SPONGE-ASSOCIATED DEHALOGENATING MICROORGANISMS AND ISOTOPE ANALYSIS OF THEIR DEHALOGENATION OF BROMINATED PHENOLS

By

ISABEL DEL CARMEN HORNA-GRAY

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And Approved by

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ABSTRACT OF DISSERTATION

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ISABEL DEL CARMEN GRAY

Dissertation Director

Professor Max Häggblom

Sponges are filter feeders, ancient animals that have been extremely successful in surviving for over 600 million years almost unchanged. These metazoa can harbor microbes comprising almost 35% of their bodies at densities of 10^9 cells/g. Sponges are also rich in organohalides, usually with bioactive cytostatic and/or cytotoxic characteristics. Some of these organohalides resemble anthropogenic pollutants, such as halogenated dioxins and flame retardants. Thus, in nature some sponges contain both a high number of microbes as well as a high concentration of organohalides. This includes the sponge *Aplysina aerophoba,* from where *Desulfoluna spongiiphila* was isolated, a sulfate reducing Deltaproteobacteria that can reductively dehalogenate bromophenols. This observation led to the interest of enriching for sponge-associated microbes capable of dehalogenation from different ecoregions of the temperate and tropical oceans. The hypothesis is that dehalogenating bacteria are widespread among sponge species regardless of geographical location and form stable populations within the sponge animal that function in the cycling of organohalide compounds. Anaerobic dehalogenating activity was found to be widespread among sponges. In addition, new

isolates obtained were closely related to *D. spongiiphila*. Compound Specific Isotope Analysis (CSIA) of 2,6-dibromophenol and its dehalogenation products revealed that carbon isotopic enrichment factors for sponge-associated bacterial dehalogenation are identical, probably due to a high similarity in the dehalogenating mechanism. The results support the hypothesis that dehalogenating microorganisms are widely distributed within marine sponges. The association between host sponge and associated microbiota is probably driven by the unique organohalide chemistry. At the same time the research of dehalogenating sponge-associated microorganism provided a unique setting for a deeper understanding into microbe-animal associations that could potentially be an ancient symbiosis.

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DEDICATION

To Gladys and Esteban, my parents for instilling in me the love for the life sciences and their unwavering support throughout all of these years.

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Unlocking the Secrets of Sponge-Associated Dehalogenating Microbes

1.1. Marine sponges survival traits: Symbionts and Organohalides

Marine sponges (*Porifera*) are the simplest organized metazoans. Most of these sessile creatures are filter feeders found worldwide, thriving in temperate and tropical waters in shallow and deep water environments. It is believed that the ability to survive highly diverse habitats is due to the capacity of sponges to establish relationships with diverse microbial communities (Hentschel *et al*., 2006). In some sponges the microbial symbionts can reach 35% of the animal biomass (Hentschel *et al*., 2012). In addition, these sessile organisms are known for their production of bioactive molecules as part of their general strategy for survival (Becerro *et al*., 2003). There are hundreds of compounds registered as bioactive molecules obtained from sponges each year. In the latest review of marine natural products (Blunt *et al*., 2014), 355 new bioactive compounds from marine sponges were published in the year 2012 alone. The sponge microbiology, bioactive compounds and microbial reductive dehalogenation fields were connected with the discovery of *Desulfoluna spongiiphila.* This strict anaerobic microorganism was enriched and isolated from *Aplysina aerophoba* (Ahn *et al*., 2003, 2009), a sponge that produces brominated tyrosines that can reach 7-12% of the sponge's body weight (Teeyapant *et al*., 1993). *Desulfoluna spongiiphila* is capable of reductively dehalogenating brominated and iodinated orthophenols to phenol (Ahn *et al*., 2003, 2009). *D. spongiiphila* is a member of the *Deltaproteobacteria,* a sulfate reducer most closely related to *Desulfoluna butyratoxydans* (Ahn *et al*., 2009),

There are many excellent reviews pertaining to this work, which focus on marine sponge microbiology (Hentschel *et al*., 2006; 2012; Vogel, 2008; Siegl *et al*., 2008; Taylor *et al*., 2007; Webster *et al.,* 2010; 2012), sponge bioactive molecules (Gribble 2000; 2003;

Forte *et al*., 2009;Thomas *et al*., 2010; Blunt *et al*., 2015), or microbial reductive dehalogenation (Smidt and de Vos, 2004, Häggblom and Bossert, 2003; Maphosa *et al.,* 2010; Zanaroli *et al*., 2015). This chapter reviews marine sponges, their microbiology and known halogenated bioactive molecules, bacterial dehalogenation, and the organohalide respirers.

1.2. Marine sponges

Sponges have survived through all 5 mass extinctions, and at the time of the Permian extinction 250 million years ago, they comprised part of only 4% of the species left on the whole planet. No doubt their design and strategies for survival are impressive. Most sponges are filter feeders and they have no clear differentiation of tissues and no central nervous system (Hooper *et al.,* 2002). Sponges belong to the phylum *Porifera*, the earliest branching phylum of the metazoa, and are considered the last extant multicellular creatures that connect us (metazoan) with the Urmetazoan, the first evolved multicellular organism (Müller, 2003). The oldest physical evidence of sponges is from 580 million years ago (Li *et al.*, 1998). The earlier Urmetazoa are thought to have started as colonial organisms with cell adhesion molecules and later developed an immune and apoptotic system into an integrated organism (Müller, 2003). The study of the genes that govern these characteristics in metazoans is of great importance to oncology because cancer seems to originate from a malfunction of those same features. In addition, sponge bioactive molecules are being used to inhibit the growth and proliferation of cancerous cells (Schumacher *et al.*, 2011), and marine sponges appear to produce a wide variety of molecules that can interfere with the cancerous cells and even kill them.

The roles of sponges in the marine environment are diverse. They can be classified for their a) impacts on substrate, (b) bentho-pelagic nutrient cycling; and (c) associations

with other organisms. In the latter case, sponges are facilitators of primary production, secondary production, providers of microhabitat, and enhanced predation protection, they also can act as biological disturbances, and releasers of chemicals (Bell, 2008). The role of sponges is often underestimated, but their resilience and persistence is evident in the incredible range of habitats that they inhabit.

Sponges are found in all oceans, as well as in freshwater, spanning from tropical, Arctic and Antarctic ecoregions (Van Soest, 2012). They have adapted to survive at a wide range of depths in the marine environment. Some are thought to live for millennia, such as *Xestospongia* sp. (McMurray *et al*., 2008). Sponges are simply organized (see Figure 1.1). They are contained within the pinacorderm, a sort of skin, made of pynacocites, which covers the outside of the sponge and its internal chambers. Within its body these chambers are lined with choanocytes, flagellated cells that create the water flow necessary for the filtering system that feeds the sponge. In between these canals and chambers, sponges contain a matrix called the mesohyl, containing cells like the archaeocytes (totipotent cells), sclerocyte (spicule producers), and porocyte (some can contract like a myocyte and regulate flow of water). Organic filaments and spicules (calcium carbonate, or siliceous) give sponges their shapes (Van Soest, 2012; Van Soest and Hooper, 2002).

The *phylum Porifera* is divided into 3 subclasses: *Calcarea*, characterized for the presence of calcium carbonate spicules; *Hexactinellidae*, also known as glass sponges, considered ancient and common deep water dwellers, which contain distinct six rayed siliceous spicules; and *Demospongiae*, the most diverse extant sponges represented by approximately 90% of the described species today. They contain spongin and siliceous spicules (Van Soest, 2012; Hooper, and Van Soest, 2002).

The allure of marine sponges to scientists is based mainly on: the clues they hold to the development of multicellular animals (Adamska *et al*., 2011); their associations with microbes (Siegl *et al.*, 2008; Taylor *et al.*, 2007); and the production of bioactive compounds (Faulkner *et al.,* 2000; Gribble *et al.*, 2003; Blunt *et al.*, 2014). Sponge resilience over millions of years is possibly due to their capacity to associate with different microbes and the bioactive molecules they produce for cellular control (cytotoxic, cytostatic) that deter predators or parasitic invasions. Sponge bioactive molecules often contain halogenated substitutions of chlorine, bromine or even iodine. These molecules conform a great percentage of the natural or biogenic organohalides (Gribble, 2003). There have been reports of certain marine top predators like pigmy sperm whales accumulating dibromophenoxyanisoles found in sponges (Vetter *et al*., 2002).

Sponge Symbioses

Sponge symbiosis is part of the strategy thought to explain the survival of sponges through all known mass extinction events. These metazoans have been fascinating subjects since microscopy allowed scientists to find great numbers of microbes in healthy sponge mesohyl (Dosse, 1939). Later, Wilkinson suggested a possible ancient symbiosis between sponges and microorganisms (1984). To date we do not know how most of the symbiotic relationships work, or whether the sponges or microorganisms govern the whole process, but most likely it is an interaction of both.

Initial research was constrained, as in other fields of microbiology, by the fact that only a limited number of sponge-associated microorganisms could be cultivated. However we are now free to study sponge symbionts at a different level with the use of molecular biological tools. The resulting observation was that sponges in general contained a great

number of different microorganisms (Hentschel *et al*., 2002). Although sponges are filter feeders, all these bacteria were not being consumed. Is it possible that a primitive and barely structured animal could contain these microorganisms by accident? On the contrary, sponges are actually capable of even differentiating between the types of bacteria on which they feed (Wilkinson *et al*., 1984; Wehrl *et al*., 2007). They show autoinmune responses after exposure to lipopolysaccharide (LPS) (Müller *et al*., 2004). Sponges can select for the microorganisms that survive in their mesohyl, and certain bacteria can survive inside the sponge (perhaps a combination of both). These relationships are analyzed in detail in the review of Taylor *et al*. (2007). In addition, almost 40% of some sponges were demonstrated to be microbial symbionts (Webster *et al.,* 2001). The next questions were what type of microorganism are they; and, what is their purpose?

In the search answers to these questions, studies have revealed a complex microbial community found in sponges that varied with each host species. However, a core group of diverse microorganisms was uniformly found in sponges from all over the world (Hentschel *et al*., 2002; 2006; Webster *et al*., 2010; Lafi *et al*., 2009; Olson and Gao, 2013). Within this core group of common microorganisms were the *Poribacteria* (Fieseler *et al*., 2004), a group of yet uncultured microorganisms. These were studied with single cell genomics, and are probably mixotrophs, with specific characteristics that aid them in the colonization of the sponge mesohyl (Siegl *et al*., 2011). It is clear that the complex microbial community in sponges must exert an important role. At the same time, when the sponges were studied for their microbial content a pattern started to appear - sponges clearly had high or low microbial abundance in their mesohyl (Weisz *et al*., 2008 a, b).

Microbial numbers and diversity are used to separate sponges into low microbial abundance (LMA) and high microbial abundance (HMA) species (Vacelet and Donadey, 1977; Hentschel *et al*., 2003; Gloeckner *et al*., 2014). Differences between LMA and HMA sponges impact their overall physiologies. It is believed that LMA sponges filter small particulate organic matter from large volumes of seawater, while HMA sponges promote symbiotic associations with diverse prokaryotes in order to thrive. HMA sponges contain 2 to 4 orders of magnitude more *Chloroflexi*, *Actinobacteria*, *Poribacteria* and archaeal ammonia oxidizers than LMA and surrounding water (Schmitt *et al*, 2011; Giles *et al*., 2013; Gloeckner *et al.,* 2014). The differences have been observed in sponges from tropical and temperate ecoregions, as well as different depths all over the world.

The use of gene sequencing allowed greater data retrieval from environmental samples, and therefore more information about the microorganisms associated with sponges (Webster *et al*., 2010). One finding was that some bacteria, which were thought to be sponge-exclusive such as the candidatus phylum *Poribacteria*, were also found in seawater and LMA sponges (Webster *et al*., 2010). This was elucidated by Moitinho-Silva *et al*. (2014), when the study of transcriptomic data revealed that *Poribacteria* were not active unless living inside HMA sponges. Along with clarification that, the microbial community present in LMA, even though at much lower numbers, is very specific to each host (Blanquer *et al*., 2014; Moitinho Silva *et al*., 2014b; Burgsdorf *et al.,* 2014).

Certain groups of microbes, such as members of the *Chloroflexi* (Schmitt *et al*., 2011) are considered enriched in HMA, more closely related to another HMA Chloroflexi, than to other microbial communities from sponges in the same region, or even a closer sponge species. Conversely, microbial sponge symbiont communities are more similar

in genetically distinct *Petrosia ficiformis* from the same location, than genetically similar *P. ficiformis* from distant locations (Burgsdorf *et al*., 2014). Oddly, then geography can matter or not depending on the microbial group and, the type of sponge (HMA or LMA) being studied.

It is believed that heterotrophy in LMA sponges is greater than in HMA sponges (Poppell *et al.,* 2014), as well as the number of choanocytes and pumping activity (Weisz *et al*., 2008). Sponges such as deep water *Geodia sp*. and *Aplysina aerophoba* can create anoxic pockets within their tissues, probably favoring the anaerobic and microaerophilic symbionts. It is hypothesized that sponges purposely create anoxic conditions in order to benefit from the potential detoxification that symbionts can perform (Hoffmann *et al*., 2005; 2008; Bruck *et al*., 2010). For a summary of interesting facts on sponge microbiology see Table 1.1.

1.2. Organohalides

Anthropogenic organohalides have been very important for the development of our industrial society due to their usefulness in energy production, agriculture, disease control and a myriad of other applications. The halogen substituents generally reduce the biodegradability of an organic substrate by changing the physical or chemical attributes (Häggblom and Bossert, 2003).

The physicochemical properties of organobromides cause their accumulation in anoxic sediments. These compounds have ecotoxicological effects (Reineke *et al*., 2006) and/or biomagnify in the food chain (De Wit *et al*., 2002; Fernie and Letcher, 2010; Bartrons *et al*., 2012). An example of economically and ecologically important organobromides are the brominated flame retardants (BFR) widely used to avoid fire

accidents in electronics, indoor household fabrics and, even in children's clothing (De Wit, 2002, 2010; BSEF, 2015). Early studies of human serum, adipose tissue, and breast milk showed that polybrominated diphenyl ethers (PBDEs) were prevalent at 17 times higher concentrations in North American versus European individuals (Hites, 2004). It is estimated that inadvertent ingestion results in uptake of 0.02-0.2 grams/day by young children ages 1-4 years (Stapleton *et al*., 2005). Several BFRs are known thyroid and estrogen disruptors, including PBDE and TBBPA (Meerts et al., 2001; Hamers *et al*., 2006, 2008). There are also reports of the increasing presence of organobrominated compounds in marine biota including whales, seals and, dolphins from the Artic and North Atlantic regions (Rotander *et al*., 2012; Johnson-Restrepo *et al*., 2005). There is concern, supported by this evidence, that the increasing anthropogenic use of organobromides is reaching the marine environment. The same characteristics of organobromides that make them useful for industrial society also gives them their toxicity and bioaccumulation features. Therefore, it is important to determine their fate and transport in the environment.

The presence of organohalides in the environment is not all from anthropogenic sources. The marine environment including marine sponges, is a particularly rich source, of biogenic organohalides, which are structurally diverse, including indoles, phenol derivatives, biphenyl ethers, dioxins, and brominated pyrroles like the simple oroidin and 12-ring stylissadines A and B (Figure 1.2), each possessing 16 stereo centers, which are very difficult to synthetize in the laboratory (Gribble, 2003; Beukes, 1998; Utkina *et al*., 1998; Bowden *et al*., 2000; Utkina, 2006; Utkina *et al.*, 2001; Forte *et al*., 2009).

Natural Organohalides in the Marine Environment

Although estuarine and marine sediments are considered significant sinks for anthropogenic organohalides, the marine environment is also a great source of natural organohalides that are produced by marine plants and animals (Neidleman and Geigert, 1986; Gribble, 2000). The abundance of organohalides is believed to be part of the strategy to survive predation, competition and biofouling (Bakus *et al*., 1986; Pawlik, 1995; Fusetani, 2004; Paul & Puglisi, 2004). Brominated compounds can account for over 10% of the sponge dry weight (Teeyapant *et al*., 1993; Turon *et al.,* 2000). These bioactive molecules can affect other metazoans behavior mostly as feeding deterrents against fishes and crabs (Thoms *et al*., 2004; Waddel and Pawlick, 2000), and function as antifouling agents effective against barnacles (Hertiani *et al*., 2010). Some studies of deterrence have shown that bioactive molecules alone would not provide defense against predation (Hill *et al*., 2005) and in some cases it is instead a combination of spicules and other characteristics that protects sponges against certain predators.

Production of bioactive compounds is an inherent characteristic of the metazoans, supported by the effectiveness of the sponge bioactive molecules collected from different habitats (Becerro *et al*., 2003). Marine sponges produce diverse and multifunctional bioactive compounds (see Table 1.2). These sessile organisms rely on their chemical and physical arsenal to deter predators and control microbial growth. The simple organization of sponges is counterbalanced by the incredible variety and scope of the bioactive molecules they produce that can have biotechnological and pharmaceutical purposes (see Figure 1.3) (Gordaliza *et al*., 2010). Based on the reviewed data, almost every sponge tested contains bioactive compounds. Even species found in the Antarctic show bioactive activity (Abbas *et al*., 2011), but only molecules that show a strong

potential are typically extracted and identified. Many of the natural organohalides are probably not toxic or highly specific, which explains the gap in knowledge of sponge organohalide production.

1.3. Dehalogenation

It is believed that natural organohalides in the environment have given rise to the peculiar dehalogenating microbes that we now study (Krzmarzick *et al.*, 2011). Microorganisms have had billions of years to evolve their metabolic capabilities. A variety of metabolic strategies have been found to cleave the carbon-halogen bond (Häggblom and Bossert, 2003).

Among the metabolic strategies of dehalogenation, one of the most interesting is reductive dehalogenation, first demonstrated in sediments by Suflita *et al*. (1982) leading to the isolation of the dehalogenating bacterium (Shelton and Tiedje, 1984). Reductive dehalogenation allows microorganisms to use the organohalide as an electron acceptor and obtain energy from this respiration. This process is known now as organohalide respiration (Mohn and Tiedje, 1992).

Respiratory Reductive Dehalogenation

Microbial flexibility in using different electron acceptors other than oxygen is well documented for the main classes of prokaryotes (for reviews see Schmitz *et al*., 2006; Häggblom and Bossert, 2003; Smidt and de Vos, 2004). Among them, is the use of organohalides as electron acceptors and if the dehalogenation is dissimilatory it is considered organohalide respiration (Mohn and Tiedje, 1992; El Fantroussi *et al*., 1998; Holliger *et al*., 1998; Häggblom and Bossert, 2003; Smidt and de Vos, 2004).

Organohalide respiring bacteria are anaerobes that gain energy from transferring electrons to the organohalides which results in a reductive cleavage of the carbon-halogen bond. These bacteria, such as *Dehalococcoides*, *Dehalobacter* and *Desulfitobacterium* spp. are widespread. They occupy a metabolic redox niche between the iron reducers and the sulfate reducers, It is believed they compete with the sulfate reducers for the reductant (H_2) (Bossert *et al*., 2003; Smidt and de Vos, 2004) and are found in proximity to fermenters, which provide them with hydrogen as a source of electrons. The organohalide respiring bacteria are mostly known for their capability to dechlorinate tetrachloroethene (PCE), trichloroethene (TCE), chlorobenzenes, and chlorophenols, polychlorinated dibenzo-p-dioxins (Holliger *et al*., 1993; Neumann *et al*., 1994; Cole *et al*., 1994, Miller *et al*., 1996; Beurskens *et al*., 1995; Liu *et al*., 2014). See Table 1.3, for examples of dehalogenation of halogenated aromatics.

Dehalogenase enzymes exhibit high diversity (Holscher *et al*., 2004; Richardson, 2013; Hug *et al*., 2013). However few dehalogenases have been characterized, and only recently published is the first complete structure of Pce A, the model dehalogenase from *Sulfurospirillum multivorans* (Bommer *et al*., 2014; Edwards *et al.,* 2014). There is still much we do not know about dehalogenases and their substrate scope. But, the recent description of x-ray crystal structure of PceA from *S. multivorans* show that the dehalogenase conforms to a vitamin B_{12} variant containing a reactive cobalt atom crucial for dehaloelimination and a selective entrance to the reactive site shaped as a letterbox. The reactive site is located deep inside the structure of the protein, which is accessed after a long hydrophobic channel (Bommer *et al*., 2014). This specialized selection of of different halogenated substrates through slection by access to the active site, might explain the high number of dehalogenases found in the genome of *Dehalococcoides mccartyi* strains allowing them a wider substrate range (Seshadri *et al*., 2005; Poritz *et*

al., 2013; Maphosa *et al*., 2010). The high sequence similarity of dehalogenases does not ensure high functional similarity. Also, dehalogenases appear to be a monophyletic group and dehalogenate chlorinated ethenes and 1,2-dichloroethane with the exception of TceA, Bvc A, VcrA (Hug *et al*., 2013). Some of these dechlorinating bacteria, such as *D. mccartyi* CBDB1 (Wagner *et al*., 2012, and *Desulfitobacterium chlororespirans* (Cupples *et al*., 2005), can also dehalogenate brominated compounds (see Table 1.4). This functionality supports the genomic data related to the diversity of dehalogenases found within dechlorinating microorganisms.

Other organobrominated compounds of interest include tetrabromobisphenol A (TBBPA) that can be anaerobically dehalogenated to bisphenol A (BPA) by enrichment cultures from sediments (Ronen and Abielovich, 2000; Voordeckers *et al.,* 2002, Ravit *et al.,* 2005; Zhang *et al.*, 2013). Deca-PBDEs are dehalogenated; He *et al*. (2006) found that. *D. mccartyi 195* transformed the octa-BDE mixture into five or six and seven- bromine forms, but did not alter deca-BDE. When other microbes were added to *D. mccartyi 195*, the mixed culture continued debromination generating two- and four- bromine PBDEs. This is a perfect example of how microorganisms can work together to utilize a substrate that each alone could not have done. The generation of lower halogenated compounds and biomagnification has been proven in polybrominated diphenyl ethers (PBDEs) (Bartrons *et al*., 2012). Instead dehalogenated product of TBBPA, BPA was not more toxic to zebra fish embryos (McCormick *et al*., 2010). Reductive debromination seems to occur widely, and one of the most singular findings was dehalogenating activity in enrichments from marine invertebrates.

Most of the described organohalide respiring microorganisms come from polluted sediments, freshwater sediments (Smidt and de Vos, 2004; Maphosa *et al.*, 2010). In

comparison there are few studies about dehalogenation in marine environments (Häggblom *et al*., 2003; Zanaroli *et al*., 2015).

Desulfoluna spongiiphila, is a curved gram-negative microorganism isolated from an HMA sponge, *Aplysina aerophoba* from the Mediterranean Sea, after successive enrichments in 2-BP in anaerobic media supplied with lactate. This microorganism is capable of dehalogenating 2,4,6-tribromophenol (2,4,6-TBP) and 2,6-dibromophenol (2,6-DBP), as well as, 2- and 3-iodophenols, but not fluorophenol (see Table 1.4). It is also a sulfate reducer, and sulfate reduction occurred at the same time of dehalogenation and increasing biomass in comparison of cultures grown only with 2-BP as electron acceptor (Ahn *et al*., 2009).

Isotope Fractionation

The microbial dehalogenating activity is catalyzed by dehalogenases. The study of these enzymes can be achieved by the analyzing their genotype (molecular techniques) and the phenotype (substrate profiles and isotope fractionation). Carbon isotope fractionation is a powerful tool in environmental science because it allows for assessment of natural attenuation *in situ* and the elucidation of dilution or degradation in a moving plume. Another important use of compound specific isotope analysis (CSIA) is to clarify the differences that exist in the dehalogenating mechanisms of pollutants, such as PCE, TCE and chlorobenzenes (for reviews see Meckenstock *et al*., 2004; Elsner, 2005; Bombach *et al*., 2010).

The studies of carbon isotope fractionation are based on the fact that cleavage of chemical bonds containing heavier isotopes requires more energy than cleavage of bonds containing lighter isotopes. During microbial biotransformations ^{12}C containing

molecules will react faster, resulting in an enrichment of ^{13}C in the residual substrate. The change of the isotope ratio δ ($^{13}C/^{12}C$) is related to the changes in concentrations, and is expressed as an isotope fractionation factor (α), derived using the Rayleigh equation (Meckenstock *et al*., 1999). Different isotope fractionation factors can potentially be linked to specific biochemical reactions (Sherwood-Lollar *et al.,* 1999). CSIA has been used extensively to study the degradation and fate of aquatic pollutants (see Table 1.5), including chloroethenes (Hunkeler *et al*., 1999; Slater *et al*., 2001; 2003; Vanstone *et al*., 2004; Nijenhuis *et al*., 2005; Liang *et al*., 2007; Liu *et al*., 2014), chloroethanes (Hunkeler and Aravena, 2000; Hirschorn *et al*., 2004; 2007; Vanstone *et al*., 2007), aromatics including benzene, toluene, chlorobenzenes, chlorinated phenols, dioxins (Meckenstock *et al*., 1999; Griebler *et al*., 2004; Aeppli *et al*., 2013; Ewald *et al*., 2007; Liang *et al*., 2014), fuel oxygenates such as MTBE (Somsamak *et al*., 2005, 2006; Youngster *et al.*, 2010; Rosell Linares *et al*., 2012), and field studies of natural or assisted bioremediation (Sherwood-Lollar *et al*., 2001; Nijenhuis *et al*., 2007; Imfeld *et al*., 2008; Stelzer *et al*., 2006; 2009; Braeckevelt *et al*., 2012). The aim is to develop methods for monitoring bioremediation of polluted sites by determining if the native microbial communities can degrade the pollutant, or by monitoring isotope fractions of the pollutant and its intermediaries. Another important use of CSIA is to clarify the differences that exist in the dehalogenating mechanisms. There are instances were the same compound is degraded differently by the same microorganism under different conditions because of a change in the reactive mechanism. Also there are other subtle aspects of the fractionation of a compound that create variation of the isotope enrichment factor even when using the same microorganism and same conditions. Numerous studies have addressed this issue (Nijenhuis *et al*., 2005; Cichocka *et al*., 2007; 2008; Kampara *et al*., 2008; Aeppli *et al*., 2009). Most recently Renpenning *et al.* (2014, 2015) showed clear differences in the reaction of dehalogenases Pce and Tce

based on the type of corrinoid in the dehalogenase and membrane characteristics of the microorganisms involved. There are other studies that have observed small variations in isotopic enrichment values due to membrane transport effects, enzyme activity and availability (Slater *et al*., 2003; Nijenhuis *et al.,* 2005; Lee *et al*., 2007; Renpenning *et al*., 2014, 2015).

Use of CSIA for bromophenols

CSIA has been used for many years in the study of dechlorination (see Table 1.5), but there has been relatively little research on brominated aromatics. Bernstein *et al*. (2013) studied Br isotope fractionation during debromination of 2,4,6-TBP and Rosenfelder *et al.* (2011) the UV degradation of PBDEs. Most recently, the dual analysis of C and Br isotopes of the abiotic degradation of tetrabromoneopenthyl, alcohol (TBNPA) by Kozzell *et al*., (2015) demonstrated that organobromides can be studied in a much similar fashion then the organochlorides. With the advent of brominated substances like the BFRs and their ubiquity in marine environments, it is pertinent to explore this reliable method for the study of debromination.

