CONTROLS ON NITROGEN FIXATION AND NITROGEN RELEASE IN A DIAZOTROPHIC ENDOSYMBIONT OF SHIPWORMS

A Dissertation
Presented to
The Academic Faculty

by

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In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy in the
School of Biology

Georgia Institute of Technology
December 2010

CONTROLS ON NITROGEN FIXATION AND NITROGEN RELEASE IN A DIAZOTROPHIC ENDOSYMBIONT OF SHIPWORMS

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To Steven Kudravi

Never before had I seen a professor so passionate for improving undergraduate student learning and training his teaching assistants. Before he left this world prematurely, he inspired me to pursue a career in university teaching.

ACKNOWLEDGEMENTS

I would like to first thank my advisor, **Joe Montoya**. This dissertation and its subsequent publications are major outputs from his co-authored NSF-sponsored grant about the enigmatic shipworm symbiosis. Besides lab techniques, data analysis, and the like, he has taught me patience in the research process, critical academic survival skills for a large research university, and how to be a hard-working ocean researcher. I am grateful for the opportunity to learn from a scientist who is so well-respected by his peers.

My dissertation committee has challenged me through my years in the School of Biology to become a better scientist, and for that I am grateful. I could not think of a better group of "science role models" than what I have assembled for my committee. **Tom DiChristina** provided very helpful insights into microbial physiology, provided feedback on experimental design, and allowed me to use his pricey Zeiss epifluorescence microscope. **Julia Kubanek** never failed to ask me thought-provoking research questions and provided helpful feedback to prepare for presentations. I just met **Brian Hammer** this year, and he had an immediate impact on my project design. I am thrilled that **Mandy Joye** took the time to be an active member of the committee. Thank you for inviting me to your UGA lab and discussing microbial biogeochemical cycles with me. I would also like to extend my appreciation to **Dan Distel**, who was an early committee member and helped guide my early study of the shipworm symbiosis.

If my committee were the coaching staff, I had a small but very important group of people that I call "Team Rachel" cheerleaders, support staff, and therapists. I just met **Tim Petrie** (Dr. F) two years ago, but without his help and support, I'm not sure that this product would have been completed. Without hesitation, he assisted to my needs when I

forgot about them (bringing me food or taking me for some exercise), gave me reality checks, or a kick in the pants, sometimes simultaneously. I do not know of a better science peer to show me the light at the end of the Ph.D. tunnel. I thank him for helping me through the roller coaster of the dissertation years and providing a wonderful environment in which to complete this final product. On a similar note, anyone who does not believe in animal therapy should watch us play with our 3 ferrets (Tomasa, Mr. Stinky, Pancho Leon) after a long day at work – I cannot overestimate the power of these furballs to relieve my graduate school stress.

My immediate family and best friend, Brenda (Dr. B), have been a huge source of almost daily support, strength, and encouragement. Even when I didn't believe in myself, they did, and never failed to be there in any capacity. My **mom** and **dad** went to great lengths to be sure that I remained as happy as possible during this challenging degree. They realized the importance of my education to me and always encouraged me to work to achieve my dreams. Upon hearing of my difficulties with the oral exams, **my late grandpa** told me to brush it off, persevere, and stay in school, which is what I did. I was very fortunate to have the most awesome girlfriend ever in Atlanta with me, **Brenda**. It was quite a neat consequence that we could continue our friendship from Davidson College while pursuing science careers together. She knows the meaning of the phrase "there's nothing that a flat iron or a glass of wine can't fix" and looks out for me like no other girlfriend.

There are many other members of the School of Biology who influenced and improved this work along the way. Some of them may not realize how important their actions and discussions were to me, so they deserve recognition. I thank the School of

hands-on laboratory research during this project were (from present time): **Sarah Weber, Ludmila Tsai, Bryce Taylor, Munroop Dhada, Maria-Rosa Padilla, Pammie Ku, Adrian Martinez, Viniya Patidar, Emily McClendon, Tiffany Andras,** and **Madison Hall**.

A big thank you to the **graduate students in the Montoya lab**. You have listened to me talk at length about N_2-fixation in shipworm symbionts in our weekly lab meetings. I will never forget how **Jason Landrum** donated almost an entire afternoon (over scotch) to help me prepare for a critical committee meeting one Friday in 2009. I thank the Montoya lab technicians, Poneh Davoodi, Samantha Allen, and Beth Van Gessel, for running samples on the mass spec for me, and for being excellent conversationalists!

Some of our lab "neighbors" from around the corner provided direct invaluable contributions. **Justin Burns** trained me to perform DAPI-staining and cell counts on the Zeiss epifluorescence microscope. **Christine Fennessey** served as the "bacteria-sitter" while I was away at sea this year.

There are several faculty at CETL who have helped me along the way. I was pleased to work as a GTA for **Lydia Soleil** and **Donna Llewellyn** for a year; thank you for providing funding and training me in outside-the-lab academic skills, such as teaching. One of the most useful courses I took during my graduate career was "Academic Writing" with **Karen Head**. Karen helped make the writing process less strenuous and more fun, and her course positively transformed my approach towards this written dissertation.

In sum, I could not have completed this long project and graduate career alone. It truly took a village to raise this scientist.

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LIST OF SYMBOLS AND ABBREVIATIONS

AMP	Adenosine monophosphate
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
atm	Atmospheres
ATP	Adenosine triphosphate
DAPI	4', 6-diamidino-2-phenylindole
DIN	Dissolved inorganic nitrogen
DIP	Dissolved inorganic phosphorus
DON	Dissolved organic nitrogen
EPS	Exopolymeric substances
GF/F	Glass fiber filter, size F, 0.7 µM pore
LB	Luria Bertani medium
PN	Particulate nitrogen
PP	Particulate phosphorus
SBM	Shipworm basal medium
SD	Standard deviation
SE	Standard error
VO_2	Respiration rate

SUMMARY

Nitrogen fixation is an ecologically important microbial process that can contribute bioavailable combined N to habitats low in N. Shipworms, or wood-boring bivalves, host N_2-fixing and cellulolytic symbiotic bacteria in gill bacteriocytes, which have been implicated as a necessary adaptation to an N-poor C-rich (wooden) diet. Shipworm symbionts are known to fix N within the gill habitat and newly fixed N is subsequently incorporated into non-symbiont containing host tissue. The presence of N_2-fixation in gill bacteriocytes presents a conundrum because N_2-fixation is tightly regulated by oxygen in most other diazotrophic microbes. Also, the direct evidence of new N being incorporated into the host tissue indicates that there are potentially complex nutrient cycles in this symbiosis, which have not been investigated. We used the cultivated symbiont *Teredinibacter turnerae*, which has been isolated from many shipworm species, as a model organism to elucidate controls on N_2-fixation and N release in the shipworm symbiosis. Our results indicate that headspace oxygen concentration does not control biomass specific N_2-fixation and respiration activity in *T. turnerae*, but it does influence the magnitude of the growth rate and timing of culture growth. Also, we examined the controls of oxygen on inorganic nutrient uptake rates, and documented a small amount of dissolved inorganic nitrogen release. While the N budget is only partially balanced, we provide indirect evidence for the allocation of fixed N to the excretion of exopolymeric substances and dissolved organic nitrogen; future studies that measure these additional N sinks are necessary to close the N budget. Although there are limitations of using pure cultures to investigate a complex symbiotic system, this study

provides direct experimental evidence that *T. turnerae* has adaptations that are conducive to N_2-fixation in gill bacteriocytes.

CHAPTER 1

INTRODUCTION

1.1 N_2-fixation is an important source of N to the global marine nitrogen cycle

Nitrogen (N) is an important component in organic matter, and is found in the largest abundance in cellular protein. The low availability of combined N (NH_4^+, NO_3^-, amino acids) can limit primary production in oceanic ecosystems (Vitousek and Howarth, 1991; Falkowski et al., 1998). Microbial N_2-fixation, the microbially mediated conversion of atmospheric N_2 into bioavailable N (e.g. NH_4^+), by diazotrophs can help to overcome N limitation and support primary production in the low-nutrient (oligotrophic) ocean (Karl et al., 1997; Karl et al., 2002; Montoya et al., 2004; Capone et al., 2005; Moisander et al., 2010).

Only within the past 20 years have oceanographers begun to realize the tremendous importance of N_2-fixation as a source of N to the marine N cycle. Karl et al. (1997) examined rates of N_2-fixation, nutrient concentrations, and constructed an N:P mass-balance model and showed that N_2-fixation by cyanobacteria supported at least 50% of the primary production in the subtropical North Pacific Ocean gyre. Using stable isotope abundances (^{15}N) of suspended particulates and zooplankton, Montoya et al. (2002) determined that N_2-fixation makes a significant contribution to the N budget of the oligotrophic North Atlantic. A similar situation also occurs in the oligotrophic areas of the Gulf of Mexico. Using biogeochemical measurements, including stable isotopes, and rates of $^{15}N_2$-fixation, Holl et al. (2007) suggested that at least 60% of the macrozooplankton C and N is derived from the diazotrophic (N_2-fixing) cyanobacterium

Trichodesmium. Studies that involve the contribution of N_2-fixation to marine biogeochemical cycles are ongoing across ocean basins, and continue to produce exciting new results.

Recent research on the global marine N cycle indicates that the N budget is not currently in balance. Although absolute numbers vary somewhat, researchers generally agree that the total N outputs outweigh the N inputs, and some estimates indicate that N outputs may be double N inputs (Capone, 2008). The imbalance may be driven by an overestimation of denitrification and anammox (anaerobic ammonium oxidation) as a sink of combined N, an underestimation of N_2-fixation as a source of N, and/or some combination of the two. In addition, an incomplete understanding of the controls upon denitrification, anammox, and N_2-fixation contributes to the large variation in N budget estimates among researchers and to the uncertainty on whether the present-day marine N budget reflects human perturbation or is not in steady state.

A great deal of the current effort to balance the marine N budget involves finding and quantifying novel sources of N_2-fixation in the oceans. Many new diazotrophic organisms are now recognized as important contributors to the marine N cycle. Unicellular cyanobacteria were first recognized as important N_2-fixers in the subtropical North Pacific Ocean by Zehr et al. (2001), and later recognized to fix N at rates equal to or higher than larger diazotrophs, such as the filamentous cyanobacterium *Trichodesmium*, thus contributing significantly to the marine N cycle (Montoya et al., 2004). Recent research indicates that these unicellular N_2-fixing cyanobacteria are more broadly distributed across the South Pacific Ocean than larger diazotrophs, and thus may be major contributors to the N cycle across oceanic basins (Moisander et al., 2010).

Diatom-diazotroph associations (DDAs) are also garnering much interest among

oceanographers as major contributors to the marine N cycle. The controls on their

distribution are poorly understood, so the estimates of the DDA contribution to the

marine N cycle are less constrained than for other diazotrophs. Recently published and

ongoing field studies indicate that they are important N_2-fixers in the Amazon River

plume (Subramaniam et al., 2008). A final source of new N to the marine N cycle that has

been previously recognized, but not well characterized, quantified, nor understood, is N_2-

fixation in wood-boring bivalves (shipworms).

1.2   Shipworms

1.2.1 Biology

Wood-boring bivalves, commonly referred to as shipworms or teredos, are

common inhabitants of marine drift wood in both coastal areas and in the deep-sea. The

name shipworm originated from their ability to cause major destruction to marine

wooden structures, such as ships or dams, by forming extensive porous burrows in solid

wood (Greene, 1994; Steinmayer Jr and MacIntosh Turfa, 1996). Shipworms provide a

critical link in the C cycle by converting woody material into organic matter more easily

utilized by heterotrophic organisms, especially in the deep-sea (Turner, 1973).

Many shipworms release large numbers of planktotrophic larvae, and can disperse

over long ranges to new wood sources (Turner, 1966; Distel, 2003). Metamorphosis

occurs when larvae settle on a wood source, and shipworm burrowing activity

commences in the adult stage. Individuals will inhabit a single piece of wood for their

lifetime, and they are attached to the calcareous-lined bore hole by a ligament near the

burrow opening (Turner, 1966; Distel, 2003). Shipworms have several unique

morphological adaptations that make shipworms identifiable as bivalves only to trained specialists. For example, they use modified shells as "raspers" to bore into wood (Turner, 1966).

1.2.2 Nutrition

Shipworm nutrition has been studied by biologists since the early 1700s, but still not fully understood (Turner, 1988). A seminal paper by Gallager et al. (1981) was the first to suggest that shipworms can survive and reproduce on a wood-only diet. Morton (1983) provided additional evidence for wood digestion by shipworms. He found that particles in shipworm feces had a lower cellulose content than the pre-chewed wood and that caecal wood fragments have a higher reducing sugar load than pre-chewed wood (Morton, 1983). Major complications of xylotrophy (wood-eating) metabolism include the ability to chew woody tissue, the ability to break down woody tissue for digestion, and the extremely low N content of woody tissue.

Using wood as a food source requires both enzymes to break down the woody substrate and a mechanism to cope with its low N content. Woody tissue has two major structural polymers, lignin and cellulose, and is commonly referred to as lignocellulose. Cellulose (($C_6H_{10}O_5)_n$) is the largest constituent of lignocellulose, and lignocellulose is largely nonnitrogenous (Breznak and Brune, 1994). Gallager et al. (1981) identified a large N imbalance between food source and adult tissue in *Lyrodus pedicellatus*. Based on energy budgeting, Gallager et al. (1981) suggested that wood could contribute only 1.83% of the total N required for adult shipworm growth and reproduction, and thus must rely on other sources of N.

Table 1.1 Nitrogen nutrition imbalance between food source and shipworm tissue

	%N	C:N
Wood hosting shipworms	0.2 ± 0.01	229.0
Shipworm tissue	5.8 ± 1.12	7.0

With the possible exception of phylogenetically "higher" termites (Ohkuma, 2001), animals are not capable of synthesizing enzymes (class of cellulases) capable of breaking down cellulose. In shipworms, the most widely accepted, hence the most-studied, mechanism, of coping with cellulose digestion and a lack of N in the diet involves bacterial endosymbionts residing within shipworm gills.

1.2.3 Symbiosis

The first experimental evidence that demonstrated how shipworms cope with the low N wooden diet came from a study by Carpenter and Culliney (1975). The authors found drift wood in the Sargasso Sea filled with shipworms. Puzzled by the lack of combined N in the water column, Ed Carpenter formed the hypothesis that shipworms fix N to cope with N stress in such an environment (pers. comm.). The authors conducted an acetylene reduction assay, a widely used method to measure N_2-fixation rates, with whole shipworms which were collected in the field and found measureable rates of N_2-fixation (Carpenter and Culliney, 1975).

The first report of symbiotic bacteria residing within shipworms was by Popham and Dickson (1973). Further studies indicated that bacterial symbionts reside within specialized host cells in the gills, called bacteriocytes (Distel et al., 1991), and are hence intracellular. Shipworm symbionts are not in direct contact with the host cytoplasm, but are contained within membrane-bound symbiont-containing vesicles (SCVs; Distel et al., 1991), which are typically 10-20 μm in diameter and contain several bacteria (Distel and

Roberts, 1997). There is no conclusive evidence of a connection between the shipworm SCVs and external environment (Distel and Roberts, 1997). Such a symbiont-host barrier has been suggested to limit O_2 diffusion symbiont cells in chemoautotrophic bivalve symbioses (Wittenberg, 1985). Histological cross-sections of symbiont-containing tissue show some resemblance to the sulfide-oxidizing and methanogenic symbioses of deep-sea hydrothermal vent and cold seep bivalves (for reviews, see Van Dover, 2000; Cavanaugh et al., 2004).

Shipworm symbionts provide supplemental N to the host shipworm and secrete cellulase enzymes, which allow degradation of woody tissue. Trytek and Allen (1980) were the first to suggest that isolated symbiont-containing gill tissue could synthesize essential amino acids, which are virtually unavailable on a solely wooden diet, and provided the first evidence for the presence of cellulases (Trytek and Allen, 1980). Subsequent experimental evidence for shipworm cellulolysis and N_2-fixation was obtained using transmission electron microscropy to identify bacterial symbionts within shipworms (in what they identified as the Gland of Deshayes but was later redefined as gill tissue; Waterbury et al., 1983). Cultivated symbionts, which showed both cellulolytic and N_2-fixing capabilities in pure culture (Waterbury et al., 1983), were later described as a new species within their own genera, *Teredinibacter turnerae* (discussed in detail below; Distel et al., 2002b).

Recent studies provided direct evidence for N_2-fixation by symbionts and subsequent N translocation to the host. Using multi-ion mass spectrometry (MIMS)[1] and $^{15}N_2$-fixation tracer techniques, Lechene et al. (2007) directly implicated symbionts with fixing N, thus providing a source of new N to the non-symbiont containing host tissue for biosynthesis. The authors localized ^{15}N incorporation to bacteriocytes within the gills, and provided evidence for the transport of molecules containing newly fixed N to the edge of the SCV (Lechene et al., 2007). ^{15}N was also traced to symbiont-free shipworm tissue, which indicates that newly fixed N directly supplements the shipworms diet (Lechene et al., 2007).

The contribution of N_2-fixation to host total N is not well quantified, but reports indicate that the contribution is relatively small. Using ^{15}N tracer techniques and growth rates, Luyten et al. (pers. comm) calculated that N_2-fixation by the symbiont consortia is capable of supplying at least 10% of the host's nitrogen requirements. Unpublished studies by Kamiker and colleagues estimated that 5-15% of host N could originate from symbiont N_2-fixation (Turner, 1988).

Polysymbiosis consortia

The shipworm symbiosis is not a monoculture of a single bacterial species. Distel et al. (2002a) used molecular methods to identify at least 3 other types of bacteria in the shipworm gill. Phylogenetic analysis indicated that the other symbiont types grouped within the clade of cultivated *T. turnerae*, but showed enough sequence divergence to form unique clades (Distel et al., 2002a). Further studies demonstrated that the

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[1] MIMS was developed by Lechene et al. (2006), and is a combination of secondary-ion mass spectrometry (SIMS), sophisticated ion optics, labeling with stable isotopes, and quantitative image-analysis software.

endosymbiont community composition varies among individuals of a single shipworm species, *Lydrodus pedicellatus* (Luyten et al., 2006). The authors were unable to conclusively determine the factors that contribute to individual variation of the symbiont population (Luyten et al., 2006). So far, the polysymbiosis consortia has only been comprehensively studied in shipworm *L. pedicellatus*.

Symbiont transmission

The apparent necessity of symbionts for shipworm growth and reproduction might suggest that shipworms require a mechanism to ensure that adult shipworms obtain a symbiont population. The debate about the mode of symbiont transmission, whether vertical (the acquisition of symbionts from parent) or horizontal (the acquisition of symbionts from the environment), is far from over, and is supported only by limited evidence on both sides. Sipe et al. (2000) interpreted the finding of genetically identical symbionts among geographically distinct and diverse shipworm species (Distel et al., 1991) as indirect evidence for horizontal transmission. Horizontal symbiont transmission requires a free-living population of symbionts, and at least one symbiont, *Teredinibacter turnerae*, has been inferred to be capable of living outside the shipworm in marine waters (Yang et al., 2009). However, efforts to identify *T. turnerae* in and around shipworm burrows and in the water column have failed (Waterbury et al., 1983; Trindade-Silva et al., 2009; Yang et al., 2009). It remains to be seen if *T. turnerae* exists in a marine free-living state.

On the other hand, a study with the shipworm *Bankia seacea* used symbiont-specific (for symbiont *Teredinibacter turnerae*) 16S rRNA-directed probes and detected bacterial rDNA in host gills, gonad tissue, and in recently spawned eggs, which the

authors used as evidence for vertical transmission of symbionts (Sipe et al., 2000). Sipe et al. (2000) did not specifically test for the presence of symbionts in juveniles, instead inferring that symbiont maintains a presence in individuals from fertilized egg to adult. Phylogenetic analysis of the symbiont population might also argue for vertical transmission of the symbionts. The fact that no free-living or symbiotic bacterium is closely related to the isolated symbiont *T. turnerae* (Distel et al., 2002b; Yang et al., 2009) may be additional indirect evidence for vertical transmission. Further phylogenetic studies indicated that the four other dominant "ribotypes" of bacteria present in the shipworm gills are related to, but distinct from *T. turnerae*, and they all form a single clade (Distel et al., 2002a). The coexistence of multiple distinct phylotypes within a single host, all of which are contained in the same clade, is less likely through a horizontal mode of transmission. Clearly, we do not have a good understanding of how shipworms obtain symbionts.

1.3 The regulation of N_2-fixation

1.3.1 Oxygen regulates N_2-fixation

The nitrogenase enzyme, responsible for the reduction of N_2 to reduced N, is found only in Bacteria and Archaea. The enzyme is comprised of two multi-subunit proteins, dinitrogenase and dinitrogenase reductase, which are encoded by the genes *nifHDK* in the *nif* operon (Dean and Jacobson, 1992). The triply-bonded N_2 molecule requires 16 ATP and 8 electrons for each molecule of N_2 reduced, which results in a high dissociation energy (940 kJ mol^{-1}). The substantial energy requirement of N_2-fixation and the sensitivity to deactivation by oxygen may be the strongest arguments for the evolution of tight regulation of N_2-fixation.

The phenomenon of O_2-sensitivity is well-studied among diazotrophs. Diazotrophs have a wide range of oxygen tolerances, and specific conditions that cause deactivation of nitrogenase is different among species. Most bacterial diazotrophs are only capable of fixing N under anaerobic or microaerophilic conditions, such as *Rhizobium* (Fischer, 1994), *Azospirillum brasilense* (Nelson and Knowles, 1978; Hartmann and Burris, 1987), and *Klebsiella pneumoniae* (Klucas, 1972; Poole and Hill, 1997). A small handful of bacteria can fix N in fully aerobic conditions (up to 100% air saturation) without negative consequences on nitrogenase activity, including *Gluconacetobacter diazotrophicus* (Emtiazi et al., 2003) and *Azotobacter vinelandii* (Kuhla et al., 1985; Marchal and Vanderleyden, 2000). In fact, N_2-fixation activity in *A. vinelandii* is quite aerotolerant; nitrogenase activity is not significantly affected by oxygen levels of 30% to 100% air saturation (Sabra et al., 2000). In bacterial batch cultures, the degree to which nitrogenase activity is affected by oxygen is probably influenced by both cultivation conditions and age of the culture (Gallon, 1992).

The sensitivity of the nitrogenase enzyme is a direct result of its structure. Dinitrogenase reductase has an exposed surface [4Fe-4S] cluster that bridges the two subunits of the enzyme; interaction with molecular oxygen readily oxidizes the [4Fe-4S] cluster, causing a conformational change that renders the enzyme inactive (Hill, 1988; Peters et al., 1995). The dinitrogenase subunit (FeMo protein) has 2 metal centers, a P cluster and a FeMo cofactor, which is used for substrate reduction; each of these subunits can be oxidized by molecular oxygen (Hill, 1988; Dixon and Kahn, 2004). Given that nitrogenase has a relatively slow turnover time and that it comprises a large percentage

(20%) of cellular protein, diazotrophs have complex genetic regulatory systems to prevent nitrogenase transcription under unfavorable conditions (Dixon and Kahn, 2004).

The genes for N_2-fixation are co-transcribed within the *nif* operon and collectively regulated in response to cellular combined N and oxygen levels in a complex regulatory cascade. Some bacteria and cyanobacteria have additional posttranslational regulation that allow short-term regulation of nitrogenase (Merrick, 2004). The specifics of the genetic controls of *nif* operon regulation differs between free-living bacteria and symbiotic bacteria (Dixon and Kahn, 2004). There are two levels of genetic regulation in most N_2-fixing bacteria: a general N control system (Ntr) and a *nif*-specific mechanism. The complex regulatory cascades for the control of N_2-fixation have been well-reviewed elsewhere (Hill, 1988; Gallon, 1992; Dixon, 1998; Dixon and Kahn, 2004). For the purposes of this review, we will briefly discuss "typical" genetic regulation of nitrogenase synthesis through the two-component regulatory system NifA/NifL. The two-component regulatory system is highly modified in the well-studied *Rhizobium* symbiosis, but still relies on NifA/NifL interaction (Dixon and Kahn, 2004).

The transcriptional activation of the *nif* operon is largely dependent upon the activity of two proteins, NifA and NifL, which are co-transcribed from an operon separate from nitrogenase. The activity of the inhibitor, NifL, determines whether the activator NifA, an enhancer binding protein, binds the *nif* operon and promotes transcription (Dixon and Kahn, 2004). The NifL protein itself is probably a redox-sensitive regulator (Hill et al., 1996). Some evidence indicates that NifL is sequestered in the membrane during "favorable" redox conditions, and is released into the cytoplasm when redox conditions are unfavorable for N_2-fixation (Dixon and Kahn, 2004). Upon

release into the cytoplasm, a protein-protein complex between NifA and NifL forms and inactivates transcription of the *nif* operon (Dixon, 1998; Dixon and Kahn, 2004).

1.3.2 Free-living N₂-fixing microbes use protection against oxygen

In many diazotrophic bacteria, the intracellular oxygen concentration can differ greatly from the extracellular oxygen concentration (Gallon, 1992). The bacteria that fix N in aerobic conditions have several mechanisms to maintain low intracellular oxygen and protect nitrogenase from inactivation. These mechanisms are best described in the aerobic bacterium *Azotobacter vinelandii,* which is capable of fixing N at very high oxygen concentrations, including at 100% saturation. In fact, *A. vinelandii* uses a wide variety of adaptations to oxygen stress adaptations and some researchers disagree on the importance of each mechanism.

Five major mechanisms of protection are known in to facilitate N₂-fixation in the presence of high oxygen in *A. vinelandii*. First, *A. vinelandii* has a terminal oxidase that consumes oxygen during respiration to maintain low intracellular oxygen concentrations ("respiration protection"; Poole and Hill, 1997). Second, *A. vinelandii* forms alginate, a type of slimy extracellular coating, that serves as an oxygen barrier to the cell surface so that *A. vinelandii* can grow in agitated broth cultures in N-free media (Sabra et al., 2000). Third, the formation of cell clusters or aggregation may increase O_2 consumption which exceeds the rate at which O_2 diffuses into the cluster, hence forming zones of anoxia or microoxia (Bergersen, 1984). Fourth, a FeSII protein can stabilize and protect the nitrogenase enzyme from inactivation and degradation in high oxygen conditions ("confirmation protection"; Moshiri et al., 1994). Last, the activity of oxygen-scavenging enzymes such as superoxide dismutase and catalase effectively bind intracellular oxygen,

reducing the levels of intracellular oxygen (Dingler and Oelze, 1987). Qurollo et al.

(2001) attempted to create an A. vinelandii superoxide dismutase mutant by disrupting

the gene encoding FeSOD (the cytoplasmic iron-containing superoxide dismutase); the

authors suggest that superoxide dismutase is essential for growth and survival in N_2-

fixing cultures. These mechanisms are probably not mutually exclusive in *A. vinelandii*,

and the dependence to which *A. vinelandii* relies on specific mechanisms is likely

influenced by environmental conditions.

1.3.3 Mechanisms of O_2 protection in N_2-fixing symbioses

The location of diazotrophic symbionts within gill bacteriocytes does not provide

an obvious known barrier against oxygen from the external environment. This is an

aspect of the symbiosis that is unique among other animal- or plant-diazotrophic

symbioses; most other diazotrophic symbioses separate the N_2-fixing microbes from the

host cells with an anoxic compartment. In the phylogenetically "lower" termite

symbiosis, a diverse community of nitrogen-fixing Bacteria and Archaea thrives within

the termite's anoxic gut (Ohkuma et al., 1996; Brune and Friedrich, 2000). Nitrogen-

fixing *Rhizobium* are held within specialized nodules of legumous plants. Here, there is a

steep oxygen gradient through a cortex diffusion barrier, and the free oxygen

concentration is less than 50 nM in the central N_2-fixation zone (Dixon and Kahn, 2004).

Other non-shipworm bivalves which host endosymbionts can maintain a very low

cytoplasmic O_2 pressure in bacteriocytes through direct consumption of O_2 by the

endosymbiont, cytoplasmic hemoglobin, and physical barriers of O_2 (Wittenberg, 1985).

A physical barrier, e.g. a host-derived ciliated epithelium lining bacteriocytes, has been

proposed as a means of excluding free O_2 from bacteriocytes in many bivalves in

chemosynthetic environments (Wittenberg, 1985). In many species of bivalves, such as

Solemya velum, O_2 is delivered to symbionts bound to cytoplasmic hemoglobin

(Wittenberg, 1985; Alyakrinskaya, 2003). With a very high affinity for O_2, cytoplasmic

hemoglobin can effectively scavenge free oxygen (Wittenberg, 1985). A single study by

Manwell (1963) identified tissue hemoglobins in *Bankia* sp. and *Teredo* sp. in the

adductor muscle, but the use of hemoglobin in shipworms to maintain low O_2

concentrations in body tissue has not been examined.

Through her many years of shipworm dissections and observations, malacologist

Ruth Turner argued that symbionts are housed in the least aerated portion of the gills and

circulatory system (Turner, 1988). Some researchers suggested that the tight packing of

symbionts into clusters might cause a steep oxygen gradient from the periphery and form

microaerobic conditions *in situ* (Turner, 1988). Still, Distel (2003) argues that the

location of the N_2-fixing symbionts suggests that they must be aerotolerant. There have

been no published studies that utilize oxygen microsensors to measure the oxygen

concentration in the locale of the shipworm symbionts. With the open-ended question of

the oxygen concentration in the shipworm symbiont niche, we sought to determine the

oxygen tolerance of N_2-fixation in symbionts.

1.4   The release of combined N from diazotrophs

Even with the recent direct evidence of newly fixed N being transported to host

shipworms (discussed above), no mechanism of N transfer from symbionts to host has

been proposed. The two most probable mechanisms of N transfer from symbiont to host

are through the transfer of symbiont organic matter to the host by digestion or the

excretion of newly fixed N by the symbiont in inorganic or organic form. According to

Turner (1988), excretion from symbionts and subsequent translocation to the host is the likely mechanism of fixed N transfer. Felbeck (1983) suggested that amino acids are excreted from symbionts to host, but there has been no experimental evidence for the symbiont DON excretion. Moreover, prior to this study, experimental evidence for N excretion by cultivated shipworm symbionts was lacking. Trytek and Allen (1980) proposed that N-rich symbionts are digested by the host. The authors developed this hypothesis with the observation that bacteria appear not to multiply and that circulating phagocytes appear to contain partially digested bacteria (Trytek and Allen, 1980; Felbeck et al., 1983). While both mechanisms are likely to be important mechanisms of N transfer from symbiont to host, we sought to better understand the possibility of N release from diazotrophic symbionts.

