The sum is more than its parts

Key species in the functioning of cold-water coral reef communities

Het geheel is meer dan de som der delen
Sleutelsoorten in het functioneren van koudwaterkoraal rifgemeenschappen
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op maandag 24 februari 2014 des middags te 12.45 uur

door

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geboren op 25 september 1981 te Lich, Duitsland

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The research presented in this thesis has been primarily conducted in the department of Ecosystem Studies of the Royal Netherlands Institute of Sea Research in Yerseke and was funded under the Initial Training Network (ITN) CalMarO within Marie Curie Actions, supported by the People Specific Programme of the Seventh Framework Program of the European Community.

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General introduction

Photo by S. Zankl

Cold-water coral reefs

When we think of coral reefs we imagine warm, shallow waters, but coral reefs are not limited to tropical waters. Recently the occurrence of coral reefs in the deep, dark ocean has caught the scientific attention. The so-called cold-water coral reefs are still poorly understood and investigated in comparison to their shallow counterparts not least since they mostly thrive at depth between 200 and 1500 m (Zibrowius, 1980; Cairns, 1994; Buhl-Mortensen and Fosså, 2006). Special equipment is required to work at these depths but with the advances in technology in the last decades the extent of this ecosystem has been revealed. Cold-water coral reefs can be found worldwide from low to high latitudes (Fig. 1.1.), generally at locations with elevated current velocities (Davies and Guinotte, 2011). They can either consist of small patch of coral colonies, which typically extent several meters across or form large reef structures and gigantic carbonate mounds, which can reach up to 300 m high and several kilometers in diameter (Roberts et al., 2006; Wheeler et al., 2007).

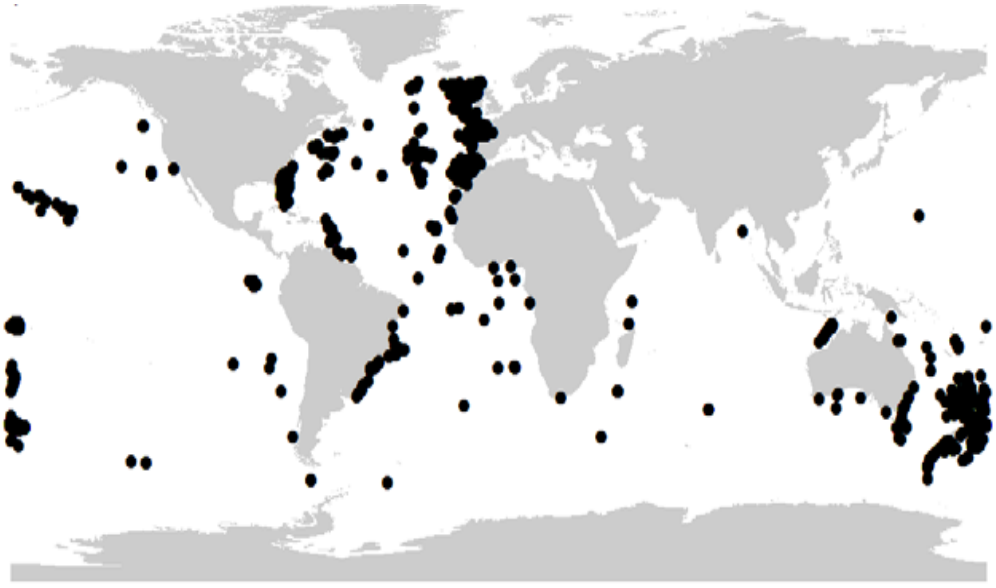

Figure 1.1. Global occurrence of cold-water corals. (Davies et al., 2011)

These structures are formed over many thousand to millions of years, representing an impressive geological archive (Roberts et al., 2006). Typically they have either

circular or elongate outlines (Buhl-Mortensen et al., 2010) and display different vertical zones with living coral atop and skeleton fragments at increasing stage of decay towards the base of the reef (Buhl-Mortensen et al., 1995, Fig 1.2.). The result is a broad variety of habitats (Buhl-Mortensen et al., 2010), which provide important refuge, feeding and nursery areas for many organisms, including commercially important fish species (Fosså et al., 2002; Husebø et al., 2002). Up to three times more species have been found on cold-water coral reefs than in the surrounding environment, characterizing them as hot spots of biodiversity and C-cycling in the deep-sea (Roberts et al., 2006; van Oevelen et al. 2009).

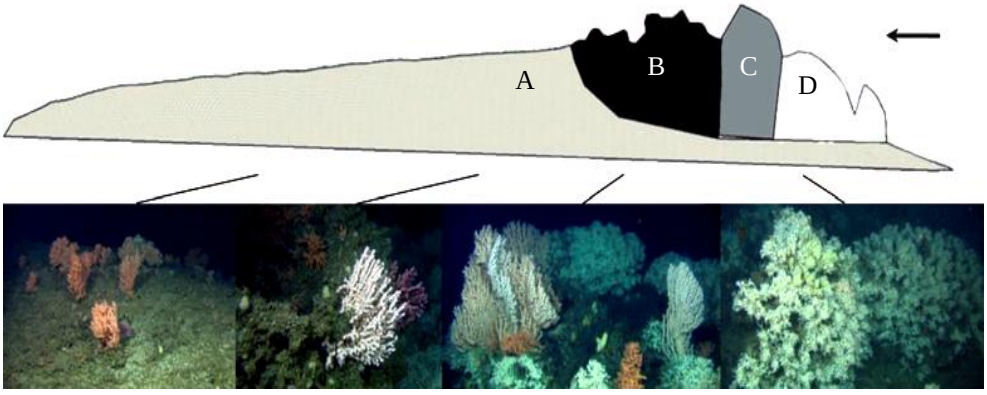

Figure 1.2. Habitat zonation along a typical reef in Norway (after Buhl-Mortensen et al., 2010). (A) Reef 'tail' consisting of coral rubble. (B) Blocks of dead coral colonies. (C) Transition zone with recently dead coral colonies. (D) The living 'head' of the reef with 'half moon'-shaped colonies facing the main current.

However, although just discovered they are already in danger by several human impacts. Trawling, undertaken by the fishing industry, causes extensive destruction of the reef framework (Fosså et al., 2002; Freiwald et al., 2002; Hall-Spencher et al., 2002; Ross and Quattrini, 2007), from which recovery might take hundreds to thousands of years (Roberts et al., 2006). The exploration of oil and gas results in the dispersal of drill-cuttings, a mixture of rock fragments and mud, which might be particularly devastating for larvae survival and therefore reef rejuvenescence (Larsson et al., 2013b). Global change, including ocean warming and ocean acidification, provides a more subtle form of impact. It might affect coral

metabolism (Dodds et al., 2007) and calcification (Maier et al., 2009; Form and Riebesell, 2012; Maier et al., 2012), which are key processes in reef functioning.
Although research efforts have drastically increased over the last decades many questions remain open, among them fundamental questions on the trophic ecology of related species. To predict future changes and design an effective protection strategy of this unique ecosystem it is however critical to understand reef functioning, starting with the metabolism of its species and the energy flow between them.

The heart of the reef: *Lophelia pertusa*

Depending on the reef location, different scleractinian corals can dominate the reef structure. In the North Atlantic Ocean *L. pertusa* (Fig. 1.3.) is the most common reef-building coral (Zibrowius, 1980). Alone at the Norwegian continental shelf Buhl-Mortensen et al. (2001) estimated the occurrence of 6000 *L. pertusa* reefs. Interestingly, the coral can occur in different colour morphs, mainly red and white. At most reef locations the white colour morph is far more abundant than the red one. Differences in bacterial communities associated with *L. pertusa* are assumed to cause the variability in colour but no final conclusion has been drawn to date (Neulinger et al., 2008). It is also unclear if these differences in bacteria community, possibly correlated with nutrient supplementation (Neulinger et al., 2008; Galkiewicz et al., 2011), might explain the differences in abundance between the morphs.

Typically both coral morphs grow in bush-like colonies, providing different microhabitats, ranging from living coral tissue to death coral skeleton (surface and cavities) and free space between coral branches (Buhl-Mortensen et al., 2010). They hereby add to the habitat diversity created by the different zones of the reef (Fig. 1.2.). The growth rate within these coral colonies however can be highly variable, ranging from 2 to 10 mm per year (Buhl-Mortensen and Rapp, 1998). So far it is not clear what causes this variability but changes in bicarbonate chemistry (Maier et al., 2009), temperature or food supply (Buhl-Mortensen and Rapp, 1998) might be factors controlling coral calcification and therefore colony growth.

Food supply and coral nutrition

It is well established that food input decreases with increasing water depth, leading to food limitation in deep sea ecosystems (Smith et al., 2009). This raises the question how *L. pertusa*, which occur at depths >1000 m, can flourish and develop extensive reef structures. It lacks the typical symbiotic photosynthetic dinoflagellates, which enhance the energy input of its tropical counterparts. Consequently, the coral is thought to rely solely on heterotrophic feeding (Buhl-Mortensen, 2001). External food input therefore appears to be a critical factor in determining coral survival and growth.

Elevated benthic velocities at reef locations (Thiem et al., 2006; Mienis et al., 2007; Davies et al., 2009) and a combination of different hydrodynamic processes (White et al., 2005), including internal waves (Frederiksen et al., 1992; Duineveld et al., 2004) and tidal pumping (Davies et al., 2009), increase the particle flux to the reef community and provide a direct linkage to surface primary production (Spiro et al., 2000; Duineveld et al., 2004; Kiriakoulakis et al., 2005). Consequently, a diverse range of particles reaches the coral, including resuspended, degraded organic matter, relatively fresh phytoplankton and zooplankton (Duineveld et al., 2004; Kiriakoulakis et al., 2005; Dodds et al., 2009; Duineveld et al., 2007). Additionally, the extensive release of mucus by *L. pertusa* fuels the microbial community in their surrounding water column, thus creating an additional food sources. Dissolved organic matter, observed in high concentrations above reef locations (van Duyl et al., 2008), might also contribute to coral nutrition (Gori et al., 2013).

Laboratory observations indeed suggested that *L. pertusa* (Fig. 1.3.) can feed on a broad range of food sources. In situ investigations using stable isotope analysis and fatty acid biomarkers revealed that its diet might depend on the availability of different food sources: At shallow locations, where zooplankton is abundant, *L. pertusa* appears to thrive mainly on this resource, whereas at deeper locations (phyto)detritus becomes more dominate in its diet (Duineveld et al., 2007; Dodds et al., 2009). However, we still know very little about the feeding ecology of *L. pertusa*, especially with respect to prey preference or food processing. Latter might be particularly relevant for the interpretation of data gained by stable isotopic and biomarker analysis (see below). So far laboratory studies have mainly focused on the capture of zooplankton (Purser et al., 2010; Tsounis et al., 2010; Larsson et al., 2013a) and less is known about other prey organisms such as algae or bacteria. Additionally, coral food uptake was studied in isolation whereas in situ many

species are associated with *L. pertusa* which might influence its behavior and metabolism.

Species interaction: Neighbours and friends?

Organisms inhabiting coral reefs never exist in isolation (Parcer and Ahmadjian, 2000) they rather interact with their surrounding environment, including neighbouring organisms. This often results in an intimate relationship between different biological species. So-called symbiotic relationships (de Bary, 1879) can seamlessly vary from beneficial (mutualism, commensalism) to harmful (parasitism) for one or both partners involved (Parcer and Ahmadjian, 2000). Besides the direct effect on involved species their relationships can crucially affect ecosystem structure and functioning (Stachowicz, 2001 and references therein). One of the best known examples for such a symbiosis is the interactions between tropical reef building corals and photosynthetic dinoflagellates, where both partners benefit from the exchange (Mueller-Parker and D'Elia, 1997; Stanley, 2006). Another popular example is the interaction between corals and various herbivores, where the coral is relieved from space competition (Burkepile and Hay, 2008; Cheal et al., 2010; Adam et al., 2011). Ultimately, both symbioses significantly contribute to the development and resilience of tropical coral reefs by enhancing coral growth and survival (Stanley, 2006; Adam et al., 2011).

Compared to tropical coral reefs where species interactions are well recognized to affect ecosystem functioning (see example above), little is known about cold-water corals and their associated fauna (Buhl-Mortensen and Buhl-Mortensen, 2004; Roberts, 2005). Until today, over 1300 species associated with cold-water coral reefs are documented, the majority of them invertebrates (Roberts et al., 2009a). So far only 29 symbiotic relations between scleractinian cold-water corals and associated invertebrates have been reported including among others polychaete, crustacean, mollusca, foraminifera, prolifera and echinodermata (Buhl-Mortensen and Buhl-Mortensen, 2004; Roberts, 2005; van Soest et al., 2007; Stevenson and Rocha, 2013). But most of these relationships are only qualitatively described and poorly understood so that their role in ecosystem functioning remains unclear (Buhl-Mortensen and Buhl-Mortensen, 2004; Roberts et al., 2009a). To investigate these interactions and their function in the community however might be crucial to understand the ecosystem and predict its response to human induced changes such as global warming or ocean acidification (Hawkins et al., 2009; Kelly and Scheibling, 2012).

In the following section we present examples for relationships between the reef building coral *L. pertusa* and associated species, including bacterial symbionts, the polychaete *Eunice norvegica* and cold-water coral reef sponges such as *Hymedesmia coriacea* (Fig. 1.3.). So far all these species interactions have been described only quantitatively if at all, even though they might crucially influence coral nutrition and growth and therefore ecosystem functioning (Fig. 1.4.).

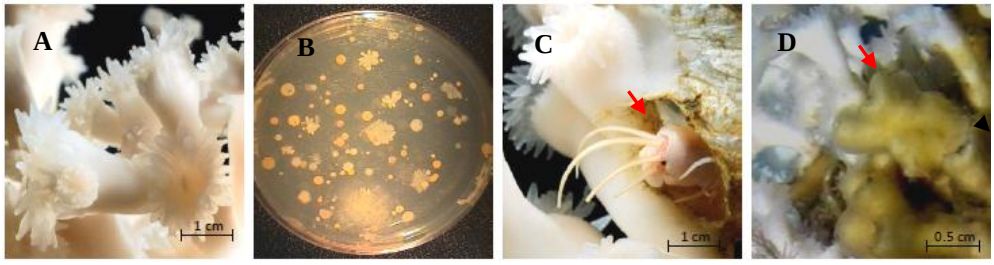

Figure 1.3. Key players in the coral reef ecosystem. (A) The coral *L. pertusa* with extended polyps. (B) Various microorganisms cultured on agar plates. (C) The polychaete *E. norvegica* poking out of its tube (red arrow). (D) The encrusting sponge *H. coriacea* (red arrow) growing next to living coral tissue. Pictures taken by Solvin Zankl (A, C), Christina Kelogg (B) and Tomas Lundälv (C).

Microcosm *L. pertusa*

Every organism is a microbial universe (Fig. 1.3.), a microcosm, and the importance of endosymbionts has long been recognised in tropical coral reefs, most notably in the symbiosis between scleractinian corals and photoautotrophic dinoflagellates. Although cold-water corals lack this specific symbiosis, they harbour a broad variety of other endosymbionts, including viruses (Maier et al., 2011), bacteria (Yakimov et al., 2006; Neulinger et al., 2008; Kellogg et al., 2009; Galkiewicz et al., 2011) and fungi (Galkiewicz et al., 2012). Recent genetic analysis of the community indicate that they might be able to supplement coral nutrition (Neulinger et al., 2008; Kellogg et al., 2009; Galkiewicz et al., 2011) by accessing inorganic nutrient sources (Fig. 1.4.). Using nitrogen fixation or ammonium assimilation coral associated microorganism might enhance N uptake in the coral holobiont (Lesser et al., 2007; Kimes et al., 2010), while chemoautotrophic processes such as nitrification or sulphur oxidation can

supplement organic C by using the energy generated during the oxidation of the respective substrate (ammonium or sulphur) for dissolved inorganic C fixation. If microbial symbionts can indeed supplement coral nutrition we must revise our picture of cold-water corals as solely heterotrophic organisms. However until now most of the microbial studies on cold-water corals are focused on molecular identification without direct measurements of the processes involved.

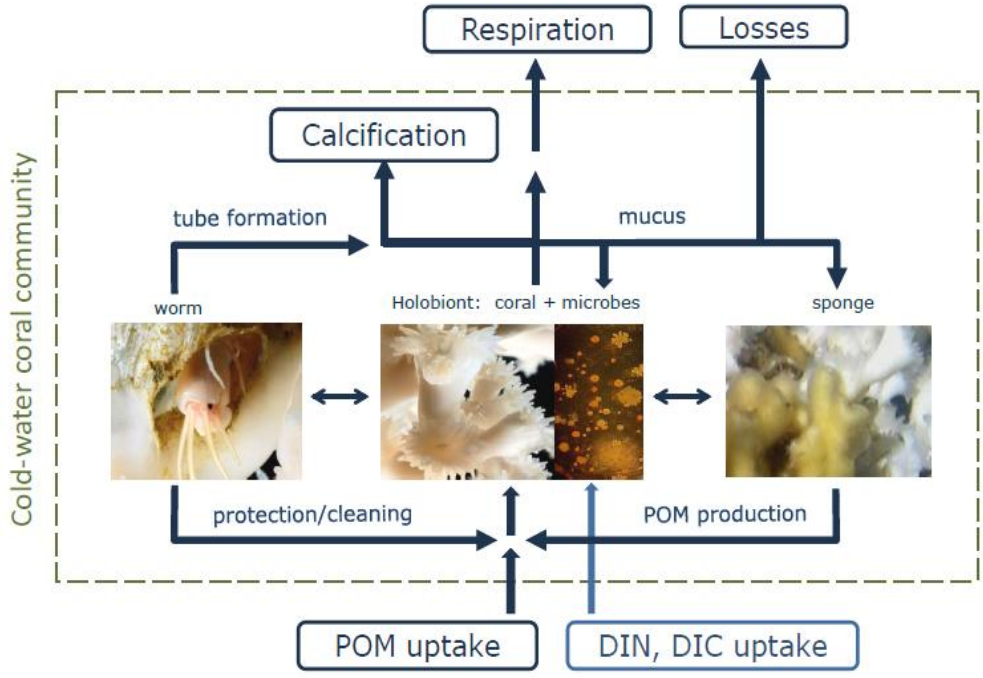

Figure 1.4. Schematic drawing of the interaction between three key species of cold-water coral reef communities. Left side: The worm *E. norvegica*, which tube-building and cleaning behavior might affect coral calcification and food uptake additional to its own food uptake. In the middle: The coral holobiont formed by the reef-building coral *L. pertusa* and associated microbes, which might supplement coral nutrition by utilizing inorganic nutrients and reduce energy loss by recycling coral mucus. Right side: The sponge *H. coriacea*, which ability to assimilated coral mucus and produce particulate organic matter (POM) might enhance food uptake by the coral and other filter feeders of the reef community. However it also competes for resources with the coral.

Eunice norvegica in symbiosis with *L. pertusa*

The polychaete *E. norvegica* (Fig. 1.3.) is most commonly found on *L. pertusa* reefs and can reach high abundances. It is also one of the few species which reside in close contact to living coral polyps (Buhl-Mortensen and Fosså, 2006). Its behaviour appears highly adapted to its living environment but has both parasitic and mutualistic elements in the interaction with *L. pertusa*. While *E. norvegica* has been observed to clean the coral surface from detritus and defend it against predators it also steals food, previously captured by the coral polyp (Buhl-Mortensen, 2001). It further builds parchment-like tubes which encore directly on the coral skeleton through the coral tissue (Buhl-Mortensen, 2001). This triggers a defence reaction by the coral causing the calcification of the worm tube. The result is not only an increased stability of the worm tube but also thicker and closely connected coral branches, which possibly enhance framework stability (Roberts, 2005). Moreover, by aggregating coral fragments the polychaete might even facilitate the development of large reef structures (Roberts, 2005). So far however, no data are available to quantify the different aspect of the relationship between the coral and polychaete, complicating a final characterisation of the relationship (parasitic versus mutualistic) and its potential affect on ecosystem functioning, including enhanced food uptake and calcification (Fig. 1.4.).

Cold-water coral reef sponges in symbiosis with *L. pertusa*

Sponges are important structural and functional components of benthic habitats along all latitudes (Diaz and Rützler, 2001; Bell, 2008 and references therein). They are extensively present on cold-water coral reefs (*e.g.* Bruntse and Tendal, 2001; Reitner and Hoffmann, 2003; Buhl-Mortensen and Fosså, 2006) with the majority living on the dead coral framework (Buhl-Mortensen et al., 1995). However, some species are closely associated with living zone of the coral reef (Jonsson et al., 2004; van Soest et al., 2007; Purser et al., 2013), growing within or on top of coral branches (Fig. 1.3.). From tropical reefs a variety of important ecological functions has been described (Bell, 2008) including important interactions with reef building corals (Wulff, 2006). These interactions can include (1) competition for food or space influenced by growth rate, growth form and chemicals (allelochemicals), (2) reef regeneration by binding lose coral pieces, (3) protection from boring organism by coating exposed coral skeleton, (3) bioerosion, (4) nutrient recycling and (4) secondary production of particulate organic matter (Wulff, 2006).

However besides the awareness of the fundamental role sponges may play in reef systems, studies of cold-water coral sponges are rare and observations on coral-sponge interactions are very limited (van Soest et al., 2007) and mainly focus on bioerosion (Beuck et al., 2007; van Soest and Beglinger, 2009). Although this is an important process in reef development, also other interactions might be crucial and should be considered to get an overall picture of the functional role of sponges in cold-water coral reef systems. This becomes especially important considering the recently discovered 'sponge-loop', which can highly impacts C and N cycling in reef ecosystems by consuming dissolved organic matter and releasing extensive amounts of particulate organic matter (de Goeij et al., 2013). Dissolved organic matter is a resource released in great amounts by cold-water corals (Wild et al., 2008) but inaccessible for most species and therefore many lost for the community. Particulate organic matter however is accessible by many species in cold-water coral reef ecosystems (Buhl-Mortensen and Buhl-Mortensen, 2004). Transformation of dissolved into particulate organic matter could enhance the food uptake in the reef community while at the same time decrease the loss of energy and nutrients from the system (Fig. 1.4.).

Methodology: Stable isotopes and fatty acid markers in trophic ecology

Stable isotopes

It is extremely difficult to study trophic interactions in deep-sea ecosystems such as cold-water coral reefs. Video observations taken by remotely operated vehicles (ROVs) or submersibles can only record the capture of organism visible by the naked eye. However, most food sources considered important in cold-water coral reef systems (mico-, nano-, picoplankton, resuspended organic matter) are too small to be observed. Video material will also not provide data about prey assimilation and processing by predators. In situ experiments like chamber incubations on the other side can provide information about community metabolism but their application is often very costly and challenging, especially given the depth and rocky structure of the environment. The analysis of stable isotopes however is a powerful method to study trophic interactions in situ or to tracer food assimilation and processing in laboratory experiments (Fry, 2006).

Stable isotopes are variants of chemical elements, differing in their numbers of neutrons and therefore in their atomic weight. In contrast to unstable radioactive isotopes, they do not undergo spontaneously radioactive decays, hence are safe to use. Biochemical reactions generally discriminated the heavy against the light isotope, a process called isotopic fractionation. Most fractionation for C occurs during primary production while only little (0 - 1 ‰) C fractionation occurs during the transfer of C from the prey to the consumer (Craig, 1953). The isotopic signature of the consumer therefore generally reflects the isotopic signature of its food source following the principle you are what you eat (deNiro and Epstein, 1978). The isotopic ratio of N however increases along the food chain (3.4 ‰ for each tropic level), and therefore can be used to reconstruct food webs and estimate tropic positions (Minigawa and Wada, 1984; Cabana and Rasmussen, 1994).

An important condition for the application of stable isotopes in tropic ecology is that sources differ distinctively in their isotopic signature (Vander Zanden and Rasmussen, 1999). However, the isotopic signatures of sources might overlap or different combination of sources might result in the same isotopic signature of the consumer. An alternative way to trace different food sources and study trophic interactions is then through tracer experiments, using stable isotope (^{13}C, ^{15}N) enriched sources (Herman et al., 2000; Boschker and Middelburg, 2002). This allows to directly following the uptake and processing of the enriched material by increasing the resolution of the different elemental pathways from the source to the consumer (Fig. 1.5.).

Fatty acids as trophic markers and biomarkers

As mentioned above the interpretation of data gained by stable isotopic analysis has its limitations. Trophic markers such as fatty acids (FAs) are commonly used in food web studies to qualitatively elucidate trophic interactions. The concept considers certain FAs as essential, especially highly unsaturated FAs (20:5ω3 and 22:6ω3), since metazoan consumer, such as corals, cannot synthesis these FAs themselves but need to acquire them with their diet (Daalsgard et al., 2003). Their unaltered transfer along the food chain, from the source to the consumer, then reflects diet assimilation and thus tropic interactions (Auel et al., 2002; Bachok et al., 2003; Budge et al., 2008).

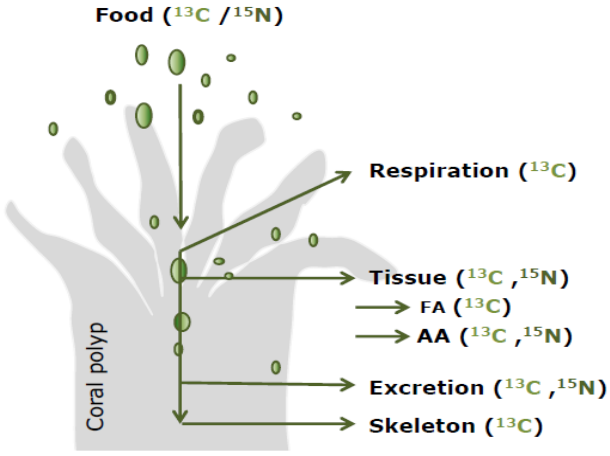

Figure 1.5. Food processing traced by stable isotope enrichment.

Additionally, biomarkers such as phospholipid-derived fatty acids (PLFAs) can enhance the discrimination of food web components by enabling the distinction between certain groups of organism (Boschker and Middelburg, 2002). In combination with stable isotope tracers they further allow to study C transfer between organisms quantitatively (van den Meersche et al., 2004). The principle for the application of PLFAs as biomarkers is the same as for trophic markers but more differentiated: Certain PLFAs are only produced by certain groups of organisms (e.g. algae, bacteria) and thus can be used to identify their presence and determine their biomass (Parrish et al., 2000).

Various studies have demonstrated the existence of such specific fatty acids for different classes of marine phyto- and bacterio- and zooplankton (Sargent and Falkpetersen, 1988; Boschker and Middelburg, 2002; Dalsgaard et al., 2003). However, recently it became apparent that some invertebrate taxa posse the ability to modify dietary fatty acids resulting in fatty acid profile which deviates from their diet (Kelly et al., 2008; Kelly et al., 2009). This complicates the application and interpretation of fatty acids as markers and raises the need to better understand the effect of dietary fatty acids on fatty acid profiles of consumers (Kelly and Scheibling, 2012; McLeod et al., 2013). By conducting stable isotope tracer experiments in which fatty acid profiles of the predator are investigated in response to certain food sources it is however possible to validate the use of fatty acids as markers (McLeod et al., 2013).

Thesis outline

Cold-water coral reefs are hotspots of biodiversity, providing important ecological functions. They are thought to rely exclusively on organic matter suspended or dissolved in the water column. Up to now only scattered information is available about possible food sources, food preferences and food processing by cold water corals and associated organisms, however these are basic information to understand ecosystem functioning. The aim of this thesis was to enhance the knowledge about cold-water coral metabolism and food selection, focusing especially on (non-) trophic interactions between key species: The reef-building coral *L. pertusa*, including its associated microbial fauna, the polychaete *E. norvegica* and the encrusting sponge *H. coriacea*. We particularly wanted to target the following questions:

1. Which resources (organic and inorganic) can be utilized by the cold-water coral *L. pertusa?*
2. How does the coral process different resources?
3. Which effects have neighboring species such as sponges and polychaetes on coral metabolism (see Fig. 1.4.)?
4. Do species interactions contribute to the high biogeochemical cycling of cold-water coral reefs?

Chapter 2 presents the uptake of various ^{13}C and ^{15}N isotopically enriched organic food sources (different sizes and biochemical composition) into the tissue and skeleton of *L. pertusa.* This chapter gives insights into the prey spectrum of the coral and provides information about the metabolic processing of resources by the coral.

Chapter 3 describes the uptake and processing of inorganic nutrients (^{13}C bicarbonate and various inorganic ^{15}N species) by the same coral species. It hereby demonstrates the mixotrophic nature of the coral holobiont and its participation in the N cycle.

Chapter 4 investigates the (non-) trophic interaction between the tube buliding polychaete *E. norvegica* and *L. pertusa* with focus on food assimilation, respiration and calcification. It also describes food selectivy between nano- and mesoplankton species (algae versus *Artemia)* by *L. pertusa* and *E. norvigica*, separate and together.

Chapter 5 investigated the effect of food concentration on the metabolism *L. pertusa* under different concentrations. It further describes food selectivity between pico- and nonoplankton (algae and bacteria) by *L. pertusa* and *H. coriacea*, separate and together. The chapter also studies the tropic interaction between *L. pertusa* and its associated sponge *H. coriacea* with respect to trophic transfer between both organisms and the production of detritus by *H. coriacea*.

Chapter 6 discusses the obtained results and their implications for ecosystem functioning.

2

Opportunistic feeding of various organic food sources by the cold-water coral *Lophelia pertusa*

Photo by S. Zankl

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Published in Biogeosciences, 11, 123-133, 2014

Abstract

The ability of the cold-water coral *Lophelia pertusa* to exploit different food sources was investigated under standardized conditions in a flume. All tested food sources, dissolved organic matter (DOM, added as dissolved free amino acids), bacteria, algae, and zooplankton (*Artemia*) were deliberately enriched in ^{13}C and ^{15}N. The incorporation of ^{13}C and ^{15}N was traced into bulk tissue, fatty acids, hydrolysable amino acids, and the skeleton (^{13}C only) of *L. pertusa*. Incorporation rates of carbon (ranging from 0.8 - 2.4 μg C g^{-1} DW d^{-1}) and nitrogen (0.2 - 0.8 μg N g^{-1} DW d^{-1}) into coral tissue did not differ significantly among food sources indicating an opportunistic feeding strategy. Although total food assimilation was comparable among sources, subsequent food processing was dependent on the type of food source ingested and recovery of assimilated C in tissue compounds ranged from 17% (algae) to 35 % (*Artemia*). De novo synthesis of individual fatty acids by *L. pertusa* occurred in all treatments as indicated by the ^{13}C enrichment of individual phospholipid-derived fatty acids (PLFAs) in the coral that were absent in the added food sources. This indicates that the coral might be less dependent on its diet as a source of specific fatty acids than expected, with direct consequences for the interpretation of in situ observations on coral nutrition based on lipid profiles.

Introduction

Cold-water corals (CWCs) form reef structures in the cold and deep oceanic waters around the world (Roberts et al., 2009a; Davies and Guinotte, 2011). These reefs form hotspots of biodiversity (Roberts et al., 2006) and are important in carbon cycling along continental margins (van Oevelen et al., 2009). The high metabolic demand of CWC communities implies high food processing rates and indeed a close relationship between food availability and the occurrence of CWC reefs has been reported in many studies (Roberts et al., 2006; Thiem et al., 2006).

In the North Atlantic Ocean, CWC reef communities are primarily formed by the scleractinian coral *Lophelia pertusa* (Roberts et al., 2006). Recent in situ investigations using stable isotope and fatty acid analyses point to a close coupling of *L. pertusa* with pelagic resources such as zooplankton and phytodetritus (Spiro et al., 2000; Duineveld et al., 2004; Kiriakoulakis et al., 2005). While tidal pumping (Davies et al., 2009) and internal waves (Frederiksen et al., 1992; Duineveld et al., 2004) deliver a diverse range of particles to the coral, ranging from fresh to resuspended material, the vertical migration (daily or seasonal) of zooplankton can also contribute to the linkage between surface-water production and CWC nutrition (Hind et al., 2000; Valle-Levinson et al., 2004; Dodds et al., 2009).

Although organic food sources of various size, type and quality reach the CWC reefs, very little is currently known about their importance to the metabolism of CWCs or the biogeochemical processing that occurs following nutritional uptake. Analysis of natural stable isotope signatures in tissues allow insight into the coral nutrition as described above (Duineveld et al., 2004; Kiriakoulakis et al., 2005; Dodds et al., 2009; Roberts et al., 2009a; van Oevelen et al., 2009), but to enhance the resolution of bulk tissue isotope data, fatty acids are often used as biomarkers in these studies (Kiriakoulakis et al., 2005; Dodds et al., 2009; Duineveld et al., 2012). However, the processing and production of fatty acids by CWCs has not yet been studied. This limits their interpretation since the use of fatty acids as biomarker for a specific food source critically depends on the assumption that these markers cannot be synthesized by the consumer itself (Kelly and Scheibling, 2012).

Feeding studies of *L. pertusa* so far have focused on uptake rates of *Artemia salina* (Purser et al., 2010; Tsounis et al., 2010) in the laboratory, but other food sources and particle sizes below 100 µm have not been considered yet. The nutritional importance of a food source does not only depend on its availability, but also on uptake and physiological processing by *L. pertusa*. In addition to particulates, dissolved resources may also contribute to coral dietary requirements.