It is possible to recognize certain groups of microorganisms depending on their unique pathways by analyzing the isotope fractionation of the compound of interest (Meckenstock *et al*., 2004). The study of bromophenols is a promising model, since the dehalogenation of orthophenols seems common for the sponge-associated bacteria. Some bacteria can also dehalogenate TBBPA, like the enrichment cultures from Negev Dessert (Arbeli *et al*., 2006) and sediment from pristine and contaminated estuaries (Ravit *et al*., 2005; Voordeckers *et al*., 2002). We therefore aimed to determine whether CSIA could be used for assessing the dehalogenation of 2,6-dibromophenol (2,6-DBP)

and for monitoring the activity of sponge-associated bacteria, and to estimate the organohalide flux *in vivo* by sponge derived cultures.

Importance

Because of their physicochemical properties organobromides can accumulate in anoxic sediments, have ecotoxicological effects (Reineke *et al*., 2006), and may biomagnify in the food chain (Fernie and Letcher, 2010). In addition, metabolic intermediaries of some polyhalogenated compounds have been found to be even more toxic and labile than the parental molecule, and so debromination processes are of great concern. The relationship between dehalogenating bacteria and marine sponges represents an important step towards understanding the fate of dangerous anthropogenic organohalides in the marine environment. Sponges had been estimated to filter as much as 24,000 liters of water/kg/day (Vogel, 1977). The impact of marine sponges filtering and dehalogenating capabilities of its microbes in the marine environment is currently unknown. The unique relationship between sponges and dehalogenating microorganisms presents a range of new possibilities. In addition, the research on this peculiar dehalogenating microbe-sponge association provides a unique setting for a deeper understanding into microbe-animal associations.

Scientific question

My goal was to determine the geographical distribution and phylogenetic relationships between the dehalogenating microorganisms associated with sponges, and to use CSIA to determine how dehalogenation by sponge-associated bacteria compares with other dehalogenating mechanisms. The overall hypothesis of my work was that dehalogenating bacteria are widespread among sponge species regardless of geographical location and form stable populations within the sponge animal that function in the cycling of organohalide compounds.

General Objective

Research objectives include: identification of dehalogenating microbes associated with marine sponges, determination of the phylogenetic relationship between these microbes, and obtaining information about mechanisms governing the debromination of bromophenols in the marine environment**.**

Specific Objectives

1. To determine whether sponge-associated dehalogenating bacteria are a cosmopolitan group found in all marine sponges and the relationship between these bacteria.
2. To develop the use of compound specific isotope analysis (CSIA) of 2,6-dibromophenol and its dehalogenation products.
3. To monitor the debromination process of sponge-associated bacteria *in vitro* and *in vivo.*

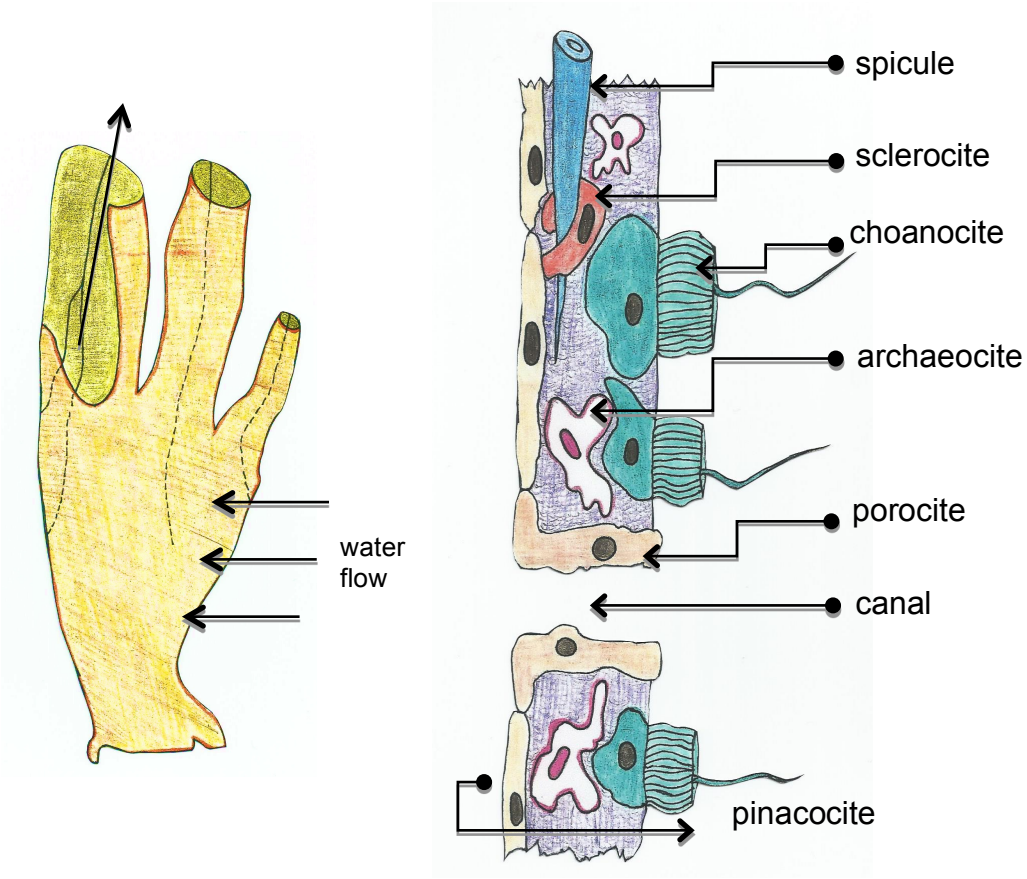

Figure 1.1. General structure of a sponge

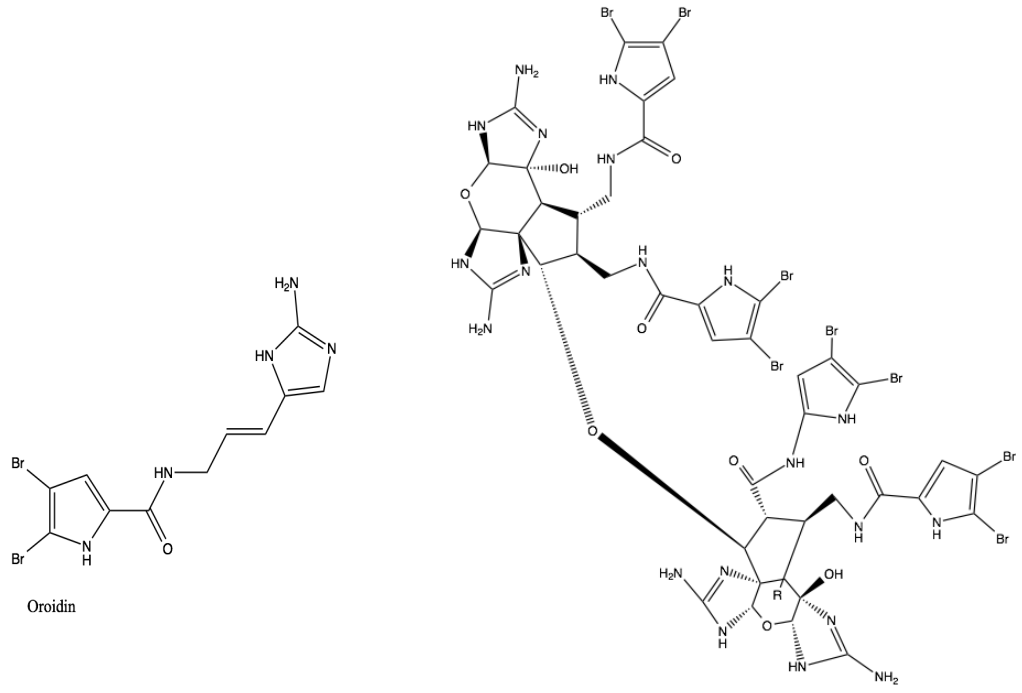

Figure 1.2. Examples of the complexity of teh sponge molecule bromopirrol, the most simple oroidin (left and the more complex stylissidine (right). (Forte *et al.*, 2009)

Table 1.1. Main breakthroughs in sponge symbiosis research

Topic	Details	Reference
Microbial abundance	New classification of sponges depending on microbial abundance.	Weisz *et al*., 2008 a
	Sponge microbial abundance correlates with pumping rates and mesohyl density	Weisz *et al*., 2008,b
	Confirmation of the different microbial community in LMA and HMA	Giles *et al*., 2012
	HMA LMA dichotomy study of micrograph	Gloeckner *et al*., 2014
	Sponges contain 35% of microorganisms	Hentschel *et al.,* 2012
	Microbial biomass 40%,	Webster *et al.,* 2001;
	Cell quantification 10^9 cells/g	Hoffman *et al*., 2005
	Non biogeographic features in affect microbial community in HMA and LMA	Blanquer *et al*., 2013
	Data confirms presence of poribacteria outside sponge but not active except in HMA	Moitinho-Silva *et al*., 2014
	Symbionts in HMA reduce heterotrophy versus that of LMA sponges with minimal bacterial communities	Poppell *et al*., 2013
Strict symbiosis	Vertical transmission of microbial consortium viviparous sponge *Ircinia felix*	Schmitt *et al*., 2007
	Vertical transmission diverse microorganisms in tropical sponge *Corticium*	Sharp *et al*., 2007
	Vertical transmission of Alphaproteobacteria *through larvae Mycale*	Enticknap *et al*., 2007
Autoinmune responses	Autoinmune response to LPS by increasing levels of production of 2 lyso-platelet activating factors in S. domuncula. Strong evidence that sponge react in an adaptive manner to exposure to LPS	Müller *et al*., 2004
HMA and LMA specific enrichments	Ammonia oxidizing archaea from *M. laxissima* (LMA) and *Ircinia* (HMA) the AOA in LMA are more closely related to sea water and sediment than HMA	Radax *et al*., 2012
	Chloroflexi, archaea	Schmitt *et al*., 2011; Bruck *et al*., 2011
	Poribacteria, *Actinobacteria* found with HMA sponges 4-6 orders of magnitude higher in HMA then LMA seawater	

Table 1.1. (continued)

Topic	Details	Reference
Interesting miscellanea	Microscopic observation, microorganisms in sponges	Dosse, 1939; Vacelet, 1977
	Hundreds of carnivorous sponges found in Barbados trench	Vacelet *et al*., 1995
	Anaerobic dehalogenating bacteria in marine sponges; isolated	Ahn *et al*., 2003, 2009
	An anaerobic world in sponges (*Geodia barretti, A. aerophoba)*	Hoffmann *et al*., 2005, 2008
	Aerophoba can differentiate food from symbiont bacteria	Wehrl *et al*., 2007
	Evidence of archaeal nitrifiers in sponges	Bayer *et al.*, 2008
	Poribacterial single cell genomics provided evidence of gram negativity, mixotrophy, adhesins	Siegl *et al*., 2011
	Core microbial community is kept in sponges at different depths (10 to 100 m)	Olson and Gao, 2013
Uniform microbial community present in sponges all over the world	Uniform microbial community in sponges from different oceans	Hentschel, 2002, 2006;
	Sponge as microbial fermenters	
	Widespread distribution of Poribacteria	Lafi *et al*., 2009
	Found Poribacteria (HMA specific) in sea water and LMA sponges with deep sequencing	Webster *et al*., 2010
	Large shifts of temperature and irradiance do not change core microbial community	Erwin *et al*., 2012
	Biogeography more important in cyanobacteria for microbial community structure	Burgsdorf *et al*., 2014

Table 1.2. Examples of borminated sponge bioactive molecules of relevance in biotechnology

Sponge	Compound(s)	Bioactivity	Reference
Agelas nakamurai	Ageladine A	Metalloproteinase inhibitor (anti-metastatic)	Fujita *et al.*, 2003
Theonella swinhoei	Poliketides	Pederin type PKS systems antitumoral	Piel *et al*., 2004a, b
Agelas oroides, and other spp.	Bromopyrroles, oroidin	Feeding deterrents	Fattorusso *et al.*,2000
	Hymenialdisine	Pan-kinase inhibitor, Type II diabetes	Forte *et al*., 2009
Cinachyrella australiensis,	Lowering colestherol	Mevinolin	Bose *et al*., 2014
Haliclona sp.	Manzamine A	Antitumoral,	Sakai *et al*., 1986
Xestospongia sp.	Manzamine E, F	Anti-malarial	Hill *et al*., 2005 Ashok *et al*., 2014
Cryptotethya crypta	Arabinofuranosyladenine A	In mouse lymphoma cells (L5178Y) reduces cell proliferation by cytostasis	Procksh *et al*., 2002
	Arabinofuranosyladenine C Fluorine derivatives are approved for use in	Ara-C is routine treatment of patients with leukemia and lymphomas. Approved for patients with cancer of the pancreas, lungs and breast	Sipkema *et al*., 2005
Dysidea avara	Avarol, and avarone, derivatives	In vitro in vivo anti-leukemia agents, in vitro anti-HIV (T-lymphotrophic cytostatic activity)	Cimino *et al*., 1982 Muller *et al*., 1985 Sarin *et al*., 1987 Sakurai *et al*., 2008

Table 1.3. Reductive debrominating bacteria in pure culture

Microorganism	Compounds dehalogenated	Reference
Desulfitobacterium chlororespirans	Bromoxynil,(3,5-Dibromo-4-Hydroxybenzonitrile) and Ioxynil, (3,5-Diiodino-4-Hydroxybenzonitrile)	Cupples *et al.*, 2005
Anaeromyxobacter dehalogenans	2-chlorophenol; 2,6-dichlorophenol; 2,5-dichlorophenol, 2-bromophenol	Sanford *et al.*, 2002
Desulfovibrio sp. TBP-1	2,4,6-tribromophenol; 4-bromophenol	Boyle *et al.*, 1998
Dehalococcoides mccartyi CBDB1	1,2,4-tribromobenzene,,all three dibrominated benzene congeners and monobromobenzene	Wagner *et al.*, 2012
Arthrobacter chlorophenolicus A6	p-nitrophenol, 4-bromophenol	Kumar *et al.*, 2011 Kumar *et al.*, 2013
Desulfoluna spongiiphila AA1	2,3, and 4 monobromophenols, 2,6-dibromophenol, 2,4,6-tribromophenol, 3,5-dibromo-4-hydroxybenzoate, 2-iodophenol, and 2-bromo-4-fluorophenol	Ahn *et al.*, 2009
Propionigenium maris strain DSL1 and MSL-1	2,4,6-tribromophenol	Watson *et al.,* 2000
Desulfovibrio dechloracetivorans	2-chlorophenol; 2,6-dichloropheno, and 2,4,6-trichlorophenol	Sun *et al.,* 2000

Table 1.4. Utilization of electron acceptor and donors by *Desulfoluna spongiiphila*

Compound	**Dehalogenation**	**End product**
2-Bromophenol, (2-BP), 3-BP, 4-BP	+	Phenol
2,4-Dibromophenol (2,4-DBP)	+	Phenol
2,6-DBP	+	Phenol
2,4,6-Tribromophenol (2,4,6-TBP)	+	4-BP, Phenol
2-Iodophenol (2-IP), 3-IP	+	Phenol
2-Bromo-4-fluorophenol	+	4-Fluorophenol
3,5-Dibromo-4-hydroxybenzoate	+	4-Hydroxybenzoate
Tetrabromobisphenol A*	+	BPA
2-Chlorophenol, 2,6-Dichlorophenol	-	-

*Liu *et al*., unpublished data
**Ahn *et al*., 2007

Table 1.5. Summary of CSIA studies of dehalogenation

Compounds	Details of the study	Reference
TCE; benzene	Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: anaerobic biodegradation of trichloroethylene (TCE), $\delta^{13}C$ values for residual TCE changed from -30.4 to -16 and α 0.9929. In contrast, toluene aerobic degradation showed by two separate mixed consortia, no change in $\delta^{13}C$	Sherwood-Lollar; *et al*., 1999
2,4,6-TBP	Bromine isotope enrichment from 2,4,6-TBP and Highly enriched debrominating culture	Bernstein *et al*., 2012
Tribromoneopentyl alcohol (TBNPA)	Dual Carbon and Bromine isotope analysis and AKIE during abiotic transformation by oxidation and reductive dehalogenation	Kozzell *et al*., 2015
1,2,4- and 1,2,3-trichlorodibenzo-p-dioxin (TrCDD)	Anaerobic enrichment culture containing *Dehalococcoides* reductively dehalogenated almost exclusively at peripheral positions forming the main products 1,3-dichloro-(DiCDD) and 2-monochlorodibenzo-p-dioxin (MCDD) from 1,2,4-TrCDD and 2,3-DiCDD from 1,2,3-TrCDD.	Ewald *et al*., 2007
1,2,3,4-tetrachlorodibenzo-p-dioxin (TeCDD)	Carbon isotope fractionation during dechlorination of TeCDD to to 1,2,4- TrCDD, and 1,3-DCDD by a *Dehalococcoides*-containing culture	Liu *et al*., 2010
1,2,3-TCB; 1,2,4-TCB	Stable carbon isotope fractionation during aerobic and anaerobic transformation of trichlorobenzene. Gave similar enrichments e of 3.1 and 3.7. While aerobic transformation by dioxygenase pathway did not	Griebler *et al*., 2004
2,4,6-TBP	Bromine isotope enrichment from 2,4,6-TBP and Highly enriched debrominating culture	Bernstein *et al*., 2012

Table 1.5 (continued)

Compounds	Details of the study	Reference
TCE	Factors controlling the carbon isotope fractionation of tetra- and trichloroethene during reductive dechlorination by *Sulfurospirillum* sp. and *Desulfitobacterium* sp. strain PCE-S	Cichocka *et al*., 2007
TCE	Chlorine and carbon isotopes fractionation during volatilization and diffusive transport of tce in the unsaturated zone. Cl atom showed a stronger fractionation then C isotopes for volatilization and diffusion, for C the volatilization showed an inverse effect	Jeannottat and Hunkeler, 2012
DCB	Distinct carbon isotope fractionation during anaerobic degradation of dichlorobenzene isomers. Non adjacent debromination AKIE more similar to each while adjacent AKIE is 2 orders of magnitude different	Liang *et al*., 2014
PCE, TCE	Combined C and Cl isotope effects indicate differences between corrinoids and enzyme (*Sulfurospirillum multivorans* PceA) for reductive dehalogenation of PCE, but not TCE	Renpenning *et al*., 2014
PCE, TCE	Rate-limiting mass transfer barriers were: cell wall, the cytoplasmic membrane in case of a cytoplasmic location of the RDase enzyme. Masking of isotope fractionation determined by: hydrophobicity of compound, properties of the cell envelope, and the localization of the reacting site enzyme	Renpenning *et al*., 2015

CHAPTER 2:

Sponge Dehalogenating Microorganisms: The curious case of *Desulfoluna*

2.1. ABSTRACT

Biogenic organohalides appear to enrich for a population of dehalogenating microorganisms within *Aplysina aerophoba* sponges in the Mediterranean Sea. We sought to determine whether the dehalogenating bacteria are found in sponges from disparate sites in temperate and tropical oceans, and if so, how are they are related. Anaerobic cultures prepared from marine sponges were amended with 2,6-dibromophenol as electron acceptor and short chain organic acids as electron donor. Dehalogenating cultures were maintained and the microorganisms further enriched by successive transfers with 2,6-dibromophenol as the electron acceptor. DNA was extracted from those cultures that showed dehalogenating activity, and the presence of *Desulfoluna* spp. like bacteria was investigated by PCR using specific 16S rRNA gene primers and terminal restriction fragment analysis polymorphism analysis (T-RFLP). Our data demonstrate the presence of *Desulfoluna spongiiphila* and its close relatives in debrominating enrichment cultures from over 20 sponge species from the temperate and tropical ecoregions located in the Atlantic and Pacific Oceans in addition to the Mediterranean Sea. The dehalogenating strains isolated to date are closely related to *D. spongiiphila* and *Desulfoluna butyratoxydans,* suggesting a cosmopolitan association between *Desulfoluna* spp. and various marine sponges. Organobromide-rich sponges may thus provide a specialized habitat for organohalide-respiring microbes.

2.2. INTRODUCTION

Marine sponges (Porifera) contain a diverse microbial community inside their bodies. These sessile filter feeders are the earliest branching phylum of the animals (at least 580 mya) making them of great interest in the study of the origins and organization of animal life (Li *et al.*, 1998; Adamska *et al*., 2011). The microbiology of marine sponges has been studied for almost 80 years (Dosse, 1939) and as in many other areas in the life sciences, the rise of molecular biology opened a myriad of questions and details about the complex structure and function of the microbial communities associated with these animals. The term sponge symbiont will be used in its most loose definition: as two or more different organisms living together in a wide range of associations (Hentschel *et al.*, 2006; Taylor *et al.*, 2007 a, b; Vogel, 2008) from symbionts transmitted vertically from parent to offspring (Schmitt *et al*., 2007) to the association of the same group of diverse microbes with different geographically disparate sponges (Webster *et al*., 2010; Fieseler *et al.*, 2004). Some sponges harbor microbes comprising 35 % (Hentschel *et al*., 2012) of their body at densities of 10^9 cells/g (Hoffman *et al.,* 2005). An exciting finding was the demonstration of stable microbial communities in marine sponges, including the unique *Candidatus phylum* Poribacteria (Hentschel *et al.,* 2002; Fieseler *et al.*, 2004, Lafi, *et al*, 2009). These bacteria are enriched in marine sponges over a range of geographical locations and host species (Fieseler *et al*., 2004; Olson and McCarthy, 2005, Lafi *et al*., 2009; Olson and Gao, 2013).

The presence of this core of associated functional and phylogenetically diverse microbial community (Weisz *et al*., 2008) gave rise to a new classification in sponges as high microbial abundance (HMA) and low microbial abundance LMA sponges. Sponges also

contain and abundance of bioactive organobromide compounds (Figure 2.1) that can reach more than 10% of their dry weight, such as in *Aplysina aerophoba* (Teeyapant *et al.,* 1993). It was hypothesized that the high bacterial biomass could include microbes with the ability to metabolize these brominated compounds. The presence of dehalogenating bacteria living within sponges was first reported by Ahn *et al.* (2003) in *Aplysina* specimens from the Mediterranean Sea. This discovery lead to the isolation of *Desulfoluna spongiiphila* strain AA1 from the marine sponge *Aplysina aerophoba*, a sulfate reducing bacterium (SRB) capable of using multiple halogenated phenols as electron acceptors under anaerobic conditions (Ahn *et al.*, 2009). It is uniquely placed among the sponge-associated bacteria since it is not part of the often considered sponge symbionts such as Cyanobacteria and Poribacteria (Usher, 2008; Fieseler *et al*., 2004). The association between host sponge and associated microbiota could be partially driven by the organohalide chemistry of the sponge animal where the organohalides select for bacteria that can utilize them as a source of carbon or energy, but also on the microbial abundance of the host. This could result in the enrichment of dehalogenating bacteria forming stable populations with the sponge animal that function in the cycling of sponge-derived organohalide compounds.

The study of this peculiar dehalogenating microbe-sponge association provides a unique setting for a deeper understanding into rare microbe-animal associations and clarification of the roles microorganisms play in the cycling of organohalides in the environment. The objective of this study was to determine whether reductive dehalogenating bacteria are associated with sponge species from different locations in temperate and tropical oceans and whether the different dehalogenating bacteria represent the same or different taxonomic groups. We used a combination of classic anaerobic microbiology and molecular tools to assess the diversity and occurrence of

the dehalogenating microbial community in different sponge species. Samples from geographically distant and taxonomically different sponges were used to establish enrichment cultures amended with 2,6-dibromophenol (2,6-DBP) as a homologue for the organohalides present in marine sponges. By measuring dehalogenating activity in anaerobic enrichments of different species of geographically disparate sponges we aimed to assess whether sponge associated dehalogenating bacteria are cosmopolitan, unique to a geographic site and/or a particular sponge species.

2.3. MATERIALS AND METHODS

2.3.1. Origin of dehalogenating cultures

Sponges were collected from sites corresponding to different marine ecoregions (Spalding *et al*., 2007) in the Mediterranean Sea and the Pacific and Atlantic Oceans. Some sponges were collected by scuba diving at Banyuls Sur Mer, France (42° 28' 59" N and 3° 07' 41" E) and Rovinj, Croatia (45° 05' N, 13° 38' E) in the Mediterranean Sea in May 2005 and April 2006; also, from the open sea in Bucco Sur, Tumbes, Peru (03° 56' 43"S, 80 ° 56' 45" W) and 03 ° 59' 02"/ 80 ° 59' 18" in July 2008 and, on Puerto Rico, Caribbean (18° 27' N and 66° 04' W). Other specimens were collected in the United States from docks from the shores of the Mullica River-Great Bay estuary, Tuckerton, New Jersey (39° 35' 31" N, 74° 20' 0.6" W) in July 2008; the tidal estuary in Connecticut (41°05' 48"N/ 72° 52' 52"W) on June 2007; Florida samples were obtained from Pourtales Terrace and Florida straits in Summer 2007 (23° 56'3" N / 80° 55'33"; 24° -81' W). The location of all sites is shown in Figure 2.2.

2.3.2. Dehalogenating enrichment culture preparation and maintenance

For the study of sponge-associated dehalogenating bacteria previously enriched cultures were used from sponges of the Mediterranean Sea (Ahn *et al.*, 2003, 2007, 2008, unpublished data) in addition to new enrichment cultures prepared with sponges collected from Puerto Rico, USA, and Peru (Gray *et al*., 2007; Ahn *et al*., 2008).

A section of the sponge was cleaned with distilled water, homogenized with mortar and pestle and 3 to 5 mL of homogenate used to inoculate 50 mL of anaerobic minimum salts media (MSM) prepared in serum flasks, with a head space of N_2/CO_2 (70%/30%, v/v) and capped with rubber stoppers and crimped as described previously (Ahn *et al*., 2003; Monserrate and Häggblom, 1997). The cultures and MSM controls were amended from deoxygenated stock solutions with 100-200 µM 2,6-dibromophenol (2,6-DBP; Sigma-Aldrich Chemical Co., Milwaukee, Wis., min. 99% purity) as the electron acceptor and 1 mM mixture each of lactate, butyrate, propionate, as electron donors. Incubation was at 25°C in the dark. Nitrogen gas flushed syringes were used for sampling or to re-spike the cultures under strict anaerobic technique.

Primary cultures that showed dehalogenation activity were maintained and dehalogenating microorganisms further enriched by successive transfers (1/10 dilution) into anaerobic MSM with or without sulfate depending on the original culture, and re-spiked with 200 µM 2,6-DBP as the electron acceptor and a mixture of short chain fatty acids (1 mM each of lactate, propionate butyrate) as electron donors. At each transfer MSM controls were also prepared and amended the same way. Sponge enriched with 2-chlorophenol,246-TBP, and iodo phenols were also prepared.

2.3.3. Sampling and analytical methods

Samples of 1 mL were taken aseptically and filtered with 0.45 µm pore size filters prior to analysis by HPLC to determine the concentrations of 2,6-DBP, 2-bromophenol (2-BP), and phenol over time. An HPLC (1100 series; Agilent) with a C18 column (Spherisorb, 4.6×250 mm, particle size 5 µm; Phenomenex) was used with methanol/water/acetic acid (50:48:2) as eluent at a flow rate of 1 mL/min, with a UV absorbance detector set to 280 nm. Concentrations were determined from 5-point standard curves in the concentration range of 100 µM to 1mM.