1.4.1 Measuring N release

There are several widely used methods to measure N release from diazotrophs. For pure cultures in a relatively N-deprived medium, researchers can use the isotope dilution method, first described by Glibert et al. (1982) and revised by Mulholland et al. (2006). In this technique, $^{15}NH_4^+$ is added to the culture medium, and rates of regeneration of NH_4^+ are calculated by isotope dilution (Glibert et al., 1982). Alternatively, researchers can use a $^{15}N_2$ tracer technique that arose from methods developed by Montoya et al. (Montoya et al., 1996). Here, N_2-fixing cultures are incubated with $^{15}N_2$ gas, and the $^{15}NH_4^+$ pool, which is newly fixed N released into the medium, is measured over time (Mulholland et al., 2004; Mulholland et al., 2006). Finally, N release can be constrained through the establishment of an N budget, using measurements of N_2-fixation and natural abundance uptake and release of nutrients, both

inorganic and organic N. This technique was recently used to constrain N release in *Trichodesmium* cultures by Holl and Montoya (2008). Mulholland (2007) gives several arguments why the direct quantification of N release by diazotrophs is difficult; to get around these problems, Mulholland (2007) recommends that N release is best constrained by the difference between net ($^{15}N_2$-fixation) and gross N_2-fixation (acetylene reduction).

1.4.2 Evidence for N release in free-living microbes

Only a few free-living diazotrophic microbes have been examined for N release. Diazotrophic cultures of *Azospirillum brasilense* and *Azospirillum amazonense* release a small amount of newly fixed N as NH_4^+ (Hartmann et al., 1988). Emtiazi et al. (2007) used the diazotrophic cultivated soil isolate *Paenibacillus* to demonstrate active NH_4^+ release when grown on a sucrose medium. Free-living *Azotobacter vinelandii* releases approximately 5% of total N fixed in the form of glutamate (Kuhla et al., 1985).

The diazotrophic cyanobacterium *Trichodesmium* has been the subject of many studies of N release because of its importance to the N cycle in the oligotrophic ocean. Direct and indirect measurements of DIN and DON release in *Trichodesmium* indicate that a significant fraction of newly fixed N is excreted. This fraction may vary between natural and laboratory populations and a function of cultivation conditions, but reported N release values range from 37 to 90% of total N fixed (Capone, 1994; Glibert and Bronk, 1994; Mulholland and Capone, 1999; Mulholland and Bernhardt, 2005). Importantly, not all authors agree that *Trichodesmium* releases a significant fraction of new N as DON. Holl and Montoya (2008) did not measure DON release directly, but used N budget constraints to conclude that *Trichodesmium* did not release DON in their continuous culture. Holl and Montoya (2005) used different light regimes and cultivation

conditions (continuous culture v. batch culture) in comparison with previous studies of DON release from *Trichodesmium* (Mulholland and Bernhardt, 2005), and conclude that these cultivation differences could account for the different DON release rates.

1.4.3 Evidence for N release in N$_2$-fixing symbioses

In diazotrophic symbioses, there is ample experimental evidence for N release by symbionts; but the amount and form of newly fixed N undergoing translocation to hosts differs among symbioses. There is wide variation in the quantity of fixed N that is released as NH$_4^+$, but in almost all cases, it appears that the symbionts transfer most of their fixed N to the host. For example, plant-cyanobacteria symbioses are known to release 40-90% of the newly fixed N to the host in the form of NH$_4^+$ (Meeks, 1998). The symbiotic cyanobacterium *Nostoc* releases 90% or more of the fixed N as NH$_4^+$ to its angiosperm host *Gunnera* (Silvester et al., 1996). Peters (1977) reports that in the cyanobacterium *Anabaena* – fern *Azolla* symbiosis, at least 50% of the fixed N is transferred to the host.

1.4.4 Why do N$_2$-fixing microbes release N?

There is much evidence for the direct release of labile inorganic N, and the reasons for inorganic N are varied. Hartmann (1988) argues that N-rich metabolites glutamate and aspartate might reduce the activity of ammonium assimilatory enzymes and increase N release in free-living microbes. The release of NH$_4^+$ from diazotrophs by outward diffusion may be unavoidable if the microbe resides in a low N environment (Dodds and Gudder, 1995; Belnap, 2001). The toxicity of the final product of N$_2$-fixation, NH$_4^+$, may cause some diazotrophs to release N if assimilation, conversion, or storage capacity is limited within the cells (Stewart and Rogers, 1977; Silvester et al., 1996;

Belnap, 2001). Viral cell lysis (Hewson et al., 2004) and cell death (Berman-Frank et al., 2004) can also result in a significant portion of newly fixed N being transferred into the dissolved inorganic or organic pools. The reasons for the release of DON are not fully understood at this time (Mulholland 2007).

Given the energetic costs of N_2-fixation and tight physiological and transcriptional regulation, it follows that N release should also be regulated, and may among diazotrophs. The regulation of N release is poorly understood; even with the well-studied cyanobacterium *Trichodesmium*, researchers have been unable to fully characterize the factors that regulate N release.

1.5 Potentially complex nutrient cycles in the shipworm symbiosis

1.5.1 Host-symbiont cycles

Prior to this study, there have been limited investigations that address the potentially complex elemental cycling (C, N, P) and regulation of processes between shipworm hosts and their symbionts. Trytek and Allen (1980) provided evidence for the synthesis of essential amino acids by the gill tissue, but failed to implicate symbionts as direct contributors to this process. The recent MIMS (multiple imaging mass spectrometry) studies, which provided conclusive evidence for symbiont N_2-fixation and N transfer to shipworm hosts (Lechene et al., 2006; Lechene et al., 2007), did not provide evidence for the form of N translocated nor elucidate physiological controls on the N cycle between hosts and symbionts.

The intracellular nature of the symbiosis and heterotrophic metabolism of the symbionts indicates that symbionts are reliant on the host for reduced C compounds. The C cycle between symbionts and hosts remains uninvestigated at this time, and it probably

affects the N cycle through C-N cycle coupling. We have no idea of the C compounds

that symbionts use for aerobic respiration. Symbionts (cultivated *Teredinibacter*

turnerae) are capable of excreting cellulases (Greene et al., 1988; Imam et al., 1993; Xu

and Distel, 2003; Ekborg et al., 2007), but the host shipworm requires symbiont-produced

cellulase enzymes in the gut for wood digestion. The method of transfer of cellulases

from the site of production (gill tissue) to site of most probable use by the shipworm

(cellulose-containing gut) remains elusive. Microscopic examination of the gill reveals no

wood tissue in the gill, so digestion of wood and the production of reduced sugars must

occur in the gut and these C compounds must be transported to gill tissue through the

circulatory system. The mechanism of this process is currently unknown.

There is some evidence from other symbiotic systems that host-symbiont

biochemical cycling can influence the amount of combined N transferred from symbionts

to hosts. In the lichen *Peltigera*-diazotrophic cyanobacterium *Nostoc* symbiosis, the

compound sarcosine acts as a negative regulator of glutamine synthetase and a positive

regulator of nitrogenase activity; the overall effect is a stimulation of N release from the

symbiont (Hallborn, 1984). The *Rhizobium*-legume symbiosis has a complicated amino

acid cycling system between symbiont and host that allows the host to obtain the most N

from N_2-fixation. The legume host provides *Rhizobium* symbionts with amino acids so

that the symbionts are able to shut down NH_4^+ assimilation; in a reciprocal altruism-like

scenario, the symbionts must secrete fixed N to the host in order to maintain the supply of

amino acids (Lodwig et al., 2003). Currently, the host-induced factors that influence the

amount of fixed N translocated from shipworm symbionts to host remains unknown.

1.5.2 Symbiont-symbiont cycles?

Environmental microbial consortia are famous for nutrient recycling among constituents. We cannot rule out the possibility that other complex nutrient feedbacks occur between the different symbiont types and the host cells. In a few other symbioses that involve more than one microbial species, the symbionts frequently exchange nutrients or waste products (engaging in syntrophy) to sustain the symbiosis. For example, in the gutless oligochaete *Olavius algarvensis*, metabolic products of endosymbiotic sulfate-reducing bacteria (sulfide) serve as the electron donor for neighboring endosymbiotic sulfide-oxidizing bacteria (Dubilier et al., 2001). Unfortunately, we do not know enough about the role and function of the multiple symbionts in shipworms to address this potentially confounding issue (Distel et al., 2002a; Luyten et al., 2006).

1.5.3 The possibility for additional nitrogen cycles in the shipworm symbiosis

In the shipworm symbiosis, all symbionts are closely related gamma-proteobacteria (Distel et al., 2002a; Luyten et al., 2006), but the exact *in situ* metabolic function of all symbionts is not yet identified. It is possible that the shipworm gills foster a more complex N cycle than just hosting microbes carrying out N_2-fixation. Several recent studies have reported microbial nitrification in invertebrate symbioses. For example, the colonial ascidian *Cystodytes dellechiajei* hosts aerobic ammonium-oxidizing *Crenarchaeota* in the tunic tissue, and it may oxidize host waste ammonium to nitrite (Martinez-Garcia et al., 2008). The authors were not able to speculate about the fate of nitrite but suspected that the *Crenarcheota* was a potential source of nutrition to the host by digestion of the symbionts (Martinez-Garcia et al., 2008). The marine sponge

Geodia barretti hosts a very complex N cycle: aerobic ammonium oxidizing *Crenarchaeota*, nitrite-oxidizing bacteria *Nitrospria*, anammox bacteria, and denitrifying bacteria were all found to form a complex syntrophic web within the sponge (Hoffmann et al., 2009). The authors suggest that the main benefit of a such a symbiosis is the efficient removal of waste products; in the sponge, ammonium removal by the aerobic and anaerobic ammonium oxidizers may exceed host ammonium excretion rates (Hoffmann et al., 2009).

Aerobic ammonium-oxidizers might use host waste ammonium as an electron donor for chemoautotrophic growth. They would also help create localized microaerobic conditions through the aerobic conversion of ammonium to nitrite. In the sponge symbiosis, nitrification can help satisfy host C demand through chemoautotrophic C fixation and subsequent digestion of C and N-rich ammonium-oxidizing microbes (Hoffmann et al., 2009). The shipworm's wood-rich diet is not expected to be lacking in C, however, C fixation by gill symbionts could be a localized C source for N_2-fixing symbionts. Also, ammonium-oxidizers may remove excess host ammonium waste, similar to the *Geodia barretti* symbiosis. The potential for additional N cycling in the shipworm symbiosis is an intriguing and completely unstudied situation.

1.6   Teredinibacter turnerae*, an ideal candidate to test hypotheses about enigmatic N$_2$-fixation and N release in shipworms*

1.6.1 Species description

The use of the cultivated shipworm symbiont *Teredinibacter turnerae* provides a useful tool for investigating the controls on N_2-fixation and N release by shipworm symbionts. Originally isolated by Waterbury et al. (1983) and later formally described by

Distel et al. (2002b), *T. turnerae* is a cosmopolitan symbiont of many species of shipworms. It has been found as a symbiont in at least 24 species of teredinids across 9 genera (Distel et al., 1991; Distel et al., 2002b). *Teredinibacter turnerae* is probably a minor constituent (about 10%) of the entire symbiotic community (Distel et al., 2002a; Luyten et al., 2006), but its cosmopolitan nature among shipworms suggests that it is important in the shipworm symbiosis. Studies of *T. turnerae* in pure culture have used isolated strains from both *Lydrodus pedicellatus* and *Psiloteredo healdi.* Unfortunately, there have been no comparative physiological studies between the 2 strains, so researchers assume that they are physiologically similar. Name variations, including *Teredinobacter* and *turnirae*, appear in the literature and could contribute to a lack of comprehensive understanding of *T. turnerae*.

  Teredinibacter turnerae is unique relative to other microbes that it occupies its own genus in the gamma-proteobacteria along with other uncultivated shipworm symbionts (Distel et al., 1991; Distel et al., 2002a; Distel et al., 2002b). Recent genome analysis suggests that *T. turnerae* has many adaptations common to a free-living state (Yang et al., 2009). In fact, many critical features of the *T. turnerae* genome do not resemble those of obligate intracellular symbionts, and it could be classified as a facultative symbiont. First, the genome of *T. turnerae* is large in comparison with other symbionts, similar to that of free-living microbes (Yang et al., 2009). Other intracellular symbionts, such as *Buchnera*, a symbiont of aphids, generally have a very reduced genome size (Moran and Baumann, 2000; van Ham et al., 2003). Second, *T. turnerae* does not have an especially low G+C content, which is often seen in obligate symbionts (Moran and Baumann, 2000; Yang et al., 2009). Finally, the first stage of genome

degeneration by obligate symbionts is usually the loss of DNA repair genes (Dale et al., 2003), but *T. turnerae* has a complete set of genes for almost all metabolic functions, including DNA repair (Yang et al., 2009). The genome sequencing project and the ability to cultivate *T. turnerae* in the laboratory suggests that *T. turnerae* may be classified as a facultative endosymbiont, and not an obligate symbiont. However, to our knowledge, *T. turnerae* has not been isolated in the free-living state despite many efforts. Efforts have been focused in and around shipworm-infested wood (Waterbury et al., 1983), mangroves that host large shipworm populations (Trindade-Silva et al., 2009), and in the water column (Yang et al., 2009).

1.6.2 Growth, physiology, and N_2-fixation studies

Teredinibacter turnerae is known as an obligate aerobic heterotroph, hence relies on aerobic respiration to supply ATP for N_2-fixation. Very few studies have investigated the biology and N_2-fixation of *T. turnerae*. Besides the original report from Waterbury et al. (1983), only 3 other studies address N_2-fixation in *T. turnerae*, and none have examined controls on N_2-fixation. Greene and Freer (1986) used the strain from shipworm *P. healdi* to show that cultures grow much slower under N_2-fixing conditions than in high N initial conditions. They reported that *T. turnerae* was not capable of growing in aerated broth cultures with SigmaCell cellulose as the C source and high initial N conditions (Greene and Freer, 1986). N_2-fixation in cultivated *T. turnerae* was a minor part of the first two reports about multiple-isotope mass spectrometry (MIMS; Lechene et al., 2006; Lechene et al., 2007). The MIMS technology allowed the researchers to demonstrate that N_2-fixation within a population of *T. turnerae* is not

homogenously distributed among individual cells, or even within single cells (Lechene et al., 2006).

The recent *T. turnerae* genome sequencing project suggests that the *nif* operon and its regulation are comparable those found in other N_2-fixing microbes. The *nif* operon contains a complete set of *nif* genes, and comprises approximately 1% of the genome (Yang et al., 2009). The organization of the *nif* operon is similar to that of aerotolerant diazotroph *Azotobacter* (Yang et al., 2009). Also similar to other diazotrophs, the *nif* operon was likely acquired through horizontal gene transfer from a Pseudomonas-like bacterium (Yang et al., 2009).

1.6.3 A unique combination: N_2-fixation and cellulolysis

Only a handful of other microbes are capable of both N_2-fixation and cellulolysis, both energetically expensive processes. The uncultivated bacterial symbionts of termites are the best known examples (Hongoh et al., 2008). *Gluconacetobacter diazotrophicus,* cultivated from corn roots, has received some attention because of its potential for bioenergy formation (Emtiazi et al., 2003). Emtiazi et al. (2007) named 7 other microbes capable of these two processes.

1.6.4 Pleomorphism in *T. turnerae*

Several different studies indicate that *Teredinibacter turnerae* is pleomorphic. First reported in Waterbury et al. (1983) and confirmed by Distel et al. (2002b) and Greene (1994), *T. turnerae* frequently exhibits a long rod or a spiraled morphology in the stationary phase. The long rod morphology is also present in the symbiotic state (Distel et al., 2002b). A different strain of *T. turnerae* exhibits aggregated morphology of cells and extracellular matrix throughout all phases of cell growth when grown on casein agar

(Ferreira et al., 2001). Ferreira et al. (2001) did not determine whether the aggregated

morphology in these cultivation conditions were the result of culture stress.

1.6.5 Biofilms in *T. turnerae*

Teredinibacter turnerae is known to secrete extracellular polymeric substances

(EPS), or a biofilm, in broth culture (Greene and Freer, 1986; Ferreira et al., 2001;

Trindade-Silva et al., 2009). In fact, biofilms can be so thick that other researchers have

found that commonly used optical density (OD) measurements are completely unsuitable

to track its growth (Greene and Freer, 1986). The amount of organic matter that comprise

biofilms in *T. turnerae* can be significant; the proteins in the EPS can account for up to

16% of the total biomass (Trindade-Silva et al., 2009).

1.6.6 Practical applications which make this an important microbe for physiological studies

Teredinibacter turnerae has perhaps received the most attention from researchers

for its cellulase enzymes. Cellulase enzymes are required to convert the most abundant

biopolymer on Earth, lignocelluloses, into a biologically usable energy source. The

genome encodes a very large number of proteins predicted to be involved in the

degradation of complex polysaccharides associated with woody materials (Yang et al.,

2009). *Teredinibacter turnerae* secretes cellulases within the family of extracellular

endoglucanases, which have been purified and characterized (Greene et al., 1988; Imam

et al., 1993; Xu and Distel, 2003; Ekborg et al., 2007). Some studies have manipulated *T.

turnerae* culture conditions in an effort to optimize cellulase activity for industrial

applications (Ahuja et al., 2004).

Teredinibacter turnerae also secretes extracellular proteases (Greene et al., 1989;

Griffin et al., 1992; Beshay, 2003). *Teredinibacter turnerae* has been shown to grow on

protein, casein, as the sole C and N source; there is increased protease production when grown on casein, which indicates that proteolytic activity could be used to sequester metabolism (Ferreira et al., 2001). Greene (1994) suggests three functions for extracellular protease activity in *T. turnerae* in the symbiosis: 1) enhance the hydrolysis of cellulosics of woody tissue and increase the availability of cellulose 2) digest protein for host shipworm, when filter feeding, and 3) post-secretory processing of enzymes. Some researchers are interested in sequestering this capability for use in detergents and in low temperature industrial applications (Distel, 2003).

Teredinibacter turnerae appears to exhibit antimicrobial activity, and this property could be sequestered and used for biomedical applications. With an isolate from shipworm *Neoteredo reynei*, Trindade-Silva et al. (2009) presented experimental evidence for the ability of *T. turnerae* to secrete compounds that inhibit the growth of both gram negative and gram positive bacteria. The *T. turnerae* genome encodes for many secondary metabolites, and Yang et al. (2009) proposed that one function of these metabolites is antimicrobial activity. It is possible that antimicrobial activity could be sequestered by the host shipworm in defense of infection from other bacterial species.

1.7 Overview of the study

We used the unique cultivated symbiont of shipworms, *Teredinibacter turnerae*, to test hypotheses concerning the controls and rates of N_2-fixation and N release by shipworm symbionts. First, we provide experimental evidence that *T. turnerae* is among the most aerotolerant of diazotrophs. Second, we provide an N budget to determine the sinks of fixed N and elucidate the amount and form of fixed N that is released. Lastly, we examined whether quick changes in oxygen concentration affects nitrogenase activity.

All of these experiments involved cultures grown in a sucrose-medium; preliminary studies that investigated the control of oxygen on N_2-fixation in cellulose-grown cultures are also included.

CHAPTER 2

EFFECTS OF HEADSPACE OXYGEN CONDITIONS ON GROWTH, N$_2$-FIXATION, AND RESPIRATION IN *TEREDINIBACTER TURNERAE*, A CULTIVATED DIAZOTROPHIC BACTERIAL SYMBIONT OF SHIPWORMS

2.1 Introduction

Shipworms are highly modified bivalves which are able to grow and reproduce on a wood-only diet (Turner, 1966; Gallager et al., 1981). Shipworms are essential remineralizers of woody material in coastal and marine environments (Turner, 1984). The high lignocellulose content and low N content necessitates that shipworms use their gill-hosted symbionts to both produce cellulase enzymes and add fixed N to the diet. The symbionts reside in specialized bacteriocytes within the gill tissue (Waterbury et al., 1983; Distel et al., 1991; Distel, 2003). Recent research indicates that symbionts indeed fix N *in situ* and newly fixed N is transported to non-symbiont-containing host tissue (Lechene et al., 2006; Lechene et al., 2007).

Gill bacteriocytes are a curious locale for N$_2$-fixation. Unlike other plant or animal-hosted N$_2$-fixing symbioses such as in termite guts (Brune and Friedrich, 2000; Ohkuma, 2001) or legume-*Rhizobium* (reviewed in Dixon and Kahn, 2004), symbionts are not housed within a defined symbiont-rich and low oxygen chamber (Waterbury et al., 1983; Distel, 2003). With the absence of an oxygen-retardant barrier in symbiont-containing tissue, N$_2$-fixation by symbionts is expected to be subject to negative regulation by oxygen. Oxygen is known to deactivate the conserved nitrogenase enzyme in many species (Hill, 1988; Dixon and Kahn, 2004) and negatively regulate transcription of the *nif* operon through its interaction with the NifA/NifL two-component regulatory system (Hill et al., 1996; Dixon, 1998). As a result, most bacterial N$_2$-fixers, such as

Rhizobium (Fischer, 1994), *Azospirillum brasilense* (Nelson and Knowles, 1978; Hartmann and Burris, 1987), and *Klebsiella pneumoniae* (Klucas, 1972; Poole and Hill, 1997), are only capable of fixing N under anaerobic or microaerobic conditions. A small handful of bacteria, including *Gluconacetobacter diazotrophicus* (Emtiazi et al., 2003) and *Azotobacter vinelandii* (Kuhla et al., 1985; Marchal and Vanderleyden, 2000), can fix N in fully aerobic conditions (up to 100% air saturation) without negative consequences on nitrogenase activity. These aerotolerant diazotrophs have a wide variety of well-studied protective mechanisms to maintain a low intracellular oxygen concentration, including high O_2 consumption (Poole and Hill, 1997), extracellular O_2-retardant layers (Sabra et al., 2000), and O_2-scavenging enzymes (Dingler and Oelze, 1987).

In this study, we aimed to elucidate how O_2 controls N_2-fixation in shipworm symbionts. We used the cultivated symbiont *Teredinibacter turnerae* (Waterbury et al., 1983; Distel et al., 2002b), a symbiont in at least 24 different shipworm species, which has previously been shown to fix N in pure culture (Waterbury et al., 1983; Greene and Freer, 1986; Lechene et al., 2006; Lechene et al., 2007). These studies indicate that *T. turnerae* is capable of respiration and N_2-fixation under a wide range of headspace oxygen conditions, from 0.07 atm to 0.21 atm.

2.2   Methods

2.2.1 Cultures

Teredinibacter turnerae (strain T7902; courtesy of D. Distel; Distel et al., 2002b), isolated from shipworm *Lyrodus pedicellatus*, was grown in soft agar (0.2% agar) shipworm basal medium (SBM) described by Waterbury et al. (1983) with sucrose as a carbon source. The medium was modified to reduce the medium ammonium by an

equimolar substitution for the iron source; we used ferric citrate instead of ferric ammonium citrate.

We grew *T. turnerae* in air-tight Bellco anaerobic test tubes (Bellco glass 2048-18150) that were crimp sealed with gas-tight rubber septa. There was 15 mL of shipworm basal medium and 13 mL headspace in each tube. Cultures were grown at room temperature (20°C) without physical disturbance. We monitored contamination with two methods. First, we struck experimental cultures on LB and Marine agar and SBM agar to actively detect growing contaminants (*T. turnerae* does not grow on these media; Distel et al., 2002b). Second, we used epifluorescence microscopy with DAPI staining.

2.2.2 Analytical methods

Oxygen treatments

We set up 4 pO_2 treatments which spanned a range from 0.07 atmosphere O_2 to normal atmospheric saturation (0.21 atmosphere). We were unable to measure N_2-fixation and respiration below this range. To vary the partial pressure of oxygen in the vessel headspace, we used a gas proportioner (Cole Palmer EW-03218-50) to mix air and N_2. The gas mixture was passed through a 0.45 µM sterile filter, then sparged into the headspace of the culture through a disposable 18 gauge needle. Cultures were sparged long enough to flush the headspace 10x. Direct measurements of dissolved pO_2 in blank media indicate that this method maintains the medium at the appropriate oxygen concentration (Appendix B).

Headspace oxygen measurements

Two mL of headspace was removed from the culture tube with an 18 gauge needle and equilibrated with 6 mL of water by agitation for 20 seconds in a gas-tight

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syringe. We measured pO_2 of the liquid phase with a StrathKelvin oxygen meter. We had to correct for residual pO_2 in the gas-liquid equilibration, and convert the concentration measured in solution to the original pO_2 in the gas phase of the equilibrium. For this, we used a standard regression with gas samples of known pO_2 concentration ranging from 0 to 0.21 atmospheres. Immediately following the sampling of the headspace for oxygen concentration, cultures were sparged with a gas mixture to restore the original pO_2 condition.

Acetylene reduction assays

Nitrogen fixation activity was measured with the acetylene reduction assay (Capone and Montoya, 2001), and incubations were carried out in the Bellco anaerobic test tubes. In brief, 3 mL of acetylene was added to each vessel, and assay incubations were carried out under standard growth conditions as noted above. The headspace ethylene concentration was measured in duplicate at hourly intervals for approximately 3-4 hours by gas chromatography (SRI model 8610c gas chromatograph, SRI Instruments, Torrance, CA USA). The gas chromatograph was fitted with a 2 m Haysep A column (Alltech Associates Inc., Deerfield, IL USA) and a flame ionization detector (SRI Instruments). We used a 4:1 C_2H_2:N_2 molar reduction ratio to convert from acetylene reduction to N_2-fixation rates.

Total protein

We measured total protein in *T. turnerae* cultures with the Bradford assay (Bradford, 1976; Coomassie Plus - The Better Bradford Assay Kit, VWR PI23236). Previous studies with *T. turnerae* have also used protein measurements to quantify culture growth (Greene and Freer, 1986). To harvest the cells from the soft agar, we

pipetted the localized cell layer from the culture tube (the cells grew in only the upper 2-3 mm of soft agar). This cell layer slurry was homogenized using ethanol-washed glass beads, vigorous shaking, and vortexing. We added 50 μL of homogenized cells to 1.5 mL Coommassie Blue, and measured the absorbance at 595 nm with a Spectronic 20 Genesys spectrophotometer. Total protein concentrations were calculated from a standard curve of bovine serum albumin.

Calculations of oxygen consumption

We determined oxygen consumption rates by measuring the depletion of oxygen following the initial poising of the cultures at their experimental pO_2 levels through sparging the headspace. We estimated the concentration of oxygen in the liquid phase using solubility constants appropriate for our culture conditions (C_o* = 374.94 μmol O_2 L^{-1} seawater atm^{-1}; Garcia and Gordon, 1992).

2.2.3 Experimental design

At regular intervals during growth, we measured headspace pO_2 (to assess oxygen depletion through aerobic respiration), N_2-fixation rates with the acetylene reduction assay, and total protein content. Acetylene reduction assays and headspace pO_2 measurements were conducted on the same subset of cultures (n = 4 for each pO_2 treatment) during growth. At each time point, 3 cultures for each pO_2 treatment were chosen at random to be sacrificed for total protein content. We conducted this assay six times during culture growth, ranging from 6 days after inoculation to 34 days of growth). Immediately following the acetylene reduction assay, we sparged the headspace of all individual cultures to the original pO_2 condition to remove acetylene and ethylene. We did not take direct action to remove acetylene and ethylene from the agar medium.

2.3 *Results*

We were able to observe culture growth and measure N_2-fixation when

Teredinibacter turnerae was incubated with headspace oxygen concentrations ranging

from 0.07 to 0.21 atm. We did not observe N_2-fixation in cultures that were incubated

with a headspace pO_2 of less than 0.07 atm at any time during growth (data not shown).

2.3.1 Headspace pO_2 affected time course of protein accumulation and μ

The headspace pO_2 significantly affected the time course of increase in the

standing stock of protein during growth (Figure 2.1, ANOVA, P = 0.039). Total protein

of the pO_2 = 0.14 atm and 0.21 atm treatments was not measurable until after day 13, but

there was measurable protein in the pO_2 = 0.07 atm and 0.10 atm treatments after day 6.

The maximum growth rate for low pO_2 cultures was similar on day 6 (0.07 atm, μ

= 0.517 ± 0.023 d^{-1}; 0.10 atm, μ = 0.517 ± 0.023 d^{-1}; data are mean ± 1 SE, n = 3). The

0.14 atm treatment had a similar maximum growth rate to the low pO_2 cultures, but

occurred later on day 13 (μ = 0.574 ± 0.023 d^{-1}; data are mean ± 1 SE, n = 3). The 0.21

atm treatment recorded a maximum growth rate double that of the other 3 treatments on

day 17 (μ = 1.289 ± 0.023 d^{-1}; data are mean ± 1 SE, n = 3). The 0.21 atm treatment had a

statistically significantly higher growth rate than the other 3 treatments (P < 0.0005, one-

way ANOVA).

2.3.2 Headspace pO_2 effects on N_2-fixation and VO_2 rates and activity

All pO_2 treatments had similar trends in absolute N_2-fixation rate during growth;

N_2-fixation increased for the first 17 days, reached a maximum at day 17, and declined

steadily after day 17 (Figure 2.2, solid lines). During the first two weeks of growth, N_2-

fixation rates in high pO_2 treatments (0.21 atm, Figure 2.2A; 0.14 atm, Figure 2.2B) were

much less than N_2-fixation rates in low pO_2 treatments (0.10 atm, Figure 2.2C; 0.07 atm, Figure 2.2D). N_2-fixation in lower pO_2 cultures (Figure 2.2C and 2.2D) ceased earlier than N_2-fixation in higher pO_2 cultures (Figure 2.2A and 2.2B). Similar to N_2-fixation rates, respiration rates in all pO_2 treatments followed a trend of steady increase early during growth, a maximal value mid-growth (day 17 or 21), and a subsequent decrease from the maximal value later in the growth curve (Figure 2.2, dotted lines).

We examined the correlation between oxygen consumption rates (VO_2) and N_2-fixation rates for all pO_2 treatments during growth. Early during growth (day 6 to 17), VO_2 and N_2-fixation rates covaried. In contrast, VO_2 and N_2-fixation did not covary late during growth (days 27-34); in all pO_2 treatments, there was a steady decrease in N_2-fixation while VO_2 was steady (Figure 2.2B, C, D) or increased (Figure 2.2A).

We compared the total N_2 fixed among all four pO_2 treatments. In similar fashion as the total protein curves, the low pO_2 treatments accumulated fixed N earlier after inoculation than high pO_2 treatments (Figure 2.3, ANOVA with Tukey's post hoc comparison test, $P < 0.0005$).