For tropical corals, it is known that dissolved organic matter (DOM) can be an important food source even under low ambient DOM concentrations (Hoegh-Guldberg and Williamson, 1999; Grover et al., 2006; Grover et al., 2008). Recently, Naumann et al. (2011) reported DOM uptake by the CWC *Desmophyllum dianthus*, thus DOM may also form an additional resource for *L. pertusa* that has not been accounted for so far.

In addition to a demand of organic resources for energy and tissue growth, *L. pertusa* also needs an inorganic carbon source to sustain calcification. Two carbon sources are possibly involved in the calcification process in corals: Dissolved inorganic carbon (DIC) from the surrounding seawater or metabolically generated CO_2. While calcification in tropical zooxanthellate scleractinian corals is mainly (70-75%) based on metabolic CO_2 (Furla et al., 2000), the opposite seems to be true for the azooxanthellate octocoral *Leptogorgia virgulata* (Lucas and Knapp, 1997). Based on stable isotope data ($\delta^{18}O$, $\delta^{13}C$), Adkins et al. (2003) suggested that C for calcification in azooxanthellate scleractinian CWCs is primarily derived externally rather than metabolically. Direct measurements distinguishing between the different calcification pathways in CWCs are however not available.

In this study we used standardized food quantities in a laboratory setting to trace the incorporation of stable isotope labelled food sources (^{13}C and ^{15}N) to measure food uptake rates and potential preferences by the CWC *L. pertusa*. Different food sources were selected to cover different particle sizes and nutritional values (C: N ratio), comprising dissolved organic matter, bacteria, algae and zooplankton (*Artemia*). Following food uptake we traced ^{13}C and ^{15}N into two of the most important biochemical components, hydrolysable amino acids (HAAs) and lipids represented by the sum of total fatty acids (TFAs) to follow metabolic processing. Within the lipid pool we especially followed food-derived C further into phospholipid-derived fatty acids (PLFAs) to have a more detailed look into the synthesis of structural and therefore functional fatty acids. Additionally, we traced food-derived C into the coral skeleton to investigate the metabolic contribution to coral calcification.

Methods

Sampling location and maintenance

All corals used in the experiment were collected at the Tisler Reef, one of the largest and shallowest inshore reefs known so far. It is located at a depth of 70 to

155 m in the Skagerrak, on a sill forming the submarine border between Norway and Sweden. The current velocity over the reef varies from 0 to 50 cm s^{-1} throughout the year while the flow direction fluctuates irregularly between NW and SE (Lavaleye et al., 2009; Wagner et al., 2011). The amount and quality of particulate organic carbon (POC) entering the reef depend on the location within the reef, and concentrations vary between 43 to 106 µg POC l^{-1} (Wagner et al., 2011). Temperature at the reef site typically fluctuates between 6 and 9 °C throughout the year (Lavaleye et al. 2009; Wagner et al. 2011).

Corals were collected from a depth of 117 m (N58°59,800' E10°58,045') using an ROV (Remotely Operated Vehicle, Sperre Subfighter 7500 DC). Within a few hours after sampling, corals were transported in cooling boxes filled with cold seawater (7 – 8 °C, salinity 31) to the laboratory at the Sven Lovén Centre for Marine Sciences, Tjärnö, Sweden. Until the start of the experiment (3 months), corals were kept in a dark thermo-stated room at 7 ºC with a flow through of sand-filtered bottom water (particle size 1 - 2 mm) from 45 m depth in the adjacent Koster-fjord (7– 8 °C, salinity 31). During this time the corals were fed with larvae (nauplii) of the Brine Shrimp *Artemia spp.* every 3 to 4 days. The Sven Lovén Centre has kept *L. pertusa* alive and growing under these conditions for a number of years.

Coral samples used in this experiment were clipped to approximately the same size with 4.5 ± 1.9 g dry weight (DW) and 8 ± 4 polyps per fragment (average ± SD) about a week before the experiments started.

Preparation of particulate labelled substrates

CWCs are thought to thrive mainly on particulate organic matter (Duineveld et al., 2012). To test for assimilation of different food particle sizes, we chose bacteria to represent picoplankton and microalgae to represent nanoplankton. *Artemia* nauplii were chosen to represent mesozooplankton because they can be cultured in high densities, are the essential food source for successfully keeping *L. pertusa* in the laboratory and have been used in earlier CWC feeding studies (Purser et al., 2010; Tsounis et al., 2010; Naumann et al., 2011). While DOM was added in the form of a commercially available algal-derived mixture of dissolved free amino acids (Cambridge Isotopes, U ^{13}C 97-99%, U ^{15}N 97-99%, CNLM-452-0.5), the labelled POM food sources were prepared by culturing the respective organisms in the presence of ^{13}C and ^{15}N labelled substrates.

A natural community of bacteria was derived from a few ml of natural seawater, obtained from the Oosterschelde estuary (salinity 30) in the SW of the Netherlands.

The water sample was added to 1 l culture medium (M63) containing 0.02 mol l^{-1} glucose (10 atom % ^{13}C, Cambridge Isotopes) and 0.01 mol l^{-1} ammonium chloride (10 atom % ^{15}N, Cambridge Isotopes). After 3 days of culturing in the dark, bacteria were concentrated by centrifugation (14500 g) and rinsed with 0.2 µm filtered seawater to remove residual labelled substrates. Bacteria in the concentrate were kept frozen until use in the experiment.

The diatom *Skeletonema costatum* was cultured axenically in 4 l f/2 culture medium containing 0.8 mmol l^{-1} $NaNO_3$ (10 atom% ^{15}N, Cambridge Isotopes) and 2 mmol l^{-1} $NaHCO_3$ (20 atom % ^{13}C , Cambridge Isotopes, 99% ^{13}C). After 3 weeks of culturing in 12 h light 12 h dark cycles (at a cell density of around $3 – 4 \times 10^6$ cells ml^{-1}), algae were concentrated by centrifugation at 450 g, rinsed three times with 0.2 µm filtered seawater to remove residual labelled substrates and kept frozen until use in the experiment.

For culturing ^{13}C and ^{15}N enriched *Artemia* nauplii, 6 times 0.1 g *Artemia* cysts (Sera) were incubated in 5 l incubation chambers filled with 0.2 µm filtered seawater under natural light conditions and light aeration. After the larvae had developed to the state that they take up particulate food (1 to 2 days after eclosion of larvae), they were fed every second day with around 7 to 10 mg C derived from ^{13}C and ^{15}N enriched pre-cultured algae (cultured as described above, 4 atom % ^{13}C, 10 atom% ^{15}N). The uptake of algae by *Artemia* was visually confirmed under the microscope. After seven days of feeding, the larvae were concentrated by filtration, rinsed with filtered seawater, counted under the binocular and stored frozen. Within the filtrate, different early larvae stages could be identified.

To standardize the amount of carbon added to the incubations, all substrates were measured for carbon and nitrogen content. ^{13}C and ^{15}N enrichment and the fatty acid composition (PLFAs) of organic food sources were also measured to trace and calculate coral food uptake (see below for methodological description).

Experimental set up and procedure

During the incubations, corals were placed in recirculation flumes (60 l) in a thermo-stated room at 7 °C (Fig. 2.1.). Water circulation was maintained by a motor-driven propeller situated in the returning pipe (for more details see Purser et al., 2010). Prior to the experiment, the flume was filled with 0.2 µm filtered seawater from 45 m depth out of the Koster-fjord (salinity 33, 7 °C, pH 7.9 on NBS) and the motor was set to ensure a flow speed of 7 cm s^{-1}, which is within the natural range found at the Tisler Reef (Lavaleye et al., 2009; Wagner et al., 2011).

Three coral fragments were randomly selected and placed in the test section of each flume (Fig. 2.1.). The three pieces were gently inserted into a 1 cm elastic silicone tube on an acrylic plate that could be attached to the flume base (Purser et al., 2010). Corals were left in the flume for 12 h to acclimatize to the conditions. After acclimation, 10 mg C of the respective food source per treatment was gently pipetted into the water column of each flume (final concentration 170 µg C l^{-1}). Visual observations confirmed that the circulating water kept the particulate food in suspension. Each flume contained three coral pieces and each food source was replicated twice. As a control treatment, corals were incubated for the same time without any food addition. After an incubation time of 4 days in darkness, coral samples were frozen at -20°C, freeze-dried and stored frozen for further analysis.

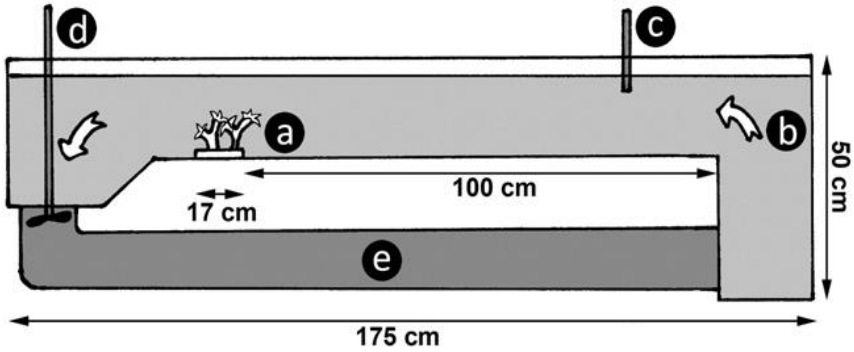


Figure 2.1. Scale diagram of recirculation Plexiglas flume setup adopted from Purser et al. (2010).
(A) Test section with coral branches in plastic mount (depth 8 cm)
(B) Direction of circulation
(C) Food delivery point
(D) Motor
(E) Opaque plastic return pipe

Sample treatment and analyses

After freeze-drying, coral samples were weighed and homogenized by grinding with a ball mill for 20 s (MM 2000, Retsch, Haan, Germany). A subsample of around 30 mg of ground material (organic and inorganic fraction) was transferred to pre-combusted silver boats and analyzed for isotopic ratio and C/N content using a Thermo Electron FlashEA 1112 analyzer (EA) coupled to a Delta V isotope ratio mass spectrometer (IRMS). Another subsample was transferred to a silver boat,

acidified stepwise with drops of increasing concentrated HCl until the inorganic C fraction was removed (no bubbling after acid addition). The remaining material was analyzed on the EA-IRMS for isotopic ratio and organic C content. Incorporation of C in the inorganic skeleton was determined by subtraction of the organic carbon fraction (tissue + organic matrix) from the total carbon pool.

Total fatty acids (TFAs) were extracted with an adjusted Blight Dyer method. A part of the total fatty acid extract was further eluted over a silica column (Merck Kieselgel 60) to isolate the phospholipid derived fatty acids (PLFAs) (Boschker et al., 1999). The TFA and PLFA extracts were then further derivatized by mild alkaline transmethylation to obtain fatty acid methyl esters (FAME). Preparation of methyl esters was carried out following the method of Boschker et al. (1999). For extraction, 0.7 g DW of coral samples, ~100µl of DOM and smaller particulate food sources, and 100 *Artemia* (equivalent to 500 µg C and 50 µg C respectively) were used. Concentration and carbon isotopic composition of individual TFAs and PLFAs were measured on a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c- IRMS) according to Boschker et al. (1999).

Hydrolysable amino acids (HAAs) were extracted and analyzed following Veuger et al. (2005). Ground coral samples were first treated with 12 mol l^{-1} HCl to dissolve the skeleton by repeated addition of HCl drops to avoid loss of sample through bubbling. The remaining material was then hydrolyzed in 6 mol l^{-1} HCl at 110 ºC for 20 h and purified by cation-exchange chromatography (Dowex 50WX8 resin). The hydrolysable amino acids were derivatized with isopropanol and pentafluoropropionic anhydride and analyzed by GC-c-IRMS for concentrations and ^{13}C and ^{15}N content.

Stable isotope data are expressed in delta notation as: $\delta X\ (‰) = (R_{sample}/R_{ref} - 1)x1000$, in which X represents C or N, R_{sample} is the heavy/light isotope ratio in the sample (e.g. $^{13}C/^{12}C$) and R_{ref} is the heavy/light isotope ratio of the reference material (R_{ref} = 0.01118 for C and R_{ref} = 0.00368 for N). The atomic% of the heavy isotope in a sample (e.g. $^{13}C/[^{13}C+^{12}C]$) was calculated as $F = R_{sample}/(R_{sample} + 1)$. The excess (above background) atom% is the difference between the F in an experimental sample and the atom% in a background (untreated) coral sample: $E = F_{sample} - F_{background}$, so that zero values of E imply no uptake of the isotopically labelled food source and positive values indicates food uptake. To correct for differences in isotope enrichment among the food sources, the excess incorporation was divided by the atom% of each specific food source, e.g. 0.09 for bacteria C and 0.1 for bacteria N respectively. To arrive at total elemental uptake, E was multiplied with C or N content of 1 g DW sample (µg C g^{-1} DW sample, µg

N g^{-1} DW sample). All results are reported as average ± SD derived from all coral pieces per treatment (n=6).

Statistical Analyses

To be able to perform statistical analyses of the obtained data we treated pseudo-replicates as true replicates if no significant different between flumes were found by using Kruskal-Wallis-Test ($p \geq 0.05$). However, all statistical values still need to be considered with care. The potential influence of food sources on C and N uptake in bulk tissue and specific components was investigated using Kruskal-Wallis-Test since a normal distribution for some data could not be achieved by data transformation. However, in cases where all requirements for an ANOVA were met, the results did not differ from the ones obtained by Kruskal-Wallis-Tests. Therefore we decided to use Kurskal-Wallis-Tests for all factors. Differences among treatments were then further investigated using Wilcoxon rank-sum test for pair wise comparison with an adjustment of *p*-values by the method of Bonferroni.

Results

Biogeochemical characteristics of particulate food sources

All food sources were significantly isotopically enriched above background and differed considerably in food quality represented by C/N ratio and PLFAs contents as well as compositions in case of particulate organic food sources (Table 2.1., Fig. 2.2.A). Accordingly, the bacteria-derived PLFA pool was dominated by C16:1ω7c/t, C16:0 and C18:1ω7c and the algae-derived PLFA pool by C16:0, C16:1ω7c, followed by C16:3ω4, C20:5ω3 and C22:6ω3 (Fig. 2.2.A). *Artemia* derived-PLFAs mainly comprised C18:3ω6, followed by C16:0, C18:1ω7c, C18:1ω9t/c and C18:2ω6t/c (Fig. 2.2.A).

Biogeochemical characteristics of *L. pertusa*

Corals used in this experiment had a total C content of 128 ± 5 mg C g^{-1} DW coral. This total C was partitioned into an organic tissue fraction of 13% and inorganic skeleton fraction of 87%. The organic C in the tissue fraction could be further partitioned into 24% of THAAs, 7% TFAs and 0.04% PLFAs (Table 2.2.). Dominant PLFAs in the coral tissue were C16:0, C18:0, C20:4ω3, C20:4ω6, C20:5ω3, C22:4ω6 and C22:5ω3 (all >5%), followed by C20:1ω9c and C22:1ω9c (~5%, Fig. 2.2.B). No significant difference in coral C content in tissue, TFAs or PLFAs was detected between the coral pieces in the different food treatments

(Kruskal-Wallis $p > 0.05$ for all comparisons). Organic N content of corals used in this experiment was 3 ± 1 mg N g^{-1} DW coral, with 12% of the N present in the THAA fraction (Table 2.2.). No significant difference in coral N content in tissue and THAAs was detected between the coral pieces in the different food treatments (Kruskal-Wallis $p > 0.05$).

Table 2.1. Characteristics of food sources used during the experiment.

Food source	**^{13}C (atm%)**	**^{15}N (atm%)**	**Molar C/N ratio**	**PLFA content (% C)**
Amino acids (DOM)	99	99	5.0	0.00
Bacteria (BAC)	9	10	3.6	0.31
Algae (ALG)	3	10	9.8	0.03
Zooplankton (ART)	3	2	4.2	0.32

Table 2.2. Total carbon and nitrogen (Total C, N), organic carbon (Organic C), total hydrolysable amino acids (THAAs), total fatty acids (TFAs) and PLFAs composition of *L. pertusa*. Values are presented in [mg C g^{-1} DW coral ± SD] and [mg N g^{-1} DW coral ± SD] respectively.

	Total	**Organic**	**THAAs**	**TFAs**	**PLFAs**
C	128.05 ± 4.95	1.60 ± 4.81	3.61± 0.55	1.02 ± 0.03	0.06 ± 0.02
N	2.79 ± 0.95		0.34 ± 0.05		

Total C and N assimilation

The assimilation of C from the food sources into coral tissue was not significantly different among treatments (Wilcoxon p > 0.05 for all comparisons, Fig. 2.3.A). *Artemia*-derived C was assimilated at a rate of 1884 ± 1067 ng C g^{-1} DW coral d^{-1}, algal-derived C with 1520 ± 498 ng C g^{-1} DW coral d^{-1}, bacterial-derived C with 750 ± 458 ng C g^{-1} DW coral $d^{-1}$and DOM with 2393 ± 1221 ng C g^{-1} DW coral d^{-1}. Also N was assimilated comparably among different food sources by *L. pertusa* (Wilcoxon p > 0.05 for all comparisons) with *Artemia*-derived N assimilated with 797 ± 399 ng N g^{-1} DW coral d^{-1}, algal-derived N with 247 ± 174 ng N g^{-1} DW coral d^{-1}, bacterial-derived N with 399 ± 200 ng N g^{-1} DW d^{-1} and DOM with 797 ± 258 ng N g^{-1} DW coral d^{-1} (Fig. 2.3.A).

With the exception of corals fed with DOM (Kruskal-Wallis p=0.006, Fig. 2.3.A), C assimilation did not differ significantly from N assimilation among corals fed with particulate sources (Kruskal-Wallis p > 0.05), regardless of different N additions per treatment due to fixed C additions and variable C/N ratios of food sources. This points to a higher retention of nitrogen during assimilation and metabolic processing (Fig. 2.3.E).

Food processing: Tracer incorporation in amino acids

Between 14 to 32% of the total assimilated carbon was incorporated into the total hydrolysable amino acid pool (THAA) of *L. pertusa*. DOM-derived C was assimilated into THAAs at a significantly higher rate (746 ± 244 ng C g^{-1} DW coral d^{-1}) than bacteria and *Artemia*-derived C (178 ± 69 ng C g^{-1} DW coral d^{-1} and 272 ± 88 ng C g^{-1} DW coral d^{-1} respectively, Wilcoxon $p_{DOM\text{-}BAC/ART}$ = 0.03). The incorporation algal-derived C (484 ± 311 ng C g^{-1} DW coral d^{-1}) did not differ significantly from that of the other food sources (Wilcoxon p> 0.05 for all comparisons).

The incorporation of N into THAAs represented up to 30% of the total N assimilated by the coral and did not differ significantly between food treatments (Kruskal-Wallis p = 0.2) with 152 ± 67 ng N g^{-1} DW coral d^{-1} for DOM, 137 ± 87 ng N g^{-1} DW coral d^{-1} for *Artemia*, 73 ± 43 ng N g^{-1} DW coral d^{-1} for algae and 72 ± 27 ng N g^{-1} DW coral d^{-1} for bacteria (Fig. 2.3.B).

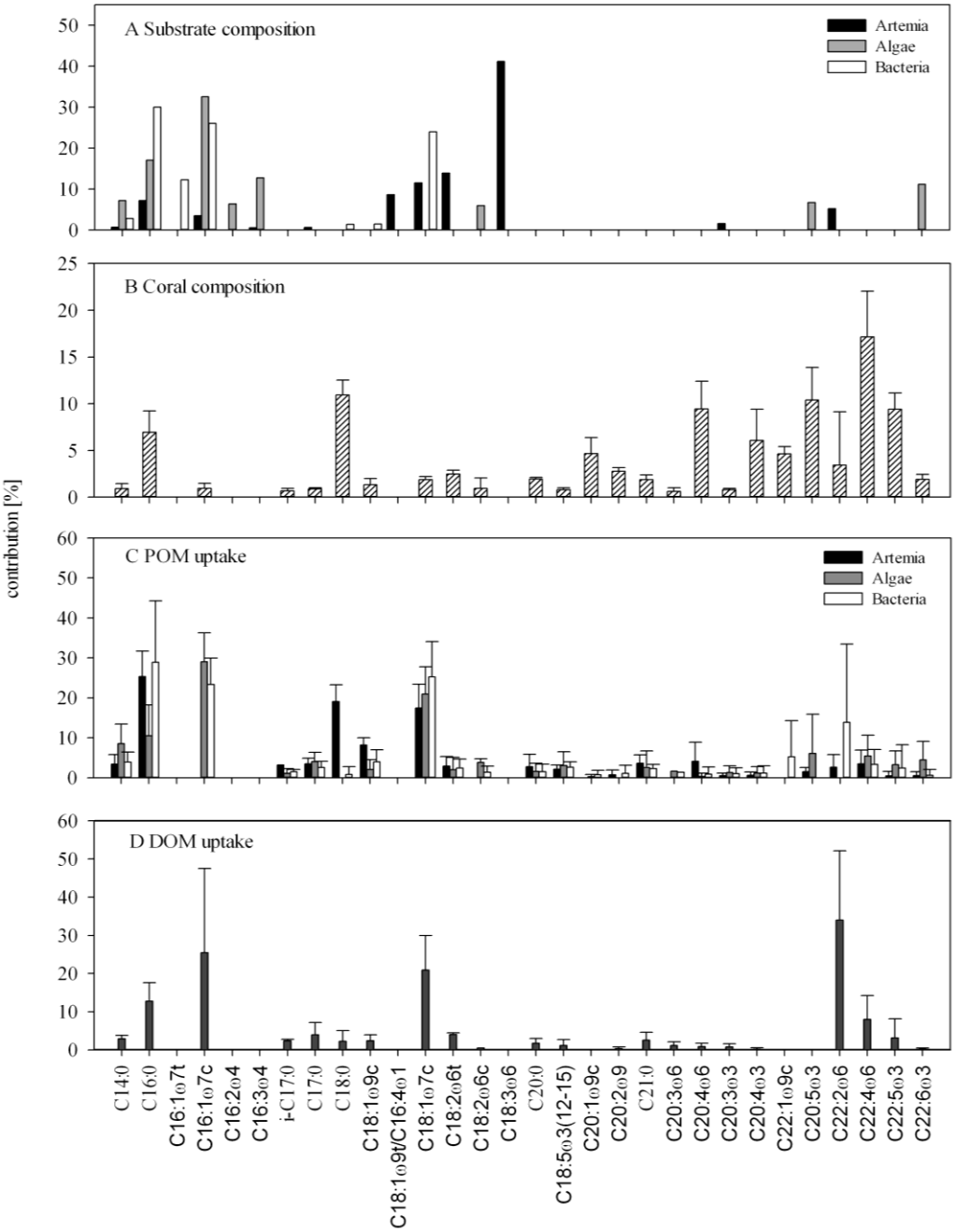


Figure 2.2. PLFA profiles of (A) POM sources (% contribution to total concentration), (B) *L. pertusa*, (C) POM-derived C incorporation into coral PLFAs by *L. pertusa* and (D) DOM transformation into coral PLFAs by *L. pertusa*. The bars in each figure represent average ± SD.

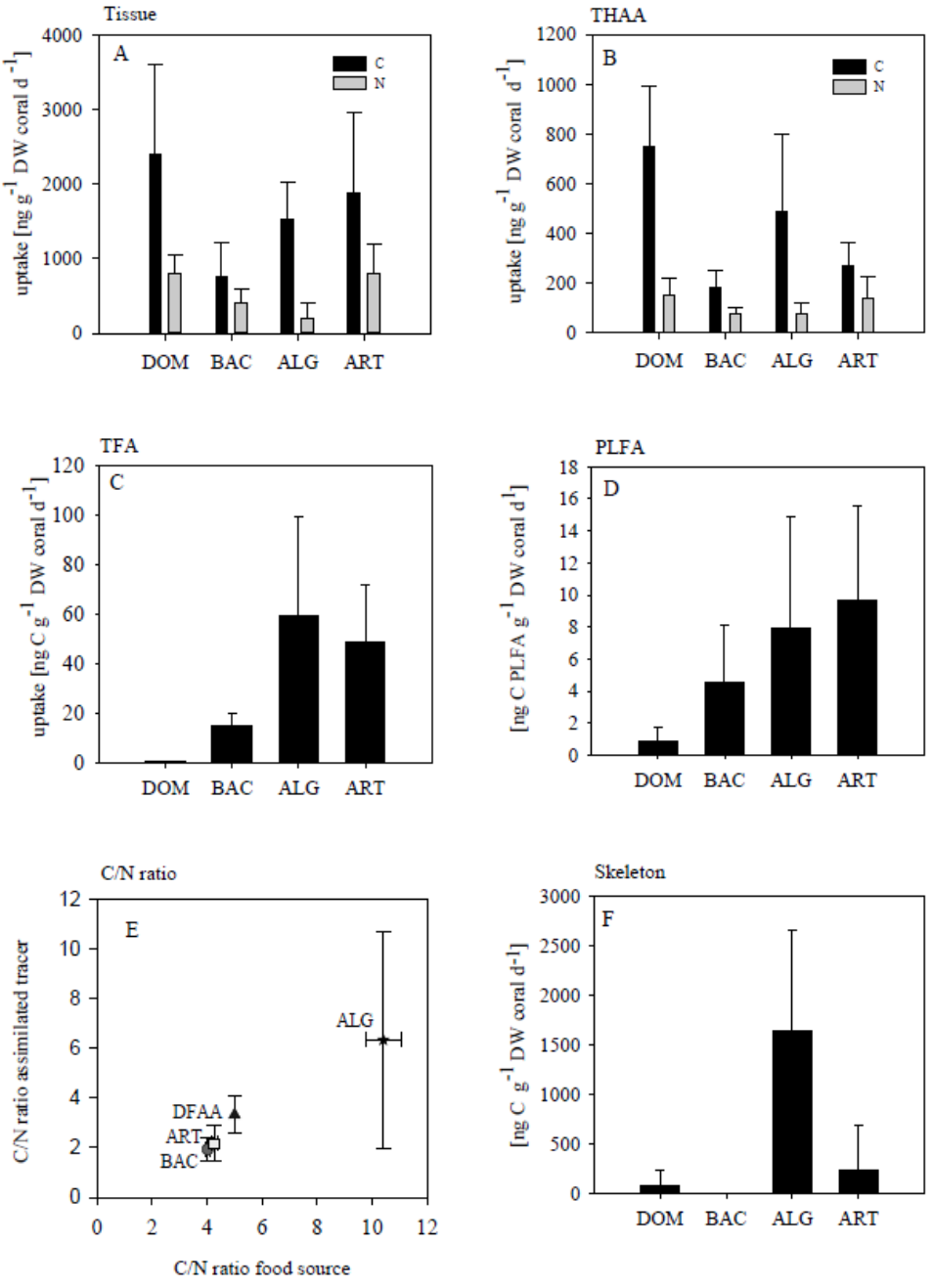


Figure 2.3. (A) C and N uptake in coral tissue (note: different C/N ratios of sources), (B), C and N uptake in THAAs of coral samples (C) C uptake in TFAs of coral samples, (D) C uptake in PLFAs of coral samples, (E) C/N ratio of food provided and of assimilation, (F) C uptake in coral skeleton. The bars in each figure represent average ± SD.

C incorporation into fatty acids

Of the total C assimilated up to 4% was traced in the total fatty acid pool (TFA) of *L. pertusa*. The ingested food source hereby significantly influenced the amount of C incorporated into TFAs. (Kruskal-Wallis $p = 0.0003$, Fig. 2.3.C). DOM-derived C incorporation was significantly lower than that of the particulate sources (0.3 ± 0.2 ng C g^{-1} DW coral d^{-1}, Wilcoxon $p_{DOM-\ ART/ALG/BAC} = 0.03$). *Artemia*- and algal-derived C (Wilcoxon $p > 0.05$) were incorporated in TFAs at comparable rates, 49 ± 23 and 59 ± 40 ng C g^{-1} DW coral d^{-1}, respectively, but bacteria-derived C was incorporated at a significantly lower rate of 15 ± 5 ng C g^{-1} DW coral d^{-1} (Wilcoxon $p_{BAC-ART} = 0.03$, Wilcoxon $p_{BAC-ALG} = 0.05$, Fig. 2.3.C).

The incorporation into the phospholipid-derived fatty acid (PLFA) C-pool accounted for 0.6% of total assimilated C. Like for TFAs, also C incorporated into PLFAs was significantly different between different food sources (Kruskal-Wallis $p = 0.002$). Again, DOM-derived C was incorporated at a significant lower rate (1 ± 1 ng C g^{-1} DW coral d^{-1}) than any of the particulate sources (Wilcoxon $p_{DOM-ART/ALG} = 0.03$, Wilcoxon $p_{DOM-BAC} = 0.05$, Fig. 2.3.D). The particulate sources however did not differ significantly in their incorporation (Wilcoxon $p > 0.05$ for all comparisons). On average *Artemia*-derived C was incorporated with 10 ± 6 ng C g^{-1} DW d^{-1}, algal-derived C with 8 ± 7 ng C g^{-1} DW coral d^{-1} and bacteria-derived C with 5 ± 4 ng C g^{-1} DW coral d^{-1}.

The incorporation of C into PLFAs in corals fed with DOM was solely caused by de novo synthesis, since the DOM source did not contain any PLFAs. The PLFAs C16:0, C16:1w7 and C18:1ω7c showed highest C incorporation but also long chain PLFAs like C22:2ω6 and C22:4ω6 incorporated tracer C in DOM fed corals (Fig. 2.2.D). For particulate sources over 40 - 60% of the assimilated PLFA-C by the corals was incorporated in PLFAs characterizing the respective food source (Fig. 2.4.). PLFAs not present in the diet but with substantial tracer incorporation (> 4 % contribution to tracer uptake) were C18:0, C18:1ω9c and C20:4ω6 in *Artemia* fed corals, C18:1ω7c and C22:4ω6 in algae fed corals and C22:2ω6 and C22:1ω9c in bacteria fed corals (Fig. 2.2.C).

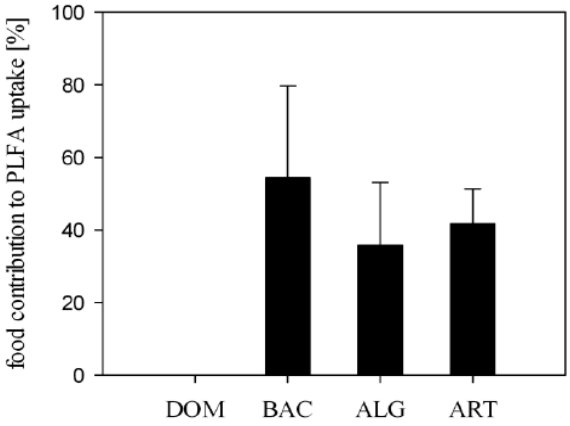


Figure 2.4. Contribution of food-derived PLFAs to total PLFA synthesis in corals directly incorporated into animal PLFAs, the food contribution was calculated by summing the uptake in food characteristic PLFAs by *L.pertusa* and dividing it by its the total PLFA uptake. The bars in the figure represent average ± SD.

Carbon incorporation into coral skeleton

Incorporation of metabolic C derived from the processing of organic food sources into the inorganic carbonate skeleton was highly variable among coral samples (Fig. 2.3.F), partly because only 1 - 2 coral pieces out of 6 showed measurable incorporation. Incorporation into the coral skeleton was highest in the algal treatment (1.6 ± 1.0 µg C g^{-1} DW coral d^{-1}), followed by the *Artemia* (0.2 ± 0.5 µg C g^{-1} DW coral d^{-1}) and DOM (0.1 ± 0.2 µg C g^{-1} DW coral d^{-1}) treatment. Coral pieces fed with bacteria did not incorporate tracer C in their skeleton.

Discussion

Biochemical characteristics of *L. pertusa*

The overall contribution of amino acids and fatty acids to C tissue is in agreement with observations on other organisms, including tropical corals, in which proteins form the largest fraction before sugars and lipids (Szmant-Froelich and Pilson, 1980; Achituv et al., 1994).

The concentration of total fatty acids (20 mg g^{-1} DW tissue with tissue DW contributing 5% to total DW, as observed in this study) however was below the

range of 55-124 mg g^{-1} DW tissue reported by Dodds et al. (2009) for *L. pertusa* from Rockall Bank, Mingulay Reef and New England Seamounts. Local differences can be responsible for this discrepancy as Larsson et al. (2013b) reported storage fatty acids concentrations of 15 to 19 mg g^{-1} DW tissue from *L. pertusa* collected at the Tisler Reef but also the maintenance in aquaria (3 month in this study) might have altered the lipid content (Larsson et al., 2013a).

Food assimilation and source preferences

In line with aquaria studies and in situ observations (Buhl-Mortensen, 2001; Freiwald et al., 2002; Tsounis et al., 2010) our study confirms that *L. pertusa* can utilize various particulate resources from a broad range of sizes including bacteria, algae and zooplankton. The assimilation of DOM by *L. pertusa* is in accordance with the observation that *Desmophyllum dianthus* took up DOM (~6 µg C g^{-1} DW d^{-1}) during core incubations at a 10-100x lower DOC concentration (Naumann et al., 2011). The comparable assimilation rates among resources hereby suggest that *L. pertusa* feeds rather unselectively at equivalent concentrations. This, together with the indication that coral food uptake is primarily driven by external factors such as food availability and current velocity (Purser et al., 2010), suggests that *L. pertusa* is an opportunistic feeder that utilizes resources depending on availability.