2.3.4. Molecular community analysis

After enrichment cultures were inoculated, 1 mL samples were taken over time and analyzed by HPLC for evidence of dehalogenation of 2,6-DBP to phenol. Cultures that dehalogenated 2,6-DBP in less than 2 weeks were considered active and prepared for microbial community analysis. Three mL samples of select enrichment cultures were filtered using 0.2 µm Nucleopore filters and DNA extracted using the UltraClean Soil DNA isolation kit (MO BIO, Solana Beach, Calif.) or a modified phenol-chloroform extraction (Kerkhof and Ward, 1993).

Terminal restriction fragment length polymorphism (TRFLP) analysis was used for comparing the dehalogenating microbial communities of the enrichment cultures. Bacterial 16S rRNA genes were amplified using primer sets 27 incorporated with a fluorescent dye (Sakano *et al.,* 2002) (Gibco life Technologies, Gathersberg, MD, USA) and unlabeled 1100 Reverse, as described previously (Ahn *et al.,* 2003). PCR amplification parameters were as follows: 94 ºC 5 min initial melt; 30 amplification cycles of 94 ºC 30 s; 55 ºC 30 s; 72 ºC 1.5 min and a final extension at 72 ºC for 7 min.

PCR products were precipitated and resuspended in formamide with a ROX standard and denatured at 95 °C (Gallagher *et al.,* 2005). Samples were analyzed on an ABI 310 automated sequencer (Applied Biosystems Instruments, Foster City, CA), which generates a TRFLP fingerprint of the microbial community. A minimum peak height cut-off value of 50 fluorescence units was used for identifying terminal restriction fragments (TRFs), effectively representing the bacterial community structure of the samples. Communities were compared using the Sorensen pairwise similarity coefficient shown in supplementary data (Murray *et al*., 1998). TRFLP electropherograms showing the common and unique terminal restriction fragments (TRFs) were prepared with 16S rRNA genes from 2,6-DBP enrichment cultures, including *D. spongiiphila* strain AA1.

PCR amplification with *D. spongiiphila*-specific primers was performed for highly enriched debrominating cultures. DNA extracted from highly enriched debrominating cultures was PCR amplified with specific primers to the *D. spongiiphila* group designed by Ahn *et al.* (2007). *D. spongiiphila*-specific 16S rRNA gene primers were AA1 F: CATGCAAGTCGAACGAGAAA and AA1 R: CAATACCCGCGACACCTAGT. Purified PCR products were sent for nucleotide sequencing performed by Genewiz Inc. (North Brunswick, NJ). The obtained sequences of around 800 bp were aligned and manually adjusted for accuracy in Geneious (Drummond *et al.*, 2012), then compared to sequences in GenBank by using BLASTN (Altschul *et al*., 1997) to obtain their closest microbial relatives. Phylogenetic trees were constructed with Geneious using the maximum likelihood and neighbor-joining methods with Jukes-Cantor distances and 1000 bootstrap replications.

2.3.5. Isolation of dehalogenating strains from sponge enrichments

Dehalogenating cultures were enriched from anaerobic microcosms containing samples of different sponges and sponge associated sediments from Croatia, France, Peru, Puerto Rico, and USA. Cultures were amended with 2-BP or 2,6-DBP and a mixture of short chain fatty acids (1 mM each of lactate, propionate butyrate) as electron donors to enrich for bacteria with debromination ability. Isolates were obtained from these enrichments using the agar-shake technique with 10 mL of MSM with 0.8% Noble Agar containing 1 mM lactate and 200 µM 2-BP placed in 27-ml anaerobic capped Balch culture tubes. After 4 to 8 weeks of incubation, colonies were harvested using a sterile syringe following strict anaerobic technique using O_2-free N_2 flushed needles. The harvested colonies were transferred to broth culture containing 100 µM 2-BP and 1 mM lactate. Broth cultures originating from the agar-shake colonies that exhibited debrominating activity were re-amended with 2-BP. When accumulating phenol concentrations exceeded 1 mM, cultures were transferred to fresh medium.

At each transfer DNA was extracted and bacterial community analysis by TRFLP was performed to analyze changes in the community structure and whether only a single TRF could be observed (as an indicator of a pure culture). If not, shake agar technique was repeated, single colonies harvested, and inoculated into MSM broth for debromination tests until a pure isolate was obtained from the cultures.

2.3.6. Phylogenetic analysis of isolates

DNA was extracted as described previously (Ahn *et al*., 2003; 2009). The 16S rRNA gene was amplified by using 27F, 519R, 1100R, 1492R, and 1525R primers (Lane, 1985). Sequences obtained were assembled with the Geneious program (Kearse *et al*.,

2012), and compared with entries in GenBank by using BLASTN (Altschul *et al*., 1997). The partial 16S rRNA gene sequences are listed in Appendix A.

2.4. RESULTS

2.4.1. Widespread distribution of anaerobic debrominating bacteria in marine sponges

Dehalogenating activity in was observed in anaerobic sponge enrichments from all geographical locations tested as summarized in Table 1. We found that most anaerobic enrichment cultures debrominated 2,6-DBP to phenol within 2 weeks. An example is shown in Figure 2.3. Dehalogenating cultures were maintained by successive transfers into anaerobic MSM amended with 2,6-DBP and lactate. *Desulfoluna spongiiphila* strain AA1 was used as the positive control for debromination, and a strain AA1 killed enrichment culture was the negative control. Enrichment cultures from two unidentified sponges PPS and PBS were inoculated simultaneously. Debromination occurred within 14 days, while the 2,6-DBP concentration remained constant in the killed control for 25 days.

The presence or absence, of bacteria highly similar to *D. spongiiphila* in over 20 different sponge species obtained from temperate and tropical oceans, was evidenced through TRFLP analysis. A TRF (195 bp) matching D*. spongiiphila* strain AA1 became progressively more enriched in the debrominating cultures as shown in Figure 2.4. After successive transfers, single TRF cultures were obtained (Figure 2.5) and used for more detailed phylogenetic analyses.

2.4.2. Phylogenetic analyses of debrominating *Desulfoluna* spp. found in marine sponges

We were able to isolate three new anaerobic debrominating bacteria in pure culture from enrichments of a *Chondrilla nucula* sponge (CHN) from the Mediterranean, and from two unidentified sponges from the South Pacific, designated PPS and PBS (Table 2.2). Phylogenetic analysis of the 16S rRNA genes (Figure 2.6) showed that the three new debrominating isolates clustered within the *Desulfoluna* genus in the Desulfobacteraceae, with strain CHN being closest to *D. spongiiphila* and strains PBS and PPS closest to *D. butyratoxydans*.

2.4.3. Worldwide distribution of debrominating *Desulfoluna* spp.

To determine whether bacteria related to *D. spongiiphila* are present and responsible for reductive debromination in different sponge species we screened the positive dehalogenating cultures with *Desulfoluna*-specific 16S rRNA gene PCR primers (Ahn *et al.,* 2008). *D. spongiiphila* and/or close relatives were found in over 20 geographically disparate marine sponge-derived dehalogenating enrichment cultures. The amplification product of around 800 bp was used to find closest relatives by using BLAST in NCBI including some uncultured bacterial sequences in the analysis for broader insight of *Desulfoluna* spp. diversity.

As observed from Figure 2.7, all debrominating sponge cultures contained bacteria closely related to *Desulfoluna*. The sequences aligned also with some environmental clones obtained from different oceans, coastal sediments, and whale falls clones, tunicate gut microbiota, showing some diversity within the *Desulfoluna* genus.

2.5. DISCUSSION

2.5.1. Dehalogenating bacteria are regularly found in marine sponges

We have found reductive dehalogenating activity in anaerobic enrichment cultures from sponges in temperate and tropical oceans (Figure 2.2 and 2.3). In all the cultures of enrichments that were tested, none showed dechlorinating activity but some did show deiodinating activity. This is the first report on the wide distribution of dehalogenating bacteria in marine sponges. Unlike other studies in sponge microbiology we worked with a very specific and small portion of microbes associated with sponges. Nevertheless, dehalogenating microbes are one of the most intriguing functional groups studied in science and this discovery imposes very interesting questions about the meaning of the presence of *Desulfoluna* in almost all tested anaerobic debrominating sponge enrichments.

The presence of dehalogenating bacteria in sponges was first demonstrated for an *Aplysina aerophoba* sponge collected in the Mediterranean Sea (Ahn *et al*., 2003). Dehalogenating bacteria in the sponge animal were thought to be selected for/enriched by organohalides such as the brominated tyrosine derivatives, aerophobin-2, aplysinamisin-1, and isofistularin-3, found in *A. aerophoba* that can reach 12% of their dry weight (Turon *et al*., 2000; Teeyapant *et al.*, 1993). Marine sponges, in general, are a source of a wide array of biogenic organohalides (Gribble, 2003), such as indoles (Beukes, 1998), phenol derivatives (Utkina *et al*., 1998), biphenyl ethers (Bowden *et al*., 2000; Utkina, 2006), and even dioxins (Utkina *et al.*, 2001) and PBDEs (Carte and Faulkner, 1981). Organobromides are found even in ancient demosponges, *Geodia* (Lindgren et al, 1986). This strongly suggests that microbes associated with marine

sponge are no strangers to organohalides, and members of the sponge microbiota could likely use these compounds for survival or benefit.

2.5.2. *Desulfoluna* is a cosmopolitan debrominating bacterium widely distributed in marine sponges

Analysis of the sponge-derived dehalogenating enrichments revealed that *Desulfoluna spongiiphila* (Ahn *et al.*, 2009) and other very closely related microorganisms were present (Figures 2.4 and 2.5). In addition, the wide distribution of *Desulfoluna* spp. in marine sponges is evident, at least one sponge enrichment from each collection site showed the presence of *Desulfoluna spp*. (Table 2.2).

The three new *Desulfoluna* isolates were obtained from a *C. nucula* sponge and two unidentified sponges (Figure 2.5 and Table 2.3). These isolates are closely related with the two existing species of the genus *Desulfoluna*: *D. spongiiphila* and *D. butyratoxydans*. The finding of anaerobic debrominating *Desulfoluna* spp. in all these enrichments could have been dismissed as cross contamination in the laboratory. However, phylogenetic analysis of the 16S rRNA gene sequences amplified from the various enrichment cultures (Figure 2.7) demonstrates the distribution of a diverse *Desulfoluna* spp. community in the various sponges, with closely related sequences also found in lugworms, tunicates, and sediments. Additionally, it appears that there is a geographical pattern, as *D. spongiiphila*-related bacteria were detected mostly in sponges from the temperate Mediterranean and the North Atlantic, while *D. butyratoxydans*-related bacteria were found in sponges from the temperate Pacific. Geographical patterns have been observed in cyanobacterial symbionts in the sponge *Petrosia ficiformis* collected from Israel and Italy (Burgsdorf *et al*., 2014), were the explanation was that the larvae of the sponge were devoid of the cyanobacteria and

therefore left the colonization to local bacterioplankton. This could be clarified with future studies to determine the location of *Desulfoluna* in the sponge mesohyl and whether debrominating *Desulfoluna* can also be found in sediments and the water column.

2.5.3. Desulfoluna and its closest relatives

Desulfoluna spp. are sulfate reducing bacteria and members of the *Desulfobacteraceae* in the *Deltaproteobacteria* most closely related to *Desulfofrigus*. *D. spongiiphila* is a mesophile growing at temperatures of 10-36 °C with and optimum growth temperature of 28 °C. It cannot grow using butyrate unlike its closest relative *D. butyratoxydans* (Ahn *et al.*, 2009). Similarly, *D. butyratoxydans* can grow at temperatures of 10–35 °C, with an optimum at 30 °C. The *Desulfofrigus* spp. are known for their sulfate reducing activity under psychrophilic conditions. *Desulfofrigus oceanense* and *D. fragile* described by Knoblauch *et al.* (1999) were isolated from sediments and capable of using butyrate for growth.

Other sulfate reducing bacteria (SRB) have been identified in marine sponges, including members of the *Desulfoarculus*, *Desulfomonile*, and *Syntrophus* cluster (Hoffman *et al.*, 2005). Brück *et al*. (2010) isolated almost a hundred anaerobic bacteria from sponges, including the ancient *Geodia* sponge, but only 5% were found to be strict anaerobes. Fluorescent in situ hybridization (FISH) studies of these sponge-microorganisms revealed the presence of a great number of sulfate reducing bacteria (SRB) within the *Deltaproteobacteria*. No evidence of *Desulfoluna* spp. was found in their samples. So far, *Desulfoluna spongiiphila* has only been found in anaerobic enrichments from marine sponges (Ahn *et al*., 2009; this work).

Only a very few of the sequences obtained from the debrominating sponge enrichment do not align with *Desulfoluna* spp. (Figure 2.7), and appear to be more related to *Desulfofrigus* spp. These microorganisms are important sulfate reducers in cold environments (Knoblauch *et al*., 1999) playing a key role in the S cycle and anaerobic photosynthesis in deep environments. Future isolation and description of these group microorganisms can further clarify their phylogenetic functional and roles in the marine sponges.

2.5.4. *Desulfoluna* and other organohalide respiring bacteria

Anaerobic dehalogenating bacteria are a diverse and specialized group of microbes considered “extremophiles” for their ability to use organohalides as electron acceptors for their respiration. This metabolic group includes strict organohalide respirers, such as *Dehalococcoides spp*., as well as rather flexible organisms such as *Anaeromyxobacter* and *Desulfovibrio* that can obtain energy from respiration of other electron acceptors like sulfate (Smidt and de Vos, 2004; Maphosa *et al*., 2010). Among the dehalogenating bacteria, *Desulfoluna* branches out as a distinct closely related phylogenetic group (Fig. 2.8). The dehalogenating *Desulfoluna* are more closely related to *Desulfomonile*, *Anaeromyxobacter* and *Geobacter*. There is evidence that the sponge-associated dehalogenases represent a new group (Lopez-Chiaffarelli and Liu *et al*; unpublished data). These new putative dehalogenases found in sponge-associated microorganisms expand our knowledge of reductive dehalogenation in marine environments and its possible ancient involvement with marine sponges.

2.5.5. Microbial abundance and enrichment of *Desulfoluna*

The sponge microbe association is used to classify sponges according to microbial abundance in their bodies, separating these into low microbial abundance (LMA) and high microbial abundance (HMA) sponges (Vacelet and Donadey, 1977; Hentschel *et al*., 2003; Gloeckner *et al*., 2014). Differences between LMA and HMA sponges have impact on their overall physiologies and it is believed that LMA sponges filter small particulate organic matter from large volumes of seawater, while HMA sponges promote symbiotic associations with diverse prokaryotes in order to thrive. The microbial communities in LMA, on the other hand, are characterized by a predominant single large clade of either *Cyanobacteria* or *Proteobacteria* and a lower diversity compared to HMA sponges, containing little or none of the typical phyla found in HMA sponges (Naim *et al*., 2014), such as *Acidobacteria*, *Chloroflexi*, and *Gemmatimonadetes* and *candidatus phyllum Poribacteria* (Schmitt *et al*., 2011; Simister *et al*., 2012; Naim *et al*., 2014).

Most of the sponges used in this study were HMA *Demospongiae* but there are also some LMA sponges like the *Poecilosclerida*e, *Dysidea*, *Mycale*, and *Crambe*, whose enrichments showed dehalogenation. However, only *Mycale* showed the presence of *Desulfoluna* spp. (see Tables 2.2 and 2.4). The other species considered LMA* by inference (Maldonado *et al*., 2012; Gloeckner *et al*., 2014), e.g. *Haliclona* and *Halichondria* collected from the tidal estuary sites, also showed debrominating activity and the presence of *Desulfoluna* spp. (Tables 2.2 and 2.4). The *Desulfoluna* sequences obtained from the LMA sponges clustered with HMA sponges of similar sites, except for that from *Halichondria* (Figure 2.7). Other studies of LMA microbial communities show more similarity with the surroundings than with other sponges from the same location (Blanquer *et al*., 2013). Therefore, the microbial abundance of the sponges studied does

not seem to exert influence over the finding/presence of *Desulfoluna* spp. or apparent co-speciation.

There are two generalizations that can be made about microbes in marine sponges: *Cyanobacteria* are the most common symbiont present in sponges regardless of HMA or LMA species (Usher and Kayley, 2008); and other prokaryotes like *Archaea*, *Chloroflexi* and *Poribacteria* are influenced by the microbial abundance of the sponge. HMA sponges contain *Archaea* and *Chloroflexi* phylotypes that are more closely related to those of other HMA sponges than with those from LMA sponges or environmental samples (Schmitt *et al.*, 2011; Bayer *et al.*, 2014; Blanquer *et al.*, 2013; Zhang *et al.*, 2014). HMA symbionts have been shown to be closely related to regardless of geographical location and even host sponge species. In the present study the debrominating *D. spongiiphila* strains isolated from *A. aerophoba* and *Ch. nucula,* both HMA sponges from the Mediterranean Sea (Temperate Atlantic Marine Ecoregion), cluster together (Fig 2.6). While *Desulfoluna* strains PPS and PBS (from the temperate-tropical Pacific marine ecoregion) (Table 2.4,) cluster with non-dehalogenating *D. butyratoxydans* isolated from sediment of the temperate Northern Pacific ecoregion. Since we do not know the HMA or LMA condition of the sponges hosting strains PPS and PBS, further conclusions could not be reached.

Previous studies of sponge biogeography by Spalding and Van Soest (2012) present data of sponges from all the different ecoregions of the world reported in the World of Porifera Project (Davidson *et al*., 2015). Sponges from the Mediterranean Sea and the North Atlantic Ecoregions were more similar to each other, similar to what was seen with the *Desulfoluna* strains/sequences obtained. Sponges in the East Pacific (were PPS and PBS are from) are more closely related to the eastern Indo Pacific and the Temperate

Northern Pacific from were *D. butyratoxidans* was isolated. *Desulfoluna* spp. found in sponges also showed similar clustering. Future studies of the sponge microbiology would benefit from studying the ecoregions similarities, especially for microbial species that seem to have a bio-geographical clustering.

A comparison of all 16S rRNA sequences (isolates and enrichment cultures) of *Desulfoluna* (Figure 2.7) shows no clustering related to a HMA or LMA sponge origin. Also, *Desulfoluna* from LMA *Halichondria* and *C. prolifera* collected in the temperate North Western Atlantic branch out as a likely member of a different species or even genus. The clustering of *Desulfoluna* may be due of other (a)biotic factors affecting the selection/enrichment of *Desulfoluna* spp. in marine sponges.

2.5.6. Sponges, the natural niche for a new type of dehalogenating bacteria

We have found a natural source of dehalogenating microbes within marine sponges. In 2000, Häggblom theorized that the wide variety of natural halogenated compounds has potentially selected for microbial populations capable of dehalogenation. This is exemplified by dehalorespiring *Dehalococcoides* spp. found in pristine terrestrial environments (Krzmarzick *et al.,* 2012). In addition organohalide dehalogenation had been reported under different anaerobic reducing conditions (Häggblom *et al*., 2000; Smidt and de Vos, 2004). Marine sponges represent a relatively safe and bountiful habitat for any marine microorganism a home with different reducing conditions and a range of substrates providing everything for the subsistence of *Desulfoluna*.

Within microbe-sponge associations there are examples for each type of relationship, from opportunistic to strict vertical symbioses (Taylor *et al*., 2007). In certain sponges, vertical transmission of microorganisms has been demonstrated, for example in

Corticium sp. (Sharp *et al*., 2007). There are bacterial species that are vertically transmitted from the sponge to its larvae, as shown for *Mycale laxissima* (Florida), *C. prolifera* (Chesapeake Bay) and *Axinella corrugata* (Florida), that containing similar Alphaproteobacteria that were transmitted to larvae (Enticknapp *et al.*, 2006). The detection of these bacteria at such early stages in the life of a sponge suggests that these are symbionts of the sponges and needed for their survival from its early stages.

As shown in this study, *Desulfoluna* is a common dehalogenating microbe found in sponges. If *Desulfoluna* spp. are opportunistic microorganisms that find a suitable environment in sponges then we should be able to isolate these species also from seawater and sediments. So far only *D. butyratoxydans* is the closest species isolated from sediments, but it is incapable of dehalogenation (Suzuki *et al*., 2008; Ahn *et al*., 2009). The *Desulfoluna* sp. strains PPS and PBS are more closely related to *D. butyratoxydans* than *D. spongiiphila.* To date, there is not sufficient data to establish whether *Desulfoluna* is an opportunistic microbe or if it is vertically transmitted. More isolates would be needed and more details of the host sponges.

Similar to the *Cyanobacteria*, *Desulfoluna* spp. appear to be widely present within different sponges, HMA or LMA, and the presence of organohalides could be central in driving the selection. Sponge brominated compounds are generally believed to be an inherent evolved survival characteristic of these metazoans (Müller *et al*., 2000, 2004; Thakur *et al.,* 2004). The identification of new halogenated compounds from sponge species is far from complete, and new compounds are found in sponges each year. The presence of brominated compounds in the sponge mesohyl is believed to have a specific selection for the *Desulfoluna*-like microorganisms since dechlorination was not observed in sponge enrichment cultures Table 2.2.

2.5.7. A possible role of dehalogenating bacteria in sponges

The capacity of *Desulfoluna* to remove the halogens from organohalides in marine sponges could effectively change the behavior of the molecule. This has already been tested with Barettin, a brominated compound from sponges*,* which exerts noticeable bioactivity and none for the debrominated analogue (Lind *et al*., 2013). It is still to be determined when dehalogenating microbes would be most active within a sponge, and what their role would be. From other studies in *Geodia* and *Aplysina* (Hoffmann *et al*., 2005; 2008) anaerobic pockets can be found in the animal body at minimum pumping rates, which would provide favorable conditions for the growth and activity of dehalogenating/sulfate-reducing *Desulfoluna* spp*.,* which could potentially be involved in the decrease or increase activity of the organohalides present in the sponges.

In summary, *Desulfoluna* spp*.* appear to be the dehalogenating bacteria enriched in marine sponges found in temperate and tropical oceans. We detected *Desulfoluna* spp. in both HMA or LMA sponges with no apparent correlation. The clustering appeared derived from geographical differences between Atlantic temperate and Tropical marine ecoregions from were the sponges came from. The initial set of isolates and sequences suggests a geographical pattern to the species being enriched: *D. spongiiphila*-like strains were found in the Mediterranean and Atlantic, while *D. butyratoxydans*-like strains may be more prevalent in the Pacific. Furthermore, the *Desulfoluna* spp. found in all sponges tested are diverse, a probable indication of their specific enrichment from seawater and/or sediment.

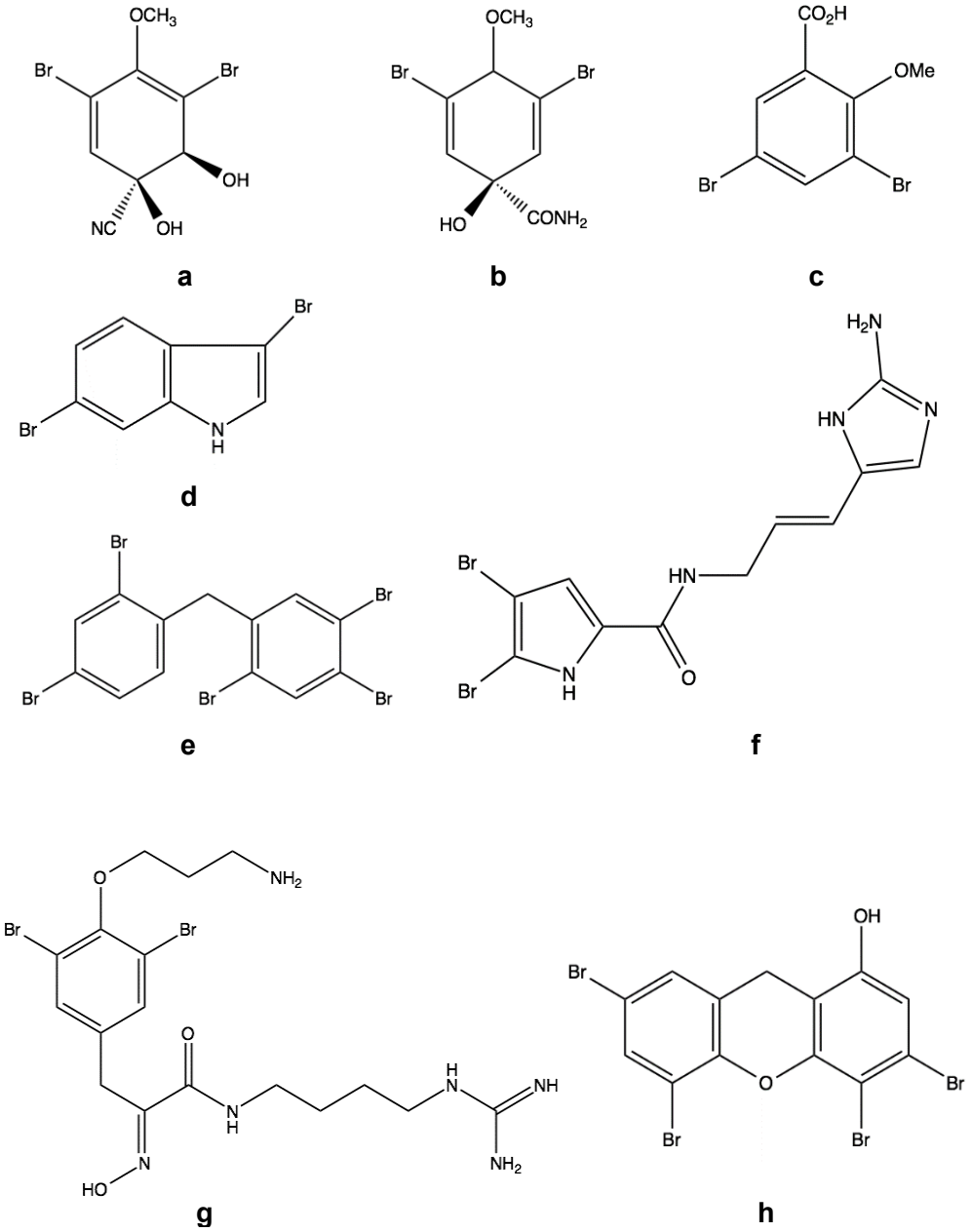

Figure 2.1. Brominated sponge bioactive molecules form Gribble *et al*., 2003 included phenols (a,b,c), indols (d) diphenyl etheres (e), tyrosine derivatives (f), pirrol (g), and dioxins (h)

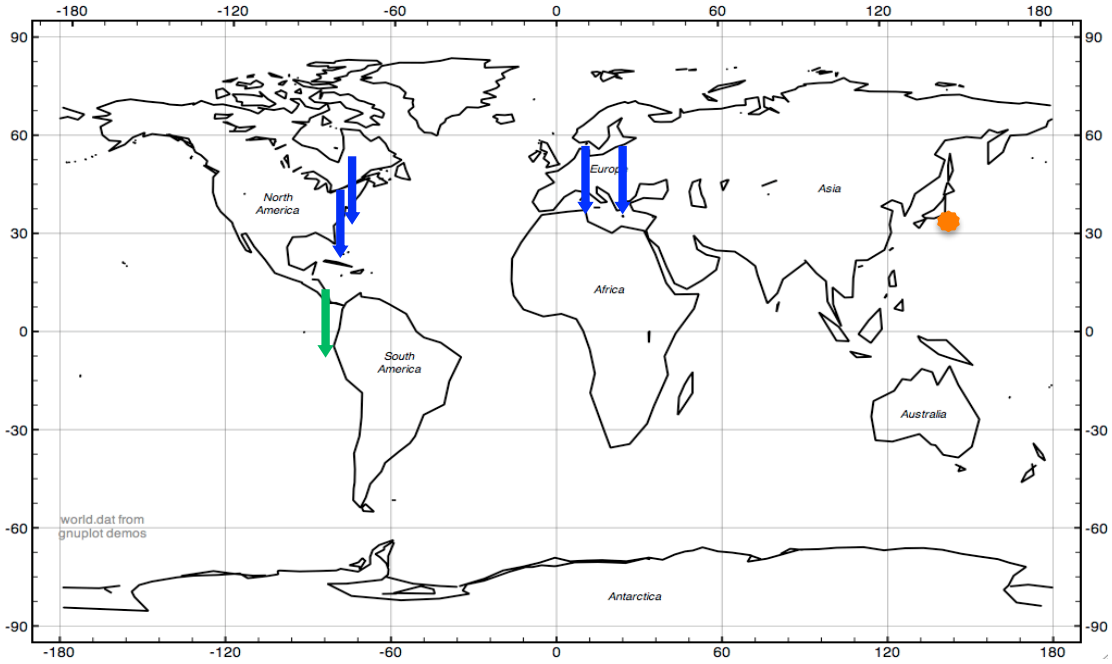

Figure 2.2. Sponge sampling points in Atlantic (blue arrows) Pacific (green) were *D. spongiiphila* has been found, and *D. butyratoxydans* original sampling point (orange)

