MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Wanted - Dead or Alive! The search for the chemosynthetic symbiont of the lucinid clam *Loripes orbiculatus*"

verfasst von / submitted by

Jennifer Windisch, BSc

angestrebter akademischer Grad / in partial fulfillment of the requirements for the degree of

Master of Science (MSc)

Wien, 2022/ Vienna, 2022

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 830
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
Betreut von / Supervisor:	Assoz. Prof. Dr. Jillian Petersen

Acknowledgements

I would like to start by thanking Jillian Petersen, who gave me the opportunity to be part of her research group and gain so much experience in the lab. I would also like to sincerely thank the staff throughout The University of Vienna and all other Domies that continuously supported me and helped me when I had issues in the lab. A big thanks also to Jay Osvatic, who let me develop my scientific skills and never got tired when I asked for help and needed him to look over things again. Also a huge thanks to the whole symbioses group that I could always ask for help and advice and to Dr. Bela Hausmann for doing the sequence processing and helping with the statistical analysis. And thanks to all the smoking and non- smoking buddies for all the fun and deep conversations on the rooftop.

Thanks to all my friends who never complained when I talked for hours about scientific projects or issues in the lab and always had an open ear. I would also like to acknowledge my dog Kira at this point, who always laid patiently for hours under my desk and on the couch during my studies at home and encouraged me by just looking incredibly sweet.

Finally, Mom and Dad, thank you so much for supporting me throughout the last 10 years. No matter if it was financially, or way more important your mental support, there was never a time where you wouldn't let me know that I am able to achieve anything I want to. Thanks for encouraging me all the time, going through all sorts of emotions with me and pushing me a little bit when I needed it. It’s been a long journey, and I could have never done it without you.

Table of Contents

1. Abstract

The evolutionary success of many animal lineages[1] as well as the functioning of marine ecosystems is highly conjoined with symbiotic associations between invertebrates and chemosynthetic microorganisms[2]. In a subset of these symbioses, the invertebrate host gains energy in the form of organic carbon through their symbionts which are able to synthesize carbon by oxidizing reduced sulfur compounds from the sediment[3]. Within these associations, symbiont transmission mode can vary but it plays an important role in symbiosis and has been elucidated for only a few species[4]. Lucinidae, a speciose family of chemosymbiotic bivalves, alongside their horizontally transmitted symbiont, have been the subject of intense study. A recent study has described high abundances of symbiont-related bacteria in association with seagrass, but sites with well studied Lucinids were not well represented[5]. We conducted this study*, t*o locate any environmental reservoirs of symbionts in association with *Loripes orbiculatus.* Environmental samples from pore water and sediment from the Bay of Fetovaia, Elba, Italy which were sampled in 2016 and 2018 and samples from *Loripes orbiculatu*s mucus burrow tubes from Sant Carles de la Rapita, Spain which were sampled in 2019 were taken and analyzed for the presence of the symbiont and the bacterial community composition. To investigate the release of symbionts from both dead and live clams, 16S rRNA amplicon survey was conducted on sediment and pore water samples from a twelve-day Symbiont Release Experiment. The number of symbionts was measured by ddPCR with the sulfur oxidizing enzyme B gene, which is essential for sulfur oxidation[6], serving as target. Symbiont related rRNA gene copies were detected from pore water filters and mucus burrow tubes which are built by the clam during sulfide mining and general burrowing activities. The sediment samples from the Bay of Fetovaia uncovered no matching rRNA sequences of the symbiont group. The release of the target symbiont could be detected in sediment samples from the dead and live clam treatment and in one water sample from the live clam treatment. These findings suggest that lucinid clams are creating adequate habitats for their symbionts through burrowing, and that clams could be, although infrequently, a source of environmental symbionts.

2. Zusammenfassung

Der evolutionäre Erfolg vieler Tierlinien[1] sowie das Funktionieren mariner Ökosysteme ist stark mit symbiotischen Assoziationen zwischen Wirbellosen und chemosynthetischen Mikroorganismen[2] verbunden. In einer Untergruppe dieser Symbiosen gewinnt der wirbellose Wirt Energie in Form von organischem Kohlenstoff durch seine Symbionten, die Kohlenstoff synthetisieren können, indem sie reduzierte Schwefelverbindungen aus dem Sediment oxidieren[3]. Bei diesen Assoziationen kann der Übertragungsmodus der Symbionten variieren, aber er spielt eine wichtige Rolle bei der Symbiose und wurde nur für wenige Arten aufgeklärt[4]. Lucinidae, eine artenreiche Familie chemosymbiotischer Muscheln, waren zusammen mit ihrem horizontal übertragenen Symbionten Gegenstand intensiver Studien. Eine kürzlich durchgeführte Studie hat eine hohe Häufigkeit von mit Symbionten verwandten Bakterien in Verbindung mit Seegras beschrieben, aber Standorte mit gut untersuchten Luciniden waren nicht gut vertreten[5]. Wir haben diese Studie durchgeführt, um herauszufinden, ob es Reservoire von Symbionten in Verbindung mit Loripes orbiculatus gibt. Umweltproben aus Porenwasser und Sedimenten aus der Bucht von Fetovaia, Elba, Italien, die 2016 und 2018 gesammelt wurden, und Proben aus Schleimröhren von *Loripes orbiculatu*s aus Sant Carles de la Rapita, Spanien, die 2019 gesammelt wurden, sind auf die Anwesenheit des Symbionten und der Bakteriengemeinschaft analysiert worden. Um die Freisetzung von Symbionten aus toten und lebenden Muscheln zu untersuchen, wurde eine 16S-rRNA-Amplikon-Untersuchung an Sediment- und Porenwasserproben aus einem zwölftägigen Symbionten Freisetzungsexperiment durchgeführt. Die Anzahl der Symbionten wurde mittels ddPCR gemessen, wobei das Gen des schwefeloxidierenden Enzyms B, das für die Schwefeloxidation essentiell ist[5], als Ziel diente. Symbiont-rRNA-Genkopien wurden in Porenwasserfiltern und Schleimröhren nachgewiesen, die von der Muschel während des Sulfidabbaus und allgemeiner Grabaktivitäten gebaut werden. Die Sedimentproben aus der Bucht von Fetovaia enthielten keine übereinstimmenden rRNA Sequenzen des Genus in dem sich unser Symbiont befindet. Die Freisetzung des Symbionten konnte in Sedimentproben mit toten und lebenden Muscheln und einer Wasserprobe aus der Behandlung mit lebenden Muscheln nachgewiesen werden. Diese Ergebnisse deuten darauf hin, dass lucinide Muscheln durch Grabenaktivitäten angemessene Bedingungen für Symbionten schaffen und dass Muscheln, wenn auch unregelmäßig, eine Quelle für Umweltsymbionten sind.

3. List of Abbreviations

ASV	Amplicon sequence variants
ddPCR	Digital Droplet Polymerase Chain Reaction
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
FISH	Fluorescence in situ hybridization
FSW	Filtered Seawater
JMF	Joint Microbiome Facility
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde solution
PW	Porewater
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
SOX	Sulfur oxidation
soxB	Sulfur oxidizing enzyme B
µm	mikrometer
µl	mikroliter
µg	mikrogram

4. Introduction

Chemoautotrophic symbiosis are nutritionally based associations between chemosynthetic bacteria and marine invertebrate or protist hosts. In the case of sulfur-oxidizing symbioses, the host provides their symbionts with access to the required substrates, such as hydrogen sulfide and oxygen. In exchange the host gains a portion of the fixed carbon for biosynthesis and energy production[7]. The first symbiosis of this kind to be discovered was in the deep-sea hydrothermal vent ecosystem in the giant tube worm, *Riftia pachyptila*[8], whose lack of mouth and gut [2] makes them dependent on their internal symbionts for their nutrition. They build large standing crops in the deep sea in the Pacific Ocean and possess a unique morphological adaptation to accommodate their symbionts, the trophosome, which encloses the endosymbionts in so-called bacteriocytes[9] . This discovery led to an exploration that brought forth a variety of different hosts and habitats which harbor similar kinds of symbioses[10,11] (Fig. 1, from Dubilier et. al., 2008, with hosts from 7 phyla). These numerous habitats include deep sea hydrothermal vent faunas[12] to shallow water habitats like seagrass meadows[13] or mangrove swamps[14,15] (Fig. 1) . Sulfur oxidizing symbioses dominate the biomass in deep sea habitats like vents and seeps but in some shallow water environments, even with extremely low sulphide concentrations (<5 μm),the abundance can be similar or even higher than in vents and seeps[16]. In seagrass sediments decomposition of organic matter produces sulphide and chemosynthetic bacteria are essential for detoxification of the sediment[17]. Here, chemosynthetic symbioses are thought to underpin key ecosystem functions [18–20] by altering the sediments available nutrients and providing most of the organic carbon needed for the animal host's nutrition.

In addition to nutritional supply, symbiont acquisition has many other advantages for the host and enables evolutionary and ecological expansion[21,22]. The symbionts also benefit from this association as they are free from predation and competition with free living bacteria[23] and are provided with a secure habitat. The mode of symbiont transmission can vary between different hosts. There are obligate associations with microorganisms that are transmitted vertically between generations[21], from parents to offspring, which may include incorporation of symbionts in or on the gametes[24] . This is the case for the vesicomyid deep-sea clam *Calyptogena okutanii,* where symbionts are transmitted via the egg and located on the outer surface on the egg plasma membrane[25]. Vertically transmitted

symbionts as well as those hosted inside the cell often form clonal populations within the host and form highly specific relationships with the host[26–28] . Symbionts can also be acquired horizontally, which includes the spread of symbionts from contemporary hosts or from an environmental stock of free-living symbionts [29]. During horizontal transmission, partners of a symbiosis have to reassociate anew each host generation[30], which drives the need for both partners to keep their offspring in close proximity. In *Bathymodiolus brooksi* the uptake of environmental bacteria is a restricted process and self infection in newly formed gill tissue dominates the bacterial colonization dynamics in newly formed filaments[31]. In *Riftia pachyptila,* symbionts are acquired during a small time window in an early developmental stage[9] and no additional symbiont uptake or release takes place during lifetime[23]. Symbionts get released and spread to the environment when the host dies and these spreader events constitute possible infection events for free living larvae and contribute to the abundance of the environmental population of the symbiont[32].

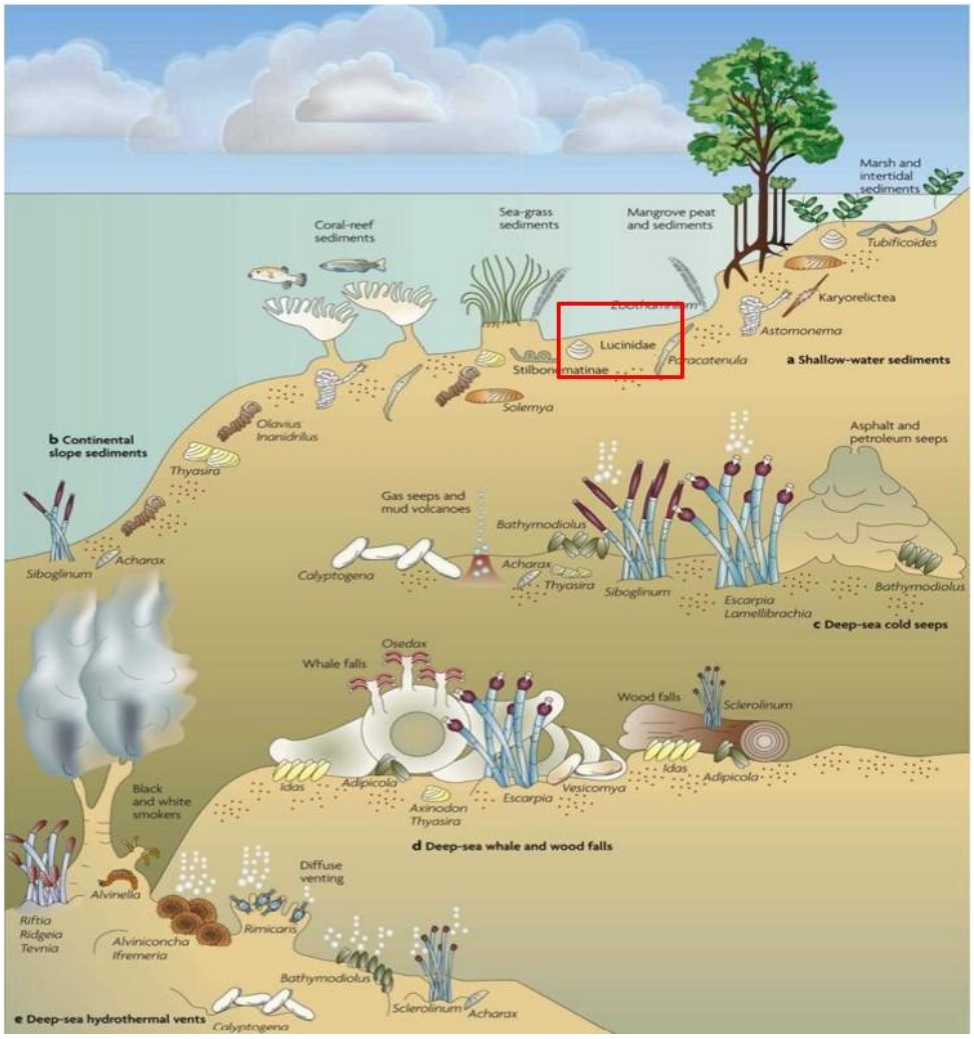


Figure 1: Different habitats where chemosynthetic symbiosis occurs with various host species. The family Lucinidae is highlighted with a red rectangle. Altered from Dubilier, 2008[16].

In the coastal zone invertebrate hosts belong to annelids, bivalves, gastropods and protozoans[33]. The greatest diversity of this intercellular thioautotrophic symbioses is found in five families among the Bivalvia - Lucinidiae, Mactridae, Solemyidae, Thyrasidae and Vesicomyidae[4]. With over 400 living species distributed from the intertidal zone down to around 2500m[34] , Lucinidae is one of the most species rich and widely distributed group of chemosymbiotic bivalve families[35]. They are found in shallow water environments with up to 1000 species per square meter[36] which makes them easily accessible and a preferred study system for chemosynthetic symbiosis. Lucinid bivalves are slow deep burrowers that live in the interface of oxic and anoxic organic rich sediment zones that supply oxygen from the oxic water column and reduced chemical entities from the sediment which get oxidized by symbionts in the bivalves gills[22,37,38]. Lucinid clams have a number of morphological adaptations resulting from their nutritional strategy[35]. They have a poorly developed digestive system, simplified feeding structures[39] and simplified but thick, large gills[38]. The

lucinid foot is also modified to become vermiform and highly extensible[39]. Besides the burrowing and locomotory activities , the foot builds a connecting mucus tube for the clam to the sediment - water interface[39] and constructs tubes which are mining the sediment below the clam for sulphide in order to supply their endosymbionts metabolism[40] (Fig. 2).

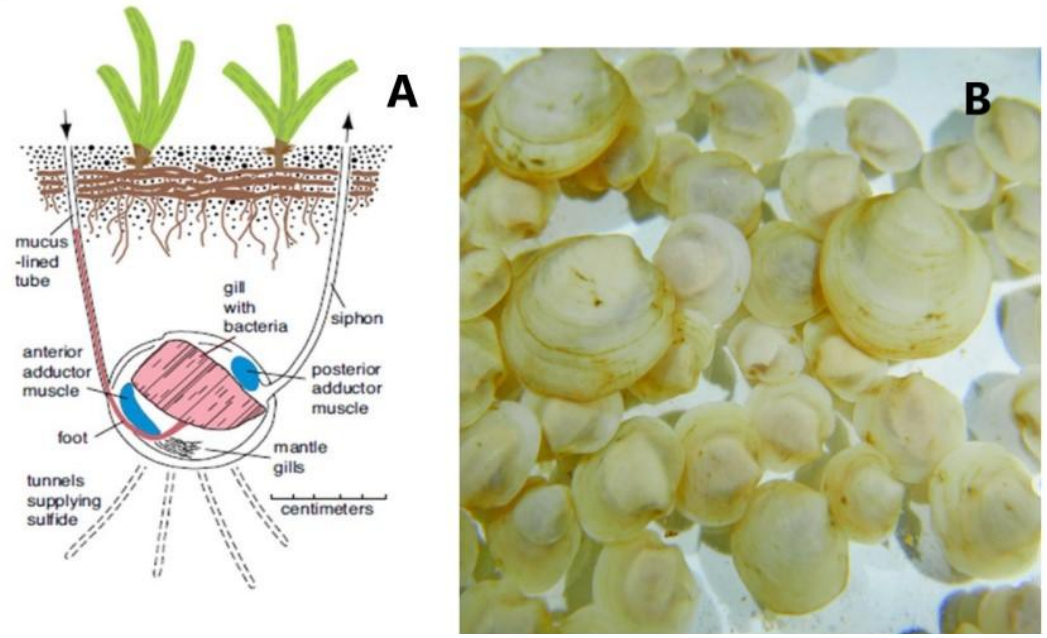


Figure 2: (A) Schematic picture of a lucinid of the genus Codakia. The U-shaped burrows are dug with a highly extensible foot. The clam is drawn in its natural habitat in seagrass sediment. From Stanley 2014[22]. (B) Individuals of Loripes orbiculatus from Mauritania, photo taken by Sarah Zauner.

Nutritionally based symbiosis in marine environments has created opportunities for adaptations to take place over much shorter timescales in contrast to traditional mutation- and competition-driven adaptations[41]. Acquiring symbionts from the environment enables the hosts to associate with locally- well adapted symbionts. To accommodate the symbionts all species of the family Lucinidae possess symbiont specific features like bacteriocytes[42,43], which are specialized modified gill epithelial cells. The gills are infected with symbionts from the environment by chance[44,45] in settled larvae, which leads to the classification of an aposymbiotic and a symbiotic phase of life (Fig. 3). All lucinid endosymbionts are thioautotrophic and belong to the bacterial class known as Gammaproteobacteria[46]. Based on their 16S rRNA sequences they were organized in three phylogenetic clades: A, B and C[2,44]. Symbionts associated with hosts that are primarily inhabiting sediments from seagrass beds belong to the largest Clade, A[45]. Compared with that, Clade B and C symbionts are mainly found in hosts originating from sediments in mangrove - rich areas[44]. In contrast to the original belief that each clade corresponds to one species of endosymbiont it was shown that

especially Clade A symbionts are made up of many different strains and have a higher diversity than originally thought [47–49]. The previously described Clade A - endosymbiont *Ca.* Thiodiazotropha endoloripes, which was found to be the only symbiont associated with *Loripes orbiculatus*[50] along Mediterranean and Atlantic coasts in Europe[51] was recently reanalysed and it was discovered that it is actually composed of two distinct species: *Ca.* Thiodiazotropha weberae and *Ca.* Thiodiazotropha lotti[51].

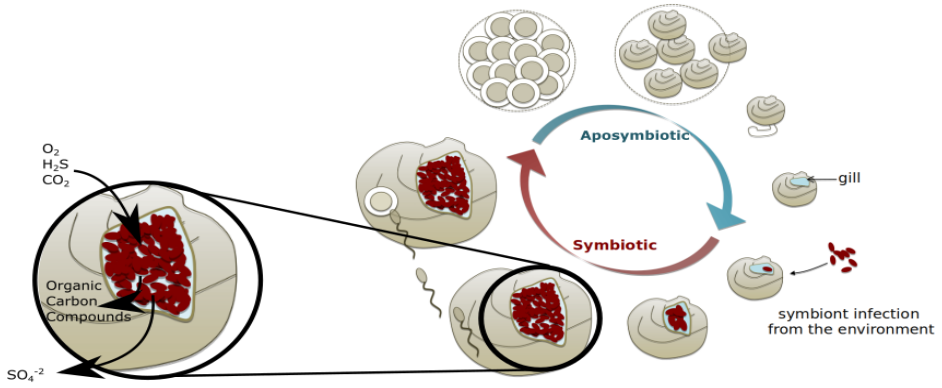


Figure 3: Life cycle of Loripes orbiculatus and its symbiont Candidatus Thiodiazotropha. Aposymbiotic clam in juvenile life phase and symbiotic clam after infection of the larvae. Illustrated by Dr. Jillian Petersen.