The differences in timing of protein content, growth rate, and N_2-fixation among pO_2 treatments complicated the comparative analysis of physiological measurements (N_2-fixation and respiration) among pO_2 treatments over the duration of the experiment. Therefore, we compared maximal values of physiological measurements among pO_2 treatments, rather than mean values from inoculation until the final day. Comparing maximal values measured during growth reduced the influence of the growth timing differences so that we could directly compare the effects of oxygen upon the physiology of *T. turnerae*.

The maximum absolute N_2-fixation rate of the $pO_2 = 0.21$ atm treatment was double that of the $pO_2 = 0.07$ atm treatment (one-way ANOVA with Tukey's post hoc pairwise comparison, $P = 0.033$), while the mid-pO_2 treatments (0.10 atm and 0.14 atm) were not statistically significantly different from the highest and lowest pO_2 treatments (Figure 2.4A). The same pattern occurred when we compared maximum absolute VO_2 rates: high pO_2 cultures had higher maximum VO_2 values (0.21 atm cultures = 166.36 ± 11.42 nmol O_2 cm^{-2} h^{-1}) than low pO_2 cultures (Figure 2.4B; 0.07 atm cultures = 99.40 ± 11.42 nmol O_2 cm^{-2} h^{-1}; one-way ANOVA with Tukey's post hoc pairwise comparison; $P = 0.020$).

We compared the specific rates of respiration and N_2-fixation for days 17 to 34, when measurable protein was present in all cultures. In contrast to absolute rates, maximum protein-specific rates of N_2-fixation and respiration were not significantly affected by headspace pO_2 (Figure 2.5; N_2-fixation activity: one-way ANOVA with Tukey's post hoc pairwise comparison, $P = 0.253$; respiration activity: one-way ANOVA with Tukey's post hoc pairwise comparison, $P = 0.292$). The maximum specific N_2-fixation activity and maximum specific VO_2 activity did not co-occur for the individual pO_2 treatments. The day of maximum N_2-fixation activity co-occurred with the maximum growth rate (0.07 and 0.10 atm: day 6; 0.14 atm: day 13; 0.21 atm: day 17). The day of maximum specific VO_2 activity co-occurred with the day of maximum absolute VO_2 (0.07, 0.10, 0.14 atm: day 17; 0.21 atm: day 21).

Specific rates of VO_2 and N_2-fixation were not coupled during days 17-34 when all cultures had measurable protein (Figure 2.6, linear regression, $R^2 = 0.46$, $P = 0.053$).

A linear regression of specific respiration and N_2-fixation for each individual pO_2 treatment yielded non-significant results.

For each culture during growth (day 17 to 34), we compared the mean pO_2 experienced by the culture during each interval to the instantaneous specific VO_2. There was a weak positive correlation between the headspace pO_2 and specific VO_2, but it was not significant (Figure 2.7, linear regression, $R^2 = 0.128$, $P = 0.078$).
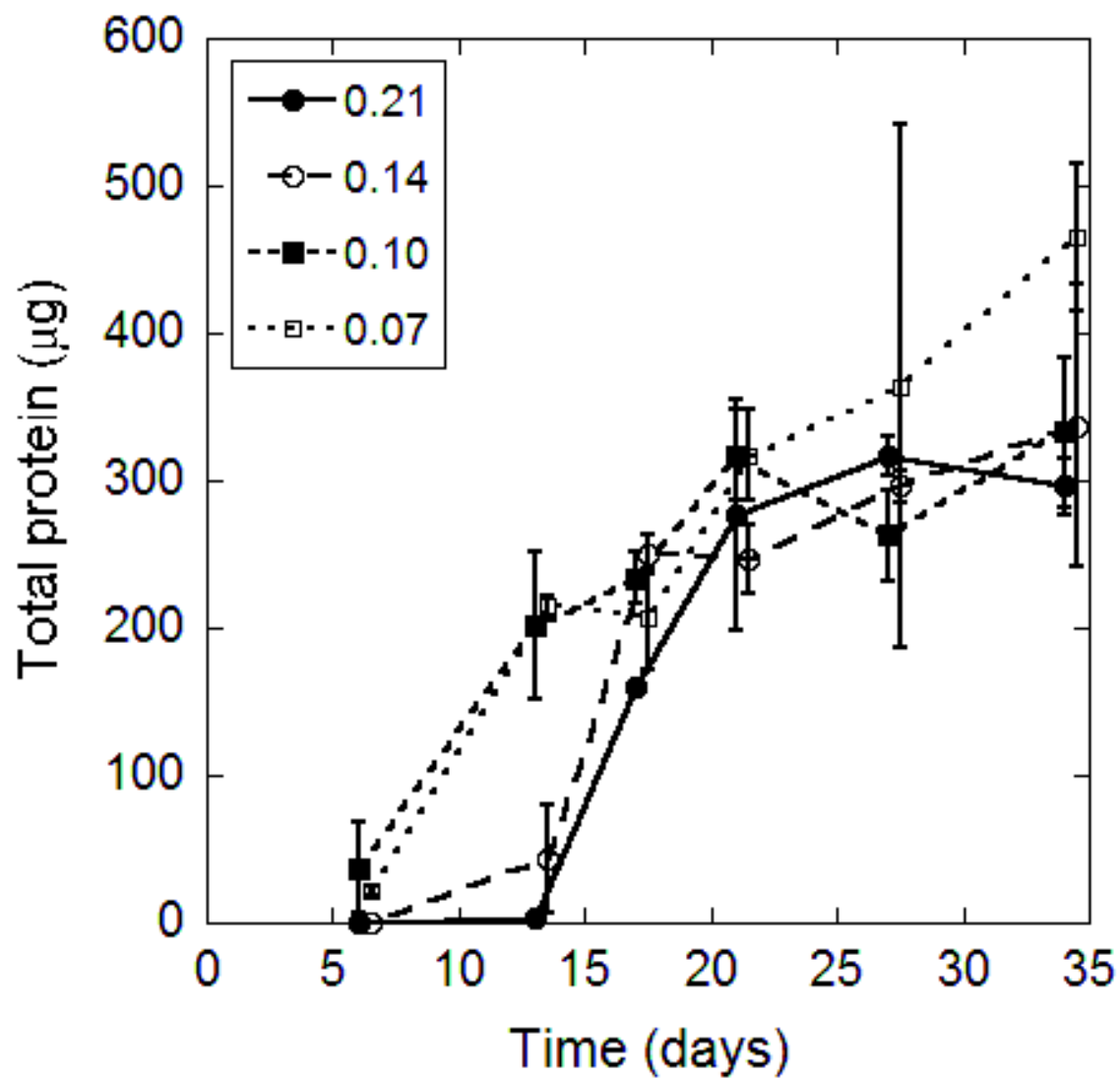
Figure 2.1. Total protein (Bradford Assay) during growth of *T. turnerae* for 4 pO$_2$ treatments. Inset legend indicates culture pO$_2$ treatment. Data are mean ± SD, n = 3 for each day and treatment.
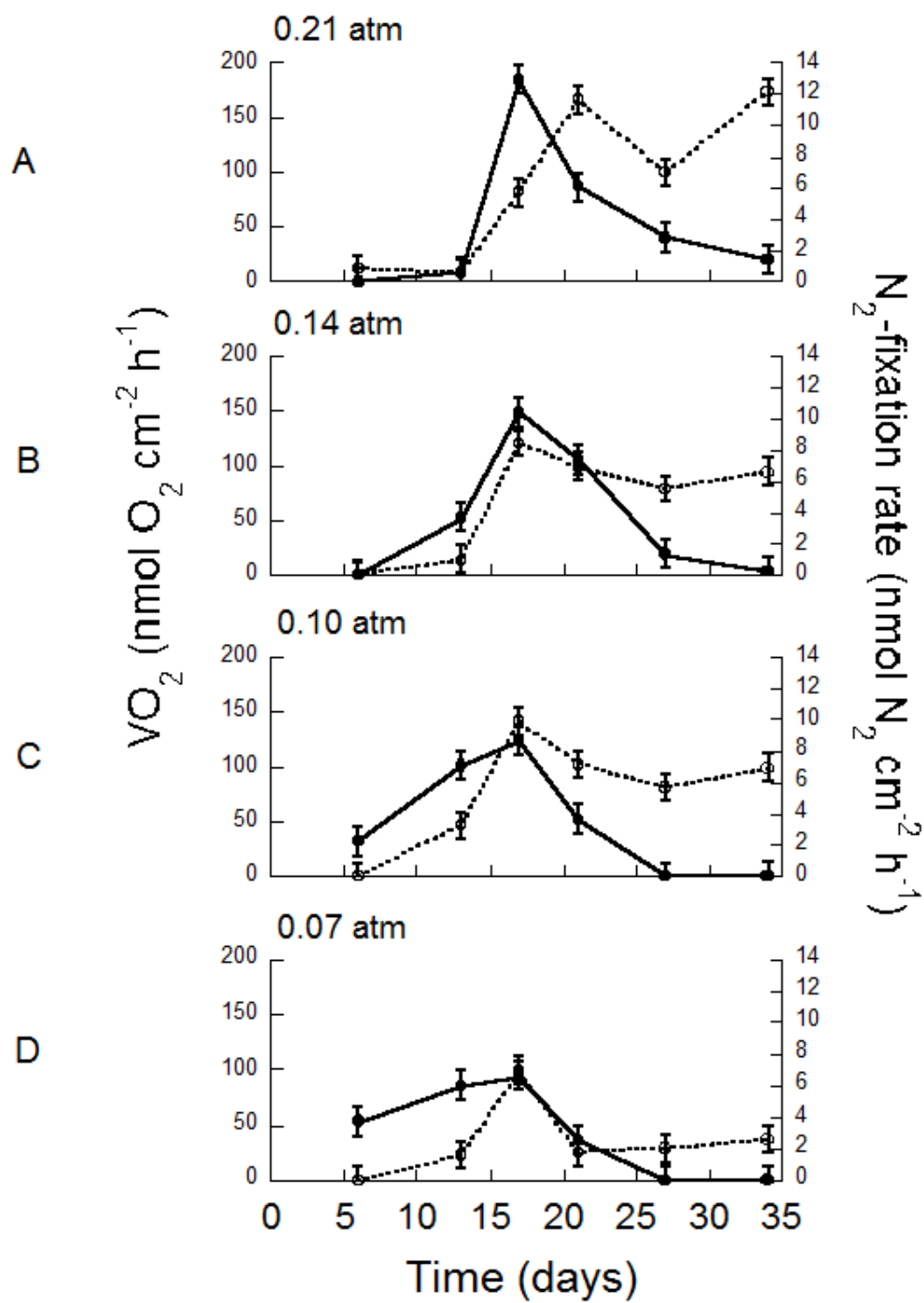
Figure 2.1. Respiration rate (dotted lines and open circles) and N_2-fixation rate (solid lines and solid circles) in *T. turnerae* cultures during growth. VO_2 and N_2-fixation covaried until approximately day 21 for all pO_2 treatments, and do not covary late during growth.
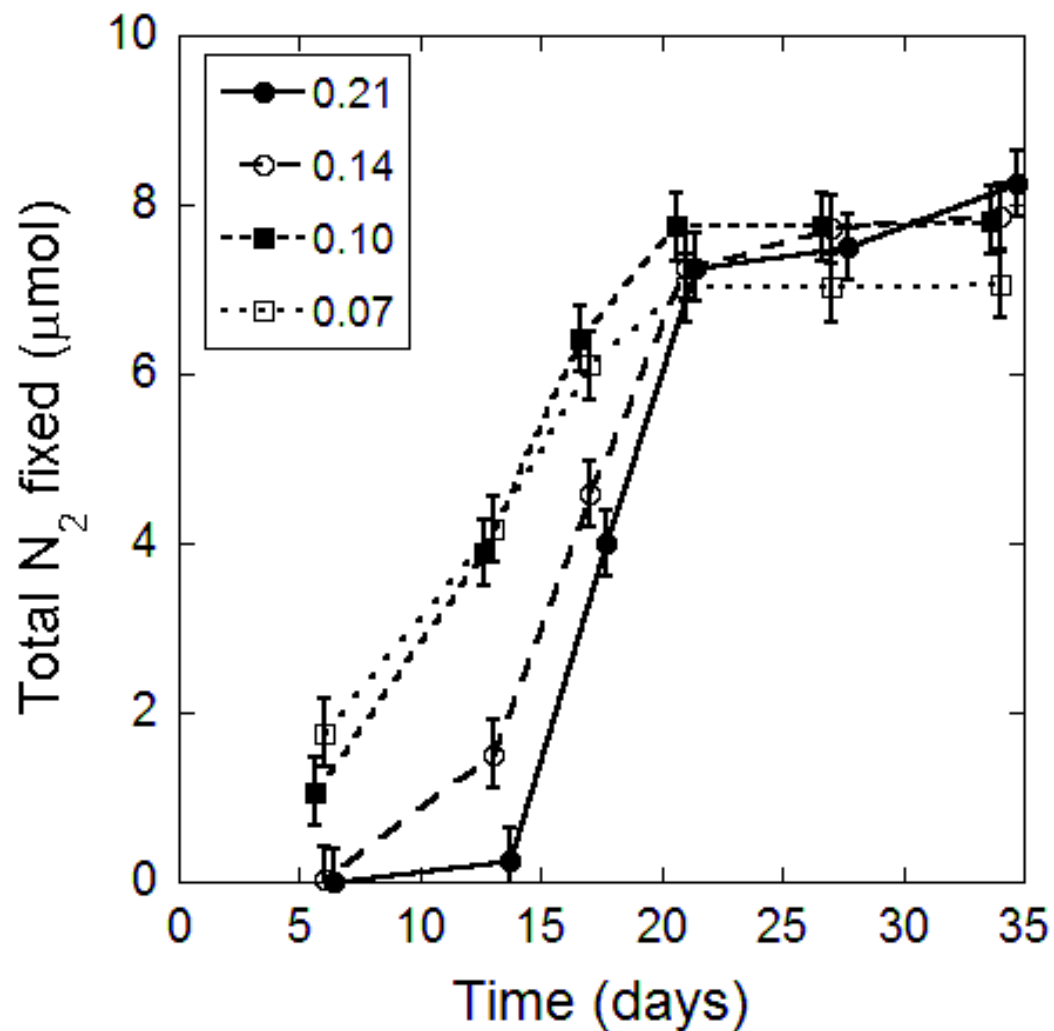
Figure 2.2. Integrated N$_2$-fixation for the pO$_2$ treatments. The differences in phasing of the N growth curve among pO$_2$ treatments were the result of N$_2$-fixation phasing. Inset legend indicates culture pO$_2$ treatment. Data are mean ± SE, n = 4 for each day and treatment.
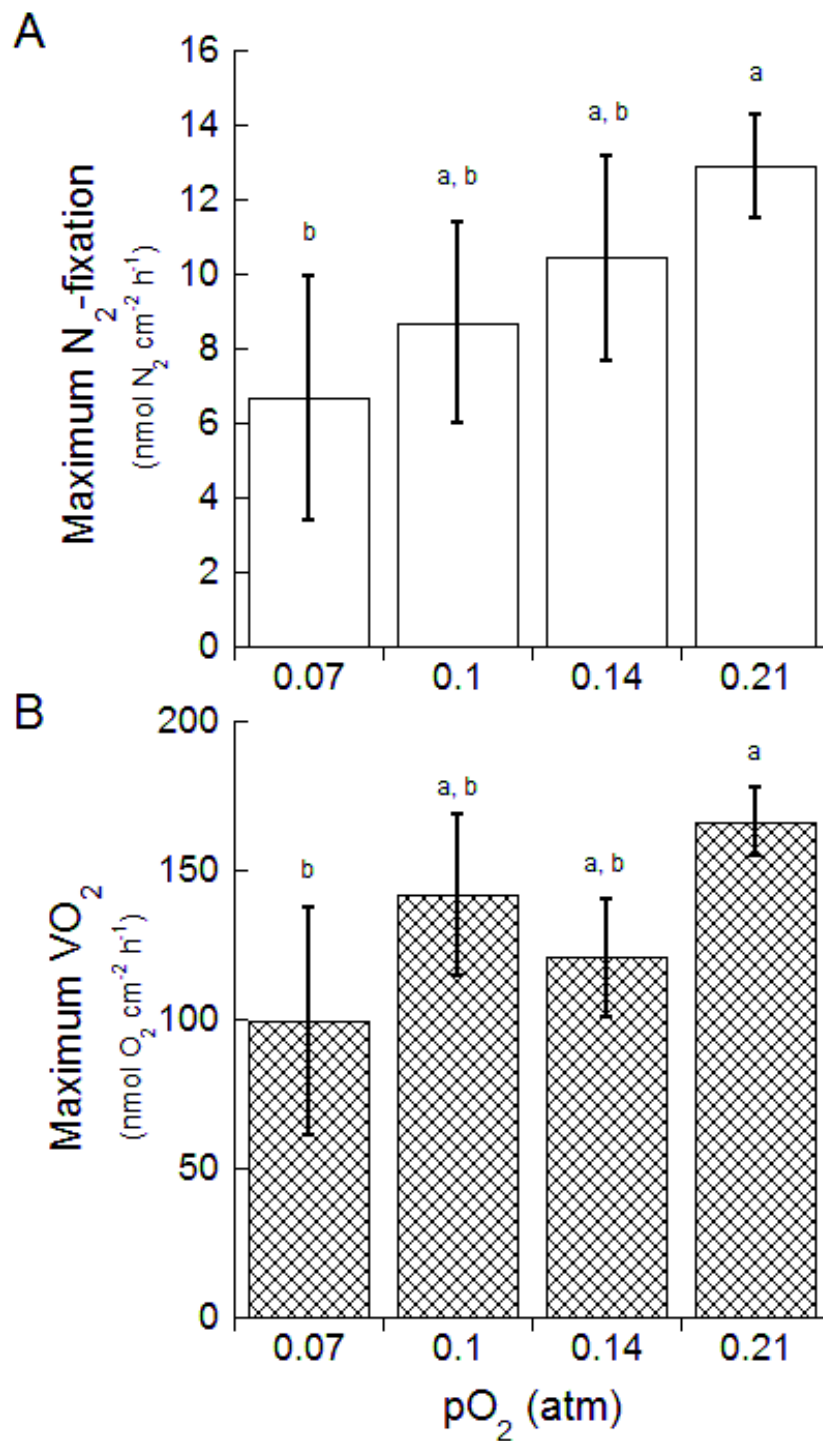
Figure 2.3. The maximum N_2-fixation rates (panel A) and VO_2 rates (panel B) were significantly affected by headspace pO_2 in *T. turnerae*. (A) Maximum N_2-fixation occurred on day 17 for all pO_2 treatments. Data are mean ± 1 SD, n = 4. (B) Maximum VO_2 for 0.07, 0.10, and 0.14 atm cultures are on day 17 and on day 21 for 0.21 cultures. Data are mean ± 1 SD, n = 4. Subscripts indicate significant groupings (ANOVA with Tukey's post hoc pairwise comparison, $P < 0.05$)
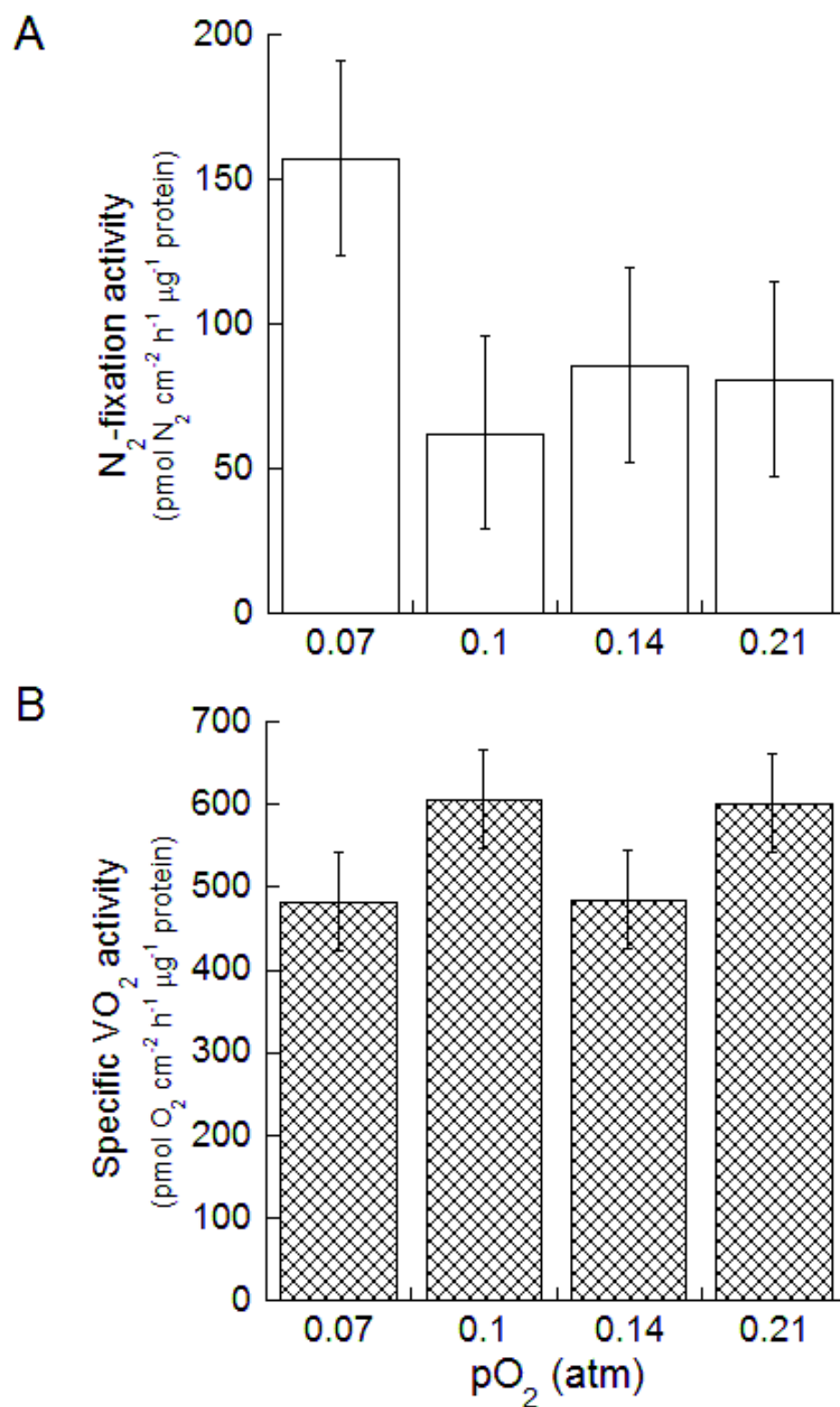
Figure 2.4. Headspace pO$_2$ did not affect the maximum biomass-specific N$_2$-fixation nor VO$_2$ rates. Data are mean ± 1 SE, n = 4.
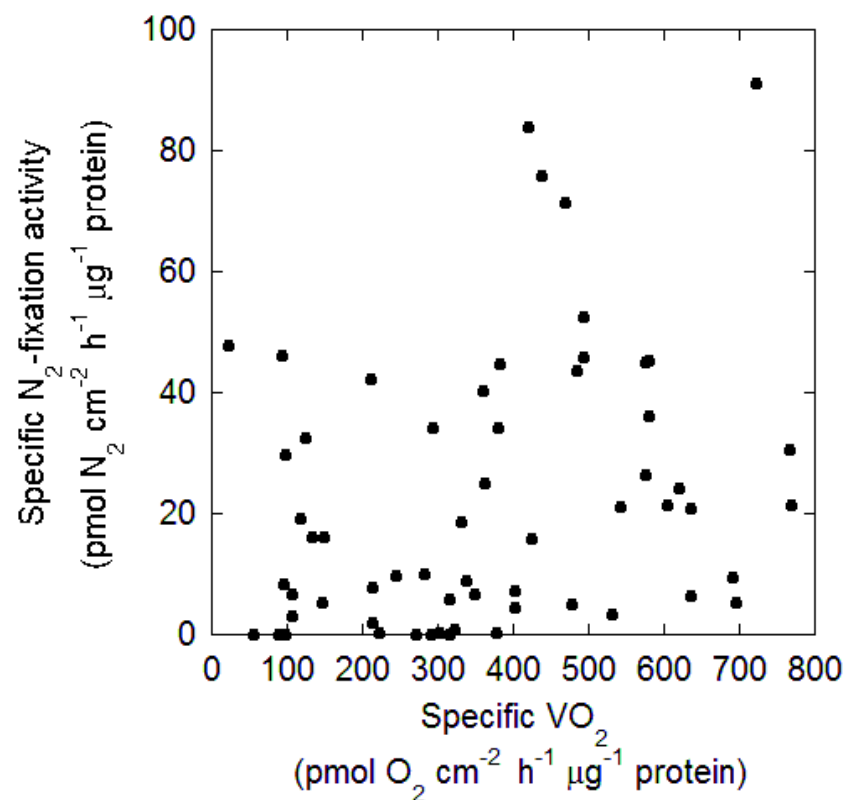
Figure 2.5. Specific VO₂ activity was not correlated with specific N₂-fixation activity. Data are one culture between days 17 to 34.
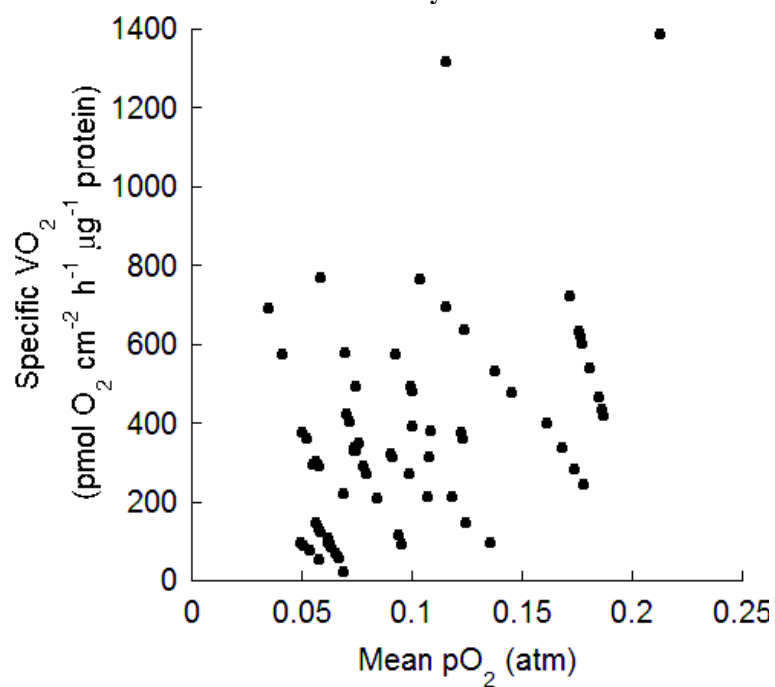
Figure 2.6. There was a positive but nonsignificant correlation between mean pO₂ experienced by *T. turnerae* and specific VO₂. The data are individual cultures.

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2.4   *Discussion*

To our knowledge, this is the first comprehensive investigation of the effects of oxygen on growth rate, N_2-fixation, and respiration in a cultivated diazotrophic endosymbiont of shipworms. Our results demonstrate that *Teredinibacter turnerae* grows and fixes N under a wide range headspace oxygen conditions, from 0.07 atm to 0.21 atm. For the 0.21 atm treatment, the growth curves were similar in duration to that obtained by Greene and Freer (1986). Our experimental design probably set up conditions which facilitated the development of a medium O_2 gradient through respiration and helped to maintain low dissolved oxygen in the culture during growth. As a consequence, the headspace oxygen conditions affected the time course of growth but not specific N_2-fixation or VO_2 activity.

2.4.1   Growth rate and specific N_2-fixation are correlated

In these batch culture conditions, the co-occurrence of elevated growth rate and specific N_2-fixation activity within each pO_2 treatment reflects the reliance of *T. turnerae* upon N_2-fixation for exponential growth in all pO_2 treatments. We compared maximum growth rate and specific N_2-fixation rates among pO_2 treatment groups, and expected that the highest growth rates would be supported by the cultures with the highest rates of specific N_2-fixation activity. Recently, such a positive correlation between specific N_2-fixation and growth rate was demonstrated in chemostat cultures of *Trichodesmium* (Holl and Montoya, 2008).

In our experiment, the 0.21 atm treatment showed a maximum growth rate double that of other treatments (all of which had comparable growth rates), but the 0.21 atm treatment did not have a significantly higher maximum specific N_2-fixation activity. Our experiment was unable to account for the additional N during this period of rapid growth.

It is possible that our measurements were not taken at short enough intervals in time to identify the highest specific N_2-fixation rate during the period of maximal growth rate. In addition, the experiment only produced two comparisons of growth rate to specific N_2-fixation, which limits our ability to test correlations, thus additional research is needed

2.4.2 Shut down of N_2-fixation in stationary phase

After day 17, N_2-fixation and VO_2 did not co-vary: there is a decrease in N_2-fixation without a similar drop in respiration, there was a lack of a trend of specific VO_2 with specific N_2-fixation, and cultures in the lower pO_2 treatments continued to respire after N_2-fixation ceased. Collectively, these results suggest that *T. turnerae* does not depend on active N_2-fixation to remain viable and physiologically active during stationary phase (indicated by a constant protein content during this period). N requirements during this phase may be reduced, for example, we expect N requirements to be lower during stationary phase in comparison to exponential phase because of a decreased need for RNA, the second largest sink for N in bacteria (RNA = 20.5% of cell weight; Neidhardt and Umbarger, 1996). Other possible sources of N that *T. turnerae* may utilize include remineralization of cellular biomass (cell mortality) and uptake of excreted dissolved inorganic or organic N.

2.4.3 Evidence for the development of O_2 gradients and microaerobic medium conditions

The capability of *T. turnerae* to sustain high N_2-fixation under a wide range of headspace oxygen conditions, including under conditions of 100% oxygen saturation, contrasts with the observations of Waterbury et al. (1983) and Greene and Freer (1986), who found that N_2-fixation did not occur under oxygenated conditions (gentle shaking) in broth medium, although they did not report the specific oxygen tolerance of *T. turnerae*.

We attribute this difference to the unique cultivation and assay methods used in this study. We maintained cultures in soft agar medium (0.2% agar) in air-tight vessels under controlled headspace oxygen conditions. This design established conditions where the dissolved oxygen concentration in the medium was probably not in equilibrium with the headspace. Instead, respiration in *T. turnerae* would create an oxygen gradient and microaerobic conditions in the non-mixed soft agar. Measurements of the medium oxygen concentration late during growth support this hypothesis (Appendix B). Briefly, we used a Clark oxygen microelectrode to measure the dissolved oxygen concentration in pO_2-controlled cultures and in blank medium under the same headspace oxygen conditions. Our measurements indicated a suboxic to anoxic medium under the pO_2-controlled headspace in *T. turnerae* cultures. We propose that development of O_2 gradients in the medium affected the time course of growth by different pO_2 treatments and explains why headspace pO_2 did not affect maximum N_2-fixation and VO_2 specific activity.