Rates of C assimilation into the tissue/biomass of *L. pertusa* in this study (~2 µg POC g^{-1} DW coral d^{-1}) are lower than the *Artemia* capture rates measured by Purser et al. (2010) under comparable flow and food conditions (324 µg POC g^{-1} DW coral d^{-1}). These measurements however are difficult to compare, because capture rates may overestimate actual ingestion if not every prey item is successfully transferred to the gut (Purser et al., 2010) and tissue assimilation only represents a fraction of the total uptake as respiration and mucus excretion are ignored. Especially latter can be a significant component of the energy budget of *L. pertusa* (Wild et al., 2008; Maier et al., 2011).

Food composition governs transfer into amino acids and lipids

Although the food sources were unselectively assimilated, there were clear differences in the metabolic processing. The most pronounced difference was observed between DOM- and POM-derived C incorporation. While POM-derived C was incorporated at a higher rate than DOM-derived C in fatty acids, DOM-derived C was incorporated at higher rates in amino acids than POM-derived C, excluding algal-derived C. This difference was most likely caused by the differences in composition between the sources. The DOM consisted solely of

dissolved free amino acids, which can be directly incorporated into coral tissue amino acids whereas fatty acids had to be produced de novo using amino acids as C-substrate. The POM sources contained among others amino acids and fatty acids. The comparatively high POM incorporation into coral fatty acids most likely results from their availability in the source and their (direct) assimilation by the coral as illustrated by the effective incorporation of dietary PLFAs into coral PLFAs (Fig. 2.4.).

Our results further indicate that not only the quantity (concentration) of amino acids / fatty acids, present in a food sources, but also their quality (composition) might affect food source processing. This is especially illustrated by the assimilation of algae in comparison to other POM sources. Algal-derived C was incorporated into coral PLFAs at a comparable rate to other POM sources, despite containing 10 times less PLFA-derived C (Table 2.1.). Additionally, algal-derived N incorporation into coral tissue and amino acids did not significantly differ from the incorporation of other POM sources despite a higher C/N ratio, i.e. a lower N concentration in the algal source. This suggests that while total assimilation can be comparable among sources, their nutritional importance in sustaining tissue components can still differ.

Assimilation and synthesis of PLFAs

Fatty acids are often used as biomarkers to infer the diet of animals (Braeckman et al., 2012; Kelly and Scheibling, 2012). This approach relies on the assumption that the relative composition of the PLFA pool in the animal reflects that of its food source(s) and hence that there is no alteration/modification or production of PLFAs occurring during the transfer from food source to animal. However this approach is only valid if the respective animal cannot synthesize the biomarker.

Overall, PLFA assimilation profiles (Fig 2.2.C, D) followed the biomarker concept and more closely reflected the dietary PLFA profiles (Fig. 2.2A) than that of the coral (Fig. 2.2.B) with highest tracer recovery in PLFAs < C20:0 chain length. However, a detailed profile comparison revealed that direct assimilation of dietary PLFAs from particulate sources could only explain 40 to 60% of the total C incorporation into the coral PLFAs. The remainder of the incorporation was due to de novo synthesis or alteration of dietary PLFAs. This capability of *L. pertusa* of de novo synthesis was also evident from the assimilation of DOM into coral PLFAs because this resource solely contained amino acids and no PLFAs. The assumption of the biomarker concept ‘you are what you eat’ therefore might not be completely true for *L. pertusa*.

Additionally, the de novo synthesis of FAs by *L. pertusa* includes also FAs which have previously been used as biomarkers. For example, C20:5ω3 and C22:6ω3 (biomarkers for diatoms and dinoflagellates, respectively (Harwood and Russell, 1984)) synthesized by *L. pertusa* from *Artemia*. Furthermore C22:1ω9c, a zooplankton biomarker (Sargent and Falkpetersen, 1988), was synthesized by *L. pertusa* when fed bacteria (detected in one of three samples). Although the variation in de novo synthesis is high, it may complicate the interpretation of fatty acid profiles from field collected specimens, because presumed "unique" fatty acids may have been (partly) synthesized by *L. pertusa*. Our study thus adds to the growing evidence that care should be taken using fatty acids as dietary tracers in benthic food webs (Kelly and Scheibling, 2012; McLeod et al., 2013).

Metabolic versus external C incorporation into coral skeleton

Inorganic carbon to sustain calcification can either originate from an external (dissolved inorganic C) or from an internal pool (metabolic-derived C). In this study we directly measured the transfer of metabolic-derived C transfer into the coral skeleton, which ranged from 0.1 – 1.6 µg C day^{-1} g^{-1} DW coral depending on the food source. This rate is considerably lower than separate, but comparable, incubations with ^{13}C-bicarbonate that showed a direct external inorganic C uptake of 46 ± 25 µg C g^{-1} DW coral d^{-1} (C. E. Mueller et al. unpubl. data). Maier et al. (2009) measured a total calcification rate, i.e. sum of internal and external usage, of 23 – 78 µg C day^{-1} g^{-1} DW coral with ^{45}Ca on freshly collected corals. These results indicate that metabolic derived C only plays a minor role as C source for calcification, which confirms the suggestion by Adkins et al. (2003) based on isotopic data analysis in the coral skeleton ($\delta^{18}O$, $\delta^{13}C$), that calcification of *L. pertusa* mainly relies on an external DIC source which decreases the effect of isotopic fractionation during the calcification process. Together with the lack of autotrophic symbionts in the coral this makes *L. pertusa* skeletons an excellent study object for climatic reconstructions (Adkins et al., 2003).

Conclusions and implications

In this study we investigated the capability of *L. pertusa* to take up different food sources ranging from dissolved organic matter, bacteria and algae to zooplankton. The comparable assimilation rates of the different food sources hereby indicated that *L. pertusa* is an opportunistic feeder that is able to exploit a wide variety of food sources even including DOM.

Our main focus was to investigate the uptake of food sources across a broad size spectrum, but the implications of these findings for field conditions depend partly on how representative the experimental food sources are for natural conditions. Zooplankton can be an important food item for CWCs (Freiwald, 2002; Kiriakoulakis et al., 2005; Dodds et al, 2009; Duineveld et al., 2012), but it is logistically challenging to use local and living copepod species in laboratory studies, especially when these need to be enriched with stable isotopes. Therefore, we decided to use laboratory-reared *Artemia* as substitute for natural zooplankton, like most other feeding studies (Purser et al. 2010, Tsounis et al., 2010; Larsson et al., 2013b). Although *Artemia* may differ from copepods in their biochemical composition (Evjemo and Olsen, 1996; Helland et al., 2003), they are in the same size range as natural copepods. The microalgae selected for experimentation, *Skeletonema costatum*, contributes to phytoplankton blooms in the Skagerrak (Lange et al., 1992) and, together with dissolved organic matter, may reach the *Lophelia* reefs during downwelling events (Wagner et al. 2011). The cultured bacteria used in the experiment likely differed in composition from that in nature, but it gives first-order information about how *L. pertusa* deals with the natural picoplankton fraction comprising a broad variety of microbes (bacteria, eukarya and archaea, Jensen et al., 2012). We are therefore confident that our experimental food sources are good representatives for natural food items and are able to illustrate the opportunistic feeding strategy of the coral.

The ability to utilize DOM hereby underlines the nutritional flexibility of *L. pertusa* and might be especially relevant for natural reefs like on Rockall bank, where POM concentrations can be very low during several months (Duineveld et al., 2007). The assimilation of external DOM and the re-assimilation of DOM from coral mucus release, responsible for high labile DOM concentrations above CWC reefs (van Duyl et al., 2008; Wild et al., 2009) might help the coral to withstand several months without POM supply.

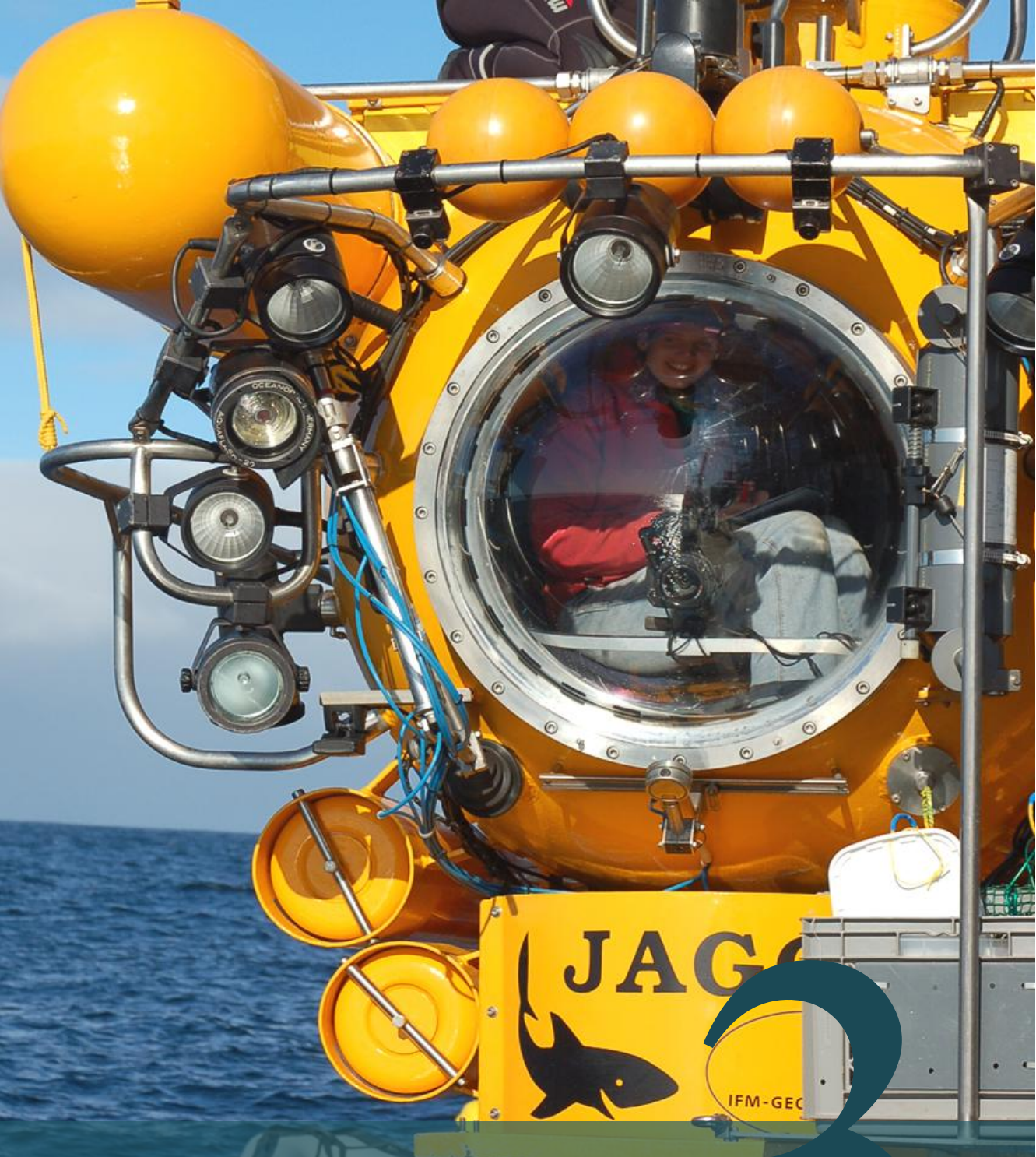
Next to the uptake of different sized food source, we also explored for the first time the processing of these sources by *L. pertusa*. The observed differences in food processing hereby suggest that the nutritional value of a food source is at least partly determined by its composition (quantity and quality of fatty acids and amino acids). Our findings further indicate that phytoplankton is a valuable resource for *L. pertusa* due to the efficient transformation into coral fatty acids. This might be especially relevant in locations where downwelling events (Tisler Reef, Mingulay Reef) supply the reefs with a high availability of phytoplankton (Duineveld et al., 2004; Davies et al., 2009; Wagner et al., 2011). Furthermore, the high flow

velocities characterizing many CWC reef locations (Messing, 1990; Thiem et al., 2006; White et al., 2007) might also favour the uptake of smaller particles such as algae, since particle retention is negatively affected by particle size especially at higher flow velocities (Shimeta and Koehl, 1997).

Additionally we found de novo synthesis within the fatty acid metabolism, indicating that corals do not only rely on dietary fatty acids to obtain certain fatty acids. This especially concerns bacteria, since they are often considered as low quality food based on their lack of long-chain fatty acids (Phillips, 1984; Leroy et al., 2012). However, given the ability of the coral to synthesized long chain fatty acids such as C22:2ω6 and C22:1ω9c from bacteria, our results suggest that bacteria can be a valuable addition to coral nutrition. This might be especially relevant since bacteria occur in high abundance around cold-water coral reefs and food uptake in *L. pertusa* is positively correlated with prey abundance (Purser et al., 2010). Additionally, bacteria are constantly available to the coral since they are fertilized by the coral itself via mucus production (Wild et al., 2009; Maier et al., 2011), while POM availability can vary spatially and temporally (Duineveld et al., 2007).

Acknowledgements

This research was supported by the CALMARO project (FP7/2008-2012), funded by the European Community's Seventh Framework Programme (FP7/2008-2012) and the CORAMM project funded by Statoil. Tomas Lundälv is thanked for collecting the corals by ROV and providing lab space and equipment. We thank the analytical lab of NIOZ-Yerseke for sample analysis and Pieter van Rijswijk for his help during the culturing work.



3

Microbial symbionts processing inorganic C and N support the cold-water coral *Lophelia pertusa*

Photo by M. López Correa

C. E. Mueller, B. Veuger, A. I. Larsson, A. Form, J. J. Middelburg and D. van Oevelen

In preparation

Abstract

In this study we investigated the processing of inorganic carbon and nitrogen by the cold-water coral *Lophelia pertusa* and its microbial symbionts. By using stable isotopes we were able to trace important processes of the nitrogen cycle (assimilation, nitrification, denitrification, and nitrogen-fixation) in two different colour morphs of the coral. Ammonium-assimilation and bicarbonate-fixation in white *L. pertusa* was studied in detail by following tracer uptake in the coral tissue and specific compounds including amino acids, total fatty acids and phospholipids-derived fatty acids (PLFAs). The latter were used in their function as biomarkers to reveal bacterial mediation in the uptake process. We also measured the incorporation of external DIC into the coral skeleton to quantify calcification. Our results show that the cold-water coral *L. pertusa* can use inorganic substrates not only to build up its skeleton but also to supplement its organic carbon and nitrogen requirement by nitrogen-fixation, ammonium assimilation and bicarbonate-fixation, most likely with the help of symbiotic microbes. At the same time *L. pertusa* and associated microbes can also contribute to N cycling within the cold-water coral reef system by nitrification and denitrification.

Introduction

The importance of symbiotic relationships is well established in tropical coral reefs, most notably in the interaction between tropical corals and photoautotrophic dinoflagellates (zooxantellae). Recent studies also highlight the potential of microorganisms associated with tropical corals to increase nutrient cycling (Wegley et al., 2007; Kimes et al., 2010): cyanobacteria fix nitrogen by reducing dinitrogen gas (N_2) to ammonia that is subsequently used by the dinoflagellate-coral association (Lesser et al., 2004; Lesser et al., 2007; Lema et al., 2012). These complex symbiotic interactions enable the tropical shallow-water coral holobionts (coral+ microbial symbionts) to increase nutrient uptake in an oligotrophic environment.

Cold-water corals (CWC) are widely distributed in the deep, dark oceans around the world (Davies and Guinotte, 2011). In contrast to their tropical counterparts, cold-water corals are assumed to rely solely on heterotrophic feeding due to the lack of solar radiation and photosynthetic symbionts. The utilization of inorganic substrates therefore appears restricted to the calcification process, where inorganic C is required to build up the coral skeleton. CWCs however harbour a distinct and diverse community of symbiotic microbes (coral holobiont). Archaea, (Galkiewicz, 2011; Emblem et al., 2012), viruses (Maier et al., 2011), bacteria (Yakimov et al., 2006; Neulinger et al., 2008; Kellogg et al., 2009; Galkiewicz et al., 2011) and fungi (Galkiewicz et al., 2012) have been observed associated with the cold-water coral *L. pertusa*, with distinctive differences between the two colour morphs, white and red corals, possible explaining their differences in abundance (Neulinger et al., 2008).

Although the role of the diverse microbial community associated with the coral is largely unknown, phylogenetic analyses indicate among others the presence of cyanobacteria, bacteria related to the genus *Vibrio* and the class *Nitrospira*, sulphur oxidizing bacteria and mixotrophic Rodobacteraceae (Neulinger et al., 2008; Galkiewicz et al., 2011). If metabolically active, these microbes may have a symbiotic function and supplement the heterotrophic feeding of their host coral (Neulinger et al., 2008; Galkiewicz et al., 2011) (Fig. 3.1.). Nitrogen fixers and microbes able to assimilate ammonium could enhance the N supply to the coral holobiont, which is often a limiting factor of biological production in the deep-sea. Ammonium-oxidizing and sulphur-oxidizing microbes, in turn can use the energy generated during the oxidation of the respective substrate (ammonium or sulphur) for dissolved inorganic C fixation (chemoautotrophy). Thus, symbiotic microbes can potentially provide energy (C) and nutrients (N) to the coral host. So far

however it is still unclear whether these processes actually occur and whether the corals benefit from these suspected microbial symbionts.

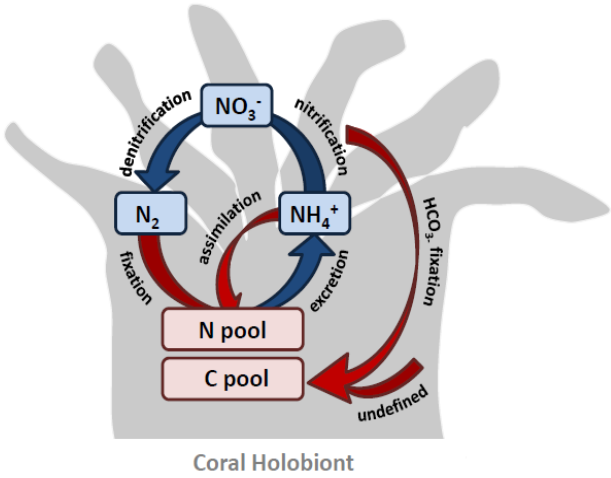


Figure 3.1. Schematic overview of N cycling related processes including HCO_3^- fixation by chemoautotrophy measured during this study. Red arrows mark the processes supplementing the N/C supply of the coral holobiont while blue arrows mark the processes which do not affect the N/C supply of the holobiont.

In this study we explored the capability of *L. pertusa* with their associated microbes to use inorganic substrates from the surrounding seawater for their energy and nitrogen requirements. In particular we tested the activity of N-related processes including nitrogen fixation, nitrification, denitrification and ammonium assimilation. We also investigated whether inorganic carbon was fixed by chemoautotrophs and transferred into coral tissue and different tissue components including total hydrolysable amino acids (THAAs), total fatty acids (TFAs) and phospholipid-derived fatty acids (PLFAs). Since microbial activity can be low and accompanying concentration changes are often difficult to detect we used stable isotope tracers, since they are excellent tools to detect even small changes and activities. In combination with PLFAs, which can be used as biomarkers for specific bacteria groups, they further allowed us to separate the activity of the bacteria from the one of the coral in the holobiont.

Methods

The experiments were either carried out on board of the Poseidon during the cruise P420 to the Trondheim-fjord (Norway) or in the laboratory at the Sven Lovén Centre in Tjärnö, Sweden (Table 3.1.).

Table 3.1. Experimental set ups and procedures. All experiments were carried out either during the Poseidon cruise P420 or at the Sven Lovén Centre in Tjärnö (TMBL).

Process	Method	Measurements	Trondheim-fjord (P420)	Tisler Reef (TMBL)
N_2-fixation	$^{15}N_2$ addition	Tissue, $^{15}NH_4^+$, $^{15}NO_3^-$	*L. pertusa* white, red	
Nitrification	$^{15}NH_4^+$ addition	$^{15}NO_3^-$	*L. pertusa* white, red	
Denitrification	$^{15}NO_3^-$ addition	$^{15}N_2$	*L. pertusa* white, red	
NH_4^+-assimilation	$^{15}NH_4^+$ addition	^{15}N Tissue*	*L. pertusa* white, red	*L. pertusa* white*
HCO_3^--fixation	$^{13}HCO_3^-$ addition	^{13}CTissue*	*L. pertusa* white, red	*L. pertusa* white*
Calcification	$^{13}HCO_3^-$ addition	^{13}C Skeleton	*L. pertusa* white, red	*L. pertusa* white

*detailed tissue analysis including ^{15}N/ ^{13}C THAAs, ^{13}C TFAs and ^{13}C PLFAs were only conducted on corals harvested at the Tisler Reef

Sampling locations and maintenance

The corals used in this study were harvested from two different locations (see details in Table 3.1.). Red and white *L. pertusa* was collected at 30 – 40 m deep in the Trondheim-fjord using the manned submersible JAGO during the Poseidon cruise P420 in September 2011. Coral fragments were cut on-board in small pieces (~2 - 4 g DW (dry weight) piece^{-1} and ~7 – 11 polyps piece^{-1}) to fit the incubation

bottles and were kept 2 to 3 days for acclimation in a 500 l tank filled with seawater at a temperature of 7-8 ºC. No food was supplied during the acclimation period. Red and white corals from Trondheim-fjord were used for a detailed nitrogen cycling study: N_2-fixation, nitrification, denitrification and NH_4^+ assimilation.

White *L. pertusa* branches were collected at the Tisler Reef (for details see Lavaleye et al., 2009; Wagner et al., 2011) and used to investigate the assimilation of NH_4^+ and the fixation of HCO_3^- in more detail by following tracers incorporation into coral tissue components (fatty acids, amino acids). The Tisler Reef is located at a water depth of 110 m at the border between Norway and Sweden, and was sampled using the remotely operated vehicle Sperre Subfighter 7500 DC. After transporting the corals in cooling boxes filled with cold seawater (7 - 8 ºC) to the Sven Lovén Centre, samples were clipped to a similar size as those from Trondheim-fjord (3.9 ± 4.3 g DW piece^{-1} and 9.3 ± 1.1 polyps piece^{-1}). The Tisler corals were maintained in aquaria (10 l) placed in a dark thermo-constant room (7 ºC) for 3 months. The aquaria were continuously flushed with sand-filtered (1-2 mm particle size) water from 45 m depth out of the adjacent Koster-fjord (salinity 31) (~1 l min^{-1}). Corals were fed with larvae (nauplii) of the brine shrimp *Artemia spp.* every 3 to 4 days following common procedures at Tjärnö.

Experimental set ups and procedures

N_2 fixation

$^{15}N_2$ enriched seawater was produced prior to the experiment by injecting $^{15}N_2$ gas in degassed artificial seawater following the protocol of Mohr et al. (2010). Red (2.6 ± 0.6 g DW piece^{-1}, 8.7 ± 3.1 polyps piece^{-1}) and white *L. pertusa* pieces (2.3 ± 0.1 g DW piece^{-1}, 7.3 ± 0.6 polyps piece^{-1}) were placed separately in gas-tight glass bottles (70 ml) filled with GF/F filtered seawater. After closing the bottles, 7 ml of the $^{15}N_2$ enriched seawater was injected through the rubber septum of the lid (replacing an equal volume of unlabelled water), resulting in an enrichment of 10 atom% $^{15}N_2$ in the incubation vial. Control corals were incubated without $^{15}N_2$ enriched seawater while controls for nutrient and background isotopic values were incubated without coral and with and without label addition. The bottles containing the coral pieces were incubated for 24 h at 7 ºC in the dark. The experiment was replicated three times for each colour morph. Besides the shaking provided by the movement of the ship every 6 to 8 hours each incubation bottle was gently shaken by hand to allow further mixing. The total incubation time was chosen to give the coral-associated microbes enough time to process the N_2, while at the same time

avoiding anoxic conditions (50% expected depletion during the incubation) as estimated from oxygen consumption measurements (3.6 µmol O_2 g^{-1}DW d^{-1} at 7°C) reported by Dodds et al. (2007) and an oxygen solubility in seawater of 280 µM (7°C). At the end of the incubations coral pieces were removed from the bottles and stored frozen for later analysis of incorporation of ^{15}N in the host and symbiont tissue. The water was filtered, pooled per treatment (to obtain enough material for analysis) and stored frozen for analysis of nutrient concentrations and ^{15}N enrichment of ammonium and nitrate.

Nitrification

Nitrification was investigated by incubating small pieces of red *L. pertusa* (3.7 ± 0.6 g DW $piece^{-1}$, 11.3 ± 1.5 polyps $piece^{-1}$) and white *L. pertusa* (4.4 ± 1.1 g DW $piece^{-1}$, 10.4 ± 2.5 polyps $piece^{-1}$) in 250 ml glass bottles filled with 200 ml GF/F filtered sea water enriched with $^{15}NH_4^+$ at two different concentrations (1 µM and 3 µM plus background of ~ 0.5 µM respectively). The control treatment (without coral) contained either filtered seawater or filtered seawater enriched with $^{15}NH_4^+$ at two different concentrations. All bottles were incubated for 48 h at 7 ºC in the dark. For each colour morph the incubations were replicated 3 times. Besides the shaking provided by the movement of the ship every 6 to 8 hours each incubation bottle was gently shaken by hand to allow further mixing. At the end of the incubation corals were removed, the water was filtered (GF/F) and frozen for further analysis of nutrients and ^{15}N-enrichment of NO_3^- and NH_4^+. The concentration and isotopic enrichment of NH_4^+ was hereby used to determine the isotope dilution of the $^{15}NH_4^+$ pools during the incubations and to estimate total NH_4^+ production using the isotopic dilution model described by Blackburn (1979).

Denitrification

Red (1.7 ± 0.6 g DW $piece^{-1}$, 3.5 ± 1.3 polyps $piece^{-1}$) and white *L. pertusa* pieces (1.7 ± 0.7 g DW $piece^{-1}$, 3.5 ± 1.3 polyps $piece^{-1}$) were placed in gas tight glass bottles (70 ml) filled with GF/F filtered sea water enriched with two different concentrations of $^{15}NO_3^-$ (1 µM, 3 µM). After closure bottles were incubated for 24 h at 7 ºC in the dark. The control treatment (no coral) contained only filtered sea water or filtered sea water enriched with $^{15}NO_3^-$ at two concentrations and was incubated in parallel. Besides the shaking provided by the movement of the ship every 6 to 8 hours each incubation bottle was softly shaken by hand to allow further mixing. The total incubation time was chosen to give the coral enough time to process the $^{15}NO_3^-$ while at the same time avoiding anoxic conditions (see N_2-fixation). Incubations were terminated by injection of $HgCl_2$ and bottles were

stored upside down for analysis of $^{15}N_2$. Each colour morph was replicated three times.

NH_4^+-assimilation and HCO3$^-$-fixation

NH_4^+-assimilation and HCO_3^--fixation were measured during the cruise with material from Trondheim-fjord and in the laboratory using corals from Tisler Reef. During the cruise red and white *L. pertusa* samples were placed separately in incubation chambers (4 l) filled with GF/F filtered sea water and maintained at 8ºC in a water bath. A stirrer in the middle of the chamber maintained water circulation. After an acclimation period of 12 hrs, ^{13}C-HCO_3^- and $^{15}NH_4^+$ were added to the water to result in an enrichment of 30 atom% for both ^{15}N and ^{13}C. The treatment was replicated three times. Control corals were incubated in parallel without label addition for isotopic background measurements. Water was exchanged every 2.5 days and new label was added. After a total incubation time of 5 to 10 days, coral samples were frozen at -20 °C, freeze-dried and stored frozen for further analysis. Incubations lasted for 5 to 10 days to obtain an idea about time dependence in substrate incorporation into tissue and carbonate skeleton.

In the laboratory (Tjärnö, Table 3.1.) only white *L. pertusa* samples were placed in incubation chambers (10 l) in a thermo-stated room at 7 °C. A motor-driven paddle on top of the chamber (2 rpm) maintained water circulation (Fig. 3.2.). Prior to the experiment, chambers were filled with 0.2 µm filtered seawater from 45 m depth out of the Koster-fjord (salinity of 33, 7 °C). Three coral fragments were randomly selected and placed in a single chamber (Fig. 3.2.). After an acclimation period of 12 hrs, ^{13}C-HCO_3^- and $^{15}NH_4^+$ were added to the water to result in an enrichment of 10 atom% for both substrates. The experiment was duplicated. Control corals were incubated in parallel without label addition for isotopic background measurements. After an incubation time of 4 days in darkness, coral samples were frozen at -20 °C, freeze-dried and stored frozen for further analysis.

Sample treatment and analyses

N_2 gas analysis

Concentration and isotopic composition of dissolved N_2 ($^{28}N_2$, $^{29}N_2$, $^{30}N_2$) were determined in the headspace gas of the incubation bottle after injection of He (replacing 5 ml of sample water) and vigorous shaking using a Thermo Electron Flash EA 1112 analyzer (EA) coupled to a Delta V isotope ratio mass spectrometer (EA-IRMS) as described in Gribsholt et al. (2005).

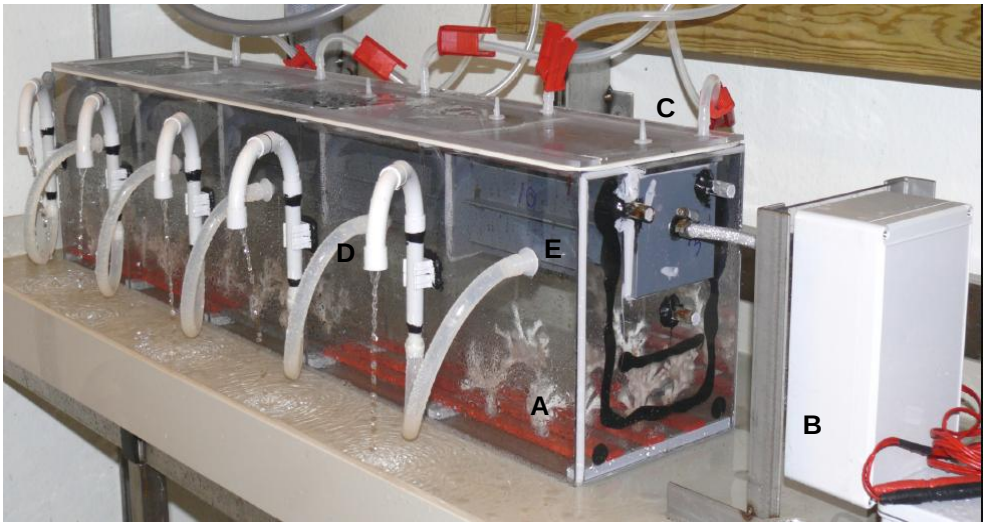


Figure 3.2. Experimental set up (Mississippi chambers), picture by A. I. Larsson. (A) chamber, (B) motor, (C) water inflow, (D) water outflow and (E) paddle.

Nutrient analysis and ^{15}N enrichment of NH_4^+ and NO_3^-

NH_4^+, NO_2^- and NO_3^- concentration in water samples were determined using automated colourimetric techniques (precision NH4+ ± 2% SD, NO2-/NO3- ± 3% SD). The isotopic composition of NH_4^+ and NO_3^- in the sample was determined in two steps following the ammonium-diffusion method as described in Gribsholt et al. (2005). In the first step MgO is added to the water sample to convert the NH_4^+ to NH_3, which is then trapped on an acidified (H_2SO_4) GF/D filter packed between two Teflon filters floating on the sample surface. In the second step the remaining NO_3^- is converted to NH_4^+ by the addition of Devarda's Alloy, which again is trapped on an acidified GF/D filter package. Finally, both filters were then measured for their isotopic composition by isotope ratio mass spectrometry (IRMS).

Tissue analysis

For isotopic analysis of coral tissues, frozen corals were freeze-dried, weighed and homogenized by grinding with a ball Mill for 20 seconds (MM 2000, Retsch, Haan, Germany). A subsample (~30 mg) of ground coral material was decalcified by stepwise acidification with 12M HCl and the remaining organic fraction (tissue + organic skeleton matrix) was measured for C and N concentration and isotopic composition by EA-IRMS.

Tissue samples were also analyzed for tracer incorporation into total fatty acids (TFA), phospholipid-derived fatty acids (PLFA) and hydrolysable amino acids (HAAs). For the extraction a total of 0.7 g DW of each grounded coral sample was used. TFAs were extracted with a modified Bligh and Dyer method. The PLFA fraction of the total fatty acid extract was separated by silica column (Merck Kieselgel 60) (Boschker et al., 1999). The TFA and PLFA extracts were derivatized by mild alkaline transmethylation to obtain fatty acid methyl esters (FAME). Preparation of methyl esters was carried out following the method of Boschker et al. (1999). Concentration and carbon isotopic composition of individual TFAs and PLFAs were measured on a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c- IRMS) (Middelburg et al., 2000).

HAAs were extracted and analyzed using a modification of the protocol from Veuger et al. (2005). Ground coral samples were first decalcified by repeated addition of 12 M HCl drops. The remaining material was then hydrolyzed in 6M HCl at 110ºC for 20 h and purified by cation exchange chromatography (Dowex 50WX8 resin). HAAs were derivatized with isopropanol and pentafluoropropionic anhydride and analyzed by GC-c-IRMS for individual AAs concentrations and ^{13}C and ^{15}N enrichment.

