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BOEM 2015-051

Coastal Marine Institute

Biomass and Mass-balance Isotope Content of Seep Populations on the Upper Slope Gulf of Mexico, Determined from Archived Samples

U.S. Department of the Interior
Bureau of Ocean Energy Management
Gulf of Mexico OCS Region

Cooperative Agreement
Coastal Marine Institute
Louisiana State University

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ABOUT THE COVER

The photograph taken from the submersible *Johnson Sea Link* shows a typical Bush Hill mussel bed. *Bathymodiolus childressi* cover a large portion of the exposed carbonate substrate and *Bathynerita naticoidea* move about grazing on detritus and organic films.

ACKNOWLEDGEMENTS

This project would not have been possible without funding by the Bureau of Ocean Energy Management (BOEM) during both establishment of the specimen archive and the analyses presented herein. This report is derived in very large part from the 2012 Master's thesis of Philip Riekenberg in the department of Oceanography and Coastal Sciences of Louisiana State University. Analyses were carried out in the laboratory of Dr. Brian Fry. The specimens in the archive were collected with the help of the Johnson SeaLink operating team and the captain and the crew of the Research Vessels *Seward Johnson* and *Edwin Link*. Conversations and collaboration with Chuck Fisher, Ian Macdonald, Craig Young, and Shawn Arellano increased our understanding of seep mussel-bed ecology. The study has benefitted from the project management provided by BOEM, especially Greg Boland, Dr. Maureen Mulino, and Dr. Agatha-Marie Kaller.

ABSTRACT

Methods are needed to assess the health of chemosynthetic communities so that anthropogenic impacts can be separated from natural changes associated with succession. The work reported successfully demonstrated the utility of analyses of stable isotopes $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ for the purpose of such an assessment. The specimens analyzed were contained in a LSU archive of material collected during BOEM-supported surveys in 1989–92 and NOAA-supported collecting in 2006. Two sites of know differences were examined. Bush Hill is at the junction of Green Canyon block 184/5 and Brine Pool NR-1 in Green Canyon 233. The former is a topographic high developed along a major fault. The latter is interpreted as a mud volcano located in a region of slope failure. The species analyzed were the foundation species of mussel *Bathymodiolus childressi* (tissue analyses = 253; shell analyses = 154) and an associated snail *Bathynerita naticoidea* (tissue analyses =247). The former depends on methanotrophic microbial symbionts in its gills for its primary nutrition although filter feeding is possible. The latter is fully heterotrophic and grazes on the surface of the mussel shell consuming any available detritus.

Bush Hill mussels and snails differ greatly from the Brine Pool NR-1 in a manner consistent with thermogenic methane at Bush Hill and biogenic methane at the Brine Pool. As a carbon source, methane alone cannot explain an equally dramatic difference in $\delta^{15}N$ and $\delta^{34}S$ values. The link among $\delta^{15}N$, $\delta^{13}C$, and $\delta^{34}S$ remains poorly understood but can be explained on the basis of four trophic resources being consumed. *Bathymodiolus childressi* is a mixotrophic rather than strictly chemoautotrophic foundation species. The four trophic resources modeled indicated that the mussel populations at both seeps consume methane through two symbiotic pathways and detritus through two additional pathways. The dominant detrital source appeared to be sulfide oxidizing microbes. The isotope compositions of all the sources except phytoplankton detritus are seep-specific. Sufficient methane-derived respiratory carbon was found in the shells for dead shell assemblages to be used for assessment of methane metabolism. Future research based on shells from dead seep communities may be able to trace the time course of the loss of the methane resource.

Recommendations for future research center around the management benefit of being able to establish the condition of the major foundation species. For mussel population, the existence of and the manner of feeding on the thiotrophic detritus can be undertaken in the field and in the laboratory. Application of stable isotope analyses to dead shell material should be undertaken to gain an understanding of the causes of extinction for the many dead-shell assemblages encountered during the exploration for seep systems.

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1. INTRODUCTION

1.1. STATEMENT OF PURPOSE

The upper slope off Louisiana exhibits a very complex topography caused by tectonic movement of salt layers on the long term and slope failure events on the shorter term (Figure 1). This combination of factors produces a region with vast areas of typical benthic soft bottom and embedded chemically-extreme communities shaped by various combinations of hydrocarbon and brine seepage (Roberts 2011). The purpose of this study is to better understand the natural variation of the trophic state within seep mussel populations, based on the stable isotope analysis of a relatively large number of specimens from two geochemically contrasting sites, Bush Hill and the Brine Pool NR1.