Higher pO_2 treatments had a longer lag growth phase, as measured by protein growth, N_2-fixation, and respiration, and this difference in timing of the growth curve compared with lower pO_2 treatments was significant. If N_2-fixation is subject to negative regulation by oxygen, we would expect N_2-fixation to occur only after the creation of microaerobic conditions in the medium. A longer lag phase for high pO_2 treatments may occur because culture growth and respiration need more time to draw down enough O_2 to create a favorable dissolved oxygen concentration within the agar and allowing N_2-fixation to commence. Our finding of a lack of correlation between headspace pO_2 and respiration rate supports this hypothesis; without an increase in respiration by high pO_2

cultures, a longer time would required for them to establish the same medium oxygen conditions as low pO_2 cultures. Studies of the development of O_2 gradients (timing, steepness, and magnitude) among the pO_2 treatments would help to elucidate this hypothesis and remain for future work.

In order to evaluate the effect of headspace pO_2 on metabolic activity, we compared N_2-fixation and VO_2 on the day of maximum activity for each pO_2 treatment; this reduced the possible effect of differences in growth rate on the comparison. Maximum N_2-fixation and VO_2 activity did not vary among pO_2 treatments, so these processes were not directly inhibited by higher headspace O_2 concentrations or limited by oxygen availability by lower headspace pO_2 concentrations. Nitrogenase enzyme activity and expression in the overwhelming majority of N_2-fixing bacteria is subject to negative regulation by oxygen (Marchal and Vanderleyden, 2000; Dixon and Kahn, 2004), though Marchal and Vanderleyden (2000) reviewed that N_2-fixation is not sensitive to external oxygen concentration in two non-heterocystous bacteria, *Azotobacter vinelandii* and *Gluconacetobacter diazotrophicus* (Emtiazi et al., 2003). In our system, the apparent absence of O_2-inhibition of N_2-fixation activity likely reflects the physical structure of the culture environment. In fact, the presence of an O_2-gradient in the agar, variation in the time course of growth with pO_2, and the time required for creation of microaerobic to anoxic conditions in the medium all suggest that *T. turnerae* is sensitive to oxygen during growth. The insensitivity of N_2-fixation activity to headspace pO_2 most likely reflects the presence of respiration-generated microaerobic conditions within the agar, with some contribution from protective mechanisms to maintain low intracellular oxygen.

2.4.4 Oxygen protective mechanisms

Respiration-generated O_2-gradients are only one way to establish microaerobic conditions that are conducive for N_2-fixation. It is very possible that *T. turnerae* has mechanisms similar to other diazotrophs which help to maintain low intracellular oxygen tension and sustain N_2-fixation during periods of oxygen stress. Respiratory protection is used by *Azotobacter* and some microaerobic diazotrophs to protect against inactivation of nitrogenase by oxygen (reviewed in Poole and Hill, 1997). For example, in the cyanobacterium *Gloeothece*, respiratory oxygen consumption was stimulated under superoxic conditions (oxygen concentrations higher than 0.21 atm); Gallon and Hamadi (1984) suggest that this elevated respiration protects the nitrogenase enzyme from inactivation. *Azotobacter* adjusts its respiration rate to match the supply of O_2 (Drozd and Postgate, 1970) and has one of the highest rates of respiration known for bacteria (Poole and Hill, 1997). Although there was a slightly positive correlation in *T. turnerae*, respiration activity was not significantly correlated with headspace pO_2 conditions, which may suggest that respiration is unlikely to be a mechanism of O_2 protection in our system. However, with the presence of an O_2 gradient in the agar, it is possible that respiratory protection could be a protective mechanism for cells outside of the pocket of respiration-generated microaerobic conditions.

Another way that diazotrophs cope with oxygen stress is through growth adaptations, including production of thick biofilms and by forming cellular aggregates, which retard oxygen penetration to interior cells. In our system, the respiration-generated microaerobic conditions in the agar medium functioned in a similar fashion. Prosperi (1994) attributes the ability of the cyanobacterium *Nostoc cordubensis* to fix N at oxygen concentrations higher than 20% air saturation to a "mucilaginous" coating (similar in

function to bacterial biofilms). Also, *Azotobacter vinelandii* (in a combined nitrogen-free continuous culture) can change the formation rate and composition of alginate, a major component of the noncellular part of biofilms, in response to oxygen tension; the barrier is thicker under conditions of higher oxygen tension (Sabra et al., 2000). *Teredinibacter turnerae* excretes thick biofilms and cells aggregate readily in broth culture (Ferreira et al., 2001; Trindade-Silva et al., 2009; D. Distel and R. Horak, pers. obs). An O_2-protective barrier would be especially important early during growth when culture respiration may not be high enough to create microaerobic conditions within the medium. Future studies are needed to evaluate the exact role of biofilms and cell aggregations as protective mechanisms of nitrogenase from oxygen deactivation in cultures of *T. turnerae*, which our agar medium study indicates is an essential feature for growth and N_2-fixation.

2.5 Conclusions

Unlike other animal or plant N_2-fixing symbioses, shipworms symbionts are not maintained in a symbiont-rich, micro-oxic compartment within the host. Instead, shipworm symbionts residing within gill bacteriocytes may be exposed to oxygen and subject to inhibition of N_2-fixation. Collectively, this study of the shipworm symbiont *T. turnerae* suggests that it is subject to negative regulation by oxygen during growth in N-limiting conditions, similar to most other diazotrophic bacteria. Future studies are needed to examine the mechanisms used by symbionts in gill bacteriocytes to sustain N_2-fixation in a potentially O_2-rich environment.

2.6 Future directions

2.6.1 Resolve the O_2 gradient in the agar and optimum dissolved oxygen concentration for the establishment of N_2-fixation

The establishment of an O_2-gradient and proper dissolved oxygen conditions within the medium are probably necessary for N_2-fixation and high growth. We propose to conduct a more thorough study of oxygen gradients in soft agar cultures with the oxygen microelectrode. We would incorporate a time component, measuring the gradient at different points during growth, and look for differences in the development (time and depth) of the gradient among pO_2 treatments. A critical goal of this survey would be to determine the optimal dissolved oxygen concentration at which cells begin to fix N. Given the results presented in this chapter and in Appendix B, we expect that oxygen gradients in lower pO_2 treatments would develop faster and be steeper than cultures in higher pO_2 treatments. Also, in all pO_2 treatments, we expect the gradient to become shallower as the culture accumulates more biomass and has a higher rate of respiration.

2.6.2 Design an assay where the medium pO_2 is in equilibrium with headspace pO_2

A drawback to our experimental design was that O2 gradients formed in the soft agar, and the cultures were aerated constantly with the headspace. Instead, we propose to grow broth cultures in a larger batch and use airtight vessels (capable of undergoing headspace oxygen manipulation) larger than the Bellco tubes used here. The larger broth volume necessitates that the cultures undergo agitation to foster a high biomass. A setup that involves a Bellco roller drum and larger volume crimp seal tubes would suffice for this purpose.

2.6.3 Establishment of a chemostat system

Future studies for a direct study of the effects of growth rate on specific N_2-fixation activity would employ a chemostat system, where the growth rate can be directly controlled. The headspace of the chemostat could also be manipulated to be maintained at a constant pO_2. Laboratory-based chemostats also probably best model the physiology of shipworm symbionts in their natural niche. Although we expect a significant time investment to establish a chemostat with *T. turnerae*, a chemostat system would be essential to directly relate the results of an experimental manipulation to the physiology of symbionts in gill bacteriocytes.

2.6.4 Nitrogenase protection in *T. turnerae*: Biofilms and EPS

Future studies should also address the most probable mechanism that cells use to maintain intracellular oxygen low while in the presence of high headspace oxygen. These mechanisms would be especially important when the VO_2 rate is not high enough to create a microaerophilic zone within the agar. While there are other potential mechanisms to maintain low intracellular O_2 in *T. turnerae* (to be discussed in sections 4.4.2 and 4.4.3), we consider the potential role of the biofilm as a protective barrier to be most critical to this study.

An informative study would test the effects of oxygen on the composition and thickness of the matrix. If EPS does play a critical role in nitrogenase protection in *T. turnerae*, we might expect that an increase in oxygen concentration would result in a thicker matrix with higher molecular weight polysaccharides. A critical first step would be to gather direct evidence for the role of EPS in protecting nitrogenase. After rearing *T. turnerae* in broth culture under atmospheres of differing oxygen concentrations, the EPS could be separated from the cells, and quantified (methods in Azeredo et al., 1999;

Delatolla et al., 2008; Takahashi et al., 2009). Then, RT-PCR could be used to examine the expression profile of the EPS genes among pO_2 treatments. It would also be useful to compare EPS growth rate and elemental and compound composition among oxygen treatments. These methods were used in broth cultures, so it would be necessary to move to a broth culture O_2-controlled system in order to extract noncellular matrices.

Alternatively, we could create genetic knockout strains of *T. turnerae* that are unable to produce EPS. If EPS plays a critical role in nitrogenase protection from oxygen, these knockout strains would have significantly lower N_2-fixation rates when compared to the wildtype.

CHAPTER 3

NUTRIENT UPTAKE AND RELEASE UNDER DIFFERENT OXYGEN REGIMES IN THE SHIPWORM SYMBIONT *TEREDINIBACTER TURNERAE*

3.1   Introduction

Wood-boring bivalves, or shipworms, can be called "termites of the sea" (Greene, 1994) with of their voracious appetite for N-poor wood. In fact, they are capable of living on a diet of only wood (Gallager et al., 1981), primarily because of an additional N supplement to their diet from N_2-fixing symbionts (Lechene et al., 2006; Lechene et al., 2007). As N_2-fixation occurs within gill bacteriocytes, this symbiosis presents a unique biogeochemical conundrum: the presence of N_2-fixation without a host-symbiont oxygen barrier (Waterbury et al., 1983) and the need for exchange of N products between symbiont and host.

Like other diazotrophs, symbionts might be expected to be susceptible to negative regulation of N_2-fixation by ambient oxygen (reviewed in Dixon and Kahn, 2004). Hence, the host might obtain less fixed N from symbionts if the symbionts were kept in a highly oxygenated part of the gill. Many diazotrophs release dissolved N products, so it is possible that symbionts translocate newly fixed N in the form of dissolved inorganic nitrogen (DIN) or dissolved organic nitrogen (DON) to the host tissue. For example, the symbiotic cyanobacterium *Nostoc* releases 90% or more of the fixed N as NH_4^+ to its angiosperm host *Gunnera* (Silvester et al., 1996). Also, the free-living *Azotobacter vinelandii* releases approximately 5% of total N fixed in the form of glutamate (Kuhla et al., 1985). The release of nitrogenous products appears to be species-dependent and heavily controlled by ambient conditions. Neither the release of dissolved N products nor

the controls on inorganic nutrient use and release have been investigated by shipworm symbionts.

There is limited evidence that newly fixed N may not be translocated to the host in a dissolved nitrogenous form. Instead, fixed N may be incorporated into essential amino acids in the bacteria and the host subsequently digests the N-rich bacteria. Trytek and Allen (1980) incubated isolated gills from the shipworm *Bankia setacea* (Tryon) with [U-^{14}C]glucose, and free and protein-bound pools of essential amino acids become labeled. Moreover, using electron microscopy, they suggested that bacteria inside host bacteriocytes do not multiply, and are digested in the posterior gill mass by phagocytes (Trytek and Allen, 1980). Further work confirming this finding was not published, so the true fate and pathway of new N from diazotrophic symbionts to host tissue is still not well understood.

We used the cultivated symbiont *Teredinibacter turnerae* as a model organism to begin to address the complex nutrient cycling between shipworm symbionts and hosts. This study used biogeochemical approaches to construct an N budget for this species and investigate the role of oxygen on inorganic nutrient use.

3.2 Methods

3.2.1 Cultures

Teredinibacter turnerae (strain T7902; Distel et al., 2002b) was grown in soft agar (0.2% agar) shipworm basal medium (SBM) with sucrose as a carbon source (Waterbury et al. 1983). The medium was modified to reduce the medium ammonium content by an equimolar substitution of ferric citrate for ferric ammonium citrate. We monitored contamination with two methods. First, we struck experimental cultures on LB

(no growth on LB; Distel et al., 2002b) and SBM agar to actively detect growing

contaminants. Second, we used epifluorescence microscopy with DAPI staining.

3.2.2 Experimental Design

Biomass PN, C:N, and δ^{15}N

We used a sterile loop to inoculate 50 mL of broth SBM with cells from a viable

5-day old agar plate. One subset of cultures grew in high concentrations of combined N

(DIN = 5 mM), and a second subset grew in relatively N-depleted conditions (DIN = 18

μM). Every day for 6 days, we sacrificed two replicate cultures for filtration and

measurement of elemental and stable isotopic composition. Cells were collected on a

precombusted (450°C, 4 h) 25 mm Whatman GF/F filter (nominal pore size = 0.7 μm) by

gentle vacuum filtration. The filters were dried in a 60°C oven and pelletized for analysis.

Measurements were made using a CE Elantech NA2400 elemental analyzer interfaced to

a Micromass Optima mass spectrometer for continuous-flow isotope-ratio mass

spectrometry (CF-IRMS).

Particulate N:P ratio

We used a sterile loop to inoculate 50 mL of broth SBM with cells from a viable

5-day old agar plate. One subset of cultures grew in high concentrations of combined N

(NH_4^+ = 5 mM), and a second subset grew in relatively N-depleted conditions (NH_4^+ = 16

μM). We assayed two different initial medium NH_4^+ concentrations in order to determine

the effect of medium NH_4^+ on the time course of particulate N:P during growth. At

regular intervals during growth (approximately every 2-3 days), cultures were sacrificed

in duplicate. The cultures were transferred to a 50 mL centrifuge tube, shaken vigorously

by hand and vortex to homogenize the cells, and were collected on a precombusted

(450^oC, 4 h) 25 mm Whatman GF/F filter (nominal pore size = 0.7 µm) by gentle vacuum filtration. We cannot rule out the possibility that the homogenization process did not cause cells to burst, but the cells and the possible fragments would have been collected on the GF/F filter. The filters were dried in a 60^oC oven.

The particulate N:P ratio was measured on a portion of the filter with the persulfate wet-oxidation method (Raimbault et al., 1999). Briefly, a portion of the filter was added to a glass 250 mL bottle (Wheaton glass) with 40 mL of Milli-Q$^{®}$ water and 5 mL of 6% potassium persulfate. The bottles were autoclaved for 30 minutes with tightly sealed caps to allow for complete conversion of the organic N and P to inorganic NO_3^- and PO_4^{-3}, respectively. After allowing the solution to cool to room temperature, the solution was analyzed for NO_3^- and PO_4^{-3} by flow-injection analysis (Lachat QuikChem 8000; Strickland and Parsons, 1972). We prepared and measured the N:P ratio for a set of peptone (PN) and AMP (PP and PN) standards and filter blanks with each batch of persulfate solution.

Effects of oxygen on nutrient cycling by *T. turnerae*

Teredinibacter turnerae cultures were grown at room temperature in a soft agar medium (0.2% agar) in 28 mL Bellco anaerobic test tubes (Bellco glass 2048-18150) that were sealed with a crimp seal gas-tight rubber septa. There was 15 mL of SBM and 13 mL headspace in each tube. Cultures were grown at room temperature without physical disturbance.

Approximately every 6 days during growth (34 days total), we measured the headspace pO_2, N_2-fixation rate with the acetylene reduction assay on 4 cultures, and sacrificed 3 cultures for cell counts and inorganic nutrient analysis. We carried out

repeated acetylene reduction assays on a subset of cultures during growth. Immediately following the acetylene reduction assay, we sparged the headspace of these cultures to the original pO_2 condition to remove acetylene and ethylene.

3.2.3 Analytical methods

Headspace oxygen conditions

We set up 4 pO_2 treatments which spanned a range from 0.07 atmospheres O_2 to normal atmospheric saturation (0.21 atmospheres). To vary the partial pressure of oxygen in the vessel headspace, we used a gas proportioner (Cole Palmer EW-03218-50) to mix air and N_2. The gas mixture was passed through a 0.45 μM sterile filter, then sparged into the headspace of the culture through a disposable 18 gauge needle. Cultures were sparged long enough to flush the headspace 10x. Direct measurements of dissolved pO_2 in blank media indicate that this method maintains the medium at the appropriate oxygen concentration (Appendix B).

Headspace oxygen measurements

Two mL of headspace was removed from the culture tube with an 18 gauge needle and equilibrated with 6 mL of water in a gas-tight syringe. We measured pO_2 of the liquid phase with a StrathKelvin oxygen meter. We had to correct for residual pO_2 in the gas-liquid equilibration, and convert the concentration measured in solution to the original pO_2 in the gas phase of the equilibrium. For this, we used a standard regression with gas samples of known pO_2 concentration ranging from 0 to 0.21 atmospheres. Immediately following the sampling of the headspace for oxygen concentration, cultures were sparged with a gas mixture to restore the original pO_2 condition.

Acetylene reduction assay

Nitrogen fixation activity was measured with the acetylene reduction assay (Capone and Montoya, 2001), and incubations were carried out in the Bellco anaerobic test tubes. In brief, 3 mL acetylene was added to each vessel, and assay incubations were carried out under standard growth conditions as noted above. The headspace ethylene concentration was measured in duplicate at hourly intervals for approximately 3-4 hours by gas chromatography (SRI model 8610c gas chromatograph, SRI Instruments, Torrance, CA USA). The gas chromatograph was fitted with a 2 m Haysep A column (Alltech Associates Inc., Deerfield, IL USA) and a flame ionization detector (SRI Instruments). We used a 4:1 $C_2H_2:N_2$ molar reduction ratio to convert from acetylene reduction to N_2- fixation rates. We used trapezoidal integration to obtain total N_2 fixed by each culture during growth.

Total protein

We measured total protein in *T. turnerae* cultures with the Bradford assay (Coomassie Plus - The Better Bradford Assay Kit, VWR PI23236; Bradford, 1976). Previous studies with *T. turnerae* have also used protein measurements to quantify culture growth, instead of optical density (Greene and Freer, 1986). To harvest the cells from the soft agar, we pipetted the cell layer from the culture tube. This cell layer slurry was homogenized using ethanol-washed glass beads, vigorous shaking, and vortexing. We added 50 µL of homogenized cells to 1.5 mL Coommassie Blue, and measured the absorbance at 595 nm with a Spectronic 20 Genesys spectrophotometer. Absorbance values of blank medium were subtracted from samples. Total protein values were calculated from a standard curve of bovine serum albumin.

Cell number

After homogenizing the cell layer slurry, we used a DAPI (4', 6-diamidino-2-phenylindole) stain (Sambrook, 2001) to enumerate the culture with a hemocytometer under epifluorescence microscopy. We calculated cellular protein concentration for all days of growth by dividing total protein by cell concentration from the hemocytometer counts.

Dissolved inorganic nutrient analysis

Concentrations of dissolved inorganic nutrients (PO_4^{-3}, NH_4^+, and $NO_3^- + NO_2^-$) were determined by flow-injection analysis (Lachat QuikChem 8000; Strickland and Parsons, 1972). Whole soft agar medium was unsuitable for nutrient analysis with flow-injection analysis because of its high viscosity, so we equilibrated an agar sample with Milli-Q® water, then measured the nutrient content of the supernatant water. Sequential equilibration of standards with DI water allowed us to determine the partitioning coefficient for nutrients in the agar-water system.

Rates of inorganic nutrient uptake and release

Rates of nutrient uptake and release were calculated using a linear regression analysis of the medium nutrient concentration over time. Periods of nutrient decrease reflected nutrient uptake and periods of increased nutrient concentration over time reflected net nutrient release.

3.3 Results

3.3.1 Biomass PN, C:N, $\delta^{15}N$

Teredinibacter turnerae cultures grown in initially high DIN conditions accumulate biomass particulate nitrogen (PN) faster than cultures grown in relatively

DIN-depleted initial conditions (Figure 3.1A, filled circles). Cultures under high initial

DIN conditions (initially 5 mM DIN) appear to accumulate a maximum of 3-4 mmol N L^{-1} biomass. We also measured dissolved inorganic phosphate (DIP) and nitrate (NO_3^-) for

this experiment (data not shown). The cultures depleted DIP (initial concentration 92 ± 5

μM) on the first day, but drawdown of DIP was undetected after day 1. There was no

significant drawdown or accumulation of nitrate in the medium. Cultures grown in

relatively DIN-depleted initial conditions accumulated PN, but did not reach the levels of

high DIN cultures (Figure 3.1A, open circles).

The biomass carbon:nitrogen (C:N) ratio in relatively DIN-depleted cultures

increased steadily during culture growth (Figure 3.1B, open circles). Cultures in high

DIN initial conditions showed an increase in biomass C:N until day 3, and remained

steady thereafter (Figure 3.1B, filled circles).

The initial DIN conditions also heavily influenced biomass stable isotopic

composition ($\delta^{15}N$). Cultures in high initial DIN conditions initially accumulated biomass

that had very low $\delta^{15}N$ values; after day 3, the biomass $\delta^{15}N$ value was enriched as

compared with the blank medium ($\delta^{15}N$ = +1.4; Figure 3.1C, filled circles). Cultures

under relatively DIN-depleted initial conditions had a range $\delta^{15}N$ = -1 to +0.21, which is

similar to the isotopic ratio of other diazotrophic organisms (Figure 3.1C, open circles).

3.3.2 N:P ratios

We investigated the change in biomass N:P ratio during growth with broth

cultures, either under initial high DIN conditions or relatively deplete DIN conditions.

Teredinibacter turnerae broth cultures grown under initially high DIN conditions (5 mM)

accumulated particulate P (PP) rapidly within the first 6 days of growth and the

subsequent decline in PP indicates that P was remineralized (Figure 3.2, open squares). However, we did not detect a significant increase in DIP late during growth. The particulate N:P ratio significantly increased during the period of PP remineralization from 10 on day 3 to over 200 on day 17 (Figure 3.2, closed circles, linear regression, $R^2 = 0.74$, $P < 0.0005$).

The Bradford protein assay was unsuitable for protein measurements of broth cultures under relatively depleted initial DIN conditions (18 μM), most likely as a result of low cell densities during growth. In addition, our cultivation method led to an inconsistent growth pattern such that replicates sacrificed on a single day did not have consistent PP values. Given these cultivation difficulties, we used PP as our measure of culture growth under these initially low DIN culture conditions. Our results showed no clear indication of PP remineralization at any time during this assay (data not shown).

Unlike high DIN cultures, PP concentration and N:P were positively correlated in the relatively depleted DIN cultures for the same time period (Figure 3.3, linear regression, slope = 1.03, $R^2 = 0.57$, $P < 0.0005$). In this batch culture system (assuming that dissolved inorganic phosphate (DIP) is the sole P source), N:P increases when DIP becomes limiting.

We did not measure inorganic nutrient content for this experiment, but similar experiments, under the same culture conditions and for similar length of time, did not provide evidence for DIN release (data not shown).
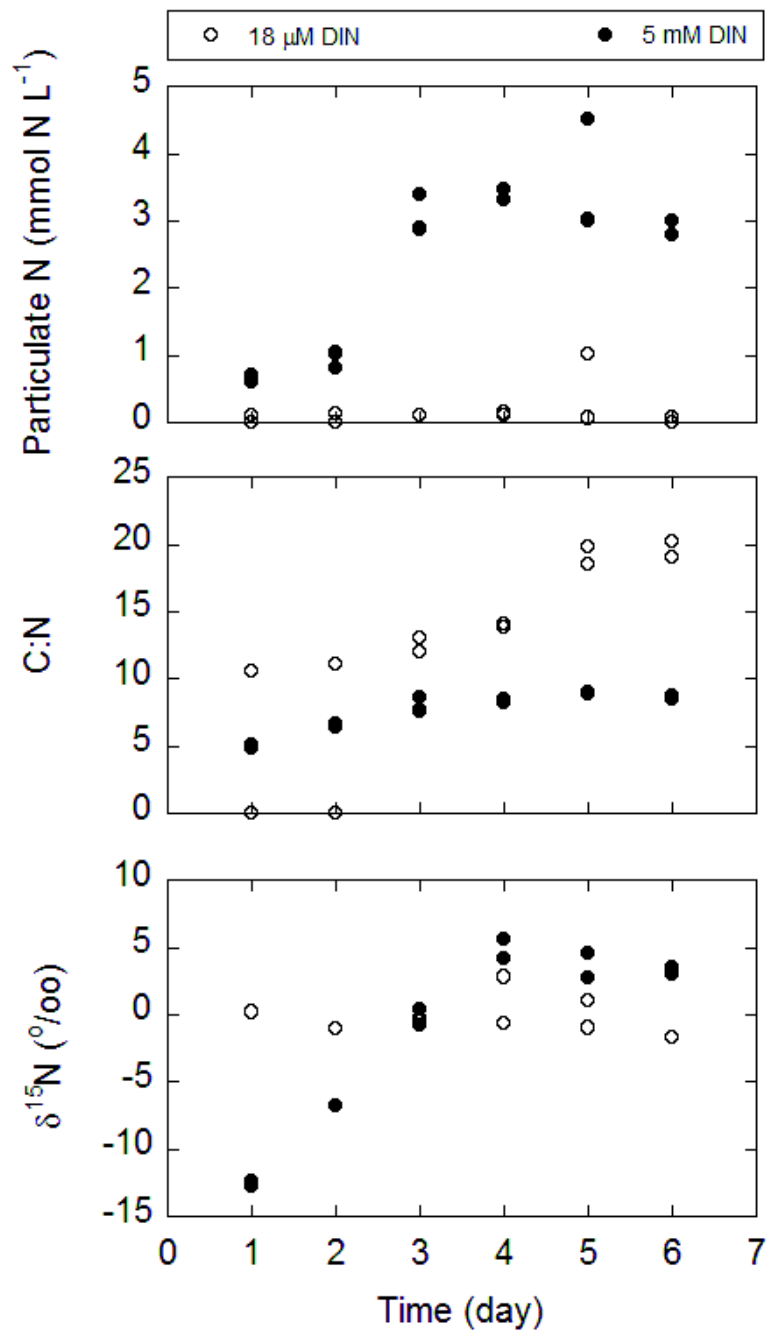
Figure 3.1.The particulate N, particulate C:N, and particulate δ^{15}N of *T. turnerae* biomass was influenced by culture conditions. Legend indicates treatment of the initial DIN conditions in blank medium. Data are measurements from a single culture, n = 2 for each day. In (C), isotopic ratios are measured with respect to atmospheric N.
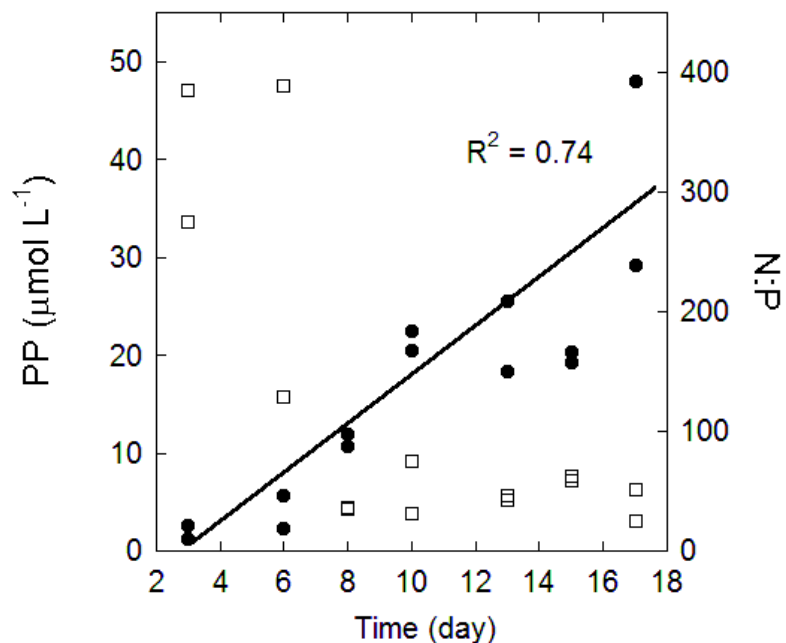
Figure 3.2. The relationship of particulate P (open squares) and particulate N:P (closed circles) during broth culture growth under high DIN initial conditions. Under these DIN conditions, *T. turnerae* rapidly accumulates PP in the first 6 days of growth. After this time, P is preferentially remineralized, and N:P significantly increases (linear regression, $R^2 = 0.74$, P < 0.0005). Data are measurements from single cultures.
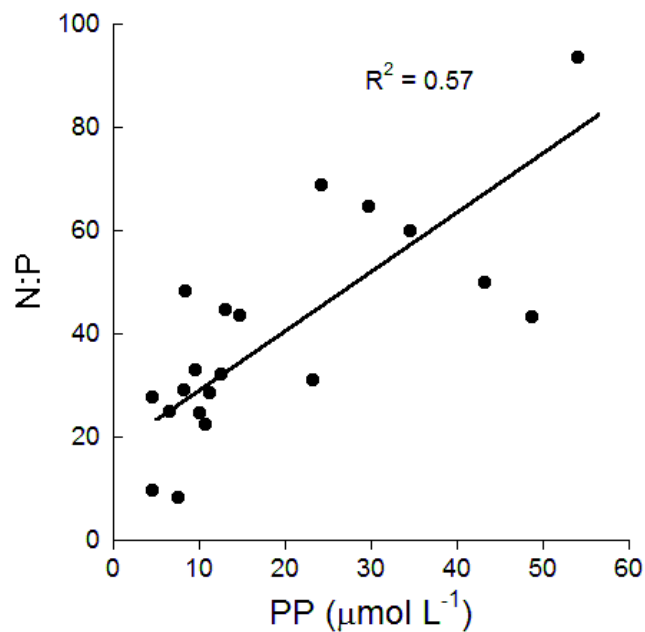
Figure 3.3. For *T. turnerae* broth cultures under relatively N-depleted initial conditions, particulate N:P ratio increased when DIP became limiting (linear regression, $R^2 = 0.56$, P < 0.0005, slope = 1.03). Data are single culture measurements.

3.3.3 Oxygen controls on nutrient usage in soft agar

The cellular protein content in the lower pO_2 treatments was positively correlated with the growth rate during culture growth (Figure 3.4C, D, open triangles; ANOVA with Tukey's post hoc pairwise comparison; 0.07 atm, $P = 0.004$; 0.10 atm, $P = 0.004$). In contrast, the cellular protein content in the higher pO_2 treatments did not significantly change with a decrease in growth rate (Figure 3.4A, B, open triangles with dotted line (mean); ANOVA; 0.14 atm: $P = 0.30$; 0.21 atm: $P = 0.495$).

