Molecular investigation of the microbial community associated with the fire sponge, *Tedania ignis*, in Bermuda

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The complex, phylogenetically diverse, and specific microbial communities associated with marine sponges are a key aspect of the ecology and evolution of the metazoan host and the endosymbiotic microbes. Using fluorescence *in situ* hybridization (FISH) methods, terminal restriction fragment length polymorphism (T RFLP), and functional gene probing via PCR, the current study investigates the microbial associations in the common Caribbean fire sponge, *Tedania ignis*. Sponge and water samples were collected from different sites around Bermuda from 2012 to 2014 in order to assess their respective microbial communities. Using FISH, SAR202 (*Chloroflexi*) (5.82% ± 0.59%) and Crenarchaea (7.97% ± 1.08%) were identified as the most abundant contributors to the microbial assemblage of *T. ignis* while the Alphaproteobacterium SAR11 (30.68% ± 1.68%) was identified as the most dominant species in the surrounding seawater. Due to the presence of Crenarchaea, the Archaeal gene for ammonia oxidation (*amoA*) was probed via PCR and found to be present. T RFLP identified the most abundant fragment length present in the sponge as 336 bp (>60% of T RFLP peak abundance). The sponge community was consistent and markedly distinct from that of the ambient seawater as identified by both FISH and T RFLP. Epifluorescent microscopy with DAPI staining also identified *T. ignis* as a high microbial abundance (HMA) sponge, in contrast to previous studies. Together, these data characterize the microbiome of *T. ignis* in much further detail than has previously been described.

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INTRODUCTION: Marine sponges (Porifera) are a significant component of benthic systems. They provide a number of ecosystemic services, including reef formation and accretion (Diaz & Rutzler 2001), nutrient recycling (for a review, see Bell 2008; Hoffman et al. 2009), contributing to primary productivity (Wilkinson 1983; Cheshire & Wilkinson 1999) and concentrating DOM on reefs (de Goeji et al. 2013). They are important competitors in coral reef communities (Suchanek et al. 1983; for a review, see Wulff 2006), and they are host to a considerable amount of invertebrate (Magnino et al. 1999; Wulff 2006) and microbial life (for a review, see Taylor et al. 2007).

Many of the metabolic pathways traditionally attributed to sponges are in truth ascribable to their endosymbiotic microbes (reviewed in Lee et al. 2001; Hentschel 2004; Weisz et al. 2010). It is suggested that the microbes confer nitrogen fixation, nitrification, and autotrophy unto their hosts, as well as provide chemicals employed in defense (Hoffmann et al. 2005; Freeman & Thacker 2011; Jiminez & Ribes 2007; Southwell et al. 2008; Corredor et al. 1988; Diaz & Ward 1997; Siegl et al. 2008). In some extreme examples, such as offshore sponges, the endosymbiotic bacteria may be responsible for obtaining up to 90% of the energy for the sponge (Wilkinson 1987). Endosymbiotic microbes can be so numerous that they can account for up to 60% of the total sponge volume and 40% of the total biomass (Vacelet 1975; Vacelet & Donadey 1977; Wilkinson 1978; Schmitt et al. 2007).

With the development and refinement of culture independent techniques, and the knowledge that sponges are rich sources of secondary metabolites (Lee et al. 2001; Kelecom 2002; reviewed in Thomas & LokaBharathi 2010), interest in the sponge-associated microbial community has increased significantly in the past decade (see Taylor et al. 2007). Initially, a uniform and phylogenetically complex sponge-specific hypothesis was suggested (Hentschel et al. 2002; Hill 2004), as the phylogenetic signatures of sponge and water communities differed significantly. It seemed that sponges must impose strong selective pressures to account for this profound difference. This was supported by the identification of the candidate phylum *Poribacteria*, owing its name to a seeming requirement for residing exclusively in sponge hosts (Fieseler et al. 2004). However, later studies contrast the uniform community hypothesis (Hardoim et al. 2012) and instead focus on the idea of core, variable and species-specific members of sponge-associated microbial communities (Schmitt et al. 2012; Simister et al. 2012). Recently, Next gen sequencing investigations have shown that sponge microbiomes are generally host-specific (Hardoim et al. 2012; White et al. 2012; Cleary et al. 2013; Easson & Thacker 2014). Additionally, due to deep sequencing technology, *Poribacteria* have been found to be present in ambient environmental samples, albeit at very low levels and likely inactive (Simister et al. 2012; Taylor et al. 2012). Thusly, it is apparent that sponge-endosymbiont understanding is an ongoing process.

Tedania ignis (Duchassaing and Michelotti 1864), colloquially known as the common Caribbean or mangrove fire sponge, is well known for inducing contact dermatitis (Yaffee & Stargardter 1963) and is known to produce anti-fouling compounds, potent cytotoxins, antimicrobials and the antitumor compound tedanolide (Schmitz et al. 1983; Schmitz et al. 1984; Muricy 1993). Due to the sponge's abundance and ease of collection, it has served as a model organism for studying Poriferan planula (Wulff 2006; Weyrer et al. 1999 and references therein). According to a survey study, *T. ignis* is the most abundant organism by volume in Caribbean mangroves; it consistently accounted for 50% of the total biovolume at three sites surveyed (Wulff 2009). In Bermuda, this ubiquitous sponge is found in protected inshore environments and prefers dead coral and other rubble as a substrate. It is also associated with mangrove roots (Sutherland 1980; Wulff 2005). *T. ignis* appears to prefer the absence

of predatory fish (family Scaridae) within mangrove environments (pers. obs. Jouett) and coral reef systems (Pawlik et al. 2013).

This study investigated the microbial consortium of *T. ignis*. Sponge and water samples were collected from around Bermuda and the microbial community was analyzed using FISH, T RFLP and PCR targeting the ammonia monooxygenase gene in order to assess *T. ignis*' putative role in the environment. Our findings show that, in Bermuda, *T. ignis* is a high microbial abundance sponge with the potential to contribute to nitrification processes due to an abundance of Crenarchaea and the ammonia monooxygenase gene. FISH and T RFLP analysis confirm that the microbial consortium within this sponge is markedly different from that of the surrounding seawater. T RFLP has also identified the most abundant fragment length (336 bp at >60%) in the sponge consortium, which is currently not ascribable to a known bacterium.

MATERIALS & METHODS:

Sample collection and preparation

Samples were collected from 5 different sites located around Bermuda during the period of November 2012 to July 2014. Sponges are only protected in Bermuda when they reside within a marine sanctuary, and none of the sites were located within protected areas. The coordinates and details for each site can be found in Table 1. At each site, four *T. ignis* specimens were retrieved by a gloved hand and transferred to a plastic bag while still at depth. Two 1 liter water samples were taken from depth a few meters from the sponge. Sponges and water were never taken from more than 2 meters deep, as *T. ignis* is a shallowly residing sponge. Samples were immediately put on ice, transferred to the lab, and fixed with formalin for all subsequent analyses. Sponge samples were stored at 4°C until further use.

