particulate food might be more seasonally variable, as observed in shallow-water lucinids (van der Geest *et al.* 2014), and as suggested by temporal differences in the cytology of digestive cells in *T. flexuosa* (Donval *et al.* 1989). In this study, stable isotope ratios varied among samples (particularly for $\delta^{15}N$), possibly as a result of small-scale patchiness in sulfur or organic matter within sediments influencing feeding processes. However, there was no statistically significant effect of sampling date on stable isotope signatures of the thyasirids examined. A larger sample size would help to resolve any temporal trends in thyasirid stable isotopic composition.

The asymbiotic thyasirids examined in this study were comparable to symbiotic *Thyasira* cf. *gouldi* in their $\delta^{13}C$ composition, but had heavier and less variable $\delta^{15}N$ signatures. The mixing models provided evidence for a predominantly SPOM-based diet in asymbiotic *T.* cf. *gouldi* (average SPOM contribution: 50-60%; Fig. 2-2). In contrast, suspension-feeding contributed a small proportion (approximately 10%) of the diet of *Parathyasira* sp., with most of the diet (approximately 70%) apparently derived from chemoautotrophic bacteria. Surprisingly, the relative dietary contributions of chemoautotrophic bacteria were higher for *Parathyasira* sp. than for symbiotic *T.* cf.

gouldi. *Parathyasira* sp. from Bonne Bay lack symbionts (Batstone 2012), but may nonetheless consume large quantities of free-living, chemoautotrophic sulfur-oxidizing bacteria. Similarly, another *Parathyasira* species from the Sea of Japan are thought to feed mainly on sedimentary organic matter and associated bacteria, on the basis of their fatty acid composition (Kiyashko *et al.* 2014); therefore, a subsurface deposit feeding mode may be common among *Parathyasira* species, and potentially also other asymbiotic thyasirids.

Parathyasira sp. was recently shown to construct numerous, elongate pedal tracts in surrounding sediments (Chapter 1). The pedal tracts of *Parathyasira* and other asymbiotic thyasirids could be optimal microenvironments for sulfur-oxidizing chemoautotrophic bacteria, which are known to seek redox boundaries in sediments (Lefèvre and Bazylinski, 2013). The isotopic signature of *Parathyasira* sp. (and, to some extent, asymbiotic *Thyasira* cf. *gouldi*) is concordant with the hypothesis of "microbial gardening" and pedal feeding. In Bonne Bay, *Parathyasira* sp. appears to rely more on "microbial gardening" than does asymbiotic *T.* cf. *gouldi*.

Conclusion

This study has shown that feeding strategies can be more variable than previously thought in thyasirids, and that asymbiotic species are not exclusively suspension-feeders. The food sources of two asymbiotic thyasirid species (asymbiotic *Thyasira* cf. *gouldi* and *Parathyasira* sp.) were remarkably different. Interestingly, the closely related symbiotic and asymbiotic *T.* cf. *gouldi* appear to vary widely in their (non-symbiotic) feeding modes, with the latter appearing to get a limited input from sediment-derived organic

matter even though they do construct pedal tracts. Further work examining sulfur isotopic composition of the sources (cf. Peterson & Fry 1987) could help discriminate potential food sources in these thyasirids. Additionally, characterizing the fatty acid and lipid signatures of these bivalves would be useful in evaluating the contribution of bacteria (free-living or symbiotic) to their diet.

Thesis Summary

The two chapters within this thesis provide information on a group of bivalves that is globally distributed yet rarely studied. The combination of behavioural investigations and dietary analysis help to answer some of the questions that have been unresolved in these organisms: what do they eat, and how do they do it?

Symbiotic *Thyasira* cf. *gouldi*

Symbiotic thyasirids bivalves have been of particular interest to researchers because they possess extracellular symbionts, which differ from most other symbiotic bivalve groups. It has been known for decades that some symbiotic thyasirids are mixotrophs and do not rely solely on their symbionts for their nutrition (Dando & Spiro 1993, Le Pennec *et al.* 1995). The current research indicates that the majority of the diet of symbiotic *Thyasira* cf. *gouldi* is derived from chemoautotrophic bacteria, yet there are other likely components, mainly organic matter within the sediment as well as other bacterial sources in the sediment. The latter dietary source makes sense given the burrowing behaviour of that species because although pedal tracts in symbiotic thyasirids are considered to result from the active search for the reduced sulfur that the bacteria need, they may also collect organic material within the sediments and consume it. There do not appear to be seasonal changes in the consumption of chemoautotrophic bacteria within this taxon (Laurich *et al*. submitted), so burrowing activities may need to be maintained throughout the year to gain access to all the nutrients they require.