The maximum growth rate for lower pO_2 cultures was similar to each other (0.07, 0.10, and 0.14 atm) on day 6 and steadily declined thereafter (Figure 3.4C, D, solid lines and closed circles). The 0.14 atm treatment had a similar growth rate to the low pO_2 cultures, but occurred later on day 13 (Figure 3.4B). The 0.21 atm treatment recorded a growth rate double that of the other 3 treatments on day 17 (Figure 3.4A).

We measured DIP concentration in the medium during growth for all pO_2 treatments, then we quantified rates of DIP uptake using linear regression of the DIP concentration between time points and correlated DIP uptake with growth rate. The maximum protein growth rate for the 0.21 atm cultures was significantly higher than the maximum growth rate for the 0.07 atm and 0.10 atm cultures (Table 3.1, Figure 3.5, solid lines; one-way ANOVA, $P < 0.05$). We were able to measure DIP uptake for 3 of the pO_2 treatments (Figure 3.5, dotted lines) during periods of elevated protein growth rate (Figure 3.5, solid lines) and the data is summarized in Table 3.1. The 0.14 atm treatment did not show a significant uptake in DIP during any interval. There was no difference in the interval DIP uptake rates among the 3 pO_2 treatments (ANCOVA, $P = 0.207$, $R^2 = 0.63$).

Shipworm basal medium contained a small amount of $NO_3^- + NO_2^-$ (1.22 µM). In all pO_2 treatments, *Teredinibacter turnerae* consumed $NO_3^- + NO_2^-$ (Table 3.2 and Figure 3.6; linear regression). However, there was no effect of the pO_2 treatment on $NO_3^- + NO_2^-$ uptake rate (ANCOVA, P = 0.975).

Patterns of medium NH_4^+ concentration during *T. turnerae* growth were similar among pO_2 treatments. All pO_2 treatments had a high rate of NH_4^+ uptake during early growth when N_2-fixation was low and NH_4^+ was reduced within the first 6 days. Low NH_4^+ levels persisted during periods of elevated N_2-fixation, and a reappearance of NH_4^+ occurred late in growth when N_2-fixation had declined (Figure 3.7). Low pO_2 ($pO_2 = 0.07$ and 0.10 atm) cultures exhibited a higher concentration of ammonium in the medium on the final day as compared with high pO_2 cultures ($pO_2 = 0.14$ and 0.21 atm; Figure 3.7).

We did not measure DIN concentration or N_2-fixation during the first 6 days of growth and N_2-fixation commences within this period, presumably after a period of DIN depletion. We performed a regression analysis on the NH_4^+ which accumulated in the medium late in growth for each pO_2 treatment to quantify the amount of NH_4^+ released by *T. turnerae*. There was a significant accumulation of NH_4^+ in the medium for all treatments except the 0.14 atm treatment (Table 3.3)
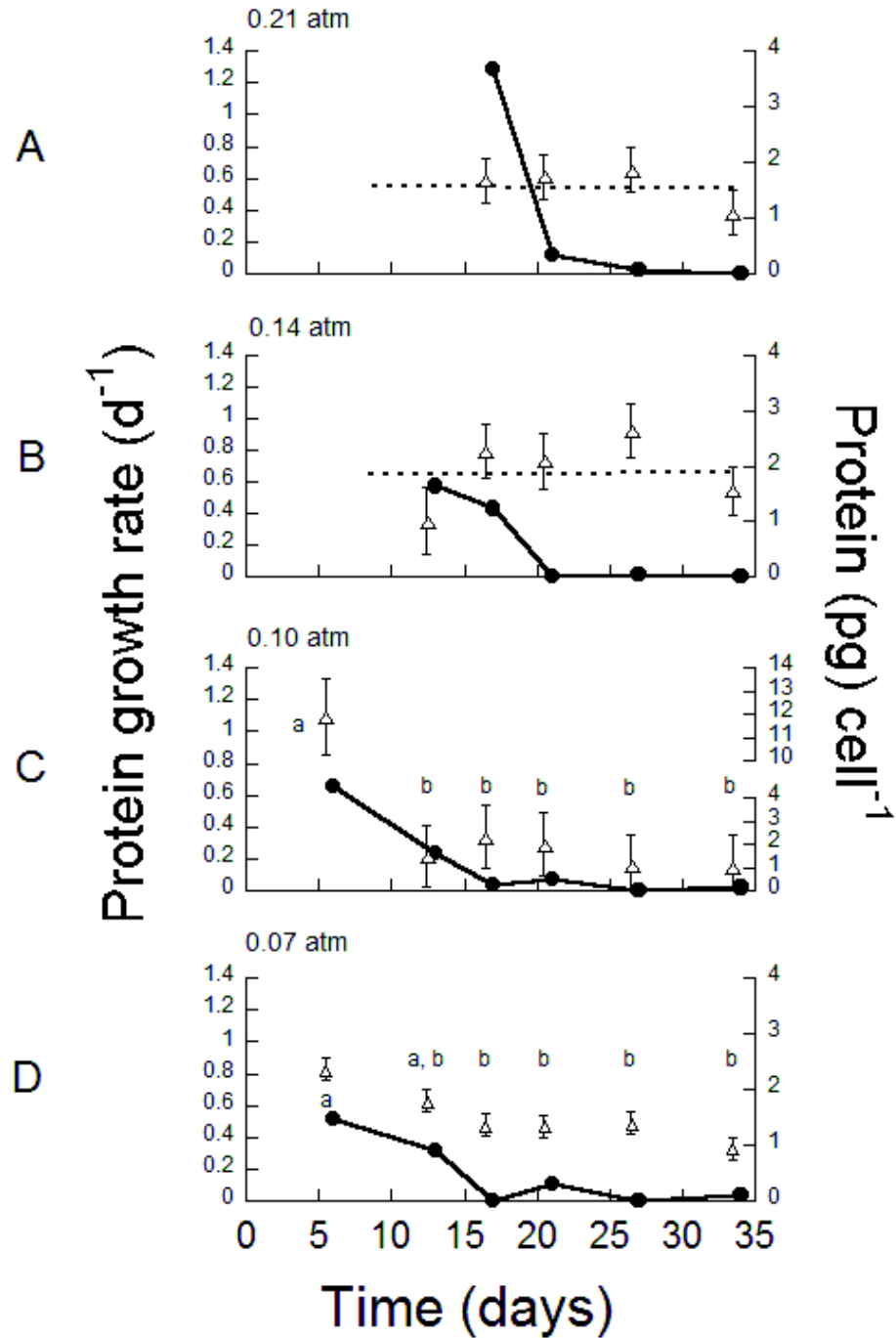
Figure 3.4. Headspace pO_2 affected the timing of the growth rate and the change in cellular protein content during growth. Growth rate: solid circles and solid lines, mean ± 1 SE (note the small error bars), n = 3; protein cell^{-1}: open triangles, mean ± 1 SE, n = 3. Dotted lines indicate the mean cellular protein content during growth. Subscripts indicate statistically significant groupings of cellular protein content for each pO_2 treatment. Data for growth rate and protein cell^{-1} were measured on the same days, and are plotted slightly offset for clarity. Note the broken protein cell^{-1} axis and scale in panel (C).

Table 3.1 Protein growth rate and DIP uptake in *T. turnerae*. Summary of the data in Figure 3.5. "Day" indicates the time period over which that value was calculated. Data for protein growth rate are mean ± 1 SD, n = 3. An asterisk indicates a significant difference in that treatment from the other treatments. P and R^2 values are for the linear regression of DIP disappearance over the "time period" indicated.

	Protein growth rate (d^{-1}) day	DIP uptake (μmol PO_3^{-4} d^{-1}) Time period (days)	P	R^2
0.07 atm	0.52 ± 0.02 6	0.017 0 - 13	0.013	0.61
0.10 atm	0.66 ± 0.02 6	0.012 0 - 13	0.002	0.59
0.14 atm	0.58 ± 0.02 13	NS	NS	NS
0.21 atm	1.28 ± 0.02* 17	0.023 13 - 21	0.002	0.76

Table 3.2 NO_3^- + NO_2^- uptake in *T. turnerae*. Summary of data in Figure 3.6. We used linear regression to quantify the uptake rate. The time period (days), P, and R^2 for each regression are noted.

	NO_3^- + NO_2^- uptake (μmol d^{-1})	Time period	P	R^2
0.07 atm	0.0024	0 to 13	0.045	0.41
0.10 atm	0.0059	0 to 6	0.001	0.89
0.14 atm	0.0027	0 to 13	<0.0005	0.80
0.21 atm	0.0019	0 to 13	0.046	0.46
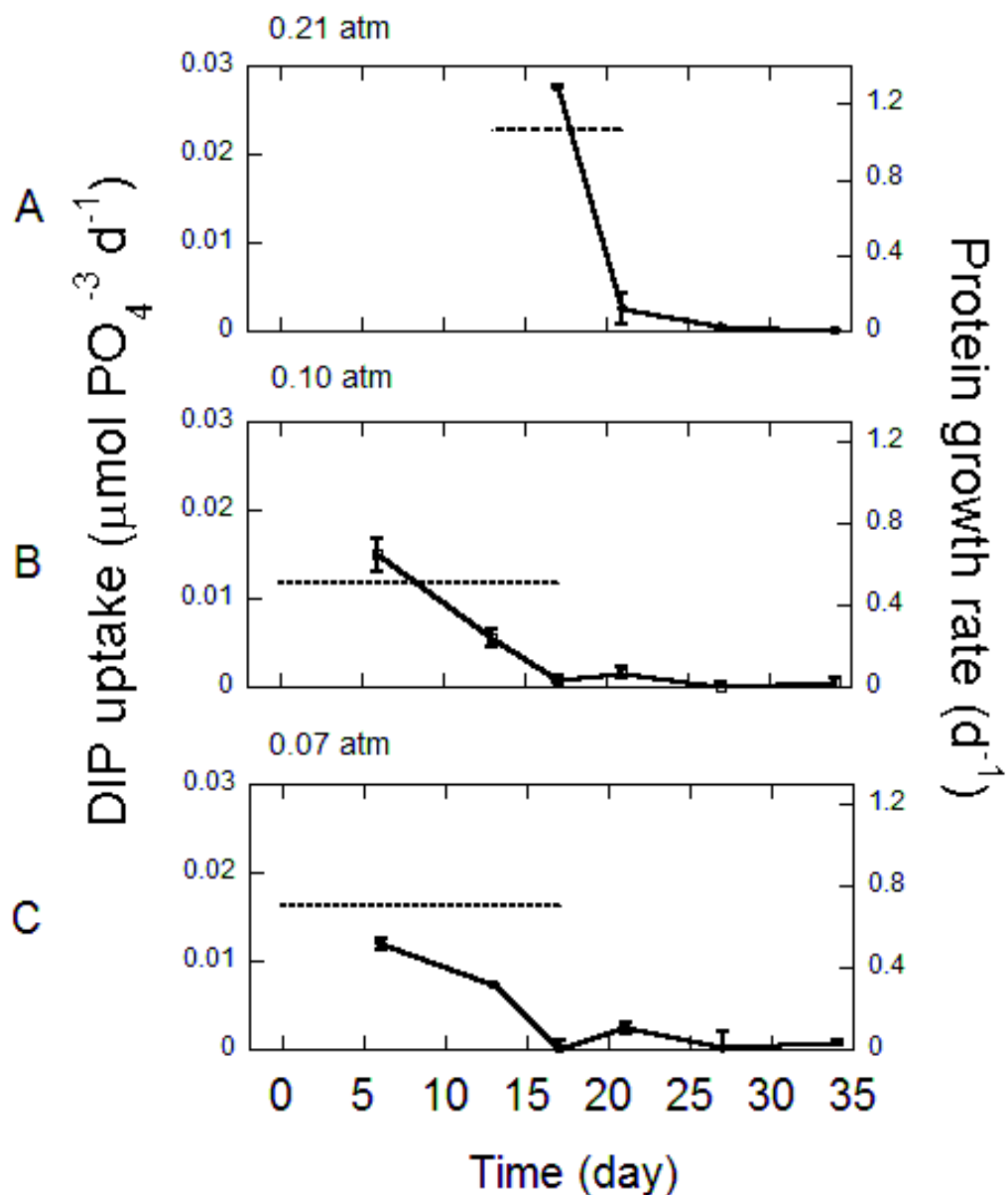
Figure 3.5. DIP uptake covaried with protein growth rate in 3 pO$_2$ treatments. Dotted line = DIP uptake rate for that interval; solid line = protein growth rate (n = 3, mean ± 1 S.D.). DIP uptake was calculated from the linear regression of DIP (n = 3 cultures) during growth and is expressed as constant rate for that interval.
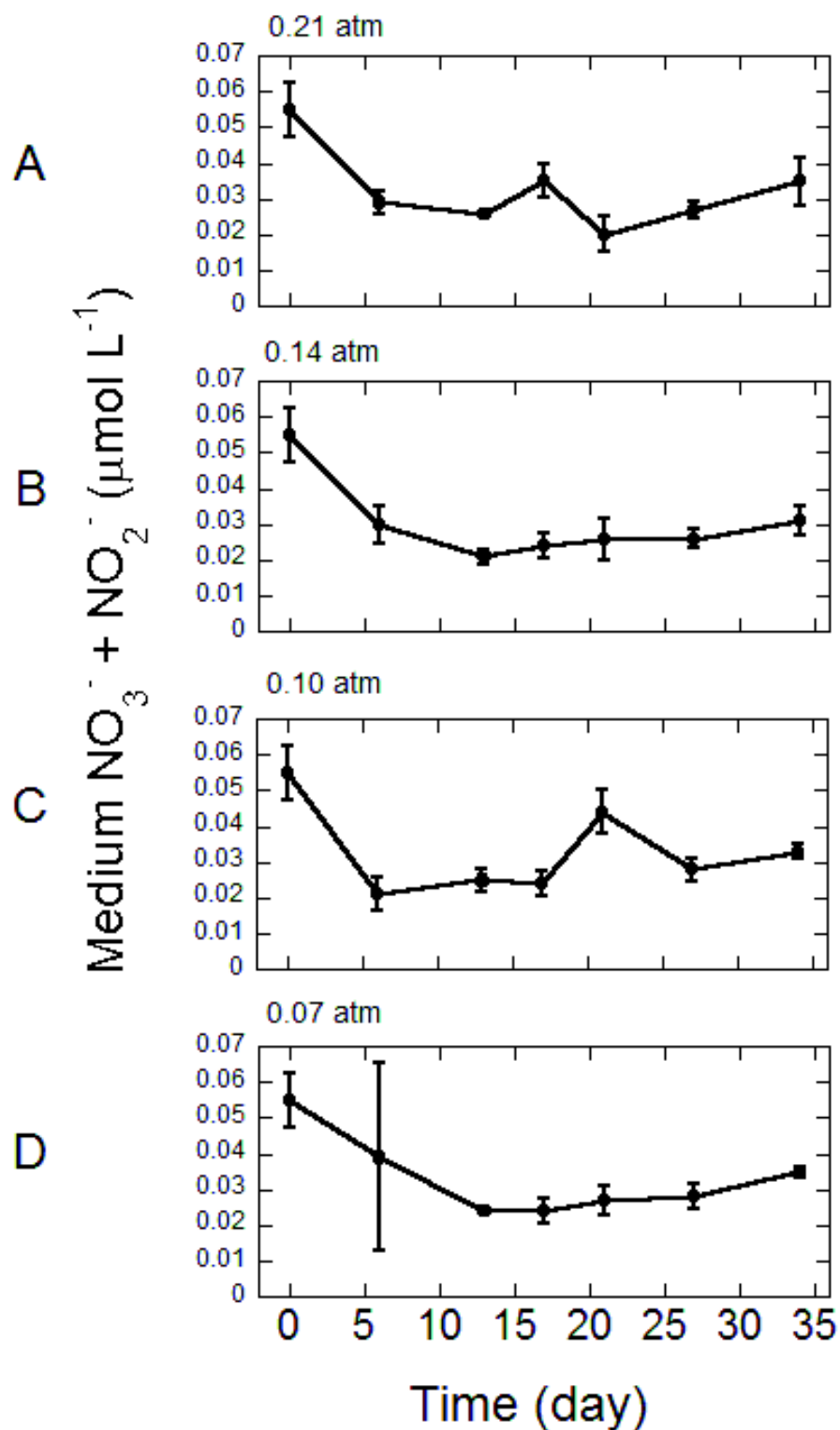
Figure 3.6. *Teredinibacter turnerae* was capable of using $NO_3^- + NO_2^-$ as a source of combined N. Data are mean ± 1 S.D, n = 3.

Table 3.3 NH_4^+ accumulation rate late during growth. We used linear regression to quantify the NH_4^+ release rate. The time period (days), P, and R^2 for each regression are noted.

	NH_4^+ release (μmol d^{-1})	Time period (days)	P	R^2
0.07 atm	0.0225	21 to 34	0.002	0.80
0.10 atm	0.0228	21 to 34	<0.0005	0.77
0.14 atm	ND			
0.21 atm	0.0071	27 to 34	0.032	0.63
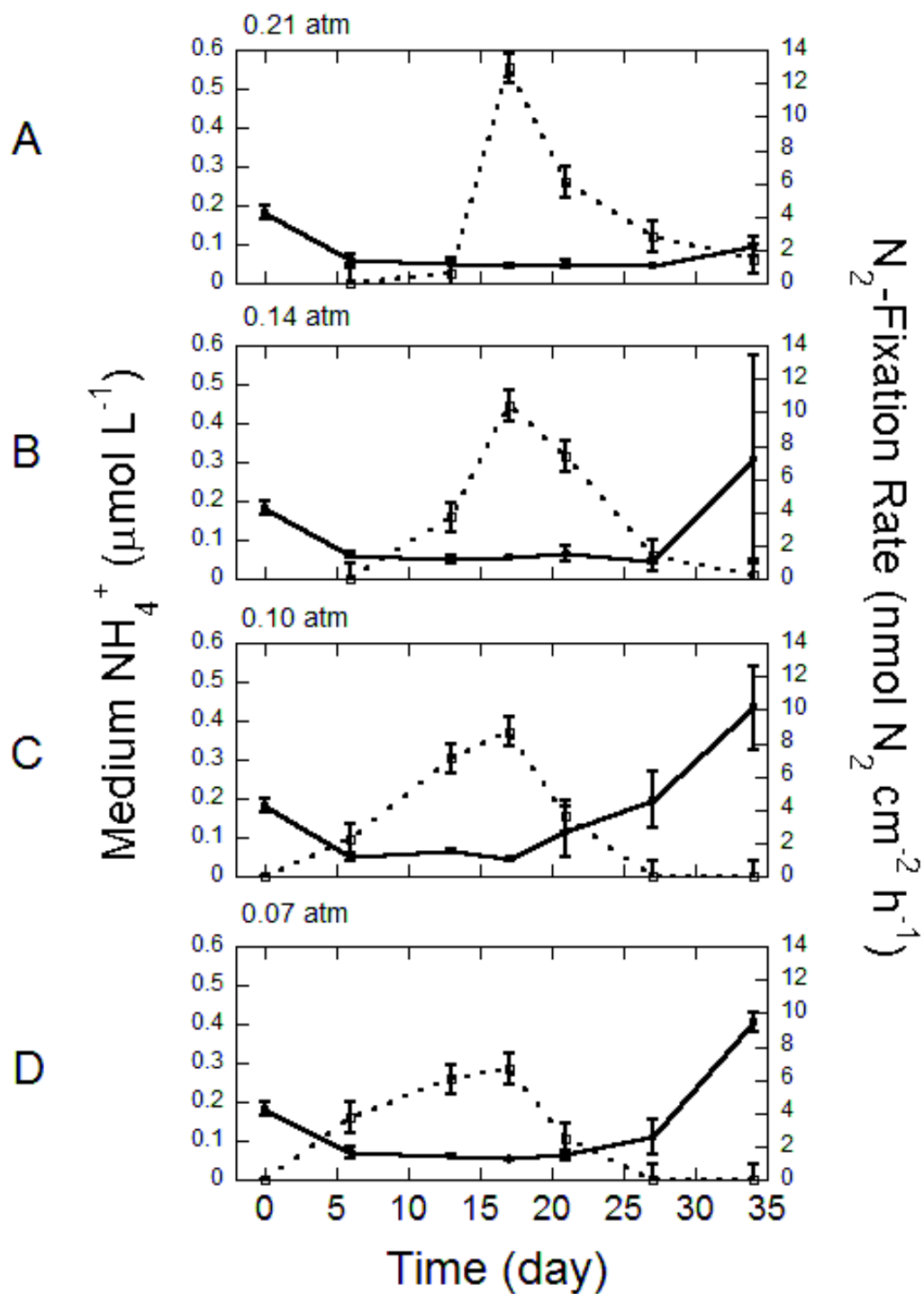
Figure 3.7. The effect of headspace pO_2 conditions and growth upon medium NH_4^+ concentration. Solid lines = medium NH_4^+ (mean ± 1 SD, n = 3); dotted lines = N_2-fixation rate (mean ± 1 SE, n = 4). Cultures that are grown under low pO_2 conditions accumulate a higher concentration of NH_4^+ late during growth than cultures grown at high pO_2 conditions.

3.3.4 Calculations for the allocation of newly fixed N to N pools

Using the assumption that there are only two sources of N available for N-biomass, either DIN or N_2-fixation, we calculated the relative importance of DIN and N_2-fixation for the production of N-biomass. The contribution of N_2-fixation to N-biomass varied with pO_2 treatment. During early growth (day 6) N_2-fixation contributed a higher percentage of N to N-biomass in low pO_2 treatments (0.07 atm and 0.10 atm) compared to high pO_2 treatments (0.14 atm and 0.21 atm; Figure 3.8). The 0.21 atm treatment contined to use DIN for N-biomass until day 17, when the N_2-fixation contribution to N-biomass was 100%.

We then quantified the amount of NH_4^+ released by *T. turnerae* by using linear regression, and we compared this molar quantity to the amount of N_2 fixed by the culture during growth. NH_4^+ released into the medium represented a very small percentage of the total N_2 fixed by the cultures. Moreover, low pO_2 cultures ($pO_2 = 0.07$ atm and 0.10 atm) released a higher percentage of newly fixed N_2 compared with higher pO_2 cultures (Figure 3.9).

We used an N-protein ratio of 4.78 to convert the measured total protein into N present in cellular protein (average for 12 marine microalgae species at all growth phases: 4.78 +/- 0.62; Lourenco et al., 2004). This is lower than the "traditional" N-protein conversion factor of 6.25, first published for N-protein conversion for foodstuffs by Jones (1931). Many authors have argued that the 6.25 conversion factor, based on an N-content of 16% in total protein, is an overestimate for living organisms and does not account for non-proteinaceous cellular nitrogen (Gnaiger and Bitterlich, 1984; Lourenco et al., 2004; Mariotti et al., 2008).

We compared the total protein with the integrated N fixed at each time point in order to estimate how much newly fixed N is converted into protein. There is a significant positive correlation between integrated N from fixation and N in protein for all pO_2 treatments (Table 3.4 and Figure 3.10, linear regression). The slope of the regression line indicates the average amount of newly fixed N that is converted into cellular protein N. All pO_2 treatments convert approximately 25-43% of the total N fixed into protein (Table 3.4). Interestingly, headspace pO_2 does not affect the percentage of N_2 fixed that is converted into N-cellular protein (ANCOVA, $p = 0.109$).

To summarize these data, we constructed an N budget for all measured sources and sinks in each pO_2 treatment (Table 3.5). For cultures grown at $pO_2 = 0.07$ atm, our measurements of N in protein and DIN release ($NH_4^+ + NO_3^-/NO_2^-$) can account for 51.5% of the total N fixed fixed, thus 49.5% of N fixed was not measured. In the 0.10 atm and 0.14 atm cultures, we quantified only 33% of total N fixed in N sinks, thus 67% of total N fixed was unquantified. Cultures grown at 0.21 atm have the highest percentage of newly fixed N unaccounted for in N pools that we measured (73.3%).
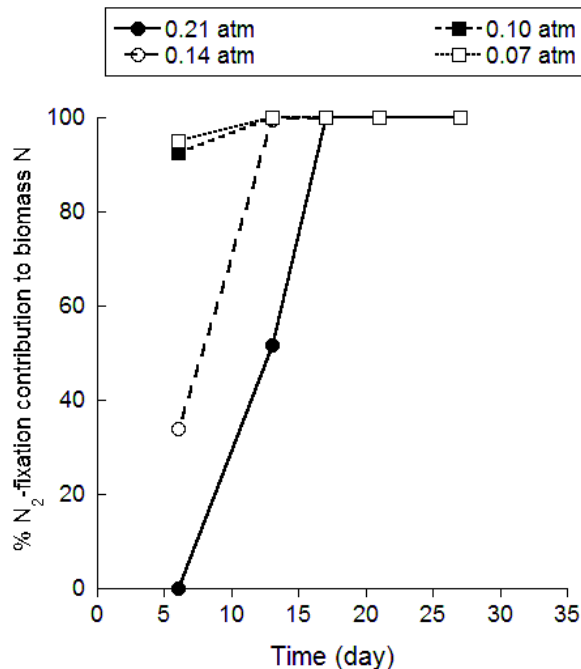
Figure 3.8. Headspace pO$_2$ affected the timing of contribution of N$_2$-fixation to N-biomass during growth. Cultures grown under high pO$_2$ conditions (0.14 and 0.21 atm) are dependent upon medium NH$_4^+$ for biomass early during growth, but use only N$_2$-fixation later during growth. Cultures grown under low pO$_2$ conditions (0.07 and 0.10 atm) derive almost all of N-biomass from N$_2$-fixation for all days measured.
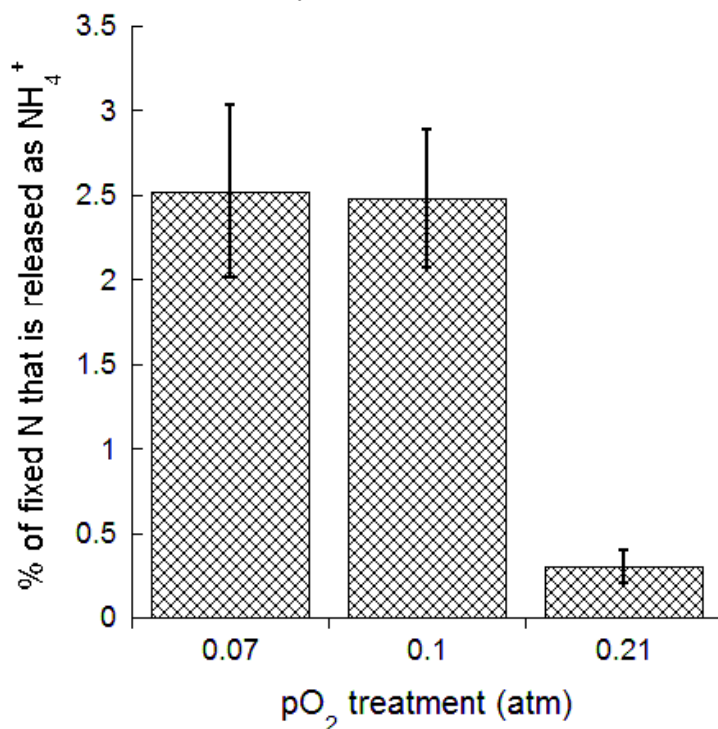
Figure 3.9 A higher percentage of fixed N was released as NH$_4^+$ in cultures grown under low pO$_2$ conditions. We performed a regression analysis of NH$_4^+$ in the medium late during growth, and expressed the released N as a percentage of total N fixed during growth. Error bars represent 95% confidence intervals.

Table 3.4. A significant linear regression of N fixed and N in protein indicates the percentage of fixed N converted into protein.

	% of fixed N converted to protein	P	R^2
0.07 atm	43%	<0.001	0.73
0.10 atm	25%	<0.001	0.77
0.14 atm	28%	<0.001	0.86
0.21 atm	30%	<0.001	0.96
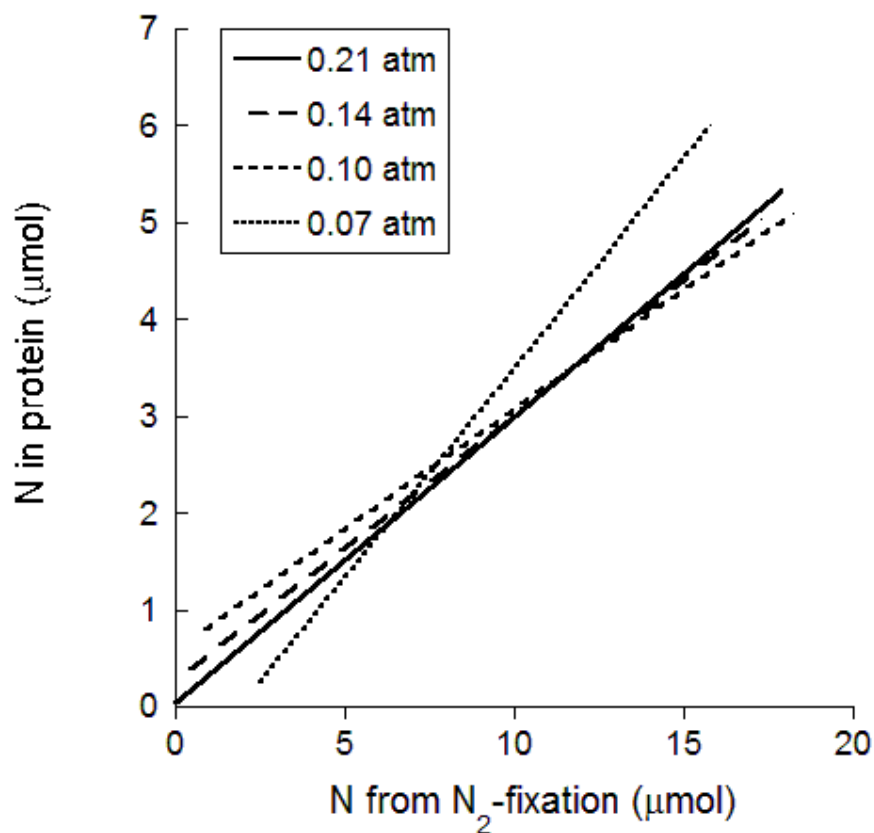
Figure 3.10. Headspace pO_2 did not affect the percentage of N from N_2-fixation that is converted into protein. Each line represents the linear regression of the integrated N from N_2-fixation and instantaneous measurement of N in total protein from 3 cultures of a single pO_2 treatment during growth (6 time points).

Table 3.5. Summary of measured sources and sinks of N for this study. The % fixed N "missing" (excess fixed N) represents the difference between the mean sum of N sources and the mean sum of N sinks. Total DIN uptake and release are calculated from the linear regression of DIN ($NH_4^+ + NO_3^-$ /NO_2^-) of nutrient over time; N from N_2-fixation is an integrated rate; N in protein is the instantaneous measurement on the final day. All measurements are in μmol N.