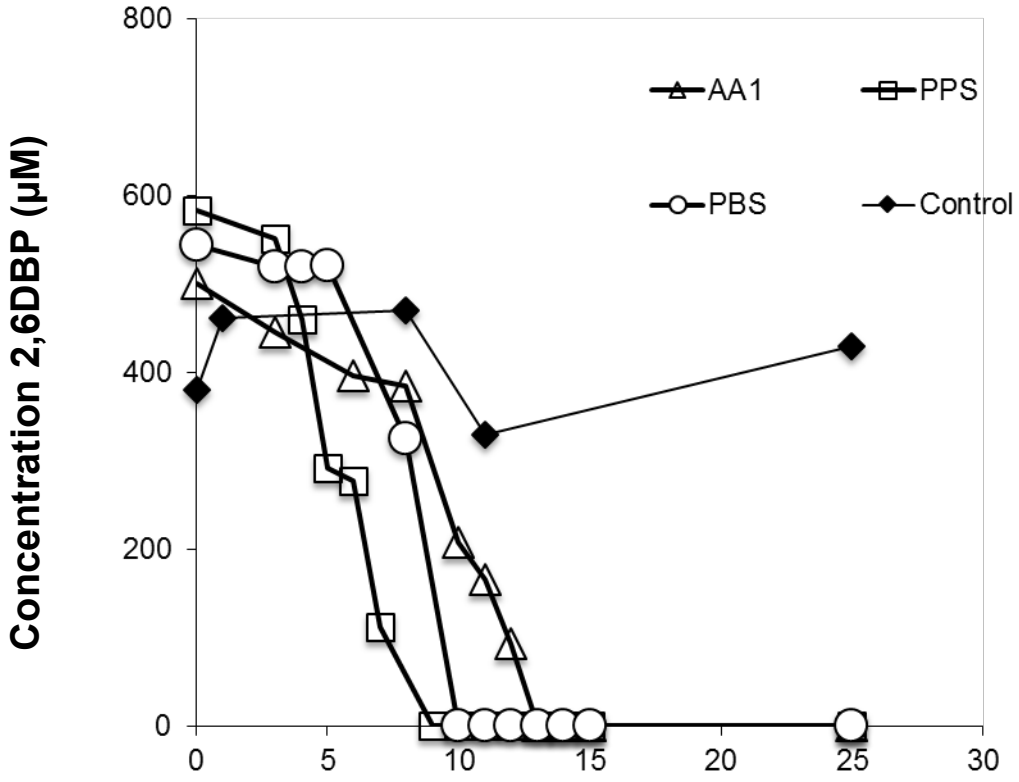

Figure 2.3. Debromination of 2,6-DBP by *D. spongiiphila* AA1, anaerobic sponge enrichment cultures PPS, PBS and killed control over time

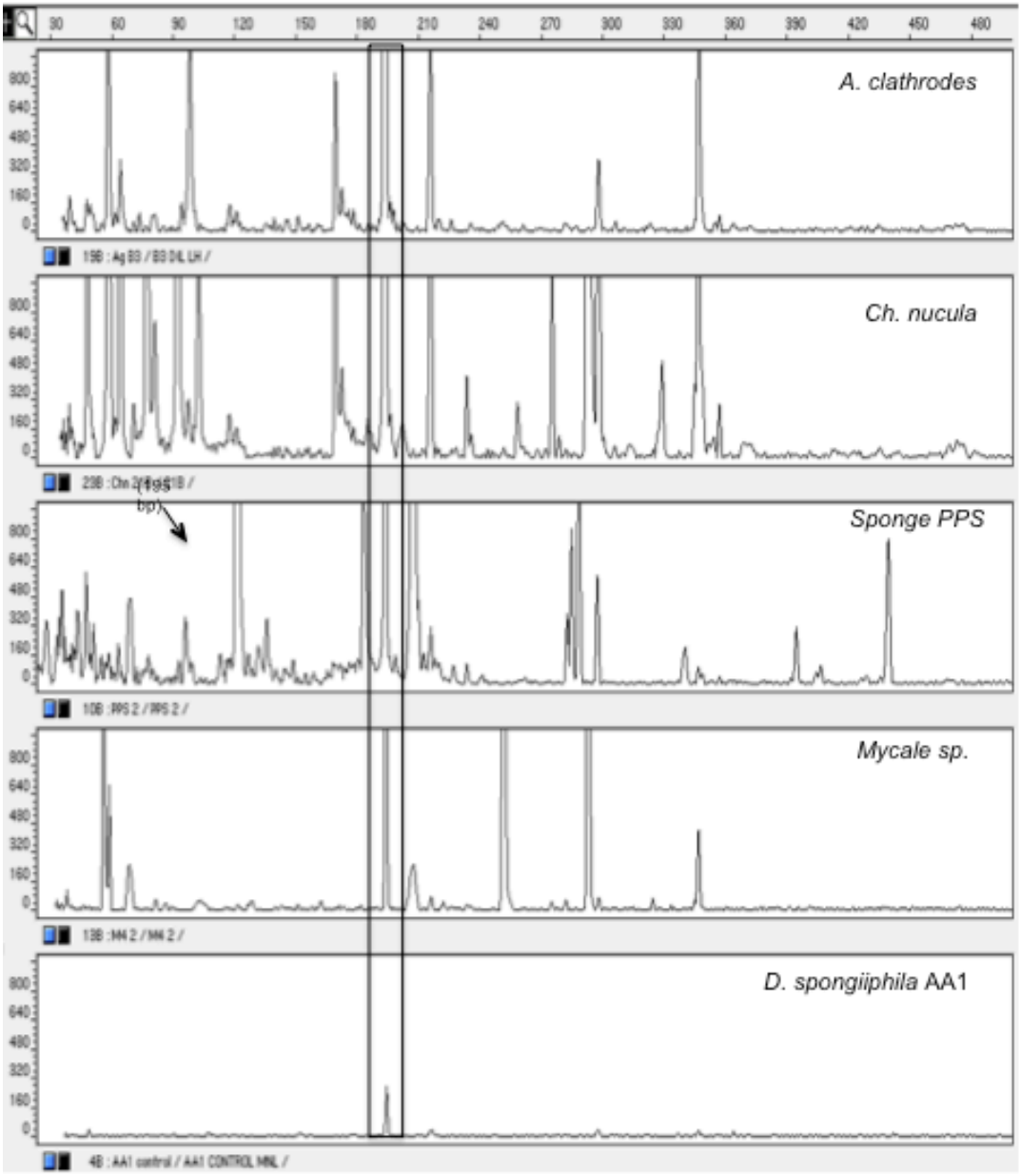

Figure 2.4. TRFLP of actively dehalogenating enrichment cultures showing the presence of *Desulfoluna* sp. 195 bp in enrichment cultures of sponges from temprate and Tropical Oceans

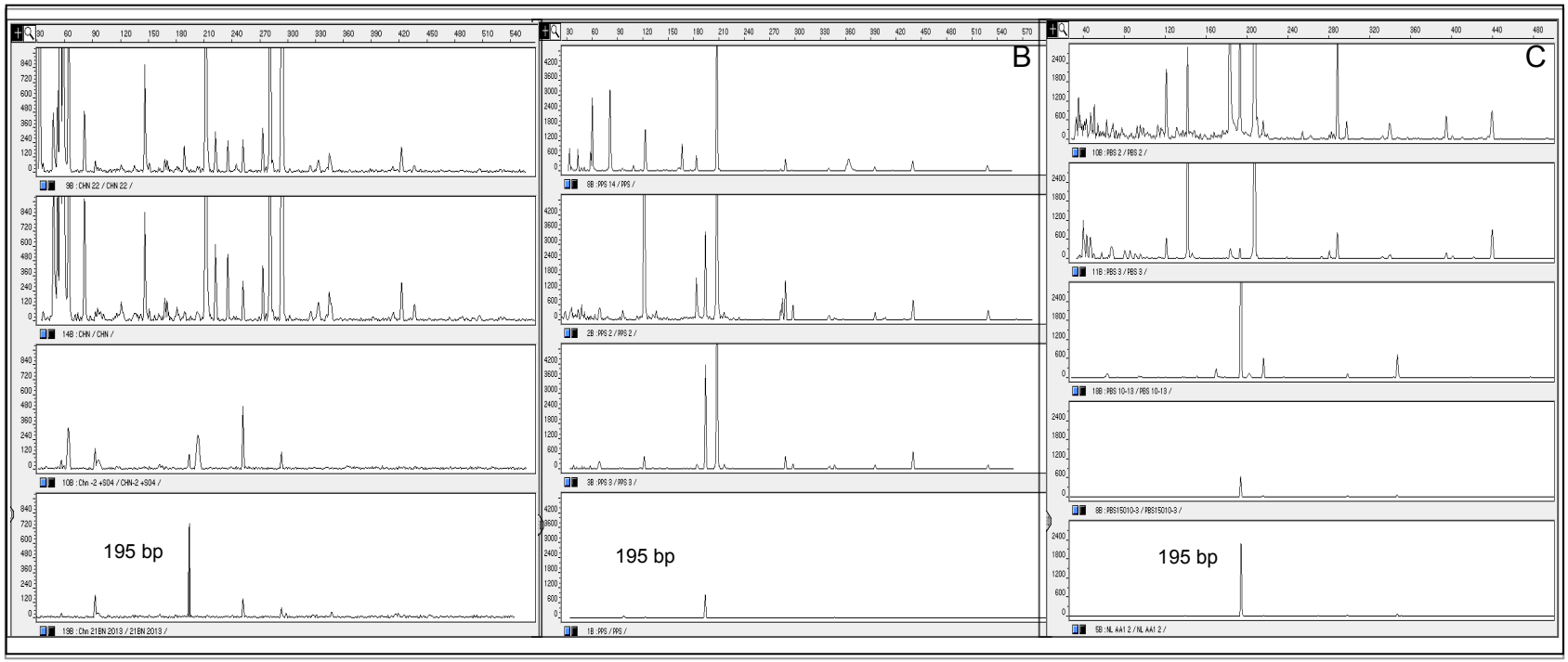

Figure 2.5. T-RFLP analysis of dehalogenating enrichment cultures showing isolation of *D. spongiiphila* like microorganisms (195 bp) from *Ch. nucula* (A), PPS sponge (B), and PBS sponge (C). Enrichments were transferred at 1 to 4 month intervals and fed 2,6-DBP 4 to 5 times in between transfers

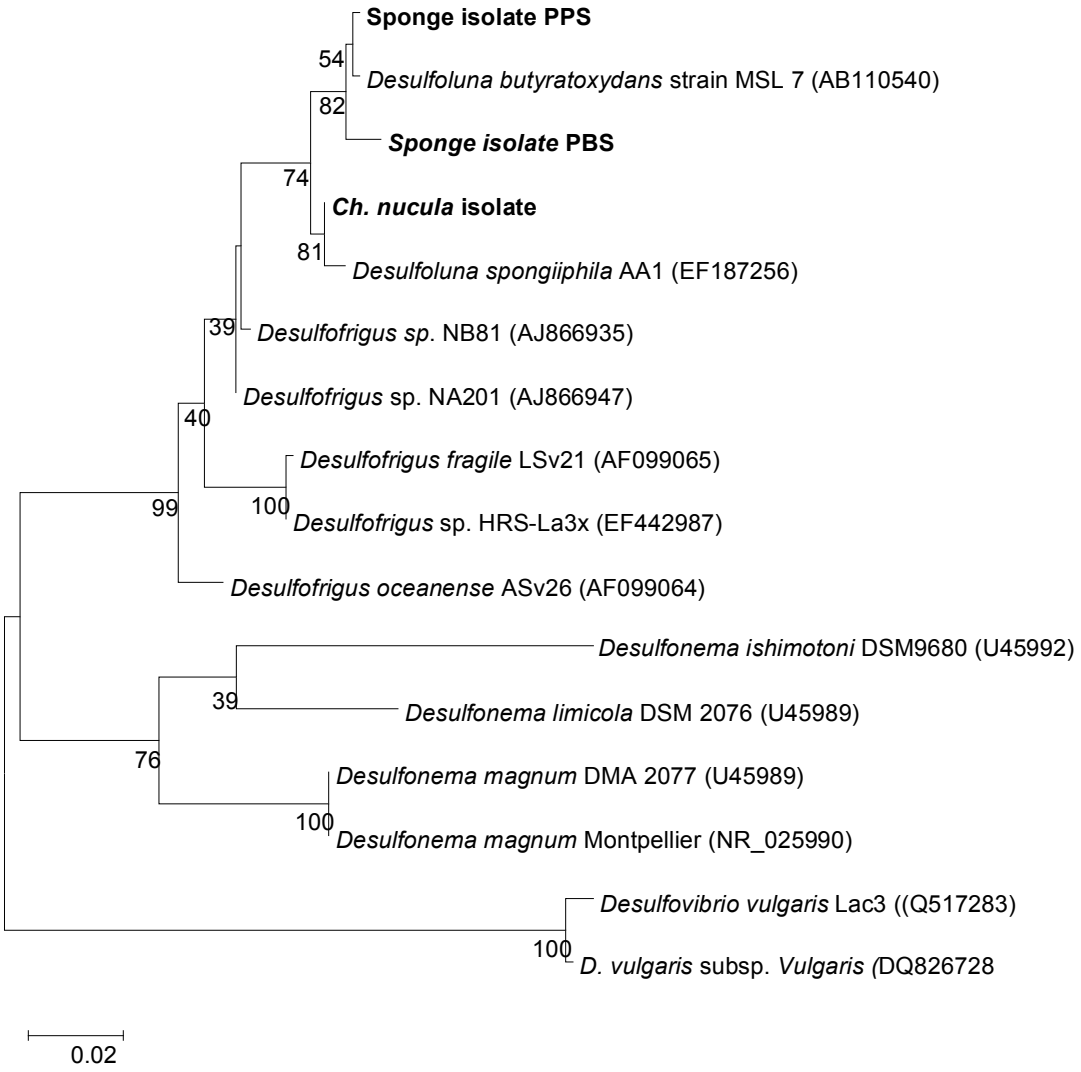

Figure 2.6. ML tree evidences relationships between debrominting sponge isolates and other dehalogenating bacteria, constructed with 16Sr RNA partial genes of 689 positions

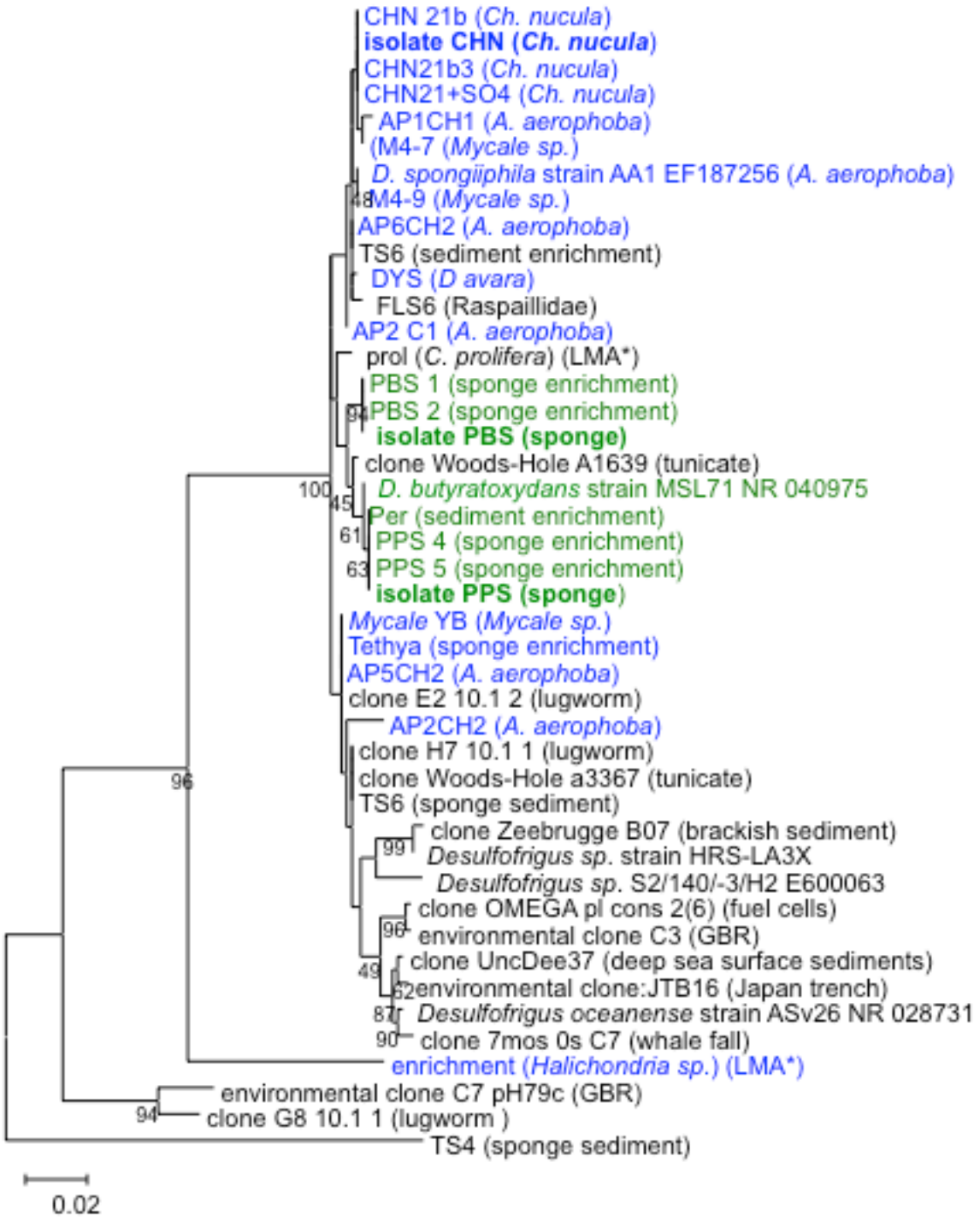

Figure 2.7. ML tree evidences a diverse group of *Desulfoluna* spp.found in different and geographically distant HMA sponges. Drawn 16S rRNA partial genes (587 positions)

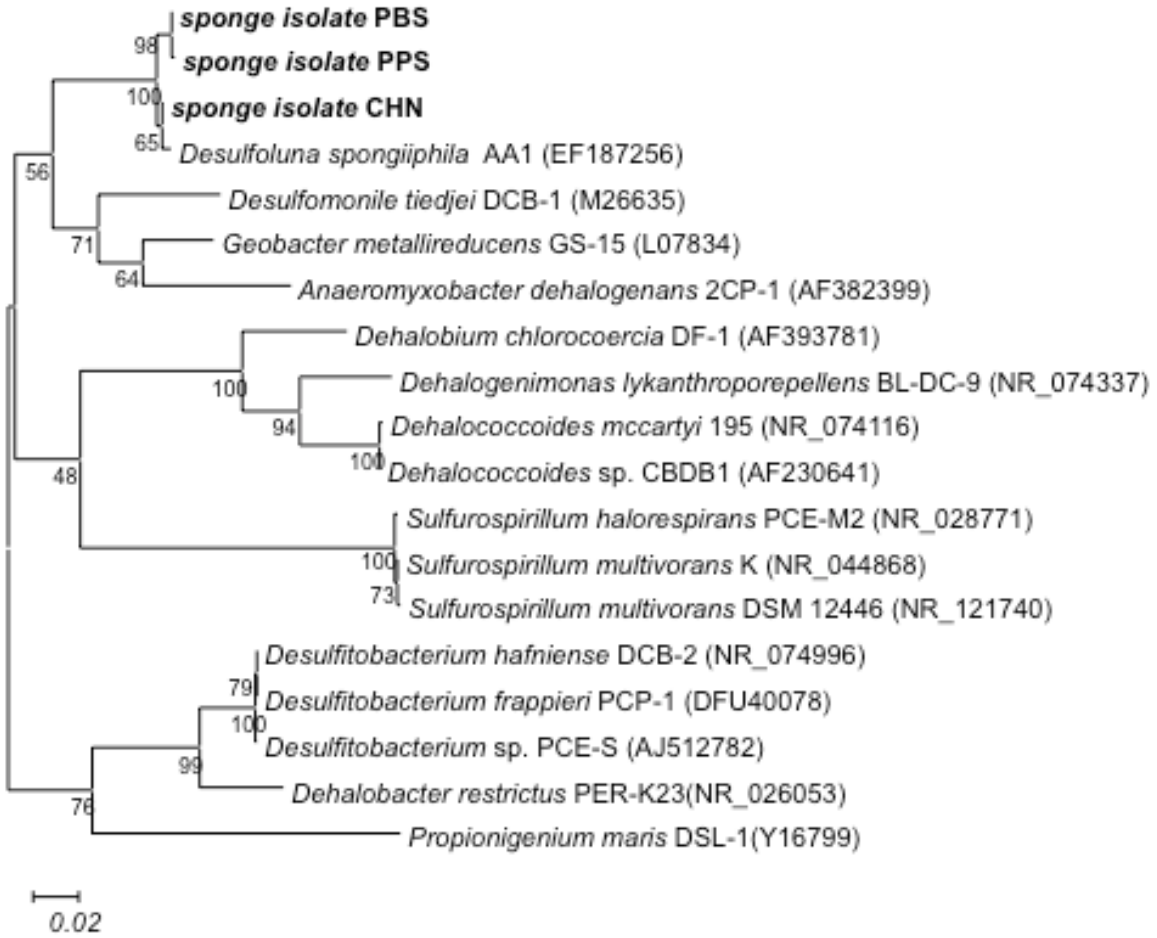

Figure 2.8. ML tree evidences relationship between debrominating sponge derived isolates and other dehalogenating bacteria, constructed with 16S rRNA partial genes (789 positions)

Table 2.1. Sponge species collected, sample collection sites and correspondent marine ecoregion

Sponge species	Location	Marine ecoregion
Aplysina aerophoba *Acanthella acuta* *Axinella polypoides* *Chondrilla nucula* *Chondrosia reniformis* *Crambe crambe* *Dysidea avara* *Ircinia* sp. *Mycale sp.* *Oscarella lobularis* *Tethya* cf. *limski* *Agelas* sp.	Banyuls sur Mer, France Rovinj, Croatia	Temperate North Atlantic (Mediterranean Sea)
Haliclona sp. *Halichondria* sp. *Clathria prolifera*	Long Island Sound ,USA	Temperate North Atlantic(Temperat e North Western)
Raspaillidae sp. Axinellidae Gorgonacea *Siphonodictyon mucosum* *Leiodermatium* sp *Plakortis* sp. Desmacellidae Spongosorites sp.	Florida, USA (depth between 200 and 900 m)	Tropical Atlantic (Tropical North Western Atlantic
Agelas clathrodes A *A. clathrodes* B *A. clathrodes* C *A. clathrodes* D *A. clathrodes* E *A. clathrodes* F	Puerto Rico	Tropical Atlantic (Tropical North Western Atlantic
PPS PBS PRS PAS PGS	Tumbes, Peru (depths 5 and 20 m)	Tropical East Pacific (Tropical Eastern Pacific)

Table 2.2. Dehalogenating capability with brominated, iodinated and chlorinated phenols (BP, IP, CP) in different sponge derived cultures phenols (BP, IP, CP) in different sponges

Sponge	2,6-DBP	2,4,6-TBP	2, and 4-IP	2-CP; 2,6-DCP
A. aerophoba	+	+	+	-
Acanthella acuta	+	-	-	nt
Chondrilla nucula	+	+	-	nt
Crambe crambe	+	-	-	nt
Dysidea avara	-	+	-	nt
ircinia sp.	+	-	-	nt
Mycale sp.	+	-	-	nt
Tethya sp.	-	+	-	nt
C. prolifera	+	+	nt	nt
Halichondria bowerbanki	+	+	nt	nt
Haliclona loosanoffi	+	+	nt	nt
Unidentified PPS, PBS, PRS, PAS, sponges	+	+/-	nt	-

Table 2.3. Anaerobic debrominating isolates from marine sponges, origin and sponge description

Isolate	**Sponge description**
sponge isolate PPS	Obtained from pink tubular shape sponge with needle like spicules (oxeas) acerated at both ends of around 40 μm, suspected to be a member of the *Haploscleridae*
sponge isolate PBS	Obtained from a cluster of barreled small sponges of pinkish color with oxeas between 40 and 50 μm
Isolate CHN	Chicken liver sponge (Schmidt, 1862) *Chondrilla nucula* sponge, collected from Rovinj, Croatia

Table 2.4. Presence or absence of *Desulfoluna*, in different sponge species from different orders and class, including referred and inferred (*, **) microbial abundnace classification (HMA or LMA), and natural organobromide production

Sponge species	Microbial abundance/natural organobromide	16S rRNA AA1	Order	Class
PPS unidentified	nd	+	*"Haplosclerida"*	*Demospongia*
PBS unidentified	nd	+		*Demospongia*
Agelas clathrodes (A)	HMA / +	+	*Agelasida*	*Demospongia*
A. clathrodes (B)	HMA / +	+	*Agelasida*	*Demospongia*
Haliclona sp.	LMA	+	*Haplosclerida*	*Demospongia*
Halichondria sp.	LMA*	+	*Halichondrida*	*Demospongia*
Clathria prolifera	LMA**	+	*Poecilosclerida*	*Demospongia*
Leiodermatium sp	HMA**	-	*Lithistida'*	*Demospongia*
Plakortis sp.	HMA*	-	*Homosclerophorida*	*Homoscleromorpha*
Raspaillidae sp.	HMA**	+	*Raspailiidae*	*Demospongia*
Acanthella acuta	LMA	nd	*Halichondrida*	*Demospongia*
Aplysina aerophoba	HMA / +	+	*Verongida*	*Demospongia*
Chondrilla nucula	HMA	+	*Chondrosida*	*Demospongia*
Chondrosia reniformis	HMA	+	*Chondrosida*	*Demospongia*
Crambe crambe	LMA	-	*Poecilosclerida*	*Demospongia*
Dysidea avara	LMA	+	*Dictyoceratida*	*Demospongia*
Ircinia sp.	HMA*	-	*Dictyoceratida*	*Demospongia*
Mycale sp.	LMA*	+	*Poecilosclerida*	*Demospongia*
Tethya cf. limski	LMA*	+	*Hadromerida*	*Demospongia*

*Other species in the genus are known HMA or LMA
** Other genus in the order are known HMA or LMA
+ known natural organobromide production

Table 2.5. Summary of sampling location of different sponge dehalogenating enrichments, sponge species and family, known bioactive compounds produced and microbial abundance, and presence of *Desulfoluna*

Sampling location	**Sponge species**	**Family**	**Bioact / OrgBr**	**Microbial abundance**	**Debromination**	***Desulfoluna***
Tumbes, Peru TEP	PBS unidentified	n.d.	n.d.	n.d.	+	+
	PPS unidentified	n.d.	n.d.	n.d.	+	+
Puerto Rico	*A. clathrodes (B)*	Agelasidae	+/+	HMA*	+	+
	Haliclona sp.	Chalinidae	+	LMA	+	+
(CT, USA) TeNA	*Halichondria sp.*	Halichondriidae	+	LMA*	+	+
	Clathria prolifera	Microcionidae	+	LMA*	+	+

Table 2.5 (continued)

Sampling location	Sponge species	Family	Bioact / OrgBr	Microbial abundance	Debromination	*Desulfoluna*
(FL, USA) WeTNA	*Leiodermatium sp*	Azoricidae	+	HMA*	+	-
	Plakortis sp.	Plakinidae	+	HMA*	+	-
	Raspaillidae sp.	Raspailiidae	n.d.	HMA*	+	+
Banyuls sur Mer, France, Rovinj, Croatia TeNA	*Acanthella acuta*	Dictyonellidae	+/-	LMA	+	nd
	Aplysina aerophoba	Aplysinidae	+/+	HMA	+	+
	Chondrilla nucula	Chondrillidae	+	HMA	+	+
	Chondrosia reniformis	Chondrillidae	+	HMA	+	+
	Crambe crambe	Crambeidae	+	LMA	+	-
	Dysidea avara	Dysideidae	+/+	LMA	+	+
	Ircinia sp.	Irciniidae	+	HMA	+	-
	Mycale sp.	Mycalidae	+	LMA*	+	+
	Tethya cf. limski	Tethyidae	n.d.	LMA	+	+

CHAPTER 3

Reductive Debromination by Sponge-Associated Anaerobic Bacteria is Coupled to Carbon Isotope Fractionation

3.1 ABSTRACT

The wide use and prevalence of brominated aromatic compounds, including brominated flame-retardants, has prompted the study of the fate and transport of such chemicals. Marine sponges are natural sources of many brominated compounds and the sponge habitat appears to enrich for a population of dehalogenating bacteria. The objective of this study was to establish whether reductive debromination of brominated phenols by sponge-associated bacteria, including *Desulfoluna spongiiphila,* could be monitored by compound specific stable isotope analysis. The reductive debromination of 2,6-dibromophenol to phenol by sponge-associated cultures resulted in substantial stable carbon isotope fractionation. All sponge-associated cultures showed similar enrichment factors. The AKIE (apparent kinetic isotope enrichment) values were similar to ones obtained for dehalogenation of other organohalides, such as chlorobenzenes, implicating similarities at the reaction site level of reductive dehalogenation. AKIE values for AA1 (*Aplysina aerophoba* sponge) and PPS unidentified *Haploscleridae* sponge were of 1.0359 ± 0.001 and 1.0355 ± 0.015 respectively. The TS7 culture (sponge associated sediment), exhibited a slightly different AKIE of 1.024 ± 0.0017. Data obtained from C-Br isotope fractionation can be compared with the existing C-Cl database and theoretical KIE values for C-Br and C-Cl bonds, but further studies of these microorganisms are imperative to better understand the dehalogenating mechanisms in sponge associated dehalogenating bacteria.