For microscopy, approximately 1 dry gram of sponge tissue was cut, using sterile scissors, from the main sponge sample. The sponge was then transferred to a mortar and mixed with 10 ml of 0.1 µm filtered sterile seawater (SSW) from the corresponding sample site. The sponge was homogenized and transferred to a centrifuge and spun at 6000 rpm for 5 min, in order to create a pellet of sponge and a supernatant of microbes suspended in solution. Previous studies (Friedrich et al. 1999; Thoms et al. 2003) show through TEM that bacteria are equally distributed throughout HMA sponge mesohyl, and therefore this was the justification of the 'slurry' approach (Friedrich et al. 2001). After centrifugation, the supernatant was poured off and the pellet discarded. The supernatant was then filtered through a Nitex mesh (500 µm) and then through a 3.0 µm filter to remove residual sponge tissue. This stock solution was then fixed with 10% formalin.

In order to allow for appropriate loading densities during microscopy, this twice filtered supernatant was diluted again. To make the appropriate loading dilution, 0.1 µm filtered SSW (10 ml) was mixed with sponge stock solution (200-600 µl). This created a reduction where 0.002-0.006 grams of sponge tissue was used. These dilutions constitute our sponge microbial samples and were fixed with 10% formalin. Water samples (40 ml) were also fixed with 10% formalin and stored at -80°C until sample processing.

Samples were not collected in 2012 for DNA analysis. In 2013 and 2014, the remainder of the sponge stock solution was filtered onto a 0.2 µm filter and stored in sucrose lysis buffer (1 ml) at -80°C until DNA sample processing. In 2013 and 2014, the remaining water samples (1 l) were filtered onto a 0.2 µm filter and stored in sucrose lysis buffer (1 ml) at -80°C until DNA sample processing.

Microbial cell abundance

Microbial samples were thawed (5 ml for water and 1 ml of the diluted sponge) and were filtered through a 0.2 µm filter pre-stained with Irgalan Black (0.2 g in 2% acetic acid) under gentle vacuum (~100mm Hg). The filters were post-stained with 0, 6-diamidino-2-phenyl dihydrochloride (DAPI; 5µg ml^{-1}, SIGMA-Aldrich, St. Louis, MO, USA) (Porter & Feig 1980) and then were enumerated with an AX70 epifluorescent microscope (Olympus, Tokyo, Japan) under UV excitation at 100x magnification. Ten fields were averaged per sample representing at least 250 cells.

Enumeration by FISH and CARD FISH

The FISH probes used for this study are detailed in Parsons et al. (2014) and Morris et al. (2004). Probes were validated *in silico* for specificity using Probe Match on the Ribosomal Database Project (Cole et al. 2009), and TestProbe and Probebase on the SILVA website (Loy et al. 2007). Bacterial abundance water samples (5 ml) and sponge dilutions (1-3 ml) were filtered onto 0.2 µm polycarbonate filters under gentle vacuum (~100 mmHg) and stored at -20ºC with desiccant. Quarter filters were washed in 95% ethanol for 2 min and then probed according to Morris et al. (2002) and Parsons et al. (2014). Because fluorescent signals are typically low for Archaea, Archaeal enumeration was performed using catalyzed reporter deposit (CARD) FISH (Teira et al. 2004; Herndl et al. 2005). Permeabilization of the cell membrane was conducted using 0.1N HCl with no prior embedding in agarose. The hybridization and wash conditions with all probe sequences are described in Parsons et al. (2014). The resulting filters from FISH and CARD FISH were mounted with 20 µl of 1.67 µg ml^{-1} DAPI in citifluor solution (Ted Pella, Inc., Reading, CA, USA), sealed with nail polish and stored frozen in the dark (Parsons et al. 2011). Image analysis coupled with epifluorescence microscopy (Olympus AX70 microscope) was used to process FISH and CARD FISH slides excited with Cy3 (550 nm) and UV wavelengths as previously described. Image acquisition was performed using a Toshiba (Irvine, CA, USA) CCD video camera with a Pro-series capture kit version 4.5 (Media Cybernetics, Bethesda, MD, USA) and processed with Image Pro software (version 7.0; Media Cybernetics) as previously described (Carlson et al. 2009; Parsons et al. 2011).

DNA extraction and TRFLP fingerprinting of the 16S rRNA gene

In 2013, DNA was extracted using the phenol isoamyl chloroform method (Giovannoni et al. 1990). In 2014, this was supplemented by adding a cetyltrimethylammonium bromide (CTAB) step in order to streamline the process and to bind presumed sponge polysaccharides (Villegas-Rivera et al. 2012). Templates from the mixed communities were amplified using PCR with an NEB High Fidelity 2X PCR Master Mix (NEB, MA, USA) and primers 27F-FAM (5'FAM-AGRGTTYGATYMTGGCTCAG) and 519R (GWAT TACCGCGGCKGCTG) (SIGMA Biosynthesis, St. Louis, MO, USA) (Morris et al. 2005). This yielded an amplicon approximately 500 base pairs in length using the following thermocycle: 94°C for 2 min, followed by 29 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 1 min. The reaction was held at 72°C for 10 min.

In the 2013 samples, the amplicons were purified using the Sigma GenElute product kit (Sigma-Aldrich, St. Louis, MO, USA), following the manufacturer's instructions, but with an additional drying step (10 min incubation at 37°C). In the 2014 samples, in order to increase the efficiency of DNA extraction from the gel, the PCR products were isolated using the Qiagen MinElute Gel Extraction kit (Qiagen, Vinlo, Limburg) in lieu of the Sigma kit. Extraction proceeded following the manufacturer's protocol, but with the additional drying step to improve the removal of ethanol. To elute the DNA, 20 µl (increased from the suggested 10 µl) of Buffer EB was applied and left for 1 min before a final 1

min centrifuge step. The purified amplicons were digested with restriction enzyme Hae III (NEB, Ipswich, MA, USA). Fragment analysis of denatured products in formamide with a custom 30 −600 bp size standard (Bioventures, Murfreesboro, TN, USA) was conducted at the UC Berkeley DNA Sequencing Facility on an Applied Biosystems 3730XL capillary sequencer. Data analysis proceeded according to previously published methods (Nelson 2009).

Ammonia concentrations in T. ignis

Using the salicylate method (Krom 1980), approximate ammonia levels were determined from the sponge, the sponge associated water, and the ambient seawater. The kit used was an API Ammonia Test Kit (Mars Fishcare, Chalfont, PA, USA). Ammonia levels from the sponge were assayed by squeezing water from the sponges into a collection tube.