Nevertheless, lucinids gain their symbionts horizontally, it is still unclear if they gain them from a substantial free-living symbiont population in the environment or/and from actively released symbionts from a nearby host during lifetime or a spreader event during host death. *Loripes orbiculatus* lives in close proximity to seagrass beds, where accumulation of seagrass organic matter enhances sulphide production and leads to highly reduced sediments[52]. The bivalve-sulfide-oxidizer symbiosis reduces toxic sulfide levels in these sediments and enhances seagrass production measured by biomass[53]. In return, the bivalves and their endosymbionts profit from organic matter accumulation and radial oxygen release from the seagrass roots[36].

Seagrasses are amongst the most productive ecosystems on Earth[54,55]. Their ecological success is believed to rely on the symbioses with chemosymbiotic clams[37] and their symbiont's ability to detoxify the sediment[56]. Their importance for ecosystem services, such as water quality, coastal protection, carbon sequestration and as fisheries habitat[57–61], thrives the need for healthy seagrass ecosystems as millions of people rely on them for food and livelihood[62].

Climate-based and other anthropogenically induced environmental changes are threatening whole ecosystems and symbiosis may become an even more important mechanism for driving organismal adaptation in the ocean[63–65]. The three stage symbiosis of seagrass, lucinid bivalve and chemosynthetic symbionts explains the evolutionary success of seagrasses in shallow waters and offers new prospects for seagrass ecosystem conservation [66,67]. Since Global Warming and eutrophication are projected to cause higher sulfide levels in coastal shallow sediments[68,69], gaining knowledge about this sulfide-detoxification mutualism is essential for the conservation and restoration of these important ecosystems.

Aims of this study

In the last few years chemosynthetic symbiosis has been extensively studied but the majority of the lucinid-symbiont association's characteristics are still unexplored. Using highly abundant shallow water lucinid bivalves as study systems allows for a wide variety of experimental approaches to study the sulfide-oxidizing symbiosis, and their impact in the environment, even as the symbiont remains to be cultured.

To gain more insight into the host-symbiont characteristics of this association the purpose of this study was following:

1. Screen the environmental sediment microhabitats in the Bay of Fetovaia, split up in pore water and sediment, for the presence of *Candidatus* Thiodiazotropha, especially for the *Ca.* T. weberae and *Ca.* T. lotti like rRNA sequences, and verify the existence of a substantial environmental population.
2. Analyze the mucus burrow tubes built by *Loripes orbiculatu*s and estimate the abundance of *Candidatus* Thiodiazotropha species if it is present.
3. Observe if endosymbionts are able to re-inoculate the environment after the infection of the host and if they get released during the lifetime or after death of the host.

In this study we aimed to gain insight into the host-symbiont characteristics of this association and tried to find proof of a substantial symbiont population in the environment and ability of the symbiont to re-inoculate the environment after the infection of the host either from live clams or as symbiont spreader event during host death. For this purpose environmental samples of pore water and sediment from the Bay of Fetovaia, Elba, Italy and

mucus tubes from Sant Carles de la Rapita, Spain were analyzed and sequenced to identify the bacterial community and the environment was screened for the presence of the symbiont. Additionally, live and dead clam experiments were conducted. The experiments consisted of treatments in which clams were kept primarily in sediment and another in which clams were kept only in filtered seawater. These two treatments should test whether any released symbionts attach to the sediment like in the experiments from Klose et al.[9] or are free in the surrounding water, as Gros et al. [70] states that it is unclear whether symbionts in the environment inhabit the pore water or are attached to sediment particles. All treatments were sampled at specific time points and portions of sediment and water were stored for DNA extraction. All DNA samples were used for MiSeq amplicon sequencing and ddPCR analysis was conducted on the samples from the Symbiont Release Experiment to identify the presence and bacterial load of the symbionts in each sample.

5. Materials and Methods

5.1 Sampling Site and Sample Collection

The Bay of Fetovaia is located on the island of Elba, Italy (42.73 N, 10.15 E) (Fig. 4). Samples of live *Loripes orbiculatus* clams were collected near a *Posidonia oceania* sea-grass meadow roughly 400 meters off the coast in a depth of 7 m in the Bay of Fetovaia. Clams for the Symbiont Release Experiment were collected in 2018, sediment cores and pore water filters used in this study were collected in 2016 and 2018. Clams of sizes ranging from 0,5 – 2 cm were found up to 50 cm deep in the sediment and were dug out by hand during scuba diving. The live animals were kept in an aquarium filled with oxygenated seawater and native sediment until further processing. Clams were stored in RNAlater, sediment in the soil kit tubes and stored at -20°C and delivered to the laboratory in Austria.

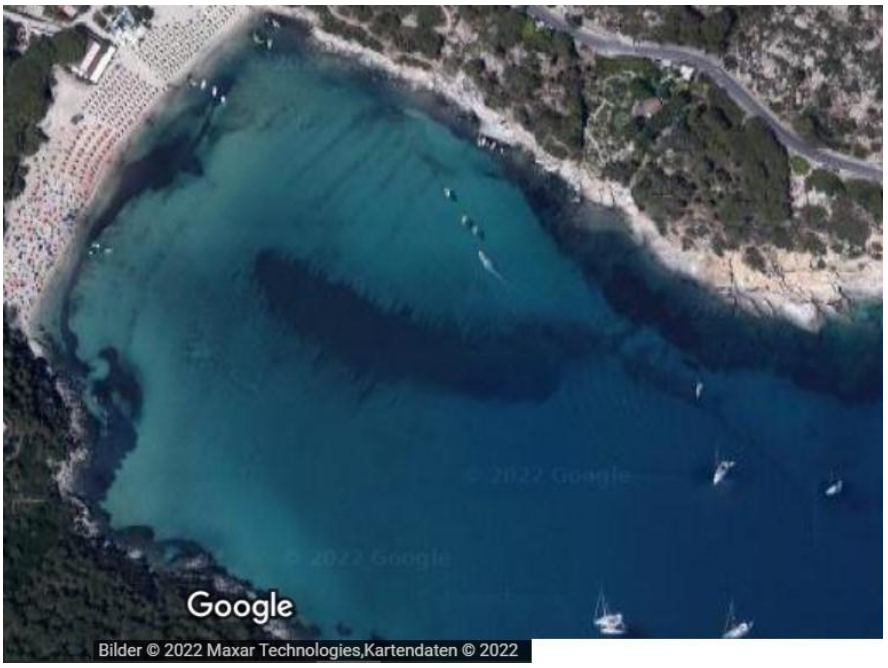


Figure 4: Sampling site, Bay of Fetovaia, Elba, Italy.

Porewater was collected in 30 ml syringes in 5 cm intervals up to a depth of 60 cm in 2016 and 45 cm in 2018 at the same site. The water samples were first filtered through a 20 µm mesh to remove any larger particles. The pre-filtered water was then transferred onto 0.2 µm hydrophilic isopore polycarbonate membrane filters with a diameter of 25 mm (Merck KGaA, Darmstadt, GER) with a vacuum pump. Samples were stored at -20°C. Four sediment cores in

a depth from 0-45 cm were taken in 2018 at the Elba sampling site. *Loripes orbiculatus* mucus tubes were sampled in Sant Carles de la Rapita, Spain (40.63 N 0.74 E) , in 2019 (Fig. 5). The study site had low water level and was inhabited by seagrass. Clams with mucus tubes were found in the first 30 cm centimeters of the sediment near *Cymodocea sp.* and were dug out by hand. The mucus tubes were collected and added to Powersoil tubes and stored at -20°C until processing.

Figure 5: Sampling site, Sant Carles de la Rapita, Spain.

5.2 Symbiont Release experiment

An aquarium experiment was conducted with live and dead clams from *Loripes orbiculatus* to gain insight in the symbiont release during lifetime and death of the host in Vienna. All clams used were allowed to adjust to aquaria conditions for 3 weeks before starting the experiment. The clams were sampled in July 2018 in Elba, Italy. Live and dead clam experiments consisted of a treatment in which clams were kept in sterile sediment within 1 cm of surface water and another in which clams were kept only in filtered seawater. For both treatment conditions control groups with either filtered sea water or sterilized sediment were set up (Fig. 6).

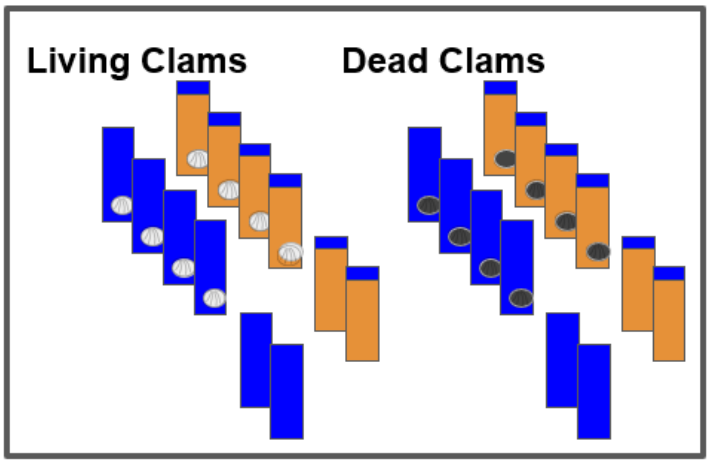


Figure 6: Symbiont Release Experiment: experimental setup: Live clams in filtered sea water (blue tubes with white clams) and sterilized sediment (brown tubes with withe clams) with controls and dead clams in filtered sea water (blue tubes with black clams) and sterilized sediment (brown tubes with black clams) with controls; illustrated by Jay Osvatic.

Clams used for the dead treatment were killed on day 1 (Fig. 7). All clams were washed three times in FSW (filter sterile seawater) before cutting. Dead clams were cut open with a sterile razor blade and the gills were severed from the visceral mass and placed in 50 ml falcon tubes. For sediment and FSW four replicates were used for the dead clam treatments, four replicates for the live clam treatments and two replicates for the control groups. All detailed materials and detailed sampling steps can be found in the supplementary information (Suppl. protocol S1). On day 1 dead and live clams were put in either sediment with FSW or in FSW. Sediment and water samples were taken on days displayed in Fig.7. Pore water samples were processed with Qiagen DNeasy PoreWater Sterivex kit . At first all water from the tube was sucked into the syringe, leaving all body parts behind. Then 35 ml were pushed through the sterivex filter and frozen at -20 after collection, until processing . Sediment samples were processed with the Qiagen DNeasy PowerSoil kit. At first clams were removed and 1 ml 2% PFA/FSW were added to a 2ml tube. Then approximately 0.5g sediment near a clam area was sampled and added to PFA/FSW tube and another 0.5g of sediment were added to a Mobio tube. The mobio tube was stored at -20° C. The sediment/PFA/FSW tube rested at 4°C overnight. The next day the sediment/PFA/FSW tube was centrifuged at 10,000 rpm for 5 minutes and the supernatant was poured off. Then 1.5 ml PBS were added and samples were resuspended. This step got repeated and then the tube got centrifuged at 10,000 rpm for 5 minutes and supernatant was poured off. 1.5 ml of 1:1 PBS/ethanol mix were added and stored at -20° C until further processing.

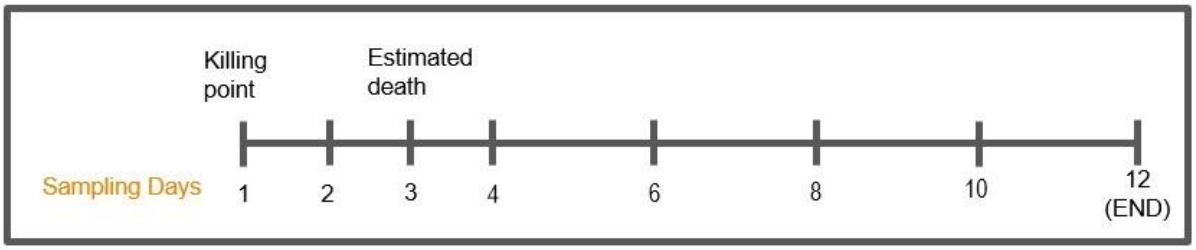


Figure 7: Sampling days for the Symbiont Release Experiment. Day 1 marks the beginning of the experiment and the killing day for clams in the dead Treatment. Day 3 is the estimated death day for clams. Samples were taken on day 2, 4, 6, 8, 10 and 12.

5.2.1 Droplet Digital PCR

ddPCR was performed on all samples with positive DNA concentration values (Suppl. table T1) from the Symbiont Release Experiment according to the protocol written by Dr. Astrid Horn (Suppl. Protocol S2). To find the best annealing temperature, a temperature gradient ddPCR with values from 45 to 75°C was performed with the positive LucA sym probe (Suppl. Fig. 1) from the Petersen Lab. 60°C was the best annealing temperature in our trial and is additionally the recommended temperature by Bio-rad so we used it for all samples. The forward and reverse Primer were originally designed to target the soxB gene of *Ca*. Thiodiazotropha endoloripes by Marta Sudo[71]. Recent publications split *Ca*. Thiodiazotropha endoloripes in two distinct species: *Ca.* Thiodiazotropha weberae and *Ca.* Thiodiazotropha lotti[51] which are both detected by the primers designed for *Ca*. Thiodiazotropha endoloripes.

The ddPCR reaction volume was 22 µl, 11 µl ddPCR EvaGreen Supermix (2X stock, Bio-Rad), 8.2 µ PCR water, 2 µ template and 0.4 µl of the soxB forward and reverse primer (Table 1). Droplet generation was performed on the Droplet Generator (Bio-Rad). ddPCR was carried out in a 20 µl reaction with 5 cycles of initial enzyme activation at 95°C for 5 min followed by 40 cycles of 30 sec denaturation at 95°C, 1 min annealing at 60°C, 5 min signal stabilization at 4°C and 5 min at 90°C. A ramp rate of 2°C per sec was added to all steps. Analysis and quantification of the soxB gene copy number were carried out on Plate Reader from Bio-Rad Droplet Digital ™ PCR and all digital analyses were done with the QuantaSoft™ Analysis Pro Software. Thresholds for each sample were set manually. The copies per ng of sample were calculated with following formula:

(starting copies per µ (copies/ µl) X (total reaction (21 µl)) / (ul of sample used(µl)) / (ng/ul of sample(ng/ µl))

Name	Sequence (5' - 3')	Target	Specificity	Reference
616V	AGAGTTTGATYMTGGCTC	16S rRNA	Prokaryotes	Müller et al. (2000) [72]
1492R	GGYTACCTTGTTACGACTT	16S rRNA	Prokaryotes	Brandl et al. (2001) [73]
soxBF	ACCGATACCCATGCACAACTCA	16S rRNA	*Ca.* Thiodiazotropha weberae + lotti	Marta Sudo (2019)[71]
soxBR	CGCTATTAGACGAGTT	16S rRNA	*Ca.* Thiodiazotropha weberae + lotti	Marta Sudo (2019)[71]
341F	CCTACGGGNGGCWGCAG	16S rRNA	Prokaryotes	Herlemann et al. (2011) [74]
785R	GACTACHVGGGTATCTAATCC	16S rRNA	Prokaryotes	Herlemann et al. (2011) [74]

Table 1: Used PCR primers (blue), ddPCR primers (orange) MiSeq PCR primers (green). All PCR primers were synthesized by Thermo Fisher Scientific.

5.2.2 Statistical Analysis

To determine the effect of Treatment and Days on soxB copies from the *L. orbiculatus* symbiont during the Symbiont Release Experiment, I constructed separate generalized linear models. The models compare each measurement of the factor Treatment and Day between lines. A Gamma log distribution was used for all models. The significance of line specific differences was assessed by comparing the predictive value of full models against null models lacking the relevant predictor. In the full model I included the day variable representing the line. For all model comparisons, the null model only included the covariate 'Day' due to our default allometric expectation that the number of soxB copies depends more on the Treatment. All models were inspected and plotted to determine if model assumptions were satisfied. All variance inflation factors were <4 and overdispersion was <0.4.

Statistical analyses were performed in R (Version 4.1.0; www.r-project.org/) and R-Studio (Version 1.4.1564; www.rstudio.com/) using the following packages and versions: performance_0.4.4, boot_1.3-22, plyr_1.8.4, readxl_1.3.1,forcats_0.4.0, ggthemes_4.2.0, car_3.0-3, carData_3.0-2, MASS_7.3-51.4, lme4_1.1-21, Matrix_1.2-17,stringr_1.4.0, dplyr_0.8.3, purrr_0.3.2, readr_1.3.1, tidyr_0.8.3, tibble_2.1.3, ggplot2_3.2.0.

5.3 Nucleic acid extractions

5.3.1 Pore Water

The extraction of nucleic acid from filtered pore water samples from the Symbiont Release Experiment as well as the filtered sea water samples from 2018 from Elba was performed with the Qiagen DNeasy PoreWater Sterivex kit according to the protocol (Suppl. protocol S3). The DNA concentration of a was quantified with the Qubit 4 14 Fluorometer (Thermo Fisher Scientific, United States). To verify that the extraction worked, we used the Quant-iT™ 1X dsDNA BR Assay kit (Thermo Fisher Scientific, United States) which measures initial sample concentrations from 200 pg/µL to 4000 ng/µL according to the manufacturer's protocol (Suppl. Protocol S4). Due to very low DNA concentrations in the filters, many samples showed negative DNA concentrations during evaluation. To compensate for this problem, all samples with negative DNA concentration values were reanalysed with the Quant-iT 1X dsDNA HS (High-Sensitivity) Assay Kit (Thermo Fisher Scientific, United States) which measures initial sample concentrations from 10 pg/µL to 100 ng/µL according to the manufacturer's protocol (Suppl. Protocol S5). After these measurements all samples were measured with Quant-iT™PicoGreen® dsDNA reagents kit (Thermo Fisher Scientific, United States) according to Suppl. Protocol S6.

5.3.2 Sediment and mucus burrow tubes

The extraction of nucleic acid from sediment samples from the Symbiont Release Experiment as well as sediment samples from the 2016 Elba trip has been extracted using the Qiagen DNeasy PowerSoil kit according to the protocol (Suppl. protocol S7).

The DNA concentration was measured on random samples with the Quant-iT™ 1X dsDNA BR Assay kit (Thermo Fisher Scientific, United States) according to the manufacturer's protocol (Suppl. Protocol S4)to verify that the extraction worked. All samples were measured with Quant-iT™PicoGreen® dsDNA reagents kit (Thermo Fisher Scientific, United States)

according to Suppl. Protocol S6. The extraction of nucleic acid from mucus tubes was done in the same way as the samples from the Symbiont Release Experiment.

5.3.3 Preparation for 16SrRNA Amplicon Sequencing

Two PCR steps were performed to prepare the samples according to an SOP based on Herbold et al., 2015[75] with following changes: equimolar pooling of the samples was conducted. In the first step PCR, the headed-primer set 341F and 785R (Table 1), which targets the small subunit rRNA of all Bacteria and Archaea were used. This PCR was performed in triplicates (25 µl reactions) with 25 cycles using 1 µl of DNA, as template. PCR amplifications were carried out on a CFX96 TouchTM Real Time PCR Detection System (Bio-Rad) with initial denaturation at 94°C for 4 min followed by 25 cycles of 30 sec denaturation at 94°C, 45 sec annealing at 52°C and 45 sec elongation at 72°C. Each reaction was terminated by an additional polymerization step of 72°C for 10 min. Samples which showed no bands in this PCR step but clearly contained DNA due to Quant-iT™PicoGreen® DNA concentrations measurements were repeated with 30 cycles using 5 µl of DNA (Suppl. Table T1) with the same PCR amplification settings as described above. Additionally, three positive controls and one negative sample containing no DNA were prepared for sequencing, for normalization and as control. The products of the first step PCR contained a head sequence at their ends which is the target binding site for the barcodes in the second step PCR. The triplicates were pooled and purified using a ZR-96 DNA cleanup-kit™ (Zymo Research, Irvine, CA, USA) after the manufacturer's protocol(Suppl. protocol S8).