Calcification (HCO_3^- incorporation in the skeleton)

The incorporation of HCO_3^- in the coral skeleton was determined following the protocol described in Mueller et al. (2013). After the determination of tracer C incorporation into the organic pool of grounded coral sample (see above), ^{13}C incorporation into the total C pool (tissue, organic matrix and skeleton) was determined accordingly without prior acidification. Tracer incorporation into the coral skeleton was then calculated by subtracting the tracer fixation in the organic C fraction (tissue and organic matrix) from the tracer incorporation in the total C pool (tissue, organic matrix and skeleton).

Calculations

The processing rates/ uptake rates of ^{15}N or ^{13}C are presented as excess ng of ^{15}N/ ^{13}C per gram of DW coral. Excess ^{15}N or ^{13}C is calculated from the difference in heavy isotope fraction (F) between sample and background multiplied by the quantity of nitrogen or carbon (Middelburg et al., 2000; Veuger et al., 2005): excess = F_{sample}-$F_{background}$ x (ng of N or C in sample), where $F = R/(R+1)$ and the isotope ratio R is calculated directly from $\delta^{15}N$ or $\delta^{13}C$ generated by the IRMS.

In order to convert process rates for the ^{15}N labelled pools to total ($^{15}N + ^{14}N$) rates, ^{15}N rates were multiplied by the ^{15}N fraction ($^{15}N/(^{15}N+^{14}N)$) of the respective substrate at the start of the incubation. For the nitrification rate measurements, the ^{15}N enrichment of the total NH_4^+ pool was strongly influenced by strong NH_4^+ production during the incubations. To compensate for the resulting isotopic dilution, we used the average ^{15}N enrichment of the NH_4^+ pool during the incubation period that was calculated from the start value (calculated from natural $^{14}NH_4^+$ concentrations and added $^{15}NH_4^+$ addition) and the end value (directly measured in the extracted NH_4^+).

Nitrification and denitrification rates determined for coral incubations were corrected for incubation water activity by subtracting the rates obtained from the control incubations. All results are reported as average ± SD.

Statistical Analyses

Differences between colour morphs of *L. pertusa* in NH_4^+ total and net-production and NH_4^+ incorporation, calculated after Balckburn (1979), and the effect of tracer addition on this processes were tested using ANOVA. Differences between colour morphs of *L. pertusa* in N_2-fixation, nitrification, and denitrification was tested using Kruskal-Wallis Test, since the requirements for ANOVA were not always met. The same test was used to analyze differences in nitrification and denitrification with respect to different substrate additions. Kruskal-Wallis Test was also used to test for differences between colour morphs and incubation times in HCO_3^- fixation and NH_4^+ assimilation. Since no significant differences could be found between 5 and 10 days of incubation, data were averaged for each colour morph for simplification.

Results

Ammonium and NO_3^- concentrations during incubations

With the addition of 1 and 3 µM $^{15}NH_4^+$ the concentrations of NH_4^+ increased respectively in the controls without corals (Table 3.2.). In incubation with corals (nitrification, N_2-fixation) the concentration of NH_4^+ increased up to 17 µM (Table 3.2.), independent of the colour of the coral or the amount of added tracer (Kruskal-Wallis $p > 0.05$). The concentration of NO_3^- increased after tracer addition in incubation with and without corals (Table 3.2.) but no significant

difference could be detected between the controls with tracer addition (Table 3.2.) and the different coral treatments (Kruskal-Wallis p = 0.3).

Table 3.2. Nutrient concentrations after the incubations with different additions of NH_4^+ (1 µM, 3 µM) and the addition of N_2. Untreated controls without corals (control water), controls with tracer addition but without corals (control water 1 µM, 3 µM).

Treatment	**n**	**DW coral [g]**	**NH_4^+ [µM]**	**NO_3^- [µM]**
Control water	2		0.5 ± 0.4	5.3 ± 3.6
Control water 1 µM	1		1.6	13.4
Control water 3 µM	1		2.3	12.1
Control N_2	1		3.1	7.7
***L. pertusa* white 1 µM**	3	3.6 ± 1.3	15.3 ± 2.7	11.0 ± 3.3
***L. pertusa* white 3 µM**	3	4.8 ± 0.5	16.2 ± 1.1	12.6 ± 1.0
***L. pertusa* red 1 µM**	3	3.5 ± 0.3	13.9 ± 3.8	8.6 ± 2.4
***L. pertusa* red 3 µM**	3	3.8 ± 1.0	8.1 ± 0.8	8.5 ± 4.6
***L. pertusa* white N_2**	3	2.3 ± 0.1	17.0*	12.5*
***L. pertusa* red N_2**	3	2.6 ± 0.6	15.6*	10.9*

*represent the average of three replicates, no standard derivations can be given due to sample pooling for analysis (see Material and Methods)

Total and Net-production of NH_4^+

Total and net-production of NH_4^+ by *L. pertusa* were comparable between colour morphs (ANOVA p > 0.05) but decreased significantly with increasing $^{15}NH_4^+$ addition for red (ANOVA p = 0.02) and white *L. pertusa* (ANOVA p = 0.04). White *L. pertusa* produced in total 6.9 ± 1.1 µg N g^{-1} DW d^{-1} and 5.0 ± 0.2 µg N g^{-1} DW d^{-1} after an addition of 1 and 3 µM NH_4^+ respectively, while red corals produced in total 6.0 ± 2.0 µg N g^{-1} DW d^{-1} and 2.4 ± 0.5 µg N g^{-1} DW d^{-1}

respectively (Table 3.3.). The net production of NH_4^+ by white *L. pertusa* was 5.7 ± 1.1 µg N g^{-1} DW d^{-1} and 4.1 ± 0.1 µg N g^{-1} DW d^{-1} after 1 and 3 µM NH_4^+ addition respectively, while red *L. pertusa* had a net production of 5.2 ± 1.8 µg N g^{-1} DW d^{-1} and 2.3 ± 0.3 µg N g^{-1} DW d^{-1} respectively (Table 3.3.).

Unlike the NH_4^+ production, the incorporation of NH_4^+, calculated after Blackburn (1979), was not significantly affected by tracer addition and comparable between coral morphs (ANOVA $p > 0.05$ for all comparisons). White *L. pertusa* incorporated 1.2 ± 0.1 µg N g^{-1} DW d^{-1} and 0.9 ± 0.3 µg N g^{-1} DW d^{-1} after an addition of 1 and 3 µM NH_4^+ respectively, while red corals incorporated 0.8 ± 0.5 µg N g^{-1} DW d^{-1} and 0.7 ± 0.6 µg N g^{-1} DW d^{-1} respectively (Table 3.3.).

Table 3.3. Total NH_4^+ and Net NH_4^+ production by red and white *L. pertusa*. Total production and incorporation were calculated following Blackburn (1979), while Net production was calculated based on concentration changes. All data were obtained during the nitrification experiment after an addition of 1 or 3 µM NH_4^+ and are expressed in µg N g^{-1} DW d^{-1}.

Treatment	Total Production (after Blackburn)	Net production	Incorporation (after Blackburn)
***L. pertusa* white 1 µM**	6.9 ± 1.1	5.7 ± 1.1	1.2 ± 0.1
***L. pertusa* white 3 µM**	5.0 ± 0.2	4.1 ± 0.1	0.9 ± 0.3
***L. pertusa* red 1 µM**	6.0 ± 2.0	5.2 ± 1.8	0.8 ± 0.5
***L. pertusa* red 3 µM**	2.4 ± 0.5	2.3 ± 0.3	0.7 ± 0.6

N_2 fixation

N originating from N_2 was found in coral tissue of both colour morph and in NH_4^+ and NO_3^- in the water column (Fig. 3.3.). Incorporation rates of N_2 in coral tissue were comparable between white and red of *L. pertusa* (Kruskal-Wallis $p = 0.3$) with 614 ± 203 ng N g^{-1} DW d^{-1} and 720 ± 257 ng N g^{-1} DW d^{-1} respectively (Fig. 3.3.). On average 10 ng N g^{-1} DW d^{-1} and 26 ng N g^{-1} DW d^{-1} were transformed into NH_4^+ in incubations with white and red *L. pertusa* respectively. NH_4^+ was

further nitrified to NO_3^- at an average rate of 93 ng N g^{-1} DW d^{-1} and 50 ng N g^{-1} DW d^{-1} by white and red *L. pertusa*, respectively.

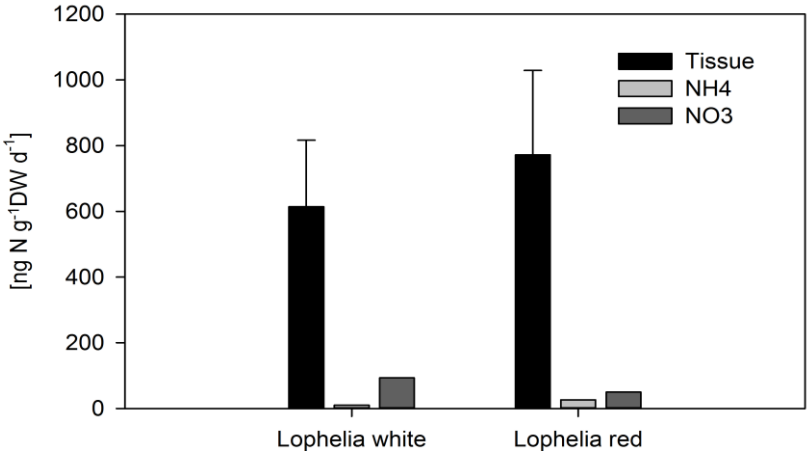


Figure 3.3. Fixation of N_2 based on tracer incorporation in coral tissue of red and white *L. pertusa* (Trondheim-fjord) and in NH_4^+ and NO_3^- in the water column.

Nitrification

Nitrification rates did not differ significantly between the colour morphs (Kruskal-Wallis $p > 0.05$) and were independent of concentration (Kruskal-Wallis $p > 0.05$). In white *L. pertusa* nitrification ranged from 0 to 31 ng N g^{-1} DW d^{-1} and from 0 to maximum of 13 ng N g^{-1} DW d^{-1} in incubation with 1 and 3µM NH_4^+, respectively. Red *L. pertusa* incubated with 1 µM and 3 µM NH_4^+ showed nitrification rates between 0 to 9 ng N g^{-1} DW d^{-1} and 0 to 4 ng N g^{-1} DW d^{-1} respectively (Fig. 3.4.).

Denitrification

Denitrification was detected at both NO_3^- concentrations and was significantly different between red and white *L. pertusa*, but only at 1 µM NO_3^-, with an order of magnitude higher rates in white *L. pertusa* (Kruskal-Wallis $p = 0.049$, Fig. 3.4.). The addition of NO_3^- however did not significantly influence denitrification rates in both colour morphs (Kruskal-Wallis $p > 0.05$), which ranged from 138 to 335 ng N g^{-1} DW d^{-1} in white *L. pertusa* in incubation with 1 µM NO_3^- and from 0 to 478 ng N g^{-1} DW d^{-1} in incubation with 3µM NO_3^-. Red *L. pertusa* incubated with 1 µM

and 3 µM NO_3^- showed denitrification rates between 12 to 22 ng N g^{-1} DW d^{-1} and 0 to 70 ng N g^{-1} DW d^{-1} respectively (Fig. 3.4.).

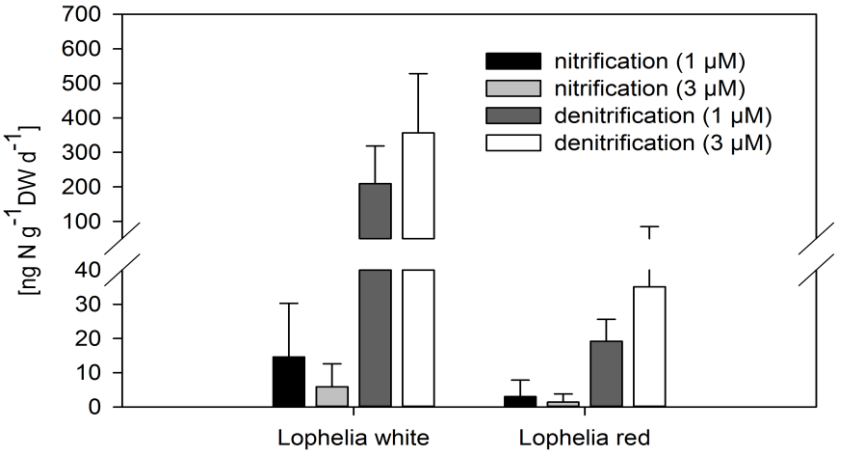


Figure 3.4. Nitrification and denitrification associated with white and red *L. pertusa* from the Trondheim-fjord (average± SD).

NH_4^+-assimilation

Corals assimilated NH_4^+ into their tissue and no significant differences in the assimilation rates were found between locations, colour morphs or incubation duration (Kruskal-Wallis $p > 0.05$). White and red *L. pertusa* from the Trondheim-fjord assimilated NH_4^+ at an average rate (5 + 10 days) of 139 ± 22 ng N g^{-1} DW d^{-1} and 191 ± 59 ng N g^{-1} DW d^{-1} respectively while corals from the Tisler Reef assimilated NH_4^+ at a rate of with 261 ± 155 ng N g^{-1} DW d^{-1} (Fig. 3.5.). These rates are in the same order of magnitude as the assimilation rates calculated after Blackburn (1979) from data gained during the nitrification experiment (Table 3.3.). The slightly higher values after Blackburn most likely result from differences in NH_4^+ availability between the experiments (~ 1 to ~ 3 µM NH_4^+ during the nitrification experiment versus ~0.5 µM during the assimilation experiment).
Assimilation of NH_4^+ was however not only detected in the coral tissue but also in the hydrolysable amino acids (HAAs) of white *L. pertusa* from the Tisler Reef. NH_4^+ was incorporated into THAAs at a rate of 27 ± 13 ng N g^{-1} DW d^{-1} (Fig. 3.6.A), corresponding to about 10% of total N incorporation. Hereby methionine,

asparagine and glutamine showed the highest N tracer incorporation and accounted for 65% of the N uptake in HAAs (Fig. 3.6.B).

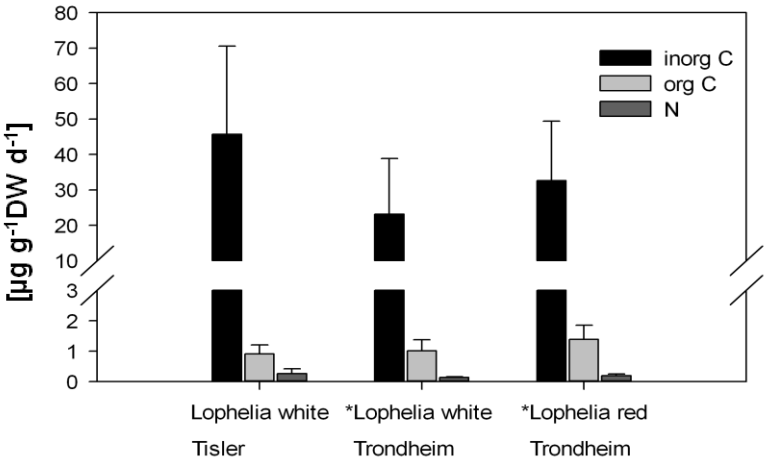


Figure 3.5. Assimilation of NH_4^+ and fixation of HCO_3^- in bulk tissue and HCO_3^- incorporation into the coral skeleton of white and red *L. pertusa* from the Trondheim-fjord and white *L. pertusa* from the Tisler Reef.

HCO_3^--fixation in coral tissue and skeleton

Coral calcification based on HCO_3^- incorporation into coral skeleton was not significantly different between location, colour morphs or incubation time (Kruskal-Wallis $p > 0.05$). White and red *L. pertusa* from the Trondheim-fjord incorporated tracer C into coral skeleton at an average rate (5 + 10 days) of 23 ± 16 µg C g^{-1} DW $d^{-1}$and 33 ± 17 µg C g^{-1} DW d^{-1} respectively while corals from the Tisler Reef showed an incorporation rate of 46 ± 25 µg C g^{-1} DW d^{-1} (Fig. 3.5.).
Dissolved inorganic carbon fixation in white and red *L. pertusa* tissue from the Trondheim-fjord and in tissue from white *L. pertusa* from the Tisler Reef was not significant different between locations, colour morphs or incubation time (Kruskal-Wallis $p > 0.05$). HCO_3^- was fixed into the coral tissue at a rate of 1016 ± 363 ng C g^{-1} DW $d^{-1}$and 1394 ± 464 ng C g^{-1} DW d^{-1} by white and red *L. pertusa* from the Trondheim-fjord and with 913 ± 294 ng C g^{-1} DW d^{-1} by white corals from the Tisler Reef (Fig. 3.5.).

The fixation of HCO_3^- was also evident in tissue components investigated in white *L. pertusa* from the Tisler Reef, where a total of 255±78 ng C g^{-1} DW d^{-1} was incorporated into the THAAs pool (Fig. 3.6.A), corresponding to about 28% of total C assimilated. Highest tracer incorporation was observed in asparagine, methionine and glutamine (77% of total HAA uptake, Fig. 3.6.B). HCO_3^- was also incorporated into TFAs with 5.5 ± 2.8 ng C g^{-1} DW d^{-1} and into PLFAs with 1.2 ± 0.3 ng C g^{-1} DW d^{-1}. Within the PLFA pool tracer C incorporation was highest for PLFAs with a chain length ≤ C20:0 (67% of the total PLFA uptake). Among them especially C16:0, C16:1ω7, C18:1ω7c, C18:1ω9c and C20:0 showed highest tracer incorporation (Fig. 3.6.C). But also PLFA with a chain length >20 showed tracer incorporation, mainly represented by C22:1ω9c and C22:4ω6 (each ~5% contribution to uptake) followed by C22:5ω3 and C20:5ω3 (each ~4% contribution to uptake).

Discussion

Cold-water corals are living in a food-depleted environment and here we show that inorganic C was not only used for calcification, but also fixed by the coral holobiont through chemoautotrophy. Moreover, we directly demonstrated that all targeted processes of the nitrogen cycle were actively mediated by the cold-water coral holobiont *L. pertusa* (Fig. 3.1.). Microbes which were found associated with the coral can perform a lot of these processes (Neulinger et al., 2008; Kellogg et al., 2009; Galkiewicz et al., 2011), but the activity of the processes has not been investigated so far. Here it is shown for the first time that these processes co-occur and that white and red *L. pertusa* holobionts function similarly for the investigated processes (N_2 fixation, nitrification, ammonium assimilation, growth and calcification), but for denitrification.

N_2-fixation

N_2-fixation in the ocean is mostly associated with cyanobacteria in surface water that use solar energy to drive the energy demanding process. In this study we demonstrated that both colour morphs of the cold-water coral *L. pertusa* perform N_2 fixation in the dark ocean. Since nitrogen fixation is mediated exclusively by microbes, this hints at the existence of a symbiotic relationship within the holobiont. Lesser et al. (2007) showed the presence of symbiotic cyanobacteria in a tropical corals. Recently Neulinger et al. (2008), Kellogg et al. (2009) and Galkiewicz et al. (2011) found gene sequences of cyanobacteria and the bacterial

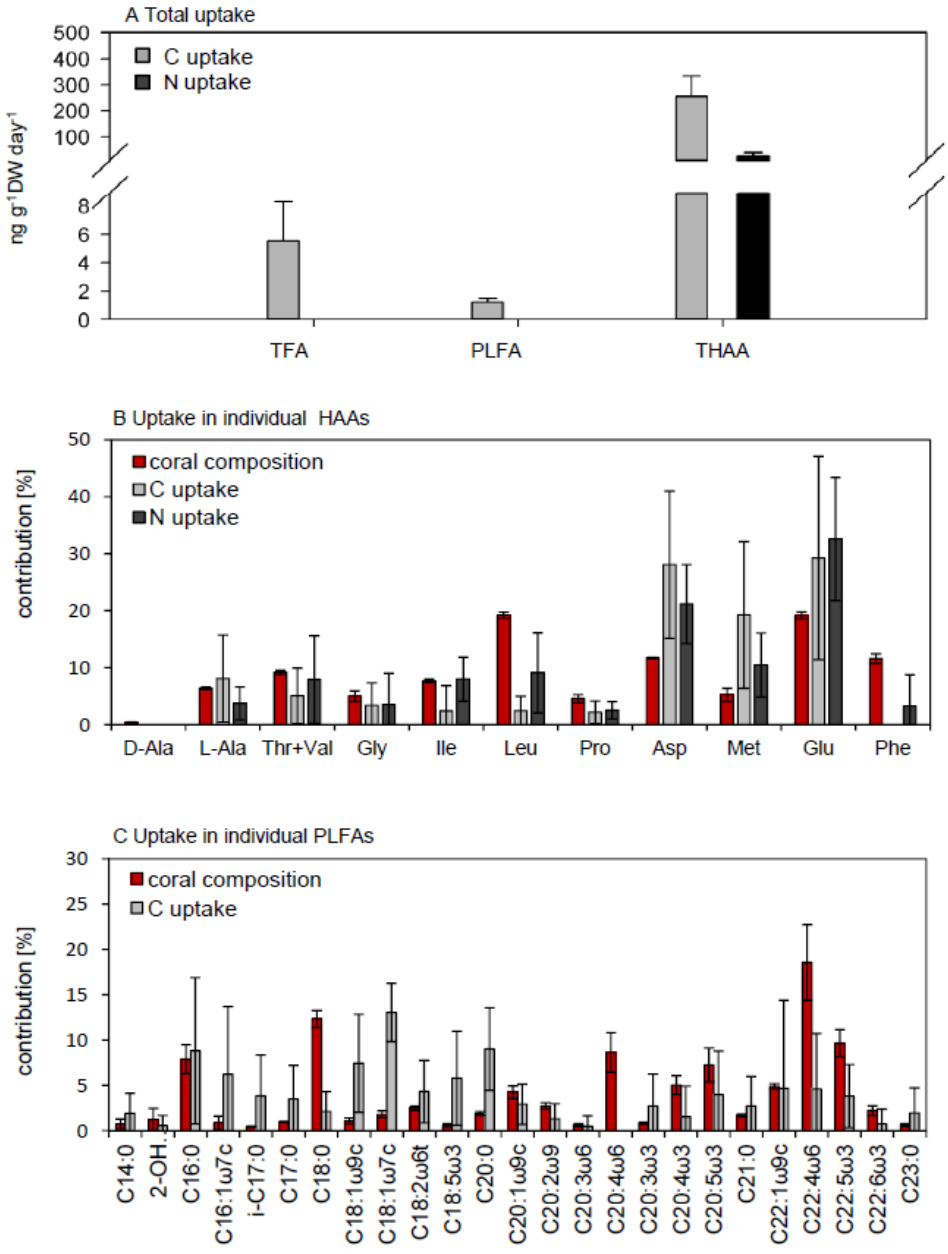


Figure 3.6. Assimilation of NH_4^+ and fixation of HCO_3^- into tissue components (TFAs, PLFAs, THAAs) of white *L. pertusa* from the Tisler Reef. (A) Total assimilation of NH_4^+ and fixation of HCO_3^-, (B) assimilation of NH_4^+ and fixation of HCO_3^- into individual HAAs and (C) HCO_3^- fixation into individual PLFAs.

genus *Vibrio* in *L. pertusa* samples, each of them able to perform the required metabolic pathways (Urdaci et al., 1988; Lesser et al., 2007). These or related strains may be involved in N_2 fixation by the cold-water coral holobiont.

However, the microbial symbionts in *L. pertusa* are most likely supported by organic compounds released by their coral host to fuel the N_2 fixation rather than by photoautotrophy. While heterotrophy favours the activity of the oxygen sensitive N_2-fixation enzyme nitrogenase by lowering oxygen concentrations due to respiration (Lesser et al., 2007), the reutilization of coral released organic waste products (mucus) might also reduces the loss of C via symbiotic recycling. Accordingly, observations by Wild et al. (2009) suggest that the microbial community inhibiting coral mucus might play a key role in its degradation and recycling. Heterotrophic N_2 fixation therefore might not only increase the N supply but at the same time might conserve energy within the coral holobiont, probably of significant importance in a food-limited environment such as cold-water coral reefs.

Nitrification

Nitrification in both colour morphs of the cold-water coral *L. pertusa* was presumably mediated by nitrifying microbes. *Nitrospira*, a nitrite-oxidiser, might be involved in the process, since related bacterial sequences were found by Yakimov et al. (2006) and Galkiewicz et al. (2011) in *L. pertusa*. The rate of nitrification observed in this study was 45 nmol N $polyp^{-1}$ d^{-1}, which is one order of magnitude smaller than the one (~500 nmol N $polyp^{-1}$ d^{-1}) inferred from nutrient fluxes (NO_3^-) reported by Maier et al. (2011). It is however possible that we have underestimated nitrification rates with our approach if most of the ammonium utilized comes directly from the coral instead as from the surrounding water, where the tracer was injected. This might be the case when the respective microbes are hosted in the coral tissue, where they come primarily in contact with NH_4^+ excreted by the coral in contrast to microbes inhibiting the mucus layer of the coral with direct access to inorganic nutrients from the surrounding water.

Interestingly our result indicate that NH_4^+ is primarily assimilated rather than nitrified as the rate of assimilation exceeded the one of nitrification by an order of magnitude. This may either be due to different sites of assimilation and nitrification (tissue/ mucus layer) and therefore different ammonium sources (external versus internal) or because assimilating microbes outcompete nitrifying microbes for ammonium (e.g., Verhagen et al., 1992).

Denitrification

Denitrification occurs primarily under anaerobic conditions, however denitrification was observed simultaneously with nitrification in both colour morphs of *L. pertusa*. The higher rates in white *L. pertusa* are hereby consistent with the finding of mixotrophic Rodobacteraceae predominantly in white *L. pertusa* (Neulinger et al., 2008), a family which includes denitrifiers (Swingley et al., 2007).

It is unclear where denitrification occurs in *L. pertusa*, but the required anaerobic conditions may indicate that denitrification occurs during polyp retraction (Buhl-Mortensen, 2001), in micro-niches in the coral mucus layer as assumed in tropical corals (Kellogg, 2004), or in the gut system.

NH_4^+-assimilation

We showed for the first time that the cold-water coral *L. pertusa* assimilates ammonium into its amino acids and tissue, comparable to their tropical counterparts (Muscatine and D'Elia, 1978; Wegley et al., 2007; Kimes et al., 2010). *L. pertusa* is known to release ammonium into the surrounding seawater, but apparently the reassimilation reduces some of the gross losses. The high tracer recovery in glutamine is in agreement with the main assimilation pathway of NH_4^+ involving the activity of the enzyme complex glutamine synthetase glutamate synthase (Anderson and Burris, 1987). The high tracer recovery in asparagines and methionine follows the further processing in the amino acids metabolism, in which glutamine among others is used as amino acid group donor for production of other amino acids such as asparagine.

The formation of methionine in this pathways is particularly interesting, because it is considered an essential amino acid, which many animals are considered either incapable of synthesizing or only synthesizing it in insufficient amounts to meet their metabolic needs (Fitzgerald and Szmant, 1997). However our results are in agreement with observation on tropical corals by Fitzgerald and Szmant (1997), who also found de novo methionine synthesis in corals, indicating that the coral holobiont is indeed able to synthesize putative "essential" amino acids.

Fixation of HCO_3^- in coral tissue

L. pertusa showed significant rates of inorganic carbon fixation in tissue, which suggested that up to 2% of coral C requirements could be obtained by this process (comparing Dodds et al., 2007 and data of this study). Fixation of HCO_3^- into

individual PLFAs of white *L. pertusa* tissue further indicated that bacteria are probably involved in this autotrophic fixation. ^{13}C tracer was mostly recovered in the PLFA C16:1ω7 and C18:1ω7 which are characteristically dominant in nitrifying bacteria (Guezennec and Fiala-Medioni, 1996; Lipski et al., 2001; de Bie et al., 2002), consistent with the observed nitrification activity (see above), and in sulphur-oxidizing bacteria (Zhang et al., 2005; van Gaever et al., 2009). The observed stoichiometry of 100 µmol HCO_3^- fixed for about 1 µmol NO_3^- produced differs from the typical stoichiometry of 0.1 µmol HCO_3^- fixed for 1 µmol NO_3^- produced of nitrifiers (Belser et al., 1984). We therefore suggest that other chemoautotrophs, such as sulphur oxidizing bacteria of which sequences have been observed in *L. pertusa* (Neulinger et al., 2008), may also have contributed to the fixation of HCO_3^- in the coral tissue.

Interestingly, we also found long chain PLFAs such as C22:1ω9c, C22:4ω6, C22:5ω3 and C20:5ω3 labelled within 4 days. These PLFAs have been produced de novo by the coral since bacteria generally only produce short chain PLFA which indicates that a trophic transfer of ^{13}C from the bacterial symbionts to the coral host has taken place within a short period of time. This evidently shows that chemoautotrophic bacteria can supplement coral carbon and energy demand. However, whether coral preys on chemoautotrophic bacteria as proposed for bacteria inhabiting the mucus layer (Coles and Strathma, 1973; Weinbauer et al., 2012) or whether the bacteria release certain products, which are taken up by the coral still need to be resolved.

Calcification

Corals can obtain dissolved inorganic carbon for calcification from the surrounding seawater or from metabolically impacted internal pools. As expected *L. pertusa* used inorganic C to sustain calcification at rates of 40 ± 22 µg C g^{-1} DW d^{-1}. These calcification rates are very similar to those reported for *L. pertusa* by Maier et al. (2009) based on ^{45}Ca technique (23-78 µg C g^{-1} DW d^{-1}). Mueller et al. (2013) reported calcification based on metabolically derived carbon and obtained rates varying from 0.1-1.6 µg C g^{-1} DW d^{-1} for corals from Tisler Reef indicating that most carbon used for calcification comes from the environment.

Interestingly, both colour morphs had comparable calcification rates, although it was formerly suggested that the differences between the microbial community structure of red and white *L. pertusa* might affect coral nutrition and therefore competiveness and abundance in situ (Neulinger et al., 2008). Based on our results however it appears that calcification and therefore coral growth does not differ

between colour morphs and is therefore not affected by the differences in the microbial community. Nevertheless metabolic differences might still exist, especially since calcification in *L. pertusa* seems to be less connected to other metabolic processes such as respiration (Larsson and Purser, 2011; Form and Riebesell, 2012).

Conclusion

In this study we show for the first time that the cold-water coral *L. pertusa* utilizes inorganic substrates to gain energy and produce organic C and N, presumably through symbiotic relationships with microbial symbionts. *L. pertusa* is able to perform NH_4^+ assimilation and regeneration, N_2 fixation, nitrification and denitrification but also bicarbonate fixation, independent of the colour morph investigated. Chemoautotrophs were identified to fix bicarbonate and transfer organic carbon to the coral. On the one hand, N_2-fixation, NH_4^+-assimilation and HCO_3^--fixation provides corals with organic nitrogen and carbon, while on the other hand, heterotrophic N_2-fixation, nitrification and denitrification may recycle some of the energy that is stored in the coral waste products. Our results further document the nutritional flexibility of *L. pertusa*: besides various organic food sources (Mueller et al., 2013, chapter 2), we have shown that they can obtain organic carbon and nitrogen from inorganic substrates through symbiotic relations with microbes. This metabolic diversity of the holobiont is a key to survive in oligotrophic conditions.

Acknowledgements

Lisbeth Jonsson, the crew from the Poseidon (GEOMAR, Kiel, Germany) and the JAGO- team, J. Schauer and K. Hissmann (GEOMAR, Kiel, Germany), are thanked for their help in coral sampling. Ann Larsson is thanked for providing valuable input and pictures on the experimental set up. Pieter van Rijswijk is thanked for help whenever it was needed the most. The analytical lab of NIOZ-Yerseke is thanked for sample analysis. This research was supported by the Calmaro project (FP7/2007-2013), funded by the European Community's Seventh Framework Program (FP7/2007-2013).

4

Symbiosis between *Lophelia pertusa* and *Eunice norvegica* stimulates coral calcification and worm assimilations

Photo by S. Zankl

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Published in PLoS ONE 8(3): e58660. doi:10.1371/journal.pone.0058660

Abstract

We investigated the interactions between the cold-water coral *Lophelia pertusa* and its associated polychaete *Eunice norvegica* by quantifying C and N budgets of tissue assimilation, food partitioning, calcification and respiration using ^{13}C and ^{15}N enriched algae and zooplankton as food sources. During incubations both species were kept either together or in separate chambers to study the net outcome of their interaction on the above mentioned processes. The stable isotope approach also allowed us to trace metabolically-derived tracer C further into the coral skeleton and therefore estimate the effect of the interaction on coral calcification. Results showed that food assimilation by the coral was not significantly elevated in presence of *E. norvegica* but food assimilation by the polychaete was up to 2 to 4 times higher in the presence of the coral. The corals kept assimilation constant by increasing the consumption of smaller algae particles less favoured by the polychaete while the assimilation of *Artemia* was unaffected by the interaction. Total respiration of tracer C did not differ among incubations, although *E. norvegica* enhanced coral calcification up to 4 times. These results together with the reported high abundance of *E. norvegica* in cold-water coral reefs, indicate that the interactions between *L. pertusa* and *E. norvegica* can be of high importance for ecosystem functioning.