PCR of ammonia monooxygenase

In 2014, DNA was extracted as above and the template from the mixed community was amplified using PCR to selectively identify the *amoA* gene within the sampled microbial communities. The NEB 2X PCR Master Mix was tested with three sets of PCR primers. The primers amo111F (5'-TTYTAYACHGAYTGGGCHTGGACATC-3') and amo643R (5'-TCCCACTTWGACCARGCGGCCATCCA-3') were used to amplify the amoA gene (Treusch et al. 2005) using the NEB 2X PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The positive control was template DNA from an anoxic fjord known to contain *amoA* and the negative control was nuclease-free water. The PCR was amplified using a TProfessional thermocycler (Biometra, Goettingen, Germany) using the following conditions: 98°C for 2 min, followed by 35 cycles of 98°C for 30s, 59°C for 30s, and 72°C for 45s. The reaction was held at 72°C for 10 minutes. PCR products were visualized on a 2% agarose gel. All 2014 samples were tested for *amoA*.

Statistical Analyses

Basic statistical analyses were carried out using Microsoft Excel. Standard error is used to represent error throughout the manuscript. Hierarchical clustering analysis and analysis of similarity was determined using the R package vegan (Oksanen et al. 2013). Nonmetric multidimensional scaling analysis was determined using the R programming language and the vegan and MASS packages (Vebables & Ripley 2002). Clustering dendrograms (UPGMA, Bray-Curtis) were constructed in PAST3 (Hammer et al. 2001).

RESULTS:

Microbial cell abundance

The microbial cell abundance was ~3 orders of magnitude higher in the sponge tissue than that of the surrounding water (Fig. 1a&b). Microbial cell abundance ranged from 2.31 x 10^8 cells g^{-1} of tissue in sponges collected from St. George's Harbour to 5.01 x 10^9 cells g^{-1} of tissue in sponges collected from the Reach in the fall of 2012. Microbial cell abundance ranged from 1.07 x 10^5 cells ml^{-1} in the water collected from Helena's Bay to 1.04 x 10^6 cells ml^{-1} in water collected from the Reach in the spring of 2013. The microbial cell abundance in the sponge tissue from all samples was significantly different from the surrounding seawater samples ($p<0.001$; sponge n=27; water n=14). Averaging all the samples by site, the microbial cell abundance in the sponge tissue was significantly different from the surrounding seawater ($p=0.004$; n=7).

Microbial community structure as determined by FISH and CARD-FISH

We used the probe Cren537 to enumerate the Thaumarchaeota in this study. The probe was designed to detect sequences of environmental Crenarchaea, specifically Marine Group I. Recently, the Thaumarchaeota (i.e. ammonia-oxidizing Archaea) have been recognized as their own phylum distinct from the Crenarchaea (Brochier-Armanet et al. 2008).

The majority of the microbial community within the sponge tissue remained unidentified, with the unprobed component making up 76.12% ± 2.35% of the microbial community (Fig. 2a). Thaumarchaea were the most abundant microbe identified with cell abundance averaging $1.48 \times 10^8 \pm 2.97 \times 10^7$ cells ml^{-1} contributing to 7.97% ± 1.08% of the microbial community in the sponge tissue. SAR202 was the most abundant bacteria identified with cell abundances averaging $1.25 \times 10^8 \pm 2.48 \times 10^7$ cells ml^{-1} contributing to 5.82% ± 0.59% of the total microbial community in the sponge tissue.

The SAR11 clade dominated the seawater samples (Fig. 2b). SAR11 cell abundance averaged $1.94 \times 10^5 \pm 2.65 \times 10^4$ cells ml^{-1}, contributing to 30.68% ± 1.68% of the microbial community in the seawater samples. The majority of the seawater community was identified with an average of $2.10 \times 10^5 \pm 2.96 \times 10^4$ cells ml^{-1} remaining unidentified. Reproducibility between sponge tissue and seawater samples was strong.

PERMANOVA using distance matrices was determined for sample type ($r=0.646$; $p=0.01$) and sample site ($r=0.442$; $p=0.16$). Thus, sample type explains 42% of the variance in the FISH data while sample site only explains 19% of the variance in the FISH data. ANOSIM was also determined for sample type ($r=0.714$; $p=0.001$) and sample site ($r=0.055$; $p=0.19$). Thus, sample type explains 52% of the variance in the FISH data while sample site is not a significant factor. Both analyses were performed using the R package vegan (Oksanen et al. 2013).

At the conclusion of the study, a Gammaproteobacteria probe, GAM42a, was used in an attempt to identify the large unprobed portion of the 2014 subsample from the Reach. The results are found in Fig. 3.

Bacterial community structure as determined by T RFLP

On average, the bacterial community in the sponge had a Shannon Weiner index of diversity averaging 1.36 ± 0.10, which was not statistically different from the surrounding seawater. In the sponge samples, fragment length 336 was most prevalent, making up 60.73% ± 1.63% on average (Fig. 4a). This fragment length has not yet been associated with any specific bacterial groups. It is also worth noting that SAR202 sequences made up only 1.64% ± 1.48% of the bacterial sequences identified in the sponges, and were not identified in the 2014 sponges, which is in contrast to the FISH data.

SAR11 was the most abundant member of the water community, with its known sequences representing just over a quarter of the bacterial assemblage on average, which corresponds with the FISH data. Known *Gammaproteobacteria* were also significant members, contributing 26.38% ± 2.34% (Fig. S1).

T RFLP analysis was conducted using only bacterial primers. A literature search made it possible to determine specific bacterial species corresponding to some of the fragment lengths (Morris et al. 2005; Apprill & Rappe 2011). Duplicate samples (Fig. S2) show similar T RFLP community profiles, indicating strong reproducibility in this method.

PERMANOVA using distance matrices was determined for sample type (r=0.809; p=0.01) and sample site (r=0.331; p=0.72). Thus, sample type explains 65% of the variance in the T RFLP data while sample site is not a significant factor. Analysis of similarities (ANOSIM) was also determined for sample type (r=0.928; p=0.001) and sample site (r=0.019; p=0.339). Thus, sample type explains 86% of the variance in the T RFLP data while sample site is not a significant factor. Both analyses were performed using the vegan package in R (Oksanen et al. 2013).

Additionally, Bray-Curtis similarity indices and NMDS analyses further support the marked difference between both sponge and water for both the FISH and T RFLP data (Fig. 5).

Ammonia concentrations in T. ignis

In 2014, seawater from within *T. ignis* had ~10x more ammonia (2-4 mg/l) than the ambient seawater (0.25 mg/l). Sponges were transported to the lab in ambient seawater, and this sponge associated seawater contained ~ 1 mg/L ammonia, 4x higher than normal seawater.

AmoA presence

The *amoA* gene was successfully amplified via PCR. The resulting 500 bp amplicon was observed in all the DNA extracted from the microbial community sampled from the sponge tissue. In addition, a 300 bp amplicon was consistently observed in all the DNA extracted from the microbial community sampled within the sponge tissue.