The second PCR step consisted of a 50 µl reaction with 5 PCR cycles, using 3 µl of the purified product from the first step PCR as template. The barcodes added another 8 bp on both ends of the amplicons used to identify the amplicons in a pooled sample. PCR amplification settings were equal to the first step PCR amplification settings. The control of the final barcoded products was checked on an agarose gel and purified using the ZR-96 DNA cleanup-kit™ (Suppl. Protocol S8). DNA concentrations were measured with Quant-iT™PicoGreen® after the manufacturer's protocol (Suppl. Protocol S6). All barcoded samples, except for the mucus tubes, were given to the Joint Microbiome Facility of the Medical University of Vienna and the University of Vienna (JMF) for sequencing, lead by Bela Hausmann and Petra Pjevac and pooled in equimolar amounts containing about 20 x 10^9

amplicon copies. Samples for the mucus tubes were processed by Microsynth Austria because they were done before JMF had taken care of the sequencing.

5.4 Sequence Processing and Analysis

The JMF amplicon sequencing pipeline uses DADA2 to output amplicon sequence variants (ASVs) instead of OTUs. ASV identifiers are generated based on the nucleotide sequence so that ASVs are comparable over all projects[76]. Demultiplexing, barcode, linker and primer trimming and data processing for all samples from the Symbiont Release Experiment as well as environmental sediment and pore water samples from Elba 2016 and 2018 and the mucus tubes, was performed by Dr. Bela Hausmann according to the current JMF protocol[76] . The 16S amplicon sequences were run with SINA with classification based on the current SILVA database and taxonomical tables and ASV count output files were created. To determine the abundance of *Ca.* Thiodiazotropha endoloripes in the Symbiont Release Experiment as well as in the environment samples I used the output files from the DADA2 pipeline for making graphs and statistical analysis. The beta diversity between all samples was calculated using the Bray Curtis dissimilarity which was plotted in a principal coordinates analysis (PCoA).

Statistical analyses were performed in R (Version 4.1.0; www.r-project.org/) and R-Studio (Version 1.4.1564; www.rstudio.com/) using the following packages and versions: phyloseq_1.34.0, data_table_1.14.0, readxl_1.3.1, tibbble_3.1.2, dplyr_1.0.6, microbiome_1.12.0, hrbrthemes_0.8.0, gcookbook_2.0, forcats_0.5.1, ggplot2_3.3.4. , scales_1.1.1, grid_4.0.5.

6. Results

6.1 Environmental Pore Water Community

6.1.1 Abundance of the genus *Ca.* Thiodiazotropha in the environment

To detect the symbiont in the environment, sediment samples and pore water filters from two different years were screened for the presence of *Ca.* Thiodiazotropha. We were able to obtain successful amplifications of the symbiont from the pore water filters from 2016 and 2018. The relative abundance in one sample from 2016 from the depth 45 cm obtained over 1% of the community composition (Fig. 8). In all other samples from the pore water from 2016 the relative abundance was lower than 0.25% (Fig. 8). *Ca.* Thiodiazotropha sequences

were also found in the 2018 pore water samples, but only in 3 total samples, and at relative abundances under 0.01%.

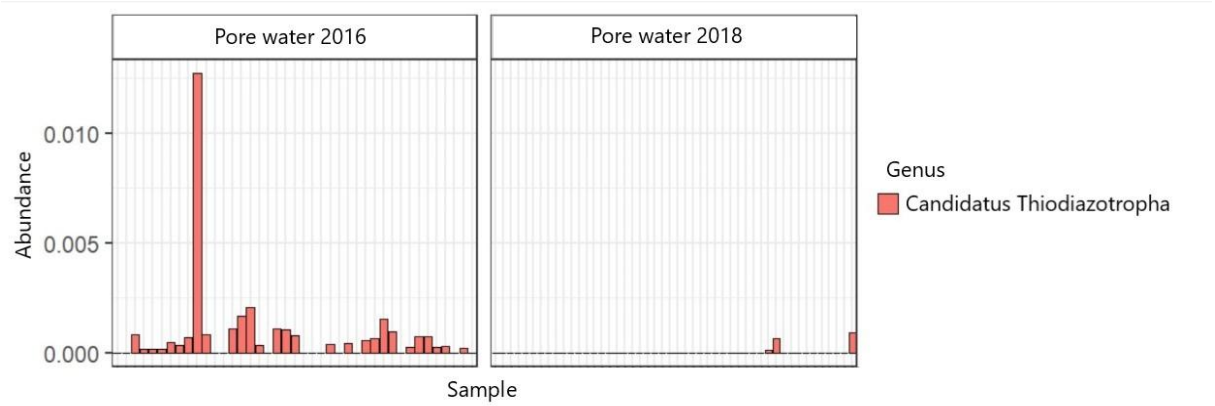


Figure 8: Relative abundance values in percentage summing um to 1% in total from members of the genus Ca. Thiodiazotropha for pore water samples from the Bay of Fetovaia, Elba, from three different spots with depth ranges from 0 - 60 cm in 2016 and 0-45cm 2018.

6.1.2 Bacterial Community in the Pore Water

Classification of the 16S rDNA sequences for pore water filters obtained in 2016 resulted in the taxonomic grouping displayed in Fig. 9. On the Phylum level, Proteobacteria, Verrumicrobiota and Desulfobacterota were identified to be most abundant in the microbial community. Also, in nearly every sample the family Desulfobacteraceae which are bacteria floating in the water column and reduce sulfates to sulfides, was found (Suppl. Fig. 2). In the upper (45-50 cm) and middle (20 cm) depth layers (Suppl. Fig. 2) but also in some of the deeper layers *Halobacteriovorax*, a bacterial predator[77] was identified to be abundant in the pore water samples. The phylum Chloroflexi also displays a high abundance, especially in the deeper layers. A huge fraction (between 10% to 26% depending on the sample) of the bacterial community is also occupied by unclassified bacteria (Suppl. Fig. 2).

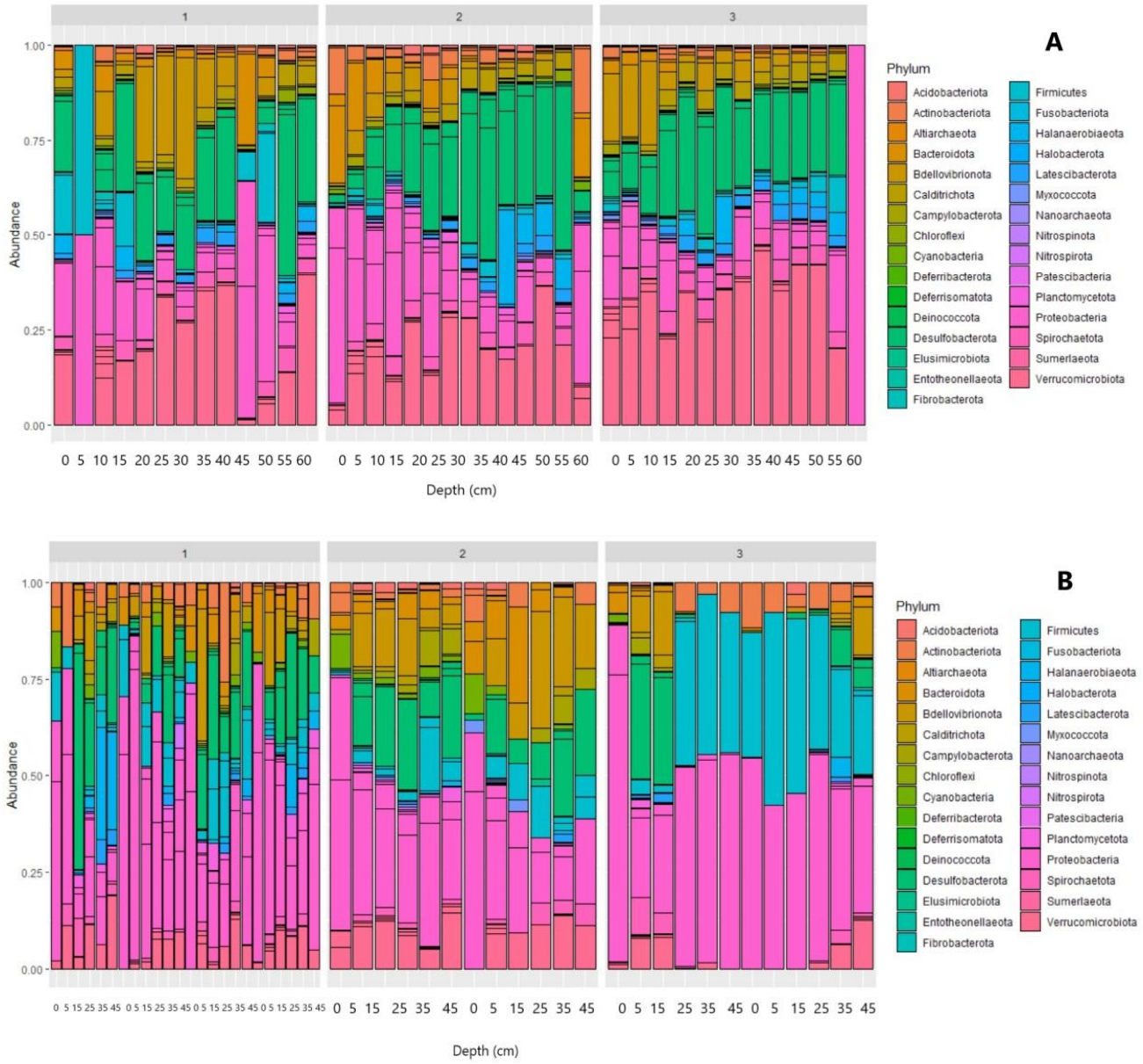


Figure 9: Relative Abundance of the bacterial community in pore water samples from the Bay of Fetovaia, Elba, 2016 (A) and 2018 (B) obtained from 16S Amplicon Sequencing. Depths ranging from 0 - 60cm in 2016 and 0-45 in 2018. The color codes showing the fraction of the phylogenetic taxa listed on the right side.

In comparison to the community composition in 2016, Protebacteria resumed to be the most abundant Phyla and also occupied a larger fraction in the overall microbial community composition of 2018 (Fig. 9). Desulfobacteraceae continue to exhibit a steady occurrence in nearly all depth layers from all four sites. In the upper layers, especially in the first sampling depths P_x_W (Suppl. Fig. 3) Rhodospirales and *Ca.* Pelagibacter, both belonging to the Alphaproteobacteria together with a small fraction of Burkholderiales belonging to the Betaproteobacteria constitute a large fraction of the community. Similar to 2016, *Halobacteriovorax* was found in the upper and middle depth layers with site specific

differences (Suppl. Fig. 3). The family Moraxellaceae was absent in all samples located in 2016 but was present in small quantities at all sites, in two pore water samples also in higher quantities, and overall distributed unevenly around the death gradient. The above described shift in the microbial pore water community between the years 2016 and 2018 is illustrated in the principal coordinates analysis of the Bray-Curtis dissimilarity in Figure 10.

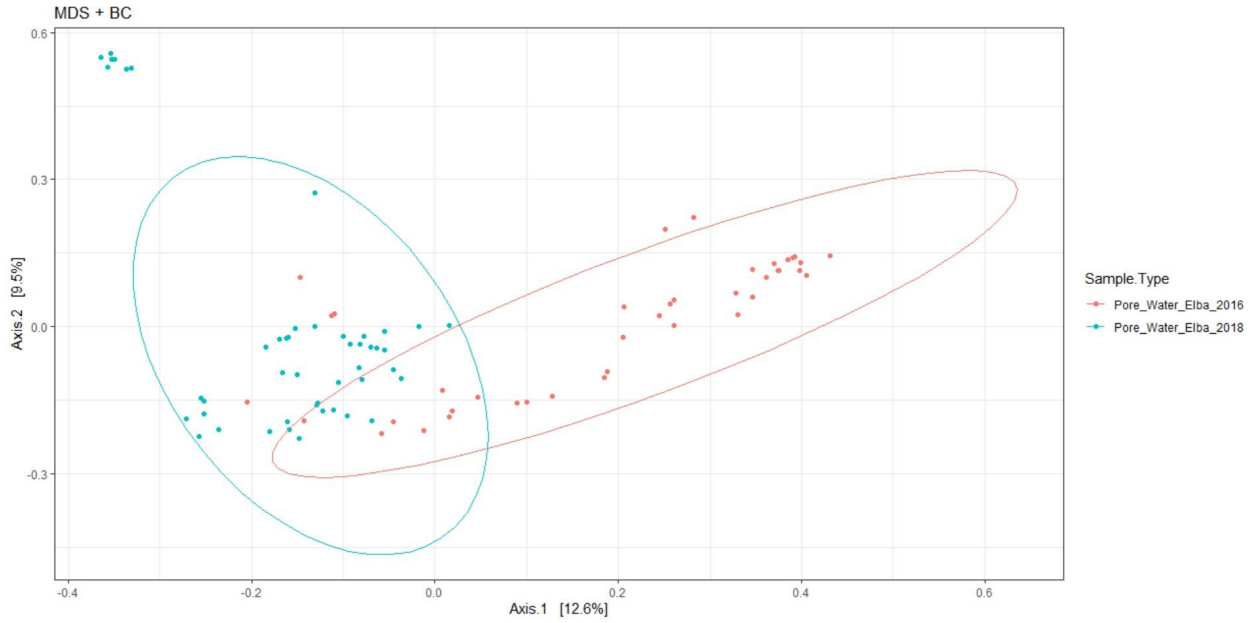


Figure 10: Principal Coordinates Analysis depicting the Bray-Curtis dissimilarity as a means to measure the beta diversity between the pore water samples of 2016 and 2018. Pore water samples from 2016 are depicted by red dots, pore water samples from 2018 are depicted by blue dots.

6.2 Environmental Sediment Community

The sediment samples were obtained from 4 different locations in the Bay of Fetovaia, Elba and ranging from depths from 0 cm – 40 cm. On phylum level community composition was dominated by Desulfobacterota and Proteobacteria (Fig. 11). In the upper layers Bacillariophyta, which are one of the dominant components of Phytoplankton, belonging to the Phyla Cyanobacteria have a high abundance and display a steady decrease towards the deeper layers (Suppl.Fig. 4) . Desulfobacteria exhibit a steady abundance throughout all sediment layers. Chromatiales which include Sedimenticolaceae belonging to the Gammaproteobacteria are present especially in the upper depth layers. Throughout the middle depth layers, Desulfovirga belonging to the Deltaproteobacteria, is distributed together with other species of the family Syntrophobacterales (Suppl. Fig. 4). *Ca*. Thiodiazotropha could not be detected in any of the depth layers in the sampling spots (Fig. 12).

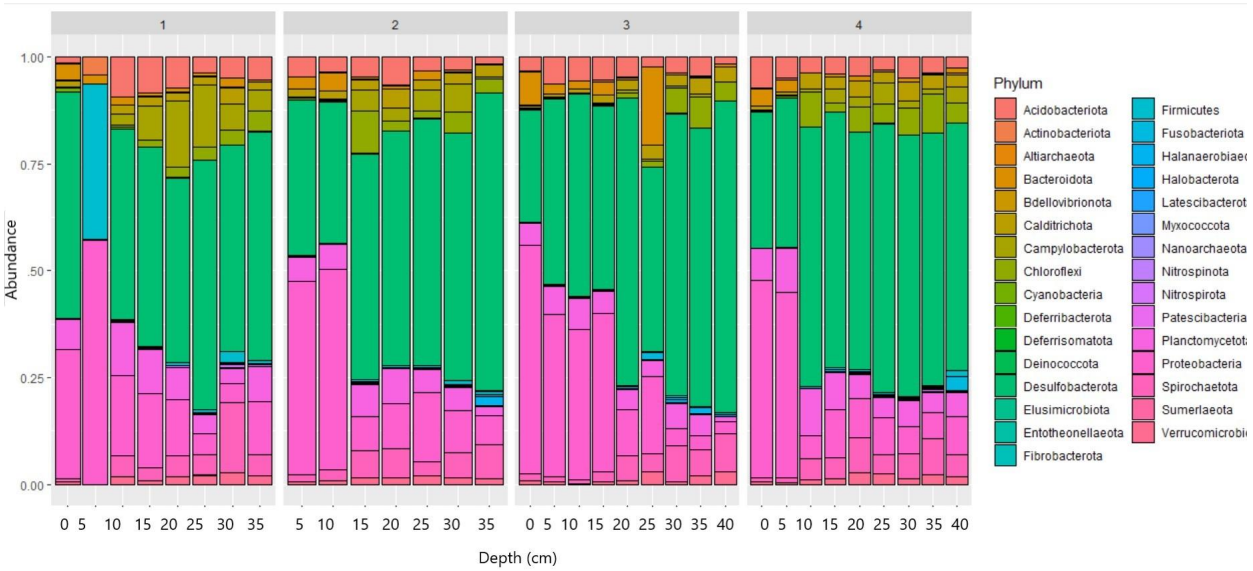


Figure 11: Sediment Community Composition from the Bay of Fetovaia, Elba, 2018 from 4 different sampling spots from depth layers from 0cm - 40cm obtained from 16S Amplicon Sequencing. The color codes showing the fraction of the phylogenetic taxa listed on the right side.

6.3 Abundance of the genus *Ca.* Thiodiazotropha in the mucus burrow tubes

To show that symbionts associated with the mucus tubes of the genus *Ca.* Thiodiazotropha are not derived from the surrounding sediment, I visualized the relative abundance values of both sample types, mucus burrow tubes and sediment, in Fig. 12. In comparison to the mucus burrow tubes where the highest relative abundance value was above 0.10 %, no DNA from the genus *Ca.* Thiodiazotropha was retrieved from the sediment samples.

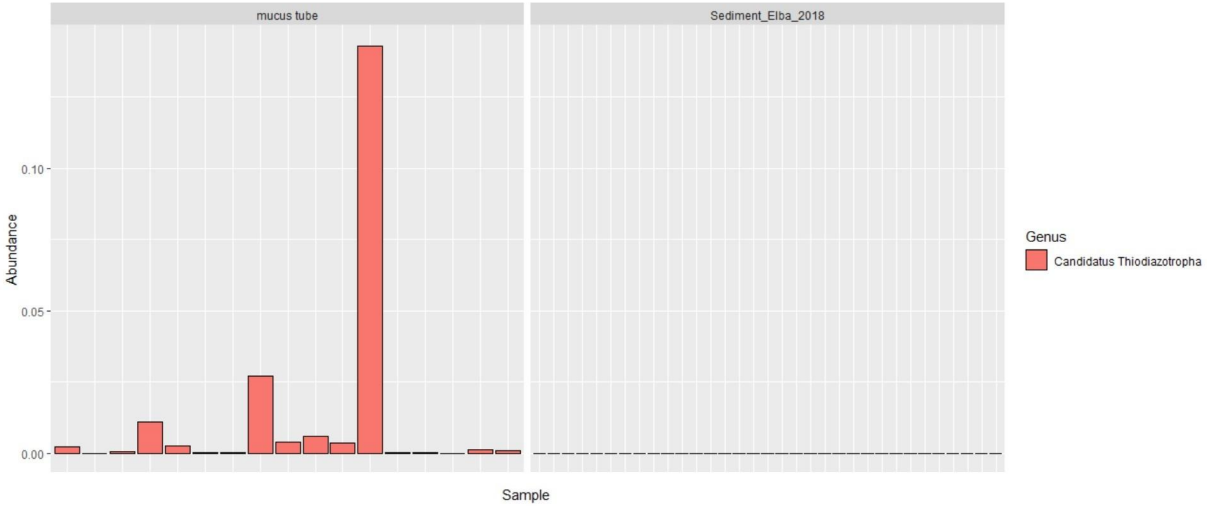


Figure 12: Relative abundance values for members from the genus Ca. Thiodiazotropha from sediment samples of the Bay of Fetovaia, Elba, 2018 and mucus burrow tubes from Sant Carles de la Ràpita, Spain, 2019 obtained from 16S Amplicon Sequencing.