	Sources of N (μmol)		Sinks of N (μmol)		% fixed N not measured
	Total DIN uptake	N from N_2-fixation	N in protein	Total DIN released	
0.07 atm	0.148	14.1	6.96	0.357	49.5%
0.10 atm	0.169	15.6	4.98	0.388	67.6%
0.14 atm	0.136	15.7	5.05	0.116	67.8%
0.21 atm	0.146	16.5	4.43	0.050	73.3%

3.4 Discussion

This study aimed to construct an N budget for pure cultures of the diazotrophic symbiont of shipworms, *Teredinibacter turnerae*, under different oxygen regimes. Our results indicated that N_2-fixation can support growth in *T. turnerae*, *T. turnerae* biomass N:P is dependent upon medium DIP, and a small quantity of newly fixed N is released as NH_4^+. In addition, headspace oxygen concentration may affect the magnitude and allocation of fixed N to N sinks which were not quantified in this study, such as extracellular biofilm and DON. These additional N sinks may be important components to the host-symbiont exchange, and deserve further consideration.

3.4.1 N:P ratio reflects growth state

Broth cultures grown under conditions of high initial NH_4^+ (5 mM) grew much more rapidly than cultures grown under conditions of relatively depleted initial NH_4^+. This result is similar to the comparison of growth curves by Greene and Freer (1986).

High initial N cultures also attained much higher N:P ratios (range: 10.4 to 239 with an outlier 392.6). We hypothesize that the higher N:P ratios reflect preferential P remineralization because the high N:P values occur later after inoculation and correspond with lower PP values. We did not measure DIP in this experiment presented here, but identical experiments conducted at a different time did show an increase in DIP concentration in the same time period, which could indicate DIP remineralization.

Cultures grown in relatively depleted initial DIN conditions displayed a range of N:P from 8.2 to 69.0, with an outlier of 93.7, and N:P was positively correlated with PP. Our assays demonstrate that N:P changes during growth, in particular, when DIP becomes limiting late during growth, N:P will increase.

This finding agrees with several studies which suggest that instantaneous particulate N:P of microbes does not conform to the Redfield N:P ratio of 16:1 (Redfield, 1934, 1958), and instead particulate N:P is sensitive to the growth state and P availability (Elser et al., 1996; Klausmeier et al., 2004). While Bratbak (1985) measured N:P ratios as low as 5:1 for healthy bacterial cells under a high growth rate, comprehensive studies of the effect of growth and inorganic nutrients on the biomass N:P ratios in cultivated aerobic N_2-fixing proteobacteria (such as *Azotobacter* and *Klebsiella*) are virtually nonexistent. As such, we draw comparisons of our data to the more widely-studied diazotrophic cyanobacterium *Trichodesmium*. Our range of N:P ratio is well within the range reported for *Trichodesmium* batch cultures by Krauk et al. (range: 16 to 140; Krauk et al., 2006) and chemostat cultures by Holl and Montoya (range: 23.4 to 45.9; Holl and Montoya, 2008). Our range is also within the stated range of the optimal N:P ratio (8.2 to 45.0) for phytoplankton, as modeled by Klausmeier et al. (2004). Much of the variation in

biomass N:P ratio can be attributed to relatively N-rich and P-rich ribosomes and N-rich

proteins (Elser et al., 1996; Klausmeier et al., 2004), so ribosome number can be a major

factor in biomass N:P in rapidly growing cultures.

In summary, our results indicate that, like other diazotrophs, *T. turnerae* biomass

N:P is influenced by growth state and DIP availability in batch culture.

3.4.2 High cellular protein

Size of rods

The cellular protein content of *T. turnerae* (range: 0.99 to 2.6 pg protein cell^{-1} and

one high outlier 11.8 pg protein cell^{-1}) was approximately one order of magnitude higher

than the "typical" protein content measured for *E. coli* (0.16 pg protein cell-1 dry weight;

Neidhardt and Umbarger, 1996). Given the typical *E. coli* cell (0.79 μm x 2.43 μm;

Grossman et al., 1982) is approximately the same size of "typical" *T. turnerae* rods (0.4 -

0.6 μm x 3 - 6 μm; Distel et al., 2002b), we calculated the volume of the typical cell

(modeled as a cylinder). The volume of *E. coli* cells is 1.2 μm^3 and the range of volume

of *T. turnerae* rods is 0.4 to 1.7 μm^3. The potential for a 50% higher cell volume in the

larger rods could account for at least some of the higher cellular protein in *T. turnerae*

rods over *E. coli*.

Pleomorphism

Although both Waterbury et al. (1983) and Distel et al. (2002b) report that *T.*

turnerae is normally found as rod with a single polar flagellum (similar morphology to *E.*

coli), and it can show pleomorphism (variation in sizes and/or shapes) and exist as a very

long rod or a spiral in the stationary phase (Waterbury et al., 1983; Distel et al., 2002b).

Neither of the previous studies systematically investigated the environmental effects on

the proportion of particular morphotypes, nor described the frequency of the secondary morphotype. In addition, dimensions for these alternative morphologies are lacking, so the non-rod morphology was not addressed in the above volume calculations. While we did conduct cell counts under epifluorescence microscopy and noticed the presence of both morphotypes under all pO_2 conditions, we did not quantify the frequency of the morphotypes during growth nor can we comment on the relative sizes. In sum, at least some of the elevated protein content relative to published values for similar rod-shaped bacteria results from the presence of a larger morphotype in the culture. Further investigations of the environmental effects on the presence of different morphotypes remain for future work. A relatively simple experiment might involve rearing *T. turnerae* under pO_2-controlled conditions (similar to our experimental set-up), and examining the frequency and distribution of the two major morphotypes during growth and a comparison among the pO_2 treatments.

Secreted extracellular proteins

Excretion of extracellular proteases and other proteins may explain at least some of the increased cellular protein content levels. Since our experimental design for the Bradford assay measured total protein, the assay measured cellular proteins and proteins that may have been secreted into the medium. *Teredinibacter turnerae* is capable of excreting extracellular proteases (Greene et al., 1989; Griffin et al., 1992; Beshay, 2003), but the controls and optimal cultivation conditions are not well-characterized. Greene (1994) suggests three functions for extracellular protease activity in *T. turnerae* in the symbiosis: 1) enhance the hydrolysis of cellulosics of woody tissue and increase the availability of cellulose 2) digest protein for host shipworm, when filter feeding, and 3)

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post-secretory processing of enzymes. Some proteins may be considered part of the dissolved organic nitrogen (DON) pool, which remains an unquantified N sink in this study (see section 3.4.4).

Relationship of cellular protein and growth rate

The frequency of cell division in bacteria shows a positive correlation with cellular protein content (Bremer and Dennis, 1987). Lower pO_2 cultures (0.07 atm and 0.10 atm) had significantly higher cellular protein content during periods of high growth rate. The cellular protein content of the 0.14 and 0.21 atm cultures did not differ during growth, even when the growth rate was much lower late during growth. A critical study of the elevated cellular protein measurements and the curious relationship of growth rate with cellular protein content would involve a revised Bradford assay (see section 3.6.4).

In sum, we detected a statistically significant higher cellular protein content than other microbes of similar size and shape. The range of sizes of T. *turnerae* rods can account for some of the excess protein as well as the presence of larger morphotypes. In addition, our assay detected secreted proteins as well as cell protein, so these may contribute to the elevated content.

3.4.3 The effects of pO_2 on uptake and release of inorganic nutrients

Teredinibacter turnerae cultures exposed to the highest pO_2, 0.21 atm, had a growth rate double that of the other pO_2 treatments over the same period. Interestingly, neither DIP uptake rates (Table 3.1) nor the specific N_2-fixation activity (Figure 2.4A) was higher in the 0.21 atm treatment over other treatments during this period of highest growth rate. Our data did not provide evidence about how an elevated growth rate could be sustained without an elevated DIP uptake and N_2-fixation activity in the 0.21 atm

treatment. Our method was sensitive enough to detect significant changes in nutrient concentration over time. It is possible that particulate C:N:P differed among the pO_2 treatments during periods of elevated growth, but we did not monitor these parameters.

Research in the previous chapter (section 2.4.1) also established that *Teredinibacter turnerae* is reliant upon N_2-fixation for growth and to meet N requirements. This dependence varies early during growth based upon pO_2 conditions, and is similar to the differences in timing of growth seen in Figures 2.1 and 2.2. The onset of N_2-fixation was delayed in cultures under high pO_2 conditions (0.14 and 0.21 atm) compared with cultures under low pO_2 conditions (0.07 and 0.10 atm). DIN was the major source of biomass N (PN) for high pO_2 cultures for a longer period after inoculation, but only because of significant phasing in culture growth.

For all pO_2 treatments, only a small percentage (0.3 to 2.5%) of new N from N_2-fixation was released as NH_4^+ in batch cultures of *T. turnerae*. We were not able to completely rule out the possibility that the increase in medium NH_4^+ may result from cell mortality and lysis. Belnap (2001) argues that in batch cultures which release N, just as much N is released from young cultures as from older cultures. Our data show net NH_4^+ accumulation only in older cultures, so further studies are required to elucidate whether *T. turnerae* actively releases fixed N as DIN (section 3.6.1).

Although there appears to be a wide variation in the quantity of fixed N that is released as NH_4^+ in comparison with other symbiotic microbes, the results of these experiments clearly place *T. turnerae* at the lowest end of N release by well-studied symbiotic microbes. In comparison, the symbiotic cyanobacterium *Nostoc* releases 90% or more of the fixed N as NH_4^+ to its angiosperm host *Gunnera* (Silvester et al., 1996).

Peters (1977) reports that in the cyanobacterium *Anabaena* – fern *Azolla* symbiosis, at least 50% of the fixed N is not incorporated into the symbiont *Anabaena* but is released to the host. However, *T. turnerae* may be more similar to free-living microbes with respect to the amount of N release that was measured in this study. Free-living *A. vinelandii* releases approximately 5% of total N fixed (Kuhla et al., 1985)

For the duration of our assay, cultures reared in low pO_2 conditions (0.07 and 0.10 atm) released a higher percentage of fixed N in the form of DIN into the medium (2.5%) relative to cultures reared at 0.21 atm (0.3%). It is possible that headspace pO_2 could influence NH_4^+ release; diazotrophic *A. vinelandii* releases a lower concentration of NH_4^+ into the medium under higher oxygen conditions (0.168 atm) as compared with lower oxygen conditions (0.021 atm; Kuhla et al., 1985). However, differences in growth rate among the different pO_2 treatments make a direct comparison of DIN release rates difficult. While the low pO_2 treatments accumulated DIN in the medium after N_2-fixation ceased (days 27-34), N_2-fixation was declining, but still measurable, in the 0.21 atm cultures during this interval. A direct comparison of the percentage of N_2-fixation released as DIN by pO_2 treatment would require a comparison of DIN release for cultures under the same physiological conditions.

For purposes of this study, we can compare net release among the different pO_2 cultures as a function of time elapsed since peak N_2-fixation. Both 0.07 atm and 0.21 atm treatments had maximum absolute N_2-fixation on day 17. In a comparison of DIN released after that date, the 0.07 atm treatment did release more DIN than cultures exposed to 0.21 atm O_2. Future studies are necessary to determine if the higher pO_2 cultures excrete the same amount of DIN as lower pO_2 cultures, only later after

inoculation, or if oxygen concentration truly has a direct effect on the efflux of DIN from

T. turnerae.

3.4.4 The allocation of newly fixed N to N pools

The dry weight of an "average" *E. coli* B/r cell is approximately 55% protein

(Neidhardt and Umbarger, 1996), and nitrogen is an important element in the other 45%

of the cell by weight. The biggest cellular non-proteinaceous sinks for new N are N-rich

RNA (20.5%), lipids (9.1%), and metabolites (3.5%; Neidhardt and Umbarger, 1996),

none of which were measured directly in this study. We used the expected 9:11 ratio of

cellular protein:nonprotein to estimate the amount of fixed N in the cellular nonprotein

pool.

We attempted to use elemental stoichiometry and nutrient measurements to

balance the N budget. We were unable to balance the N budget with DIP uptake based on

biomass N:P for two reasons: *T. turnerae* biomass N:P is dependent on growth state and

our experiment was conducted in soft agar, which is suboptimal for biomass N:P

measurements.

To determine the error in our N budget, we used an error propagation approach

for all measurements (integrated N fixed during growth, N in protein, and N in non-

protein). We expressed the quantity of fixed N not accounted for in biomass as a percent

of the total fixed N ("fixed N excess", Table 3.6). We did not include DIN uptake or DIN

release in the error analysis of the N budget because their contribution as an N source and

N sink was small. Our calculations indicate that the N budget may be close to balanced in

the low pO2 treatment (0.07 atm, Table 3.6), and there is significant excess of fixed N

relative to biomass in the other pO_2 treatments (Table 3.6). We consider two additional

sinks for new N that were not measured in this study, which may help close the N budget:

exopolymeric substances, or excretion as dissolved organic nitrogen (DON).

Table 3.6. Revised estimation of fixed N excess in pO$_2$ treatments of *T. turnerae* based on error analysis. Ranges were calculated using an error propagation approach. All measurements are units μmol N.

	N source (μmol)	N sinks (μmol)		Mean Estimated fixed N excess	Range of fixed N excess
	Total N fixed	N in protein	N in non-protein		
0.07 atm	10.8 – 17.4	5.6 – 8.3	4.6 – 6.9	10%	2 – 19%
0.10 atm	12.8 – 18.5	4.0 – 6.0	3.3 – 4.9	42%	36 – 47%
0.14 atm	12.4 – 19.0	3.5 – 6.6	2.9 – 5.4	41%	34 – 48%
0.21 atm	15.6 – 17.4	3.8 – 5.1	3.1 – 4.1	51%	49 – 53%

Unquantified N sink: Protective extracellular O$_2$-barrier

Newly fixed N may also have been incorporated into the nonproteinaceous part of the biofilm matrix, termed exopolymeric substances (EPS). Since the EPS is mostly comprised of polysaccharides and would not be measured with the Bradford assay, we did not quantify it as an N sink. Most N$_2$-fixing aerobes have some form of EPS (Jarman et al., 1978), which forms much of the structural components of biofilms and are responsible for its functional integrity (Wingender et al., 1999; Davey and O'Toole, 2000). EPS are a major part of most bacterial communities, and the structure, composition, and thickness of the EPS are not static and can be influenced by culture conditions. EPS can account for 50 – 90% of the total organic matter (Characklis and Wilderer, 1990). However, the N content of the noncellular biofilm matrix is not static,

and the matrix N content can be approximately 2.0 – 2.2% of the dry weight for non diazotrophic organisms (Delatolla et al., 2008).

EPS has been directly implicated with protection of nitrogenase. In one study, Prosperi (1994) found the cyanobacterium *Nostoc cordubensis* to be capable of maintaining nitrogenase activity under atmospheric conditions only when heterocysts are enveloped in a biofilm layer (the author uses the term "mucilage"). The rate of EPS synthesis in *Azotobacter vinelandii* increases during N-limited growth (Jarman et al., 1978). Also in using *A. vinelandii*, Sabra et al. (2000) demonstrated that a diazotrophic bacterium can increase the thickness and composition of the EPS barrier under conditions of elevated oxygen. Similarly, Dewanti and Wong (1995) found that *E. coli* O157:H7 formed thicker biofilms in a minimal salts medium as compared to trypicase soy broth and Bacto peptone medium. Previous research with other diazotrophic microbes indicates that EPS can be an important component to the culture and is influenced by cultivation conditions.

Several studies reported the presence of biofilms in *T. turnerae* cultures (Ferreira et al., 2001; Distel et al., 2002b; Trindade-Silva et al., 2009), and the extracellular proteins can account for up to 16% of the biomass weight (Ferreira et al., 2001). No study has yet determined a function of these biofilms in *T. turnerae*, although we propose that it serves as an O_2-protective layer. This experiment provides indirect evidence that *T. turnerae* secretes extracellular proteins and polysaccharides to form a biofilm that serves as an O_2-protective barrier. First, the C-rich EPS layer would contribute to a high C:N through the increased C:N ratio of *T. turnerae* under relatively DIN-depleted conditions over cultures in high initial DIN conditions and its increase during growth.

Second, the results of the effect of oxygen on cellular protein content give some indication that fixed N from *T. turnerae* may be deposited as EPS and O_2-protective barrier. Since our Bradford assay measured total protein, it included cellular proteins and proteins that may have been secreted into the medium. Also, we cannot rule out the possibility that *T. turnerae* may have an extra demand for protein to conduct N_2-fixation; in fact, up to 20% of cellular protein may be involved with N_2-fixation in other diazotrophs (Dixon and Kahn, 2004). Even with this possibility taken into account, there is still excess protein in cultures of *T. turnerae*.

Third, the presence of a trend of an increasing pool of missing fixed N as a function of headspace pO_2 makes an intriguing argument for a high proportion of newly fixed N being secreted as EPS. It is plausible that cultures under an atmosphere of high O_2 may secrete a thicker EPS layer, hence using more new N, to serve as a protective barrier for nitrogenase. In fact, this phenomenon of increased extracellular protective layer under high oxygen conditions has been demonstrated in *A. vinelandii* (Sabra et al., 2000).

Unquantified N sink: Dissolved organic nitrogen (DON)

The fact remains that shipworm symbionts provide newly fixed N to the host shipworm and the mechanism is unclear. There is little evidence that new N is transferred from symbionts to host through phagocytosis of N-rich symbionts (Trytek and Allen, 1980). Also, the digestion of symbionts is known as a method of nutrient exchange in the symbiotic tubeworm *Riftia pachyptila* (Van Dover, 2000) and in bivalves with methanotrophic mussels (Streams et al., 1997).

The results of this study indicate that the symbiont *T. turnerae* is capable of excreting only a very small percentage of newly fixed N as DIN. A final, yet uncharacterized, method for N transfer from symbionts to host could be the translocation of excreted DON from N_2-fixing symbionts.

Bacteria are better known as remineralizers of DIN, but some are capable of excreting DON. *Azotobacter vinelandii* can excrete up to 11% of its total protein in the form of glutamate (Kuhla et al., 1985). Interestingly, cultures under a higher concentration of oxygen excrete more glutamate per cell (Kuhla et al., 1985). N_2-fixing symbionts of soybeans excrete alanine from the bacteroid nodules (Waters et al., 1998). Several studies suggest that a significant fraction of new N is excreted as DON in the cyanobacterium *Trichodesmium*. This fraction may vary among natural and laboratory populations and with cultivation conditions, and reported values range from 37 to 90% of total N fixed (Capone, 1994; Glibert and Bronk, 1994; Mulholland and Capone, 1999; Mulholland and Bernhardt, 2005). Not all authors agree that *Trichodesmium* releases a significant fraction of new N as DON; Holl and Montoya (2008) used N budget constraints to conclude that *Trichodesmium* did not release DON in their continuous culture conditions. Collectively, these studies show that the amount of DON release depends on the organism and is strongly influenced by cultivation conditions.

We attempted to quantify DON excretion on several occasions in broth cultures under both initially high and relatively deplete N conditions. Although we used the well-known persulfate oxidation technique to quantify total dissolved nitrogen (TDN; Valderrama, 1981; Glibert and Bronk, 1994; Bronk et al., 2000; Hagedorn and Schleppi, 2000), this technique proved suboptimal in shipworm basal medium (SBM) in several

trials. Briefly, after persulfate digestion of sterile SBM (blank medium), an abnormally high measurement was reported for NH_4^+ during flow-injection analysis (Lachat QuikChem). Such increases in free NH_4^+ did not occur in a series of DON standards with glycine and urea. We used the methods of Sigman (1997) with sterile SBM to determine that it was indeed an excess combined N which caused high NH_4^+ readings, and not the presence of another chemical that absorbed maximally at the same wavelength. The NH_4^+ concentration increased with the addition of persulfate reagent, and the presence of sucrose contributes the most to this rise. The curious increase in NH_4^+ after persulfate digestion of sterile SBM is probably an interference, and it prevented us from accurate interpretation of DON measurements. We analyzed a few samples using the high temperature oxidation method in the E. Ingall lab at GT, but the experimental design was flawed, and the sample size was small.

Even without direct evidence for the release of DON, our study provides several lines of indirect evidence for the production of DON. First, DON was an unquantified N pool in our study, and, a pool of N is unaccounted for in the higher pO_2 treatments.

Second, the data from $\delta^{15}N$ of biomass indicates that N is excreted from *T. turnerae*. Cultures under high initial DIN conditions exhibit a pattern consistent with isotopic fractionation during uptake from a finite DIN pool. Early during growth, biomass $\delta^{15}N$ increases for 3 days, but biomass $\delta^{15}N$ exhibits a different pattern after day 3. Biomass is actually enriched in ^{15}N over that of the blank medium during stationary phase. This pattern likely reflects excretion of an N-rich product depleted in ^{15}N, which leaves the remaining biomass enriched in ^{15}N.

3.5   Conclusions

To our knowledge, this is the first study to investigate the elemental stoichiometry, stable isotopic composition, and inorganic nutrient use by a symbiont of shipworms. In addition, we examined the effect of oxygen on nutrient cycling and established a partial N budget based on N_2-fixation rates, protein deposition, and inorganic nutrients. Our study provides direct evidence that *T. turnerae* can use combined N for biosynthesis and that a small amount (0.3 to 2.5%) of newly fixed N is excreted as NH_4^+. There is some excess fixed N that we were not able to measure in this assay, but the N budget is partially balanced. Future studies are required to balance the N budget by identifying the remaining N sinks, such as DON excretion and incorporation into EPS, and quantifying their magnitude, which may be influenced by oxygen concentration. Given the small amount of NH_4^+ release and potential for a significant DON pool, we suggest that future studies focus on the possibility of DON excretion or the digestion of symbionts as methods of N translocation to hosts.

3.6   Future directions

3.6.1 Gather direct evidence for the release of fixed N as DIN

Further experiments, especially isotopic tracer experiments, are required to help elucidate whether newly fixed N is indeed actively released as DIN or if the accumulation in DIN results from cell lysis in old cultures. The $^{15}N_2$-fixation technique (Montoya et al., 1996) could be used to trace new N into organic matter during culture growth. At the same time, the isotopic value of the DIN ($\delta^{15}N$) could be measured during growth (Sigman et al., 1997). A significant increase in the isotopic composition of the DIN would indicate release of newly fixed N. Based on our results, we would compare the

amount of fixed N released among pO_2 treatment groups to determine if headspace O_2 has an effect on DIN release.

3.6.2 DON quantification

Further analyses should begin by attempting to use another technique to analyze the DON concentration through the measurement of TDN and subtraction of dissolved inorganic N (Lachat QuikChem). Both the UV oxidation and high temperature oxidation methods (Bronk et al., 2000) appear to be good starting points to measure TDN; however, Bronk et al. (2000) reported that these techniques have lower yields. In addition, the high temperature catalytic oxidation method of TDN quantification (Alvarez-Salgado and Miller, 1998) has been used successfully on marine samples using a Shimadzu TOC machine coupled to an Antek NO analyzer (Weston et al., 2006). Future studies that investigate the ability of *T. turnerae* to excrete DON, and particularly under which culture conditions, may not only help elucidate how new N is transferred to shipworm hosts, but also may contribute insights into some of the controversy of DON release by diazotrophs.

3.6.3 EPS quantification

Future studies to quantify the noncellular biofilm matrix as an N sink should focus on extracting the matrix from the cells and quantifying the weight and total N content, using methods similar to those presented in Delatolla et al. (2008). Once the technique for separation of EPS from cells has been adapted for *T. turnerae*, a comparison of weight and N content among pO_2 treatments would help elucidate whether more new N is deposited as EPS and used as a protective barrier under higher oxygen conditions.

3.6.4 Close the N budget

Direct measurements of N sinks

Future studies of nutrient use and controls of environmental conditions on N_2-fixation and N release by *T. turnerae* need to focus on the potential N budget gap under high pO_2 conditions. Our data suggests that the allocation of new N to N sinks depends upon oxygen concentration.

First, the *T. turnerae* N-protein ratio should be established, as this is possible source of error in our calculations. We conducted an experiment to measure this ratio in May 2010 by correlating the Bradford total protein concentration to biomass N content. We obtained an N-protein conversion factor of 2.59 ± 1.01 during growth of *T. turnerae*, which is much lower than the published range of N-protein conversion factors for microbes. Our experimentally determined N-protein conversion factor assay and outcome was not adequate for use this study.

Second, other important N sinks should be clarified and quantified. There are three major components to this study: quantification of N incorporated as EPS (discussed in 2.6.4), a revision of the protein measurement that was used in this study, and quantification of DON excretion.

We applied the Bradford assay to the whole culture, after homogenization with glass beads to break up the biofilms. We interpreted the resultant measurement to represent the "total protein," inclusive of any extracellular secreted proteins and EPS. It would be useful to establish a protein measurement for the cells and another for noncellular components. In the revised protocol, we would induce cell lysis through chemical treatment, heat shock, and centrifuge, then collect the cells for protein measurement. Using *T. turnerae*, Greene et al. (1989) distinguished the cell protein from

cell-free protein after centrifuging the culture; they applied the Lowry method for cell protein measurement and the Bradford assay for cell-free protein.

Direct measurements of the DON pool is necessary, and revisions to the techniques in these experiments are discussed in section 3.6.2. Additionally, a more balanced N budget using the above revised measurements and elemental stoichiometry would help constrain the DON pool without direct measurement.

Use elemental stoichiometry

In addition, the elemental stoichiometry also needs to be considered to confirm our partially balanced the N budget. Our research showed that cellular N:P ratio was dependent upon DIP availability, and we cannot rule out the possibility that N:P is also influenced by oxygen concentration. C:N and N:P stoichiometry can influence the release of NH_4^+, and it appears that the stoichiometry conducive for NH_4^+ release is not consistent among species (Goldman, 1987; Tezuka, 1990; Elser et al., 1995). We were unable to find any studies that directly investigated the influence of oxygen on the C:N or N:P ratio in N_2-fixing bacteria.

Any future experiment to investigate the effect of growth rate, nutrient limitation, and N_2-fixation on biomass N:P ratio in *T. turnerae* would require the use of broth cultures. We were unable to obtain biomass N:P ratios from the soft agar cultures in this study because of the difficulty of filtering the culture on a GF/F (a widely accepted method to collect biomass and measure N:P with the persulfate oxidation method). Although our broth culture investigations clearly demonstrated a dependence of N:P on DIP and growth state, these cultures were physically quite different from our pO_2-controlled soft agar cultures. Hence, we were not able to calculate an accurate mass

balance calculation of DIP drawdown using the broth culture N:P ratios. A more accurate

N budget would require a significant investment of time in order to develop optimal

cultivation techniques and techniques for manipulation of the broth cultures, which form

thick biofilms.

CHAPTER 4

NITROGEN FIXATION IN THE DIAZOTROPHIC SHIPWORM SYMBIONT *TEREDINIBACTER TURNERAE* IS INSENSITIVE TO CHANGES IN HEADSPACE OXYGEN CONCENTRATION

4.1   Introduction

In the shipworm symbiosis, N_2-fixation from symbionts provides new N to host shipworms to supplement the host's N-poor wood diet (Gallager et al., 1981; Lechene et al., 2006; Lechene et al., 2007). Symbionts reside within specialized gill bacteriocytes (Waterbury et al., 1983; Distel, 2003); the locale provides a unique N_2-fixation/oxygen conundrum because there is no clear mechanism to protect symbionts from oxygen, either in host tissue or in the surrounding seawater (Turner, 1988). Turner (1988) suggests that the oxygen conditions in the area of the symbionts are likely to be reflective of the oxic conditions inside the shipworm burrow. There is a strong possibility that those oxic conditions in the burrow will change according to the host activity and respiration or flushing rate of the burrow with ambient seawater.

In most other diazotrophic microbes, an increase or decrease in oxygen concentration has rapid and dramatic effects on the nitrogenase enzyme and transcription of the *nif* operon. Exposure to oxygen causes irreversible conformational changes in the highly conserved nitrogenase enzyme. Dinitrogenase reductase has a surface exposed [4Fe-4S] cluster that bridges the two subunits of the nitrogenase; interaction with molecular oxygen readily oxidizes the [4Fe-4S] cluster, causing a conformational change, and rendering the enzyme inactive (Hill, 1988; Peters et al., 1995). The dinitrogenase subunit (FeMo protein) has 2 metal centers, a P cluster and a FeMo cofactor, used for substrate reduction, each of which are oxidized by molecular oxygen (Hill, 1988; Dixon

and Kahn, 2004). Also in the presence of elevated ambient oxygen, the two-component

regulatory proteins NifA and NifL interact to inhibit the transcription of the *nif* operon

(Dixon, 1998). A decrease in oxygen concentration, or exposure to anaerobic conditions,

is usually expected to have a negative impact on N_2-fixation in aerophilic and

microaerophilic diazotrophs as well because ATP generation through aerobic respiration

will decrease in an oxygen-depleted atmosphere (Linkerhagner and Oelze, 1995;

Linkerhagner and Oelze, 1997). Although the exact magnitude of response and amount of

oxygen necessary to affect the nitrogenase enzyme differs somewhat among species, the

rapid (timescale of minutes) shut off of N_2-fixation in response to oxygen is well-

documented among diazotrophic bacteria (Hill, 1988; Marchal and Vanderleyden, 2000;

Dixon and Kahn, 2004).

We used the cultivated symbiont *Teredinibacter turnerae* (Waterbury et al., 1983;

Distel et al., 2002b) as a model shipworm symbiont to investigate the response of

symbiont N_2-fixation to altering oxygen conditions. Our results indicate that *T. turnerae*

N_2-fixation activity is unaffected by an abrupt manipulation in headspace oxygen. We

discuss the probable mechanisms that *T. turnerae* may use to protect the nitrogenase

enzyme. This study gives us more insight that shipworm symbionts may be capable of

sustaining N_2-fixation in the face of changing environmental conditions.

4.2   Methods

4.2.1 Cultures

Teredinibacter turnerae (strain T7902; courtesy of D. Distel; Distel et al., 2002b)

was grown in soft agar (0.2% agar) shipworm basal medium (SBM) described by

Waterbury et al. (1983) with sucrose as a carbon source. The medium was modified to

reduce its ammonium content by an equimolar substitution for the iron source; we used ferric iron citrate instead of ferric ammonium citrate. We grew *T. turnerae* in air-tight Bellco anaerobic test tubes (Bellco glass 2048-18150) that were sealed with a crimp seal gas-tight rubber septa. Cultures were grown at room temperature without physical disturbance. We monitored contamination with two methods. First, we struck experimental cultures on LB (does not grow on LB; Distel et al., 2002b) and SBM agar to actively detect growing contaminants. Second, we used epifluorescence microscopy with DAPI staining.