DISCUSSION:

T. ignis *as an HMA sponge*

Our results indicate that *T. ignis* is an HMA sponge. *T. ignis* has previously been identified as a low microbial abundance (LMA) sponge using transmission electron microscopy (Schiller 2006; Wehrl 2006; Gloeckner et al. 2014). Gloeckner et al. (2014) only investigated three samples of *T. ignis* and used TEM on tissue sections. This study investigated 27 replicate samples using epifluorescent microscopy with DAPI enumeration. One gram of sponge tissue was homogenized and diluted with sterile seawater and analyzed within 48 hours. Cell counts were significantly higher than water counts and well within the HMA range of 10^8-10^{10} cells/g in all instances ($p<0.001$; sponge n=27; water n=14).

It is important to note that the Gloeckner et al. (2014) *T. ignis* samples were collected in Florida, while our samples were collected in Bermuda. Phenotypic plasticity has thus far not been found to extend to HMA/LMA status (Gloeckner et al. 2014), but perhaps this is the first identified instance. Alternatively, this discrepancy may be an artefact of the difference between TEM and epifluorescent microscopy for ascribing HMA/LMA status to certain sponges. For a thorough review of the physiological implications for the sponge holobiont concerning its microbial status, see Gloeckner et al. (2014) and references therein.

Archaeal abundance and anoxic microhabitats

Hoffman et al. (2005) suggested that anoxic biochemistry can appreciably influence the sponge holobiont. The discovery of anaerobic microbes in *Porifera* suggests anoxic microhabitats within sponges, which have been known for some time (Riisgad et al. 1983; Hoffman et al. 2005). Most Archaeal species found in sponges have been the methanogenic Euryarchaea (Preston et al. 1996; Webster et al. 2001). Thaumarchaeota, formerly Crenarchaeota, are known ammonia oxidizing

microbes that are prominent in oxyclines (Wright et al. 2012). Both Crenarchaea and Euryarchaea were found within the *T. ignis* sponge tissue via CARD FISH. Both these Archaea can tolerate low oxygen to anoxic conditions (Wright et al. 2012).The presence of both Crenarchaea and Euryarchaea, in addition to the finding that HMA sponges have lower pumping rates (Siegl et al. 2008), provides adequate circumstantial evidence that *T. ignis* has anoxic microhabitats.

T RFLP and FISH comparison

In accordance with previous findings (Wilkinson 1984; Hentschel et al. 2002; Montalvo & Hill 2011; Ribes et al. 2012; Schmitt et al. 2012), the microbial associations of *T. ignis* differed markedly from the seawater. This suggests that sponge species offer alternative niches and/or apply strong selective pressures for microbial symbionts.

TRFLP analysis was specific to marine bacteria while FISH analysis included both bacteria and Archaea. The percent contributions of the bacterial species determined by FISH were adjusted accordingly (Fig. 6a&b).

The sponge associated bacteria were dominated by fragment length 336 using the TRFLP analysis. This fragment length has yet to be identified to a specific bacterial taxon. With the sponge associated bacteria dominated by the unprobed fraction using FISH, this suggests that the bacterial taxon associated with fragment length 336 is unknown. In terms of the microbes identified using FISH, SAR202 contributed 6.60% ± 0.84% to the bacterial community, whereas the T RFLP results indicated little to none present. SAR202 is a clade of bacteria, and in this study, the fragment lengths of 155 and 258 bp were used to identify these diverse *Chloroflexi*. It is possible that there are some clades of SAR202 specific to sponges that have yet to be identified. Thusly, their resulting T RFLP fragment lengths have yet to be associated with the SAR202 clade.

SAR11 is a clade of bacteria, and the FISH probes used in this study were designed to cover the majority of clades (Morris et al. 2002; Carlson et al. 2009). SAR11 was the dominant bacteria within the surrounding seawater at all sites, comprising 34.35% ± 1.81 of the bacterial community. It is also possible that some T RFLP fragment lengths associated with the SAR11 clade have yet to be identified, meaning that contribution by T RFLP could be an underestimation. Previous studies (Morris et al. 2005; Apprill & Rappe 2011), using the same digestion methodology as our study, have attributed fragment lengths 113, 227, and 292 to SAR11.

T. ignis' *putative nitrogen cycling role*

The results from the salicylate test suggest that *T. ignis* has high levels of ammonia within the sponge tissue and may passively leak ammonia into the environment. This is expected due to oxidation of ingested particulate organic matter (see Ribes et al. 2012). Sponges are the first animal reported to release significant amounts of nitrate and nitrite (Corredor et al. 1988; Diaz & Ward 1997; Southwell et al. 2008). It has been known for some time that they also excrete ammonia (Hyman 1940; Brusca & Brusca 1990; Ruppert & Barnes 1994; Bell 2008).

This study has shown high levels of ammonia, the presence of Thaumarchaeota, and the *amoA* gene within the sponge tissue. Thus, ammonia oxidation is a possibility within T. *ignis.* There were two amplicons associated with the *amoA* gene PCR: the expected 500 bp product and an additional 300 bp product. A previous study using the same primer pair found the same sized product from a sediment sample in an Australian estuary (Abell et al. 2010). Though perhaps not originally intended for

amplification, it appears to be an Archaeal *amoA* fragment (GenBank accession no. HQ247867). Interestingly, in this study, this band was only present in the sponge samples.

It has been suggested that the Crenarchaea are responsible for the first step of nitrification in sponges, and *amoA* has been linked to the nitrogen cycle (Lopez-Legentil et al. 2010; Turque et al. 2010). This phenomenon is also suggested for other sponges (Hoffmann et al. 2009 and references therein; Radax et al. 2012). Additionally, a significant amount of *amoA* in an environment may serve as a buffer towards pollution; in this way, *T. ignis* may be a staple for healthy mangrove systems, especially in terms of resisting eutrophication (Turque et al. 2010).

Previous studies have found that Crenarchaea have 1-3 copies of the *amoA* gene per cell (Wuchter et al. 2006; Coolen et al. 2007; Bemen et al. 2008). One study (Bemen et al. 2008) found that for every ~3000 copies of the Crenarchaea 16S rRNA gene, approximately 40 nmol liter^{-1} day^{-1} of NH_4 was oxidized. If this assumption is applied to a single gram of *T. ignis* tissue (assuming 1 *amoA* gene copy per cell), it appears as if this sponge has the potential to oxidize approximately 1700 nmol N cm^{-3} day^{-1}, which is similar to previous results based on similar methods (Radax et al. 2012), though our results are not taking into account possible denitrification processes.

T. ignis can encrust large surfaces with a layer about 1 cm thick (Kaplan 1988) and grow in vertical forms up to 30 cm in height (Voss 1980). This considerable biomass suggests that there is significant potential for *T. ignis* to oxidize all available ammonia, possibly ensuring that the first rate-limiting step of nitrification can proceed unimpeded. Due to its requirement for shallow water and its majority of the total biovolume in mangroves, this makes *T. ignis* a potentially previously unidentified contributor to nitrification in mangrove and inshore environments.