6.4 Symbiont Release Experiment

6.4.1 Abundance of *Ca.* Thiodiazotropha in the Symbiont Release Experiment

Successful amplifications of or target symbiont were obtained from treatments with dead clams in sediment as well as live clams in sediment and live clams in water. The highest numbers of soxB copies were obtained in the dead clams in sediment in the first days (day 2 to day 4) of the experiment. The treatment with live clams in water showed successful amplifications on day 2 with an abundance value of 0.4% for that specific sample. The sediment treatment with living clams displayed successful amplifications on day 10 of the experiment. No sample in all treatments had more than 3 % as *Ca.* Thiodiazotropha (Fig. 13). Only two samples (Suppl. Fig. 5) displayed very high values compared to the overall low abundance of the symbiont in the sediment treatment with dead clams. The sample which contributes to the highest relative abundance value is a sample which was taken right at the beginning of the Symbiont Release Experiment on day 2. The sample with the second highest relative abundance values is from the same sample and was taken on day 4 of the experiment. The Abundance of *Ca.* Thiodiazotropha differed clearly between the treatments dead and live and was also affected by the type of element. Relative abundance values were highest in the sediment samples obtained from the dead clam treatment group. All samples except for one outlier, which is also responsible for the high percentage value over 3 % in Fig. 13, exhibit relative abundance values beneath 0.5 % and much lower per sample.

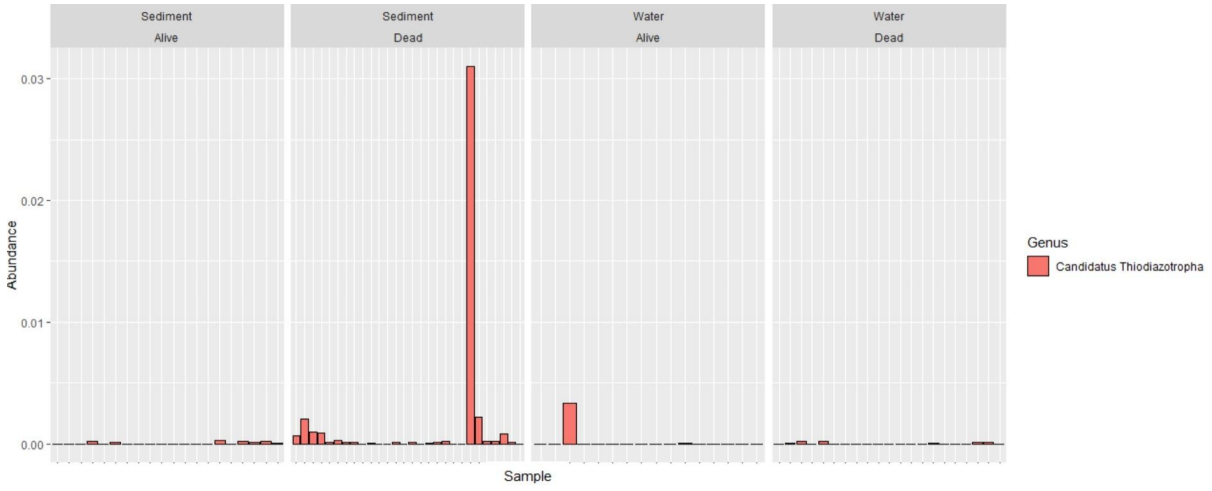


Figure 13: Relative Abundance summing up to 1% in total for target Ca. Thiodiazotropha species in samples from the Symbiont Release experiment. The highest value exhibits the relative abundance of 3% from Ca. Thiodiazotropha.

The experimental control group for sediment shows no amplifications of *Ca*. Thiodiazotropha (Suppl. Fig. 5). In contrast the experimental control group for water exhibits a number of 6 total standardized ASV counts in one sample and one count in another sample (Suppl. Fig. 6). The sediment treatment with dead clams displayed the highest abundance in comparison to the other ones (Fig. 13). In the sediment treatment 20 samples from the dead clam group and 7 samples from the live clam group exhibit soxB copy numbers compared to the nearly sterile sediment control treatment. In the water treatment with live clams one sample showed higher soxB copy numbers than the soxB copy numbers in the water control treatments(Suppl. Fig. 6).

6.4.2 Droplet Digital PCR

Overall, the number of soxB gene copies was very low and the treatment with dead clams in sediment displayed the highest soxB copy numbers (Fig. 14) . The treatment dead clams in water was significantly different to all other treatments except for the control treatments whereas the treatment dead sediment differed highly significant to all other treatments (Table 2). No treatment for sediment and water was significantly different to the experimental control treatments.

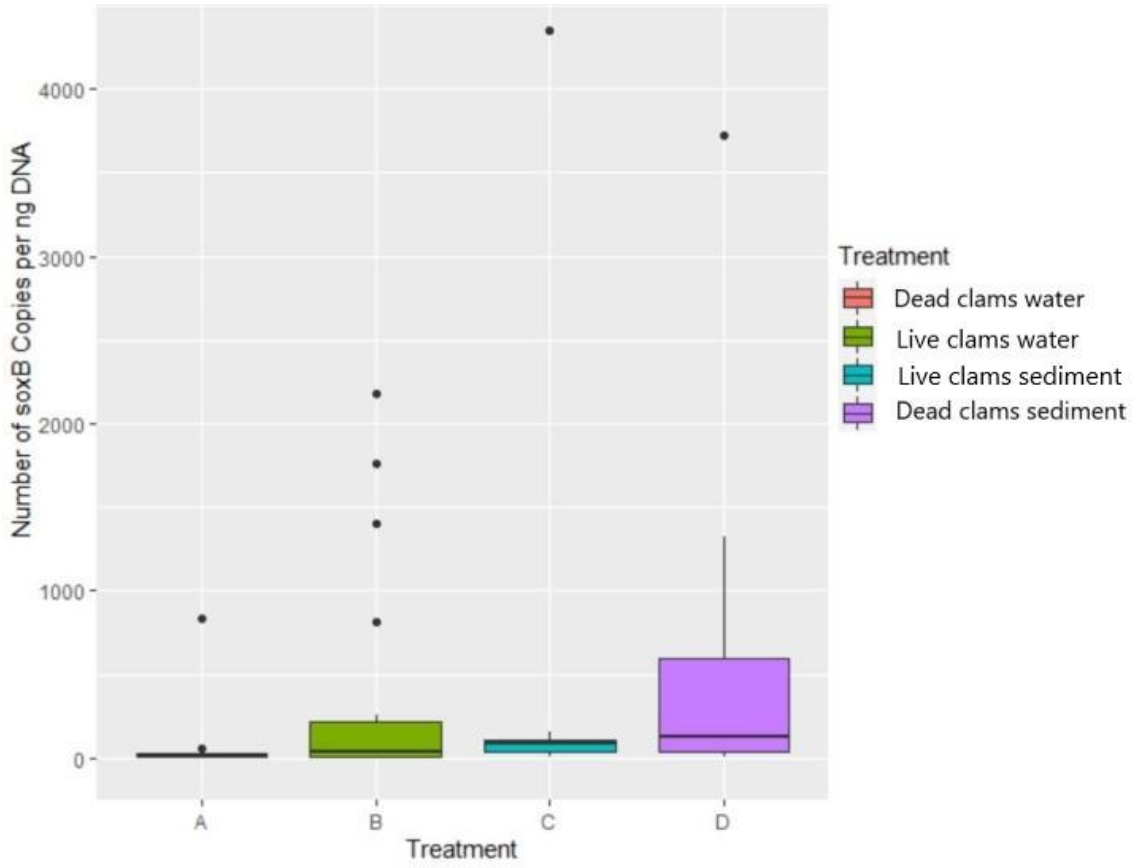


Figure 14: Boxplot displaying number of soxB copies per ng DNA from the target symbiont depending on treatment of the Symbiont Release Experiment , A= dead clams water, B= live clams water, C=live clams sediment, D= dead clams sediment.

The fullmodel for treatment was significantly better than the nullmodel. Therefore, the differences between the treatments show that the number of soxB gene copies is dependent on the life cycle of the clam. Water samples from tubes with live clams had nearly no copies in them implying that there is a possibility of slight symbiont release. The quantity of soxB in the water and sediment samples with dead clams was significantly higher compared to the other treatments (Table 2) except for the control treatments.

Table 2: Null/full model comparison and summary of the model for treatment. Water treatment with dead clams is included in the intercept.

Treatment				
full/null model comparison			treatment day	Pr(>Chi) 0.01161 * 0.05139
	estimate	**std. error**	**t value**	**Pr(>|t|)**
intercept	4.42203	0.52291	8.457	4.99e-13 ***
live clams water	1.61505	0.68045	2.373	0.01977 *
live clams sediment	2.92735	0.94426	3.100	0.00259 **
dead clams sediment	2.35438	0.68391	3.443	0.00088 ***
control water 1	-2.24235	1.44319	-1.554	0.1238
control water 2	1.10195	1.01646	1.084	0.28125
control sediment 1	-0.4269	1.73651	-0.246	0.80637
control sediment 2	1.13448	1.77394	0.640	0.52412
Day	-0.16099	0.07159	-2.249	0.02701 *

6.4.3 Bacterial Communities Symbiont Release Experiment

The overall microbial community composition in the sediment with live clams is dominated by Proteobacteria and Bacteriodetes on the phylum level (Fig. 15). In contrast to live clam, the dead clam treatment exhibits a higher percentage of the phyla Desulfobaterota and Campylobacterota. At the beginning of the experiment, we could identify a high abundance of the Genus Vibrio, belonging to the Proteobacteria, which declined with the time of the experiment. Lutibacter, belonging to the Bacteroidetes, has also a high abundance especially in the middle (day 4 to 6) and towards the end (day 10 to 12) of the experiment (Fig. 16). A constant presence in every sample was also identified for Oleiphilus, Acrobacter and Desulfobacter which all three belong to the Phylum Proteobacter.

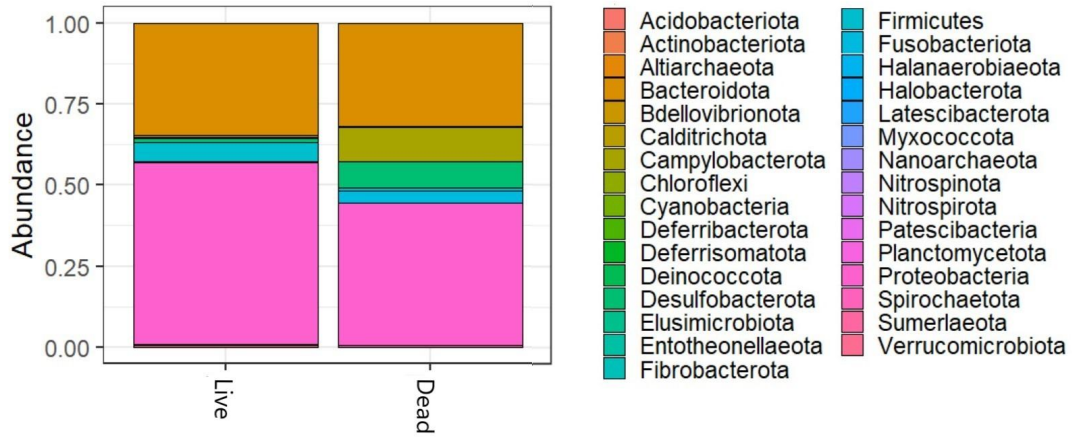


Figure 15: Relative abundance of Phyla present in samples from the Symbiont Release Experiment. Comparison on phyla level between treatments dead and live.

In contrast, the microbial community composition in sediment with dead clams shows a shift in abundance of the appearance of two Bacteria, both belonging to Bacteroidetes, which were not present in the sediment with live clams, Prolixibacteraceae and Marinilabilaceae. At the beginning of the experiment the microbial community was also dominated by the genus *Vibrio* which decreased through the end. *Arcobacter* is also represented in every sample but with less abundance than in the sediment with live clams.

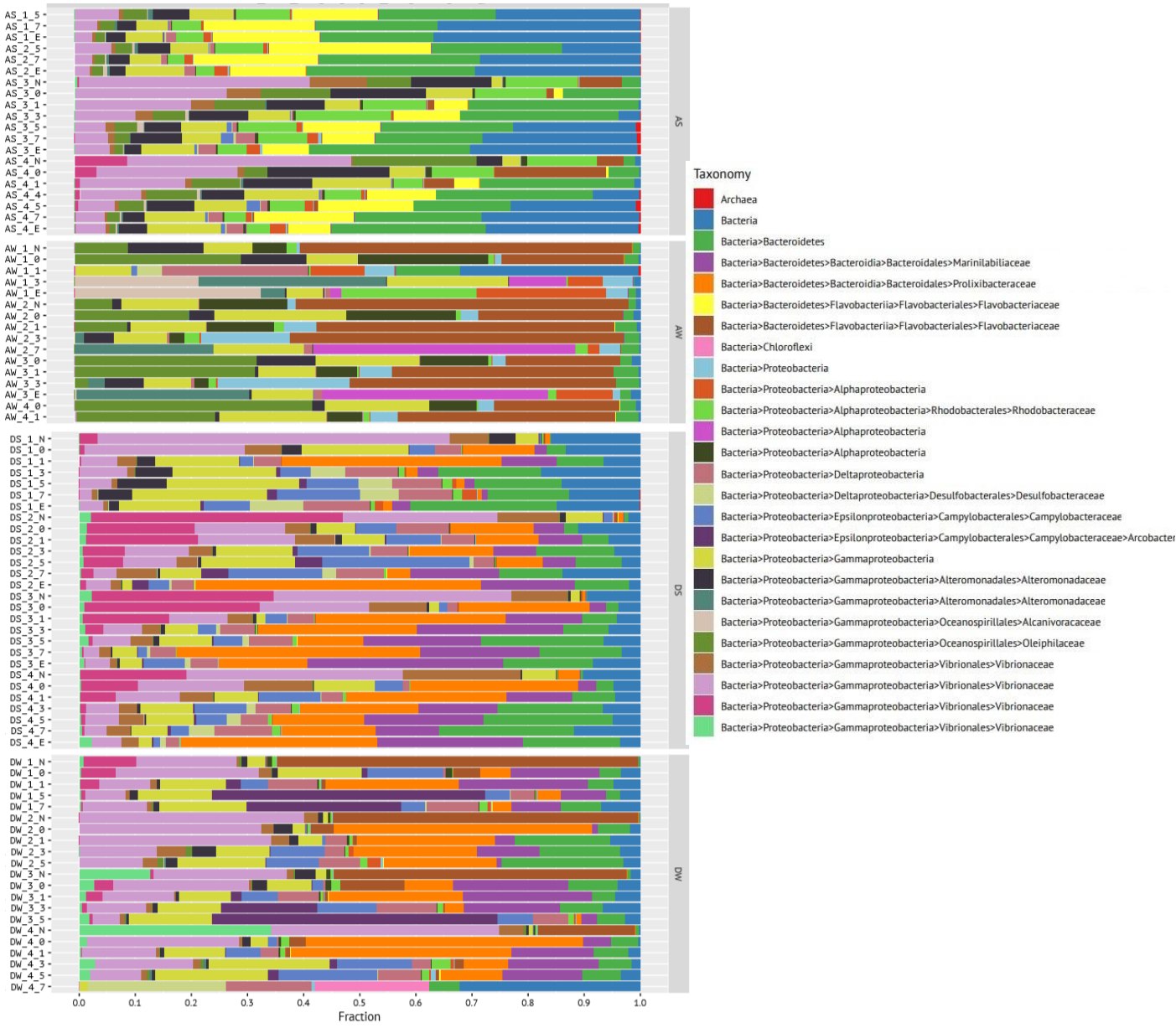


Figure 16: Fractioning of the bacterial community from the Symbiont Release Experiment , AS = live sediment, AW =live water, CS= Control Sediment, CW= Control water, obtained from 16S Amplicon Sequencing. The first numbers of the sample day are referring to the replicate number of the treatment. The second number represents the sampling day : N= start of the experiment and killing of the clams from the ´dead´ treatment, 0 = day were killed clams believed to be officially dead, , E= end of the experiment.

In the water samples with dead clams, we obtained a shift in the microbial community composition from the genus *Vibrio* at the beginning which declined in the course of the experiment (Fig. 16). On the first day the community consisted mainly of the genus *Vibrio* and an unclassified Flavobacteriaceae. The abundance of the genus *Vibrio* declined highly at the day the clams were killed and vanished one day after the killing of the clams and were replaced by an unclassified Prolixibacteraceae and an unclassified Marinilabiliaceae. Arcobacter were identified in 2 samples and showed a high abundance in the middle stage of

the experiment. Gammaproteobacteria exhibit a steady abundance in all samples and obtain nearly the same fraction (nearly 50%) of the community compared to the water samples with live clams (Suppl. Fig. 4).

7. Discussion

We investigated a part of the life cycle of *L. orbiculatus* for excretion of its symbiont, finding that there is a small release of the target symbiont during lifetime in the treatments with live clams in water as well as in sediment compared to the experimental control groups indicating that there is a potential for symbiont spreading during lifetime. In treatments with dead clams we obtained the highest number of endosymbionts in the sediment samples compared to all other treatments of the Symbiont Release Experiment. However, the control treatments were not significantly different to any of the experimental treatments. The mucus burrow tubes also revealed a number of symbionts from the genus *Ca*. Thiodizotropha in contrast to the symbiont free sediment core samples from the environment. It was possible to identify the genus *Ca.* Thiodiazotropha in the environment outside of its clam host. The low number of ASV counts for *Ca.* Thiodizotropha in the environmental samples implies that the symbiont may belong to the rare biosphere of highly diverse, low abundance microbial communities in the environment. As genetic analyses delivered strong indications for a potential extracellular lifestyle[78], we expected to find a substantial population attached to the sediment or floating in the pore water column but no sequence retrieved from the environment perfectly matched the endosymbionts sequences from *L. orbiculatus*.

7.1 A number of environmental niches narrows down the search for a substantial symbiont population outside the host

Former studies revealed that the same species of lucinid hosts harbor different strains of symbionts in different locations[47,78,79]. This could be advantageous for the host as it seems apparent that symbiont acquisition from the environment provides the hosts with locally optimal adapted symbionts. These symbiont strains are rather restricted to a certain habitat and are not as host specific as previously thought except for one cosmopolitan bacterial symbiont *Candidatus* Thiodiazotropha taylori, which is associated with multiple lucinid hosts around the globe[51]. It belongs to the Clade A of lucinid endosymbionts and is closely related

to *Ca.* Thiodiazotropha lotti and *Ca.* Thiodiazotropha weberae. To survive the contrasting conditions of a symbiotic and free-living lifestyle, the symbionts' metabolic capabilities are well adapted to changing environmental sulfide concentrations[71] and they possess functional traits typical of free-living gammaproteobacteria such as heterotrophic metabolism[80]. The symbionts genome size can indicate whether it is obligate and only lives in the clam host or if it is facultative and symbionts are also able to survive in the environment. Due to genome reduction, obligate symbionts tend to lose genes which are needed to survive in the environment and therefore have a smaller genome size than free-living bacteria[81–83]. The size of the *Ca*. Thiodiazotropha lotti and *Ca*. Thiodiazotropha weberae genome with a range from ~4,450,000 bp to 5,125,948 bp [78] indicates a potential for a free living life-style, as the genome sizes fit into the spectrum typically for bacteria with a free-living life-style[84]. Considering the lack of genes for the synthesis and secretion of extracellular polysaccharides and matrix proteins required for the formation of biofilms[78] which may limit the symbionts ability to attach to surfaces, the chance to find the symbionts in the pore water filters seemed more promising than to find them in the sediment samples. The absence of the symbiont in the germ line and the horizontal transmission mode also promote the existence of a substantial free-living environmental population. Unfortunately, we could not find any sequences that matched the symbionts 16S rRNA in the sediment core samples from the Bay of Fetovaia. Other studies also encountered that the symbiont is very hard to detect in the surrounding sediment of the clam[85]. We may have been inefficient in detecting *Ca.* Thiodiazotropha species because the symbiont is present in the sediment only in very small quantities and may belong to the rare biosphere (<0.1% of total abundance)[86]. Microbial communities mainly consist of a large number of rare species and a limited number of abundant species[87]. When the rare biosphere was discovered, it was believed that many rare species are metabolically inactive and act as insurance for the community if other species disappear or environmental conditions change[88]. In contrast, a vast number of recently described studies suggested that many of the low abundant species are active and playing an important role in community functions[89–92]. The existence of a low abundant active symbiont population in the sediment is still possible and the chosen method of Illumina sequencing could have been biased by common DNA- extraction and PCR - biases during sample preparation.