Introduction

In the North East Atlantic, the scleractinian cold-water coral *Lophelia pertusa* is the dominating reef forming species. Its complex framework offers a multitude of different habitats (Buhl-Mortensen et al., 1995; Freiwald et al., 2004; Buhl-Mortensen et al., 2010) used by a great variety of species (Frederiksen et al., 1992; Jonsson et al., 2004; Henry and Roberts, 2007). Among 1300 documented species (Roberts et al., 2006) 29 symbiotic relations between scleractinian cold-water corals and associated invertebrates have been reported (Buhl-Mortensen and Buhl-Mortensen, 2004). So far, most of these relationships are not clearly defined and their role in the functioning of the ecosystem is poorly understood (Buhl-Mortensen and Buhl-Mortensen, 2004; Roberts et al., 2009a).

One ubiquitous species that is abundantly (12-17 ind. m^{-2}, based on our data and the following references (Buhl-Mortensen and Fosså 2006; van Oevelen et al., 2009) observed in close contact with the cold-water coral *L. pertusa* is the polychaete *Eunice norvegica* (Zibrowius H. et al., 1975; Wilson, 1979; Winsnes, 1989). It forms parchment-like tubes within living coral branches which later are calcified by its coral host (Buhl-Mortensen, 2001). Roberts (2005) suggested that *E. norvegica* strengthens the reef framework by thickening and connecting coral branches. Moreover, by aggregating coral fragments the polychaete might enhance the development of large reef structures (Roberts, 2005). However, the relationship might come at a metabolic cost for the coral due to enhanced precipitation of $CaCO_3$. So far however, no data is available to quantify this aspect of the relationship between coral and polychaete.

Additional to the indirect metabolic effect via calcification the polychaete also might have a more direct effect on coral metabolism: Aquaria observations by Buhl-Mortensen (2001) have shown that *E. norvegica* occasionally steals food from its host coral while at the same time it cleans the living coral framework from detritus and protects it from potential predators through aggressive territorial behaviour. Again, the net outcome of these different processes on the metabolism of the coral and the polychaete has never been quantified.

Symbiosis are long term interactions between different biological species (de Bary, 1879; Paracer and Ahmadjian, 2000) which can involve positive (mutualism (++), commensalism (+0)) and negative feedbacks (competition (--), parasitism (-+)) between the species (Dales, 1957; Addicott, 1984). Based on qualitative observations the relations between *L. pertusa* and *E. norvegica* range from parasitic (food stealing) to mutualistic (cleaning and protection of coral branches) (Buhl-Mortensen, 2001; Roberts, 2005). However to better estimate the net outcome of

the interplay of the different process involved quantitative data, especially with respect to species metabolism are necessary (Martin, 1988). These data will further help to assess the significance of this interaction for the structure and functioning of cold-water coral reefs, which can be crucially influenced by species interactions (Stachowicz, 2001).

In this study we directly quantify the interactions between *L. pertusa* and *E. norvegica* with respect to food assimilation, calcification and respiration, key processes in species metabolism and highly involved in the interaction between *L. pertusa* and *E. norvegica.* To trace and quantify these processes with respect to the interaction between both species we used two ^{13}C and ^{15}N labelled food sources that are considered important for cold-water coral reef communities, i.e. algae and zooplankton (Duineveld et al., 2004; Dodds et al., 2009; Duineveld et al., 2012). The use of two food sources also allowed us to investigate food competition and niche segregations between *L. pertusa* and *E. norvegica*. During the experiment corals and polychaetes were either kept separate or in association with each other to allow singling out observe the net outcome of the influence of association on different metabolic processes. By using isotopically enriched food sources we were able to directly trace not only carbon and nitrogen tissue assimilation by *L. pertusa* and *E. norvegica,* but also calcification based on metabolically derived C-deposition in coral skeleton. Metabolically derived C, i.e. inorganic C originating from respired food, is one of the two sources that sustain the demand for inorganic C by calcification (Furla et al., 2000). Since ^{13}C-labelled food was used in our experimental design, the subsequent deposition of respired ^{13}C into the coral skeleton was used as proxy for calcification as discussed below.

Methods

Sampling location and maintenance

All coral pieces and polychaetes used in the experiments were obtained from the Norwegian Tisler Reef, with all necessary permits obtained from the Directorate of Fisheries, Norway to conduct the described study. The reef is situated at 70 to 155 m depth in the NE Skagerrak, close to the border between Norway and Sweden. Throughout the year, the current velocity over the reef normally varies from 0 to 50 cm s^{-1}, with peaks in excess of 70 cm s^{-1}, while the flow direction fluctuates irregularly between NW and SE (Lavaleye et al., 2009; Wagner et al., 2011). Temperature at the reef site typically varies between 6 to 9ºC throughout the year (Lavaleye et al., 2009; Wagner et al., 2011). The amount and quality of particulate

organic carbon (POC) reaching the reef depends on the location within the reef, so that POC concentrations can range from 43.5 to 106.3 µg C l^{-1} (Wagner et al., 2011).

Specimens were collected from a depth of around 110 m (N58°59,800' E10°58,045') with the remotely operated vehicle (ROV) Sperre Subfighter 7500 DC. Within a few hours they arrived in the laboratory at the Sven Lovén Centre in Tjärnö (Sweden) in cooling boxes filled with Koster-fjord seawater (7ºC, salinity 31). Before used in the experiment organisms were kept 1 - 8 days for acclimation in a dark temperature-controlled laboratory at 7ºC with sand-filtered running seawater from 45 m depth out of the adjacent Koster-fjord (sand particle size 1 - 2 mm, water exchange rate of ca 1 l min^{-1}). No additional feeding was offered during the maintenance period since the sand-filtered Koster-fjord water still contained a lot of small particles (pers. observation) which could be used as food source. During the acclimation phase corals were kept without polychaetes, while polychaetes were kept in aquaria with coral rubble including also living colonies in response to their need of shelter. One day before used in the experiment larger coral colonies were clipped to approximately the same size, determined by dimension and buoyant weight, to allow using comparable coral samples per chamber. Polychaetes were selected solely on the base of their dimension. After the experiment all samples were also measured for dry weight (DW) to allow standardization among treatments.

Preparation of particulate labelled substrates

Cold-water corals are exposed to various food particles and they are considered to feed on a mixed diet including phyto- and zooplankton (Duineveld et al., 2004; Kiriakoulakis et al., 2005; Duineveld et al., 2007; Dodds et al., 2009). The diatom *Thalassiosira pseudonana* (5µm) was chosen to represent small particulate organic matter (POM) substrates reaching cold-water coral reefs (Duineveld et al., 2004). They were cultured axenically in f/2 medium adapted after Guillard (1975) on the base of artificial seawater in which 90 % of either the unlabelled $NaHCO_3$ or $NaNO_3$ were exchanged with the isotopically enriched equivalent (Cambridge Isotopes, 99 % ^{13}C, 99 % ^{15}N). After 3 weeks of culturing cell densities were around $3\text{-}4*10^6$ cells ml^{-1} and the algal suspension was concentrated by centrifugation at 450 g. The concentrates were rinsed three times with 0.2 µm filtered seawater to remove residual label. The isotopic enrichment of the algal concentrates were 59 % ^{13}C ($\delta^{13}C$ 125908 ‰) and 64% ^{15}N ($\delta^{15}N$ 472590 ‰),

respectively. They were stored frozen until use in the experiment (for details on isotope analysis, see below).

Artemia spp. nauplii (~300 µm) were chosen to represent large zooplankton-derived POM substrates reaching cold-water coral reefs (Dodds et al. 2009). They resemble natural zooplankton and have been successfully used in food studies on *Lophelia pertusa* before (Purser et al., 2010; Tsounis et al., 2010; Naumann et al., 2011). ^{13}C and ^{15}N enriched nauplii were cultured by hatching 0.6 g *Artemia* cysts (Sera) in 10 l incubation chambers filled with 0.2 µm filtered seawater under natural light conditions and mild aeration. After the larvae had developed to a state at which they take up particulate food (1 to 2 days after eclosion of larvae), larvae were fed every second day with a suspension containing ^{13}C or ^{15}N enriched pre-cultured algae (at around 1.5 mg C l^{-1} and 0.15 mg N l^{-1} respectively). The uptake of algae was confirmed visually under the microscope as green food particles in the animal guts. After seven days of feeding, larvae were concentrated by filtration on a 200 µm filter, rinsed off the filter with filtered seawater, counted under the binocular and stored frozen. Within the *Artemia* concentrate different early larval stages could be detected. The final isotopic enrichment of the larvae was 4 % ($\delta^{13}C$ 2909 ‰) for ^{13}C and 7 % ($\delta^{15}N$ 21800 ‰) for ^{15}N respectively.

To standardize the amount of carbon added to the incubations, substrates were analyzed for carbon and nitrogen content (see below for methodological description). The 1 : 1 mixture of ^{13}C Artemia : ^{15}N algae and ^{13}C algae : ^{15}N *Artemia*, both at total concentration of 800 µg C l^{-1}, represented the two food treatments. Together with the treatments "*Lophelia* separate", "*Eunice* separate" and "*Lophelia* - *Eunice* together" gives a total of 6 treatments, each of them performed in triplicate.

Experimental set up and procedure

Incubation chambers (Fig. 4.1.) were placed in a thermo-controlled room at 7 °C and filled with 5 µm filtered Koster-fjord bottom water prior to the start of the experiment. A total of 29 coral fragments (2 to 3 coral pieces $chamber^{-1}$, 3.2 ± 1.33g DW $piece^{-1}$; 9.78 ± 1.16g DW $chamber^{-1}$; 16.55 ± 5.43 polyps $chamber^{-1}$, with no significant difference of coral weight between treatments) and 12 polychaete specimens (1 polychaete $chamber^{-1}$, 0.48 ± 0.12g DW $polychaete^{-1}$, with no significant difference of polychaete weight between treatments) were selected and placed separately or together in the middle of the incubation chambers. To stabilize the corals fragments in an upright position, they were gently inserted into a 1 cm elastic silicone tube on an acrylic plate that was attached on the chamber

base. To provide refuge space in treatments with only polychaetes present bleached coral skeleton and plastic tubes were placed in the chambers. Both substitutes were indeed used as refuge by the polychaetes during the experiment, although the tubes were not present during the acclimation phase. Chambers with corals and polychaetes contained only living coral specimens, used as shelter by the polychaete during the incubation. Water circulation was maintained during the experimental period by a motor-driven paddle in the upper part of the incubation chamber (Fig. 4.1., rotor speed 30 rpm). The motor section was not directly attached to the incubation chamber and effectively avoided heating of the chambers during the incubations. Corals and polychaetes were left in the chamber for 12 h for acclimatization prior to feeding.

At the start of the experiment, 400 µg C l^{-1} of each food source was gently pipetted into the water column of each chamber. The chambers were closed from flow through for 2.5 days to allow feeding (feeding period). Visual observation confirmed that the circulating water kept the food particles in suspension. After the feeding period, the chambers were flushed to remove remaining food particles and waste products by pumping 5 µm filtered Koster-fjord bottom water through the chambers at a flow speed of 140 ml min^{-1} for 12 h. This pattern was conducted twice. After the last flushing period (140 ml min^{-1}, 5 µm filtered Koster-fjord water, lasting12h), incubation chambers were closed without food addition for another 24 h. During this period the respiration of the added ^{13}C enriched food substrates was quantified by measuring the production of dissolved inorganic ^{13}C in the water (^{13}C-DIC) (Moodley et al., 2000; de Goeij et al., 2008; Gontikaki et al., 2011). Water samples were taken before (control) and after the respiration incubation, and filtered (GF/F) in a 20 ml headspace vial. Each sample was poisoned with 10 µl $HgCl_2$, closed with an aluminum cap fitted with a rubber septum and stored upside down.

In parallel to the main experiment, 3 control corals and 3 control polychaetes were incubated without food for stable isotope ^{13}C and ^{15}N background measurements. After a total experimental time of 7.5 days, coral and polychaete samples were frozen at -20°C and transported to the Royal Netherlands Institute for Sea Research-Yerseke, where they were freeze-dried and stored frozen for further analysis.

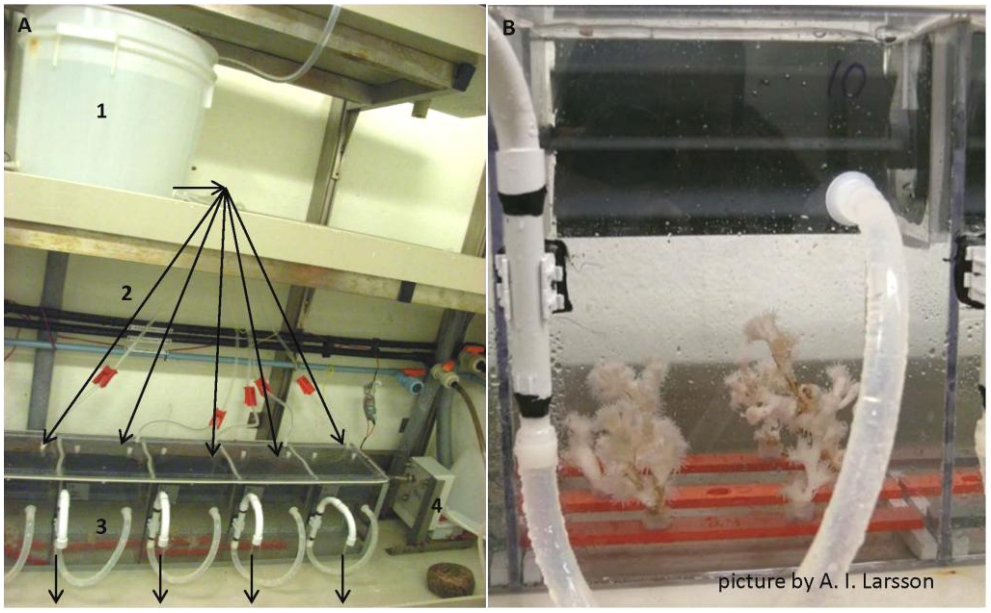


Figure 4.1. Experimental set up (Mississippi chambers). (A) Experimental set up including the water reservoir for filtered sea water (1), the direction of the water flow indicated with arrows (2), 5 incubation chambers (3) and the motor driving the chamber paddles (4). (B) Close-up of one incubation chamber (10 l) provided by A. I. Larsson.

Sample treatments and analysis

Tissue assimilation

Prior to isotopic analysis frozen coral and polychaete samples were freeze-dried, weighed and homogenized by grinding with a ball Mill for 20 s (MM 2000, Retsch, Haan, Germany). A subsample of around 30 mg of grinded coral material and 2 - 3 mg grinded polychaete material was transferred to pre-combusted silver boats and decalcified by acidification. While polychaete samples were directly acidified with concentrated HCl (12 mol l^{-1}), coral samples were first placed in an acidic fume for 3 to 4 days to remove most of the inorganic C. Coral samples were then further acidified by stepwise addition of HCl with increasing concentration (maximum concentration 12 mol l^{-1}) until the inorganic carbon fraction (skeleton) was fully removed (as evidenced by the absence of bubbling after further acid addition). The remaining fraction after acidification resembled the organic fraction of each samples, which in case of the coral samples includes the coral tissue and the organic matrix in the skeleton that represents only a very small organic fraction

(Allemand et al., 1998). After complete decalcification each sample was measured for total ^{13}C and ^{15}N using a thermo Electron Flash EA 1112 analyzer (EA) coupled to a Delta V isotope ratio mass spectrometer (IRMS).

All obtained stable isotope data were expressed in µg C/ g C biomass and µg N/ g N biomass. They were calculated as following on the base of the delta notations obtained from the IRMS: δX (‰) = $(R_{sample}/R_{ref} -1)*1000$, where X is the element, R_{sample} is the heavy : light isotope ratio in the sample and R_{ref} is the heavy:light isotope ratio in the reference material (Vienna Pee Dee Belemnite standard for C and atmospheric nitrogen for N). When used for C the R_{ref} = 0.0111797 and when used for N then R_{ref} = 0.0036765. The atomic % of heavy isotope in a sample is calculated as $F = R_{sample}/(R_{sample}+1)$. The excess (above background) atm% is the difference between the F in an experimental sample and the atm% in a control sample: $E = F_{sample} - F_{control}$. To correct for differences in enrichments of the food sources, the excess incorporation was divided by the atm% of each specific food source.

Calcification (C incorporation in coral skeleton)

To measure the incorporation of C in coral skeleton 30 mg of each coral sample (including tissue, organic matrix and skeleton) was directly transferred to a silver boat and measured on the EA-IRMS for total ^{13}C content. The same calculations used to calculate tracer C tissue assimilation were then used to calculate tracer C incorporation in the total C pool of the coral sample. Incorporation of tracer C in the inorganic skeleton was finally determined by subtracting tracer C assimilation in the organic carbon fraction (tissue and organic matrix) from the tracer C incorporation in the total C pool (tissue, organic matrix and skeleton). This allowed us to trace the metabolically derived C, i.e. from food respiration, into the coral skeleton and to quantify calcification rates based on this C source. Although this calcification process may be of limited importance to total calcification by cold-water corals (Adkins et al., 2003), a comparison can reveal calcification differences between treatments. Another advantage is that small changes within a small time period can be detected, similar to the ^{45}Ca labelling method (Tambutté et al., 1995; Maier et al., 2009), but without the necessity of radioactive isotopes.

Respiration

Respiration of labelled food substrates was measured by analyzing the concentration and isotopic ratio of the CO_2 in the water samples taken at the beginning and the end of the 24 h incubation at the end of the experiment. After creating a headspace of 3 ml in each sample vial by injecting N_2 gas through the

vial septum (Miyajima et al., 1995; Moodley et al., 2000), samples were acidified with 20 µl of concentrated H_3PO_4 to transform DIC into CO_2. After CO_2 had exchanged with the vial headspace 10 µl sample of the headspace gas was injected into an elemental analyzer isotope-ratio mass spectrometer (EA-IRMS). The final calculations for tracer C respiration followed the description for tracer C tissue assimilation.

Statistics

The program PERMANOVA (Anderson, 2005) was used to investigate interactions between the different factors (food and treatment) by permutational multivariate analysis of variance (PERMANOVA). The outcome of each PERMANOVA test was expressed in Monte Carlo P-values, which are more robust in case of smaller numbers of replicates. If the variance between data was not homogeneous (tested using Fligner-Killeen test) a Kruskal-Wallis test was used as true non-parametric approach.

Results

Tissue assimilation and respiration by *Eunice norvegica*

E. norvegica assimilated in total 95 ± 67 µg C (day * g C biomass polychaete)$^{-1}$ and 175.51 ± 83.05 µg N (day * g N biomass polychaete)$^{-1}$ in the absence of *L. pertusa* (Fig. 4.2.A). However when *L. pertusa* was present, the assimilation of *E. norvegica* was significantly enhanced 4 times for C and 2 times for N (PERMANOVA p=0.03, Fig. 4.2.A). C partitioning between different food sources did not differ significantly among treatments (PERMANOVA $p \geq 0.1$), although a trend of higher *Artemia* uptake in the presence of the coral was recorded (Fig. 4.2. B). However in both, absence and presence of corals, *Artemia* was the dominant N-source for *E. norvegica*, accounting for 87% of total assimilated N in polychaete tissue when corals were present to 91% when corals were absent (PERMANOVA $p \leq 0.02$, Fig. 4.2.B).

During incubations without corals, *E. norvegica* respired 1423 ± 1431 µg C (day * g C biomass polychaete)$^{-1}$. Most of the C respired by the polychaete in these incubations was derived from its feeding on *Artemia* (Fig. 4.3.A). Total respiration in incubations with only the polychaete present did not differ from incubations with *E. norvegica* and *L. pertusa* present (PERMANOVA p= 0.7, Fig. 4.3.A).

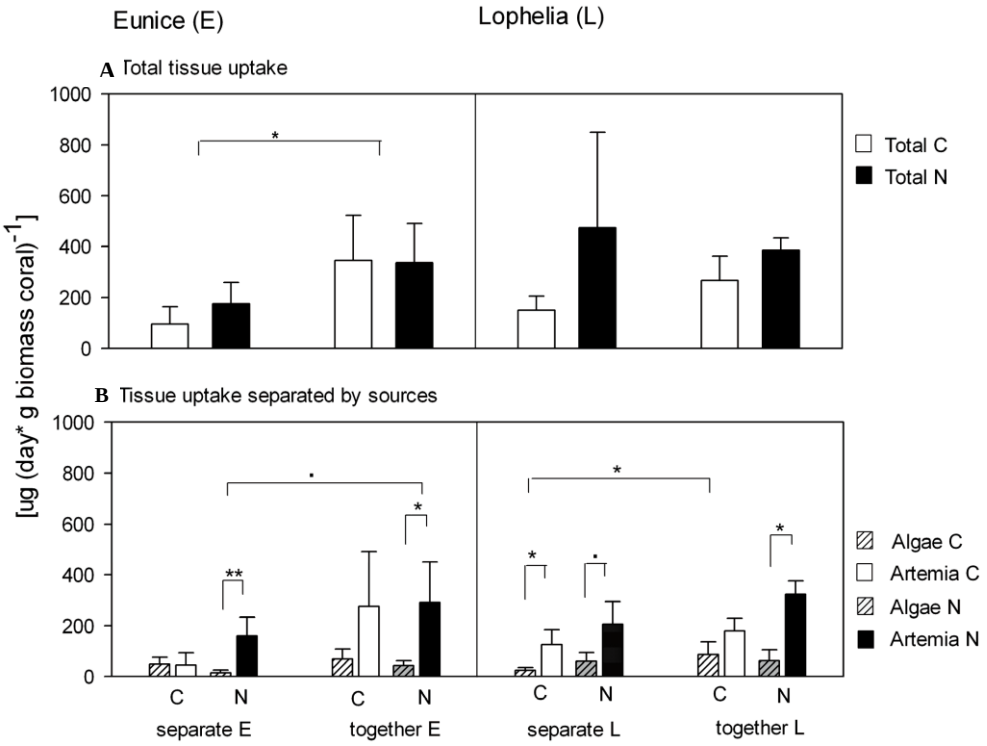


Figure 4.2. Tissue uptake by *E. norvegica* (E) and *L. pertusa* (L.). **(**A) Total C and N tissue uptake, (B) C and N tissue uptake separated by food substrate (algae, *Artemia*). Animals were incubated separate or together in one chamber; statistical significance between treatments is indicated as followed: ** $p<0.009$, * $p<0.05$, · $0.05<p<0.06$. The bars in each figure represent average ± SD.

Tissue assimilation, calcification and respiration by *Lophelia pertusa*

In total *L. pertusa* assimilated 149 ± 56 µg C (day * g C biomass coral)$^{-1}$ and 473 ± 375µg N (day * g N biomass coral)$^{-1}$ when *E. norvegica* was absent (Fig. 4.2.A). Neither the uptake of C nor the uptake of N was significantly affected by the presence of the polychaete (PERMANOVA p=0.3, Fig. 4.2.A). Even though the presence of *E. norvegica* did not change total C and N tissue assimilation of *L. pertusa*, it did change the contribution of food sources (Fig 4.2.). In the absence of the polychaete, *L. pertusa* significant partitioned between food sources and preferentially consumed *Artemia* (PERMANOVA p=0.02, Fig 4.2.B). In the presence of *E. norvegica* however *L. pertusa* increased the assimilation of algal-

derived C from 16% to 33% (PERMANOVA p=0.07, Fig. 4.2.B), which resulted in equal assimilation rates of both food sources (PERMANOVA p=0.1, Fig. 4.2.B). This trend was not visible for N assimilation, where no significant influence by *E. norvegica* on food utilization by *L. pertusa* could be observed (PERMANOVA p=1) and *Artemia* remained the dominant source of N for the coral independently of *E. norvegica* presence or absence (PERMANOVA p≤0.5, Fig. 4.2.B).

Coral respiration in the absence of *E. norvegica* accounted for 1242 ± 699 µg C (day * g C biomass coral)$^{-1}$. *Artemia* was the primary C source 15% (Fig. 4.3.A, 4.3.B). Respiration in the absence of the polychaete was not significantly different from incubations where *E. norvegica* was present (PERMANOVA p=0.5, Fig. 4.3.A) and supplied 85% of the respired C, in contrast to algae, which contributed only 15% (Fig. 4.3.A, 4.3.B). Respiration in the absence of the polychaete was not significantly different from incubations where *E. norvegica* was present (PERMANOVA p=0.5, Fig. 4.3.A).

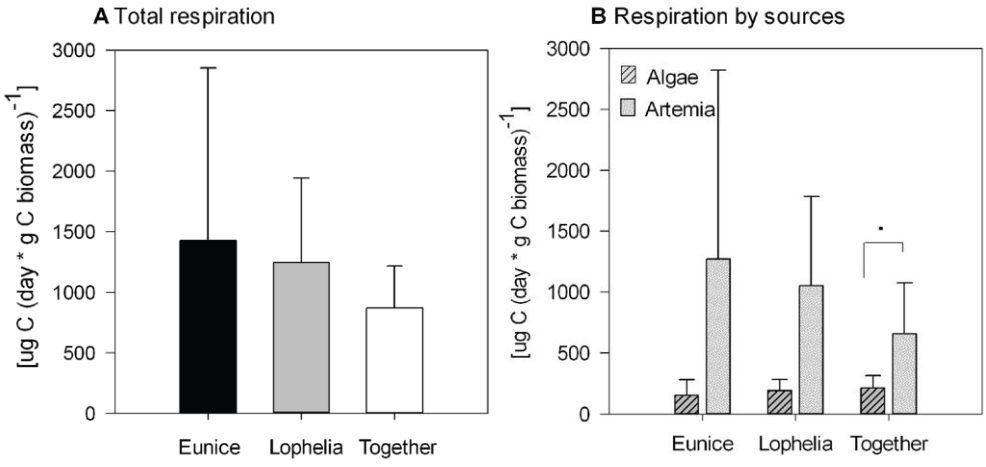


Figure 4.3. Respiration by *E. norvegica* and *L. pertusa*. (A) Total C respiration, (B) C respiration separated by food substrate (algae, *Artemia*). Animals were incubated separate or together in one chamber; statistical significance between treatments is indicated as followed: ** p<0.009, * p<0.05, · 0.05<p<0.06. The bars in each figure represent average ± SD.

Metabolic derived coral calcification was significantly enhanced up to 4 times by the presence of the polychaete (Kruskal-Wallis p=0.05, Fig. 4.4. A). On average,

Artemia contributed 68% and 85% to total inorganic C formation, whereas algae accounted for 32 % and 15 % in absence and presence of the polychaete respectively (Fig. 4.4.B). These contribution were however not significantly different (Kruskal-Wallis p=0.2, Fig. 4.4.B).

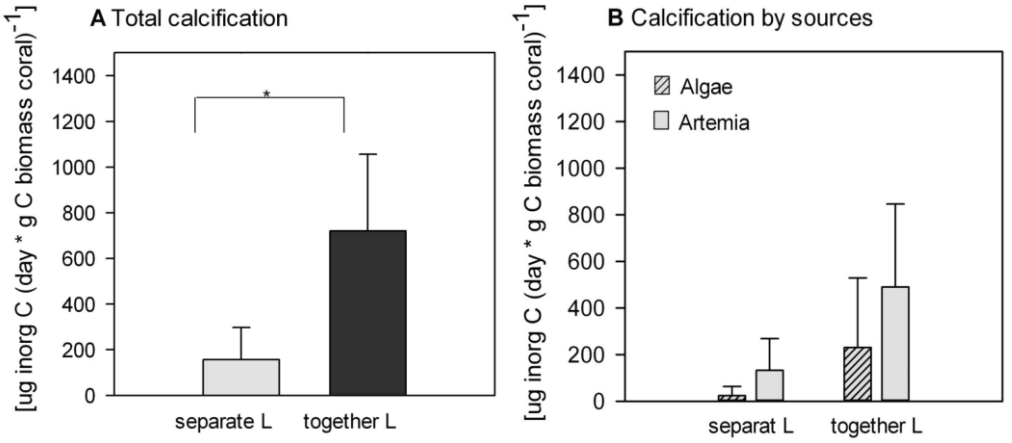


Figure 4.4. Calcification by *L. pertusa*. (A) Total calcification, (B) calcification separated by food substrate (algae, *Artemia*). *L. pertusa* was kept with and without *E. norvegica*; statistical significance between treatments is indicated as followed: ** $p<0.009$, * $p<0.05$, · $0.05<p<0.06$. The bars in each figure represent average ± SD.

Carbon budget of *Lophelia pertusa* and *Eunice norvegica* separately and together

Partitioning of C between tissue assimilation, calcification and respiration was merged into a C budget for each treatment based on total C tracer uptake by *L. pertusa* and *E. norvegica*. This revealed that in the absence of *E. norvegica, L. pertusa* invests 10% of total acquired C in tissue, 10% in calcification and 80% in respiration (Fig. 4.5.A, Table 4.1.). With *E. norvegica* present, however, this picture changed, mainly due to higher calcification rates stimulated by the presence of the polychaete. On average 14% of total acquired C was transferred into tissue, 39% was recovered in carbonate and 47% lost by respiration (Fig. 4.5.B, Table 4.1.).

For *E. norvegica,* the main change in the C budget was higher food assimilation in the presence of the coral. When *L. pertusa* was absent *E. norvegica* assimilated

6% of total acquired C in the tissue while 94% of total acquired C was lost by respiration (Fig. 4.5.C, Table 4.1.). With *L. pertusa* present *E. norvegica* increased its tissue C-uptake up to 28% while only 72% of acquired C was used for respiration (Fig. 4.5.D, Table 4.1.).

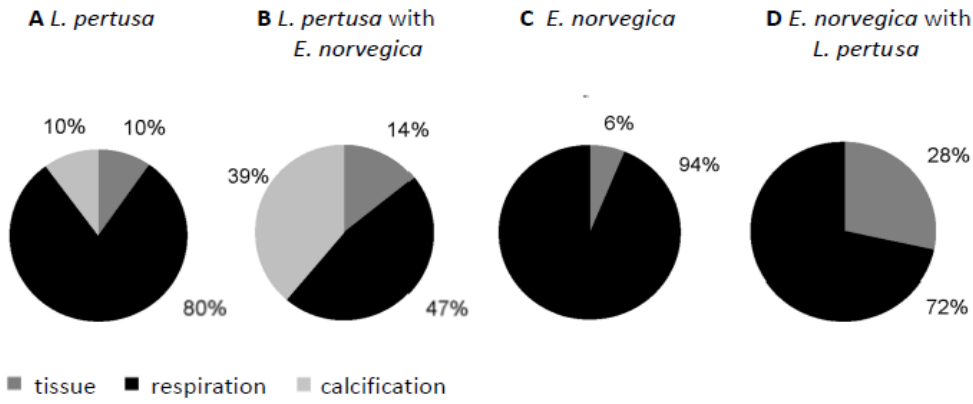


Figure 4.5. Carbon budget of *L. pertusa* and *E. norvegica*. (A) C-budget for *L. pertusa* without *E. norvegica*, (B) C-budget for *L. pertusa* with *E. norvegica*, (C) C-budget for *E. norvegica* without *L. pertusa*, (D) C-budget for *E. norvegica* with *L. pertusa*. Each budget is based on total tracer recovery. The partitioning between tissue assimilation, respiration and calcification are expressed relatively to total C uptake (sum of assimilation, respiration and calcification).

Table 4.1. C-partitioning between metabolic components of *L. pertusa* and *E. norvegica* separate and together. (n=3). Values presented in [µg C(day*g C biomass)$^{-1}$].

Component	***L. pertusa***	***L. pertusa* + *E. norvegica***	***E. norvegica***	***E. norvegica* + *L. pertusa***
Tissue	150 ± 56	266 ± 95	95 ± 68	345 ± 177
Respiration	1243 ± 700	869 ± 345	1423 ± 1431	869 ± 45
Calcification	156 ± 141	720 ± 336		

Discussion

Assimilation and calcification in the symbiotic coral-polychaete relation

Eunice norvegica

In this study we quantified the qualitative observations of the interaction between *E. norvegica* and *L. pertusa* to infer the importance of this interaction for cold-water coral ecosystems. Results revealed that *E. norvegica* assimilated 4 times more C and 2 times more N in the presence of the coral. Respiration however was independent of coral presence but well within the range of former observations (van Oevelen et al., 2009). The polychaete further tended to switch to a more selective food uptake, preferentially taking up bigger particles when *L. pertusa* was present (Fig. 4.2.B). These results are in agreement with previous behavioural observations, where *E. norvegica* has been reported to steal mainly bigger food items from its coral host (Buhl-Mortensen, 2001). We hypothesize that larger particles cause longer handling times by the coral during the process of feeding (Shimeta and Jumars, 1991; Shimeta and Koehl, 1997), which gives the polychaete more time to remove these particles from the polyp surface. Small particles might be consumed faster by the coral host and also might be more effectively anchored within the mucus layer of the coral (Sanderson, 1996; Shimeta and Koehl, 1997; Naumann et al., 2009). Since mucus is used by the coral not only to trap, but also to transport particles to its mouth, (Herndl et al., 1985; Lewis and Price, 1975), a weaker binding of larger particles within the coral mucus layer would make it easier for the polychaete to access and remove those from the coral surface.

The observed high influence of coral presence on polychaete nutrition evidences that the interaction not only provides settlement and shelter but also increases the fitness of the polychaete. Coral rubble and dead framework can also provide shelter, but they are neither able to help in tube strengthening by calcification nor in food supply, since the coral is dead. The increased food input by proximity to living corals might explain the common occurrence of *E. norvegica* within living coral branches as one of two species so far documented living in direct contact to coral tissue (Buhl-Mortensen and Fosså, 2006). The advantage of living within the live coral becomes even clearer with regard to the location of the tube selected by the polychaete and its reef aggregating behaviour described by Roberts (2005). To ensure its benefits, the polychaete places its tube openings close to big coral polyps (Buhl-Mortensen, 2001; Buhl-Mortensen et al., 2010) and moves small broken coral branches within reach of its tube (Roberts, 2005).