Ammonia oxidizing Archaea have been identified as the main nitrifiers in other *Porifera*, such as the deep sea sponge *Lamellomorpha* (Li et al. 2014). In *Geodia barretti*, the microbial community was also found to be capable of denitrification and anammox reactions, both of which are potential sources and sinks of inorganic nitrogen (Hoffmann et al. 2009). Sponges often ingest nitrogen and excrete NH_4^+, which can then be oxidized for recirculation by other symbiotic microbes (Brusca & Brusca 1990). Nitrogen removal by sponges can be 2-10x higher than that of sediment (Middleburg et al. 1996; Seitzinger & Giblin 1996), and so it was posited that mineralization processes occurring within sponges might be the most important in some marine environments (Hoffmann et al. 2009). Another study tested ten species of sponges, and all but one of them were found to host nitrification (Southwell et al. 2007). The frequency of this association suggests that nitrification is a vital component of the metabolism of most sponge species. This means that in ecosystems where sponges are abundant, their population size and microbial composition has the potential to significantly impact the concentration of available dissolved inorganic nitrogen, a crucial nutrient (Southwell et al. 2007). Based on the evidence presented herein, this may be applicable to *T. ignis*.

In conclusion, the data herein have either newly characterized *T. ignis* as an HMA sponge or demonstrated the first observed instance of phenotypic plasticity extending to microbial abundance within a sponge species. Additionally, our results have suggested the putative role this sponge may have in mangroves in regards to its considerable ammonia oxidation potential, and shown that the single greatest contributor to the microbial consortium, using the aforementioned T RFLP methodology, is 336 bp. Additionally, there is adequate circumstantial evidence for anoxic or hypoxic environments in the sponge's mesohyl, which could support denitrifying populations. Previous trends,

such as marked difference between the sponge and water community and a relatively stable community, have been reinforced. However, more remains to be understood; it is suggested that future investigation includes FISH analysis using the *Poribacteria* probe described in Fieseler et al. (2004). Additionally, clone library analysis should be employed to discern the identity of the 336 bp T RFLP product, as this bacterium's presence is so significant that it likely is the best definer of the holobiont's biochemistry. Direct nitrogen quantification should also be employed.

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705
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709 Table 1

Site Name, Year (Abbreviation)	Coordinates
Ferry Reach, 2012 (R12)	32°22’13.27” N, 64°41’43.63 W
Ferry Reach, 2013 (R13)	
Ferry Reach, 2014 (R14)	
Ferry Reach Mangrove, 2013 (RM)	32°22’16.69” N, 64°41’39.33 W
St. George Harbour, 2013 (SGH)	32°22’18.50” N, 64°41’09.98 W
Helena’s Bay, 2014 (HB)	32°22’21.55” N, 64°41’26.42 W
Bailey’s Bay, 2014 (BB)	32°21’01.51” N, 64°43’22.51 W

710

Figure 1(on next page)

Average microbial abundances of samples

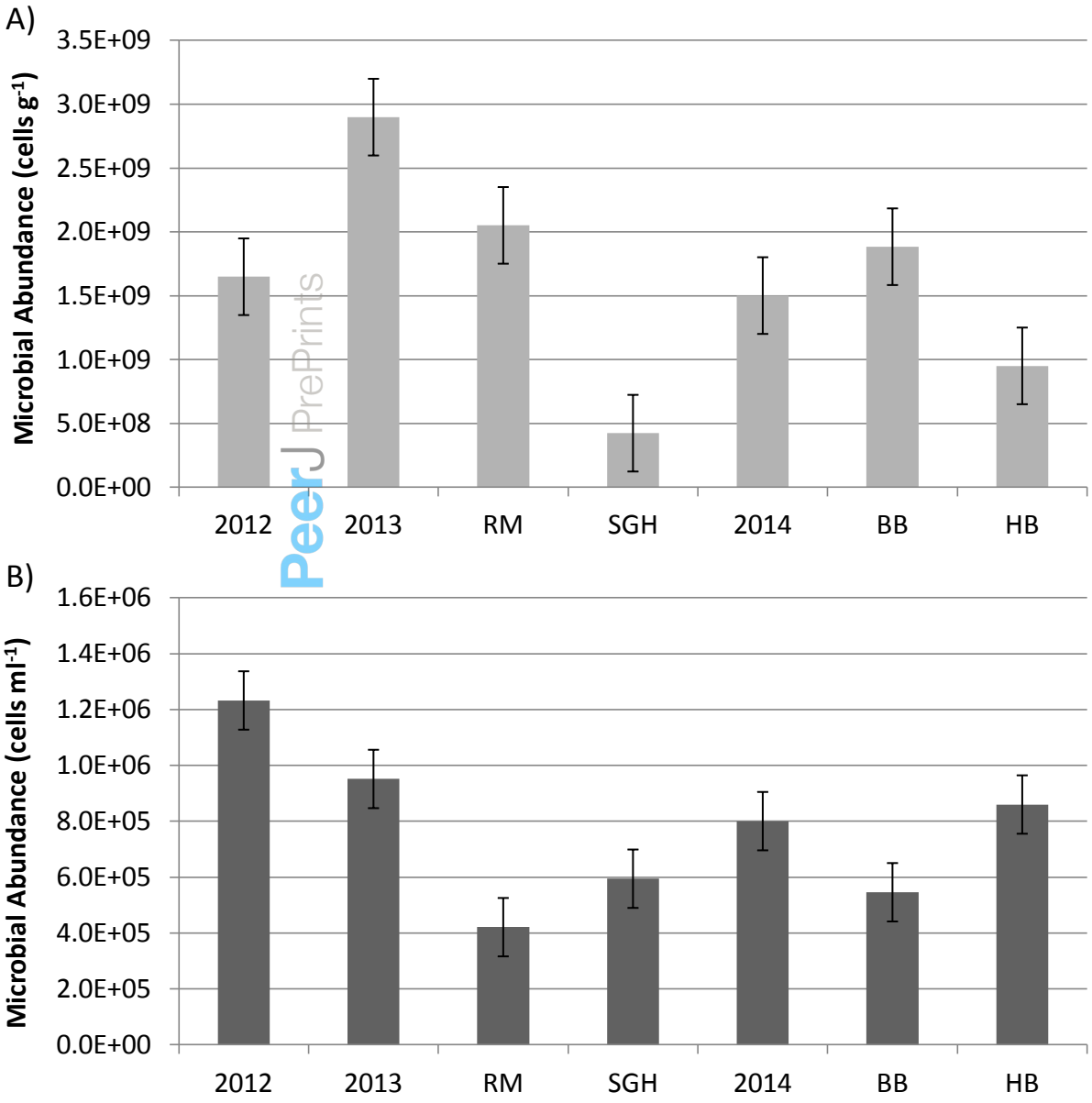

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Figure 2(on next page)

Averages of FISH probing for different samples

Figure 2: The averages of FISH probing at each site for A) sponge tissue and B) seawater