4.2.2 Analytical methods

Oxygen treatments

We set up 4 pO_2 treatments which spanned a range from 0.07 atmospheres O_2 to normal atmospheric saturation (0.21 atmospheres). To vary the partial pressure of oxygen in the vessel headspace, we used a gas proportioner (Cole Palmer EW-03218-50) to mix air and N_2. The gas mixture was passed through a 0.45 μM sterile filter, then sparged into the headspace of the culture through a disposable 18 gauge needle. Cultures were sparged long enough to flush the headspace 10x. The cultures were sparged frequently enough to maintain a constant headspace pO_2 (every 2-3 days). Direct measurements of dissolved pO_2 in blank media indicate that this method maintains the medium at the appropriate oxygen concentration (Appendix B).

Headspace oxygen measurements

Two mL of headspace was removed from the culture tube with an 18 gauge needle and equilibrated with 6 mL of water in a gas-tight syringe. We measured pO_2 of the liquid phase with a StrathKelvin oxygen meter. We had to correct for residual pO_2 in

the gas-liquid equilibration, and convert the concentration measured in solution to the original pO_2 in the gas phase of the equilibrium. For this, we used a standard regression with gas samples of known pO_2 concentration ranging from 0 to 0.21 atmospheres. Immediately following the sampling of the headspace for oxygen concentration, cultures were sparged with a gas mixture to restore the original pO_2 condition.

Acetylene reduction assays

Nitrogen fixation activity was measured with the acetylene reduction assay (Capone and Montoya, 2001), and incubations were carried out in the Bellco anaerobic test tubes. In brief, 3 mL acetylene was added to each vessel, and assay incubations were carried out under standard growth conditions as noted above. The headspace ethylene concentration was measured in duplicate at hourly intervals for approximately 3-4 hours by gas chromatography (SRI model 8610c gas chromatograph, SRI Instruments, Torrance, CA USA). The gas chromatograph was fitted with a 2 m Haysep A column (Alltech Associates Inc., Deerfield, IL USA) and a flame ionization detector (SRI Instruments). We used a 4:1 C_2H_2:N_2 molar reduction ratio to convert from acetylene reduction to N_2- fixation rates.

Total protein

We measured total protein in *T. teredinibacter* cultures with the Bradford assay (Coomassie Plus - The Better Bradford Assay Kit, VWR PI23236; Bradford, 1976). To harvest the cells from the soft agar, we pipetted the cell layer from the culture tube. This cell layer slurry was homogenized using ethanol-washed glass beads, vigorous shaking, and vortexing. We added 50 µL of homogenized cells to 1.5 mL Coommassie Blue, and

measured the absorbance at 595 nm with a Spectronic 20 Genesys spectrophotometer. Total protein values were calculated from a standard curve of bovine serum albumin.

4.2.3 Experimental design

First, we measured headspace pO_2 to ensure that all replicate cultures experienced the same initial pO_2. Then, we randomly divided the replicates into the following treatments: control (same headspace pO_2) and one or two pO_2 treatments higher and/or lower than the original pO_2. The culture headspace was sparged with the appropriate pO_2 treatment to flush the headspace 10x. We calculated that T. turnerae should experience the change in O_2 concentration in less than 1 minute after the change in headspace pO_2, based on the diffusivity of O_2 in the agar medium and the position of the cells in the agar ($D = 2.05$ x 10^{-5} cm^{-2} s^{-1}; Nelson et al., 1986). We conducted an acetylene reduction assay and measured ethylene production within the first hour and for 2-3 h thereafter. After the acetylene reduction assay, we measured the headspace pO_2 as a check to ensure that the treatments did experience different pO_2 conditions. Finally, all cultures were sacrificed after the acetylene reduction assay to measure total protein content.

We carried out a headspace manipulation experiment on four separate occasions. On each occasion, we used a different initial pO_2. We used cultures that were 7 – 14 days old. In the treatment groups, we manipulated the headspace oxygen so the treatment groups experienced large increases or decreases in pO_2.

We did a pairwise comparison of the specific N_2-fixation activity between control and each treatment group (compare control group for each pO_2 treatment to corresponding increase in headspace pO_2, likewise, compare control group to decrease in headspace pO_2) to investigate whether the increase or decrease of oxygen had an effect

on N_2-fixation. Because the treatment samples are otherwise identical to the control and the biological effect upon N_2-fixation of an increase in pO_2 differs from that a decrease in pO_2, paired t-tests between control and treatment groups were most appropriate.

4.3   Results

Cultures reared at $pO_2 = 0.21$ atm were subsequently treated with headspace pO_2 $= 0$ atm and 0.10 atm did not have a significantly different specific N_2-fixation activity than the control group (Figure 4.1A; two sample t-test; Final $pO_2 = 0$ atm, P = 0.316; Final $pO_2 = 0.10$ atm, P = 0.190; n = 3 for each treatment).

Cultures that were maintained at $pO_2 = 0.14$ atm and were treated with headspace $pO_2 = 0$ atm and 0.21 atm also did not have a significantly different specific N_2-fixation activity than the control group (Figure 4.1B; two sample t-test; Final $pO_2 = 0$ atm, P = 0.323, Final $pO_2 = 0.21$ atm, P= 0.227; n = 4 for each treatment). However, we note that two replicates in the final $pO_2 = 0.21$ atm treatment did not reduce acetylene.

Similar to the outcome for the initial higher pO_2 treatments, N_2-fixation activity in cultures originally under lower headspace pO_2 conditions was unaffected by increases and decreases in headspace pO_2. Cultures that were maintained at $pO_2 = 0.10$ atm and then treated with headspace $pO_2 = 0$ atm and 0.21 atm did not have a significantly different specific N_2-fixation activity than the control group (Figure 4.1C; two sample t-test; Final $pO_2 = 0$ atm, P = 0.190, Final $pO_2 = 0.21$ atm, P= 0.719; n = 3 for each treatment). Although cultures reared at headspace $pO_2 = 0.07$ atm and treated with a headspace $pO_2 = 0$ atm had a slightly depressed specific N_2-fixation activity compared with the control group (mean ± SE; control = 0.504 ± 0.094, n = 5; treatment = 0.253 ±

0.039, n = 4), the difference was not significant (Figure 4.1D; two sample t-test; P = 0.069).

4.4 Discussion

For all treatment groups over the time course of the experiment (up to 3 h), there was no statistically significant deviation from a linear accumulation of ethylene. This indicates that neither control nor treatment groups experienced a change in nitrogenase activity during the acetylene reduction assay. When we compared nitrogenase activity between the control and treatment groups, there was no effect of increasing or decreasing the oxygen conditions on N_2-fixation activity in *Teredinibacter turnerae*.

No other non-heterocystous bacterium has been described in the literature that is capable of sustaining N_2-fixation under such rapid changes in oxygen concentration. In comparison, the N_2-fixing bacterium *Azospirillum brasilense* is typical in its response to changes in oxygen. An increase of 0.2 kPa dissolved oxygen to 2.0 kPa dissolved oxygen shut down N_2-fixation within 5 minutes; *A. brasilense* experienced partial recovery to 60% original levels when the dissolved oxygen levels were returned to 0.2 kPa (Hartmann and Burris, 1987). When Hartmann and Burris (1987) flushed the medium with N_2 and removed all O_2, nitrogenase activity completely ceased, but the readdition of 0.2 kPa restored N_2-fixation to original levels. The symbiotic diazotroph *Frankia* had a similar response as *A. brasilense* to changes in oxygen concentration. While in symbiosis with its plant host at 21 kPa, nitrogenase activity dropped quickly then recovered to initial activity levels when the plant was transferred to 5 kPa and 40 kPa (Kleeman et al., 1994). Possible reasons for such insensitivity of nitrogenase activity to the manipulation
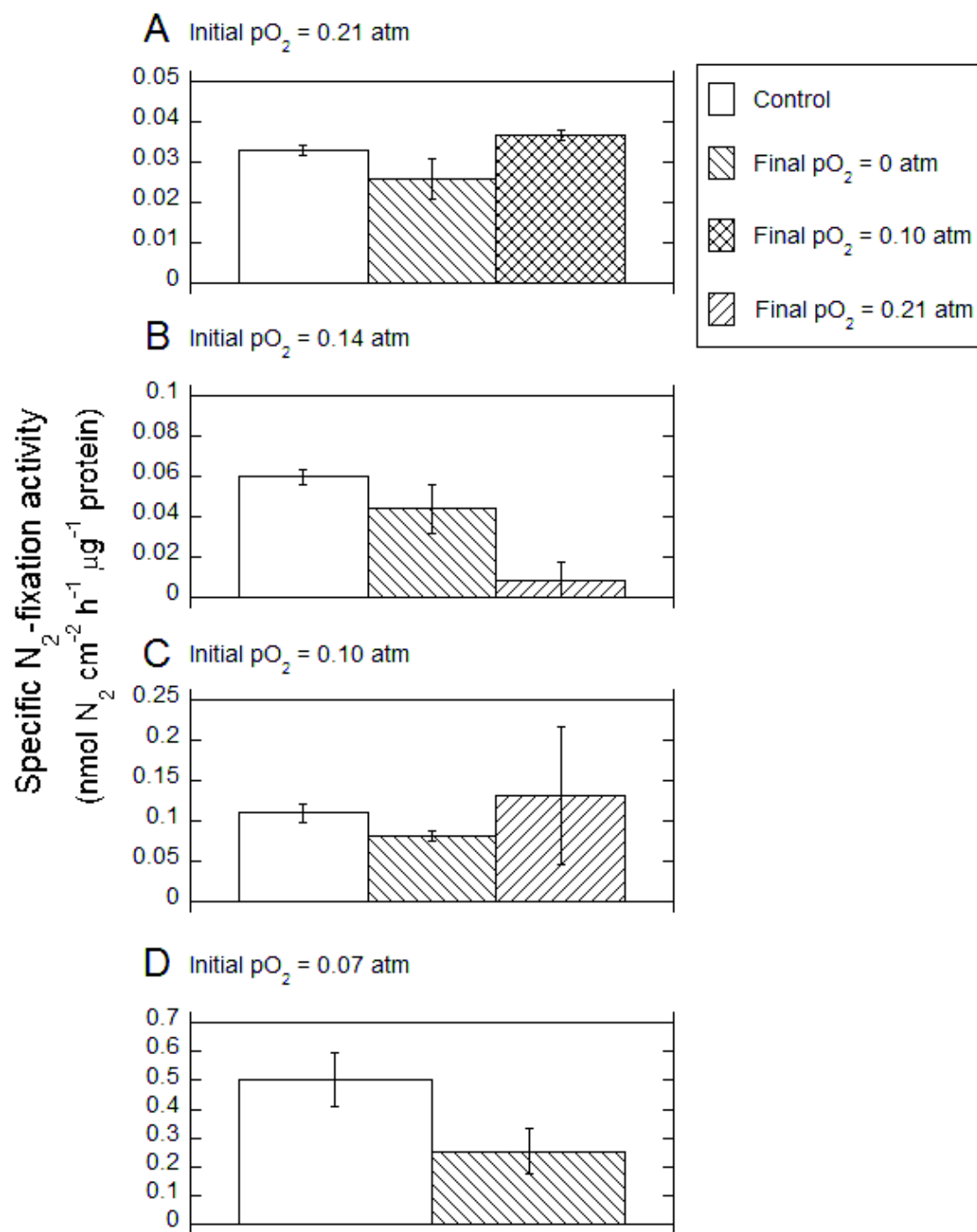
Figure 4.1. Increases or decreases in headspace pO_2 did not affect specific N_2-fixation activity in *T. turnerae*. N_2-fixation activity was measured for 3 h following a change in headspace pO_2. Data are mean ± 1 SE.

of oxygen are: a different N_2-fixation regulatory cascade or protective mechanisms, such as swimming away, O_2-retardant barriers, or biochemical protections.

4.4.1 Assay Design

We originally assayed for the effects of oxygen changes on N_2-fixation using a different protocol. In these trials, we incubated a culture of *T. turnerae* that contained a known pO_2 with acetylene for 3 h to assay for ethylene production, flushed the headspace with a different pO_2 treatment, and again assayed for ethylene production immediately after the headspace pO_2 was changed. We assumed that the difference in ethylene production between the original and treatment measurements would be attributable to the effect of changing oxygen on nitrogenase activity. However, the process exposed acetylene to the culture for an extended period of time (over 8 h). Such lengthy acetylene incubations are thought to affect measurements of nitrogenase activity because of prolonged N-starvation (David and Fay, 1977). Hence, we modified the assay to minimize the length of acetylene exposure.

4.4.2 Possible difference in N_2-fixation regulation

All N_2-fixing proteobacteria use the two-component signal transduction system NifA/NifL to control transcription of the *nif* operon and hence nitrogenase enzyme under conditions of oxygen stress (reviewed in Dixon, 1998). The *nif* operon is generally considered to be highly conserved (reviewed in Klipp, 2004), and the *nif* operon of *T. turnerae* is probably no exception. For instance, the recent genome sequencing project implied that the *T. turnerae nif* operon was horizontally acquired from a *Pseudomonas-* like bacterium (Yang et al., 2009). The sequence of the catalytic domains for *nif* operon regulators NifA and NifL have not been compared to test whether they are conserved and

thus regulate the *nif* operon in the same fashion as other NifA/NifL systems. In other diazotrophs, NifA has a central conserved domain required for binding, and has a GAF domain, whose precise mechanism of regulation is unclear across diazotrophs and an have many different roles. Partly due to this variability, Halbleib and Ludden (2000) argue that the specifics of N_2-fixation transcriptional control should be elucidated separately for each species because of the potentially wide range of variability.

4.4.3 Possible mechanisms of protection against sudden O_2 changes

Escape oxygen by swimming

Distel et al. (2002) reported that *T. turnerae* is motile by means of a polar flagellum Our estimations of oxygen gradients, presumed depth of cells, and swimming speed of similar bacteria suggest that *T. turnerae* could swim down an oxygen gradient to a location with an optimal dissolved oxygen concentration and continue to fix N at the original rate when headspace oxygen is manipulated. An oxygen gradient forms in the soft agar in response to bacterial growth and is dependent upon the oxygen concentration in the headspace. We expect *T. turnerae* to grow at the optimal dissolved oxygen concentration to balance the need for enough oxygen to maintain respiration against the need for relatively low ambient oxygen to prevent nitrogenase deactivation.

We attempted to measure the oxygen gradient in pO_2-controlled cultures of *T. turnerae* with oxygen microelectrodes, but were unable to resolve the steep gradient (Appendix B). The oxygen microelectrode had a resolution of 100 μm and showed anoxic conditions from the air-agar interface (probe touching the medium) through a depth of 5 cm. Hence, the steep gradient from oxic to anoxic conditions was within the top 100 μm and below our limits of resolution.

Other diazotrophic bacteria, such as *Azospirillum brasilense*, are known to use

aerotaxis (chemotaxis for oxygen) in order to move to a preferred oxygen concentration

(Taylor, 1983b; Taylor, 1983a; Zhulin et al., 1996). For example, Zhulin et al. (1996)

demonstrated that *A. brasilense* can use aerotaxis in a temporal oxygen gradient and that

the preferred oxygen concentration for aerotaxis was not distinct from the preferred

concentration for N_2-fixation. The results of this study suggest that diazotrophic bacteria

can use aerotaxis to find the optimal zone of oxygenation for N_2-fixation, so it is possible

that *T. turnerae* can sense a difference in oxygen concentration and respond.

Zhulin et al. (1996) report that swimming speeds for *A. brasilense* are affected by

oxygen concentration, and range from 14 to 49 μm sec^{-1}. If *T. turnerae* swims at the

average of this range, approximately 31 μm sec^{-1}, it could travel 1.89 mm in the first

minute after a change in headspace pO_2. Given such a swimming speed, the very steep

measured O_2 gradient (extending over <100 μm), and the estimated range of cell depth (0

to 3 mm; based on observations of turbidity in the medium), *T. turnerae* would be

capable of using aerotaxis to migrate within the medium during a change in headspace

pO_2.

O_2-retardant barriers

Teredinibacter turnerae may have a structural mechanism, either through biofilms

or cellular clumping, which buffers the diffusion of oxygen into the cells and prevents

quick nitrogenase activity inhibition. Prosperi (1994) reported on the cyanobacterium

Nostoc cordubensis which requires an extracellular "mucilage" (biofilm) for nitrogenase

activity at high oxygen levels. Likewise, Sabra et al (2000) argues that *Azotobacter*

vinelandii forms an extracellular alginate (polysaccharide coating) that is an effective

oxygen barrier. *Teredinibacter turnerae* forms a visible biofilm in broth culture, and it is plausible that this barrier limits diffusion of oxygen into the cells. The possibility for O_2-retardant barriers is discussed elsewhere in this study (sections 2.4.4, 3.4.4).

Biochemical protections against intracellular O_2

Alternatively, but not exclusively, it is possible that there are biochemical adaptations of *T. turnerae* that allow N_2-fixation to be sustained when ambient oxygen levels are increased. For example, in *A. vinelandii*, the energy supply to nitrogenase is a major factor to sustaining N_2-fixation activity at high oxygen concentrations; nitrogenase activity can be sustained at high oxygen concentrations if the enzyme is supplied with enough ATP (Linkerhagner and Oelze, 1995; Linkerhagner and Oelze, 1997). Dingler and Oelze (1987) reported increased specific activities of the oxygen-scavenging enzymes superoxide dismutase and catalase when the oxygen concentration is increased in diazotrophically grown cultures of *A. vinelandii*; they suggested that the enzymes are responsible for helping the bacterium maintain N_2-fixation when exposed to increased oxygen concentration. Similar studies with diazotrophic *Azotobacter brasilense* showed an increase in the specific activity of superoxide dismutase when oxygen was increased (Nur et al., 1982). The mechanisms of O_2 scavenging by reactive oxygen species has recently been well reviewed in Winterbourn (2008). Indeed, cultivated *T. turnerae* has demonstrated oxidase and catalase activity (Distel et al., 2002b). However, we gathered no evidence for the activity of O_2-scavenging enzymes in this project.

4.5 Conclusions

The recent finding of a lack of strong negative headspace oxygen control over N_2-fixation during *T. turnerae* growth (Chapter 2) prompted this study to investigate whether

N$_2$-fixation is affected by a sudden of manipulation of headspace oxygen concentration. Our assay did not show any change in specific N$_2$-fixation activity after an alteration, increase or decrease, in culture headspace oxygen. We propose that *T. turnerae* is capable of 1) either swimming to escape O$_2$ changes in the headspace or 2) using EPS as an oxygen protective barrier and/or 3) using biochemical adaptations that keep intracellular O$_2$ levels constant. Further studies are required to determine the mechanism(s) *T. turnerae* uses to prevent nitrogenase shut-off in the wake of rapid O$_2$ alterations.

4.6 Future directions

4.6.1 Do O$_2$-scavenging enzymes help to protect nitrogenase?

Distel et al. (2002b) reported the presence of O$_2$-scavenging enzymes catalase and oxidase in *T. turnerae*, and it is possible that *T. turnerae* uses these to maintain low intracellular O$_2$. We propose to identify instantaneous increases or decreases in O$_2$-scavenging enzyme transcription, protein levels, and enzymatic activity upon a manipulation in headspace oxygen. When pO$_2$-controlled N$_2$-fixing cultures are exposed to a change in oxygen, we could sacrifice a subset of cultures for RT-PCR, Western Blots, and enzyme activity assays. Western Blots would be useful to determine the presence or absence of catalase and oxidase under different pO$_2$ conditions. RT-PCR would help resolve differences in the amount of mRNA transcripts for the O$_2$-scavenging enzymes in the changed headspace pO$_2$ conditions as compared with the original pO$_2$. There are many assays to measure catalase activity in bacteria that we could adapt for *T. turnerae*, most based on the methods of Sinha (1972). If these assays indicate increased levels of catalase and oxidase transcripts and enzymatic activity under increased pO$_2$ headspace conditions while maintaining the same specific N$_2$-fixation activity, we would

conclude that the O_2-scavenging enzymes help to maintain O_2 conditions conducive for N_2-fixation.

CHAPTER 5

PROJECT CONCLUSIONS

The shipworm symbiosis is quite a unique bacteria-animal relationship. It is similar in function to the termite symbiosis, so much so that shipworms have been called "termites of the sea" (Greene, 1994), yet, the symbiosis is similar in anatomy to that of chemoautotrophic bivalve symbioses, which are frequently seen at deep-sea hydrothermal vent and cold seeps (reviewed in Cavanaugh et al., 2004). Many prior studies indicate negative regulation of N_2-fixation in conditions similar to those we expect in the gill habitat. Hence, the anatomy of the symbiosis does not appear to provide an optimal environment to maximize N_2-fixation rates. In addition, we expect a complicated nutrient cycle between shipworms and their symbionts, involving the transfer of essential elements, such as C, N, and P. These uninvestigated cycles have the potential to negatively regulate N_2-fixation in the symbiosis.

Given the complexity of the symbiosis and difficulties of working with intact shipworms for experimental studies, it would be difficult to manipulate variables and test hypotheses in whole shipworms. Instead, we used the sole cultivated shipworm symbiont, *Teredinibacter turnerae*, to begin to elucidate some of the mechanisms which allow gill-hosted N_2-fixation to contribute to the shipworm N-poor wooden diet.

5.1 Summary of major findings

5.1.1 Nitrogenase activity in *T. turnerae* is insensitive to headspace oxygen conditions

Our results indicate that *T. turnerae* is capable of N_2-fixation under a wide range of headspace oxygen conditions (0.07 to 0.21 atm). Maximum nitrogenase activity does

not differ among cultures reared in different oxygen conditions, and a change in headspace oxygen does not bring about an immediate change in nitrogenase activity.

All diazotrophs must maintain low intracellular oxygen conditions in order to carry out N_2-fixation to protect the nitrogenase enzyme from oxygen deactivation and/or prevent negative transcriptional regulation of the *nif* operon. Based on our results, we reject the possibility of increased cellular respiration as a mechanism for nitrogenase protection in *T. turnerae* based on an absence of coupling between respiration and N_2-fixation in our experiments. However, it is possible that respiration aided in maintaining a low dissolved oxygen concentration in the medium. Prior demonstration of catalase and oxidase activity in *T. turnerae* lends evidence that oxygen-scavenging enzymes could play a role in nitrogenase protection.

Although *T. turnerae* probably uses EPS and cell aggregations for O_2 resistance in pure culture, the same is unlikely in the symbiotic state. Conditions are not conducive for the formation of biofilms in the gill bacteriocytes. Here, symbionts are sequestered as aggregations of a few cells within host bacteriocytes. Given these results and limitations, we are cautious to make conjectures about the mechanisms of protection of the bacteriocytes-dwelling symbionts. Certainly, the resistance of N_2-fixation to oxygen would be a good physiological adaptation of *T. turnerae* and other symbionts to their niche. In order to truly apply the pure culture study to the real gill niche, the mechanism of O_2 protection and biogeochemical habitat of the gill needs to be better characterized.

5.1.2 N budget under different O_2 regimes

We used direct measurements to construct a partially balanced N budget for *T. turnerae* under N_2-fixing conditions in different headspace oxygen conditions. Our

measurements indicated that *T. turnerae* can use combined N for biosynthesis and a small percentage of total N fixed can be released as NH_4^+. Oxygen concentration control N release, but further characterization is needed for conclusive evidence. We provided indirect evidence for the release of DON through stable isotopic composition and N budget constraints. The potential for DON excretion by *T. turnerae* remains an exciting future direction because the controls on DON release in diazotrophs are debated and we know of only a few ecologically important microbes that can release DON.

Whether shipworm symbionts are capable of DIN or DON excretion *in situ* remains an outstanding question. Since controls and rates of N excretion in other diazotrophs appears to be species-specific and cultivation-dependent and the gill habitat remains uncharacterized, it is difficult to extrapolate our results to the symbiotic state. Given our discovery of a very small amount of NH_4^+ release, I propose that future studies should address DON translocation or the feasibility of N-rich symbiont digestion as mechanisms of N transfer from symbionts to hosts.

5.2 Study limitations

5.2.1 Unaddressed exchange cycles in the symbiosis

Our experimental set-up does have inherent limitations which may prevent this study's results from being direct applied towards understanding the real-world shipworm symbiosis. We reared *T. turnerae* on a C diet of sucrose. However, the C source of the symbionts *in situ* is undetermined and has the potential to regulate the amount of N supplemented to the host through C- and N-cycle coupling. We chose to use sucrose because of prior success in growing *T. turnerae* on this C source and the ability to measure both N_2-fixation and inorganic nutrient uptake and release in these culture

conditions. Our preliminary results (Appendix A) showed major differences in N_2-fixation activity between sucrose-grown and cellulose-grown cultures. This provides some evidence of the potential influence of the host-symbiont C cycle on the host-symbiont N cycle.

Also, we cannot rule out the possibility that there are probably several other complex nutrient or compound cycles among the different symbiont types and with the host cells that we could not account for. This cycling could affect symbiotic N_2-fixation in ways which we could not assay in this study nor begin to address. Given the surprising results of our study, we cannot assume that the controls on symbiont physiology will be the same as controls in other microbial systems. Unfortunately, we do not know enough about the role and function of the multiple symbionts in shipworms to address this confounding issue (Distel et al., 2002a; Luyten et al., 2006).

5.2.2 Limitations of batch cultures as models

We used batch cultures for experimental analysis of N_2-fixation and N release, but the symbiotic system *in situ* probably best modeled as a chemostat system. Our experiments confirmed a tight coupling between the growth rate and N_2-fixation activity; factors that influence the growth rate will in turn affect N_2-fixation. It is possible that the effects of oxygen on N_2-fixation may vary during growth of a batch culture. Chemostats maintain a constant growth rate for the cultivated organism, and serve as excellent model systems for testing hypotheses about specific controls on a physiological process. For example, a chemostat system has been established in the Montoya lab to examine the controls of combined N on N_2-fixation in *Trichodesmium* (Holl and Montoya, 2005). The establishment of a *T. turnerae* chemostat under controlled O_2 conditions would be a

worthwhile endeavor. Not only would a comparison of this study's batch culture results to similar O_2 manipulations in a chemostat be useful, but growth conditions of experimental studies would be more reflective of the shipworm symbiosis.

5.3 Future directions for studying the shipworm symbiosis

5.3.1 Characterization of the gill habitat

A good step towards understanding controls on the N_2-fixation in the shipworm symbiosis would be to directly measure and model oxygen concentrations in the gill niche. Oxygen microelectrodes and modeling could be used on intact shipworms to correlate symbiont-containing bacteriocytes with oxygen concentration. Also, it is unclear how environmental conditions, such as depth, curvature, or flushing rate of the bore hole, could affect the oxygen concentration in the gill bacteriocytes. All of these variables would need to be accounted for when making new hypotheses about the control of oxygen on N_2-fixation in shipworms.

5.3.2 Potential cycling between host-symbiont and symbiont-symbiont

The host could transfer compounds to the symbionts, or engage in a cycling of compounds between host and symbiont (for discussion, see section 1.5.1) in order to promote N_2-fixation and N translocation to the host. For example, in *Azotobacter vinelandii*, the energy supply to nitrogenase is a major factor to sustaining N_2-fixation activity at high oxygen concentrations; nitrogenase activity can be sustained at high oxygen concentrations if the enzyme is supplied with enough ATP (Linkerhagner and Oelze, 1995; Linkerhagner and Oelze, 1997).

The shipworm symbiosis may require a systems biology approach and expression profiling in order to better elucidate the potentially complex host-symbiont and symbiont-

symbiont relationships. Charles (2010) argues that metagenomics is a useful tool to uncover relationships between hosts and symbionts in plant/microbe symbioses, especially because the symbionts are unable to be cultivated. Certainly, metaproteomics and metatranscriptomics could also be useful towards discovering nutrient exchange relationships between these uncultivable microbes and their host; these could elucidate the key protein players that facilitate the symbiosis. A "Symbiosis Chip" was developed by Barnett et al. (2004) in order to examine gene expression profiling in both symbionts and hosts simultaneously. The metaproteome of the hindgut bacterial community of the termite *Nasutitermes corniger* was recently analyzed (Burnum et al., 2010); through their analysis, Burnum et al. (2010) constructed complete metabolic pathways and were able to elucidate the role of the bacterial symbionts to the host. The multi-species symbiosis presents additional challenges to these techniques; the number of each symbiont type can vary greatly among individuals of species (Luyten et al., 2006). Thus, a large sample size of individuals would probably be required for the "-omics" studies.

It is not well understood if fixed N could be transferred to shipworm hosts through the digestion of N-rich symbionts. We propose that this is a feasible alternative, or may be used in conjunction with, to the active secretion of newly fixed N compounds by symbionts to shipworm hosts. To test this hypothesis, we could conduct a microscopy-based study of the general condition of bacterial cells in gill bacteriocytes. Barry et al. (2005) used a microscopy study of the intracellular methanotrophic symbionts of *Bathymodiolus platifrons*, and suggested that the presence of symbionts in multiple stages of digestion is consistent with nutrient transfer from symbiont to host through symbiont digestion.

5.4 Conclusion

We used the cultivated symbiont *Teredinibacter turnerae* to begin to test hypotheses about the controls of N_2-fixation and N release in the unique and enigmatic shipworm symbiosis. Our results indicate that N_2-fixation and respiration in *T. turnerae* is remarkably tolerant of a wide range of headspace oxygen conditions, and N_2-fixation and respiration activity is unaffected by changes in oxygen concentration. *Teredinibacter turnerae* is capable of excreting a small amount of fixed N as DIN, possibly for transfer to the host shipworm. The N budget is partially balanced, and there may be additional N sinks that need to be characterized and quantified in future studies. In addition, further studies are warranted to reveal insights about the potentially complex host-symbiont exchange nutrient/compound cycles among host and symbionts in the symbiosis. The shipworm symbiosis remains an excellent model system for investigating novel microbial biogeochemical cycles, the interactions of microbes and hosts, and the importance of N_2-fixation to discrete environments.

APPENDIX A

N$_2$-FIXATION IN CELLULOSE MEDIUM

A.1 Introduction

The form of C that shipworm symbionts receive from their hosts is currently unknown. Shipworm symbiont *Teredinibacter turnerae* is capable of growth on sucrose and cellulose (Waterbury et al., 1983; Distel et al., 2002b). Cellulose is a complex polymer, and microbes require cellulases in order to use it as a C source. The genome of *T. turnerae* encodes for a wide variety of cellulase enzymes (Yang et al., 2009), and *T. turnerae* excretes them for extracellular cellulose digestion (Greene et al., 1988; Imam et al., 1993; Xu and Distel, 2003).