3.2. INTRODUCTION

The marine environment is considered a sink for many pollutants, including brominated flame retardants (BFR), a group of organohalides widely used to avoid fire accidents in electronics, indoor house-hold fabrics and, even in clothing (De Wit, 2002, 2010; BSEF, 2015). Brominated aromatics, such as tetrabromobisphenol A (TBBPA) and polybrominated diphenyl ethers, are among the most widely used BFRs (Birnbaum and Staskal, 2005; EPA, BSEF, 2015). Because of their physicochemical properties organobromides can accumulate in anoxic sediments, have ecotoxicological effects (Reineke et al., 2006) or may biomagnify in the food chain (De Wit, 2002; Fernie and Letcher, 2010; Bartrons *et al*., 2012). Several BFRs are thyroid and estrogen disruptors (Hamers *et al*., 2006, 2008). The wide use and prevalence of these compounds has prompted the study of the fate and transport of brominated chemicals in the environment.

One of the most important strategies for degradation of halogenated aromatics is reductive dehalogenation, which decreases the number of halogens in the molecule because the brominated substance is used as an electron acceptor (Häggblom and Bossert, 2003). Microorganisms play an important role in the dehalogenation of brominated aromatics (e.g., Monserrate and Häggblom, 1997; Fennell *et al*., 2003; Ravit *et al*., 2005; Tokarz *et al.,* 2008; Smidt and de Vos, 2004; Maphosa *et al*., 2010). Marine sponges are rich in organohalides usually with bioactive, antifouling or/and antibiotic characteristics (Blunt *et al*., 2014). Some of these organohalides resemble anthropogenic pollutants, such as halogenated dioxins and flame retardants. The use of tri- and dibromophenols-, less hydrophobic analogues of BFRs, provides laboratory

conditions to study debrominating microbes from diverse sources in the environment, including marine sponges.

Microorganisms have historically been exposed to organohalides from biogenic sources, and this exposure is believed to support the survival of reductive dehalogenating bacteria (Krzmarzick *et al*., 2012). The oceans are a particularly rich source of biogenic halogenated aromatics, with more than 1600 marine organohalides identified (Gribble *et al*., 2003) and more than 1200 bioactive molecules of marine origin (Blunt *et al*., 2014). Some of the most prolific organohalide producers are marine sponges. Sponges are filter feeders, and although microorganisms are a major component of the sponge diet, complex sponge-specific microbial communities thrive within sponge tissue (Hentschel *et al*., 2006, 2012; Taylor *et al*., 2007a,b; Thacker *et al*., 2012; Webster *et al.*, 2012). The combined abundance of organohalides and the high density of their associated microbes, for example in the sponge *Aplysina aerophoba,* prompted Ahn *et al.* (2003) to determine whether sponge-associated microbes were capable of dehalogenation. This eventually lead to the isolation of a novel sponge-associated bacterial species, *Desulfoluna spongiiphila*, capable of reductive debromination of tri-, di-, and mono-bromophenols (Ahn *et al.*, 2009). Sponge-produced organohalides thus appear to select for microorganisms that can utilize these compounds as a source of energy (Chapter 2).

Compound specific isotope analysis (CSIA) has been used extensively to study degradation and fate of aquatic pollutants for more than a decade (Meckenstock *et al.*, 1999; Sherwood-Lollar *et al.*, 1999). Among compounds analyzed are aromatic hydrocarbons (Richnow *et al.*, 2003; Braeckevelt, *et al.*, 2012), methyl *tert*-butyl ether (Somsamak *et al.*, 2005), organohalides including tetrachoroethene (Nijenhuis *et al.*, 2005; Fletcher *et al.*, 2011), dichoromethane (Nikolauz *et al.*, 2006) and polychlorinated

dibenzo-*p*-dioxins (Ewald *et al.*, 2007; Liu *et al*., 2010). CSIA represents a powerful tool for assessing biodegradation of organic chemicals in field studies (Meckenstock *et al*., 2004; Stelzer *et al.*, 2006; Fischer *et al*., 2007; Rosell-Linares *et al*., 2007; Imfeld *et al.,* 2008) and as a tool to differentiate transformations pathways, such as in the biodegradation of 1,2-dichloroethane (Hirschhorn *et al*., 2007), and degradation of tribromopenthyl alcohol (Birgkit *et al*., 2015).

In order to understand the roles and metabolic activities of the endomesohyl microbiota in sponges I studied the use of CSIA to analyze organobromide substrates and dehalogenation products. CSIA is based on the different reaction kinetics of light vs. heavy isotopes. Chemical bonds containing heavier isotopes need more energy for cleavage and in some biological transformations there is a preferential transformation of ^{12}C substrate, resulting in an enrichment of ^{13}C and thus producing a change in the C isotope ratio ($^{13}C/^{12}C$) in the remnant substrate over time. Biological transformation in certain cases will lead to an enrichment of heavier isotopes in the residual fraction of the substrate (for reviews, Meckenstock *et al*., 2004; Schmidt *et al*., 2004; Bombach *et al*., 2010; Elsner, 2010). Different isotope fractionation factors can potentially be linked to specific biochemical reactions because the extent of fractionation is linked to the degradation pathway of the targeted compound and type of bonds being affected during initial cleavage.

I tested whether CSIA could be used for assessing the dehalogenation of brominated phenols and for monitoring the activity of sponge-associated bacteria and eventually estimating the organohalide flux *in vivo*. 2,6-dibromophenol (2,6-DBP) was used as a model compound to assess whether temporal analysis of carbon isotopic composition during reductive debromination by sponge derived cultures can be used to determine the

isotopic enrichment factor (ε) for this process. This is the first report of carbon compound specific isotope effect analysis (CSIA) of brominated phenols during microbial degradation in a marine system.

3.3. MATERIALS AND METHODS

Chemicals. 2,6-DBP was used as substrate and other phenolic compounds (2-bromophenol, 2-BP; 4-chlorophenol, 4-CP; 4-methylphenol; 2,4-dichlorophenol; phenol) used as standards were obtained from Aldrich Chemical Co., Milwaukee, Wis**.** and had a minimum of 99% purity.

Origin and cultivation of reductively debrominating bacterial cultures. Anaerobic dehalogenating cultures of *Desulfoluna spongiiphila* strain AA1, sponge enrichment culture (PPS,) of an unidentified sponge, presumed to be a *Haploscleridae*), and sediment enrichment culture (TS7), prepared from sediment underneath a marine sponge) were maintained by successive transfers in anaerobic minimum salts media (MSM). The cultures were amended with 200 µM 2,6-DBP as the electron acceptor and a mixture of short chain fatty acids (1 mM each of lactate, propionate and butyrate) as the electron donor (Ahn *et al.*, 2003; Chapter 2 of this thesis).

Experimental set up. The triplicate cultures of the dehalogenating AA1, PPS and TS7 (1 L) were established in MSM with respective killed controls;. All cultures were amended with 2,6-DBP and 1 mM of lactate under strict anaerobic conditions. Samples (1 mL and 30 mL) were taken over a time course and stored at -20 ^{o}C until analyses of dehalogenation product concentrations and carbon isotope composition were performed.

***In vivo* sponge experiments.** Whole *Clatheria prolifera* sponges were collected from Long Island Sound, CT, stored in zip-lock bags filled with seawater, and transported back to the laboratory. Intact sponges were placed in 2 L tanks filled with artificial seawater and aerated with an aquarium air pump. To monitor *in vivo* sponge debrominating activity the tanks were amended with 500 µM 2,6-DBP and maintained at room temperature. Water controls without sponges and 500 µM 2,6-DBP were also established. Samples of the water in the tank were taken to analyze 2,6-DBP, dehalogenation product concentrations and carbon isotope composition of the phenols. A 1 mL sample was used for HPLC analysis. Samples for CSIA (between 10 to 100 mL) were stored at -80 ^{o}C prior to analysis.

Bromophenol Analysis. Samples were filtered (0.45 µm pore size filter) prior to analysis, Concentrations of 2,6-DBP, 2-bromophenol and phenol were determined using a 1100 series (Agilent) HPLC equipped with a C18 column (Spherisorb, 4.6×250 mm, particle size 5 µm; Phenomenex) with methanol/water/acetic acid (40:58:2; vol:vol:vol) as the eluent at a flow rate of 1 mL/min and a UV detector set to 280 nm.

Compound specific isotope analysis. Samples taken from the microbial cultures and *in vivo* sponge experiments were stored at -20 ^{o}C and transported to the Helmholtz Center for Environmental Research in Leipzig, Germany for compound specific isotope analysis (CSIA). A mixture of 2,6-DBP, 4-CP, 2-BP, and phenol was used to determine:1) the best non-fractionating sample preparation method from solid phase extraction; 2) sample concentration; and 3) solvent extractions by either methanol or dichloromethane (DCM). Extraction in DCM was found to be the most suitable for CSIA (Appendix B). The samples (3-50 mL) were centrifuged for 20 minutes at 14000X g and the supernatant was separated and acidified with 0.02 mL concentrated HCl. After

acidification, 0.5 mL of DCM was added and mixed thoroughly by vortex. After a minimum of 10 minutes settling the DCM fraction was collected with a Pasteur pipette and transferred into GC vials.

The carbon isotope composition of aromatic hydrocarbons was measured with a GC-C-IRMS (gas chromatography combustion isotope-ratio-monitoring mass spectrometry) system (FINNIGAN MAT, Bremen, Germany) The system consisted of a GC unit connected to a FINNIGAN MAT combustion device with a water removal assembly coupled to a FINNIGAN MAT 252 mass spectrometer. The bromophenols in the GC effluent stream were oxidized to CO_2 in the combustion interface and were transferred to the mass spectrometer to determine $^{13}CO_2/^{12}CO_2$ ratios (Merritt *et al*., 1994). The instrument was calibrated using a reference gas (CO_2) with an isotopic composition of 41.95‰ PDB described previously (Richnow *et al*., 2003; Ewald *et al*., 2007). Bromophenols extracted in DCM were injected into a gas chromatograph (HP 6890, Agilent, Palo Alto, CA) using a split/ splitless injector held at 280 °C. The split was adjusted to obtain suitable peak areas for the reproducible determination of the $^{13}CO_2/^{12}CO_2$ mass ratio. At least 3 replicate measurements were made for each sample. For chromatographic separation, a ZB-5 capillary column (60 m × 0.32 mm, 0.5 µm film thickness; Phenomenex Inc., Torrance, CA) with the following oven temperature program was used: 2 min at 70 °C; 10 °C/min to 200 °C; 20°C/min to 300 °C; and hold for 2 min with helium as the gas carrier.

A mixture of substituted phenols (2-chlorophenol, 2-methylphenol and 2,4-dichlorophenol) in addition to 2,6-DBP, 2-BP and phenol were tested to establish the ideal internal standards for our samples, the detection limits and the linearity (i.e.,

isotope ratio obtained is independent of the concentration of the analyte) of the isotope ratio mass spectrometer (IRMS). The detection limits for 2,6-DBP, 4-CP, 2-BP and phenol were 300, 700, 400 and 900 µM, respectively. The detection limits for 2,6-DBP, 4-CP, 2-BP and phenol were 300, 700, 400 and 900 µM, respectively. The linearity for isotope analysis was satisfactory only in a narrow range between 500 to 1200 µM (Appendix A).

In isotope analysis, stability rather than sensitivity is sought (Sessions *et al.*, 2006). Therefore, the minimum concentration of the compound was used were the accuracy and reproducibility of the stable isotope composition ($\delta^{13}C$) value remained for 2,6-DBP within acceptable limits (0.47 ‰ of standard deviation) for CSIA of C the limit is 0.5 ‰. These conditions were met for all of the phenolic compounds in the range of 500 to 1200 µM, except for 2,6 DBP, for which the limit was 500 µM (see Appendix A).

The $^{13}C/^{12}C$ in the sample was measured, R_{sample}, which was then normalized to $R_{standard}$, the $^{13}C/^{12}C$ ratio of the standard reference material V-PDB. using the following formulae:

$$\delta^{13}C = \left(R_{sample} / R_{standard} - 1\right) * 1000 \quad (1)$$

$$\ln \frac{R_t}{R_0} = \left(1/\alpha C - 1\right) \ln \frac{C_t}{C_0} \quad (2)$$

$$\varepsilon_{reactive} = \varepsilon \left(n/x\right) \quad (3)$$

$$\varepsilon C = \left(\alpha C - 1\right) * 1000 \quad (4)$$

$$AKIE = \frac{1}{\left(1 + \left(z * \varepsilon_{reactive} / 1000\right)\right)} \quad (5)$$

$$AKIE = \frac{1}{1 + z * \left(\frac{n}{x}\right) * \frac{\varepsilon}{1000}} \quad (6)$$

Where R and Ro are the $^{13}C/^{12}C$ ratios in 2,6-DBP at time t, and C is the concentration. The Rayleigh equation was used as described previously by Elsner *et al*. (2005) to relate the changes in $\delta^{13}C$ as a consequence of debromination (Equation 1). The fractionation factor α relates the changes in $\delta^{13}C$ to changes in the concentration (Equation 2). Another factor used to describe this process is the enrichment factor ε (Equation 3).

The α and ε determined with GC-C-IRMS can mask the isotopic effect at the reaction site (Elsner, 2010). To characterize the reactive position of 2,6-DBP, kinetic isotope effect (KIE) values were calculated for a particular molecular position. The apparent KIE was calculated using Equations 4 and 5 according to Elsner *et al. (*2005), where "n" is the number of C atoms of the molecule, "x" the number of reactive positions, and "z" is the number of positions in intramolecular competition. In the case of 2,6-DBP, n = 6 for carbon atoms, x =1 from the stepwise debromination of 2,6-DBP via 2-BP to phenol as represented in Figure 3.1, and z = 2 because of the symmetrical positions (2 or 6) available for debromination.

3.4. RESULTS

3.4.1. CSIA of debrominating cultures *D. spongiiphila* AA1, PPS and TS7

Three sponge-derived dehalogenating cultures were compared to determine carbon isotopic fractionation during debromination of 2,6-DBP. All cultures showed sequential debromination of 2,6-DBP to 2BP and finally to phenol in less than 14 days (Figs. 3.1

and 3.2). In cultures of *D. spongiiphila* AA1 the $\delta^{13}C$ of 2,6-DBP increased from an initial value of -30 to -25.6 ‰ at 79% transformation. Similarly, the $\delta^{13}C$ of 2,6-DBP in the Peruvian sponge enrichment culture (PPS) and the Tuckerton NJ sediment culture (TS7) increased from -30.2 to -25.4 ‰ at 79% transformation and, from -31.1 to -28.2 ‰ at 81% degradation, respectively (Fig. 3.2).

Equations 1-6 were used to determine the isotopic fractionation (α) and isotopic enrichment (ε) factors as well as the AKIE for each debrominating culture as summarized in Table 3.2. The α for the sponge-associated debrominating cultures AA1, PPS and TS7 were similar, at 1.0359, 1.0355, and 1.0237, respectively. The ε factor was -3.0 ‰ for the AA1 and PPS cultures, and -2.0 ‰ for the TS7 culture. The $\varepsilon_{reactive}$ value also shows the same trend for AA1 and PPS, which were almost identical, as seen in Table 3.2.

3.4.2. Carbon isotope fractionation during debromination of 2,6-DBP by sponges *in vivo*

Intact *C. prolifera* sponges were incubated in aerated artificial seawater tanks, amended with 500 µM 2,6-DBP and monitored for debrominating activity over two weeks. Complete loss of 2,6-DBP was observed within 13 days, with concomitant carbon isotope enrichment in the residual 2,6-DBP from an initial $\delta^{13}C$ value of -31.1 to -29.8 ‰ with a standard deviation of 0.3 ‰ (Fig. 3.4). Neither dehalogenation products, nor substantial change in the $\delta^{13}C$ of 2,6-DBP were observed in the no-sponge water column controls. Enrichment factors (α and ε) were not calculated because the Rayleigh equation is restrained in general to closed systems (Griebler, 2004; Meckensock *et al.*,

2004). In summary, reductive dehalogenation of 2,6-DBP was observed in the *in vivo* sponge experiment with concomitant carbon isotopic enrichment of 2,6-DBP.

3.5. DISCUSSION

CSIA of 2,6-DBP was used to gain further understanding of the mechanisms of dehalogenation by sponge-associated bacteria. To our knowledge this is the first study investigating the ^{13}C isotopic enrichment during anaerobic reductive debromination by sponge-associated microorganisms *in vitro* and *in vivo*. Reductive debromination of 2,6-DBP resulted in measurable carbon isotope fractionation.

Similar α 1.003±0.0016 and 1.003±0.004 ε of -3.1 ± 1.5 and -3.0 ± 0.5 AKIE of 1.0359 ± 0.001 and 1.0355 ± 0.015 values were observed for the sponge derived-cultures: AA1 (*Aplysina aerophoba* sponge) and PPS (unidentified *Haploscleridae* sponge), respectively. The TS7 culture (sponge associated sediment), exhibited a slightly different α = 1.002±0.00026 and ε = -2.0 ± 0.3 2 values; AKIE = 1.024±0.0017 (Table 3.1). Taking into account the analytical variability, the α, and ε values obtained were essentially identical for the dehalogenating obtained from marine sponges enrichment cultures.

The ε values obtained express the isotope enrichment factor of the whole molecule, while the AKIE focuses on the effect of the ^{13}C isotope enrichment at the reactive site. It has been shown that AKIE values can reflect the similarities of dehalogenating pathways by similar microorganisms, by eliminating the dilution of the isotope effect of higher number of non reactive carbon atoms and intramolecular competition (Abe *et al.*, 2009).

In this study, the identical AKIE values are in correlation with previously published theoretical KIE values published for C-Br bond, 1.042 (Kozzell *et al*., 2015) and within the limits of the nucleofilic substitutions of the first (1.00 to 1.03) and second order (1.03 to 1.09).

The similar values observed for the sponge-associated cultures are indicative of a transformation of 2,6-DBP mediated by the same mechanisms comprising similar steps. Differences in carbon isotope fractionation can provide evidence for differences in the dehalogenation mechanism (Nijenhuis et al., 2005; Bombach *et al,* 2010; Elsner *et al.,* 2005). The AKIE value is indicative of isotope fractionation due to the transformation pathway and the type of the bonds affected. To a lesser extent, AKIE value also reflects differences in mass transfer, enzymatic commitment to catalysis, multiple chemical reaction steps or limited bioavailability of the compound (Elsner, 2010). From the microbiological point of view, subtle differences would reflect microbial membrane transport effects, location of the enzyme in the cell (membrane or cytoplasm), enzymatic activity, and as recently demonstrated, the growth stages and type of membranes (Gram positive vs. Gram negative) of the microorganisms involved (Nijenhuis *et al*., 2005; Cichocka *et al*., 2007; Hirschorn *et al*., 2007; Liang *et al*., 2014; Renpenning *et al*., 2015). The practically identical AKIE values observed for the sponge-associated cultures indicate that the whole 2,6-DBP transformation process by these dehalogenating cultures is identical.

Based on 16S rRNA gene phylogeny the debrominating bacteria in the PPS culture are related to *Desulfoluna spongiiphila* AA1 at the genus level, while the TS7 debrominating culture is not (Chapter 2; Girer, 2011). Isotope fractionation may be different because of subtle differences during the reductive debromination by different microorganisms. In

this case the results from CSIA and AKIE confirm the similarity between the sponge-derived dehalogenating *Desulfoluna* spp. and TS7.

The ε and AKIE values obtained from dehalogenation of various organohalides are compiled in Table 3.2. The C-Br and C-Cl bond breakage of organobromides and organochlorides show a wide range. The AKIE for C-Br bond cleavage calculated in this study is within values found previously for $\varepsilon_{reactive}$ C-Cl. One previous published study on brominated aromatics (bromodiphenyl ether 47; BDE 47) examined C-Br fractionation for abiotic photolytic reactions with UV light (Rosenfelder *et al.,* 2011). The values for microbial dehalogenation ranged from AKIE = 0.0008 for PCE by *S. multivorans* to AKIE = 1.039 for dehalogenation of TCE by *S. multivorans* (Figure 3.5). The apparent kinetic isotope effects for reductive debromination fall in the higher range of the AKIE calculated for the diverse array of organohalides previously studied.

Recently, a study of dichlorobenzene (DCB) dechlorination by methanogenic microcosms (Liang *et al.,* 2014), showed that the AKIE values for 1,3-DCB and 1,4-DCB (1,034 and 1,039) were identical in comparison to the value obtained for 1,2 DCB (1.005). The AKIE values for DCB are close to the values we obtained for 2,6-DBP dehalogenation by the sponge-derived cultures. The aromatic compounds 1,3 and 1,4-DCB and 2,6-DBP are structurally alike, containing non-adjacent halogen molecules. Thus we can postulate that the DBP and DCB dehalogenases are similar and could render similar isotopic fractionation values. In the case of the 1,2-DCB, the AKIE values are logarithmic representations of the differences in isotope fractionation (Equation 2). Therefore, the logarithmic difference between 1.034 and 1.005 is more then 1.5, which is almost 2 orders of magnitude, and is due to changes at the level of the

substrate/reactive site and microorganisms involved (Liang *et al.,* 2014). Most of the organohalides studied with *Dehalococcoides* and *Desulfitobacterium* spp. tested compounds with adjacent organohalides, e.g., PCE and TCE, 1,2 DCB, and 1,2,3 TCB> Hence,. It is possible that an enzyme which can only dehalogenate molecules with non-adjacent halogens would contain a different reaction site.

The explanation for similar AKIE values from more studied organohalides could lie with other reported reductive dehalogenation mechanisms. It is rare common to find phylogenetically distinct anaerobes that show dehalogenases with mechanistic and structural similarities (Rhee *et al*., 2003; Smidt and de Vos, 2004; Bommer *et al*., 2014; Payne *et al*., 2015). At the same time, sequence similarity and substrate specificity are generally not correlated, making functional predictions from rRNA gene sequence information difficult (Hug *et al*., 2014). In a CSIA study of dehalogenases, ε values of dehalogenation of TCE obtained from *Dehalococcoides* spp. were similar, while the ε of PCE for *Dehalococcoides* sp. strain 195 was different despite the high similarity of pceA gene responsible for the dechlorination (Urrea *et al.,* 2011). This was later corroborated for the dehalogenase pceA from *Sulfurospirillum multivorans* (Renpenning *et al.,* 2014). It was subsequently observed that differences between TCE and PCE fractionation were due different hydrophobicity, cell membrane, and localization of the reaction site (Renpenning *et al*., 2015). Some of these findings can be applied to dehalogenating microorganisms found in marine sponges. Ahn *et al.,* (2003) evidenced that the dehalogenases were a new deeply branched group within other dehalogenases from *Desulfitobacterium sp*. Y51 PceA, *D. dehalogenans* Cpr A, *Sulfurospirillum multivorans* PceA, *D. mcartyi* TecA with similarities of 23 to 44%. *Desulfoluna* debrominase is a corrinoid-based dehalogenase, with Fe-S clusters (Lopez-Chiaffarelli *et al*., unpublished

data). There is no description of the dehalogenase from *Desulfoluna* spp. yet, but this study adds to the general knowledge about these enzymes.

It has been established that some dechlorinating organisms like *D. mccartyi* CBDB1 are capable of dehalogenation of organobromides, such as 1,2,4-TBB and, hepta or lower brominated BDEs (Wagner *et al*., 2012; Xu *et al*., 2014). Soon we should be able to characterize how similar or different the isotope fractionation is for these compounds. So far only Sohn *et al*. (unpublished data) has measured isotope fractionation during dehalogenation of bromobenzenes by cultures comprising *Dehalococcoides* spp.

Although α and ε were not calculated for the *in vivo* sponge cultures of 2,6-DBP loss and isotopic enrichment was observed in contrast with the control (Fig. 3.4). Phenol or 2-BP were not detected in the open tank cultures, although this could potentially be due to volatilization (the tank was aerated to keep the sponge animals alive). However, I hypothesize that 2,6-DBP fractionation of the *in vivo* experiment was due to the activity of sponge-associated dehalogenating bacteria, since the change of 2,6-DBP isotope composition ($\Delta\delta^{13}C$) of the aerated water control was negligible ($\Delta\delta^{13}C$ =0.2) compared to the sponge system ($\Delta\delta\ ^{13}C$= 4.5). Nonetheless, the effect of sorption into the animal body of the $\delta\ ^{13}C$ 2,6-DBP was not evaluated. Several processes can change the isotope composition of a compound such as adsorption, dispersion, and dilution. Sorption is not thought to affect isotope composition significantly (Meckenstock *et al.*, 2004), but further studies will be needed to understand how the isotopes of organohalides react in contact with live animals.