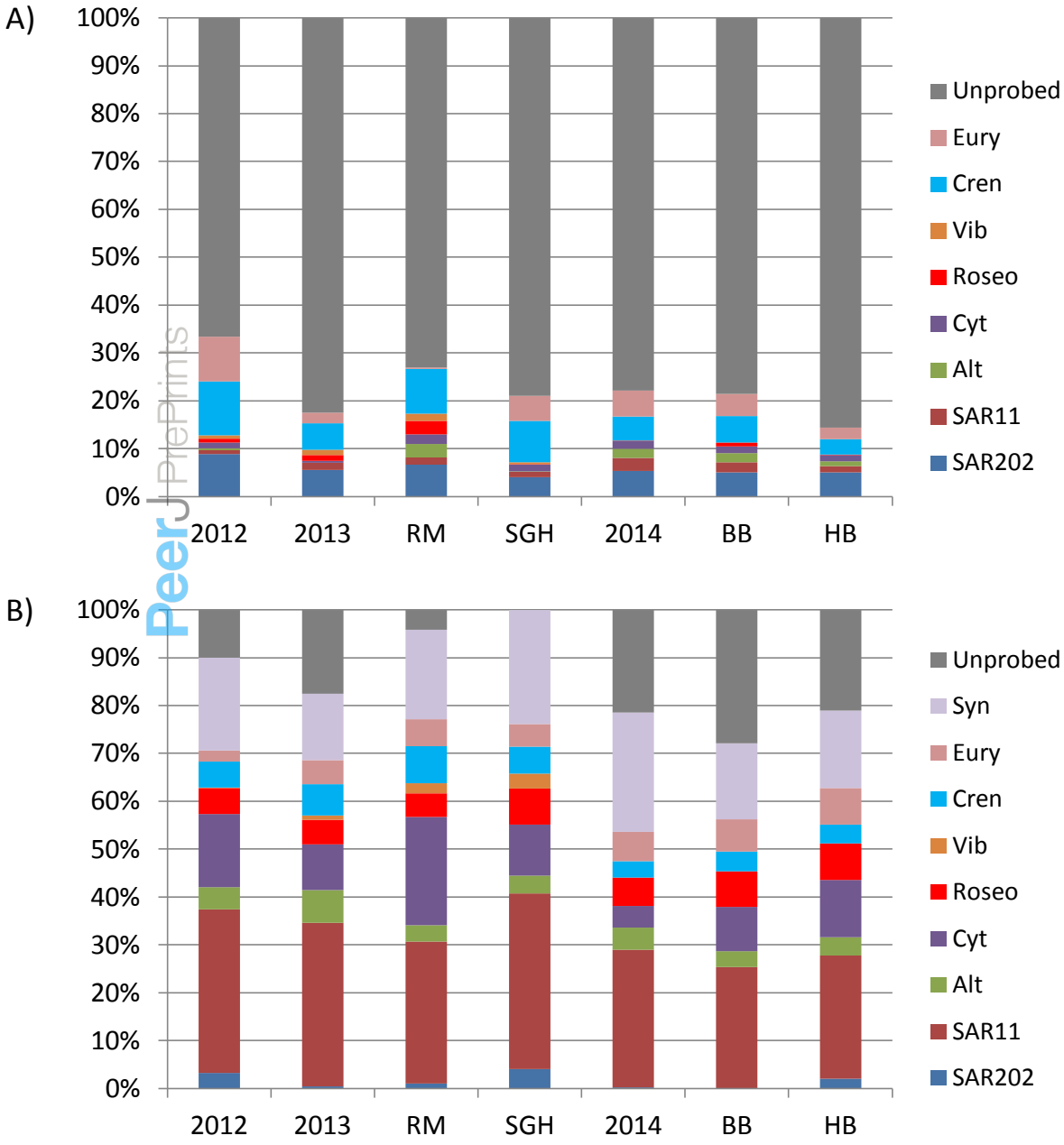

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Figure 3(on next page)

FISH as microbial taxons

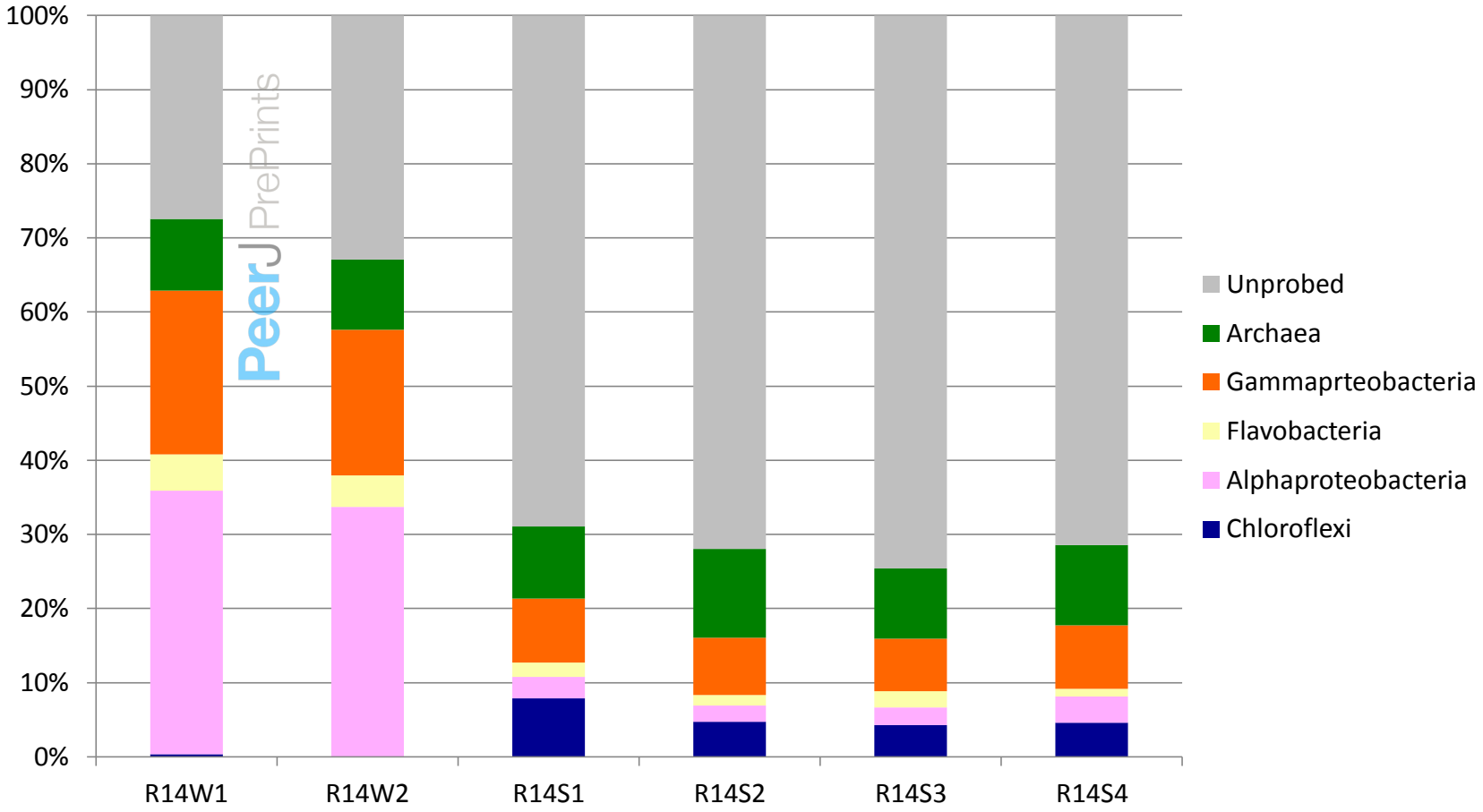

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Figure 4(on next page)