In the pore water samples from the Bay of Fetovaia we have been able to identify a small number of symbionts from the genus *Ca.* Thiodiazotropha at all three sampling sites from 2016 by MiSeq amplicon sequencing. It seems that the symbionts distribution around the depth range exhibits a large variability between the sites. At one site the related symbiont can be found between 10 and 15 cm and then again at 40 -50 cm. At the second site it is only represented in low quantities at 45 and 55 cm and at the third side it is found in upper layers and also in layers from 30- 50 cm. Assuming that higher numbers of related symbionts can be found next to a host clam, this high unevenness might be produced by the distribution pattern of the clam host. In contrast to pore water samples from 2016, we were not able to find the same abundance of related symbionts in pore water samples in 2018. Only three samples out of 48 contained the genus *Ca*. Thiodiazotropha. All three samples displayed a relative abundance beneath 0.01% of the genus *Ca.* Thiodiazotropha (Fig. 14). The sampling spots were the same as in 2016 but the usage of different filters could have led to the difference in abundance patterns in the samples from 2018. Altogether we found that less than 0.05% of the bacterial population in both of the sediment microhabitats belong to the family Sedimenticolaceae, which includes *Ca.* Thiodiazotropha and other lucinid symbionts. From over 400,000 analyzed sequences we were not able to obtain any sequences that perfectly matched the symbionts 16S rRNA sequence. Another possibility for the differences in abundance could be fluctuations and changes in the environment caused by storms, seasonality, or other unknown factors. A recent study showed that *Ca.* Thiodiazotropha species are associated with seagrass roots and are hard to detect in the surrounding sediment[85]. The study also states that many large and productive seagrass meadows grow independently from the occurrence of Lucinids in the habitat. The symbionts are attached to the seagrass root and *Ca*. Thiodiazotrphoa together with other bacteria are building up the detoxifying root microbiota. The relative abundance of *Ca*. Thiodiazotropha in seagrass roots was not strongly correlated with the presence or absence of Lucinids in the surrounding sediment. This suggests that *Ca.* Thiodiazotropha colonizes seagrass roots worldwide regardless of the presence or absence of Lucinids. With their morphological features Lucinids act as a suitable niche for the symbiont and assure a stable incoming nutrient supply which is needed for symbiont growth. The seagrass root environment is very similar to the habitat provided by the clam in respect to the mosaic of chemical gradients like oxygen, pH, metals and nutrients[93,94]. This explains how the symbiont is able to grow in the seagrass root

habitat and reveals how the root environment also acts as a suitable niche for Ca. Thiodiazotropha alongside many other bacteria. Since the decline of seagrass meadows is a huge and fast-moving problem in marine ecosystems around the globe[95], our sampling spot in Elba also experienced a decline in seagrass densities. Considering the close relationship between *Ca.* Thiodiazotropha, *L. orbiculatus* and seagrass growth, it appears likely that the fast decline of seagrass has led to a smaller number of symbionts in the environment.

7.2 Mucus burrow tubes could act as a suitable habitat for symbionts outside the clam

In contrast to the symbiont free sediment, we were able to amplify 16S rRNA genes from the genus *Ca.* Thiodiazotropha from mucus tubes built by the clam. Previous studies proposed that Lucinids take up the majority of their symbionts at an early developmental stage from only one symbiont population but are still able to take up symbionts throughout their lifespan[78,79]. As *L. orbiculatus* harbors more than one symbiont species, it is yet unclear if all of them are taken up together in the juvenile phase and the dominant strain outcompetes the non-dominant strains or if one strain is taken up at the beginning of the recruitment and the non-dominant strains are taken up when the dominant symbiont population is already established. The presence of the symbionts in the mucus tubes of the clam could represent a possible entry site for symbiotic reinfection of the host. The mucus tube could also act as a crucial site for microbial sorting, symbiont attraction, and recognition as it is in the case in deep sea dwelling tubeworms[96] or in the marine squid bioluminescent Vibrio symbiosis[97,98]. Some polychaete worms also use their mucus burrows to attract and farm bacteria that are used as nutrition through ingestion by the mouth[99]. Lucinids could utilize a similar strategy to attract and farm the symbionts and when seasonal changes alter the availability of resources in the environment. Given the absence of detectable symbionts in the sediment samples and the uneven depth distribution pattern of symbionts from the pore water in 2016, it seems likely that these patterns are created by the general burrowing activities by the clam, the mucus tube could possibly act as chemoattractant to recruit new symbionts. If this unevenness is really caused by clam distribution this would also support the crucial role of mucus tubes in symbiont colonization dynamics and host symbiont dynamics. When environmental conditions change, mechanisms to farm bacteria outside the host and control bacterial uptake could be beneficial for survival. Horizontally transmitted symbionts and

symbionts housed outside the cell often form single or mixed populations with either one or more bacterial strain[28,47]. Possessing a variety of bacterial strains might be very helpful in changing environmental conditions, as co-occurring bacterial strains have been shown to differ extensively in key functions, such as the use of energy and nutrient sources, electron acceptors and viral defense mechanisms[31]. During the life cycle of the host, bacterial colonization dynamics determine the genetic diversity of horizontally transmitted microbial populations which can be influenced by stochastic effects which also may manifest themselves in differences in microbiota strain composition amongst hosts[31,100]. Insight in the possible attraction and symbiont selection of symbionts through mucus can be critical to understand how hosts might respond to environmental changes as mixed populations tend to extend the hosts metabolic capabilities [101].

7.3 Symbiont-host relationship a dead end?

Another interesting question was if symbionts are actively released from the host during lifetime. Usually, marine invertebrates, which do not acquire their symbionts vertically from their parents, acquire their symbionts as juveniles from an environmental population. Often this symbiont uptake is restricted to early developmental stages[102] as it is the case for *Riftia pachyptila*. When the larvae of *Riftia pachyptila* takes up a symbiont from the environment it loses its digestive organs and gets sessile[96,103]. From this point on no symbionts are taken up or released from the tubeworm during lifetime[104]. Symbionts occur in very small abundances in the surrounding environment and huge abundances of the symbiont only occur during host death when symbionts get released[32]. Up to now, we know from several sulphide starving experiments[105,71] that Lucinids, after symbiont depletion, are capable to reacquire symbionts in an adult stage from the environment[33,79]. The question remains if the symbionts are able to escape the clam during lifetime and re-inoculate the ambient environment and are able to reinfect juvenile host individuals like symbionts in many other chemosynthetic relationships. If not, the evolutionary advantage of the endosymbiont is questionable. The host clam acts as a niche and provides space and protection for symbiont growth but if the bacteria cannot escape the clam, this association is a dead end for the symbiont from an evolutionary aspect.

To investigate this for *L. orbiculatus* and *Ca.* Thiodiazotropha MiSeq amplicon sequencing was conducted on all samples from the 12 day symbiont release experiment. The target symbiont

`LuCD` which is able to target *Ca.* Thiodiazotropha weberae and lotti, was found in treatments with dead and live clams in sediment. The dead sediment treatment differed significantly to all other treatments (Table 2) and the majority of the samples displayed a relative abundance beneath 0.25% with one exception with a relative abundance above 3% (Fig. 14). However, no treatment differed significantly to the nearly sterile control treatments for sediment. DNA extraction for many of the control samples did not work and have not been included in the statistical analyses. Therefore we had an unproportional number of control samples and treatment samples. In treatments with live clams, the target symbiont was found in one sample in higher abundances than in the water control and in seven sediment samples which could indicate that the symbiont is released, although infrequently, during lifetime but only in very small quantities. It is also possible that the symbionts found in the water treatment with live clams may be caused by contamination as they have not been found in the same falcon tube during the next sampling step. Since some samples from the water control groups have been contaminated with the symbiont DNA and the dead sediment treatment did not differ significantly from the control groups we can not be certain about the results and further experiments should be conducted.. These findings lead me to the conclusion that there is a possibility of small spreading of symbionts during lifetime which could be able to reinfect juveniles and contribute to a small population in the environment. The fact that symbionts in the Symbiont Release Experiment are found in the sediment treatment with dead and alive clams and also in the water treatment with live clams but are only present in pore water in the processed environment samples is also interesting. The presence of the symbionts in the sediment treatment with dead clams suggest that the symbiont is able to attach to the sediment but no amplifications were found in sediment samples from the environment. As previously mentioned, a reason for this could be that the symbiont is part of the rare biosphere and therefore could not be detected in the environment. The reason why we were able to find them in the symbiont release experiment could be that symbionts display a higher abundance in the falcon tubes and therefore were detectable. Another explanation could be that the released symbionts from the dead clams were also dead or just starting to degrade and therefore just surrounded the decomposing clam in the sediment. To confirm that the surrounding cells are still intact and viable and not just eDNA, FISH could be performed to confirm intact cells, further studies should be conducted.

Horizontally acquired bacteria in chemosynthetic environments tend to possess escape strategies or defense mechanisms that allow them to re-enter the environment and contribute to their overall fitness and avoid exploitation[106]. These findings and the well described evolutionary stable association between *L. orbiculatus* and its chemosynthetic symbiont also promotes the theory of small symbiont spreading during lifetime.

Learning more about the uptake and release of the symbiont during lifetime is essential to understand the characteristics of the symbioses and the impact on the environment. When environmental conditions get unfavorable for the symbiont, the clam host could be an insurance for the survival of the symbiont population if symbionts are able to escape the clam after infection. These unfavorable conditions could be atypical seasonal changes or destruction of the habitat through an abrupt change of environmental conditions. Together with transmission mode of the symbiont and the site of bacterial housing, symbiont uptake can affect the composition of the symbiont population[107].

8. Outlook

Since we were not able to identify a substantial symbiont population in the Bay of Fetovaia during our study, much more can be done to investigate this further. If the symbionts can not be detected in the environment, neither in the sediment nor in the porewater, there could be another reservoir for symbionts. It could be that a symbiont spreader event is the host's death as shown for *Riftia pachyptila*[23]. This would drive the need for a stable death rate in each population. Given the presence of the symbiont in the mucus tubes in relatively high numbers compared to the other microhabitats, it could be considered that the “free-living” symbiont population is living in structures built by the clam and therefore also highly dependent on the host. Symbionts associated with the mucus tubes may already include selected symbionts which are pre-filtered by the chemoattractant mucus tube. Further research has to be done to gain more knowledge about these mechanisms.

We were able to find our target symbionts relatives in small amounts in the pore water of samples from 2016 and 2018. With the primers which target *Ca.* Thiodiazotropha weberae and lotti, we were able to detect the smyiont in sediment and water samples of the dead clam treatments and the live clam water treatment but the question remains if symbionts which are released after host death are still live. Therefore I would suggest investigating this

more by conducting dead and live clam Symbiont Release Experiments and investigate if released symbionts are still live after the death of the host. This could be achieved by a number of different approaches. Sediment samples could be analyzed by Flow Cytometry or viablePCR with a target for *Ca.* Thiodiazotropha at different time points during the experiment. Recent studies[5,85] unveiled that many free-living forms of sulfur-oxidizing chemolithoautotrophic symbionts and lucinid endosymbiotic bacteria are associated with seagrass roots worldwide with absence of the clam hosts in the entire habitat. The screening of the rhizosphere of seagrass in lucinid habitats could also bring new insights in this chemosymbiotic mutualism and the distribution of possible infectors for aposymbiotic juvenile hosts.

9. References

1. Lorion, J. *et al.* Adaptive radiation of chemosymbiotic deep-sea mussels. *Proc. Biol. Sci.* **281**, 20132972 (2014).
2. Cavanaugh, C. M., McKiness, Z. P., Newton, I. L. G. & Stewart, F. J. Marine chemosynthetic symbioses. *The prokaryotes* **1**, 475–507 (2006).
3. König, S. *et al.* Nitrogen fixation in a chemoautotrophic lucinid symbiosis. *Nature Microbiology* vol. 2 (2017).
4. Gros, O., Liberge, M. & Felbeck, H. Interspecific infection of aposymbiotic juveniles of Codakia orbicularis by various tropical lucinid gill-endosymbionts. *Mar. Biol.* **142**, 57–66 (2003).
5. Cúcio, C., Overmars, L., Engelen, A. H. & Muyzer, G. Metagenomic analysis shows the presence of bacteria related to free-living forms of sulfur-oxidizing chemolithoautotrophic symbionts in the rhizosphere of the seagrass Zostera marina. *Front. Mar. Sci.* **5**, (2018).
6. Wodara, C., Kostka, S., Egert, M., Kelly, D. P. & Friedrich, C. G. Identification and sequence analysis of the soxB gene essential for sulfur oxidation of Paracoccus denitrificans GB17. *J. Bacteriol.* **176**, 6188–6191 (1994).
7. Stewart, F. J., Newton, I. L. G. & Cavanaugh, C. M. Chemosynthetic endosymbioses: adaptations to oxic–anoxic interfaces. *Trends Microbiol.* **13**, 439–448 (2005).
8. Felbeck, H. & Childress, J. J. Riftia pachyptila: a highly integrated symbiosis. *Oceanologica Acta, Special issue* (1988).
9. Klose, J. *et al.* Trophosome of the Deep-Sea Tubeworm Riftia pachyptila Inhibits Bacterial Growth. *PLoS One* **11**, e0146446 (2016).
10. Distel, D. L. *et al.* Discovery of chemoautotrophic symbiosis in the giant shipworm Kuphus polythalamia (Bivalvia: Teredinidae) extends wooden-steps theory. *Proc. Natl. Acad. Sci. U. S. A.* **114**, E3652–E3658 (2017).
11. Dubilier, N., Giere, O., Distel, D. L. & Cavanaugh, C. M. Characterization of chemoautotrophic bacterial symbionts in a gutless marine worm Oligochaeta, Annelida) by phylogenetic 16S rRNA

sequence analysis and in situ hybridization. *Appl. Environ. Microbiol.* **61**, 2346–2350 (1995).

12. Dick, G. J. The microbiomes of deep-sea hydrothermal vents: distributed globally, shaped locally. *Nat. Rev. Microbiol.* **17**, 271–283 (2019).
13. Barnes, P. A. G., Hickman, C. S., Walker, D. I. & Wells, F. E. Lucinid bivalves and marine angiosperms: a search for causal relationships. *The Seagrass Flora and Fauna of Rottnest Island, Western Australia. Western Australian Museum, Perth* 215–238 (1999).
14. Lebata, M. J. Oxygen, sulphide and nutrient uptake of the mangrove mud clam Anodontia edentula (Family: Lucinidae). *Mar. Pollut. Bull.* **42**, 1133–1138 (2001).
15. Schweimanns, M. & Felbeck, H. Significance of the occurrence of chemoautotrophic bacterial endosymbionts in lucinid clams from Bermuda. *Marine ecology progress series. Oldendorf* **24**, 113–120 (1985).
16. Dubilier, N., Bergin, C. & Lott, C. Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat. Rev. Microbiol.* **6**, 725–740 (2008).
17. Govers, L. L. *et al.* Marine Phytophthora species can hamper conservation and restoration of vegetated coastal ecosystems. *Proc. Biol. Sci.* **283**, (2016).
18. Cardini, U. *et al.* Chemosymbiotic bivalves contribute to the nitrogen budget of seagrass ecosystems. *ISME J.* **13**, 3131–3134 (2019).
19. Higgs, N. D., Newton, J. & Attrill, M. J. Caribbean Spiny Lobster Fishery Is Underpinned by Trophic Subsidies from Chemosynthetic Primary Production. *Curr. Biol.* **26**, 3393–3398 (2016).
20. de Fouw, J. *et al.* Drought, Mutualism Breakdown, and Landscape-Scale Degradation of Seagrass Beds. *Curr. Biol.* **26**, 1051–1056 (2016).
21. Bennett, G. M. & Moran, N. A. Heritable symbiosis: The advantages and perils of an evolutionary rabbit hole. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 10169–10176 (2015).
22. Stanley, S. M. Evolutionary radiation of shallow-water Lucinidae (Bivalvia with endosymbionts) as a result of the rise of seagrasses and mangroves. *Geology* **42**, 803–806 (2014).
23. Sato, M. & Sasaki, A. Evolution and Maintenance of Mutualism between Tubeworms and Sulfur-Oxidizing Bacteria. *Am. Nat.* **197**, 351–365 (2021).

24. Gros, O., Darrasse, A., Durand, P., Frenkiel, L. & Mouëza, M. Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve Codakia orbicularis. *Appl. Environ. Microbiol.* **62**, 2324–2330 (1996).
25. Ikuta, T. *et al.* Surfing the vegetal pole in a small population: extracellular vertical transmission of an 'intracellular' deep-sea clam symbiont. *R. Soc. Open Sci.* **3**, 160130 (2016).
26. Goffredi, S., Hurtado, L., Hallam, S. & Vrijenhoek, R. Evolutionary relationships of deep-sea vent and cold seep clams (Mollusca: Vesicomyidae) of the 'pacifica/lepta' species complex. *Mar. Biol.* **142**, 311–320 (2003).
27. Wernegreen, J. J. For better or worse: genomic consequences of intracellular mutualism and parasitism. *Curr. Opin. Genet. Dev.* **15**, 572–583 (2005).
28. Duperron, S., Halary, S., Lorion, J., Sibuet, M. & Gaill, F. Unexpected co-occurrence of six bacterial symbionts in the gills of the cold seep mussel Idas sp. (Bivalvia: Mytilidae). *Environmental Microbiology* vol. 10 433–445 (2008).
29. Petersen, J. M. & Yuen, B. The Symbiotic 'All-Rounders': Partnerships between Marine Animals and Chemosynthetic Nitrogen-Fixing Bacteria. *Applied and Environmental Microbiology* vol. 87 (2021).
30. Bright, M. & Bulgheresi, S. A complex journey: transmission of microbial symbionts. *Nat. Rev. Microbiol.* **8**, 218–230 (2010).
31. Picazo, D. R. *et al.* Horizontally transmitted symbiont populations in deep-sea mussels are genetically isolated. *ISME J.* **13**, 2954–2968 (2019).
32. Klose, J. *et al.* Endosymbionts escape dead hydrothermal vent tubeworms to enrich the free-living population. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 11300–11305 (2015).
33. Elisabeth, N. H., Caro, A. & Césaire, T. Comparative modifications in bacterial gill-endosymbiotic populations of the two bivalves Codakia orbiculata and Lucina pensylvanica during bacterial loss and …. *FEMS microbiology* (2014).
34. Taylor, J. D. & Glover, E. A. Lucinidae (Bivalvia)–the most diverse group of chemosymbiotic molluscs. *Zool. J. Linn. Soc.* **148**, 421–438 (2006).