Some studies have assessed fractionation in open systems (Van Breukelen, 2007) by adapting the established Rayleigh equation to account for dilution. OthersBy performing a multitracer test with bromide and deuterium labeled toluene in a BTEX polluted aquifer (Abe and Hunkeler, 2006), others have proposed that the Rayleigh equation systematically, but only slightly, underestimates the extent of degradation in physical heterogeneous systems, However, Fisher *et al*. (2006) concluded that the Rayleigh equation holds in some open systems. ^{13}C-labelled chlorobenzene studies have been employed to characterize microbial degradation *in situ* in a constructed wetland (Braeckevelt *et al*., 2007). These approaches could be modified to study the dehalogenating communities in sponges. Perhaps to study debromination in living sponges we could use a more hydrophobic brominated substrate to avoid potential evaporation. Additional controls to account for sorption might include killed sponge tissue, as well as sampling sponge tissue for the brominated substrate and analysis of the isotope composition. Another possibility is the use of LC-IRMS (Birkigt *et al.*, 2015) to study more hydrophilic organobromides in sponges in the future.

The results of these experiments show that CSIA can be used to analyze microbial reductive debromination. All sponge-derived cultures showed similar enrichment and fractionation factors (Figure 3.5). The AKIE values can now be used to compare the fractionation at the reaction site level with other similar dehalogenating mechanisms. *Desulfoluna*, is a peculiar group of dehalogenating microorganisms active in ancient metazoans and, represent an interesting contrasting group from the other known dehalogenating microorganisms. The data obtained from C-Br isotope fractionation to can be related to the more extensive C-Cl database. Much has been clarified regarding organochloride dehalogenation, but there is still much to understand about organobromides. These studies used 2,6-DBP as a model, but it is feasible to assess

other brominated aromatics dehalogenation with CSIA, such as TBBPA and PBDE, compounds of urgent interest because of their wide occurrence and large production

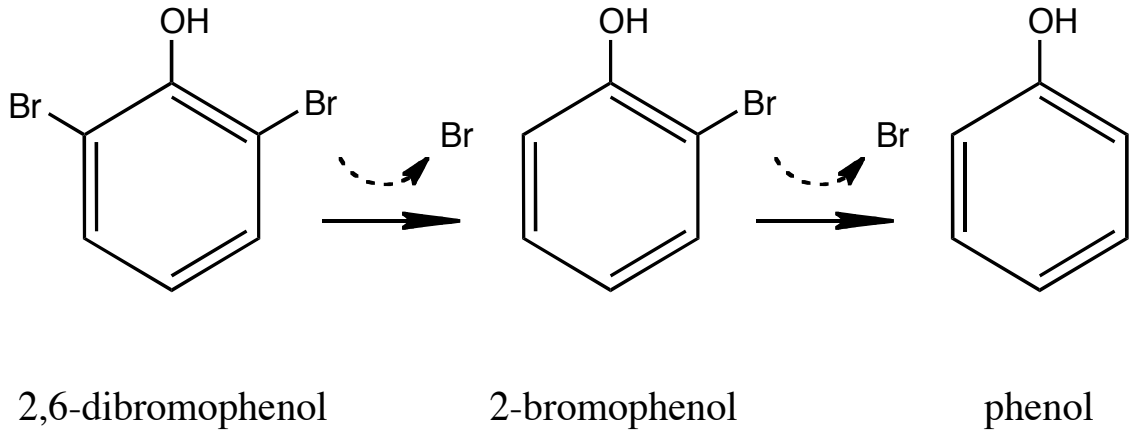

Figure 3.1. Sequential dehalogenation of 2,6-DBP to 2-BP and phenol by sponge derived bacterial cultures

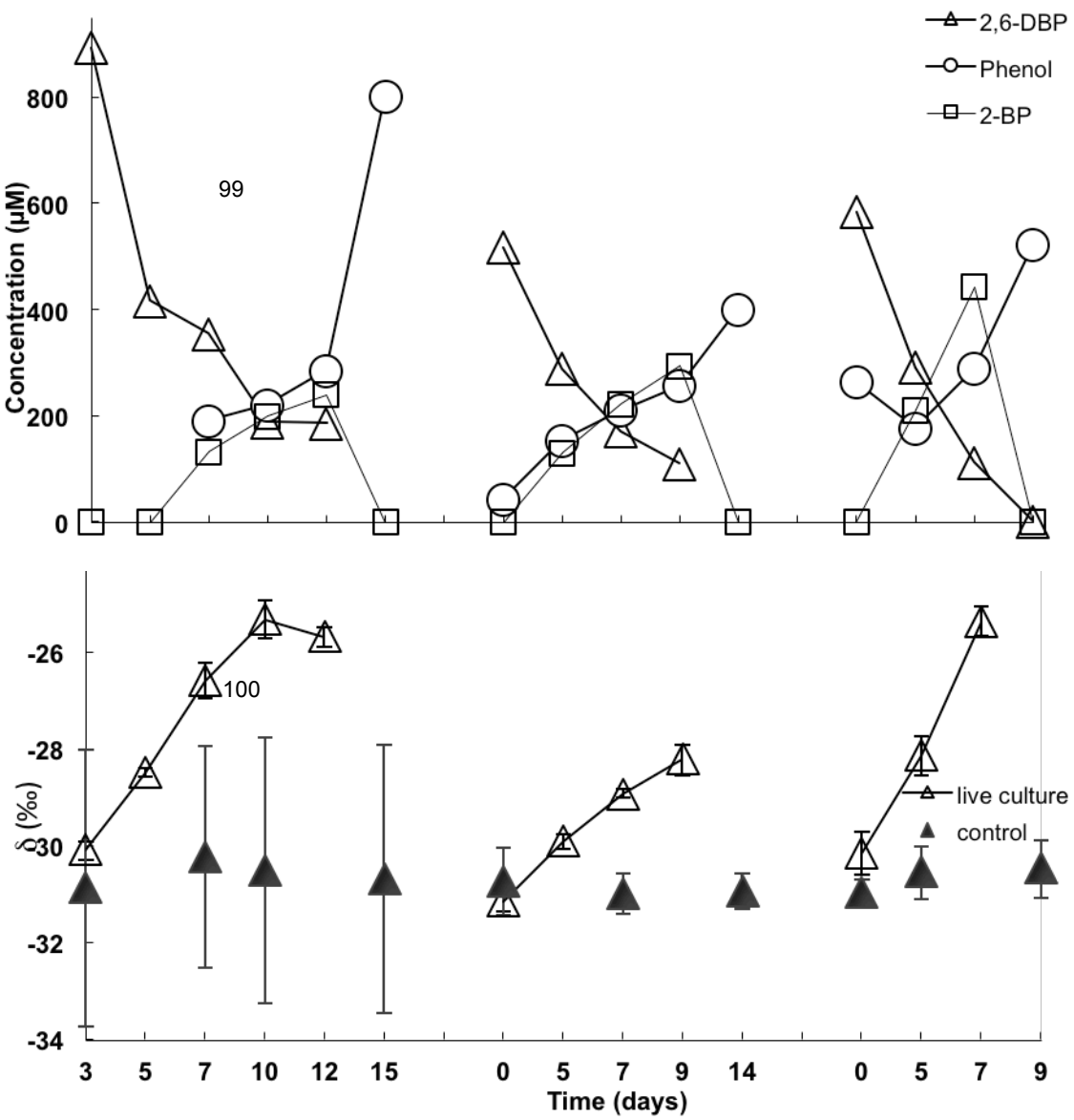

Figure 3.2. Dehalogenation of 2,6-DBP via 2-BP with accumulation of phenol as final product and $\delta^{13}C$ values from 2,6-DBP dehalogenation by of *D. spongiiphila* AA1, sponge enrichment culture PPS, and sponge sediment culture TS7

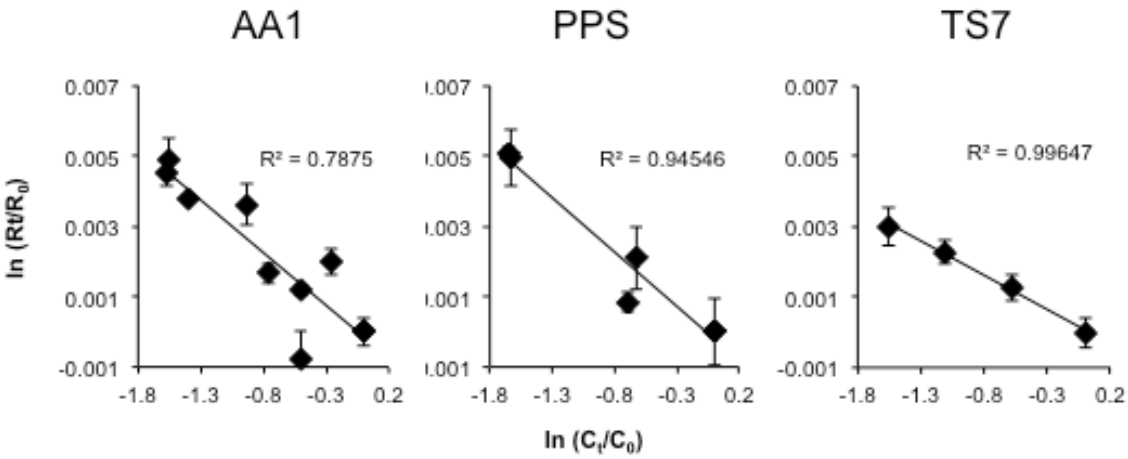

Figure 3.3. Relationship between isotope composition and concentration of 2,6-DBP analyzed in *D. spongiiphila* AA1, and enrichment cultures PPS and TS7

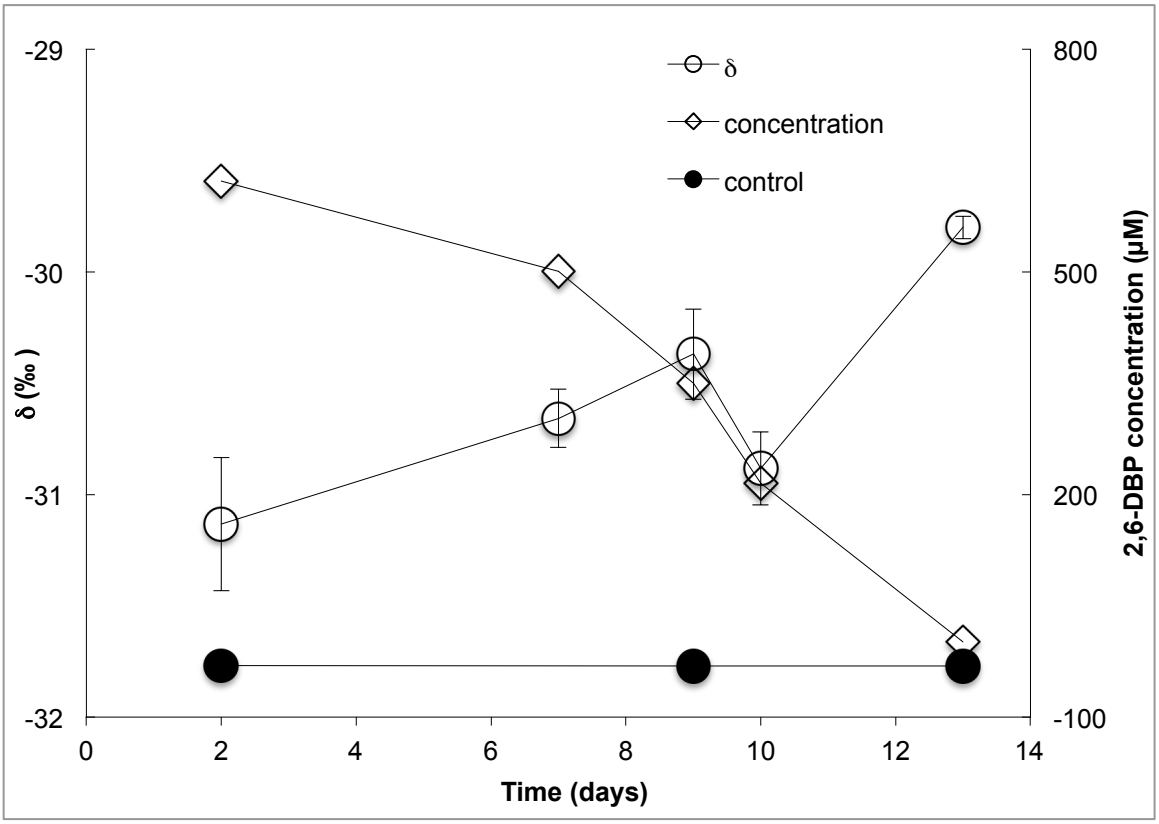

Figure 3.4. Concentration of 2,6-DBP and its $\delta^{13}C$ from dehalogenating experiment *in vivo* of sponge *Clatheria*

Table 3.1. The isotopic fractionation factor (α) and isotopic (ε) as well as the apparent kinetic isotope effect (AKIE) for debromiantion of 2,6-DBP by sponge associated cultures AA1, PPS, TS7

Culture	α	ε	**AKIE ‰**
AA1	1.00301±0.00158	-3.1 ± 1.5	1.0359 ± 0.00998
PPS	1.003±0.00406	-3.0 ± 0.5	1.0355 ± 0.0149
TS7	1.00195±0.00026	-2.0 ± 0.3	1.0237 ± 0.00167

Table 3.2. Carbon ε and AKIE (‰) values obtained for biotic and abiotic dehalogenations of different organohalides

Compound	Cultures	ε bulk ‰	AKIE ‰	References
2,6-DBP	AA1	-3.11 R^2= 0.95	1.038 R^2=0.93	This study
2,6-DBP	PPS	-2.9 R^2= 0.92	1.036 R^2=0.92	This study
2,6-DBP	TS7	-1.95 R^2=0.99	1.024 R^2=0.99	This study
TBNPA	nZVI reductive dehalogenation	-7.6 ± 0.7	1.038 ± 0.0035	Kozell *et al*., 2015
TBNPA	alkaline solution	-10.4 ± 1.6	1.052 ± 0.008	Kozell *et al*., 2016
TBNPA	oxidation with H_2O_2 nCUO	-2.4 ± 0.3	1.012 ± 0.0015	Kozell *et al*., 2017
1,4-DCB	Methanogenic Dupont NJ sediment	-6.3	1.039 ± 0.001	Liang *et al.*, 2014
1,3-DCB	Methanogenic Dupont NJ sediment	-5.4	1.034 ± 0.003	Liang *et al.*, 2014
1,2-DCB	Methanogenic Dupont NJ sediment	-0.8	1.005 ± 0.001	Liang *et al.*, 2014
1,2 DCPA	culture RC (*Dehalococcoides*)	-10.8 ± 0.9	1.0325 ± 0.0029	Fletcher *et al.*, 2009
1,2 DCPA	culture KS (*Dehalococcoides*)	-11.3 ± 0.8	1.0332 ± 0.0027	Fletcher *et al.*, 2009
1.2-DCA	cultures A and B	-7.3 ± 0.2‰	1.0148±0.0004	Hirschorn et al., 2007
1.2-DCA	cultures C and D	-16.7±0.5	1.0346± 0.0010*a*	Hirschorn et al., 2007
PCE	*S.multivorans*	-0.4*	1.0008	Cichocka *et al.*, 2007
PCE	Crude extract of *S. multivorans*	-1*	1.0020	Nijenhuis *et al.*, 2005
PCE	*Desulfitobacterium restrictus*	-4*	1.0081	Renpenning et al., 2015
PCE	*D. hafniense* PCE-S	-5.2*	1.0105	Cichocka *et al.*, 2007
PCE	*Geobacter lovley*	-2.3*	1.0046	Renpenning *et al*., 2015
PCE	*D. michiganensis* (crude extract)	-2.6*	1.0052	Renpenning *et al*., 2015
PCE	*D. michiganensis*	-1.7*	1.0034	Renpenning *et al*., 2015

* Values approximated with equations 6 and 5, from previously published ε

Table 3.2. continued

Compound	Cultures	ε bulk ‰	AKIE ‰	References
PCE	*Dehalobacter restrictus*	-6.3*	1.0128	Renpenning *et al*., 2015
PCE	*Dehalococcoides mccartyi*	-6*	1.0121	Cichocka *et al*., 2008
1,2,4-TCB	*Dehalococcoides* sp.CBDB1	-3.2*	1.0399	Griebler *et al*., 2004
1,2,3-TCB	*Dehalococcoides* sp. CBDB1	-3.5*	1.0215	Griebler *et al*., 2004
TCE	*S. multivorans*	-18.7*	1.0808	Cichocka *et al*., 2007
TCE	*D. mccartyi*	-13.7*	1.0282	Cichocka *et al*., 2008
TCE	*D. mccartyi*	-13.7*	1.0282	Cichocka *et al*., 2008
	Methanogenic consortium	-5.5*	1.0225	Slater *et al.,* 2001
PCE	cyanocobalamine	-22.4*	1.0469	Renpenning *et al*., 2014
PCE	nospseudo B12	-25.3*	1.0533	Renpenning *et al*., 2014
BDE 47	UV radiation	-2.11± 0.45	1.0260	Rosenfelder *et al.*, 2011
1,2-DBE	Fe-S and H2S	-31*	1.0097	Kuder et al., 2012

* Values approximated with equations 6 and 5, from previously published ε

CHAPTER 4:

General Discussion

4.1. General findings

In this study, my goal was to determine the geographical distribution and phylogenetic relationships between the dehalogenating microorganisms associated with sponges and whether the dehalogenation by sponge-associated bacteria can be monitored using compound specific stable isotope analysis (CSIA). A combination of techniques was used to elucidate these questions. Anaerobic enrichments and chronological quantification of bromophenols and their metabolites were used to determine dehalogenating activity. The microbial community analysis of sponge-derived enrichments was based on TRFLP fingerprinting and 16S rRNA gene sequence analysis, while CSIA was used to monitor dehalogenation by sponge-associated bacteria.

Debrominating activity was demonstrated in enrichment cultures from over 20 geographically distant marine sponge species from the temperate and tropical ecoregions. Most of dehalogenating microorganisms were closely related to the genus *Desulfoluna*. Three new anaerobic debrominating bacteria were isolated in pure culture. Strain CHN came from a *Chondrilla nucula* sponge, and is most closely related to *D. spongiiphila* (Ahn *et al*., 2009). The strains PPS and PBS were obtained from two unidentified sponges, and are more closely related to *D. butyratoxydans*, the non-dehalogenating member of the genus (Suzuki *et al*., 2009).

To characterize the dehalogenating mechanism of *Desulfoluna* spp. and to develop methodologies to study marine organisms *in situ*, we aimed to determine if 2,6-DBP dehalogenation would result in carbon isotope fractionation. In addition, the sponge

Clatheria prolifera was tested for *in vivo* dehalogenation and isotope fractionation. Similar isotope enrichment factors for dehalogenation were obtained for all three sponge-associated bacteria, AA1, PPS and TS7, suggesting a similar dehalogenation mechanism. The extent of isotopic enrichment depends on the biodegradation mechanism and the types of enzymes involved (Meckenstock *et al*., 2004). The AKIE values were similar to ones obtained for dehalogenation of other organohalides, such as chlorobenzenes, implicating similarities at the reaction site level of reductive dehalogenation.

4.2. Desulfoluna as a sponge symbiont

As a sponge dweller, *Desulfoluna* spp. would be subject to different selective pressures. Those that generally govern sponge symbionts are: the overall microbial abundance, biogeography of the host, and phylogeny of the sponge.

Hypotheses on how *Desulfoluna* might function in sponges

The *Desulfoluna* bacteria are in low numbers in comparison to the total sponge microbial community and could, so far, only be studied after initial enrichment. Anaerobic conditions are readily established in sediments, which can explain the numerous organohalide respiring bacteria enriched from fresh water and marine sediments (for reviews, see Smidt and de Vos, 2004; Häggblom and Bossert, 2003). In contrast, how does the strict anaerobe *Desulfoluna* survive inside an animal that feeds on microbes while filtering oxygenated seawater? It is widely thought that organohalide respirers grow best in complex communities with fermenters and methanogenic microorganisms

(Dolfing *et al*., 2003; Smidt and de Vos, 2004; Edwards, 2014), where they act as the sink for electron while using hydrogen or other electron donors provided by the associated microbes. *Aplysina aerophoba*'s body characteristics enable them to keep a level of anaerobiosis by reducing their pumping activity, and thus subjecting its symbionts to periods of anoxia. This has also been observed in the ancient sponge *Geodia barretti*, were sulfate reduction was also measured (Hoffmann *et al*., 2005, 2008). These sponges possess great differences in habitat but both contain sulfate-reducing bacteria in their microbial communities. It is thought that the maintenance of their anaerobic symbionts is a characteristic in these sponges. The low pumping in sponges is also generally observed during winter months, were they also undergo body size reduction. Anaerobic symbionts could allow the sponge to survive under such unfavorable conditions by providing some sustenance through anaerobic photosynthesis (Bruck *et al*., 2010), or a detoxification role like anaerobic ammonia oxidation (Zhang *et al*., 2014), or perhaps dehalogenation of (potentially toxic) organobromide metabolites. Numerous studies have shown that sponges rely on their symbionts to survive (Hentschel *et al*., 2006), therefore the *Desulfoluna* probably have a function within the sponge microbial community, which is yet to be demonstrated.

The capacity of *Desulfoluna* to remove the halogens from bioactive organohalides, therefore change the performance of these molecules, is still to be determined. Potentially increasing in numbers and activity during minimum oxygenation of the animal and, be involved in the transformation of the organohalides present in the sponge. An example of the change in activity exerted by dehalogenation has been studied with Barettin [cyclo-(6-bromo-8-en-tryptophan)-arginine], a bioactive molecule from *Geodia barretti* (Lind *et al*., 2013) in which dehalogenation decreased its activity.

Brominated diphenylethers (BDE) found in *D. herbacea* and *D. granulosa* have been linked their cyanobacterial symbiont *Oscillatoria*, and shown to increase or diminish with light exposure (Becerro *et al*, 2004). The inhibitory effects of two brominated diphenyl ethers isolated from *D. granulosa* revealed that the pentabrominated compound showed stronger inhibitory effects than the tetrabrominated BDE, and inhibited a smaller range of kinases, thus indicating a slightly higher specificity (Putz, 2009). This corroborated enhanced bioactivity effects of halogenation in natural products and drugs (Neumann *et al*., 2008). The biogenic PBDEs in *Dysidea* sponge provides evidence that parent hydroxylated-BDEs can be converted to mixed halogenated congeners (Agarwal *et al*., 2015) having a direct effect increasing hydrophobicity and membrane permeability (Gerebtzoff *et al*., 2004). Lowering the amount of halogens by reductive dehalogenation of naturally produced bioactive organohalides seems an effective way of decreasing the activity while, the increase polarity could also mean increasing the chances to remove the compounds from sponge bodies more easily as a detoxification function. It is still to be determined how these compounds are employed and processed in a sponge afterwards.

4.3. Desulfoluna as organohalide respirer

While reductive dehalogenation of brominated phenolics has been observed before in microorganisms (Suflita *et al*., 1982; Monserrrate and Häggblom 1997; Bedard and Van Dort, 1998; Watson *et al*., 2000; Fennell *et al*., 2004), the closest similar reactions to *Desulfoluna* are that of *Desulfovibrio* TBP-1 which dehalogenates 2,4,6-TBP, 2,6-DBP, 2,4-DBP, 2-BP and 4-BP to phenol, and was isolated from NY/NJ harbor polluted sediments (Boyle *et al*., 1998). In addition, enriched sediments contaminated from a small ephemeral stream in the Negev Desert, that debrominated 2,4,6-TBP, 2,4-DBP, 4-

BP to phenol in a medium with 2-3% NaCl (Arbeli *et al*., 2006; Bernstein *et al*., 2012). All of these microbes have a tolerance to high salinity or marine conditions. More studies into the physiologies of the marine dehalogenating bacteria would certainly be of great interest.

Most of the knowledge about dehalogenation comes from studies of dechlorination by organohalide respiring bacteria such as *Dehalococcoides*, *Dehalobacter*, *Desulfitobacterium* and others (Fetzner and Lingens, 1994, Häggblom and Bossert, 2003; Holliger *et al*., 1998; Smidt and de Vos, 2004; Hug *et al.*, 2013). Some of the dechlorinating microorganisms show debrominating capacity: brominated ethenes are dehalogenated by *Sulfurospirillum multivorans and Desulfitobacterium hafniense PCE* (Ye *et al*., 2010)*;* the pesticide bromoxynil (2,6-dibromo-4-cyanophenol) is dehalogenated by *D. chlororespirans* (Cupples *et al*., 2005); polybrominated dioxins are dehalogenated by and enrichment culture comprised of *Dehalococcoides* and *Desulfovibrio* species (Lee *et al*., 2011); and bromobenzenes are dehalogenated by *Dehalococcoides mccartyi* strain CBDB1 (Wagner *et al.*, 2012). In conclusion, some dechlorinating bacteria can also debrominate the bromide versions of the chlorinating compounds they respire. On the other hand, *Desulfoluna spongiiphila* dehalogenates bromo- and iodophenols but not chlorophenols (Ahn *et al.,* 2009). This supports the hypothesis that sponge-associated microbial dehalogenases are different from the characterized dehalogenases to date, as first indicated by Ahn *et al.,* (2003).

In nature the diversity of function in the microbial dehalogenases is probably large, so far all *Dehalococcoides* strains contain multiple dehalogenases (Maphosa *et al*., 2010; Hug *et al*., 2014: Marshal *et al*., 2014; Fricker *et al*., 2015). Characterization and structural analysis has been completed for only a few of these dehalogenases, the latest study

published is Pce A, the “model” dehalogenase from *Sulfurospirillum multivorans* (Bommer *et al*., 2015). So far, a high sequence similarity between dehalogenases does not ensure high functional similarity with the exception of TceA, Bvc A, VcrA dehalogenases that appear to be a monophyletic group and dehalogenate chlorinated aliphatics (Hug *et al*., 2013). There is still much we do not know about dehalogenases and their substrate range. The recent description of the x-ray crystal structure of PceA from *S. multivorans* demonstrates that the dehalogenase consists of a vitamin B_{12} variant containing reactive cobalt atom crucial for dehaloelimination, and a selective entrance to the reactive site shaped as a letterbox. The reactive site is deep inside the protein, after passing a long hydrophobic channel (Bommer *et al*., 2014). The wide range of substrates utilized by some dehalogenating bacteria is likely due the high number of dehalogenases that can be found in their genomes, such as in *Dehalococcoides sp*. VS (Maphosa *et al*., 2010). The dehalogenase structure seem to allow for highly diverse reaction site explaining the number of different dehalogenases a microorganism would need to survive as a strict organohalide respirer (Bommer *et al*., 2014; Edwards *et al*., 2014).