Lophelia pertusa

In contrast to *E. norvegica* total C or N uptake by *L. pertusa* was not influenced by the presence of the polychaete. Instead, *L. pertusa* switched from preferential feeding on *Artemia* to more opportunistic feeding by enhancing the uptake of smaller particles in the presence of the polychaete. The higher contribution of smaller particles in the presence of the polychaete is most likely caused by the preferential stealing of bigger particles by the polychaete (see above), leaving the coral to feed on what is left over. This implies that the success of *L. pertusa* to exploit a certain food source depends not only on the availability of the source but also on the interference with other species living in close association with the coral.

While experiments conducted on *L. pertusa* in isolation will help to understand its capabilities and potential, interactions between species have to be elucidated to advance our understanding of the species in the context of its natural environment. The interaction with *E. norvegica* here implies that laboratory studies done only with corals and large food particles, might overestimate the importance of these sources for the coral in its natural environment. However, the ability to utilize a broad range of food sources probably ensures that *L. pertusa* does not suffer from stealing by *E. norvegica*, as indicated by the observation that total assimilation and respiration were not affected by the polychaetes' presence. We further show that calcification by corals increased in the presence of the polychaete up to 4 times compared to treatments with only corals present, confirming the assumption that the coral enhanced calcification while interacting with *E. norvegica* (Roberts, 2005). Hence, this interaction may influence total calcification in a reef and therefore reef development. The coral pieces in our study showed no previous impact of polychaete presence like old tube remains, polyp-malformation or thickening. This indicates that the observed higher calcification rate is related to the initial phase of the polychaete-coral relationship during the polychaetes' tube formation. It is however likely that the positive feedback on calcification continues during the entire coral-polychaete relationship, since the polychaete keeps on elongating and rearranging its tube around coral branches with time (Roberts, 2005).

Surprisingly, however, this enhanced calcification was not accompanied by higher metabolic activity represented by total respiration. Naumann et al. (2011) found that changes in calcification in the cold-water coral *Desmophyllum dianthus* are correlated to changes in respiration. In contrast to that however and in agreement with our observations Form and Riebesell (2012) found that enhanced calcification by *L. pertusa* under high CO_2 exposure did not entail enhanced

respiration. This implies species-specific differences in calcification as suggested by Adkins et al. (2003) resulting in a more conservative calcification by *L. pertusa* then by *D. dianthus*.

Implications for ecosystem functioning

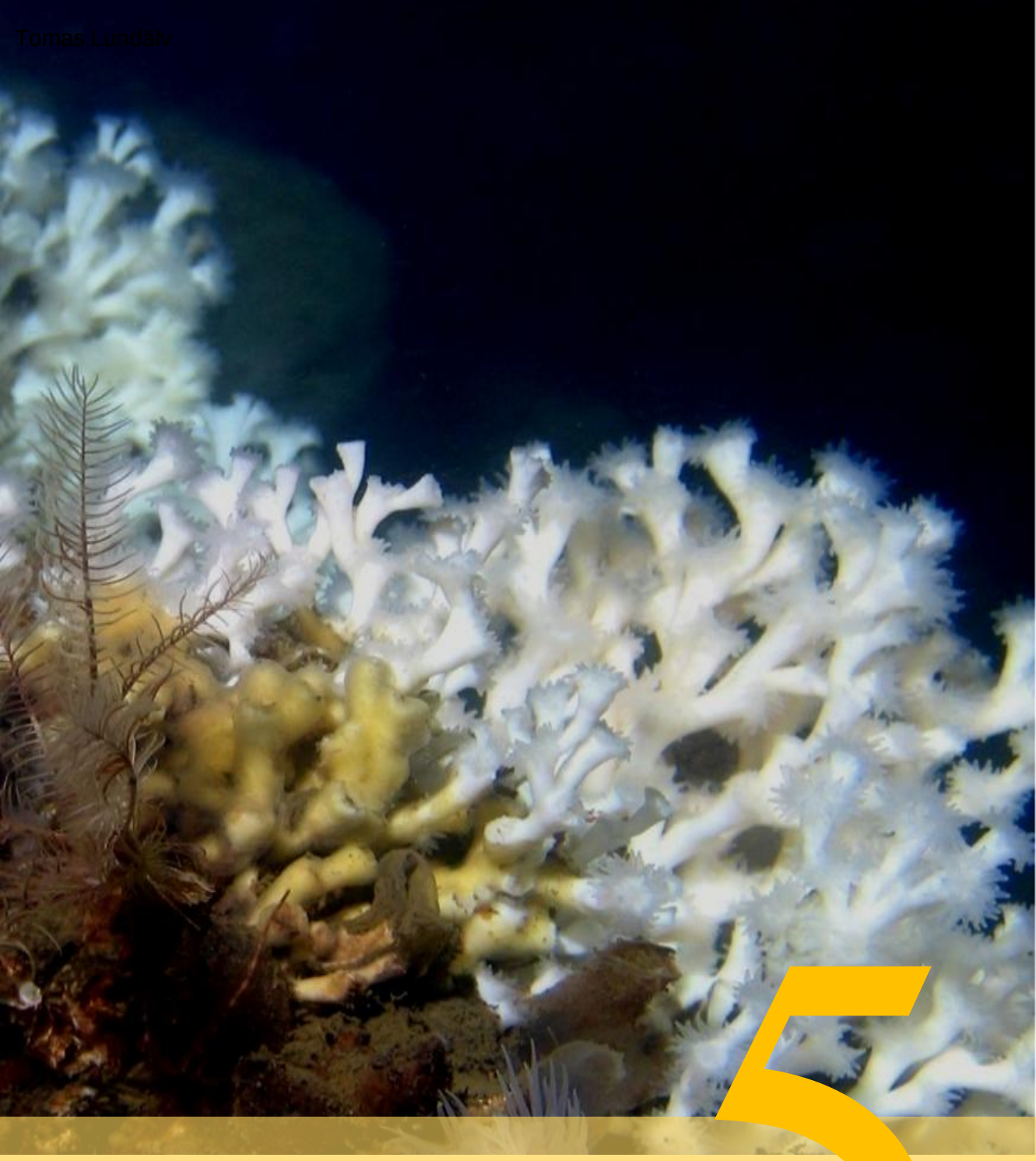
Cold-water coral reefs have been described as hotspots of carbon cycling (van Oevelen et al., 2009) and biodiversity along continental margins (Jonsson et al., 2004; Henry and Roberts, 2007; Roberts et al., 2009). So far, most aquaria studies focused on individual key species within the system, in particular the cold-water coral *Lophelia pertusa* (Dodds et al., 2007; Purser et al., 2010; Maier et al., 2012). Here we evidently show that interactions between species may substantially contribute to the development and functioning of a reef. Apparently, not only competition between species, but also facilitation can shape ecosystems by cascading throughout the community and so affect ecosystem functioning (Stachowicz, 2001; Gochfeld, 2010; Bergsma and Martinez, 2011) and persistence, especially under changing environmental conditions (Suttle et al., 2007).

In this study we showed that *Eunice norvegica* positively influences coral calcification and changes food partitioning, however without impacting total energy uptake by its coral host. So far, calcification of cold-water corals has been studied in an isolated single-species setting and quantified in the context of environmental changes (Osinga et al., 2011, and references therein), but knowledge on the influence of biological interactions is limited and qualitative (Patton, 1967; Rinkevich and Loya, 1985). Our results suggest, however, that the importance of biological interactions for the process of calcification in a reef environment might have been underestimated, since we measured a quadruplicate increase of calcification when interaction between *L. pertusa* and *E. norvegica* could take place. Enhanced calcification results in branch thickening and anastomosis, which facilitates reef growth and framework strength and thus can enhance ecosystem development and persistence, since the development of coral skeleton is essential for this ecosystem (Roberts, 2005).

It is however yet unclear how this interaction is affected by changing environmental conditions, such as ocean acidification and warming, or how this interaction reflects upon the impact of such changes on reef development. To improve our prediction of the future of cold-water coral reefs it is not only necessary to study the coral itself under various conditions but also to account for the many organisms living in association with the coral and contributing to the formation of this unique ecosystem.

Acknowledgements

Lisbeth Jonsson is thanked for helping in coral sampling. Pieter van Rijswijk is thanked for help whenever it was needed the most. The analytical lab of NIOZ-Yerseke is thanked for sample analysis. Ann I. Larsson is thanked for providing the pictures of the experimental set up. The editor and the two reviewers are thanked for their valuable input on the manuscript.



5

Food processing and recycling by a cold-water coral and its associated sponge

Photo by T. Lundälv

C. E. Mueller, T. Lundälv, F. C. van Duyl, J. M. de Goeij, J. J. Middelburg and D. van Oevelen

In preparation

Abstract

In this study we investigated processing of ^{13}C and ^{15}N labelled bacteria and algae by the cold-water coral *Lophelia pertusa*, i.e. respiration, tissue and metabolic derived C incorporation in the skeleton, in the presence and absence of the encrusting sponge *Hymedesmia coriacea*. Different concentrations (100, 300, 1300 µg C l^{-1}) and ratios of bacterial and algal biomass (1:1, 3:1) were supplied to the coral without the sponge. The processing activity of *L. pertusa* was enhanced with increased food supply and the contribution of algae and bacteria to total coral assimilation was proportional to the food mixture. This proportionality was not observed in respiration and skeleton incorporation, indicating a decoupling for these processes. The co-occurrence of coral and sponge, tested in one food regime (3 bacteria : 1 algae biomass), did not influence the processing rates of both species. Finally, we tested whether a trophic transfer of dissolved or particulate mucus existed from the cold-water coral to the sponge using ^{13}C and ^{15}N isotope labelling. A clear trophic transfer from the coral to the sponge was observed as evidenced from an isotope enrichment of specific bacterial and sponge fatty acids in the sponge tissue. The sponge utilized the coral mucus in their metabolism, which resulted in the production of sponge-derived particulate detritus. This indicated that the recently detected sponge loop might also function in cold-water coral reefs.

Introduction

Cold-water coral reefs are widely distributed in the deep-sea (Davies and Guinotte, 2011) and typically found at locations with high bottom-water velocities such as continental margins, seamounts and mid-ocean ridges (Freiwald and Roberts 2005; Roberts et al., 2009a). Although food supply is limited to external input and can be highly variable due to the physically dynamic environment (Duineveld et al., 2007; Kiriakoulakis et al., 2007) cold-water coral reefs are highly active, extensive and spatially complex ecosystems, harbouring a multitude of different species, characterizing them as hotspots of biodiversity and carbon cycling (Roberts et. al., 2009; van Oevelen et al., 2009).

Reef-building scleractinian corals are the key species of cold-water coral reefs, because they produce the three dimensional reef framework which provides settlement, refuge and feeding ground for many associated organism (Roberts et al., 2009a). In the North Atlantic Ocean, *Lophelia pertusa* is the dominant reef building species and the most studied cold-water coral to date (Roberts et al., 2006). Field observations indicate that the coral can feed on a broad range of different food sources ranging from particulate suspended matter to bacterio-, phyto and zooplankton (Kiriakoulakis et al., 2005; Duineveld et al., 2007; Roberts et al., 2009a; Duineveld et al., 2012). From laboratory studies on zooplankton uptake it is known that food concentration can impact coral nutrition and metabolism (Purser et al., 2010, Tsounis et al., 2011, Larsson et al., 2013a) and that species interactions can alter coral growth (Roberts, 2005; Mueller et al., 2013). It is however still unclear whether corals have food preferences or respond to changes in the availability of small particles (algae and bacteria), that can reach high abundances around the reef (Maier et al., 2011; Jensen et al., 2012).

Another key element in the functioning of cold-water coral reefs is the diverse group of sponges, in particular with respect to their interactions with reef-building corals (Wulff, 2006). Sponges are equipped with a high filtering capability that allows them to change particle quantity and composition within their neighborhood (Gili and Coma, 1998). Accordingly, they may be able to reduce coral food uptake when thriving in close contact to living coral branches. The nature of the relationships between reef-building corals and associated sponges is however largely unclear (van Soest and Lavaleye, 2005; van Soest et al., 2007).

One of the sponge species thriving in close contact with living coral tissue, possibly resulting in an interaction with *L. pertusa*, is the encrusting sponge *Hymedesmia coriacea*, which sometimes overgrows the coral (Buhl-Mortensen, 2001). The sponge-coral interaction is usually explained in terms of competition

for space and food (Rützler and Muzik, 1993; Hill, 1998; Rützler, 2002), but the sponge also might simply protect the coral by covering its dead skeleton (Goreau and Hartman, 1966, Wulff and Buss, 1979). Moreover, the close proximity to living coral tissue might also give the sponge excess to an additional energy source. Tropical coral-reef sponges are known for their ability to consume dissolved organic matter (DOM) (de Goeij et al., 2008), a resource excreted in large quantities by the coral (Wild et al. 2009), but inaccessible by most higher organisms. Sponges can transform this DOM into substantial amounts of particulate organic matter (POM) (Witte et al., 1997; de Goeij et al., 2009), which may then become available to the detritivore food web. The conservation and recycling of energy and nutrients has been observed in tropical coral-reef systems (de Goeij et al., 2013), but the sponge loop has not yet been investigated in cold-water coral reefs.

Food availability as well as food quality (composition) plays a major role in cold-water coral nutrition. Moreover, interactions between corals and associated sponges may impact cold-water reef functioning. We therefore investigated the physiological response of *L. pertusa* to different food conditions (quantity and quality) and the presence of the sponge *H. coriacea*. We used a mixed diet of algae and bacteria to study metabolic processing (respiration, assimilation, coral skeletal incorporation) of smaller particles by *L. pertusa* and *H. coriacea*. Both species were either kept separate or together in an aquarium. A novel dual labelling approach, in which one source was either enriched with ^{13}C or ^{15}N (^{13}C bacteria + ^{15}N algae, ^{13}C algae + ^{15}N bacteria, Fig. 5.1.) was carried out in all experiments to examine food preferences of each species and potential niche segregation. To test for a transfer of coral derived material through the sponge we offered ^{13}C and ^{15}N labelled coral mucus to *H. coriacea* and followed its assimilation into sponge tissue and sponge-derived POM. The assimilation of tracer C into phospholipid-derived fatty acids, including biomarkers for bacteria and sponge tissue, was then used to determine the role of bacteria and sponge cells in the mucus uptake.

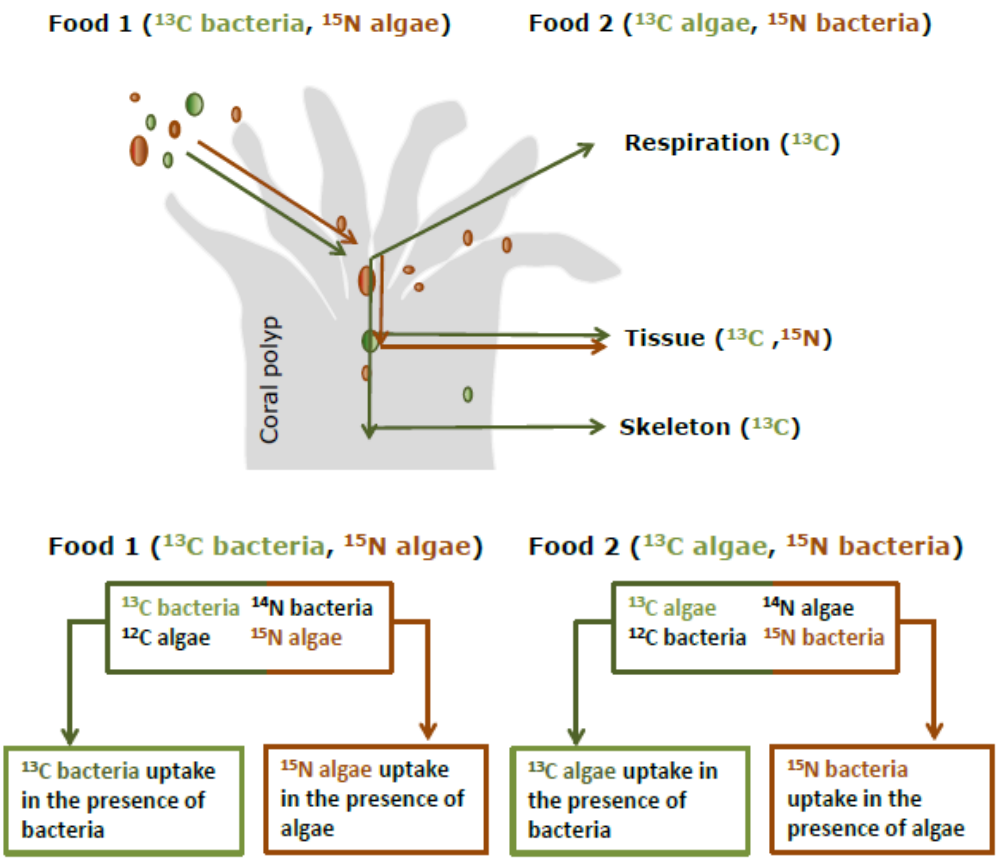


Figure 5.1. Concept of ^{13}C and ^{15}N dual-labelling used in this study with both food combinations of algae and bacteria respectively enriched in ^{13}C or ^{15}N (Food 1, 2).

Material and Methods

Sampling location and maintenance

The corals and sponges used in the experiments were harvested at the Tisler Reef, located 70 to 155 m deep in the Skagerrak, at the border between Norway and Sweden. The velocity over the reef varies from 0 to 50 cm s^{-1}, with peaks in excess of 70 cm s^{-1}, while the flow direction fluctuates irregularly between NW and SE (Lavaleye et al., 2009; Wagner et al., 2011). Temperature over the reef typically varies between 6 to 9 ºC throughout the year (Lavaleye et al., 2009; Wagner et al., 2011). The amount and quality of organic carbon (POC) available at the reef

depends on the location within the reef and varies between 43.5 to 106.3 µg POC l^{-1} (Wagner et al., 2011).

Both species were collected from a depth of around 110 m using the remotely operated vehicle Sperre Subfighter 7500 DC. Specimens were placed in cooling boxes filled beforehand with cold seawater (7 - 8 ºC) and transported within a few hours to the laboratory at the Sven Lovén Centre in Tjärnö (Sweden). After arrival coral samples were clipped to approximately the same size and living coral polyps from sponge samples were removed to prepare sponge specimens. All specimens were maintained in aquaria (~20 l) placed in a dark thermo-constant room (7 ºC) for up to 6 weeks before used in the experiment as acclimation time. Only some of the sponge specimens in the recycling experiment were used within one week. Sand-filtered (1-2 mm particle size) bottom water from 45 m depth out of the adjacent Koster-fjord (salinity 31) was continuously flushed through the aquaria (~1 l min^{-1}). From experience at the station, it is known that the sand-filtered water still contains sufficient organic particles so that no extra food had to be provided during the acclimation period.

Preparation of labelled food substrates

Bacteria (~1 µm) were cultured by adding a few ml of natural seawater from the Oosterschelde estuary (Netherlands) to M63 culture medium adjusted after Miller (1972). In the medium either 50% of glucose (3 g l^{-1}) or 50% of NH_4Cl (1.125 g l^{-1}) was replaced by its heavy isotope equivalent (Cambridge Isotopes, 99% ^{13}C, 99% ^{15}N) to obtain ^{13}C or ^{15}N isotopically labelled bacteria. After 3 days of culturing in the dark bacteria were concentrated by centrifugation (14500 g), rinsed 3 times with 0.2 µm filtered sea water to remove residual label and kept frozen until further use.

Single or double (^{13}C, ^{15}N) isotopically labelled diatoms were cultured axenically in f/2 culture medium adjusted after Guillard (1975). In this medium either 80 % of the $NaHCO_3$ or 70 % of the $NaNO_3$ or both were replaced by its heavy isotope equivalent (Cambridge Isotopes, 99% ^{13}C, 99% ^{15}N) before a sterile culture inoculum of the diatom *Thalassiosira pseudonana* (~5µm) was injected. After 3 weeks of culturing with a 12 h light-dark cycle (at a cell density of around 3 - $4*10^6$ cells ml^{-1}), diatoms were concentrated by centrifugation at 450 g. The concentrate was rinsed three times with 0.2 µm filtered seawater to remove residual label and kept frozen until further use.

All labelled food substrates were measured for carbon and nitrogen content and isotopic composition as described below.

Experimental set up and procedure

Resource processing by the coral *L. pertusa*

Prior to the start of the experiment incubation chambers (10 l), situated in a thermo-stated room (7 ºC), were filled with filtered (5 µm) Koster-fjord bottom water. Fragments of *L. pertusa* (2-3 fragments chamber^{-1}, 7.90 ± 2.12 g dry weight (DW) chamber^{-1}, 14.06 ± 2.43 polyps chamber^{-1}) were placed in the incubation chambers (Fig. 5.2.). An upright position of coral fragments was ensured by inserting them into elastic silicone tubes that were mounted on an acrylic plate, which was fixed to the chamber base. Steady water circulation during the experiment was maintained by a motor-driven paddle in the upper part of the incubation chamber (speed: 2 rpm).

For the duration of 10 days corals were allowed to feed 12 h per day (feeding period) on the isotopically labelled food. A food pulse was given at the beginning of the feeding period with the respective concentration and ratio of ^{13}C bacteria/^{15}N algae and ^{13}C algae/^{15}N bacteria (Fig. 5.1.). After each feeding period, chambers were flushed with filtered (5 µm) Koster-fjord bottom water (140 ml min^{-1}) for 12 h (flushing period) to avoid accumulation of waste products such as NH_4^+ and CO_2, to remove remaining food particles and to renew the O_2 supply. In the first treatment a total of 100 µg C l^{-1} d^{-1} was supplied to the corals, offered in a 1:1 ratio of bacteria to diatoms. In the second treatment 300 µg C l^{-1} d^{-1} was offered also in a 1:1 ratio of bacteria to diatoms. In the third treatment a total of 1300 µg C l^{-1} d^{-1} was offered in a 3:1 ratio of bacteria to diatoms. With exception of the first treatment (2 replicates) all treatments were replicated three times.

After the last flushing period at day 10, incubation chambers were closed for another 48 h to measure production of ^{13}C dissolved inorganic carbon (^{13}C-DIC) from food source processing as a proxy for respiration (Moodley et al., 2000; de Goeij et al., 2008; Gontikaki et al., 2011). Pilot experiments (C. E. Mueller, unpubl. data) and literature reports indicate that pH (7.8 ± 0.04 NBS), oxygen and ammonium concentration (Dodds et al., 2009) does not change considerably so that no negative effect on coral or sponge physiology is expected. Water samples for later DIC analysis were taken before (control) and after the respiration incubation and filtered (GF/F) in a 20 ml headspace vial. Each sample was poisoned with 10 µl $HgCl_2$, closed with an aluminium cap fitted with a rubber septum and stored upside down for further analysis.

Corals for background isotope measurements (controls) were incubated in parallel under ‘acclimatization’ conditions: i.e. without food addition to the sand-

filtered seawater (1-2 mm particle size). At the end of the experiment coral samples were frozen at -20°C and stored frozen for further analysis.

Resource processing by coral and sponge

To investigate food-based interactions between *H. coriacea* and *L. pertusa,* sponge-free coral fragments (2-3 fragments chamber^{-1}, 10.35 ± 1.31 g DW coral chamber^{-1}, 16.83 ± 2.79 polyps chamber^{-1}) and dead fragments overgrown by *H. coriacea* (0.03 ± 0.01 g DW sponge chamber^{-1}) were placed separately or together in incubation chambers (Fig. 5.2.). The set up was similar to the one used to study concentration dependent coral uptake.

For 10 days (12 h feeding d^{-1}, 12 h flushing d^{-1}) a total of 1300 µg C l^{-1} d^{-1} was offered consisting of ^{13}C bacteria/ ^{15}N algae or ^{13}C algae/ ^{15}N bacteria in a diatom-bacteria ratio of 1:3 (Fig. 5.1.). Each food source offered to corals, sponges and both species and replicated 3 times. Respiration was measured for 48 h after the last flushing period as described above. Control specimens were incubated in parallel without food addition. At the end of the experiment, coral and sponge samples were frozen at -20°C and stored frozen for further analysis.

Mucus recycling via sponges

In order to monitor the transfer of coral mucus to sponges, we enriched corals with ^{13}C and ^{15}N by repeated feeding with ^{13}C and ^{15}N enriched algae (1.6 mg C l^{-1} d^{-1}; 0.3 mg N l^{-1} d^{-1}), a resource that is assimilated by the coral (Mueller et al. 2013), for 3 weeks in five 10-L incubation chambers with water being exchanged every 12 h.

The transfer of mucus to *H. coriacea* was then investigated in a system of coupled cylindrical incubation chambers that were filled with filtered (GF/F) water from the Koster-fjord. Isotopically labelled coral fragments (coral chamber, 75g DW coral, Fig. 5.2.) were transferred from the feeding chamber to the cylindrical incubation chamber (Fig. 5.2.), while the chamber next-in-line contained 6 sponge specimens (0.04 ± 0.02 g DW sponge^{-1}) of which 3 had been kept in the laboratory for around 6 weeks while the rest had been kept < 1 week prior to the incubation. Two tubes connected the chambers so that a pump system could maintain a water circulation between chambers (200 ml min^{-1}). An additional water pump (150 l h^{-1}) was installed in the coral-chamber to stimulate mucus production and release (Wild et al., 2012). Every 24 h, half of the water was renewed to avoid the excessive accumulation of waste products. At the beginning and the end of the experiment water samples were taken from the coral chamber and filtered over precombusted GF/F filters to collect particulate coral mucus in the form of ^{13}C and ^{15}N enriched

POM. Control sponges were incubated in parallel, i.e. without corals and additional food. After 4 days, each sponge piece was moved into 1 l of freshly filtered seawater (GF/F) to determine the production of sponge-derived POM. After 24 h sponges were removed and frozen for tissue analysis. The incubation water was filtered over a precombusted GF/F filter to collect produced POM. At the end of the experiment all sponge, coral and filter samples were frozen at -20°C and stored frozen for further analysis.

Sample treatment and analysis

Tissue assimilation and POM analysis

The sponge, coral and POM-filter samples (coral or sponge excreted POM, mucus experiment) were freeze-dried. Sponge tissue was scraped from the coral surface, homogenized by pestle and weighed. Coral samples were weighed and homogenized by grinding with a ball Mill for 20 s (MM 2000, Retsch, Haan, Germany). 30 mg of ground coral material, 2 - 3 mg ground sponge material or a subsample of the POM-filter was transferred to pre-combusted silver boats and decalcified by acidification (Nieuwenhuize et al. 1994) to analyze the organic coral tissue, sponge tissue and POM, respectively. Subsequently, each sample was measured for ^{13}C and ^{15}N using a thermo Electron Flash EA 1112 analyzer (EA) coupled to a Delta V isotope ratio mass spectrometer (IRMS).

All stable isotope data were expressed in total elemental uptake (µg C g^{-1} DW and µg N g^{-1} DW). They were calculated as following on the base of the delta notations obtained from the IRMS: δX (‰) = $(R_{sample}/R_{ref} -1)*1000$, where X is the element, R_{sample} is the heavy : light isotope ratio in the sample and R_{ref} is the heavy : light isotope ratio in the reference material (Vienna Pee Dee Belemnite standard for C and atmospheric nitrogen for N). When used for C R_{ref} = 0.0111797 and when used for N then R_{ref} = 0.0036765. The atomic % (atm%) of heavy isotope in a sample is calculated as $F = R_{sample}/(R_{sample}+1)$. The excess (above background) atm% is the difference between the F in an experimental sample and the atm% in a control sample: $E = F_{sample} – F_{control}$. To correct for differences in enrichments of the food sources, the excess incorporation was divided by the atomic enrichment of each specific food source.

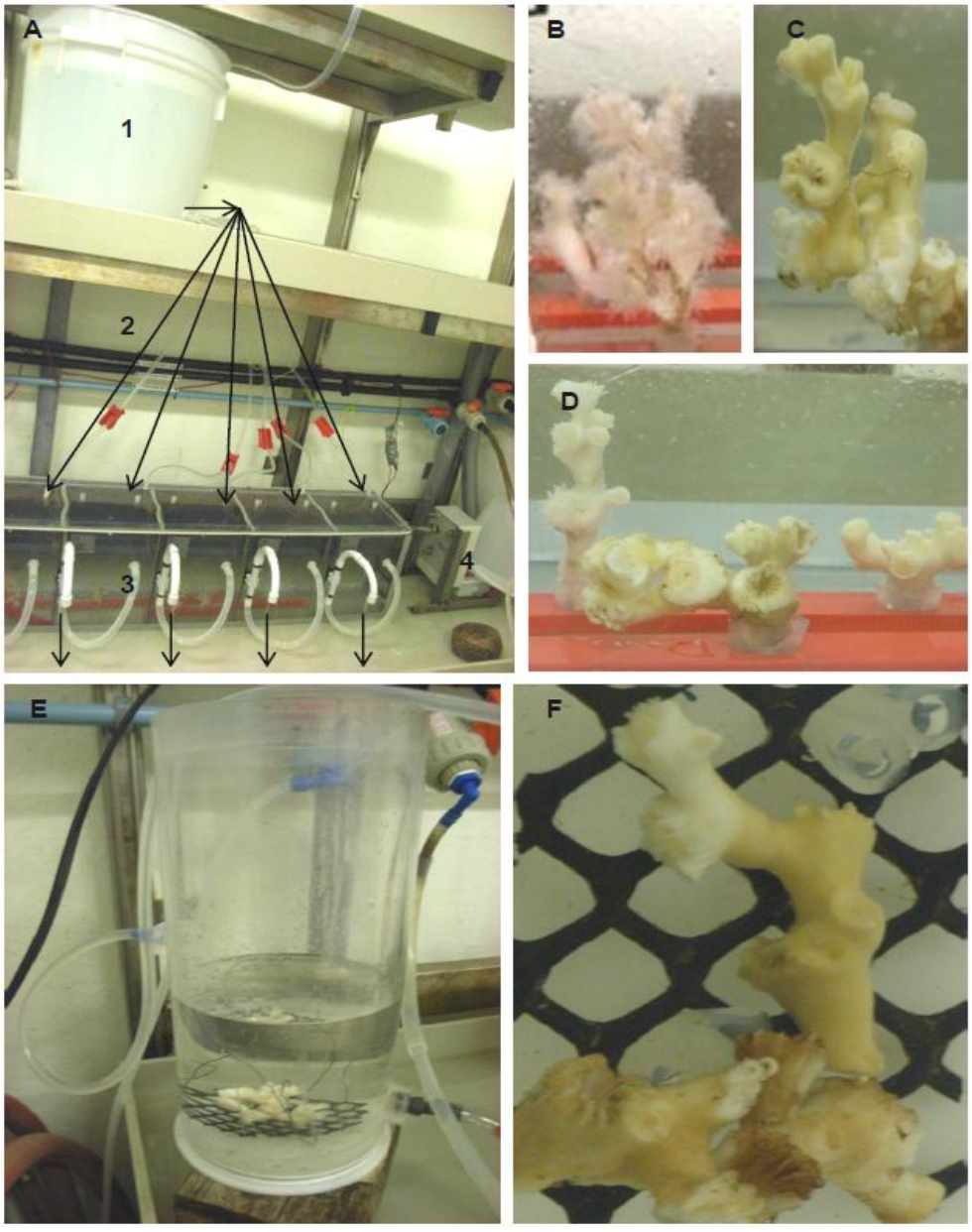


Figure 5.2. Experimental set up: (A) Mississippi chambers including the water reservoir for filtered sea water (1), the direction of the water flow indicated with arrows (2), 5 incubation chambers (3) and the motor driving the chamber paddles (4), (B) *L. pertusa* in one chambers, (C) *H. coriacea* in one chamber, (D) *L. pertusa* together with *H. coriacea* in one chamber, (E) experimental set up of the mucus experiment showing one experimental chamber, (F) Close-up of one sponge placed in the chamber.

Phospholipid-derived fatty acid analysis

Phosopholipid-derived fatty acids (PLFAs) of sponge samples incubated with coral mucus were extracted according to Boschker et al. (1999). First, total fatty acids were extracted using a modified Blight Dyer method and then further separated by a silicic-acid column (Merck Kieselgel 60) to obtain phospholipid-derived fatty acids. PLFA extracts were then further derivatized by mild alkaline transmethylation to generate fatty acid methyl esters (FAME) that are amendable to gas chromatographic analysis. Concentration and carbon isotopic composition of individual FAMEs were determined with a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS). FAME identification was based on the comparison of retention time data with known standards (12:0, 19:0) and converted to PLFAs. Additional peak identification was obtained by gas-chromatography-mass spectrometry (GC-MS) following Boschker et al. (1999). A total of 0.018 g DW sponge was used in the extraction.

Respiration

A headspace of 3 ml was created in each DIC sample vial by injecting N_2 gas through the vial septum (Miyajima et al., 1995; Moodley et al., 2000). Samples were then acidified with 20 µl of concentrated H_3PO_4 to transform DIC into CO_2. When CO_2 had exchanged with the vial headspace, 10 µl sample of the headspace gas was injected into an elemental analyzer isotope-ratio mass spectrometer (EA-IRMS). Calculations for ^{13}C respiration followed the description for ^{13}C tissue assimilation.