Asymbiotic *Thyasira* cf. *gouldi*

Asymbiotic thyasirids have gone largely unstudied. With the exception of Dufour and Felbeck (2003) who observed no pedal tracts in association with asymbiotic taxa (*Thyasira obsolete* and *T. ferruginea*), nothing else has been published on asymbiotic thyasirids, other than to confirm they lack symbionts (Dufour 2005). Here, we found that asymbiotic *T.* cf. *gouldi* burrow similarly to symbiotic *T.* cf. *gouldi*. Pedal tracts are expected to be associated with the acquisition of sulfides required by symbionts, yet the formation of such structures by asymbiotic *T.* cf. *gouldi* suggests an alternate purpose. Asymbiotic *T.* cf. *gouldi* appear to be predominantly suspension feeding with some dietary input from sedimentary organic matter. The pedal tracts may be used in searching for organic material within the sediment. While deposit-feeding, asymbiotic *T.* cf. *gouldi* may also consume some chemoautotrophic bacteria as evidenced by the small portion of their diet that comes from these bacteria. Pedal feeding is an ancestral form of feeding in bivalves that is typically only found in juveniles, yet persists to the adult stage in some small species (Reid *et al.* 1992). Asymbiotic *T.* cf. *gouldi* may have retained some ability to pedal feed into adulthood, in combination with suspension feeding, to acquire nutrients or it may be an example of how retention of juvenile trait into the adult could be the precursor to chemosymbiosis.

***Parathyasira* sp.**

As in the other asymbiotic taxon, asymbiotic *Thyasira* cf. *gouldi*, *Parathyasira* sp. burrows and creates pedal tracts similar to symbiotic *T.* cf. *gouldi*. *Parathyasira* sp. lacks chemoautotrophic bacteria, yet appears to consume the same bacteria as the symbiotic

taxon, based on isotopic analysis. “Microbial gardening” occurs where organisms maintain an environment that promotes bacterial growth (Lopez & Levinton 1987). It is possible that the pedal tracts that *Parathyasira* sp. create form “microbial gardens” for the same (or similar) sulfur-oxidizing bacteria that symbiotic *T.* cf. *gouldi* obtain from their environment and maintain as symbionts (Dufour *et al.* 2014). *Parathyasira* sp. may create and maintain pedal tracts to support the growth of bacteria, with no development of symbiotic relationship in this species. Thyasirids produce redox interfaces along pedal tract linings by pumping in oxygenated water from above, creating microenvironments in the sediment that promote bacterial growth (Hakonen *et al.* 2010, Lefèvre and Bazylinski 2013). This interpretation of the purpose of the pedal tracts in this taxon is supported by stable isotope analysis that indicates that the majority of their diet comes from the chemoautotrophic bacteria. Interestingly, *Parathyasira* sp. appears to obtain more of their nutrients from chemoautotrophic bacteria than does symbiotic *T.* cf. *gouldi.* Some thyasirids may get more nutrients from bacteria by “microbial gardening” alone than by investing the resources into maintaining a symbiotic association.

Future Work

This research brings forward many unanswered questions. A more detailed dietary analysis where different types of bacteria may help to discriminate food sources, especially in asymbiotic species. Lipid and fatty acid analysis could be useful in determining the importance of various types of bacteria to the diet of those thyasirids. Additionally, the analysis of sulfur stable isotopes could be useful because there was very little variation in carbon isotopes among sources and taxa in this study.

Regarding burrowing studies, a more frequent and regular X-radiography investigation of pedal tract formation could help to determine how thyasirids form and maintain these structures and if there is directionality in these behaviours. Additionally, measuring or manipulating the distribution of sulfide within the sediment would help to determine if the pedal tracts are oriented towards sources of sulfides. Studying ventilation patterns (the pumping action) would help to understand how often thyasirids pump oxygenated water within sediments, or sulfidic water out of their pedal tracts, thereby creating redox zones for "microbial gardening".

Finally, as this study took place in a limited region, where only a few thyasirids species were available, expanding similar research to other areas and thyasirids species would help to increase our understanding of this group and how chemosymbiosis evolved within it.

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I. Grain Size Analysis

South East Arm

Phi	mm size	Class Weight	Fraction of Dry Weight	Percent of Dry weight	Sediment Type
-1	2	0.07	0.001287877	0.128787739	Very fine gravel
0	1	0.38	0.006991334	0.699133442	Very coarse sand
1	0.5	0.93	0.017110371	1.711037109	Coarse sand
2	0.25	6.01	0.110573473	11.05734734	Medium sand
3	0.125	9.17	0.168711939	16.87119386	Fine sand
4	0.062	13.81	0.254079812	25.40798116	Very fine sand
Catchpan	0.001	23.69	0.435854507	43.58545067	Silt-clay/mud

Deer Arm

Phi	mm size	Class Weight	Fraction of Dry Weight	Percent of Dry weight	Sediment Type
-1	2	0.07	0.013781598	1.378159794	Very fine gravel
0	1	0.38	0.003626736	0.36267363	Very coarse sand
1	0.5	0.93	0.001632031	0.163203134	Coarse sand
2	0.25	6.01	0.088311029	8.831102891	Medium sand
3	0.125	9.17	0.202371886	20.23718855	Fine sand
4	0.062	13.81	0.399847677	39.98476771	Very fine sand
Catchpan	0.001	23.69	0.285605484	28.56054836	Silt-clay/mud

Neddy's Harbour

Phi	mm size	Class Weight	Fraction of Dry Weight	Percent of Dry weight	Sediment Type
-1	2	0.07	0.002328706	0.232870578	Very fine gravel
0	1	0.38	0.004836543	0.483654277	Very coarse sand
1	0.5	0.93	0.01916704	1.916703986	Coarse sand
2	0.25	6.01	0.070219436	7.021943574	Medium sand
3	0.125	9.17	0.295387371	29.53873712	Fine sand
4	0.062	13.81	0.46645768	46.64576803	Very fine sand
Catchpan	0.001	23.69	0.137035378	13.70353784	Silt-clay/mud