T RFLP data for different samples

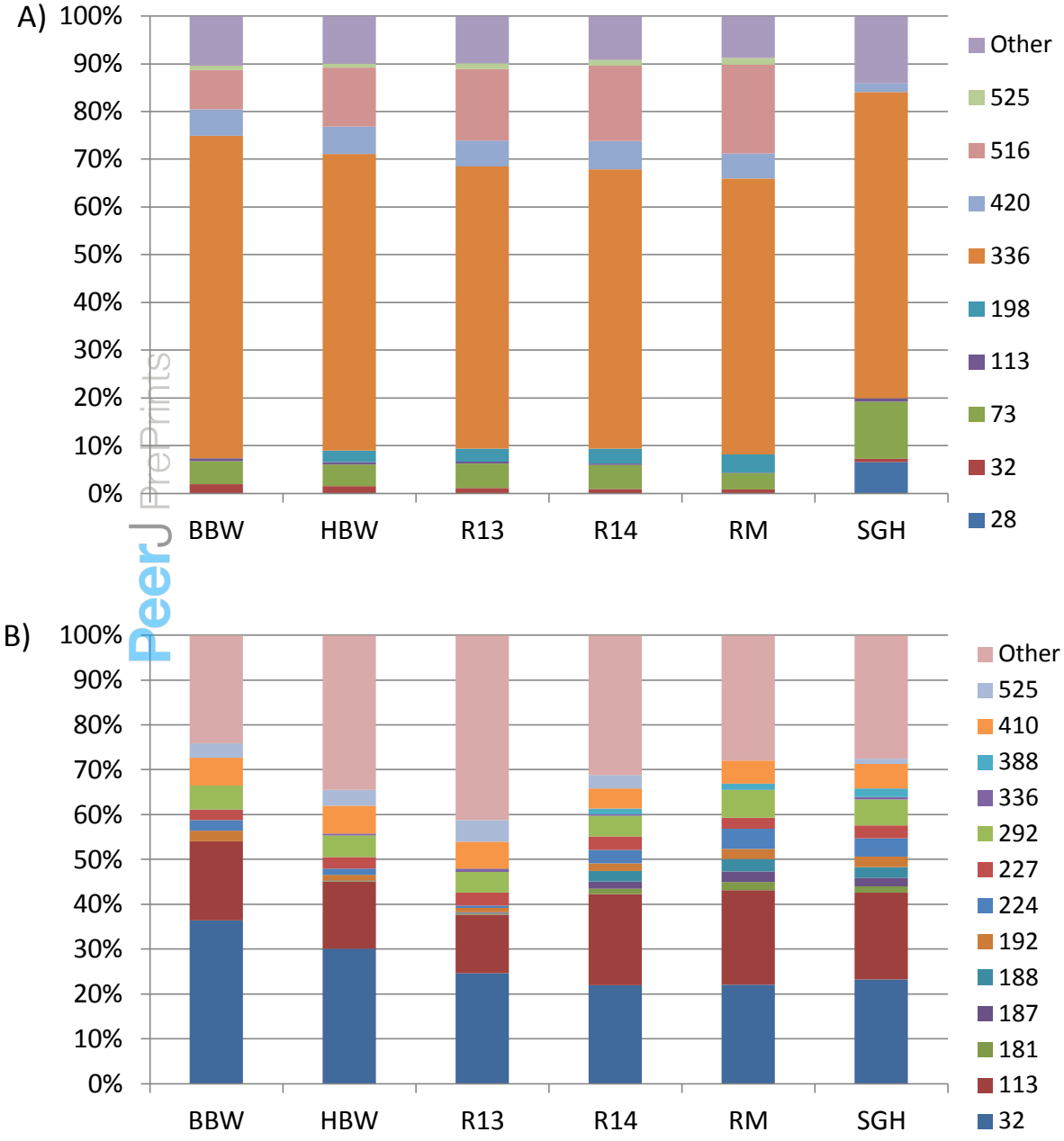
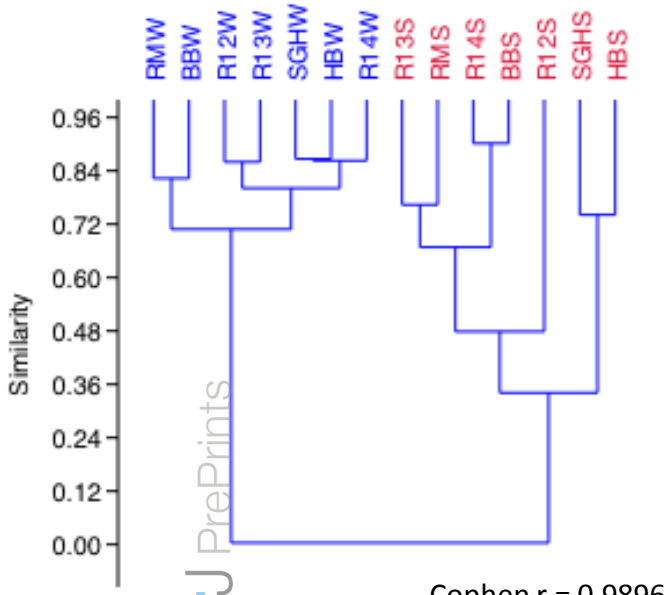


Figure 5(on next page)

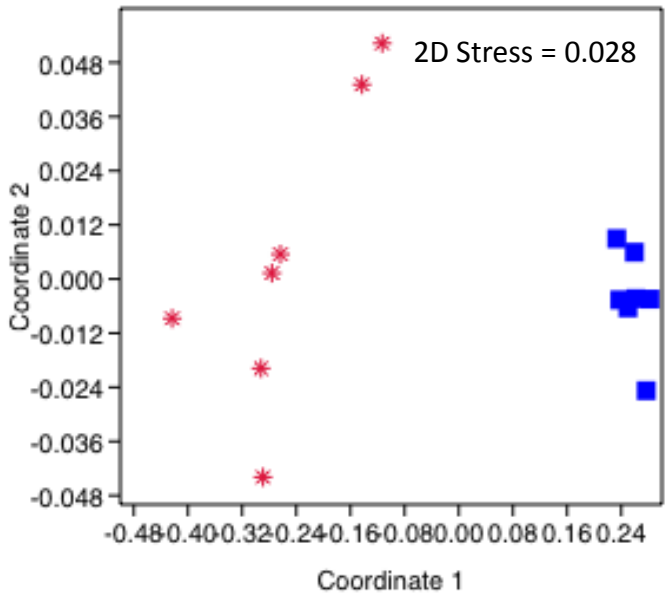
Bray-Curtis and NMDS of FISH and TRFLP for all samples