We formed the hypothesis that the recalcitrant C source, cellulose, would negatively regulate N$_2$-fixation, most likely because of the increased energy demands of cellulolysis. To our knowledge, this preliminary report is the first to examine the direct effects of a recalcitrant C source on N$_2$-fixation. We also investigated whether pO$_2$ concentration had a similar effect on N$_2$-fixation in cellulose-grown cultures, as compared with sucrose-grown cultures.

A.2 Methods

We used the same protocol from Chapter II (cultures, analytical methods, and experiment design). For this study, we made two small changes. First, the sole carbon source was cellulose (Sigmacell Cellulose, Sigma Aldrich). Second, we frequently had to extend the time acetylene incubation beyond 3 h up to 5 h to detect rates of ethylene accumulation. The methods are listed here for completion.

A.2.1 Cultures

Teredinibacter turnerae (courtesy of D. Distel; Distel et al., 2002b), isolated from

shipworm *Lyrodus pedicellatus*, was grown in soft agar (0.2% agar) shipworm basal

medium (SBM) described by Waterbury et al. (1983) with cellulose as a carbon source.

The medium was modified to reduce its ammonium by an equimolar substitution for the

iron source; we used ferric iron citrate instead of ferric ammonium citrate. We grew *T.*

turnerae in air-tight Bellco anaerobic test tubes (Bellco glass 2048-18150) that were

sealed with a crimp seal gas-tight rubber septa. There was 15 mL of shipworm basal

medium and 13 mL headspace in each tube. Cultures were grown at room temperature

without physical disturbance. We monitored contamination with two methods. First, we

struck experimental cultures on LB and Marine agar and SBM agar to actively detect

growing contaminants (T. turnerae does not grow on these media; Distel et al., 2002b).

Second, we used epifluorescence microscopy with DAPI staining.

A.2.2 Analytical methods

Oxygen treatments

We set up 4 pO_2 treatments which spanned a range from 0.07 atmospheres O_2 to

normal atmospheric saturation (0.21 atmospheres). To vary the partial pressure of oxygen

in the vessel headspace, we used a gas proportioner (Cole Palmer EW-03218-50) to mix

air and N_2. The gas mixture was passed through a 0.45 μM sterile filter, then sparged into

the headspace of the culture through a disposable 18 gauge needle. Cultures were sparged

long enough to flush the headspace 10x.

Headspace oxygen measurements

Two mL of headspace was removed from the culture tube with an 18 gauge

needle and equilibrated with 6 mL of water in a gas-tight syringe. We measured pO_2 of

the liquid phase with a StrathKelvin oxygen meter. To correct for residual pO_2 in the gas-liquid equilibration, we used a standard regression with gas samples of known pO_2 concentration from 0 to 0.21 atmospheres. Immediately following the sampling of the headspace for oxygen concentration, cultures were sparged with a gas mixture to restore the original pO_2 condition.

Acetylene reduction assays

Nitrogen fixation activity was measured with the acetylene reduction assay (Capone and Montoya, 2001), and incubations were carried out in the Bellco anaerobic test tubes. In brief, 3 mL acetylene was added to each vessel, and assay incubations were carried out under standard growth conditions as noted above. The headspace ethylene concentration was measured in duplicate at hourly intervals for approximately 3-4 hours by gas chromatography (SRI model 8610c gas chromatograph, SRI Instruments, Torrance, CA USA). The gas chromatograph was fitted with a 2 m Haysep A column (Alltech Associates Inc., Deerfield, IL USA) and a flame ionization detector (SRI Instruments). We used a 4:1 C_2H_2:N_2 molar reduction ratio to convert from acetylene reduction to N_2-fixation rates.

Total protein

We measured total protein in *T. turnerae* cultures with the Bradford assay (Bradford, 1976; Coomassie Plus - The Better Bradford Assay Kit, VWR PI23236). Previous studies with *T. turnerae* have also used protein measurements to quantify culture growth (Greene and Freer, 1986). To harvest the cells from the soft agar, we pipetted the cell layer from the culture tube. This cell layer slurry was homogenized using ethanol-washed glass beads, vigorous shaking, and vortexing. We added 50 μL of

homogenized cells to 1.5 mL Coommassie Blue, and measured the absorbance at 595 nm

with a Spectronic 20 Genesys spectrophotometer. Total protein values were calculated

from a standard curve of bovine serum albumin.

Calculations of oxygen consumption

We determined oxygen consumption rates by measuring the depletion of oxygen

following the initial poising of the cultures at their experimental pO_2 levels. We estimated

the concentration of oxygen in the liquid phase using solubility constants appropriate for

our culture conditions ($C_o^* = 374.94$ µmol O_2 L^{-1} seawater atm^{-1})(Garcia and Gordon,

1992).

A.2.3 Experimental design

At regular intervals during growth, we measured headspace pO_2 to assess oxygen

depletion through aerobic respiration, N_2-fixation rates with the acetylene reduction

assay, and total protein content. Acetylene reduction assays and headspace pO_2

measurements were conducted on the same subset of cultures (n = 4 for each pO_2

treatment) during growth, and 3 cultures for each pO_2 treatment were chosen at random

to be sacrificed for total protein content. We conducted this assay six times during culture

growth. Immediately following the acetylene reduction assay, we sparged the headspace

of all individual cultures to the original pO_2 condition and remove headspace acetylene

and ethylene.

A.3   Results

Although we did measure headspace pO_2 and medium nutrient content for all

samples, we were unable to measure both inorganic nutrient depletion and respiration rate

by all pO_2 treatments during growth. This is most likely a result of low biomass density in the thin band of biomass.

We were able to measure N_2-fixation rates in the cellulose medium for days 12 (after the first appearance of the culture band) to 37 for all pO_2 treatments (Figure A.1). The pO_2 treatment did not affect the mean N_2-fixation rate during growth (ANOVA, P = 0.467; mean ± 1 SE; 0.07 atm: 46.6 ± 7.5 pmol N_2 cm^{-2} h^{-1}; 0.10 atm: 43.4 ± 7.5 pmol N_2 cm^{-2} h^{-1}; 0.14 atm: 36.4 ± 7.5 pmol N_2 cm^{-2} h^{-1}; 0.21 atm: 53.2 ± 7.5 pmol N_2 cm^{-2} h^{-1}).

N_2-fixation rates by the pO_2 treatments were affected differently with respect to time (ANOVA with interaction term; P < 0.0005). N_2-fixation rates in lower pO_2 treatments did not statistically change during growth (Figure A.1C, A.1D, mean is represented with a dotted line; 0.07 atm: ANOVA, P = 0.128; 0.10 atm: ANOVA, P = 0.505), but there is a pronounced increase in N_2-fixation by higher pO_2 treatments) late during growth (Figure A.1A, A.1B, 0.14 atm: ANOVA, P < 0.0005; 0.21 atm: ANOVA, P = 0.031). We note that N_2-fixation rates in the pO_2 = 0.21 atm treatment were highly variable among the 4 replicates on day 12. Two replicates fixed N at rates comparable to day 18 and two replicates fixed N at the highest rates recorded during the entire experiment.

During growth, the absolute N_2-fixation rates for cultures grown in cellulose were at least 100x lower than N_2-fixation rates measured in sucrose medium (see Chapter 2). We normalized N_2-fixation activity to protein content to determine if a lower biomass in cellulose-grown cultures caused the lower N_2-fixation rates. Maximum specific N_2-fixation activity was approximately 1000x less in cellulose medium as compared with sucrose medium for all pO_2 treatments (Figure A.2, note the difference y-axis units).

Similar to sucrose-grown cultures, pO_2 did not affect maximum specific N_2-fixation activity in cellulose-grown cultures (Figure A.2A, sucrose, ANOVA with Tukey's post-hoc pairwise comparison test, $P < 0.0005$; Figure A.2B, cellulose, ANOVA with Tukey's post-hoc pairwise comparison test, $= P\ 0.685$).

Integrated N_2-fixation presents a similar trend to N_2-fixation activity with regards to the effect of C. When considered over the entire experiment, sucrose-grown cultures accumulate approximately 1000x more fixed N than cellulose-grown cultures for all pO_2 treatments (Figure A.3). The effect of pO_2 treatment on integrated N_2-fixation is less clear in cellulose-grown cultures, but it is apparent that the lowest pO_2 treatment, 0.07 atm, fixed significantly less than the highest pO_2 treatment, 0.21 atm (Figure A.3; ANOVA with Tukey's post-hoc pairwise comparison; $P < 0.01$).
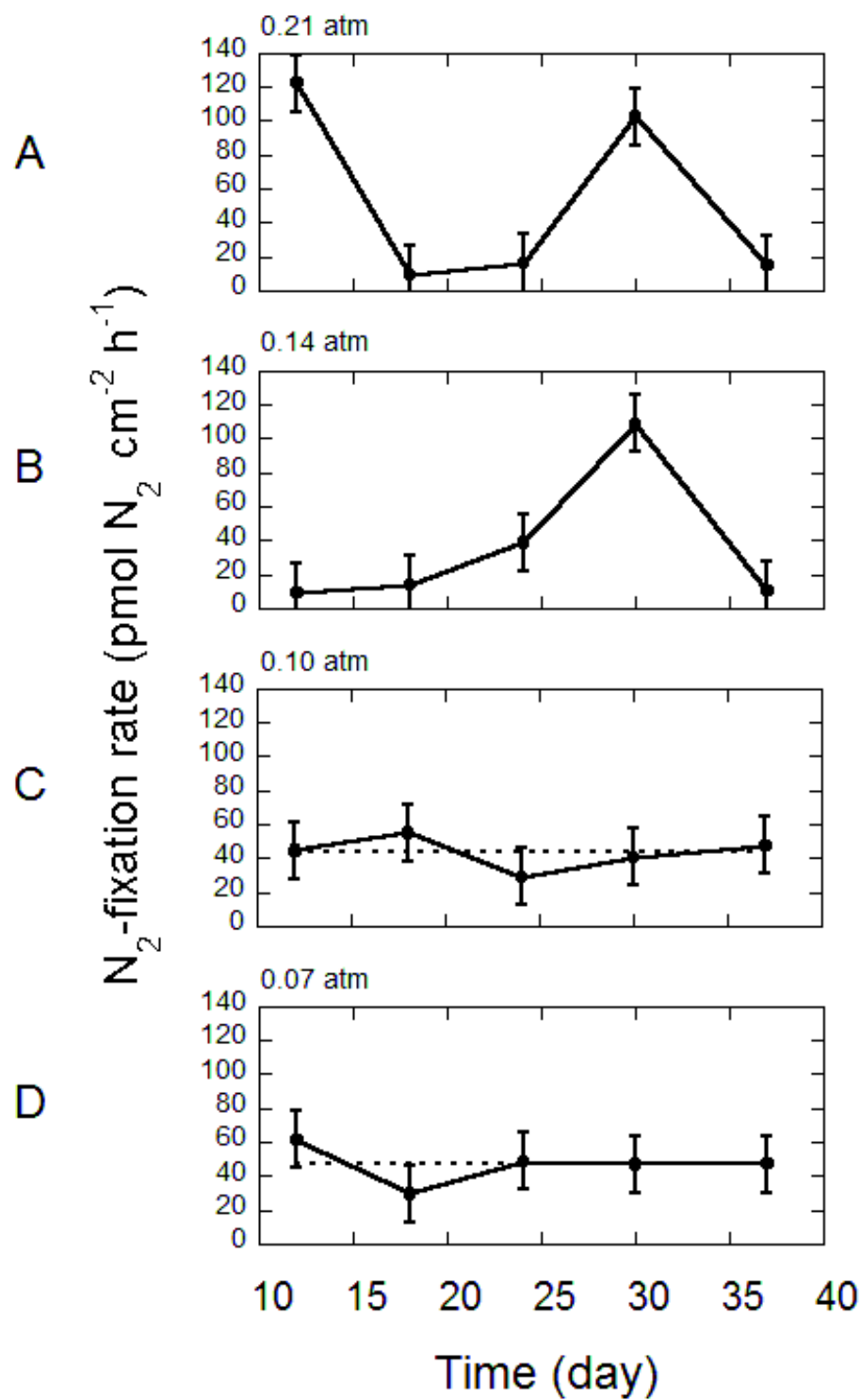
Figure A.1. N_2-fixation by *T. turnerae* in cellulose medium under different pO_2 regimes. Solid lines and closed circles: daily absolute N_2-fixation rates (n = 4, mean ± 1 SE). Dotted lines: mean N_2-fixation rate.
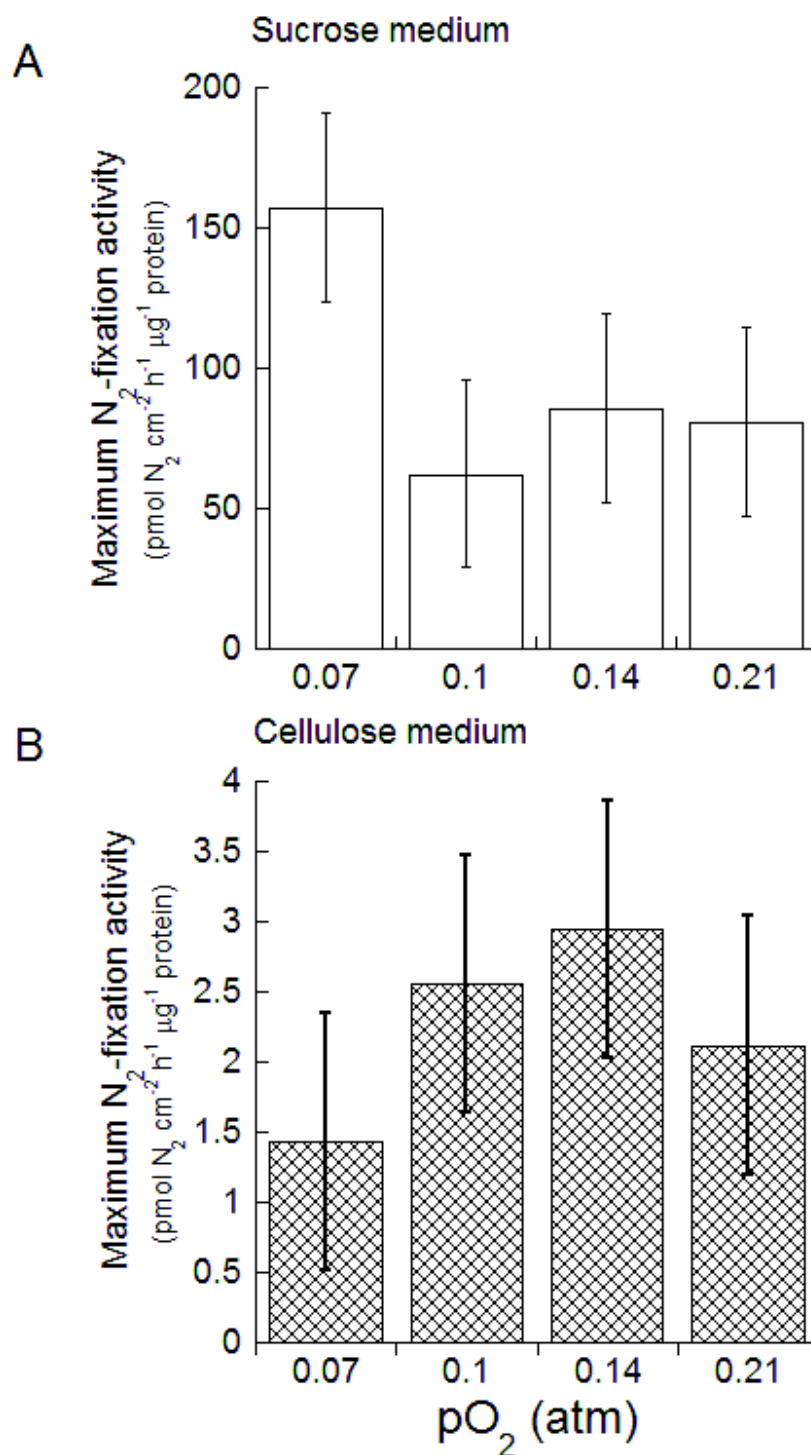
Figure A.2. The maximum specific N_2-fixation rate was much higher in sucrose-grown cultures (panel A) than in cellulose-grown cultures (panel B; note the difference in y-axis scale). pO_2 treatment does not affect N_2-fixation activity in either cultures grown in either. Data are mean ± 1 SE.
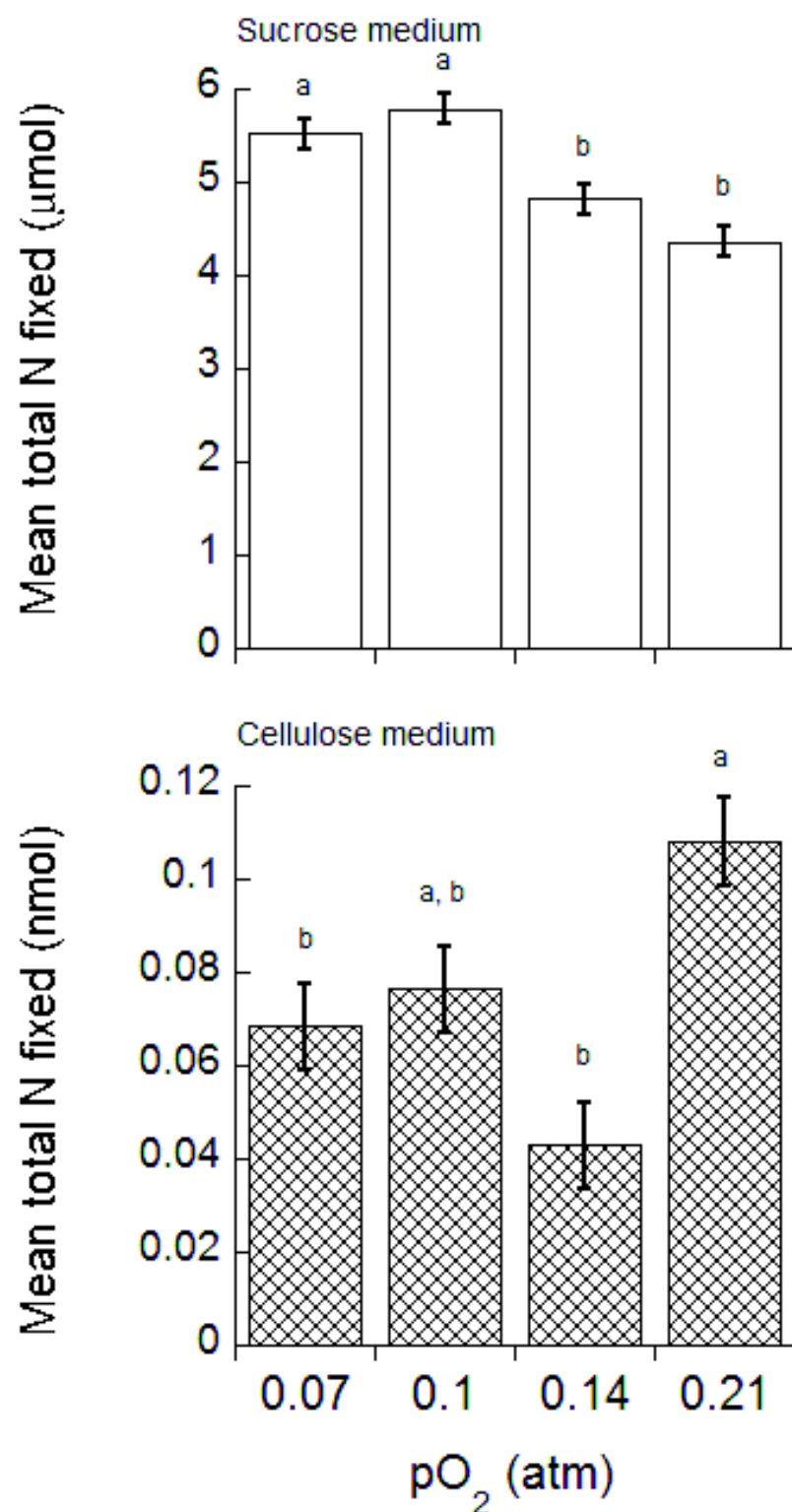
Figure A.3. Total N fixed during growth by sucrose-grown cultures (panel A) was much higher than total N fixed by cellulose-grown cultures (panel B). Data are mean ± 1 SE. Subscripts indicate statistically significant groupings based on ANOVA with Tukey's post-hoc pairwise comparison. Note the difference in y-axis scale.

A.4   Discussion

A.4.1 Experimental difficulties

Although we were able to see a visible biomass layer and evidence of cellulolysis (in the form of localized clearing of the medium), *T. turnerae* utilized inorganic nutrients and respired at a rate that was far below our limit of detection. Hence, we were unable to establish an N budget nor evaluate the effects of pO_2 on respiration for this experiment. Future experiments which investigate these parameters may benefit from incubating the cultures in a different fashion. One possibility would be to grow broth cultures in a larger batch to encourage more growth. Airtight vessels larger than the Bellco tubes that we used would be necessary. The larger broth volume necessitates that the cultures undergo agitation to foster a high biomass. A setup that involves a Bellco roller drum and larger volume crimp seal tubes would suffice for this purpose.

A.4.2 pO_2 and N_2-fixation in cellulose-grown cultures

We were unable to detect an effect of headspace oxygen concentration on N_2-fixation in cellulose-grown cultures. We obtained a similar outcome for the same study conducted in sucrose medium. We note that there were no significant differences in maximum N_2-fixation activity among all 4 pO_2 treatments, but the integrated N_2-fixation was significantly different among pO_2 treatment groups. We consider the cultures from the 0.14 atm treatment the reason for this anomaly. There was higher variability among replicates in the 0.14 atm treatment than in other treatment groups; two replicates consistently fixed N at higher rates during growth while two other replicates recorded two days each of no measurable N_2-fixation.

A.4.3 Comparing N_2-fixation between C sources

We did not directly compare *T. turnerae* physiological parameters for cellulose-grown and sucrose-grown cultures on a day-by-day basis because the measurements from the two experiments were not conducted on the same post-inoculation days. In addition, our calculations indicate that the C content is not equal between the sucrose and cellulose media. In the sucrose medium, the molar C content is 14.6 mM, and the final medium concentration is 0.5%. The final medium concentration of cellulose is 0.2%. We measured the C molarity of the cellulose medium and we are awaiting the results to complete this study (measured by mass spectrometry). An experiment to determine the effects of the C source on *T. turnerae* physiology would require equimolar C content for the media tested. For this project, we did not attempt to change the C content.

Our preliminary results indicate that as compared with sucrose-grown cultures, cellulose-grown cultures have much lower N_2-fixation rates and lower N_2-fixation activity. The ultimate result from this is that cellulose-grown cultures accumulate at least 1000x less fixed N than sucrose-grown cultures. Unlike the pO_2 treatments of sucrose-grown cultures, N_2-fixation rates in low pO_2 cellulose-grown cultures do not peak late during growth. Our results do not provide evidence why these cultures do not exhibit this peak, which is expected in a batch culture. At this time, we are unable to determine whether the differences in N_2-fixation results from C limitation in cellulose cultures or from depressed N_2-fixation in cellulose medium. We hypothesize that energetic costs related to cellulolysis may be one cause of lowered N_2-fixation in cellulose-grown cultures.

Future studies to examine the coupling of the C and N cycle in *T. turnerae* would need to include measurements of C depletion in the medium. Also, if C molarity was

lower in the cellulose medium and we can thus assume C limitation, future experiments

should assay the N cycle in equimolar C concentrations.

APPENDIX B

INVESTIGATIONS INTO THE OXYGEN CONCENTRATION IN SOFT AGAR MEDIUM

B.1 Introduction

In this study, we manipulated the oxygen concentration of the overlying headspace, and not in the medium itself. We did the following studies to examine how the headspace pO_2 can affect the distribution of cells in the medium and attempted to quantify the preferred dissolved oxygen concentration for *T. turnerae* growth.

B.2 The use of indicator dyes to demonstrate O_2-sensitivity in T. turnerae

Tetrazolium salts are frequently used as a redox indicator in microbial cultures. The salts form a colored insoluble precipitate in zones of reduction, and in turn, reveal oxygen-depleted zones (Altman, 1970; Paerl and Bland, 1982; Paerl and Prufert, 1987). We incubated *T. turnerae* in oxygen-controlled soft agar cultures with 0.01% TTC (2,3,5-triphenyl-3-tetrazolium chloride; cultures set up according to Chapter 2 methods). We found that the depth of the insoluble precipitate below the air-agar interface was affected by the headspace pO_2 (Figure B.1). This indicates that *T. turnerae* is sensitive to oxygen concentration, and that oxygen can affect the distribution of *T. turnerae* in the medium.

Pearl and Bland (1982) reported a strong correlation between the tetrazolium salt precipitate and a sharp decrease in nitrogenase activity, which they attribute to a competition between nitrogenase activity and tetrazolium reduction for reducing power. We confirmed this finding; cultures incubated with tetrazolium salts did not record measureable rates of acetylene reduction at any point during growth (data not shown). This relationship of depth of cell layer with culture headspace pO_2 was different for

sucrose-grown cultures without tetrazolium salts. Early during growth (less than 10 days), culture depth was influenced by headspace pO_2, but after that time, there was no measureable depth of culture below the air-agar interface. We attribute this finding to the fact that cultures are capable of fixing more N when tetrazolium salts are removed.
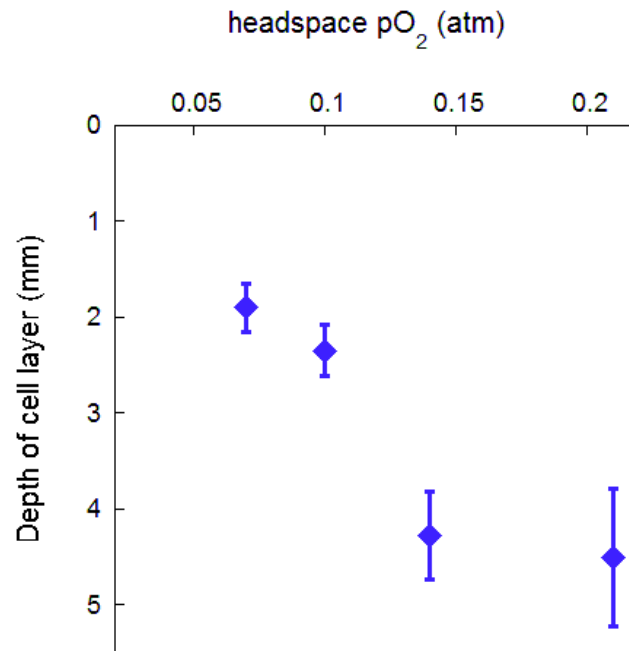
Figure B.1. The culture headspace pO_2 affected the depth oxygen-depleted zone (cell layer) in *T. turnerae* soft agar cultures when grown in the presence of tetrazolium salts. We measured the depth from the air-agar interface to the depth of the precipitate with a caliper. Data are mean ± 1 SD, n = 4.

B.3 Microelectrode studies in oxygen-controlled soft agar cultures

Given the ability of *T. turnerae* to migrate in the agar, we formed the hypothesis there is a gradient of O_2 in the soft agar medium and that cultures grow at a preferred dissolved oxygen concentration. We sought to test this hypothesis by measuring the concentration of dissolved oxygen in fully grown oxygen-controlled soft agar cultures (set up according to Chapter 2 methods). We used a Clark-type microelectrode mounted on a micromanipulator arm, minimum resolution 100 µm (courtesy of UGA, S. Joye). We used a two reference calibration: He-bubbled water and air-bubbled water.

We tested 4 different pO_2 treatments (0.07, 0.10, 0.14, and 0.21 atm), n = 3 replicates for non-inoculated control (blank medium) and n= 3 replicates for each pO_2-controlled culture. For all non-inoculated controls, the measured dissolved oxygen concentration approximated the expected value for the particular headspace pO_2, and no gradient was detected (data not shown). In contrast, measurements for all pO_2-controlled cultures indicated a completely anoxic medium from the air-agar interface to 5 cm depth (2.5 cm below the cell layer). Hence no gradient was detected, and we were unable to obtain the preferred oxygen concentration at which the cell layer will form. Future experiments should include the application of microelectrode studies to cultures at a wide range of growth phases; these studies would help elucidate the development of the oxygen gradient within the soft agar medium.

B.4 Consequences for the project

The dissolved oxygen in the medium is determined directly by the headspace pO_2. Our method for maintaining the pO_2-controlled cultures by manipulating the headspace oxygen was adequate to expose cultures to differing oxygen concentrations.

The results of the microelectrode study allow us to assume that there was no appreciable dissolved oxygen in the medium for the headspace oxygen manipulation experiments (Chapter 4). Without residual oxygen in the medium, cultures immediately experience the "new" pO_2 and are subject to its effects.

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VITA

RACHEL E. A. HORAK

Rachel grew up in Northern Virginia, land of the Washington, D.C. suburbia sprawl. She chose to attend Davidson College (Davidson, NC) based on their outstanding academic programs and because of the opportunity to compete on a Division I swim team. After 4 years of a rigorous liberal arts curriculum and 4 Southern Conference Championships, she graduated with a BS in Biology. Desperately needing an academic hiatus, she coached swimming full-time for several years, but was ultimately bored with life outside academia. Rachel completed a Master's of Arts, Biology, with Cindy Van Dover at the College of William and Mary (Williamsburg, VA). There, she got hooked for life on the excitement of research at sea, and took a dive in the submersible DSV *Alvin*. While at sea one summer, she met a group of fellow Atlanta Braves-loving microbiologists from the School of Biology at Georgia Tech who sold her on the awesomeness of the program. During her career at Georgia Tech, she has fostered a passion for astrobiology, and attended NASA/National Astrobiology Institute- sponsored courses and conferences in Hawaii, Santa Clara, and Seattle. She presented her own research about the shipworm symbiosis at several oceanographic conferences, including in Honolulu, Orlando, and Portland. Rachel logged 83 days at sea while at Georgia Tech, including 2 dives in the *Johnson SeaLink* submersible, a hurricane-shortened adventure in the Gulf of Mexico, 4 weeks measuring inorganic nutrients in the oligotrophic South Pacific, and 5 weeks in the Amazon River plume looking for diatoms. Somewhere between traveling all over the country and putting in ridiculously long hours in the lab, she worked as a GTA or supervisory TA in the School of Biology for 6 semesters and as a GTA in CETL for 2 more semesters. Rachel hopes to continue studying cool microbes which have implication for the possibility of life on other planets, and eventually find a professorship that involves lots of undergraduate teaching. She enjoys swimming, wine tours, traveling, and spending time with boyfriend Tim and her goofy 3 furballs (ferrets) in her new home of Seattle, Washington, and is waiting for another Atlanta Braves championship.