Metabolic-derived C assimilation in the skeleton

The incorporation of metabolic-derived ^{13}C in coral skeleton was measured following Mueller et al. (2013) and Tanaka (2007): 30 mg of each coral sample (including the organic fraction and inorganic skeleton) was transferred to a silver boat and measured on the EA-IRMS for total tracer C content. The incorporation into the tracer C in the inorganic skeleton was obtained by subtracting tracer C assimilation in the organic fraction from the tracer C incorporation in the total (organic fraction and inorganic skeleton). Calcification based on metabolic-derived inorganic carbon may only be a small amount of the total calcification (~8%, Adkins et al., 2003) but it still can be used as a proxy to detect changes in total calcification.

Statistical Analysis

The influence of food concentration and composition (algae or bacteria) on C and N uptake in corals, respiration and metabolic-derived assimilation in the skeleton was tested using Scheffé-Tests, since sample sizes between treatments were not equal (2 replicates in the treatment with 100 µg C l^{-1}, 3 replicates in the other treatments). The influence of sponge presence on food uptake and processing by corals and vice versa was investigated using permutational multivariate analysis of variance (PERMANOVA) with pair-wise a posteriori comparisons (Anderson, 2005). The latter analysis was also used to test whether differences existed in mucus processing between sponges that were kept in aquaria for various amounts of time. The outcome of each PERMANOVA test was expressed in Monte Carlo P-values, which is more robust in case of a smaller number of replicates.

Results

Stable isotopic substrates

The isotopic enrichment of cultured bacteria ranged from 44 - 46 atm% for C and 47 atm% for N while the isotopic enrichment of algae ranged from 47 - 58 atm% for C and from 64 - 68 atm% for N. Bacteria had a C/N ratio of 4.8 ± 0.2 while algae had a C/N ratio of 7.8 ± 0.5. Particulate coral mucus was labelled with 0.4 atm% C and 1.1 atm% N and had a C/N ratio of 5.2 ± 0.4.

Concentration and composition dependent food uptake by coral

Food concentration significantly increased assimilation and respiration by *L. pertusa* of both food sources (Fig. 5.3.A, B), while C incorporation into coral skeleton was not significantly influenced (Fig. 5.3.C). On average assimilation by *L. pertusa* increased from 0.4 ± 0.05 µg C g^{-1} DW d^{-1} at the lowest food concentration to 2.8 ±0.4 µg C g^{-1} DW d^{-1} at the highest food concentration (Fig. 5.3.A). Similarly, total N assimilation increased with food availability but was overall lower (0.1 ± 0.02 to 0.4 ± 0.1 µg N g^{-1} DW d^{-1}). Respiration by *L. pertusa* increased with food concentration from 2.7 ± 0.3 to 46.1 ± 10.2 µg C g^{-1} DW d^{-1} at the highest food concentration (Fig. 5.3.B). Skeleton C incorporation varied from 0.2 ±0.2 to 0.7 ± 0.4 µg C g^{-1} DW d^{-1} at the lowest and the highest food concentration respectively (Fig. 5.3.C). In total *L. pertusa* processed 2.6 ± 0.6 % of

added C d^{-1} to 3.6 ± 1.4% of added C d^{-1} in the lowest and highest food treatment respectively with a peak at 4.8 ± 0.8 % in the moderate food treatment (Fig. 5.3.D).

Total C assimilation by the coral was proportional to food composition (Table 5.1.). However, the contribution of algae and bacteria to total N assimilation, respiration and skeleton C incorporation did not differ with different food compositions (Table 5.1., 5.2., 5.3.).

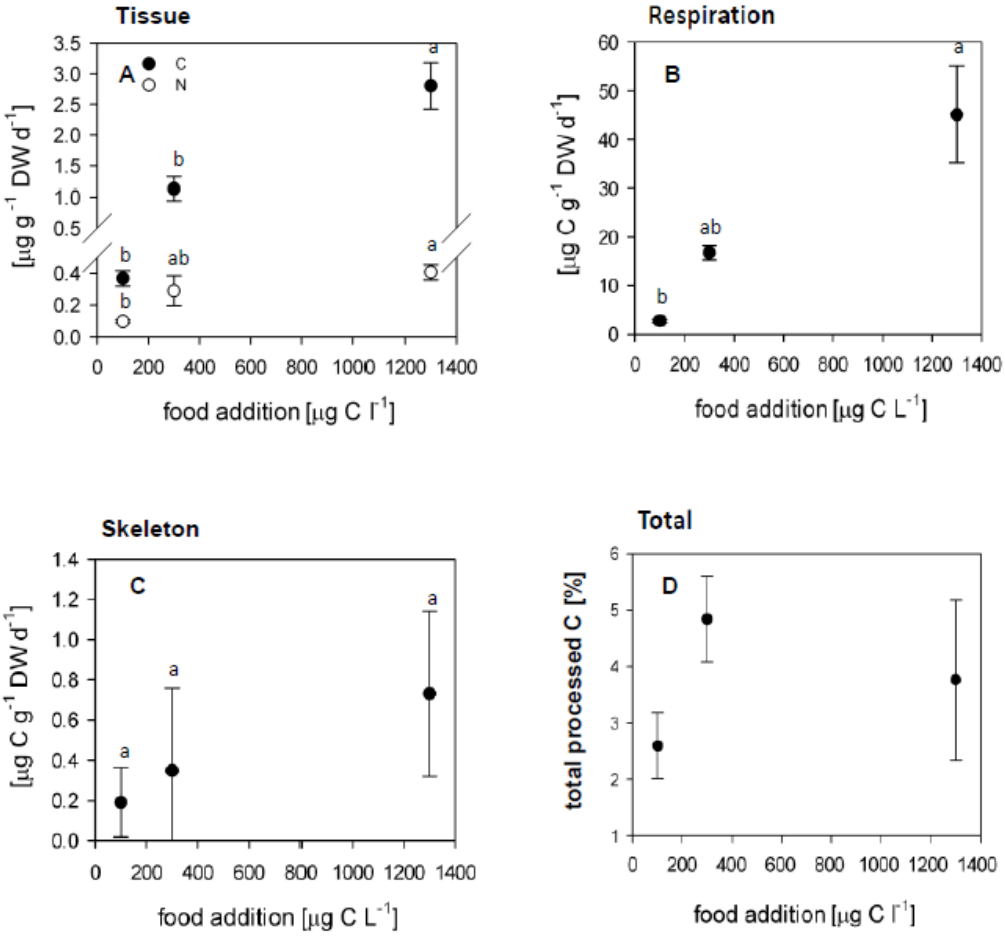


Figure 5.3. Food processing by the coral *L. pertusa* at different food concentrations. (A) Total C and N assimilation in coral tissue, (B) total C incorporation in coral skeleton, (C) total C respiration, (D) total processed C per day relative to C addition. Errors bars represent average ± SD.

Coral-sponge interactions

The presence of the sponge *H. coriacea* did not affect the processing rates of *L. pertusa* (Fig. 5.4.). On average, *L. pertusa* assimilated a total of 2.8 ± 0.4 µg C g^{-1} DW d^{-1} and 0.4 ± 0.1 µg N g^{-1} DW d^{-1} in the absence of the sponge and 2.3 ± 0.4 µg C g^{-1} DW d^{-1} and 0.4 ± 0.1 µg N g^{-1} DW d^{-1} in the presence of the sponge (Fig. 5.4.). Respiration varied between 46.1 ± 10.2 µg C g^{-1} DW d^{-1} and 57.3 ± 40.6 µg C g^{-1} DW d^{-1} (Fig. 5.4.) in the absence and presence of the sponge respectively. Incorporation of carbon into the coral skeleton ranged between from 0.7 ± 0.4 in the absence of the sponge to 1.6 ± 0.4 µg C g^{-1} DW d^{-1} in the presence of the sponge (Fig. 5.4.).

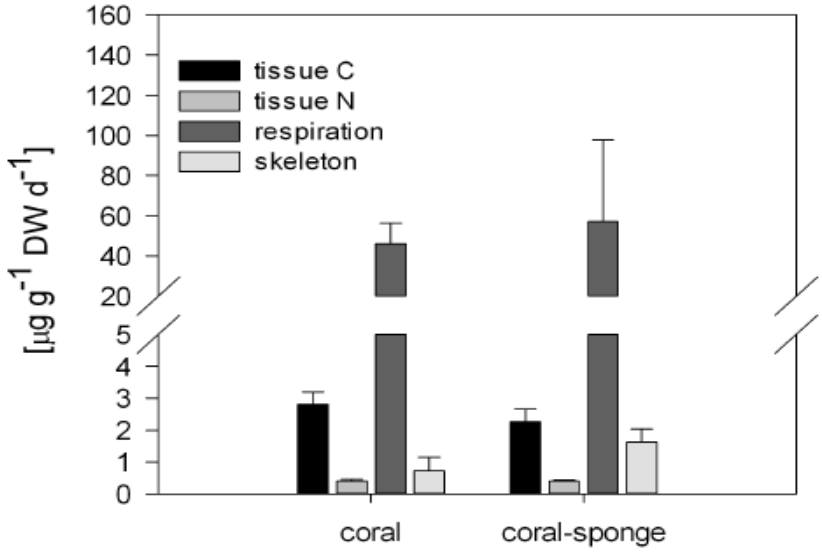


Figure 5.4. Total C and N processing by the coral *L. pertusa* kept together or separately with the sponge H. *coriacea* in aquaria. Errors bars represent average ± SD.

The presence of the sponge did not affect the contribution of algae and bacteria to total assimilation or respiration by *L. pertusa* (Table 5.1., 5.2.). Bacteria C assimilation by the coral significantly exceeded algae C assimilation independent of sponge presence, whereas bacteria and algae contributed similarly to N assimilation and total respiration in each treatment (Table 5.1.). The contribution of algal and bacterial C to metabolic carbon incorporation into coral skeleton however

differed depending on sponge presence. Corals incubated without sponges used bacteria and algae C at the same rate whereas in the presence of sponges corals increased the proportion of bacteria C allocated to the skeleton without altering the algae contribution (PERMANOVA p = 0.016, significant difference between algae and bacteria C assimilation in coral skeleton, Table 5.3.).

The sponge *H. coriacea* processed in total 5.5 ± 2.8 % of tracer C in the absence of the coral, which was equal to food processing when both species were present (3.9 ± 2.8 %.). Total assimilation of *H. coriacea* was not significantly affected by the presence of the coral and varied between 917.2 ± 586.4 µg C g^{-1} DW d^{-1} and 202.8 ± 133.2 µg N g^{-1} DW d^{-1} in the absence of the coral and 531.9 ± 55.5 µg C g^{-1} DW d^{-1} and 104.0 ± 56.8 µg N g^{-1} DW d^{-1} in the presence of the coral (Fig. 5.5.). Total respiration by *H. coriacea* averaged 19841.4 ± 6926.9 µg C g^{-1} DW d^{-1} in the absence of the coral (Fig. 5.5.), which highly exceeded rates observed in incubations with both species present (57.3 ± 40.6 µg C g^{-1} DW d^{-1}).

Table 5.1. C-and N-assimilation partitioned by food sources by *L. pertusa* and *H. coriacea*, kept separate (Coral, Sponge) or together (Coral-Sp, Sponge-Co) under various food conditions (see C-Addition). All values are given in µg C g^{-1} DW d^{-1}.

Treatment	**Coral**	**Coral**	**Coral**	**Coral Sp**	**Sponge**	**Sponge Co**
C-Addition [µg l^{-1}]	**100**	**300**	**1300**	**1300**	**1300**	**1300**
Bacteria C	0.20 ± 0.01	0.47 ± 0.15	1.72 ± 0.20	1.47 ± 0.22	442.31 ± 221.91	280.37 ± 110.27
Algae C	0.16 ± 0.04	0.66 ± 0.29	1.09 ± 0.26	0.79 ± 0.20	369.92 ± 315.41	251.57 ± 142.29
Bacteria N	0.06 ± 0.01	0.18 ± 0.05	0.23 ± 0.06	0.20 ± 0.01	159.61 ± 153.94	92.06 ± 58.12
Algae N	0.04 ± 0.01	0.11 ± 0.05	0.18 ± 0.02	0.20 ± 0.05	43.15 ± 48.45	11.91 ± 4.01

Table 5.2. Respiration partitioned by food sources by *L. pertusa* and *H. coriacea*, kept separate (Coral, Sponge) or together (Coral-Sp) under various food conditions (see C-Addition). All values are given in µg C g^{-1} DW d^{-1}.

Treatment	**Coral**	**Coral**	**Coral Sp**	**Sponge**	**Sponge Co**
C-Addition [µg l^{-1}]	**100**	**300**	**1300**	**1300**	**1300**
Bacteria	1.28 ± 0.26	9.51 ± 1.22	33.30 ± 13.24	36.86 ± 32.07	11783. 45± 3164.28
Algae	1.47 ± 0.60	7.47 ± 0.66	12.80 ± 5.29	20.46 ± 9.06	8057.96 ± 5999.23

Table 5.3. Incorporation of C tracer into coral skeleton partitioned by food sources with respect to different food conditions (see C-Addition). All values are given in µg C g^{-1} DW d^{-1}.

Treatment	**Coral**	**Coral**	**Coral Sp**	**Sponge**
C-Addition [µg l^{-1}]	**100**	**300**	**1300**	**1300**
Bacteria	0.06 ± 0.08	0.15 ± 0.13	0.35 ± 0.45	1.22 ± 0.49
Algae	0.14 ± 0.10	0.20 ± 0.30	0.38 ± 0.07	0.40 ± 0.09

The presence of the coral did not affect the contribution of algae and bacteria to total C assimilation or respiration of *H. coriacea* (Table 5.1., 5.2.). In both treatments algae and bacteria C contributed similar amounts to total C assimilation and respiration (although bacteria biomass exceeded algae biomass by three). However, the assimilation of N was affected by the presence of the coral. In the absence of the coral bacteria and algae N contributed comparable amounts to total

N assimilation of *H. coriacea*, whereas in the presence of the coral assimilation of bacteria N highly exceeded that of algae (PERMANOVA p=0.024, Table 5.1.).

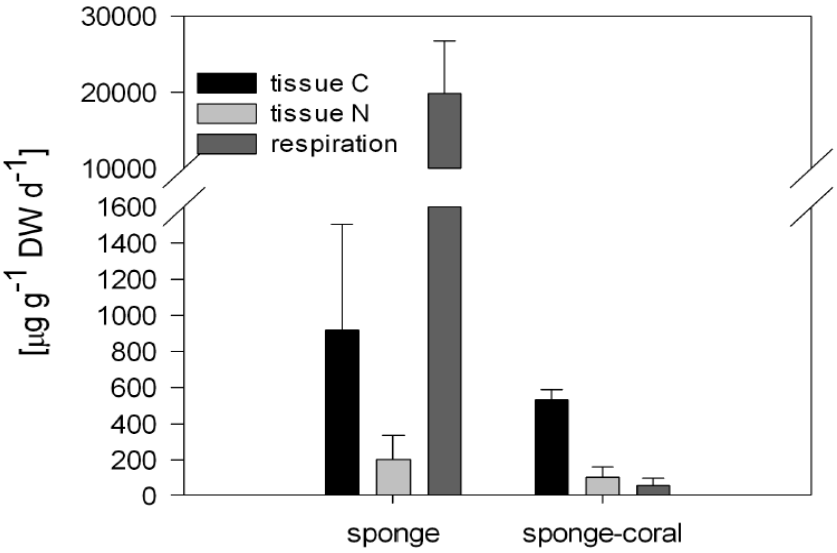


Figure 5.5. Total C and N processing by *H. coriacea* kept together or separately with the coral *L. pertusa* in aquaria. Errors bars represent average ± SD.

Coral mucus recycling via sponges

On average 611 ± 524 µg POC l^{-1} and 132 ± 105 µg PON l^{-1} was supplied to the sponge chamber in the form of coral-derived POM (mucus). This resulted in an evident incorporation of coral-derived material by the sponge *H. coriacea:* Sponges used for the experiment within a week assimilated 296.3 ± 262.2 µg C g^{-1} DW d^{-1} and 61.8 ± 40.5 µg N g^{-1} DW d^{-1} respectively. Sponges kept in aquaria for longer than 6 weeks assimilated 88.6 ± 153.4 µg C g^{-1} DW d^{-1} and 16.7 ± 15.7 µg N g^{-1} DW d^{-1} respectively (Fig, 5.6.). This difference was not significant, probably due to the high variability of the data.

All sponges produced isotopically enriched sponge-derived POM after the exposure to enriched coral mucus. Freshly harvested sponges expelled 113.5 ± 46.5 µg POC g^{-1} DW d^{-1} and 37.3 ± 28.0 µg PON g^{-1} DW d^{-1} respectively. Long kept sponges expelled 504.6 ± 350.0 µg POC g^{-1} DW d^{-1} and 79.1 ± 40.7 µg PON g^{-1} DW d^{-1} respectively (Fig, 5.6.). Like for tissue assimilation no significant effect of time in aquaria could be detected.

A significant effect of aquaria time was however observed for the incorporation of coral-derived C into individual PLFAs (Fig. 5.7.). Freshly harvested sponges assimilated 9.1 ± 2.1 µg C g^{-1} DW d^{-1} in PLFA while "older" specimens assimilated significantly less (5.0 ± 0.9 µg C g^{-1} DW d^{-1}, PERMANOVA p = 0.0216). Typical bacteria PLFA like iso-, anteiso- and methyl-branched PLFAs, C18:1ω7c and C17:1ω7c (Zelles, 1999; Boschker and Middelburg, 2002; Lazcano et al., 2013) contributed 22 % (36 % including C16:1ω7c) to assimilation in PLFAs in shortly harvested sponges and 22 % (45 % including C16:1ω7c) in longer kept specimens. Typical demosponge PLFAs (> C22:0) (Koopmans et al., 2010) contributed 9 % to C assimilation in PLFAs in shortly harvested sponges and 6 % in longer kept specimens.

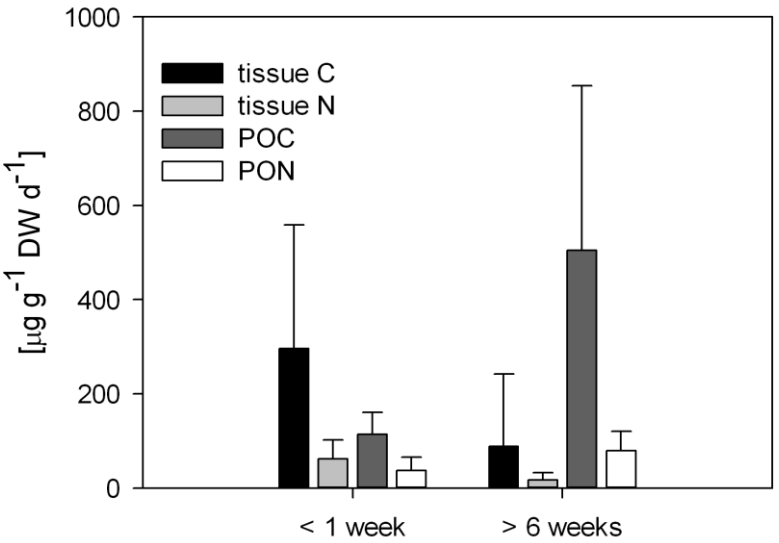


Figure 5.6. Coral-derived C and N processing by *H. coriacea*. C and N tissue assimilation and PON/ POC production. Errors bars represent average ± SD.

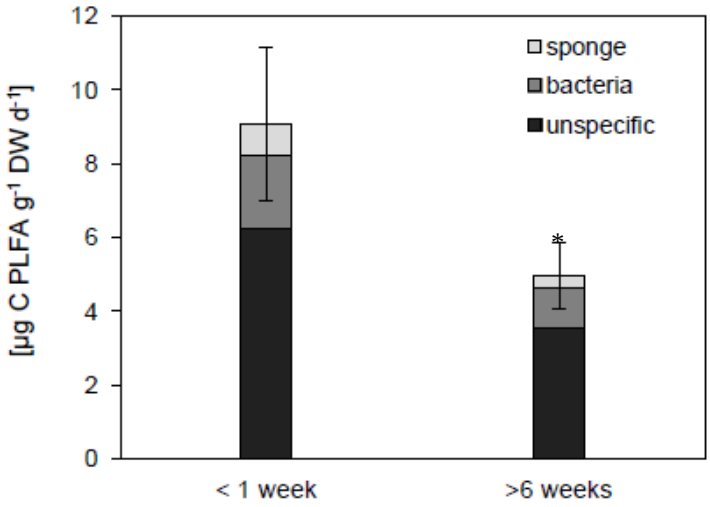


Figure 5.7. Coral-derived C and N processing by *H. coriacea*. C-assimilation in PLFAs by *H. coriacea* from coral derived organic matter. Error bars represent average ± SD of total incorporation into PLFAs (Σsponge-, bacteria- and unspecific PLFAs).

Discussion

Food concentration dependent uptake by *L. pertusa*

Higher concentrations of bacteria and algae resulted in increased respiration and assimilation rates by *L. pertusa*, indicating the metabolism of the coral is tightly coupled to food availability. This is consistent with observations by Purser et al. (2010) and Larsson et al. (2013a) showing higher respiration and removal rates of zooplankton with increased particle concentration. Interestingly, capture rates observed by Purser et al. (2010) and metabolic activity observed in this study appear to saturate at concentrations of a few hundred µg C l^{-1}. This highly exceeds average POC concentration above CWC reefs, typically varying between 20 to 100 µg C l^{-1} (Kiriakoulakis et al., 2007; Wagner et al., 2011), and it implies that *L. pertusa* is able and well adapted to use temporal pulses of high food concentration caused by internal waves or tidal cycles such as observed on Tisler and Mingulay reef (Roberts et al., 2009b; Wagner et al., 2011; Duineveld et al., 2012). This highlights the flexibility in the metabolism of *L. pertusa*, which probably also contributes to the wide ecological niche that *L. pertusa* has (Davies and Guinotte, 2011).

In contrast to assimilation and respiration, calcification was not significantly affected by higher food input. This is consistent with the lack of any response of skeletal growth of *L. pertusa* to different food conditions (Larsson et al., 2013a), who suggested that the response time of calcification to changing environmental conditions acts on a longer time scale. From tropical coral studies it is known that calcification processes can be less responsive to environmental conditions than tissue growth (Anthony and Fabricius, 2000; Tanaka et al., 2007; Tolosa et al., 2011). The relatively low metabolic costs related to calcification in *L. pertusa* (Larsson and Purser, 2011; Form and Riebesell, 2012), may explain why a longer time period is needed before a response in calcification to altered food conditions can be measured. It is therefore interesting to note that Naumann et al. (2011) did measure significant higher calcification in fed than unfed specimens of the CWC *Desmophyllum dianthus*, but *D. dianthus* is known to be a fast growing species that spends more energy on calcification.

Food composition dependent uptake by *L. pertusa*

The assimilation of food particles by *L. pertusa* responded proportionally to the composition of the offered food. This seems to indicate that *L. pertusa* is an unselective feeder. This opportunistic feeding strategy is consistent with observations that different CWC reefs are fueled by different sources depending on their availability (Dodds et al., 2009) and aquarium studies (Mueller et al., in review, see chapter 2). However when correcting assimilation for food availability (chesson index, Chesson, 1983), it was clear that *L. pertusa* selectively fed on algae (Fig. 5.8.A). Interestingly, the coral also selected algae when algae and bacteria were supplied equally at $\geq$ 300 µg C l^{-1}. This indicates that food concentration plays a role in the selection process. At concentrations of 100 µg C l^{-1} *L. pertusa* assimilates what is available while above this concentration the coral is able to select algae over bacteria, independent of the biomass ratio between bacteria and algae. This selectivity was however not observed in respiration or C incorporation into the coral skeleton, which indicates a weak coupling between assimilation and respiration/ skeleton incorporation.

Food uptake and selectivity by the sponge *H. coriacea*

Tropical and temperate sponges are known for their high metabolic rates (Gili and Coma 1998). The CWC reef sponge *H. coriacea* is equally active since respiration and assimilation was in the same order of magnitude as temperate sponges *Haliclona occulata* (Koopmans et al., 2010). *H. coriacea* did not preferentially fed

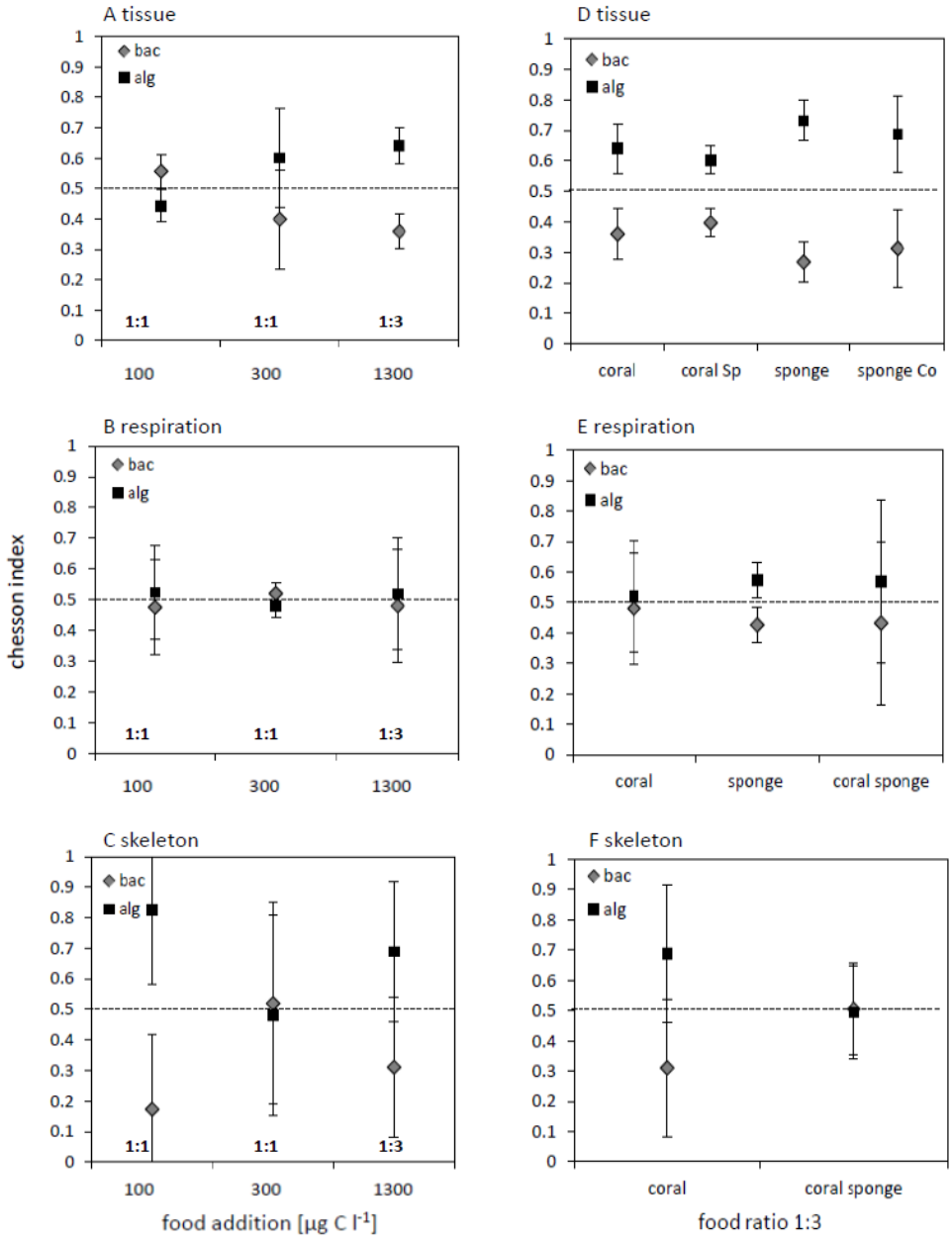


Figure 5.8. Selectivity by *L. pertusa* and *H. coriacea*. (A) Selective assimilation, (B) respiration and (C) skeleton C incorporation by *L. pertusa* depended on food concentration and composition (chesson index, Chesson 1983). (D) Selective tissue assimilation and (E) respiration by *L. pertusa* with (coral Sp) and without the sponge.

on bacterioplankton, as reported for most other sponges (Witte et al., 1997; Ribes et al., 1999; de Goeij et al., 2008). Assimilation and respiration of bacteria and algae was equal, even when algae were less abundant, indicating a preferential assimilation of algae under this condition (Fig. 5.8.D, E). A comparable assimilation of pico- (equalling bacteria) and nanoplankton (equalling algae)-derived carbon under analogue conditions has also been observed for the temperate sponge *Mycale lingua* (Pile et al., 1996), which is reported on CWC reefs (van Soest et al., 2007). Phytoplankton may therefore be an important diet contribution to CWC reef sponges.

The observed selection of relatively larger algal cells might be caused by the aquiferous canal system of the sponge and its different cell types. While particles < 4 µm are preferentially retained in the filter cell, i.e. choanocytes, of sponges, larger particles are captured by their pinacocytes, epithelial cells (Turon et al., 1997). Hence, a modulation of selective cell types might reflect the differences in assimilation rates among species and allows niche segregation between suspension feeders on the CWC coral reef. Further, histological studies are needed to confirm this hypothesis.

Processing of coral-derived C and N by *H. coriacea*

In this study we report the first evidence for a trophic transfer between an encrusting cold-water coral reef sponge and the coral-water coral *L. pertusa*. *H. coriacea* assimilated coral-derived organic matter (mucus) into its biomass and partly expels this coral-derived material as particulate organic matter. This sponge-derived POM may subsequently be used by other detritivores. This recycling pathway has recently been identified for tropical coral reefs (de Goeij et al., 2013), but our results indicate that this 'sponge loop' may also exist in cold-water coral reef communities. So far bacteria have been considered as the main consumers of the extensively released coral mucus in cold-water corals reef systems, thereby functioning as a vector for C an N cycling in cold-water coral reefs via the well established 'microbial loop'(Wild et al., 2008; Wild et al., 2009). However the retention of energy and nutrients by encrusting cold-water coral reef sponges might be an additional recycling pathway in the cold-water coral ecosystem that is currently not considered in food web studies (van Oevelen et al., 2009).

Interestingly, coral-derived organic matter was assimilated at a similar rate as bacteria and algae, suggesting that coral-derived mucus matter can be of similar importance to sustain sponge metabolism as algae and bacteria. *H. coriacea* is often seen to live very close to the living coral tissue, which may provide the

sponge with readable access to coral mucus that is produced in large amounts (Wild et al., 2008). This direct availability and accessibility may imply that the sponge *H. coriacea* uses coral-derived organic matter as the main source of energy under natural conditions, similar as its tropical counterparts (van Duyl et al., 2011). The enrichment of bacteria-specific biomarker (PLFA) in the sponge tissue suggests strongly that bacteria are involved in the assimilation of coral-derived organic matter by the sponge. However, also the sponge-specific PLFAs showed assimilation and it is possible that sponge associated bacteria mainly take up the dissolved fraction of coral mucus while the sponge itself mainly assimilates the particulate fraction. However direct assimilation of the DOM fraction by sponge cells cannot be excluded and might occur in parallel as observed for tropical sponges (de Goeij et al., 2008).

Sponge-coral interaction

H. coriacea is one of the few species that grows adjacent to living polyps of the CWC *L. pertusa* (Buhl-Mortensen and Buhl-Mortensen, 2004) and has been observed to overgrow coral polyps (Buhl-Mortensen, 2001). This indicates that the coral-sponge relationship might be parasitic or at least competitive, possibly affecting the metabolism of both species (Pawlik et al., 2007). Nevertheless, in this study we did not observe any significant changes in food uptake or metabolic processing by *L. pertusa* or *H. coriacea* when mutually present. Our data however clearly showed that *H. coriacea* can assimilate coral-derived C and N and transform it into sponge biomass (see above). This indicates a commensalistic relationship between coral and sponge in which the sponge gains energy by consuming coral mucus, whereas the coral is unharmed.

However, only a small fraction of the offered food was processed during this study and it is possible that the sponge-coral relationship is highly context dependent (Loh and Pawlik, 2012). The competitive effects, also manifested in the overgrowth of the coral by the sponge, therefore might be masked by the relatively high food availability. However, also other environmental factors might trigger coral overgrowth. Accordingly, Aerts (2000) and Rützler (2002) suggested that especially damaged and stressed corals have a higher susceptibility to sponge overgrowth which is in agreement with observations that *H. coriacea* is especially overgrowing coral polyps in reef areas impacted by human fishing activities (T. Lundälv pers. comm.). Considering that 30 to 50% of CWC reefs in Norway are impacted by fishery (Fosså et al. 2002) and indications are available that this might highly affect the coral-sponge relationship more data and experiments are needed

to better understand the characteristics, drivers and consequences of the this interaction for ecosystem functioning.

Acknowledgements

Lisbeth Jonsson is thanked for her help with ROV sampling. Ann Larsson is thanked for providing valuable input and pictures on the experimental setup. Pieter van Rijswijk is thanked for help whenever it was needed the most. The analytical lab of NIOZ-Yerseke is thanked for sample analysis. This research was supported by the Calmaro project (FP7/2007-2013), funded by the European Community's Seventh Framework Program (FP7/2007-2013). Additionally support was given by the European Community for the access provided at the Sven Lovén Centre for Marine Sciences, Tjärnö, Sweden (ASSEMBLE grant agreement no. 227799 to FCvD).