The association of bromophenol degrading bacteria with invertebrates was first studied in sediments with bromophenols produced by the acorn worm *Saccoglossus kowaleskii* (King, 1988). 2,4,6-TBP was dehalogenated under anaerobic conditions by the sediment-associated microorganisms. 2,6-DBP and 2,4-DCP were also degraded but at slower rates. To our knowledge there is only one previous case of a haloaromatic degrading microorganism associated with a metazoan that has been isolated in pure culture; these are *Propionibacterium maris* strains DSL-1 and MLS-1, anaerobic bacteria that grow by oxidation of succinate to propionate, isolated from the linings of the burrow of *Notomastus lobatus* (Watson *et al*., 2000; Schink, 2006), and are capable of

dehalogenating 2,4,6-TBP and 2,6-DBP to monobromophenols (Watson *et al*., 2000). The *Desulfoluna* dehalogenases represent a great opportunity for expanding the knowledge of the dehalogenating mechanisms in existence and the great diversity that can exist within such a specialized feature.

CSIA analyses confirm the similarity of the dehalogenation mechanism within the dehalogenating sponge-associated bacteria. The identical enrichment factors obtained with CSIA support that the 2,6-DBP dehalogenases of the sponge-associated bacteria are similar. The results add to the phylogenetic evidence that *Desulfoluna* in sponges are closely related and additionally possess similar mechanisms of dehalogenation. Ongoing studies of *D. spongiiphila* suggest that its dehalogenases likely contain a corrinoid co-factor. When in cultivation with propyl iodide in the dark inhibited dehalogenation and, later was recovered when cultures were incubated in the light (Lopez-Chiafarelli, personal communication). Future studies will unravel how similar or dissimilar the *Desulfoluna* dehalogenases are from the widely studied dechlorinases.

Since the first report of organohalide respiration (Suflita *et al*., 1982; Shelton and Tiedje, 1984), dehalogenating bacteria had been at the center of studies involved in deciphering the mechanisms governing this specialized process. The interest focused mainly for their potential use in bioremediation, and grasp at the edges how life works even in conditions previously thought to be impossible. Among the fascinating dehalogenating bacteria, *Desulfoluna* belongs to a group of “flexible” organohalide respirers (Maphosa *et al*., 2010; Smidt and de Vos, 2004). This specialized group of microorganisms is characterized by their capacity to utilize other electron acceptor in addition to organohalogens, including, nitrate, sulfate, sulfur, fumarate, or iron. The dehalogenating *Desulfoluna* spp. represent a great opportunity for studying specialized symbiotic

relationships of prokaryotes and metazoans. Sponges are ancient metazoans with physical evidence left from 600 million years, and according to molecular studies had survived probably for more than 800 my. They are considered the oldest living animals that resemble the common ancestor "urmetazoan" of all living multicellular organisms on earth (Müller, 2003). In addition, sponge proteins are more similar to humans than *Caenorhabditis elegans* (Gamulin *et al*., 2000). Any biologist involved in their study is marveled at how impressive these creatures are in terms of their wide distribution, a testament to the adaptations to new environments (Van Soest *et al*., 2012), their biochemical arsenal (Thomas *et al*., 2010), and the clues they hold on how "we, came to be" in cellular terms (Adamska *et al*., 2011; Müller, 2003).

4.4. Future Research

The sponge-symbiont conundrum

The dehalogenating *Desulfoluna*-sponge association is a puzzle. Among the questions left unanswered are if all other sponges that are known producers of brominated compounds contain *Desulfoluna* spp. and if so, what is the phylogenetic relationship with the current isolates and sequences. More detailed characterization of the dehalogenating mechanism in marine environments is of great interest and should include sampling from other marine ecoregions, including the Indo-Pacific, temperate Northern Eastern and Western Pacific, Antarctic and Arctic. Some sponge species of interest would be *Geodia barretti*, *Xestospongia muta*, and *Cladorhyzidae*, as well as additional *Dysidea* and *Agelas* spp. The later sponges are all known producers of brominated bioactive compounds, and could well be highly enriched with *Desulfoluna*

spp. The study should definitively include fresh water sponges; if *Desulfoluna* is a marine bacterium could it survive in freshwater environment? Which other dehalogenating bacteria might we find?

The genome of *Desulfoluna spongiiphila* is currently being studied (Lopez-Chiafarelli, unpublished data) and the findings will advance our understanding of these unique debrominases. The future research of *Desulfoluna* debrominases through CSIA and KIE should include the study of similar and different dehalogenating mechanisms in the marine environment. It would be interesting to compare the dehalogenating mechanisms of *Desulfovibrio* TBP-1, the well-characterized Negev Desert enrichment, and *Desulfovibrio dechloroacetivorans*. Some of these cultures can also dehalogenate TBBPA, which is a compound of great interest due its widespread use as a flame retardant.

Sponges might be used in bioremediation

Sponges are filter feeders and most microorganisms that are filtered from water serve as food. However, microbes living inside of a marine sponge find themselves in a rich environment where products of the host metabolism are a good source of amino acids and carbohydrates compared to the relatively low nutrient environment of seawater. Sponges had been used successfully as microbial filters capable of retaining 10^{10} *E. coli* cells and filter 14 L/h of water; there are studies already on how to use sponges as water filters in aquaculture (Van treek *et al*., 2003; Fu *et al*., 2006, Longo, 2010; Ledda *et al*., 2014). Moreover, it has been shown *ex situ* that whole marine sponges can dehalogenate 2,6-DBP to 2-BP and phenol (Chapter 3; Lopez-Chiafarelli, unpublished data). Further studies could elucidate if sponges have the capacity to remove other

hydrophobic pollutants from water. This is crucial since rarely is environmental pollution due to a single pollutant but rather a mixture of organic chemicals. Using sponges to improve the quality of marine water could represent a viable solution. Sponges are estimated to filter as much as 24,000 liters of water/kg/day (Vogel, 1977). The impact of the marine sponges filtering and dehalogenating capabilities of its microbes in the marine environment is currently unknown. The unique relationship between sponges and dehalogenating microbes represents a range of new possibilities. This relationship is also an important step towards understanding the fate of currently dangerous anthropogenic organohalides present in the environment.

4.5. Final conclusions

Desulfoluna is the dehalogenating bacterium found in a variety of marine sponges. The results support the hypothesis that the biogenic organohalides have enriched for a population of dehalogenating microorganisms within sponges and the association between host sponge and associated microbiota is driven by the unique organohalide chemistry. The research on this peculiar dehalogenating sponge associated microorganism provided a unique setting for a deeper understanding into microbe-animal associations that could potentially be an ancient symbiosis.

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APPENDIXES

Appendix A.

Sorensen Analysis of TRFs in marine sponge dehalogenating enrichment cultures

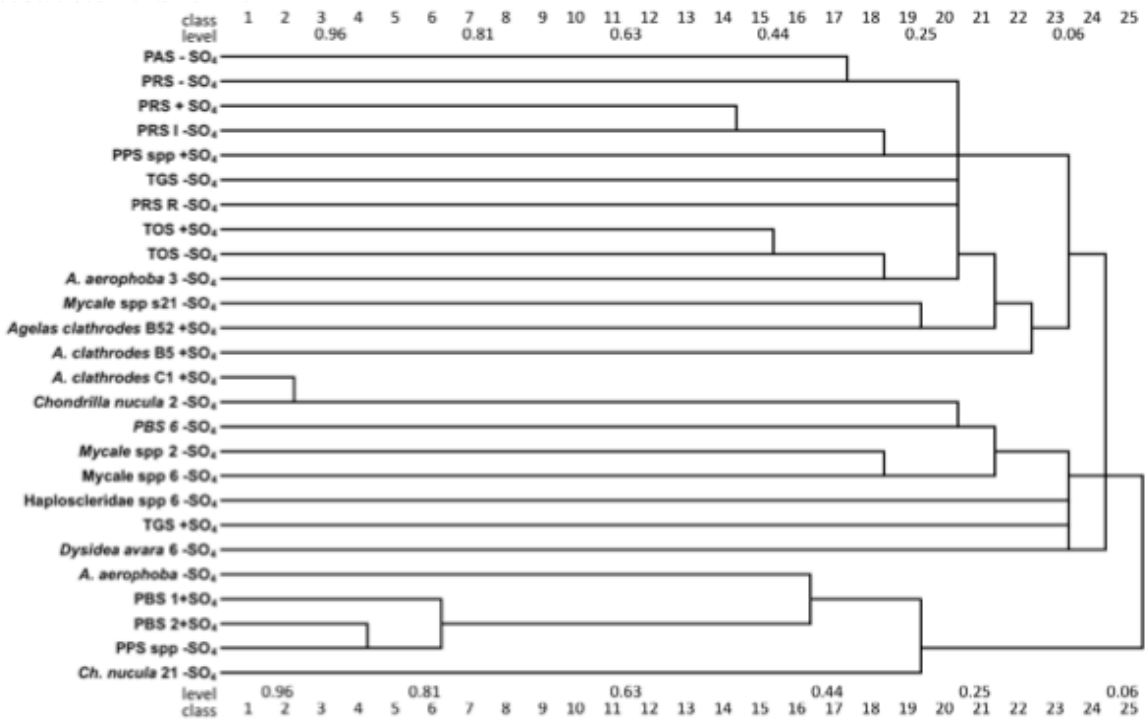

Figure A: Analysis based on presence or absence of TRFs in the samples of enriched dehalogenating cultures PRS, PAS, PBS and PPS sponges from the South Pacific, *A. aerophoba*, *Mycale* and *Dysidea* from the Mediterranean, *A. clathrodes* from Puerto Rico, *C.*

Appendix B

Partial 16S rRNA gene sequences of Desulfoluna isolates CHN, PPS, PBS, and sponge enrichment cultures

Isolate CHN from *Chondrilla nucu*la (1033 bp)

TCGGATTCCCCGAAGGGASTCTCCCGAGATGTCAAGTCCAGGTAAGGTTCTGCGCG
TTGCGTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTT
GAGTTTTAATCTTGCGACCGTACTCCCCAGGCGGACAACTTAATGCGTTAGCTCCGG
CACCGCAGGGGTCAATACCCGCGACACCTAGTTGTCAACGTTTACTGCGTGGACTA
CCAGGGTATCTAATCCTGTTTGCTCCCCACGCCTTCGCGCCTCAGCGTCAGTATTGG
TCCAGGAAGTCGCCTTCGCCACTGGTGTTCCTCCAGATATCTACGAATTTCACCTCT
ACACCTGGAATTCCACTTCCCTCTCCCATACTCAAGCCACCCAGTATCGGATGCAGT
TCCGGGGTTAAGCCCCGGGATTTCACACCCGACTTAAATGGCCGCCTACGCGCCCT
TTACGCCCAATAATTCCGAATAACGCTTGCACCCCCCGTGTTACCGCGGCTGCTGG
CACGGAGTTAGCCGGTGCTTCCTTCAGTGGTACCGTCAATTCCCTTATTTATTAAATA
AGGAAGGTTTCTTCCCACTTGACAGAGCTTTACGACCCAAGAGCCTTCATCACTCAC
GCGGCGTTGCTGCGTCAGGGTTTCCCCCATTGCGCAAAATTCCTCACTGCTGCCTC
CCGTAGGAGTCTGGACCGTGTGTCAGTTCCAGTGTGGCTGATCATCCTCTCAGACC
AGCTAACCATCGAAGCCTTGGTAGGCCATTACCCCACCAACAAGCTAATGGTACGC
GAACTCATCCCCAAACAAGAGCTTCCAAGGAGAGGCCCTCTTTGATAATCCAAACCG
TGGTAAGGATTATGTCATCCGGTATTAGCAGCCCTTTCGAACTGTTATCCCAAATTCA
GGGGTAGATTATTCACGCGTTACTCACCCGTGCGCCACTGTACTCGTTTTCCGAAGA
AAACTTTCTCGTTCGACTTGCATGTGTTAAGCACGCCGCCAGCGATCATTCWGAGC
CAGGATCAAACTC

Isolate PPS from unidentified sponge (1076 bp)

CTGACGANAGCCATGCAGCACCTGTCATCGGGCTCCCCGAAGGGCACTCTTATCTT

WARARATTCCCGAGATGTCAAGTCCAGGTAAGGTTCTGCGCGTTGCGTCGAATTAA

ACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTG

CGACCGTACTCCCCAGGCGGACAACTTAATGCGTTAGCTCCGGCACCGCAGGGGT

CAATACCCGCGACACCTAGTTGTCAACGTTTACTGCGTGGACTACCAGGGTATCTAA

TCCTGTTTGCTCCCCACGCCTTCGCGCCTCAGCGTCAGTATTGGTCCAGGAAGTCG

CCTTCGCCACTGGTGTTCCTCCAGATATCTACGAATTTCACCTCTACACCTGGAATTC

CACTTCCCTCTCCCATACTCAAGCCACCCAGTATCGGATGCAGTTCCGGGGTTAAGC

CCCGGGATTTCACACCCGACTTAAATGGCCGCCTACGCGCCCTTTACGCCCAATAAT

TCCGAATAACGCTTGCACCCCCCGTGTTACCGCGGCTGCTGGCACGGAGTTAGCCG

GTGCTTCCTTCAGTGGTACCGTCAATTCCCTTATCTATTAAATAAGGAAGGTTTCTTC

CCACTTGACAGAGCTTTACGACCCAAGAGCCTTCATCACTCACGCGGCGTTGCTGC

GTCAGGGTTTCCCCCATTGCGCAAAATTCCTCACTGCTGCCTCCCGTAGGAGTCTG

GACCGTGTGTCAGTTCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACCCATCGTT

GCCTTGGTAAGCCGTTACTCTTACCAACAAGCTAATGGGACGCGAACTCATCCCCAA

ACAAGAGCTTCCAAGGAGAGGCCCTCTTTGATAACCCAAACCGTGGTAAGGATTATG

TCATCCGGTATTAGCAGCCCTTTCGAACTGTTATCCCAAATTCAGGGGTAGATTATTC

ACGCGTTACTCACCCGTGCGCCACTGTACTCGTTTTCCGAAGAAAACTTTCTCGTTC

GACTTGCATGTGTTAAGCACGCCGCCAGCGTTCATTCTGAGCCAGGATCAAACTC

Isolate PBS from unidentified sponge (1003 bp)

CCGAGATGTCAAGTCCAGGTAAGGTTCTGCGCGTTGCGTCGAATTAAACCACATGCT
CCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAATCTTGCGACCGTACT
CCCCAGGCGGACAACTTAATGCGTTAGCTCCGGCACCGCAGGGGTCAATACCCGC
GACACCTAGTTGTCAACGTTTACTGCGTGGACTACCAGGGTATCTAATCCTGTTTGC
TCCCCACGCCTTCGCGCCTCAGCGTCAGTATTGGTCCAGGAAGTCGCCTTCGCCAC
TGGTGTTCCTCCAGATATCTACGAATTTCACCTCTACACCTGGAATTCCACTTCCCTC
TCCCATACTCAAGCCACCCAGTATCGGATGCAGTTCCGGGGTTAAGCCCCGGGATT
TCACACCCGACTTAAATGGCCGCCTACGCGCCCTTTACGCCCAATAATTCCGAATAA
CGCTTGCACCCCCCGTGTTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCT
TCAGTGGTACCGTCAGTCTCTGCATCTATTAAATACAAAGAGATTCTTCCCACTTGAC
AGAGCTTTACGACCCAAGAGCCTTCATCACTCACGCGGCGTTGCTGCGTCAGGGTT
TCCCCCATTGCGCAAAATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTG
TCAGTTCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACCCATCGTTGCCTTGGTA
AGCCGTTACCTCACCAACAAGCTAATGGGACGCGAACTCATCCCCAAACAAGAGCTT
CCAAGGAGAGGCCCTCTTTGATAATTTAAACCGTGGTAAAAATTATATCATCCGGTAT
TAGCAGCCCTTTCGAACTGTTATCCCAAATTCAGGGGTAGATTATTCACGCGTTACT
CACCCGTGCGCCANTGNACTCGTTYTCCGAASWDWACTTTCTCGNYSRACNTTGCA
TGTGTTAAGCACGCCGCCAGCGTTCATTCTGAGCCAGGA

Enrichment Peruvian sediment 2007 (779bp)

TCATGCAAGGTGAACGAGAAAGTTTTCTTCGGAAAACGAGTACAGTGGCGCACGGG
TGAGTAACGCGTGAATAATCTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGC
TAATACCGGATGACATAATCCTTACCACGGTTTGGATTATCAAAGAGGGCCTCTCCT
TGGAAGCTCTTGTTTGGGGATGAGTTCGCGTCCCATTAGCTTGTTGGTAAGGTAACG
GCTTACCAAGGCAACGATGGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAA
CTGACACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGG
GGGAAACCCTGACGCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAA
GCTCTGTCAAGTGGGAAGAAACCTTCCTTATTTAATAGATAAGGGAATTGACGGTAC
CACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGT
GCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGTAGGCGGCCATTTAAGTC
GGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGCATCCGATACTGGGTGGCTTGA
GTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGG
AGGAACACCAGTGGCGAAGGCGACTTCCTGGACCAATACTGACGCTGAGGCGCGA
AGGCGTGGGGAGCAAACAGGATTAGAACCCTGGTATCCACGCATAAAACG

Enrichment *Halichondria sp*. LIS 5 (749 bp)

TCATGCAAGTCGAACGAGAAAGGGATTGCTTGCAATCCCGAGTAGAGTGGCGCACG
GGTGAGTACACGTGGATAATCTACCTTTGAATCCGGGATAACTGTTCGAAAGGGTAG
CTAATACCGAATGAAGTCAAAATTTCTTCGGAATTTTTGATGAAAGACAGCCTCTACC
TGTAAGCTGTTGTTTGAAGATGAGTCCGCGCACCATTAGTTTGTTGGTGGGGTAACG
GCCTACCAAGACTGTGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAA
CTGACACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGG
GGGAAACCCTGACGCAGCAACGCCGCGTGAGTGAAGAAGGCCCTTGGGTCGTAAA
GCTCTGTCAACAGGGAAGAAATTATCATGTATTAATAGTGCGTGGTATTGACGGTAC
CTGTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGGT
GCAAGCGTTATTCGGAATCATTGGGCGTAAAGGGCACGCAGGCGGTCTTGCAAGTC
AGATGTGAAAGCCCGGGGCTCAACCCCGGAAGTGCATTTGAAACTACAAGACTTGA
GTACGGTAGAGGAGAGGGGAATTCCTGGTGTAGAGGTGAAATTCGTAGATATCAGG
AGGAACACCGGTGGCGAAGGCGCCTCTCTGGGCCGAAACTGACGCTGAGGTGCGA
AGGCGTGGGTAGCGAACAG

Enrichment LIS 3, *Clathria prolifera* from Lon Island Sound (759 bp)

ATGCAAGTCGAACGAGAAAGTTTTCTTCGGAAAACGAGTAAAGTGGCGCA
CGGGTGAGTAACGCGTGAATAATCTACCCCTGAATTTGGGATAACAGTTC
GAAAGGGCTGCTAATACCGGATGACATTCTTCTTACCACGGTTTGAAGAA
TCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGTC
CCATTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCAACGATGGGTAGC
TGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGTCCAGACT
CCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGAC
GCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGTC
AAGTGGGAAGAAACCTTCCATGATTAATACTTATGGGAATTGACGGTACC
ACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGG
GGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGTAGGCGGC
CATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGCATCCG
ATACTGGGTGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGA
GGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTCC
CGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATT
AGATACCCT

Enrichment FLS6, *Raspaillidae* from Florida trenches (778 bp)

TCATGCAAGTGAACGAGAAAGTTTTCTTCGGAAAGCGAGTAAAGTGGCGC

ACGGGTGAGTAACGCGTGAATAATCTACCCCTGAATTTGGGATAACAGTT

CGAAAGGGCTGCTAATACCGGATGAAATAATCCTTACCACGGTTTGGGTT

ATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGT

ACCATTAGCTTGTTGGTGGGGTAAGAGCCTACCAAGGCTTCGATGGTTAG

CTGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGTCCAGAC

TCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGA

CGCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGT

CAAGTGGGAAGAACCTCTCCTTATTTAAAAGATGGGGAGACTGACGGTAC

CACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGG

GGGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGTAGGCGG

CCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGCATCC

GATACTGGATGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGTAG

AGGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTC

CTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGAT

TAGAACCCTGGTATCCACGCATGAAAGG

Enrichment DYS*, Dysidea avara* (712 bp)

GCTGGCGCACGGGTGAGTACGCGTGATAATCTACCCCTGAATTTGGGATA
ACAGTTCGAAAGGGCTGCTAATACCGGATGACATAATCCTTACCACGGTT
TGGATTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGT
TCGCGTACCATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCATCGAT
GGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAA
CCCTGACGCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAG
CTCTGTCAAGTGGGAAGAAACCTTCCTTATTTAATAGATAAGGGAATTGA
CGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ACACGGGGGGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGT
AGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACT
GCATCCGATACTGGATGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAG
GTGTAGAGGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGC
GACTTCCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAA
CAGGATTAGATA

Enrichment CHN YB, *Chondrilla nucula* YB (721 bp)

GTCAGGGTAGTGGCGCACGGGTGAGTACGCGTGAATAATCTACCCCTGAATTTGGG
ATAACAGTTCGAAAGGGCTGCTAATACCGGATAATATGATTCTTACCACGGTTTGAAT
TATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGTACCATT
AGTTTGTTGGTGGGGTAACGGCCTACCAAGACTACGATGGTTAGCTGGTCTGAGAG
GATGATCAGCCACACTGGAACTGACACACGGTCCAGACTCCTACGGGAGGCAGCAG
TGAGGAATTTTGCGCAATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGAGTGATG
AAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAGAAACCTTCCATGCTTAATAC
GCATGGGAACTGACGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAG
CCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCG
CGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGCA
TCCGATACTGGATGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGAGG
TGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTCCTGGACCAA
TACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATA

Enrichment PBS, unidentified sponge (717 bp)

TCTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGCTAATACCGGATGATATAA

TTTTTACCACGGTTTAAATTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGG

ATGAGTTCGCGTCCCATTAGCTTGTTGGTGAGGTAACGGCTTACCAAGGCAACGAT

GGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGTCCAGAC

TCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAG

CAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAG

AATCTCTTTGTATTTAATAGATGCAGAGACTGACGGTACCACTGAAGGAAGCACCGG

CTAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATT

ATTGGGCGTAAAGGGCGCGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGG

CTTAACCCCGGAACTGCATCCGATACTGGGTGGCTTGAGTATGGGAGAGGGAAGTG

GAATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAA

GGCGACTTCCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACA

GGATTAGATACCCTGGTAGTCCACGCAGTAAACGTTGACAACTA

Enrichment CHN 21 +SO4, *Chondrilla nucula* (738 bp)

CAGGGTCTAATCCTGTTTGCTCCCCACGCCTTCGCGCCTCAGCGTCAGTATTGGTC
CAGGAAGTCGCCTTCGCCACTGGTGTTCCTCCAGATATCTACGAATTTCACCTCTAC
ACCTGGAATTCCACTTCCCTCTCCCATACTCAAGCCACCCAGTATCGGATGCAGTTC
CGGGGTTAAGCCCCGGGATTTCACACCCGACTTAAATGGCCGCCTACGCGCCCTTT
ACGCCCAATAATTCCGAATAACGCTTGCACCCCCCGTGTTACCGCGGCTGCTGGCA
CGGAGTTAGCCGGTGCTTCCTTCAGTGGTACCGTCAATTCCCTTATTTATTAAATAAG
GAAGGTTTCTTCCCACTTGACAGAGCTTTACGACCCAAGAGCCTTCATCACTCACGC
GGCGTTGCTGCGTCAGGGTTTCCCCCATTGCGCAAAATTCCTCACTGCTGCCTCCC
GTAGGAGTCTGGACCGTGTGTCAGTTCCAGTGTGGCTGATCATCCTCTCAGACCAG
CTAACCATCGAAGCCTTGGTAGGCCATTACCCCACCAACAAGCTAATGGTACGCGAA
CTCATCCCCAAACAAGAGCTTCCAAGGAGAGGCCCTCTTTGATAATCCAAACCGTGG
TAAGGATTATGTCATCCGGTATTAGCAGCCCTTTCGAACTGTTATCCCAAATTCAGG
GGTAGATTATTCACGCGTTACTCACCCGTGCGCCACTGTACTCGTTTTCCGAAGAAA
C

Enrichment CHN 21B 4, *Chondrilla nucula* (789 bp)

AAAGTTTTCTTCGGAAAACGAGTACAGTGGCGCACGGGTGAGTAACGCGTGAATAAT
CTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGCTAATACCGGATGACATAAT
CCTTACCACGGTTTGGATTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGG
ATGAGTTCGCGTACCATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCTTCGATG
GTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGTCCAGACT
CCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGC
AACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAGA
AACCTTCCTTATTTAATAAATAAGGGAATTGACGGTACCACTGAAGGAAGCACCGGC
TAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATTA
TTGGGCGTAAAGGGCGCGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGC
TTAACCCCGGAACTGCATCCGATACTGGGTGGCTTGAGTATGGGAGAGGGAAGTGG
AATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAG
GCGACTTCCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCAGTAAACGTTGACAACTAGGTGTCGNGGGGG
TAT

Enrichment CHN 21B3, *Chondrilla nucula* (775 bp)

GAGAAAGTTTTCTTCGGAAAACGAGTACAGTGGCGCACGGGTGAGTAACGCGTGAA
TAATCTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGCTAATACCGGATGACA
TAATCCTTACCACGGTTTGGATTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTT
GGGGATGAGTTCGCGTACCATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCTT
CGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGAC
GCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGG
GAAGAAACCTTCCTTATTTAATAAATAAGGGAATTGACGGTACCACTGAAGGAAGCA
CCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCG
GAATTATTGGGCGTAAAGGGCGCGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCC
GGGGCTTAACCCCGGAACTGCATCCGATACTGGGTGGCTTGAGTATGGGAGAGGG
AAGTGGAATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGGAGGAACACCAGTG
GCGAAGGCGACTTCCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGTTGACAACT

Enrichment PPS 5, Unidentified sponge (799 bp)

TCGAACGAGAAAGTTTTCTTCGGANANCGAGTACAGTGGCGCACGGGTGAGTAACG
CGTGAATAATCTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGCTAATACCGG
ATGACATAATCCTTACCACGGTTTGGGTTATCAAAGAGGGCCTCTCCTTGGAAGCTC
TTGTTTGGGGATGAGTTCGCGTCCCATTAGCTTGTTGGTAAGGTAACGGCTTACCAA
GGCAACGATGGGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACAC
GGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACC
CTGACGCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGTCA
AGTGGGAAGAAACCTTCCTTATTTAATAGATAAGGGAATTGACGGTACCACTGAAGG
AAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAGCGTT
ATTCGGAATTATTGGGCGTAAAGGGCGCGTAGGCGGCCATTTAAGTCGGGTGTGAA
ATCCCGGGGCTTAACCCCGGAACTGCATCCGATACTGGGTGGCTTGAGTATGGGAG
AGGGAAGTGGAATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGGAGGAACACC
AGTGGCGAAGGCGACTTCCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGG
GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGTTGACAACTAGGT
GTCGNGGGGGTATTG

Enrichment PPS 4, unidentified sponge (773 bp)

TAGTGTCAACGTTTACTGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCAC
GCCTTCGCGCCTCAGCGTCAGTATTGGTCCAGGAAGTCGCCTTCGCCACTGGTGTT
CCTCCAGATATCTACGAATTTCACCTCTACACCTGGAATTCCACTTCCCTCTCCCATA
CTCAAGCCACCCAGTATCGGATGCAGTTCCGGGGTTAAGCCCCGGGATTTCACACC
CGACTTAAATGGCCGCCTACGCGCCCTTTACGCCCAATAATTCCGAATAACGCTTGC
ACCCCCCGTGTTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTCCTTCAGTGG
TACCGTCAATTCCCTTATCTATTAAATAAGGAAGGTTTCTTCCCACTTGACAGAGCTT
TACGACCCAAGAGCCTTCATCACTCACGCGGCGTTGCTGCGTCAGGGTTTCCCCCA
TTGCGCAAAATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGTGTCAGTTCC
AGTGTGGCTGATCATCCTCTCAGACCAGCTACCCATCGTTGCCTTGGTAAGCCGTTA
CCTTACCAACAAGCTAATGGGACGCGAACTCATCCCCAAACAAGAGCTTCCAAGGA
GAGGCCCTCTTTGATAACCCAAACCGTGGTAAGGATTATGTCATCCGGTATTAGCAG
CCCTTTCGAACTGTTATCCCAAATTCAGGGGTAGATTATTCACGCGTTACTCACCCG
TGCGCCACTGTACTCGTTNTCCGAAGAAAACTTTC

Enrichment PBS 2, unidentified sponge (798 bp)

CGACACCTAGTTGTCAACGTTTACTGCGTGGACTACCAGGGTATCTAATCCTGTTTG
CTCCCCACGCCTTCGCGCCTCAGCGTCAGTATTGGTCCAGGAAGTCGCCTTCGCCA
CTGGTGTTCCTCCAGATATCTACGAATTTCACCTCTACACCTGGAATTCCACTTCCCT
CTCCCATACTCAAGCCACCCAGTATCGGATGCAGTTCCGGGGTTAAGCCCCGGGAT
TTCACACCCGACTTAAATGGCCGCCTACGCGCCCTTTACGCCCAATAATTCCGAATA
ACGCTTGCACCCCCCGTGTTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTC
CTTCAGTGGTACCGTCAGTCTCTGCATCTATTAAAYACAAAGAGATTCTTCCCACTTG
ACAGAGCTTTACGACCCAAGAGCCTTCATCACTCACGCGGCGTTGCTGCGTCAGGG
TTTCCCCCATTGCGCAAAATTCCTCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGT
GTCAGTTCCAGTGTGGCTGATCATCCTCTCAGACCAGCTACCCATCGTTGCCTTGGT
AAGCCGTTACCTCACCAACAAGCTAATGGGACGCGAACTCATCCCCAAACAAGAGC
TTCCAAGGAGAGGCCCTCTTTGATAATTTAAACCGTGGTAAAAATTATATCATCCGGT
ATTAGCAGCCCTTTCGAACTGTTATCCCAAATTCAGGGGTAGATTATTCACGCGTTAC
TCACCCGTGCGCCACTGTACTCGTTCTCCGAANAGAACTTTCTCGNCGAACTTTGCA
TG

Enrichment PBS 1, unidentified sponge (765 bp)

AAAGTTCGGAGAACGAGTCATGGCGCCGGGTGAGTAACGCGTGAATAATCTACCCC
TGAATTTGGGATAACAGTTCGAAAGGGCTGCTAATACCGGATGATATAATTTTTACCA
CGGTTTAAATTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTC
GCGTCCCATTAGCTTGTTGGTGAGGTAACGGCTTACCAAGGCAACGATGGGTAGCT
GGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCG
CGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAGAATCTCTTT
GTATTTAATAGATGCAGAGACTGACGGTACCACTGAAGGAAGCACCGGCTAACTCC
GTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATTATTGGGC
GTAAAGGGCGCGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCC
CGGAACTGCATCCGATACTGGGTGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCA
GGTGTAGAGGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTT
CCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCAGTAAACGTTGACAACTA

Enrichment M4 - 9, *Mycale* (808 bp)

TCATGCAAGTCGAACGAGAAAGTTTTNTTCGGANAACGAGTACAGTGGCGCACGGG
TGAGTAACGCGTGAATAATCTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGC
TAATACCGGATGACATAACCCTTACCACGGTTTGGGTTATCAAAGAGGGCCTCTCCT
TGGAAGCTCTTGTTTGGGGATGAGTTCGCGTACCATTAGCTTGTTGGTGGGGTAATG
GCCTACCAAGGCTTCGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAA
CTGACACACGGTCCAGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGG
GGGAAACCCTGACGCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAA
GCTCTGTCAAGTGGGAAGAAACCTTCCTTATTTAATAGATAAGGGAATTGACGGTAC
CACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGT
GCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGCGTAGGCGGCCATTTAAGTC
GGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGCATCCGATACTGGATGGCTTGA
GTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGG
AGGAACACCAGTGGCGAAGGCGACTTCCTGGACCAATACTGACGCTGAGGCGCGA
AGGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGTTGA
CAACTAGGTGTCGNGGGGTATTG

Enrichment M4 - 7, unidentified sponge (777 bp)

GAGAAAGTTTTCTTCGGAAAACGAGTACAGTGGCGCACGGGTGAGTAACGCGTGAA
TAATCTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGCTAATACCGGATGACA
TAACCCTTACCACGGTTTGGGTTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTT
GGGGATGAGTTCGCGTACCATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCTT
CGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGACACACGGTCC
AGACTCCTACGGGAGGCAGCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGAC
GCAGCAACGCCGCGTGAGTGATGAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGG
GAAGAAACCTTCCTTATTTAATAGATAAGGGAATTGACGGTACCACTGAAGGAAGCA
CCGGCTAACTCCGTGCCAGCAGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCG
GAATTATTGGGCGTAAAGGGCGCGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCC
GGGGCTTAACCCCGGAACTGCATCCGATACTGGGTGGCTTGAGTATGGGAGAGGG
AAGTGGAATTCCAGGTGTAGAGGTGAAATTCGTAGATATCTGGAGGAACACCAGTG
GCGAAGGCGACTTCCTGGACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGC
AAACAGGATTAGATACCCTGGTAGTCCACGCAGTAAACGTTGACAACTAG

AP6CH2, *Aplysina aerophoba* (728 bp)

GAGGTCAGTGGCGCACGGGTGAGTACGCGTGAATAATCTACCCCTGAATTTGGGAT
AACAGTTCGAAAGGGCTGCTAATACCGGATGACATAATCCTTACCACGGTTTGGRTT
ATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGTACCATTA
GCTTGTTGGTGGGGTAATGGCCTACCAAGGCTTCGATGGTTAGCTGGTCTGAGAGG
ATGATCAGCCACACTGGAACTGACACACGGTCCAGACTCCTACGGGAGGCAGCAGT
GAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGAGTGATG
AAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAGAAACCTTCCTTATTTAATAG
ATAAGGGAATTGACGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCAGC
CGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGCGC
GTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGCAT
CCGATACTGGATGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGAGGT
GAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTCCTGGACCAAT
ACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATAGCCTGGTAT

AP5CH2, *Aplysina aerophoba* (729 bp)

AACGGGTAAGTGGCGCACGGGTGAGTACGCGTGAATAATCTACCCCTGAATTTGGG
ATAACAGTTCGAAAGGGCTGCTAATACCGGATAACATGATTCTTACCACGGTTTGAA
TYATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGTACCAT
TAGTTTGTTGGTGGGGTAACGGCCTACCAAGACTACGATGGTTAGCTGGTCTGAGA
GGATGATCAGCCACACTGGAACTGACACACGGTCCAGACTCCTACGGGAGGCAGCA
GTGAGGAATTTTGCGCAATGGGCGAAAGCCTGACGCAGCAACGCCGCGTGAGTGAT
GAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAGAAACCTTTCRYACTTAATA
CGTAYGGGAACTGACGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCAGCA
GCCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGGC
GYGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGC
ATCCGATACTGGATGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGAG
GTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTCCTGGACCA
ATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATAGCCTGGT
A

AP2CH2, *Aplysina aerophoba* 704 bp

GCCGCACGGGTGAGTACGCGTGAATAATCTACCCCTGAATTTGGGATAACAGTTCG

AAAGGGCTGCTAATACCGGATAATATGATCCTTACCACGGTTTGAATTATCAAAGAG

GGCCTCTCCTTGGAAGCTCTTGTTTGGKGATGAGTTCGYGTACCATTAGWTTGTTGG

TGGGGTAACGGCCTACCAAGWYTACGATGGWTAGCTGGTCTGAGAGGATGATCAK

CCACACTGGAACTGACACACGGTCCAKACTCCTACGGGAGGCASCAGTGAGGAATT

TTGCGCAATGGGCGAAAKCCTGACGCAGCAACGCYGMGTGAGTGATGAAGGCTCTT

GGGTCGTAAAKCTYTGTCMAGTGSGAAGAAACCTTMCMYRCKTAATWYGCAYGGKA

ACTGACGGTACCWSTGAAGGAAGCWCCGGCTAACTCCGTGCCWGCAGCCGCGGT

AACACGGGGGGTGCAAGCGTTATTCGTCATTATTGGGCGTAAAGGGCGCGTAGGCG

GCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACTGCATCCGATAC

TGGATGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGAGGTGAAATTC

GTAGATATCTGGAGGAACACCATATGCGAAGGTGACTTCCCGGACCAATACTGACG

CTGAGGCGAGAAGGCGTGGGGAGCAAACAGGAT

AP2 C1, *Aplysina aerophoba* 733 bp

AAACGAGTACAGTGGCGCACGGGTGAGTACGCGTGAATAATCTACCCCTGAATTTG
GGATAACAGTTCGAAAGGGCTGCTAATACCGGATGATATAATTCTTACCACGGTTTG
GGTTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGTAC
CATTAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCTTCGATGGTTAGCTGGTCTGA
GAGGATGATCAGCCACACTGGAACTGACACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGAG
TGATGAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAGAAACCTTCCTTATTT
AATAGATAAGGGAATTGACGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAG
GGCGCGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAA
CTGCATCCGATACTGGATGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGT
AGAGGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTCCTGG
ACCAATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATAACCT
GGTATC

AP1CH1, *Aplysina aerophoba* (721 bp)

AAGGGTTAAGTGGCGCACGGGTGAGTACGCGTGAATAATCTACCCCTGAATTTGGG
ATAACAGTTCGAAAGGGCTGCTAATACCGGATGACATGACCCTTACCACGGTTCGG
GTTATCAAAGAGGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGTACCA
TTAGCTTGTTGGTGGGGTAACGGCCTACCAAGGCTTCGATGGTTAGCTGGTCTGAG
AGGATGATCAGCCACACTGGAACTGACACACGGTCCAGACTCCTACGGGAGGCAGC
AGTGAGGAATTTTGCGCAATGGGGGAAACCCTGACGCAGCAACGCCGCGTGAGTG
ATGAAGGCTCTTGGGTCGTAAAGCTCTGTCAAGTGGGAAGAAACCTTCCTTATTTAA
TAAATAAGGGAACTGACGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAACACGGGGGGTGCAAGCGTTATTCGGAATTATTGGGCGTAAAGGG
CGCGTAGGCGGCCATTTAAGTCGGGTGTGAAATCCCGGGGCTTAACCCCGGAACTG
CATCCGATACTGGGTGGCTTGAGTATGGGAGAGGGAAGTGGAATTCCAGGTGTAGA
GGTGAAATTCGTAGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTCCTGGACC
AATACTGACGCTGAGGCGCGAAGGCGTGGGGAGCAAACAGGATTAGATA

Tethya sp., sponge enrichment

AGCTGCCGCCGGGTGAGTACGCGTGTATAATCTACCCCTGAATTTGGGATAACAGTT
CGAAAGGGCTGCTAATACCGGATAATATGATTCTTACCACGGTTTGAATTATCAAAGA
GGGCCTCTCCTTGGAAGCTCTTGTTTGGGGATGAGTTCGCGTACCATTAGTTTGTTG
GTGGGGTAACGGCCTACCAAKACTACRATGGTTAGCTGGTCTGAGAGGATGATCAK
CCACACTGGAACTGACACACGGTCCAKACTCCTACGGGAGGCASCAGTGAGGAATT
TTGCRCAATGGGCGAAAKCCTGACGCAGCAACGCCGCGTGAGTGATGAAGGCTCTT
GGGTCGTAAAKCTYTGTCAAGTGGGAAGAAACCTTCCAYRMTTAATACKCATRGKAA
CTGACGGTACCACTGAAGGAAGCACCGGCTAACTCCGTGCCWGCAGCCGCGGTAA
CACGGGGGGTGCAAGCGTTATTCGKAATTATTGGGCGTAAAGGGCGYGTAGKCGGC
CATTTAAGTCGGGTGTGAWATCCCGGGGCTTAACCCCGGAMCTGCATCCGATACTG
GATGGCTTGAGTATSGGAGAGGGAAGTGGAATTCCAGGTGTAKAGGTGAAATTCGT
AGATATCTGGAGGAACACCAGTGGCGAAGGCGACTTCCTGGACCAATACTGACGCT
GAGGCGCGAAGGCGTGGGGAGCAA

FLS6, *Raspaillidae* 778 bp

TCATGCAAGTGAACGAGAAAGTTTTCTTCGGAAAGCGAGTAAAGTGGCGCACGGGT
GAGTAACGCGTGAATAATCTACCCCTGAATTTGGGATAACAGTTCGAAAGGGCTGCT
AATACCGGATGAAATAATCCTTACCACGGTTTGGGTTATCAAAGAGGGCCTCTCCTT
GGAAGCTCTTGTTTGGGGATGAGTTCGCGTACCATTAGCTTGTTGGTGGGGTAAGA
GCCTACCAAGGCTTCGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAA
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DYS enrichment*, Dysidea avara* (712 bp)

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Appendix C:

Method optimization for isotope composition analysis of bromophenols

Linearity Tests

To determine the linearity of halophenols in the GC-C-IRMS, standards were prepared at different concentrations from 500 to 2500 µM and recorded the amplitude and Isotope composition δ $^{13}C/^{12}C$ values were oobtained at least in triplicate for all compounds. Determined the limits of detection and linearity of the compounds by evaluating that the standard deviations of the concentration is less than 0.5 and amplitude signals between 30 and 3500 mV.

Table C.1. δ $^{13}C/^{12}C$ and amplitude (mV) observed for halophenol standards at different concentrations

2,6-DBP	**Amplitud**		**δ Values**	
Conc µM	Mean ampl	STDEV	Mean ampl	STDEV
500	672	14	-33.77	1.94
750	1475	9	-34.22	0.47
1000	2184	164	-33.76	0.37
2000	5052	416	-32.00	0.45
2500	5977	60	-31.94	0.36
2BP	**Amplitud**		**δ Values**	
Conc µM	Mean ampl	STDEV	Mean ampl	STDEV
500	720	30	-31.95	0.26
750	1526	53	-32.76	0.32
1000	2288	126	-33.28	0.14
2000	5582	502	-33.63	0.08
2500	7012	15	-33.75	0.15
Phenol	**Amplitud**		**δ Values**	
Conc µM	Mean ampl	STDEV	Mean ampl	STDEV
500	652	13	-31.95	0.26
750	1486	30	-32.76	0.32
1000	2439	174	-33.28	0.14
2000	4251	3258	-33.63	0.08
2500	7470	230	-33.75	0.15

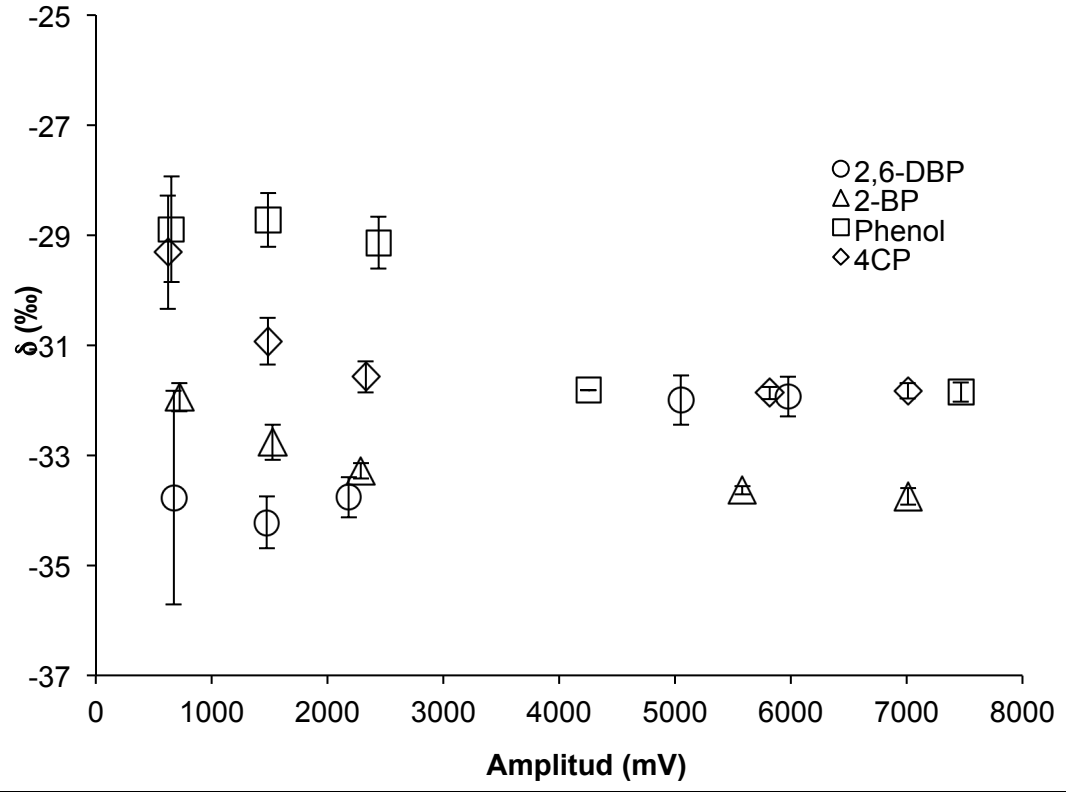

Figure C.1. δ $^{13}C/^{12}C$ values versus amplitude obtained from mixture of halophenol standards at different concentrations showing the lienarity of the compounds in GC-C-IRMS 2

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Table C.2. Detection limits of the phenol, 2-BP, 2,6-DBP and 4-chlorophenol (4-CP) utilized as internal standard

Compound	Detection Limit (µM)	Range of concentration (µM)
Phenol	900	500- 1000
2-BP	400	500- 1000
2,6-DBP	300	750- 1000
4-CP	700	750- 1000

Solid Phase Extraction of Bromophenols

It has been determined that the dete ction limit concentration of bromophenols is between 300 - 900µM. The initial amount of bromophenols inoculated with the cultures was 200µM 2,6-DBP. Furthermore the isotope fractionation is ideally studied between 90% to 10%. Which means that we must be able to concentrate 20µM to at least 300µM without changes the original isotope composition

Optimisation of the sample concentration for bromophenols:

Sample volume for isotope measurements:	3 mL
Initial concentration of bromophenols:	200 µM
90 % biodegradation concentration:	20 µM
limit of detection of bromophenols:	300 µM
limit of detection of phenol:	900 µM

→ concentration factor needed (BP, DBP): 20 (→ elution volume = 150 µL)

→ concentration factor needed (phenol): 45 (→ elution volume = 66 µL)

The samples used the optimization experiments were duplicates of aqueous samples (volume of 3 mL demineralized water) spiked with bromophenol, dibromophenol and internal standards (4-chlorophenol, 2,4-dichlorophenol), and acidified with HCl to pH 2.

Solid phase extraction (SPE) procedure:

SDB-1 cartridges (Baker) were used, after being conditioned by rinsing them twice with 3 mL methanol and 6 mL distilled water. The aqueous sample was loaded on the top of the cartridge, washed with 5x 6 mL demineralized water until pH 5-6, and dried under nitrogen and vacuum.

Eluted with methanol

Collected directly in the GC-vial

1) total collected elution volume = 200 µL
    - fraction 1 = 50 µL (1-1)
    - fraction 2 = 50 µL (1-2)
    - fraction 3 = 50 µL (1-3)
    - fraction 4 = 50 µL (1-4)
2) total collected elution volume = 200 µL
    - fraction 1 = 100 µL (2-1)
    - fraction 2 = 100 µL (2-2)
3) total collected elution volume = 200 µL

The fractions obtained were measured in the GC-MS, split 3:1 and compared recovery of SPE experiments by comparing results with external standards prepared in DCM or methanol with expected concentrations.

Calculations to approximate concentrations of standards to be used

	Extraction	
	Before	**After**
Volume	3000µL	200µL
Mass	600 nmol	600 nmol
Concentration	600nmol/3mL 200nmol/mL 200µM	600nmol/200µL 3nmol/µL 3 mM

Table C.3. Concentrations of 2,6-DBP collected (replicate order of collection) fractions of 50, 100 and 200 µL from standards concentrated with SPE

Volume (µL)	**Initial concentration (µM)**	**R.T.**	**Area SPE**	**Extract concentration (µM)**
50	200	10.32	1086364	13.24
50	200	10.32	1526809	18.71
50	200	10.32	4852285	60.03
50	200	10.32	11907933	147.7
50	200	10.32	3542302	43.76
50	200	10.32	1201873	14.68
50	200	10.32	10290423	127.6
50	200	10.32	6307721	78.12
100	200	10.32	146042	1.56
100	200	10.32	6896095	85.43
100	200	10.32	751496	9.08
100	200	10.32	8272732	102.53
200	200	10.32	1058466	12.9
200	200	10.32	827204	10.02
Standard*	750	10.32	55692840	691.73
Standard*	1500	10.32	128000000	1590.14
Standard*	3000	10.32	239000000	2969

* Standards Not extracted in SPE

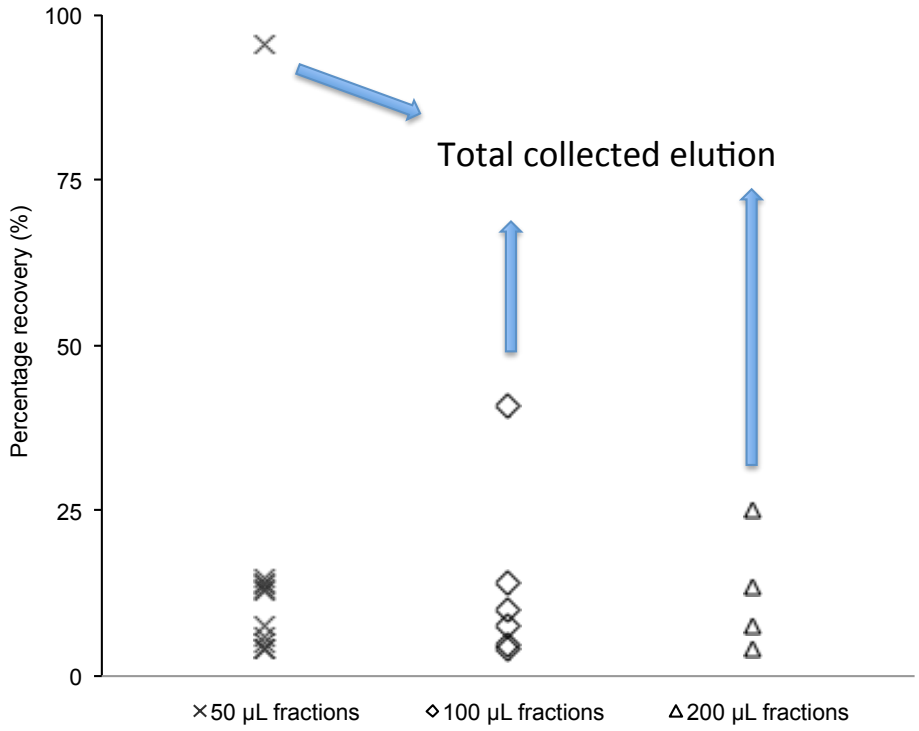

Figure C.2. Percentage of recovery ofhalophenols after using solid phase extraction (SPE) obtained form fractions of 50, 100, 200 µL

Conclusion: Obtained data evidences that solid phase extraction is not a viable method for extraction of 2,6-DBP, due to the variation within the 50 µL fractions extracted and minimum recovery obtained from the 200 µL fraction as it is evident in table 4 and figure 2. The highest recovery of halophenols (more than 97%) was achieved by, eluting 50 µL with SPE 10 times. This methodology was deemed too cumbersome and time consuming to be used as the extraction method.

Sample Concentration with liquid-liquid extractions

Extractions were first performed in methanol but all the samples evaporated with the bromophenols. Used Dichloromethane (DCM) to extract halophenols for the next set. Isotope fractionation composition results after concentrating samples with the Kamm

Apparatus (US patent 2839046) and the evaporating sample concentrator (SBH130D Digital Block Heater) for once and 3 times.

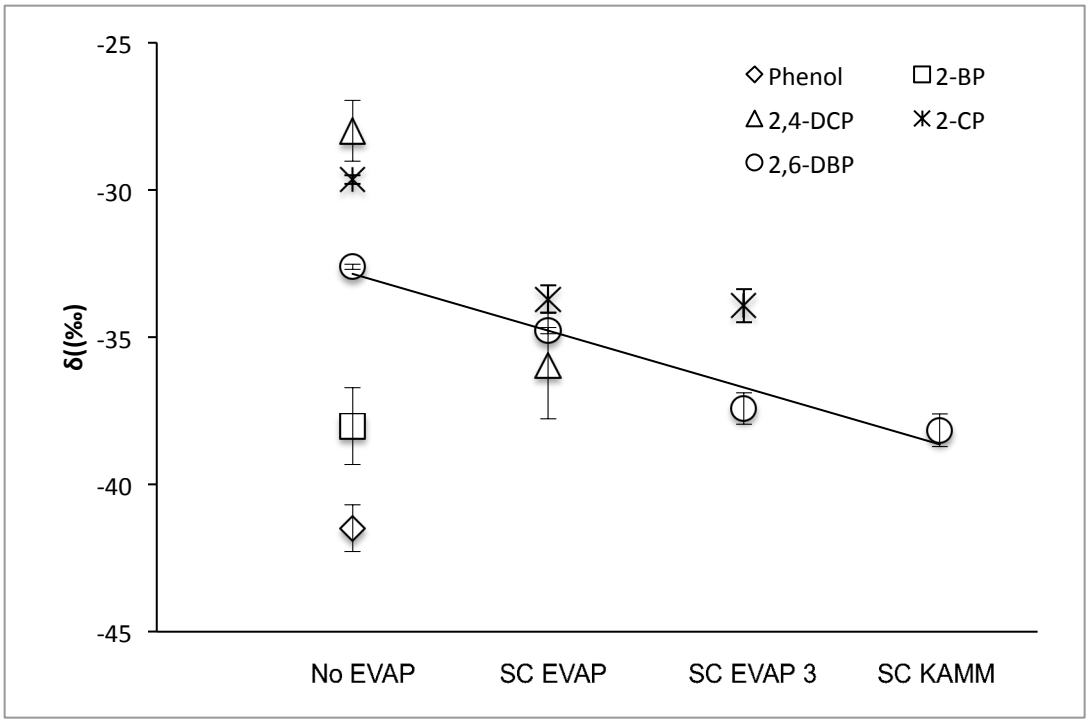

Figure C.3. Fractionation of δ $^{13}C/^{12}C$ values of bromophenols aqeous stnadards extracted in DCM with different methods of concentration

Conclusion: Both treatments to concentrate sample (CS) with evaporator and the Kamm apparatus changed the isotopic composition of 2,6-DBP. Recommend using another method. Curiously 2CP retained its isotopic composition with the sample concentrator, but was lost when the Kamm apparatus was used. Also, with evaporation, the isotope composition change becomes lighter probably because the loss of the compound regardless of the higher mass of the ^{13}C in the molecule.