35. Roeselers, G. & Newton, I. L. G. On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. *Appl. Microbiol. Biotechnol.* **94**, 1–10 (2012).
36. van der Heide, T. *et al.* A three-stage symbiosis forms the foundation of seagrass ecosystems. *Science* **336**, 1432–1434 (2012).
37. Reynolds, L. K., Berg, P. & Zieman, J. C. Lucinid clam influence on the biogeochemistry of the seagrassThalassia testudinum sediments. *Estuaries Coasts* **30**, 482–490 (2007).
38. Taylor, J. D. & Glover, E. A. Functional anatomy, chemosymbiosis and evolution of the Lucinidae. *Geological Society, London, Special Publications* **177**, 207–225 (2000).
39. Allen, J. A. On the basic form and adaptations to habitat in the Lucinacea (Eulamellibranchia). *Philos. trans. R. Soc. Lond.* **241**, 421–484 (1958).
40. Dando, P. R., Ridgway, S. A. & Spiro, B. Sulphide 'mining' by lucinid bivalve molluscs: demonstrated by stable sulphur isotope measurements and experimental models. *Mar. Ecol. Prog. Ser.* **107**, 169–175 (1994).
41. Apprill, A. The Role of Symbioses in the Adaptation and Stress Responses of Marine Organisms. *Ann. Rev. Mar. Sci.* **12**, 291–314 (2020).
42. Johnson, M. A. & Fernandez, C. Bacterial symbiosis in Loripes lucinalis (Mollusca: Bivalvia) with comments on reproductive strategy. *Journal of the Marine Biological Association of the United Kingdom* vol. 81 251–257 (2001).
43. Frenkiel, L. & Mouëza, M. Gill ultrastructure and symbiotic bacteria in Codakia orbicularis (Bivalvia, Lucinidae). *Zoomorphology* **115**, 51–61 (1995).
44. Brissac, T., Merçot, H. & Gros, O. Lucinidae/sulfur-oxidizing bacteria: ancestral heritage or opportunistic association? Further insights from the Bohol Sea (the Philippines). *FEMS Microbiol. Ecol.* **75**, 63–76 (2011).
45. Lim, S. J. *et al.* Extensive Thioautotrophic Gill Endosymbiont Diversity within a Single Ctena orbiculata (Bivalvia: Lucinidae) Population and Implications for Defining Host-Symbiont Specificity and Species Recognition. *mSystems* **4**, (2019).
46. Distel, D. L. & Felbeck, H. Endosymbiosis in the lucinid clams Lucinoma aequizonata, Lucinoma

annulata and Lucina floridana: a reexamination of the functional morphology of the gills as bacteria-bearing organs. *Mar. Biol.* **96**, 79–86 (1987).

47. Brissac, T., Higuet, D., Gros, O. & Merçot, H. Unexpected structured intraspecific diversity of thioautotrophic bacterial gill endosymbionts within the Lucinidae (Mollusca: Bivalvia). *Mar. Biol.* **163**, 176 (2016).
48. Duperron, S., Fiala-Medioni, A., Caprais, J.-C., Olu, K. & Sibuet, M. Evidence for chemoautotrophic symbiosis in a Mediterranean cold seep clam (Bivalvia: Lucinidae): comparative sequence analysis of bacterial 16S rRNA, APS reductase and RubisCO genes. *FEMS Microbiol. Ecol.* **59**, 64–70 (2007).
49. Lim, J. S. Clemson University. (2018).
50. Petersen, J. M. *et al.* Chemosynthetic symbionts of marine invertebrate animals are capable of nitrogen fixation. *Nature Microbiology* vol. 2 (2017).
51. Osvatic, J. T. *et al.* Global biogeography of chemosynthetic symbionts reveals both localized and globally distributed symbiont groups. *Proc. Natl. Acad. Sci. U. S. A.* **118**, (2021).
52. Hasler-Sheetal, H. & Holmer, M. Sulfide Intrusion and Detoxification in the Seagrass Zostera marina. *PLoS One* **10**, e0129136 (2015).
53. Papenbrock, J. Highlights in seagrasses' phylogeny, physiology, and metabolism: what makes them special? *International Scholarly Research Notices* **2012**, (2012).
54. Hemminga, M. A. & Duarte, C. M. *Seagrass Ecology*. (Cambridge University Press, 2000).
55. Domning, D. P. World Atlas of Seagrasses. By Edmund P Green and , Frederick T Short. Published by University of California Press, Berkeley (California), in association with UNEP-WCMC. $60.00. xii 298 p 14 pl; ill.; index. ISBN: 0-520-24047-2. 2003. *The Quarterly Review of Biology* vol. 80 256–256 (2005).
56. Gagnon, K. *et al.* Facilitating foundation species: The potential for plant–bivalve interactions to improve habitat restoration success. *J. Appl. Ecol.* **57**, 1161–1179 (2020).
57. Nordlund, L. M., Unsworth, R. K. F., Gullström, M. & Cullen-Unsworth, L. C. Global significance of seagrass fishery activity. *Fish Fish* **19**, 399–412 (2018).

58. Fourqurean, J. W. *et al.* Seagrass ecosystems as a globally significant carbon stock. *Nat. Geosci.* **5**, 505–509 (2012).

59. Orth, R. J. *et al.* A Global Crisis for Seagrass Ecosystems. *Bioscience* **56**, 987–996 (2006).

60. Nordlund, L. M., Koch, E. W., Barbier, E. B. & Creed, J. C. Correction: Seagrass Ecosystem Services and Their Variability across Genera and Geographical Regions. *PLOS ONE* vol. 12 e0169942 (2017).

61. de los Santos, C. B. *et al.* Seagrass ecosystem services: assessment and scale of benefits. *Out of the blue: The value of seagrasses to the environment and to people* 19–21 (2020).

62. Unsworth, R. K. F., Nordlund, L. M. & Cullen-Unsworth, L. C. Seagrass meadows support global fisheries production. *Conserv. Lett.* **12**, e12566 (2019).

63. Hughes, T. P. *et al.* Spatial and temporal patterns of mass bleaching of corals in the Anthropocene. *Science* **359**, 80–83 (2018).

64. Burrows, M. T. *et al.* The pace of shifting climate in marine and terrestrial ecosystems. *Science* **334**, 652–655 (2011).

65. Doney, S. C. *et al.* Climate change impacts on marine ecosystems. *Ann. Rev. Mar. Sci.* **4**, 11–37 (2012).

66. van der Heide, T., van Nes, E. H., van Katwijk, M. M., Olff, H. & Smolders, A. J. P. Positive feedbacks in seagrass ecosystems--evidence from large-scale empirical data. *PLoS One* **6**, e16504 (2011).

67. Maxwell, P. S. *et al.* The fundamental role of ecological feedback mechanisms for the adaptive management of seagrass ecosystems - a review. *Biol. Rev. Camb. Philos. Soc.* **92**, 1521–1538 (2017).

68. García, R., Holmer, M., Duarte, C. M. & Marbà, N. Global warming enhances sulphide stress in a key seagrass species (NW Mediterranean). *Glob. Chang. Biol.* **19**, 3629–3639 (2013).

69. Govers, L. L. The effects of biogeochemical stressors on seagrass ecosystems. ([Sl: sn], 2014).

70. Gros, O., Liberge, M., Heddi, A., Khatchadourian, C. & Felbeck, H. Detection of the Free-Living Forms of Sulfide-Oxidizing Gill Endosymbionts in the Lucinid Habitat (Thalassia testudinum

Environment). *Applied and Environmental Microbiology* vol. 69 6264–6267 (2003).

71. Sudo, M. Hidden diversity in the sulphur oxidation metabolism of lucinid symbionts as an adaptation to environmental variability. (University of Vienna, 2019).
72. Müller, M. R., Ehrmann, M. A. & Vogel, R. F. Multiplex PCR for the detection of Lactobacillus pontis and two related species in a sourdough fermentation. *Appl. Environ. Microbiol.* **66**, 2113–2116 (2000).
73. Brandl, M. T., Quiñones, B. & Lindow, S. E. Heterogeneous transcription of an indoleacetic acid biosynthetic gene in Erwinia herbicola on plant surfaces. *Proc. Natl. Acad. Sci. U. S. A.* **98**, 3454–3459 (2001).
74. Herlemann, D. P. *et al.* Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* **5**, 1571–1579 (2011).
75. Herbold, C. W. *et al.* A flexible and economical barcoding approach for highly multiplexed amplicon sequencing of diverse target genes. *Front. Microbiol.* **6**, 731 (2015).
76. Pjevac, P. *et al.* An Economical and Flexible Dual Barcoding, Two-Step PCR Approach for Highly Multiplexed Amplicon Sequencing. *Front. Microbiol.* **12**, 669776 (2021).
77. Williams, H. N. *et al.* Halobacteriovorax, an underestimated predator on bacteria: potential impact relative to viruses on bacterial mortality. *ISME J.* **10**, 491–499 (2016).
78. Hausl, B. R. Genome diversity and free-living lifestyle of chemoautotrophic lucinid symbionts. (uniwien, 2017).
79. Gros, O., Elisabeth, N. H., Gustave, S. D. D., Caro, A. & Dubilier, N. Plasticity of symbiont acquisition throughout the life cycle of the shallow-water tropical lucinid Codakia orbiculata (Mollusca: Bivalvia). *Environ. Microbiol.* **14**, 1584–1595 (2012).
80. Lim, S. J. *et al.* Taxonomic and functional heterogeneity of the gill microbiome in a symbiotic coastal mangrove lucinid species. *The ISME Journal* vol. 13 902–920 (2019).
81. Moran, N. A. Tracing the evolution of gene loss in obligate bacterial symbionts. *Curr. Opin. Microbiol.* **6**, 512–518 (2003).
82. McCutcheon, J. P. & Moran, N. A. Extreme genome reduction in symbiotic bacteria. *Nat. Rev.*

Microbiol. **10**, 13–26 (2011).

83. Moran, N. A., McCutcheon, J. P. & Nakabachi, A. Genomics and evolution of heritable bacterial symbionts. *Annu. Rev. Genet.* **42**, 165–190 (2008).
84. Koonin, E. V. & Wolf, Y. I. Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. *Nucleic Acids Res.* **36**, 6688–6719 (2008).
85. Martin, B. C. *et al.* Cutting out the middle clam: lucinid endosymbiotic bacteria are also associated with seagrass roots worldwide. *ISME J.* **14**, 2901–2905 (2020).
86. Sogin, M. L. *et al.* Microbial diversity in the deep sea and the underexplored 'rare biosphere'. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 12115–12120 (2006).
87. Lynch, M. D. J. & Neufeld, J. D. Ecology and exploration of the rare biosphere. *Nat. Rev. Microbiol.* **13**, 217–229 (2015).
88. Lennon, J. T. & Jones, S. E. Microbial seed banks: the ecological and evolutionary implications of dormancy. *Nat. Rev. Microbiol.* **9**, 119–130 (2011).
89. Pester, M., Bittner, N., Deevong, P., Wagner, M. & Loy, A. A 'rare biosphere' microorganism contributes to sulfate reduction in a peatland. *ISME J.* **4**, 1591–1602 (2010).
90. Bodelier, P. L. E. *et al.* Microbial minorities modulate methane consumption through niche partitioning. *ISME J.* **7**, 2214–2228 (2013).
91. Lawson, C. E. *et al.* Rare taxa have potential to make metabolic contributions in enhanced biological phosphorus removal ecosystems. *Environ. Microbiol.* **17**, 4979–4993 (2015).
92. Yamamoto, K. *et al.* Diversity and geochemical community assembly processes of the living rare biosphere in a sand-and-gravel aquifer ecosystem in the Midwestern United States. *Sci. Rep.* **9**, 13484 (2019).
93. Brodersen, K. E. *et al.* Seagrass-Mediated Phosphorus and Iron Solubilization in Tropical Sediments. *Environ. Sci. Technol.* **51**, 14155–14163 (2017).
94. Martin, B. C. *et al.* Oxygen loss from seagrass roots coincides with colonisation of sulphide-oxidising cable bacteria and reduces sulphide stress. *ISME J.* **13**, 707–719 (2018).
95. Dunic, J. C., Brown, C. J., Connolly, R. M., Turschwell, M. P. & Côté, I. M. Long-term declines and

recovery of meadow area across the world's seagrass bioregions. *Glob. Chang. Biol.* **27**, 4096–4109 (2021).

96. Nussbaumer, A. D., Fisher, C. R. & Bright, M. Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature* **441**, 345–348 (2006).
97. Allam, B. & Espinosa, E. P. Bivalve immunity and response to infections: Are we looking at the right place? *Fish & Shellfish Immunology* vol. 53 4–12 (2016).
98. Nyholm, S. V. & McFall-Ngai, M. The winnowing: establishing the squid–vibrio symbiosis. *Nat. Rev. Microbiol.* **2**, 632–642 (2004).
99. Papaspyrou, S., Gregersen, T., Kristensen, E., Christensen, B. & Cox, R. P. Microbial reaction rates and bacterial communities in sediment surrounding burrows of two nereidid polychaetes (Nereis diversicolor and N. virens). *Marine Biology* vol. 148 541–550 (2006).
100. Ansorge, R. *et al.* Functional diversity enables multiple symbiont strains to coexist in deep-sea mussels. *Nat Microbiol* **4**, 2487–2497 (2019).
101. Ikuta, T. *et al.* Heterogeneous composition of key metabolic gene clusters in a vent mussel symbiont population. *ISME J.* **10**, 990–1001 (2016).
102. Franke, M., Geier, B., Hammel, J. U., Dubilier, N. & Leisch, N. Coming together – symbiont acquisition and early development of Bathymodiolus mussels. doi:10.1101/2020.10.09.333211.
103. Bright, M., Eichinger, I. & von Salvini-Plawen, L. The metatrochophore of a deep-sea hydrothermal vent vestimentiferan (Polychaeta: Siboglinidae). *Org. Divers. Evol.* **13**, 163–188 (2013).
104. Polzin, J., Arevalo, P., Nussbaumer, T., Polz, M. F. & Bright, M. Polyclonal symbiont populations in hydrothermal vent tubeworms and the environment. *Proc. Biol. Sci.* **286**, 20181281 (2019).
105. Elisabeth, N. H., Gustave, S. D. D. & Gros, O. Cell proliferation and apoptosis in gill filaments of the lucinid Codakia orbiculata (Montagu, 1808) (Mollusca: Bivalvia) during bacterial decolonization and recolonization. *Microscopy Research and Technique* vol. 75 1136–1146 (2012).
106. Vrijenhoek, R. C. Genetics and Evolution of Deep-Sea Chemosynthetic Bacteria and Their

Invertebrate Hosts. *Topics in Geobiology* 15–49 (2010) doi:10.1007/978-90-481-9572-5_2.

107. McCuaig, B., Liboiron, F. & Dufour, S. C. The bivalve Thyasira cf. gouldi hosts chemoautotrophic symbiont populations with strain level diversity. *PeerJ* **5**, e3597 (2017).

108. Hindson, B. J. *et al.* High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Anal. Chem.* **83**, 8604–8610 (2011).

10. Supplementary information

10.1 Supplementary Materials and Methods

10.1.1 PCR sample amplification information list for Symbiont Release Experiment

Samples PW		template	cycles	Samples PW		template	cycles
DW_1_N	25	1 ul	25	CW_2_N	negative	1 ul	25
DW_1_0	25	1 ul	25	CW_2_0	negative	1 ul	25
DW_1_1	25	1 ul	25	CW_2_1	negative	1 ul	25
DW_1_3	30	5ul	30	CW_2_3	negative	1 ul	25
DW_1_5	25	1 ul	25	CW_2_5	negative	1 ul	25
DW_1_7	25	1 ul	25	CW_2_7	negative	1 ul	25
DW_1_E	30	5ul	30	CW_2_E	negative	1 ul	25
DW_2_N	25	1 ul	25	AW_1_N	25	1 ul	25
DW_2_0	25	1 ul	25	AW_1_0	25	1 ul	25
DW_2_1	25	1 ul	25	AW_1_1	25	1 ul	25
DW_2_3	25	1 ul	25	AW_1_3	25	1 ul	25
DW_2_5	25	1 ul	25	AW_1_5	30	5ul	30
DW_2_7	30	5ul	30	AW_1_7	30	5ul	30
DW_2_E	30	5ul	30	AW_1_E	25	1 ul	25
DW_3_N	25	1 ul	25	AW_2_N	25	1 ul	25
DW_3_0	25	1 ul	25	AW_2_0	25	1 ul	25
DW_3_1	25	1 ul	25	AW_2_1	25	1 ul	25
DW_3_3	25	1 ul	25	AW_2_3	25	1 ul	25
DW_3_5	25	1 ul	25	AW_2_5	30	5ul	30
DW_3_7	30	5ul	30	AW_2_7	25	1 ul	25
DW_3_E	30	5ul	30	AW_2_E	30	5ul	30
DW_4_N	25	1 ul	25	AW_3_N	30	5ul	30
DW_4_0	25	1 ul	25	AW_3_0	25	1 ul	25
DW_4_1	25	1 ul	25	AW_3_1	25	1 ul	25
DW_4_3	25	1 ul	25	AW_3_3	25	1 ul	25
DW_4_5	25	1 ul	25	AW_3_5	30	5ul	30
DW_4_7	25	1 ul	25	AW_3_7	30	5ul	30
DW_4_E	30	5ul	30	AW_3_E	25	1 ul	25
CW_1_N	negative	1 ul	25	AW_4_N	30	5ul	30
CW_1_0	negative	1 ul	25	AW_4_0	25	1 ul	25
CW_1_1	negative	1 ul	25	AW_4_1	25	1 ul	25
CW_1_3	negative	1 ul	25	AW_4_3	30	5ul	30
CW_1_5	negative	1 ul	25	AW_4_5	30	5ul	30
CW_1_7	negative	1 ul	25	AW_4_7	30	5ul	30
CW_1_E	negative	1 ul	25	AW_4_E	30	5ul	30
Samples soil	cycles	template	cycles	Samples soil		template	cycles

AS_1_N	30	5ul	30	CS_2_N	negative	1 ul	25
AS_1_0	30	5ul	30	CS_2_0	negative	1 ul	25
AS_1_1	30	5ul	30	CS_2_1	negative	1 ul	25
AS_1_3	30	5ul	30	CS_2_3	negative	1 ul	25
AS_1_5	25	1 ul	25	CS_2_5	negative	1 ul	25
AS_1_7	25	1 ul	25	CS_2_7	negative	1 ul	25
AS_1_E	25	1 ul	25	CS_2_E	negative	1 ul	25
AS_2_N	30	5ul	30	DS_1_N	25	1 ul	25
AS_2_0	30	5ul	30	DS_1_0	30	5ul	30
AS_2_1	30	5ul	30	DS_1_1	25	1 ul	25
AS_2_3	30	5ul	30	DS_1_3	25	1 ul	25
AS_2_5	25	1 ul	25	DS_1_5	25	1 ul	25
AS_2_7	25	1 ul	25	DS_1_7	30	5ul	30
AS_2_E	25	1 ul	25	DS_1_E	25	1 ul	25
AS_3_N	30	5ul	30	DS_2_N	30	5ul	30
AS_3_0	30	5ul	30	DS_2_0	30	5ul	30
AS_3_1	30	5ul	30	DS_2_1	25	1 ul	25
AS_3_3	30	5ul	30	DS_2_3	25	1 ul	25
AS_3_5	30	5ul	30	DS_2_5	25	1 ul	25
AS_3_7	30	5ul	30	DS_2_7	25	1 ul	25
AS_3_E	30	5ul	30	DS_2_E	25	1 ul	25
AS_4_N	30	5ul	30	DS_3_N	25	1 ul	25
AS_4_0	30	5ul	30	DS_3_0	25	1 ul	25
AS_4_1	30	5ul	30	DS_3_1	25	1 ul	25
AS_4_4	30	5ul	30	DS_3_3	25	1 ul	25
AS_4_5	30	5ul	30	DS_3_5	25	1 ul	25
AS_4_7	30	5ul	30	DS_3_7	25	1 ul	25
AS_4_E	30	5ul	30	DS_3_E	25	1 ul	25
CS_1_N	negative	1 ul	25	DS_4_N	25	1 ul	25
CS_1_0	negative	1 ul	25	DS_4_0	25	1 ul	25
CS_1_1	negative	1 ul	25	DS_4_1	25	1 ul	25
CS_1_3	negative	1 ul	25	DS_4_3	25	1 ul	25
CS_1_5	negative	1 ul	25	DS_4_5	25	1 ul	25
CS_1_7	negative	1 ul	25	DS_4_7	25	1 ul	25
CS_1_E	negative	1 ul	25	DS_4_E	25	1 ul	25

PW 2018		templ ate	cycles	PW 2018	templat e	cycles
P_1_W	negative	5ul	30	O_1_W	5ul	30
P_1_5	negative	5ul	30	O_1_5	5ul	30
P_1_15	25	5ul	30	O_1_15	5ul	30
P_1_25	negative	5ul	30	O_1_25	5ul	30
P_1_35	negative	5ul	30	O_1_35	5ul	30
P_1_45	25	5ul	30	O_1_45	5ul	30
P_2_W	negative	5ul	30	O_2_W	5ul	30