6

General discussion

Photo by S. Zankl

Biogeochemical cycling in cold-water coral reefs

Cold-water coral reefs are fuelled by the input of organic matter that is produced in the surface ocean. High bottom currents at various reefs (Thiem et al., 2006; Mienis et al., 2007; Davies et al., 2009) together with a variety of hydrodynamic mechanisms provide an enhanced food supply to the community and establish the link to surface primary production. Turbulence created by the reef framework further increases particle flux to cold-water coral reefs and the release of organic matter by the coral fuels the microbial community around reef locations. The way the food is processed within the reef community is however largely unknown. Lavaleye et al. (2009) found a preferential removal of particulate organic nitrogen above cold-water coral reefs and model observations showed a 20 times higher organic matter uptake by the cold-water coral community than by communities in the off-reef seafloor, characterizing cold-water coral reefs as hotspots of C cycling (van Oevelen et al., 2009). According to van Oevelen et al. (2009) three key components in the CWC-reef community dominate organic matter processing: The reef-building coral *Lophelia pertusa*, the polychaete *Eunice norvegica* and associated sponges. However not much is known about the feeding ecology of these species.

The aim of this thesis was to advance knowledge about the metabolism of the coral and its associated species, focusing especially on (non-) trophic interactions between them. Hereby we particularly wanted to target the following questions:

1. Which resources (organic and inorganic) can be utilized by the cold-water coral *L. pertusa?*
2. How does the coral process different resources?
3. Which effects have associated species such as sponges and polychaetes on coral metabolism?
4. Do species interactions contribute to the high biogeochemical cycling of cold-water coral reefs?

Resource utilisation by *L. pertusa*

L. pertusa is the main reef-forming cold-water coral in the North Atlantic Ocean and is thought to rely solely on heterotrophic feeding. Stable isotope analyses indicate that a broad range of organic matter sources might be used by the coral, including resuspended, degraded organic matter, relatively fresh phytoplankton and zooplankton (Duineveld et al., 2004; Kiriakoulakis et al., 2005; Duineveld et al., 2007; Dodds et al., 2009; Duineveld et al.; 2012). The uptake and processing of these resources by *L. pertusa*, and therefore their nutritional significance, is however poorly understood. So far, laboratory studies have mainly focused on the capture of zooplankton (Purser et al., 2010; Tsounis et al., 2010; Larsson et al., 2013a). Only one study investigated the clearance of dissolved organic matter (DOM) from the water column by *L. pertusa* (Gori et al., 2013) while the capture of algae and bacteria or the utilisation of inorganic nutrients has not been studied at all.

Within this thesis we investigated the uptake of various organic resources by *L. pertusa*. Our results indicate that *L. pertusa* assimilates the whole range of resource available at cold-water coral reef locations, including DOM, bacteria, algae and zooplankton. The assimilation of resources was comparable when offered in separate treatments. This indicates that cold-water corals follow an opportunistic feeding strategy and utilise what is available in its surrounding environment. However, if available in equal concentrations in the same treatment a preference for larger particles such as zooplankton over smaller particle such as phytoplankton was observed. Food availability, next to particle size, therefore appears to be important factor determining coral nutrition. This corresponds to the observation that the diet of *L. pertusa* differs between reef locations depending on the availability of resources: High zooplankton abundance at shallow reef locations leads to high diet contribution (Dodds et al., 2009) whereas at deeper locations *L. pertusa* thrives mainly on (phyto-) detritus (Duineveld et al., 2007; van Oevelen et al., 2009).

Next to the utilisation of organic resources we however also observed the utilisation of inorganic nutrients (HCO_3^-, NH_4^+, N_2) by the coral holobiont (coral+bacteria), characterizing the coral rather as mixotroph then solely heterotroph. We especially observed inorganic C fixation, N_2 fixation and NH_4^+ assimilation, processes which can supplement coral nutrition and are most likely conducted by microbial symbionts of *L. pertusa*. Although the contribution of these processes to the energy demand of the coral are not comparable to the contribution of photoautotrophic dinoflagellates in tropical corals (~2% C by chemosynthetic

bacteria versus ~95% C addition by photoautotrophic dinoflagellates, Muscatine, 1990), it still confirms the high nutritional flexibility of *L. pertusa*.

The broad utilisation of resources by *L. pertusa*, including inorganic nutrients, together with the opportunistic feeding behaviour by the coral might also partly explain its cosmopolitan distribution, since it allows *L. pertusa* to growth under a wide range of environmental conditions. Especially the assimilation of DOM or bacteria might be a good strategy to bridge periods of reduced external particulate food input, since both resources are produced within the coral ecosystem (Wild et al., 2008; Wild et al., 2009; Maier et al., 2011). Accordingly, Larsson et al. (2013a) found that *L. pertusa* can survive long periods of starvation without changes in respiration or tissue to dry weight ratio, suggesting that *L. pertusa* might be well adapted to compensate changes in food conditions, possibly by net assimilation of DOM as demonstrated for the cold-water coral *Desmophyllum dianthus* (Naumann et al., 2011). It is therefore possible that the high nutritional flexibility of *L. pertusa* might also help the coral in adapting to possible changes in external food supply (composition, concentration) triggered by a combination of global warming and ocean acidification (see Blanchard et al., 2012; Gao et al., 2012). But investigations are needed to confirm this assumption. Further studies are also needed to investigate the effect of food concentration/composition together with changes in other environmental parameter such as temperature and carbonate chemistry to better predict the response of cold-water corals to global change.

Resource processing by *L. pertusa*

Various aspects of the physiology of *L. pertusa* haven been investigated recently, including skeletal formation (Adkins et al., 2003; Maier et al., 2009; Form and Riebesell, 2012; Maier et al., 2013a), respiration (Dodds et al., 2007; Maier et al., 2013b; Larsson et al., 2013a), mucus production (Wild et al. 2008; Maier et al. 2011) and food capture (Purser et al., 2010; Tsounis et al., 2010; Larsson et al., 2013a). The metabolic processing of different food sources by the coral however is poorly understood to date.

Comparing data of food capture (300 - 700 µg C g^{-1} DW d^{-1}, depending on food addition, Purser et al., 2010), respiration (40 to 70 µg C g^{-1} DW d^{-1}, depending on the temperature, Dodds et al., 2007) and mucus production in form of total organic C (500 to 900, 300 to 500 µg C g^{-1} DW d^{-1}, Wild et al., 2008 and Maier et al., 2011 respectively) from previous studies it appears that most of the C acquired by prey capture can be lost by mucus production and respiration while less is actually

assimilated. Assimilation, respiration and skeleton incorporation data obtained from this thesis confirm these observations. Using capture rates from Purser et al. (2010) under comparable conditions to estimate C available from prey capture, we found that only ~1% of the acquired C was assimilated in the tissue, ~0.1% of the food-derived C was stored in the skeleton and ~7% was lost by respiration (Fig. 6.1. A). 92% of the C captured by *L. pertusa* as measured by Purser et al. (2010) however could not be explained by processes measured in this thesis. Although capture rates might overestimate the amount of C available to the coral, since not every prey item is ingested, the undefined amount of C was in the range of mucus release rates measured previously (Wild et al., 2008; Maier et al., 2011). However, whether mucus is indeed a big sink of C obtained from prey capture needs to be validated in further studies.

We also followed the assimilation of food source into different tissue compounds such as hydrolysable amino acids, fatty acids and phospholipid-derived fatty acids to investigate food processing within the coral. We hereby could show that the biochemical composition of food sources can affect their assimilation into tissue compounds by *L. pertusa*. This was best illustrated in the assimilation of dissolved organic matter (DOM), consisting of dissolved free amino acids versus particulate organic matter (POM), represented by bacteria, algae or zooplankton. Although both sources were assimilated into amino acids, POM assimilation into fatty acids significantly exceeded the assimilation of DOM into fatty acids (Fig. 6.1.B). Low assimilation of DOM into fatty acids could be explained by the absence of fatty acids in this source. However, that *L. pertusa* incorporated tracer C into fatty acids when offered DOM represented by free amino acids indicates at the same time that the coral is able to synthesise fatty acids de novo. *L. pertusa* therefore might not rely on dietary fatty acids alone to sustain its lipid metabolism. We found de novo synthesis of fatty acids also in POM treatments, confirming this assumption. The statement you are what you eat therefore might not be completely true for *L. pertusa* and has particular implications for the use of FA as trophic markers, which depends on the assumption that the fatty acids composition of the consumer reflects the fatty acids composition of its food source.

However, the variability in data was high and the incubation time short (4 days). To confirm the implications of these results, which are particularly relevant for studies using fatty acids as trophic markers, more extended experiments with more replicates are urgently needed.

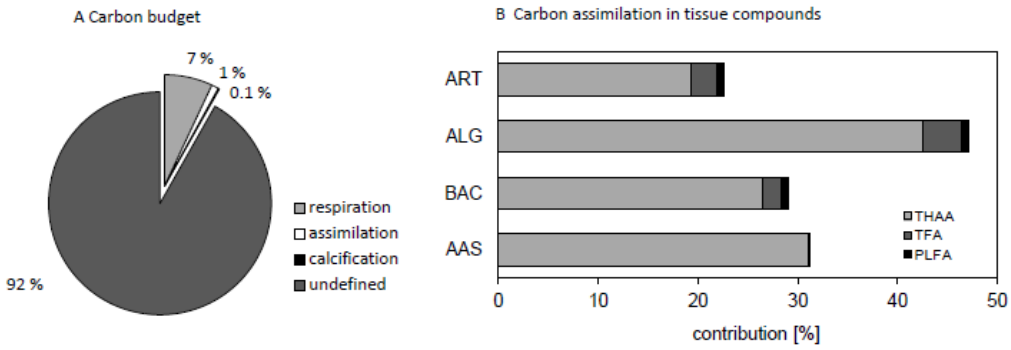


Figure 6.1. Carbon budget of *L. pertusa* obtained from tracer uptake and capture rates measured by Purser et al. (2010) under comparable conditions (A). Carbon tracer assimilation in tissue compounds by *L. pertusa* (B).

Associated species influence coral calcification, nutrient recycling and the biogeochemical cycling of the reef community

Cold-water corals such as *L. pertusa* are the heart of cold-water reef ecosystems. They build the complex framework which physically alters the surrounding environment. This framework can provide many ecological functions, which attracts numerous different species (Roberts et al., 2006; Buhl-Mortensen et al., 2010). Cold-water coral reefs are therefore not only characterized by the reef-building coral alone but also by the many species associated with it. As the coral, these species interact with their surrounding environment including neighbouring species such as the coral itself. These interactions can be manifold, including competition, symbiosis (mutualism/facilitation - parasitism) and predator-prey interactions (Buhl-Mortensen and Buhl-Mortensen 2004; Roberts et al., 2009a). It is long been recognised that such species interactions can shape ecosystems and thus define their functioning, reflected by the collective activity of organisms in exploiting and recycling available resources (Danovaro et al., 2008). Among the various interactions facilitation, the positive interactions between species, were highlighted recently as one key mechanism for enhancing ecosystem functioning (Bradley et al., 2002; Stachowicz, 2001).

Within this thesis we identified several positive interactions between the cold-water coral *L. pertusa* and associated fauna which can influence ecological processes (Fig. 6.2.). Our results therefore indicated that facilitative interactions

between key species on cold-water coral reefs enhance several key functions as describes below.

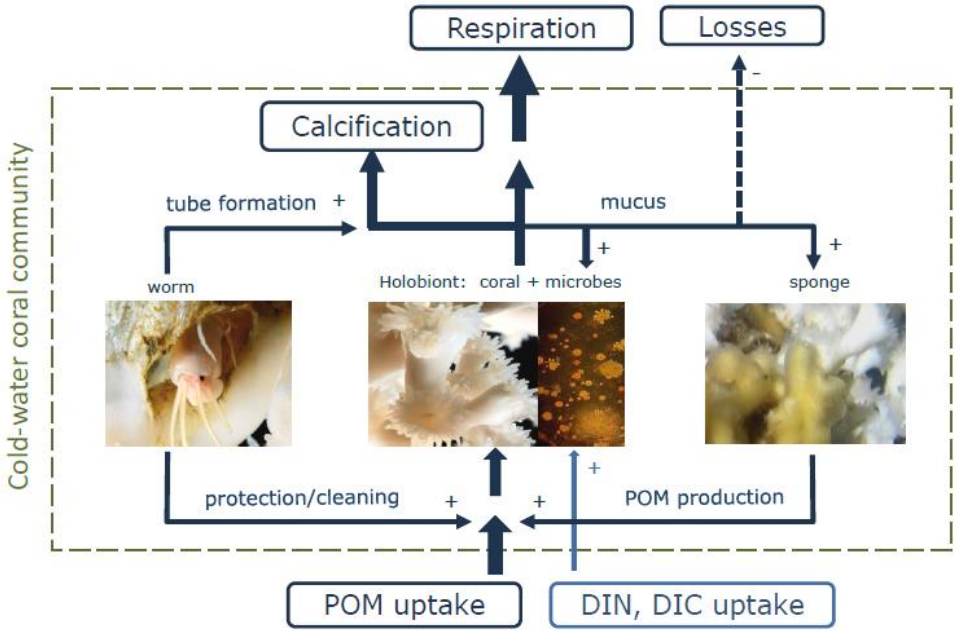


Figure 6.2. Schematic drawing of interactions between, three key species in the cold-water coral reef community: *E. norvegica*, the holobiont *L. pertusa* and *H. coriacea*. Left side: The worm *E. norvegica* enhances coral calcification through its tube construction while the worm's cleaning behavior increases its food uptake. In the middle: The coral holobiont formed by the reef-building coral *L. pertusa* and associated microbes, which supplement coral nutrition by utilizing inorganic nutrients and reduce energy loss by recycling coral mucus. Right side: The sponge *H. coriacea* assimilates coral mucus and produce particulate organic matter (POM). This POM may be taken up by the coral and other filter feeders of the reef community and thereby reduces the loss of energy and nutrients from the reef system.

Microbes

The microbial community associated with the cold-water coral *L. pertusa* appeared to supplement coral nutrition by accessing inorganic nutrients but also to contribute to mucus recycling by using it as an energy source for heterotrophic processes such as denitrification and N_2 fixation (Fig. 6.2.). The role of microbes in C recycling

within cold-water coral reefs has been recently demonstrated by Wild et al. (2008, 2009). The authors particularly highlighted the role of microbes associated with the coral mucus layer involved in coral–derived organic matter processing. This together with our findings indicate that the coral holobiont (coral + microbes) can contribute to the C recycling within the reef ecosystem. Our studies however also highlight the ability of the coral holobiont to participate in the cycling of N within the reef environment. We found nitrification, denitrification, ammonium assimilation and N_2 fixation occurring in the presence of the coral. Considering that just recently also complex N cycling has been demonstrated for the reef associated sponge holobiont *Geodia baretti* (Hoffmann et al., 2009), it is possible that cold-water coral reefs are not only hotspots of C cycling but with the contribution of microbial symbiosis might also be locations of enhanced N cycling and transformation.

Tube-building polychaetes: *Eunice norvegica*

We also observed that interactions between the coral and associated macrofauna significantly affect key processes in the reef community. Hereby we observed that the interaction between *L. pertusa* and the tube building worm *E. norvegica* enhances coral calcification and therefore reef strength, growth and resilience without significant extra metabolic cost for the coral as evident in the absence of changes in coral respiration. The coral in turn enhances the food uptake of the worm without being hindered in total food uptake itself (Fig. 6.2.). In sum the interaction between coral and worm not only fuels reef growth it also enhances the food assimilation of the community. Considering that the worm can be very abundant at reef locations (Roberts, 2005; van Oevelen et al., 2009) the interaction between coral and worm might significantly enhance cold-water coral reef functioning. The observation that the coral-worm relationship also enhances coral aggregation and increases the defence abilities of both species (Buhl-Mortensen, 2001; Roberts, 2005) further strengthens its positive effect on ecosystem functioning. Hereby, especially enhanced calcification and reef-aggregation might contribute to the development of extensive coral reefs and giant carbonate mounds (Roberts, 2005).

Cold-water coral reef sponges

Another key interaction was identified between *L. pertusa* and its associated sponge *H. coriacea*. In this thesis we observed that the interaction between both organisms was particularly characterised by trophic transfer. The coral produces

extensive amounts of mucus, mainly released as DOM (Wild et al., 2008), which in turn is consumed by the sponge. Subsequently, the sponge produces large amounts of POM, which in contrast to DOM, previously released by the coral, is accessible by many reef inhabitants (Buhl-Mortensen and Buhl-Mortensen, 2004). That indeed sponge derived-POM is consumed by higher trophic levels has recently been shown in a tropical reef ecosystems with major implications on ecosystem functioning (de Goeij et al., 2013). The so-called 'sponge loop' can reduce energy and nutrient loss from the ecosystem by retaining and recycling coral-derived organic matter while subsequently making it available to higher trophic levels. Considering that sponges are abundant in cold-water coral reef ecosystems (van Soest et al., 2007) and the assimilation of DOM appears common among them since also van Duyl et al. (2008) observed the ability of two other cold-water coral reef sponges to assimilate DOM in the form of leucine. The sponge loop together with the microbial loop (Wild et al., 2008; Wild et al., 2009) might therefore be key components in cold-water coral reef recycling and contribute to ecosystem functioning.

Conclusion: The sum is more than its parts

Within this thesis we found that the coral frames the ecosystem while its neighbours shaping it to the final picture. Important ecosystem processes such as calcification, food uptake, food processing and recycling are defined not only by the coral but by the many species which share the ecosystem and actively interact with the coral. Hereby especially facilitative species interactions appear to be essential components in the functioning of cold-water coral reef ecosystems. They also might be a key factor explaining the high biogeochemical cycling observed at reef locations (van Oevelen et al., 2009; White et al., 2012) due to their positive influence on coral calcification and their effect on C and N (re)cycling within the reef ecosystem.

However, we should keep in mind that species interactions can vary with changing environmental conditions (Bohannan and Lenski, 2000; Sanford et al., 2003) and that their effects are often context-dependent (Hoeksema and Bruna, 2000). Microbial symbionts which might increase coral health and nutrition under present conditions might decrease coral health by causing diseases under future temperature scenarios (Rosenberg et al., 2007; Vega Thurber et al., 2009). Macrofauna which increases coral calcification under current conditions might help to compensate coral dissolution under future ocean acidification scenarios but

might as well cause additional costs under these scenarios. Global change might also impact coral health and therefore trigger overgrowth of corals by sponges (Aerts and van Soest, 1997), formally been beneficial to the reef community by transforming coral-derived DOM into POM. Considering the influence of species interactions on ecosystem functioning, it appears essential to understand their nature and functioning to better predict the response of cold-water coral reefs to future environmental conditions.

7

References, summaries, acknowledgements

Photo by M. López Correa

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Summary

Cold-water coral reefs are hotspots of biodiversity and biogeochemistry in the deep-sea, solely fuelled by external energy sources. Hence, food input, processing and recycling play a major role in the functioning of these ecosystems. In this thesis I aimed to study energy flow and metabolism of cold-water coral reef ecosystems by assessing the uptake and processing of different food sources by key species of the community: The coral *Lophelia pertusa*, the worm *Eunice norvegica* and the sponge *Hymedesmia coriacea*. We further investigated the (non-) trophic interactions between these species and determined their impact on ecosystem functioning. Stable isotope as deliberately added tracers (^{13}C and ^{15}N) and bulk and compound specific isotope analysis were the major tools used throughout this study.

The scleractinian coral *L. pertusa* is the dominant reef-building species and is the basis of the complex cold-water coral ecosystem. Very limited knowledge is however available what resources this species can utilize. In chapter 2 we studied assimilation and processing of various organic food sources by *L. pertusa*, including zooplankton, algae, bacteria and dissolved organic matter. We found that all food sources were assimilated at comparable rates. This indicates that *L. pertusa* is an opportunistic feeder that can utilize a broad range of resources. However metabolic processing differed among resources, suggesting that different food sources vary in their nutritional values to the coral.

L. pertusa is a holobiont, because it harbours a variety of microbial symbionts in the external mucus layer and tissue. The functional role of these microbes is unclear, although genetic analysis has shown that they may have a role in coral nutrition. To gain a more complete picture on nutrition of the coral as a holobiont, we studied the role of microbes in the uptake and processing of various inorganic nutrient sources by *L. pertusa* in chapter 3. We found NH_4^+ assimilation, nitrification, denitrification, and nitrogen-fixation carried out by the coral holobiont. We further demonstrated the fixation of bicarbonate in coral tissue mediated by chemoautotrophic bacteria. These results indicate that the holobiont *L. pertusa* contributes to the N cycling within the reef community. Concurrently *L. pertusa* is able to utilize inorganic nutrients to supplement its C and N requirements, highlighting its previously unrecognized mixotrophic nature.

Many species live attached to and on the carbonate framework that is formed by *L. pertusa*. Some species live in close association with the coral, potentially affecting

its growth and nutrition and therefore ecosystem functioning. The worm *E. norvegica* is one such example, but it was unclear what this association entails. Aquarium observations have shown that the worm cleans and protect its coral host. It however also steals food from the coral and might enhance coral calcification during the building of its home tube. In chapter 4 we quantitatively investigated the relationship between *E. norvegica* and *L. pertusa* with respect to uptake and processing of two food sources (algae, zooplankton (Artemia)) and calcification rates by the coral. Results showed that the worm can enhance its food uptake in the presence of the coral. The coral however kept assimilation constant by increasing the consumption of smaller algae particles less favored by the polychaete. Furthermore in the presence of the worm the coral increased calcification. This did however not result in extra energetic costs as indicated by constant respiration. Consequently, the relationship is beneficial for both partners involved and might be of high importance for reef development and resilience given its effect on coral calcification.

Sponges are key species in the processing of organic matter and their interactions with reef-building coral can highly impact ecosystem functioning. In chapter 5 we studied the role of food concentration and composition on the metabolism of *L. pertusa* and its interaction with its associated sponge *H. coriacea*. Three different concentration levels and two ratios of bacterial to algal biomass were supplied to the coral without the sponge. Skeletal incorporation and metabolic activity increased with enhanced food supply. Assimilation of the different food sources was determined by their availability while respiration and skeletal incorporation did not respond to different food compositions. This indicated a decoupling for respiration and skeletal incorporation from tissue assimilation. The trophic interaction between sponge and coral was then tested at one food concentration level but neither the sponge nor the coral showed any change in food uptake or processing of bacteria and algae under mutual presence. We however could observe trophic transfer between the coral and sponge via coral mucus, partly mediated by bacteria associated with the sponge. Subsequently, the sponge utilized the coral mucus to produce sponge-derived detritus, indicating energy recycling via the sponge loop.

In conclusion (chapter 6), this thesis shows that the cosmopolitan reef building coral *L. pertusa* is an opportunistic feeder, exploiting a broad range of resources. Together with its microbial symbionts it is able to even access inorganic nutrients, characterizing it as mixotroph. Food concentration, composition and in particular

species interactions can positively affect coral nutrition and growth and thereby can enhance cold-water coral reef ecosystem functioning.

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Nederlandse samenvatting

Koudwater koraalriffen zijn hotspots van biodiversiteit en biologische activiteit in de diepzee, die uitsluitend gevoed worden door externe energiebronnen. Daarom speelt de input, verwerking en hergebruik van voedsel een belangrijke rol in het functioneren van deze ecosystemen. Dit proefschrift heeft als doel de energiestromen en het metabolisme van koudwater koraalrif ecosystemen te bestuderen door het quantificeren van de opname en verwerking van de verschillende voedselbronnen van de belangrijkste gemeenschapssoorten: het koraal *Lophelia pertusa*, de worm *Eunice norvegica* en de spons *Hymedesmia coriacea*. Daarnaast onderzochten we (non-) trofische interacties tussen deze soorten en hun invloed op het functioneren van het ecosysteem. Het toevoegen van stabiele isotopen (^{13}C en ^{15}N) als tracer in combinatie met bulk en component specifieke isotoop analyse zijn de belangrijkste tools die gebruikt werden in deze studie.

Het steenkoraal *L. pertusa* is de belangrijkste rifbouwende soort en vormt op veel plaatsen de basis van het complexe koudwater koraal ecosysteem. Er is echter zeer beperkte kennis beschikbaar over welke voedselbronnen deze soort benut. In hoofdstuk 2 bestudeerden we assimilatie en verwerking van verschillende organische voedselbronnen door *L. pertusa*, waaronder zoöplankton, algen, bacteriën en opgeloste organische aminozuren. We vonden dat alle voedselbronnen werden geassimileerd met vergelijkbare snelheden. Dit duidt erop dat *L. pertusa* een opportunistische soort is, die een breed scala aan voedselbronnen kan gebruiken. Echter, de respiratiesnelheid was niet gelijk voor de diverse bronnen, wat suggereert dat met de verschillende bronnen wel variëren in hun voedingswaarde voor het koraal.

L. pertusa is een holobiont, omdat het diverse microbiële gemeenschap bevat in de externe slijmlaag en weefsel. De functionele rol van deze microben is niet duidelijk, hoewel genetische analyse aangetoond heeft dat ze mogelijk een rol spelen bij de voedselopname van het koraal en dan met name het gebruik van anorganische nutriënten. Om een vollediger beeld van het koraal als holobiont te krijgen, bestudeerden we in hoofdstuk 3 de rol van microben in de opname en verwerking van diverse anorganische nutriënten door *L. pertusa*. We vonden dat de holobiont een breed scala aan stikstofomzetting uitvoerde, zoals NH_4^+ assimilatie, nitrificatie en stikstoffixatie. We vonden verder dat chemo-autotrofe bacteriën in het koraalweefsel, bicarbonaat konden omzetten tot organische

koolstofverbindingen. Deze resultaten tonen aan dat de holobiont *L. pertusa* niet alleen bijdraagt aan de N cyclus binnen het koraalrif, maar dat *L. pertusa* ook in staat is om anorganische voedingsstoffen te gebruiken als aanvulling op hun dieet. De gecombineerde opname van organische en anorganische bronnen duidt er op data de holobiont leeft als een mixotroof, hetgeen voorheen onbekend was.

Veel soorten leven gehecht aan de carbonaat structuur dat wordt gevormd door *L. pertusa* op de zeebodem. Sommige soorten leven in nauw contact met het koraal, met mogelijke gevolgen voor de groei en voeding en dus het functioneren van het ecosysteem. De worm *E. norvegica* is een voorbeeld hiervan, maar dusver was het niet duidelijk wat de relatie tussen beide organismen inhield. Aquarium observaties hebben aangetoond dat de worm haar koraalgastheer reinigt en beschermt. Echter, de worm ontneemt ook voedsel van het koraal. De worm bouwt ook een buisvormige schuilplaats, welke vervolgens door het koraal wordt ingebed in carbonaat. Hierdoor kan de aanwezigheid van de worm de calcificatie bevorderen. In hoofdstuk 4 hebben we kwantitatief onderzoek gedaan naar de relatie tussen *E. norvegica* en *L. pertusa* met betrekking tot de opname en verwerking van voedsel, namelijk algen en zoöplankton (*Artemia*), en de calcificatiesnelheiden van het koraal. Resultaten toonden aan dat de voedselopname van de worm hoger lijkt in aanwezigheid van het koraal zonder dat de voedselopname van het koraal negatief werd beïnvloed. In aanwezigheid van de worm, schakelt het koraal over opname van van kleinere algen deeltjes, die minder geliefd zijn bij de worm. De aanwezigheid van de worm leidde tot een duidelijke verhoging van de calcificatiesnelheid van het koraal, wat niet leidde tot extra energetische kosten, aangezien respiratie constant bleef. Derhalve is de relatie gunstig voor beide partners en van belang voor de ontwikkeling en veerkracht van het rif.

Sponzen spelen een sleutelrol in de verwerking van organisch materiaal en hun interactie met rifbouwend koraal kan van grote invloed zijn op het functioneren van het rifsysteem. In hoofdstuk 5 bestudeerden we de rol van voedselconcentratie en samenstelling op het metabolisme van *L. pertusa* en de interactie met de diepzee spons *H. coriacea*. De calcificatie en metabolische activiteit van het koraal namen toe bij verhoogde voedselbeschikbaarheid. De trofische interactie tussen spons en koraal werd vervolgens getest, maar zowel de spons als het koraal toonden geen verandering in voedselopname of verwerking van bacteriën en algen bij wederzijdse aanwezigheid. We konden echter wel een trofische transfer waarnemen tussen het koraal en de spons via koraal mucus, deels verlopend via

bacteriën die geassocieerd zijn met de spons. Daarbovenop gebruikt de spons het koraal mucus om detritus partikels te produceren, wat aantoont dat energie hergebruik plaatsvindt via een ‘spons loop’.

Samenvattend (hoofdstuk 6), laat dit proefschrift zien dat het kosmopolitische, rifbouwende koraal *L. pertusa* een opportunistische feeder is die een breed scala aan resources exploiteert. Samen met zijn microbiële symbionten is het in staat om zelfs anorganische nutriënten te gebruiken, hetgeen karakteristiek is voor een mixotroof. Voedselconcentratie, samenstelling en in het bijzonder de interactie met andere soorten kunnen een positieve invloed hebben op koraal voeding en groei en versterken daarmee het functioneren van het koud water koraalrif ecosysteem.

Acknowledgements

Interactions have been the main topic of this thesis but they were also a key factor determining my working life. Many people direct and indirect contributed to this booklet and I would like to express my deepest gratitude for this.

First and foremost I would like to thank my promoters Jack Middelburg and Karline Soetaert, who offered me the chance of a life time in choosing me for this PhD position. I will never forget the moment when you gave me the best Christmas present I ever got! Thank you for your guidance and advice ever since.

Dick van Oevelen, my co-promotor, you were my daily support and source of wisdom. Your guidance kept me straight, when I run the risk of losing focus, with all the wonders still to be discovered and investigated. I thank you deeply for your patience and support, for the many fruitful discussions and straighten sentences!

My gratitude also goes to my office mates: Leon, you always was my source of philosophical and scientific wisdom. Thank you for the many inspiring talks and for opening up the world of sponges to me! Cécile, you were my picture of energy and determination, overcoming all the ´eddies´ in your way. Thank you for a wonderful time.

Special thanks also go to my Swedish cooperation partners. Ann Larsson, Tomas Lundälv and Lisbeth Jonsson without you this thesis would not have been possible. Many thanks also go to my German partners Armin Form and Janina Büchner. Thank so much for offering me the opportunity to join your cruise, for all the support and input and friendship. Special thanks hereby also go to the crew of the Poseidon (cruise P420), Solvin Zankl (for incredible pictures!), Matthias López Correa, Sebastian Henning and Murray Roberts. It was such a pleasure meeting and working with all of you. The ultimate thanks got to Jürgen Schauer and Karen Hissmann who gave me the chance to meet my pets face to face. It definitely was an experience I will treasure forever!
My gratitude also goes to all the people of the NIOZ-Yerseke for creating such a wonderful and relaxed working atmosphere. It was a pleasure meeting and working with all of you. James, thank you so much for always having my favorite room ready and giving me a wonderful chocolate goodbye! To the ladies at the reception and the administrative department, thanks for all your help! Tanja thank you very much for translating my summary, I would have been lost otherwise! My special

thanks also go to the team of the analytical lab with an extra thank you for Peter van Breugel, who always had a solution and open ear for my many questions. I am so grateful for all your help and patience in analyzing sample, which often did not follow the general rules.

I also want to thank Bart Veuger for his great input on the N-cycling adventure and his patience in answering all my questions. It was a pleasure working with you!

Great thanks also go to Pieter van Rijswijk, who had the amazing ability to always find what was needed in his garage wonderland and who introduced me to the world of culturing. Thank you so much for everything!

I especially want to thank Lara, my dear friend, who thought me what passion was. I am so grateful that we met, thank you so much for your love and support. I don't know what I would have done without your friendship. The same thanks go to Julia. The last month would have been so much harder without you! Thank you for the many many open ears and that you shared your cats and kitchen with me!

Lucy, I am so happy that I had the chance to meet you. Thank you so much for the many wonderful talks we shared and the many cakes, which helped me to keep going!!! Without you my tropical coral work experience would have been a darker spot! Thank you for brightening this up!

I also want to thank Alessia, Yayu and Juliette for a great time in Denmark, a lovely wedding and your unconditional support. You are great!

Gratitude, which I never can express fully, which no words in the world are able to cover completely, goes to my family. Mama und Papa, danke für Eure Liebe, Euer Verständnis, Eure Begeisterungsfähigkeit, Eure Unterstützung, Euer Vertrauen und Eure Zuversicht. Egal was auch passierte, Ihr wart immer für mich da, habt mich aufgefangen, wann immer ich gestrauchelt bin! Danke, dass Ihr mich auf diesem einmaligen Abenteuer begleitet habt! Stephan, du bist der beste grosse Bruder, den man sich wünschen kann. Danke, dass ich immer auf Dich zählen kann. Ich bin so froh, dass es Dich gibt! Mein ganz besonderer Dank gilt meinem Schatz, Daniel. In den sieben Jahren, in denen wir nun zusammen sind, haben wir Höhen und Tiefen gemeistert. Du bist mit mir durch dick und dünn gegangen und bist nie von meiner Seite gewichen, egal wie tief das Tal oder wie weit der Weg gewesen ist, nichts konnte Dich erschüttern. Du bist mein Fels in der Brandung, meine Sonne am

Himmel, meine Flügel im Wind, ohne Dich wäre ich nicht vollständig, ohne Dich hätte ich das hier niemals geschafft. Danke, danke an Euch alle!

THANK YOU!