1A)

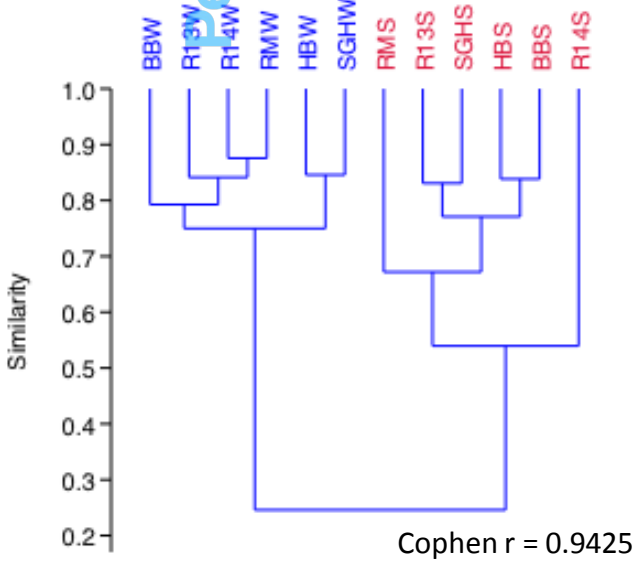


Cophen r = 0.9896

2A)



1B)



Cophen r = 0.9425

2B)

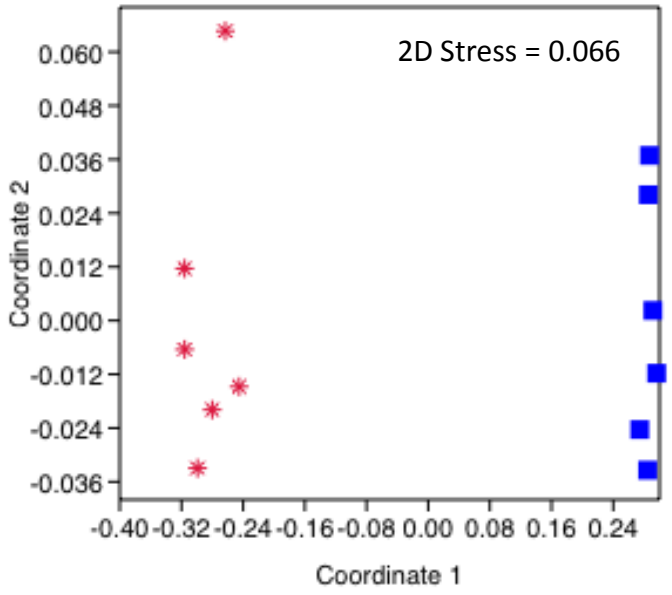
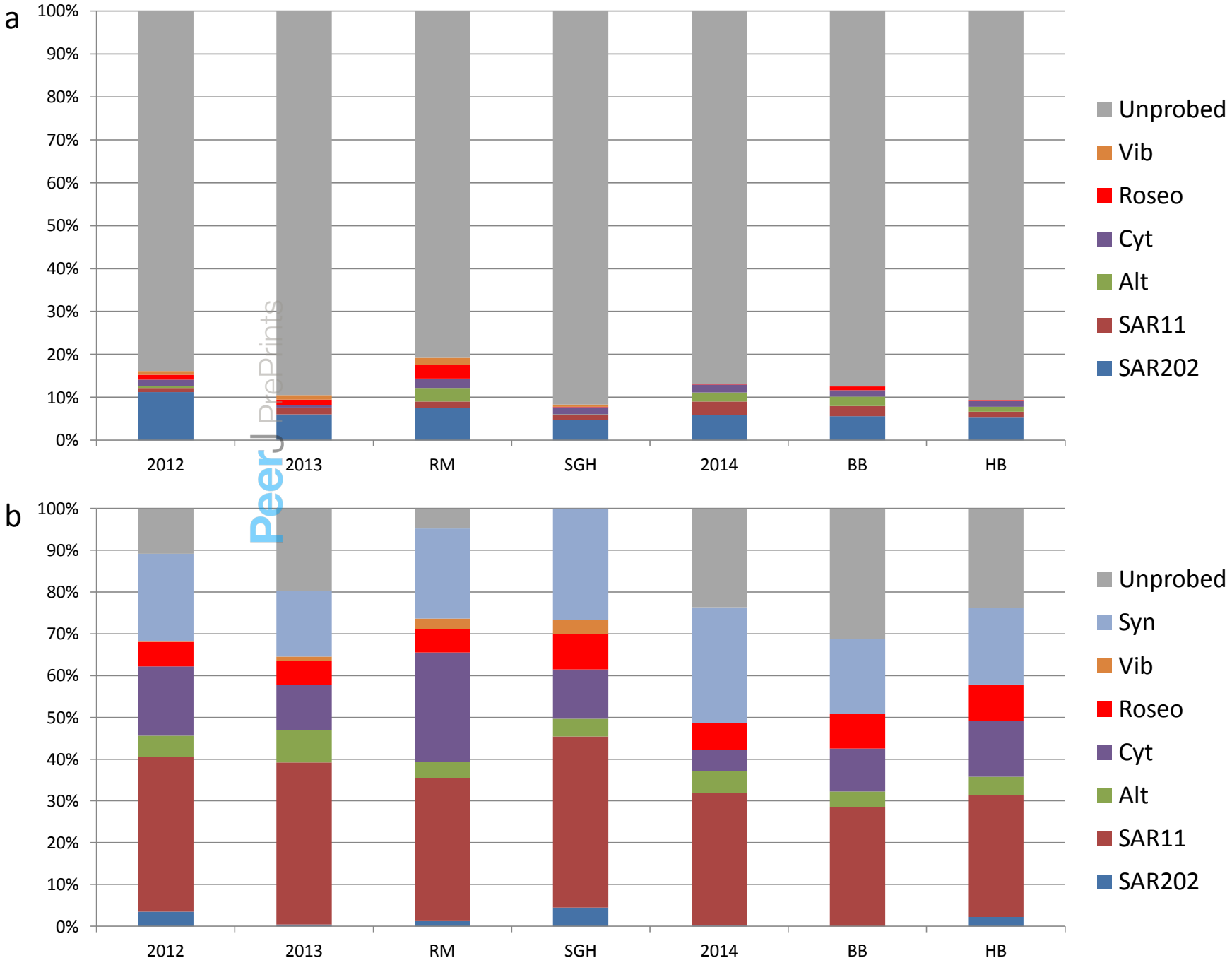


Figure 6(on next page)

FISH adjusted eubacterial percents



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