P_2_5	negative	5ul	30	O_2_5	5ul	30
P_2_15	negative	5ul	30	O_2_15	5ul	30
P_2_25	negative	5ul	30	O_2_25	5ul	30
P_2_35	negative	5ul	30	O_2_35	5ul	30
P_2_45	negative	5ul	30	O_2_45	5ul	30
P_3_W	25	5ul	30	C_1_W	5ul	25
P_3_5	25	5ul	30	C_1_5	5ul	25
P_3_15	negative	5ul	30	C_1_15	5ul	25
P_3_25	negative	5ul	30	C_1_25	5ul	negative
P_3_35	negative	5ul	30	C_1_35	5ul	negative
P_3_45	25	5ul	30	C_1_45	5ul	negative
P_4_W	25	5ul	30	C_2_W	5ul	negative
P_4_5	25	5ul	30	C_2_5	5ul	negative
P_4_15	25	5ul	30	C_2_15	5ul	negative
P_4_25	negative	5ul	30	C_2_25	5ul	negative
P_4_35	negative	5ul	30	C_2_35	5ul	negative
P_4_45	negative	5ul	30	C_2_45	5ul	negative

ElbaPW16	Spalte1	template	cycles	Spalte2	ElbaPW163	Spalte4	template5	cycles6
1_W	30	5ul	30		2_35	25	5ul	25
1_0	30	5ul	30		2_40	25	5ul	25
1_5	30	5ul	30		2_45	25	5ul	25
1_10	25	5ul	25		2_50	25	5ul	25
1_15	30	5ul	30		2_55	25	5ul	25
1_20	25	5ul	25		2_60	25	5ul	25
1_25	25	5ul	25		3_W	25	5ul	25
1_30	25	5ul	25		3_0	25	5ul	25
1_35	25	5ul	25		3_5	25	5ul	25
1_40	25	5ul	25		3_10	25	5ul	25
1_45	30	5ul	30		3_15	25	5ul	25
1_50	30	5ul	30		3_20	30	5ul	30
1_55	25	5ul	25		3_25	30	5ul	30
1_60	25	5ul	25		3_30	30	5ul	30
C1	negative	5ul	25		3_35	30	5ul	30
C2	negative	5ul	25		3_40	30	5ul	30
2_W	30	5ul	30		3_45	25	5ul	25
2_0	30	5ul	30		3_50	25	5ul	25
2_5	30	5ul	30		3_55	25	5ul	25
2_10	25	5ul	25		3_60	25	5ul	25
2_15	25	5ul	25		C1	negative	5ul	25
2_20	25	5ul	25		C2	negative	5ul	25
2_25	25	5ul	25		C3	negative	5ul	25
2_30	25	5ul	25		C4	negative	5ul	25

10.1.2 Protocol S1: Protocol for Symbiont Release Experimental Setup

Clams:

- Dead clams will be cut open with a razorblade and the gills will be severed from the visceral mass.
- All clams were washed 3 times in FSW before cutting or adding to tubes.
- Sediment tube (10, 4 dead, 4 alive, 2 control)

Materials (per tube):

- 50 ml falcon tubes
- FSW ~50ml
- Sediment ~20 ml
- 50 ml syringe

1. 20 ml of sediment will be poured into a 50 ml syringe.
(work near the flame)
(if the sediment does not come out, cut off tip with a sterile razor blade)
2. Add sediment to the Falcon tube.
3. Add enough water to the sediment that it is moist and under ~5ml of water.
(approximately 30 ml)
4. Add 3 of the needed types of clams with a (flamed) tongs.
(they should be pushed into the sediment but easily removable)
(make sure the clams stay closed)
5. Seal and place in aquarium room

Sampling Schedule and Protocol

This experiment will officially go for 12 days.

FSW sampling (10)

Materials (per tube):

- 50 ml syringe
- needle for 50 ml syringe (long ones)
- sterivex filter
- Luer-lok cap for sterivex filter (or parafilm)
- PFA 37%
- 2um 25mm filter
- 45um 25mm filter
- 25mm filter holder
- FSW

1. Suck all water from the tube into the syringe.
(leave behind any body parts)
2. Push 35 ml through the sterivex filter
(freeze after collection)
3. Add 1 ml of 37% PFA (This might actually be too concentrated)

4. Let the FSW/PFA solution set at 4 degrees C overnight
5. (Next day) FSW/PFA solution will be pushed through a 0.2µl filter, with a 0.45µl support filter
6. Push ~30ml of FSW through the filter
7. Push air through filter to quick dry
8. Let dry and store at -20 degrees C

Sediment sampling (10)

Materials (per tube):

- 2% PFA/FSW solution (made by 37% PFA)
- 2ml tube
- centrifuge
- PBS
- Mobio tube
- Ethanol (molecular grade)

1. Remove clams.
2. Add 1 ml 2% PFA/FSW to a 2ml tube
3a. Sample 0.5g sediment near clam area and add to PFA/FSW tube
3b. Sample 0.5g of sediment near the clam area and add to Mobio tube.
4. Store Mobio tube at -20 degrees C.
5. Let sediment/PFA/FSW tube rest in 4 degrees C overnight.
6. (Next day) centrifuge sediment/PFA/FSW tube at 10,000 rpm for 5 minutes. Pour off supernatant.
7. Add 1.5 ml PBS and resuspend samples.
8. Repeat steps 6 and 7.
9. Centrifuge at 10,000 rpm for 5 minutes. Pour off supernatant.
10. Add 1.5 ml of 1:1 PBS/ethanol mix and store at -20 degrees C

FSW samples were processed in Power water sterivex kit.
Sediment samples were processed in Power soil kit.

10.1.3 Protocol S2: ddPCR Protocol

1. Set-up PCR reaction (EvaGreen Supermix)

    - Use multiples of 8 for PCR set-up (all 8 wells on Cartridge must be filled for droplet generation)
    - Thaw EvaGreen Supermix
    - Template DNA should be around 66 ng (up to 100 ng) – if concentration is unknown, use different dilutions (1:10, 1:100… for template)
    - Restriction digestion of template optional to ensure better accessibility to target gene (plasmids or high amount of initial DNA); can be performed directly in Mastermix or

prior to PCR set-up (buffer should have low salt conc) without need to stop digestion; HaeIII, MseI, AluI, HindIII, CviQI work well with Supermix

- Best use more than 1 µl DNA template (dilute beforehand if necessary) – pipetting error too high
- Prepare mastermix (prepare 22 µl per sample to be able to use 20 µl for droplet generation):

reagent	**General advice**	**Conc. in PCR**	**Used for amoebae**	**Conc. in amoebae PCR**
2 x QX200 ddPCR EvaGreen Supermix	11 µl	1 x	11 µl	1 x
Forward primer	x µl	100-250 nM	0.4 µl	100 nM (5 µM stock)
Reverse primer	x µl	100-250 nM	0.4 µl	100 nM (5 µM stock)
Optional: DNA restriction enzyme	1 µl	2-5 U	-	
DNA template or PCR water	x µl	Up to 100 ng	2 µl	
PCR water	add 22 µl		8.2 µl	

- Vortex and spin down

2. Droplet generation

- Put DG8 cartridge in cartridge holder
- Always first pipette 20 µl of PCR reaction in wells in middle row
  AVOID air bubbles at bottom of wells! *Use 20 µl tips, hold in 15° angle at bottom of well during pipetting half of suspension, then slowly go up the wall of the well with the tip during pipetting the remaining suspension*
  All wells in the cartridge need to be used for droplet generation. If less than 8 samples use 1x ddPCR buffer instead of PCR reaction
- Add 70 µl droplet generation oil to all wells in bottom row of the DG8 cartridge
- Attach gasket across DG8 cartridge (not reusable)
- Put in droplet generator
- Transfer 42 µl of droplets from top row of DG8 cartridge to a 96-well plate
  AVOID droplet shearing! *Use 200 µl tips (best wide bore), hold in 30-45° angle between side wall and bottom of well, pipette slowly (~5 secs) and slowly (~5 secs) release droplets in 96-well plate on side wall near bottom*
  AVOID evaporation of droplets! *Cover filled columns in 96-well plate*
- Seal plate with pierceable foil at 180°C for around 5 secs (ours ~170°C, 8’’), red line faces upwards

Start PCR within 30 min of sealing or store sealed plate up to 4 h at 4°C

3. Droplet PCR

- Set volume at 40 µl, lid heated 105°C

- Cycling:

Enzyme activation	95°C	5’		Add ramp rate 2°C/sec to all steps
Denaturation	95°C	30’’	40x	
Annealing		1’		
Signal stabilization	4°C	5’		
	90°C	5’		
Final hold	10°C	∞		

- Plates can be left over night at 10°C or stored at 4°C for max. 3-4 d until plate reading good results in reader, when left ~30 min at 10°C after PCR has finished even better if you read the droplets the day after
- Amplicon length >400 nt, do 3-step PCR (95°C, 30’’; annealing, 30’’, 72°C, 30’’)

4. Plate reading

- Login
- Follow instructions in software
- After exchange of oil or seldom usage (= only once in 2 weeks) – do “prime” to fill system with oil
- Reading can only be started, when enough reader oil is present and waste is empty (reader starts to blink as soon as oil < 30%, but it works for quite some time from then on)
- Exchange of waste/oil: prepare 50 ml 10% bleach for waste bottle (Sodium hypochlorite; located in base cupboard in FISH lab; prepare always fresh, because it deteriorates over time and when exposed to light or heat; use 41.7 ml of 12% bleach + 8.3 ml MQ)

This protocol was written by Astrid Horn using Bio Rads Droplet Digigtal PCR Aplication Guide, Bio Rads QX200 Droplet Reader and QuantaSoft Software instruction manual and according to Hindson et al. 2011.[108]

10.1.4 Protocol S3: DNeasy® PowerWater® Sterivex™

1. Filter water sample through a Sterivex filter unit. Remove as much of the remaining liquid as possible using a syringe containing air. Cap both ends with the inlet and outlet caps.
2. Remove the inlet cap and add 0.9 ml of Solution ST1B using a pipette tip. Insert pipette completely into the inlet so that pipette tip is visible inside the unit just above the membrane.
3. Re-cap the inlet and secure the Sterivex filter unit horizontally, with the inlet facing out, to a vortex adapter.
4. Vortex at minimum speed for 5 min.
5. While still attached to the vortex adapter, rotate the Sterivex filter unit 180 degrees from the original position. Vortex at minimum speed for an additional 5 min.
6. Set the Sterivex filter unit with the inlet facing up and remove the inlet cap. Add 0.9 ml of Solution MBL using a pipette tip. Insert pipette completely into the inlet so that the pipette tip is visible inside the unit just above the membrane. Re-cap the inlet.
7. Incubate the Sterivex filter unit at 90ºC for 5 min. Ensure heat is evenly distributed. Note: Do not heat at higher temperatures or for longer than 5 min.
8. Cool the unit at room temperature for 2 min. Ensure that the caps are on tightly. 10 DNeasy PowerWater Sterivex Kit Handbook 05/2019
9. Secure the Sterivex filter unit horizontally, with the inlet facing out, to a vortex adapter.
10. Vortex at maximum speed for 5 min. Set the Sterivex filter unit with the inlet facing up and remove the inlet cap.
11. Pull back the plunger of a 3 ml syringe to fill the barrel with 1 ml of air, and then attach it to the inlet of Sterivex filter unit. Push air into the unit until there is resistance, and then release the plunger. Continue to pull back on the plunger to remove as much of the lysate as possible. Detach the syringe from the Sterivex filter unit.
12. Add the lysates to 5 ml glass PowerBead Tubes. Secure the PowerBead Tubes horizontally to a vortex adapter.
13. Vortex at maximum speed for 5 min. Centrifuge at 4000 x g for 1 min.
14. Transfer all the supernatant to a clean 2.2 ml collection tube.
15. Add 300 µl of Solution IRS and vortex briefly to mix. Incubate at 2–8°C for 5 min.
16. Centrifuge the tube at 13,000 x g for 1 min. Avoiding the pellet, transfer the supernatant to a clean 5 ml collection tube.

17. Place a tube extender firmly into an MB Spin Column.
18. Attach the tube extender/MB Spin Column unit to a VacConnector and VacValve on the QIAvac 24 Plus Manifold.
19. Add 3 ml of Solution MR to the Collection Tube containing supernatant. Vortex to mix.
20. Load the entire 4.5 ml of supernatant into the tube extender/MB Spin Column.
21. Turn on the vacuum source and open the VacValve of the port. Allow the lysate to pass through. After the lysate has passed through completely, close the VacValve of that port.
22. While keeping the MB Spin Column attached to the VacValve, remove the tube extender and discard.
23. Add 0.8 ml of ethanol to the MB Spin Column. Open the VacValve. Allow the ethanol to pass through the column completely. Close the VacValve.
24. Add 0.8 ml of Solution PW to the MB Spin Column. Open the VacValve and allow Solution PW to pass through the column completely. Continue to pull a vacuum for another minute to dry the membrane. Close the VacValve. DNeasy PowerWater Sterivex Kit Handbook 05/2019 11
25. Add 0.8 ml of ethanol to the MB Spin Column. Open the VacValve and apply a vacuum until the ethanol has passed through the MB Spin Column completely. Continue to pull a vacuum for another minute to dry the membrane. Close the VacValve.
26. Turn off the vacuum source and open an unused port to vent the manifold. If all 20 ports are in use, break the vacuum at the source.
27. Remove the MB Spin Column and place in a 2.2 ml collection tube. Centrifuge the tube at 13,000 x g for 2 min to completely dry the membrane.
28. Transfer the MB Spin Column to a new 2.2 ml collection tube and add 100 µl of Solution EB or sterile DNA-free PCR-grade water to the center of the white filter membrane.
29. Centrifuge at 13,000 x g for 1 min at room temperature. Discard the MB Spin Column. The DNA is now ready for any downstream application.

10.1.5 Protocol S4: Quant-iT 1X dsDNA BR Assay

For best results, ensure that all materials and reagents are at room temperature.

1.1 Set up the required number of 0.5-mL tubes for standards and samples. The Quant-iT™ 1X dsDNA BR Assay requires 2 standards. Note: Use only thin-wall, clear, 0.5-mL PCR tubes. Acceptable tubes include Qubit™ Assay Tubes (Cat. No. Q32856).

1.2 Label the tube lids. Note: Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit™ Fluorometer requires the standards to be inserted into the instrument in the correct order.

1.3 Add 10 µL of the 0 ng/µL and the 100 ng/µL Quant-iT™ 1X dsDNA BR Standard to the appropriate tube

1.4 Add 1–20 µL of each user sample to the appropriate tube.

1.5 Add the Quant-iT™ 1X dsDNA BR Working Solution to each tube such that the final volume is 200 µL. Note: The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Quant-iT™ working solution, and each sample tube requires anywhere from 180–199 µL.

1.6 Mix each sample vigorously by vortexing for 3–5 seconds.

1.7 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to read the standards and samples. Follow the procedure appropriate for your instrument.

10.1.6 Protocol S5: Quant-iT™ 1X dsDNA HS Assay

For best results, ensure that all materials and reagents are at room temperature.

1.1 Set up the required number of 0.5-mL tubes for standards and samples. The Quant-iT™ 1X dsDNA HS Assay requires 2 standards. Note: Use only thin-wall, clear, 0.5-mL PCR tubes. Acceptable tubes include Qubit™ assay tubes (Cat. No. Q32856).

1.2 Label the tube lids. Note: Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit™ Fluorometer requires the standards to be inserted into the instrument in the right order.

1.3 Add 10 µL of the 0 ng/µL and the 10 ng/µL Quant-iT™ 1X dsDNA HS Standard to the appropriate tube
1.4 Add 1–20 µL of each user sample to the appropriate tube.

1.5 Add the Quant-iT™ 1X dsDNA HS Working Solution to each tube such that the final volume is 200 µL. Note: The final volume in each tube must be 200 µL. Each standard tube requires 190 µL of Quant-iT™ working solution, and each sample tube requires anywhere from 180–199 µL.

1.6 Mix each sample vigorously by vortexing for 3–5 seconds.

1.7 Allow all tubes to incubate at room temperature for 2 minutes, then proceed to read the standards and samples. Follow the procedure appropriate for your instrument.

10.1.7 Protocol S6: DNA quantification with Quant-iT™PicoGreen® dsDNA reagents kit (Thermo Fisher Scientific)

This protocol is based on the manufacturers manual. Standards and samples were measured in triplicates taking the mean values for concentration calculation. The assays were prepared in black 96-well-microplates (Greiner Bio-One, Kremsmünster, AUT), measurements were carried out with an Infinite M200 microplate reader (Tecan Group AG, Männerdorf CH) .

1. Prepare 1 x TE buffer by diluting 20 x TE buffer 1:20.
2. Dilute the Standard solution (λ DNA 100 ng/µl) to a concentration of 2 ng/ µl
3. Prepare PicoGreen® working solution: 9950 µl 1 x TE + 50 µl PicoGreen®
4. Prepare the following Standard mixture in the first 3 columns of the plate:

	Std. (2 ng)	**1 x TE buffer**	**Final amount (ng)**
A	50 µl	50 µl	100
B	37,5 µl	62,5 µl	75
C	25 µl	75 µl	50
D	12,5 µl	87,5 µl	25
E	5 µl	95 µl	10

F	2,5 µl	97,5 µl	5
G	1,2 µl	98,8 µl	2,5
H	0 µl	100 µl	Blank

5. Pipette 1 µl of each sample and 99 µl of 1 x TE buffer in triplicate in remaining wells.

6. Pipette 100 µl of PicoGreen® work solution in each well.

7. Protect plate from light and incubate for 2 – 3 min at room temperature.

8. Read plate according to the following conditions:

Excitation	~ 480 nm
Emission	~ 520 nm
Integration time	40 s
Lag time	0 s
Gain	Optimal
Number of flashes	10
Claculated well	Highest standard
Shaking	5 s

9. Subtract the fluorescence value of the reagent blank from that of each of the samples. Use corrected data to generate a standard curve of fluorescence versus DNA concentration.

10. Insert the mean of the sample fluorescent values into the function of the standard curve. The resulting value is the DNA concentration in ng/µl

10.1.8 Protocol S7: Power Soil® DNA Isolation kit protocol (MO BIO Laboratories Inc.)

1. To the PowerBead Tubes provided, add 0.25 grams of soil sample.

2. Gently vortex to mix
3. Check Solutions C1. If Solution C1 is precipitated, heat solution to 60°C until dissolved before use
4. Add 60 µl of Solution C1 and invert several times or vortex briefly
5. Secure PowerBead Tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex (MO BIO Catalog# 13000-V1-24) or secure tubes horizontally on a flat bed vortex pad with tape. Vortex at maximum speed for 10 minutes. Note: If you are using the 24 place Vortex Adapter for more than 12 preps, increase the vortex time by 5-10 minutes.
6. Make sure the PowerBead Tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 10,000 x g for 30 seconds at room temperature. CAUTION: Be sure not to exceed 10,000 x g or tubes may break.
7. Transfer the supernatant to a clean 2 ml Collection Tube (provided). Note: Expect between 400 to 500 µl of supernatant. Supernatant may still contain some soil particles.
8. Add 250 µl of Solution C2 and vortex for 5 seconds. Incubate at 4°C for 5 minutes.
9. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
10. Avoiding the pellet, transfer up to, but no more than, 600 µl of supernatant to a clean 2 ml Collection Tube (provided).
11. Add 200 µl of Solution C3 and vortex briefly. Incubate at 4°C for 5 minutes.
12. Centrifuge the tubes at room temperature for 1 minute at 10,000 x g.
13. Avoiding the pellet, transfer up to, but no more than 750 µl of supernatant into a clean 2 ml Collection Tube (provided)
14. Shake to mix Solution C4 before use. Add 1200 µl of Solution C4 to the supernatant and vortex for 5 seconds
15. Load approximately 675 µl onto a Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow through and add an additional 675 µl of supernatant to the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature. Load the remaining supernatant onto the Spin Filter and centrifuge at 10,000 x g for 1 minute at room temperature.
16. Add 500 µ of Solution C5 and centrifuge at room temperature for 30 seconds at 10,000 x g
17. Discard the flow through
18. Centrifuge again at room temperature for 1 minute at 10,000 x g.
19. Carefully place spin filter in a clean 2 ml Collection Tube (provided). Avoid splashing any Solution C5 onto the Spin Filter.

20. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, sterile DNA-Free PCR Grade Water may be used for elution from the silica Spin Filter membrane at this step.
21. Centrifuge at room temperature for 30 seconds at 10,000 x g.
22. Discard the Spin Filter. The DNA in the tube is now ready for any downstream application. No further steps are required.

10.1.9 Protocol S8: ZR-96 DNA Clean-up kit protocol (ZYMO Research Corp.)

1. In a 1,5 ml microcentrifuge tube, add 2 volumes of DNA Binding Buffer to each volume of DNA sample. (e.g., 300 µl binding buffer to 150 µl sample). Mix briefly by vortexing.

2. Transfer sample mixtures to the wells of a Silicon-A™ Plate mounted onto a Collection Plate.

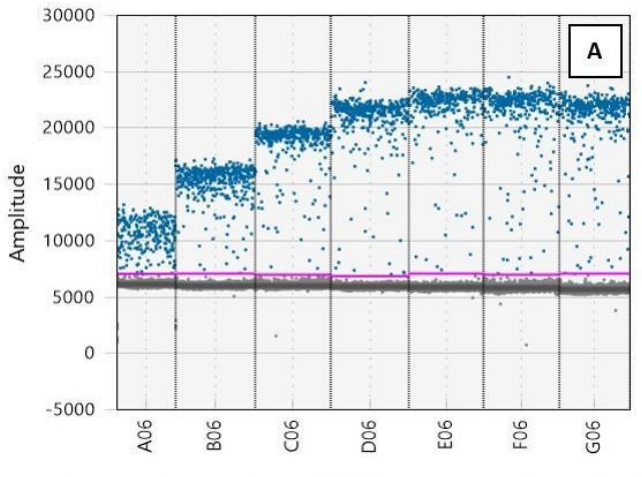
3. Centrifuge at ≥ 3,000 x g (5,000 x g max.) for 5 minutes until sample mixtures have been completely filtered. Discard the flow-through.

4. Add 300 µl Wash Buffer to each well of the Silicon-A™ Plate. Centrifuge at ≥ 3,000 x g for 5 minutes. Repeat wash step.

5. Add 30 – 40 µl water directly to the column matrix in each well. Transfer the Silicon-A™ Plate onto an Elution Plate and centrifuge at ≥ 3,000 x g for 3 minutes to elute DNA. Ultra-pure DNA in water is now ready for use.

10.2 Supplementary Figures

10.2.1 Figure 1: ddPCR temperature gradient for best annealing temperature



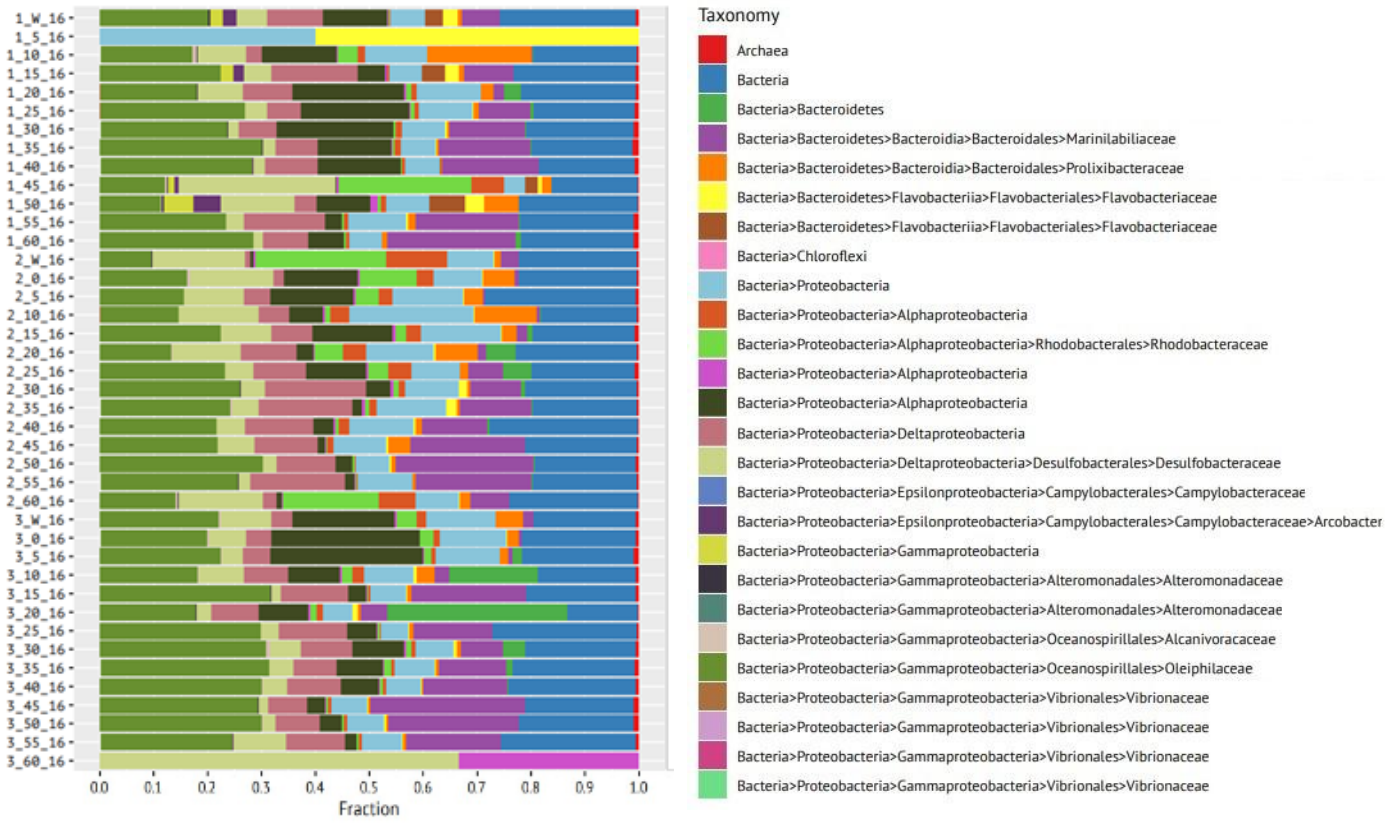
B

Well Data

Well	Sample	Target	Conc(copies/µL)	Status
A06	control	SOXB	22,5	Manual
B06	control	SOXB	25,9	Manual
C06	control	SOXB	26,4	Manual
D06	control	SOXB	26,1	Manual
E06	control	SOXB	22	Manual
F06	control	SOXB	21,6	Manual
G06	control	SOXB	25,2	Manual

Suppl. Fig. 1: (A) Amplitudes from ddPCR trial representing the soxB copy number per µl generated with different annealing temperatures. A06=45°C, B06=50°C, C06=55°C, D06= 60°C, E06=65°C, F06=70°C, G06=75°C; (B) data table with the number of soxB copies per µl in respect to different annealing temperatures. Threshold lines were set manually for each sample.

10.2.2 Figure 2: Community composition from pore water filters in 2016.

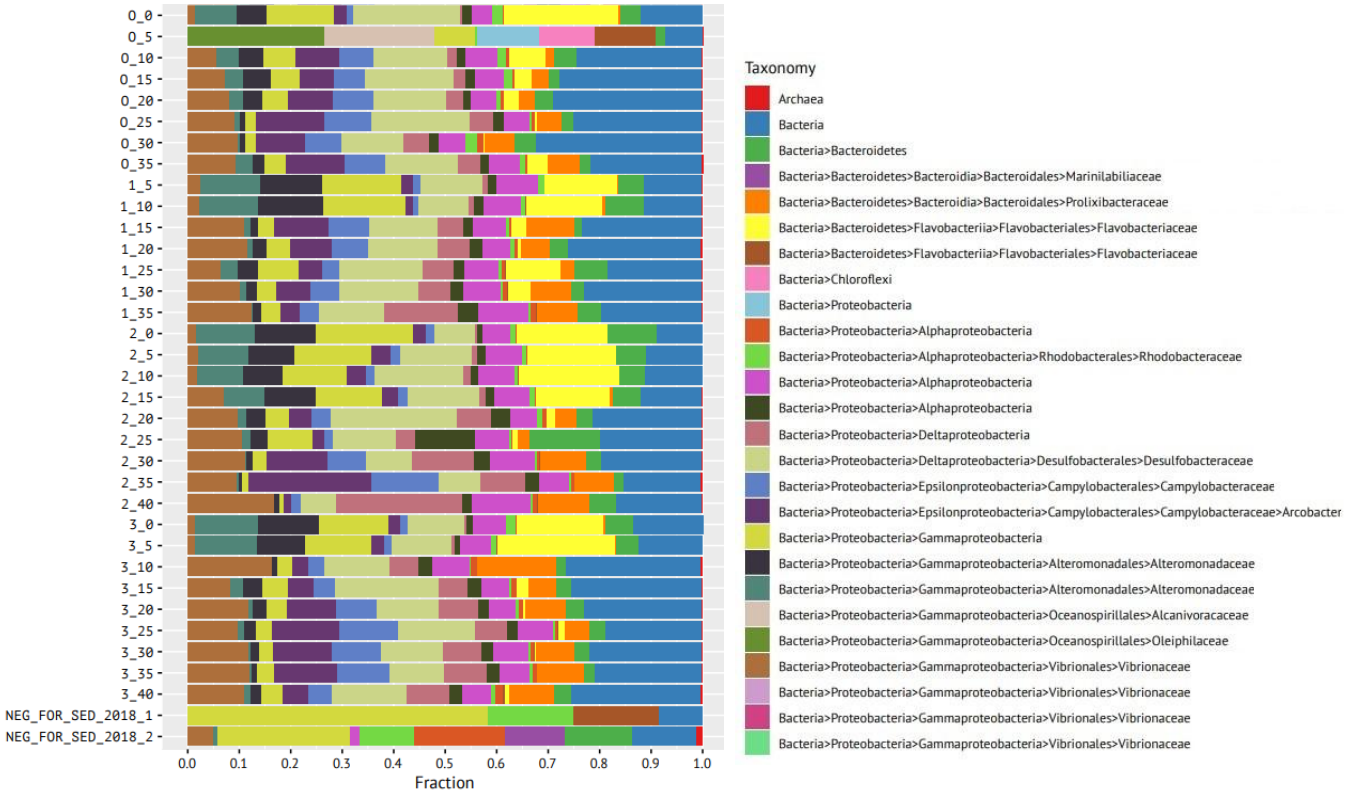


Suppl. Fig. 2: Fractioning of the bacterial community for pore water samples from the Bay of Fetovaia, Elba, 2016 obtained from 16S Amplicon Sequencing. Depths ranging from 0 - 60cm. The first numbers of the sample are referring to the sampling spot. The second number represents the sampling depth and the last number represents the year.

10.2.2 Figure 3: Community composition from pore water filters in 2018.

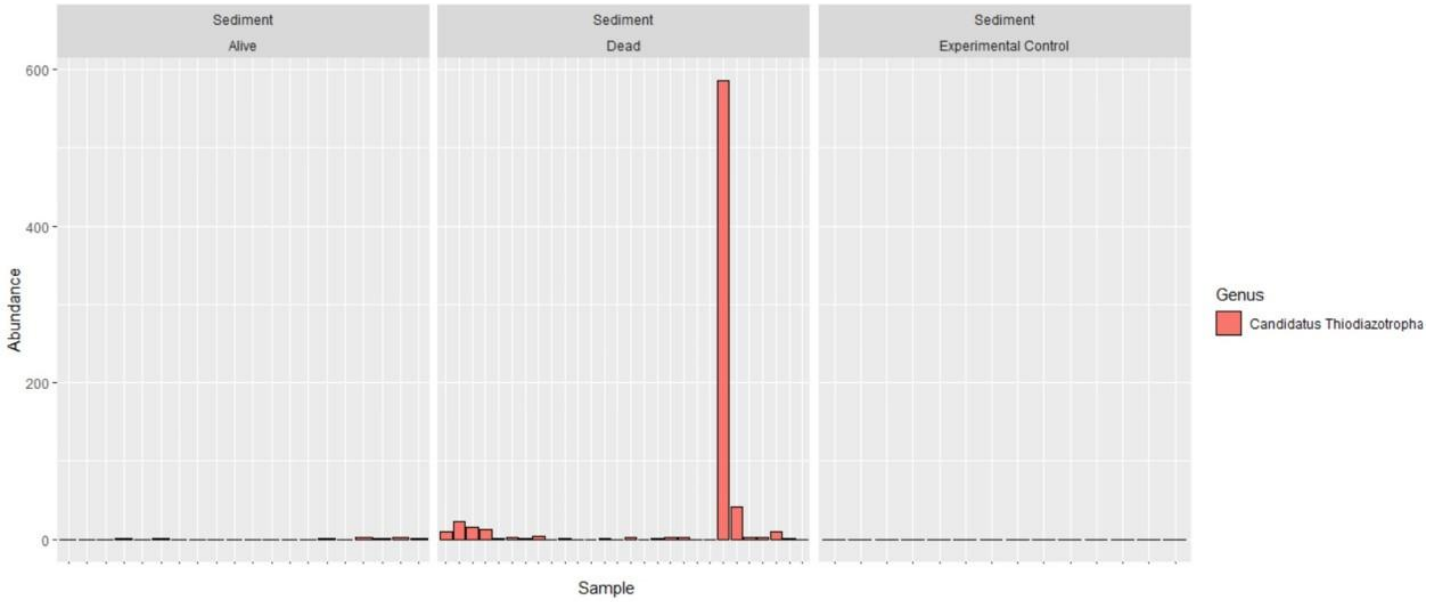
Suppl. Fig. 3 Community compositions based on 16S Amplicon from Pore Water in the Bay of Fetovaia,Elba, 2018. Depth layers ranging from 0cm - 45cm. The first numbers of the sample are referring to the sampling spot. The second number represents the sampling depth and the last number represents the year. The color codes showing the fraction of the phylogenetic taxa listed on the right side.

10.2.2 Figure 4: Sediment community composition from sediment cores in 2018



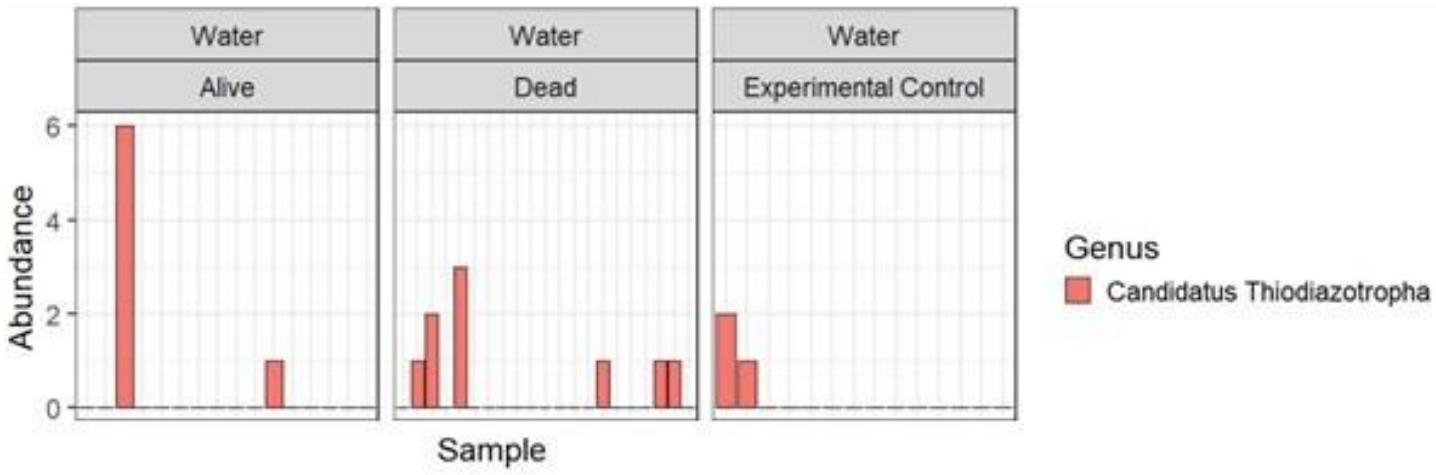
Suppl. Fig. 4: Sediment Community Composition from the Bay of Fetovaia, Elba, 2018 from depth layers from 0cm - 40cm obtained from 16S Amplicon Sequencing. The first numbers of the sample are referring to the sampling spot. The second number represents the sampling depth and the last number represents the year. The color codes showing the fraction of the phylogenetic taxa listed on the right side.

10.2.2 Figure 5: Samples with the highest total abundance values from the Symbiont Release Experiment



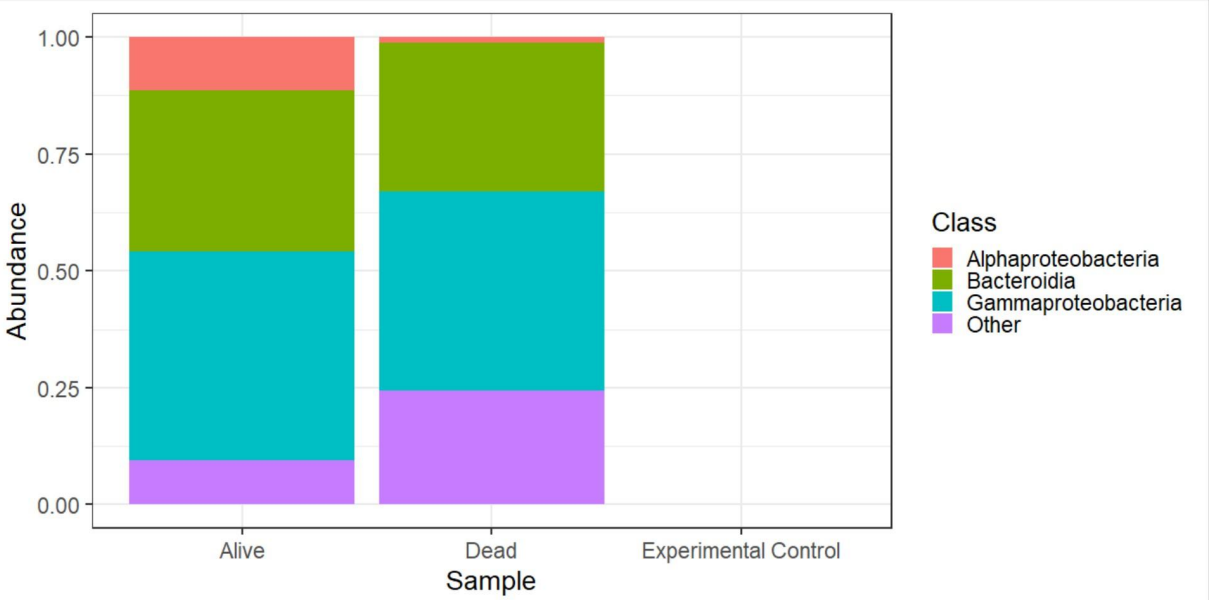
Suppl. Fig. 5: Total counts of the copies from target Ca. Thiodiazotropha species in the sediment treatment with dead and live clams and the experimental control from the Symbiont Release experiment obtained from 16S Amplicon sequencing.

10.2.4 Figure 6: Total counts of *Ca*. Thiodiazotropha in the three water treatment groups in the Symbiont Release Experiment



Suppl. Fig. 6: Comparison of the soxB copy number of target Ca. Thiodiazotropha species from the water treatments from the symbiont release experiment obtained from 16S Amplicon Sequencing.

10.2.3 Figure 7: Top 3 Classes regarding to the dead and live treatments from the Symbiont Release Experiment



Suppl. Fig. 7: Comparison of the standardized Abundance of the top 3 Classes from the dead and live treatments from the symbiont release experiment obtained from 16S Amplicon Sequencing.