The work reported in this study is a modest effort with no at-sea component to meet the information need of how the ecological status of chemosynthetic seep communities might be assessed. Rather than undertake new expensive sampling, we have used archival material at Louisiana State University's Department of Oceanography and Coastal Sciences (DOCS). The tool of choice is stable isotope analysis looking at the important isotopes ^{13}C, ^{15}N and ^{34}S. These isotopes and the total mass of the elements C, N, and S may provide a measure of condition. The species examined are the foundation mussel species and an abundant gastropod. Selected mussel shell and soft tissues are also examined to see if information can be gained from the shell remains at inactive seeps. Samples dating to 1989, 1991, and 1992 have been augmented with material from 2006. Because all analyzed material was collected before the *Deepwater Horizon* accident, the data reported here is a baseline for future assessment and monitoring.

The questions directly asked within this study are:

- Does the mass percent of carbon, nitrogen, and sulfur in tissue of the seep mussel *Bathymodiolus childressi* provide a useful tool for assessing the overall condition of populations?
- Does the stable isotope composition of carbon, nitrogen and sulfur in mussel tissue provide a useful indication of trophic condition of a population capable of both chemosynthesis and heterotrophic feeding?
- Is the determined trophic condition of a foundation species also reflected in a consumer organism, such as the snail *Bathynerita naticoidea*, in the same system?
- Does analysis of mussel shells have the potential of providing information on the trophic condition that might be used to assess past seepage condition?

2. BACKGROUND

2.1. UPPER LOUISIANA SLOPE SEEPS

2.1.1 Chemosynthetic Systems and the Atypical Deep Sea

Between 1984 and 1985, species related to hydrothermal-vent fauna were reported from the eastern Pacific and the Gulf of Mexico cold continental slopes and recognized as being supported by the seepage of methane and sulfides (Paul et al. 1984, Kennicutt et al.1985, Kulm et al. 1986). Increased and more effective exploration of slopes has verified that similar seep systems are a relatively common habitat that exists as a geochemical continuum (Levin 2012). Chemosynthetic seep communities were first reported in the northern Gulf of Mexico in 1985 (Kennicutt et al.1985). Boland (2010) provides a good historical review of participants and publications. Following subsequent BOEM studies (MacDonald et al. 1995), these systems were afforded protection as a potentially sensitive habitat. Notice to Lessees (NTL) 2009-G40 and previous NTL's recognize that chemosynthetic communities are widespread and vary in condition and ecological importance. Criteria for recognizing the level of importance of an individual seep location, however, remain incompletely determined but somehow related to density and biodiversity of the systems.

The seeps in the Gulf are the most studied in the world, due to the extensive oil and gas development in the region. The initial discoveries off Louisiana occurred during industry and Bureau of Ocean Management (BOEM)-supported investigations. A full history has been given by Boland (2010). Since the discoveries, various aspects of the biology, chemistry, and geology of Gulf seeps have been investigated, supported primarily by BOEM but also the National Oceanic and Atmospheric Agency (NOAA) and the National Science Foundation (NSF). Cordes et al. (2009) provide an informative review on the ongoing research.

Because of the depth limitation of the *Johnson Sea Link* (*JSL*) submersible, which was operated at that time by the Harbor Branch Oceanographic Institute (HBOI), the most intense investigation of the northern Gulf Upper Continental Slope was above 1000 m. *JSL* was a cost effective and highly reliable vehicle with a depth limit of 1000m. *JSL* provided an unequaled panorama of the bottom and a very large capacity for samples. Unfortunately, HBOI terminated the submersible program, and *JSL* is no longer available.

The specimens analyzed in this study were collected at either Bush Hill (Green Canyon 185) or Brine Pool NR-1 (Green Canyon 233) (see Figure 1). The sites lie between longitudes 91º 50.0 W and 91º 10.0W and latitudes 28º 0.0N and 27º 30.0N. A seismic survey of this area was carried out with a 20x20m per pixel resolution 6–8 May 2012 by the R/V *Okeanos Explorer*. Bathymetry, bottom backscatter, and water column backscatter indicative of methane bubbles were recorded. The region has been sculpted by salt diapers, withdrawal basins, and slope failure scars (Figure 1). All of these features are typical of the northern Gulf slope. The region contains numerous bubble plumes and high amplitude seafloor (Shedd et al. 2011).

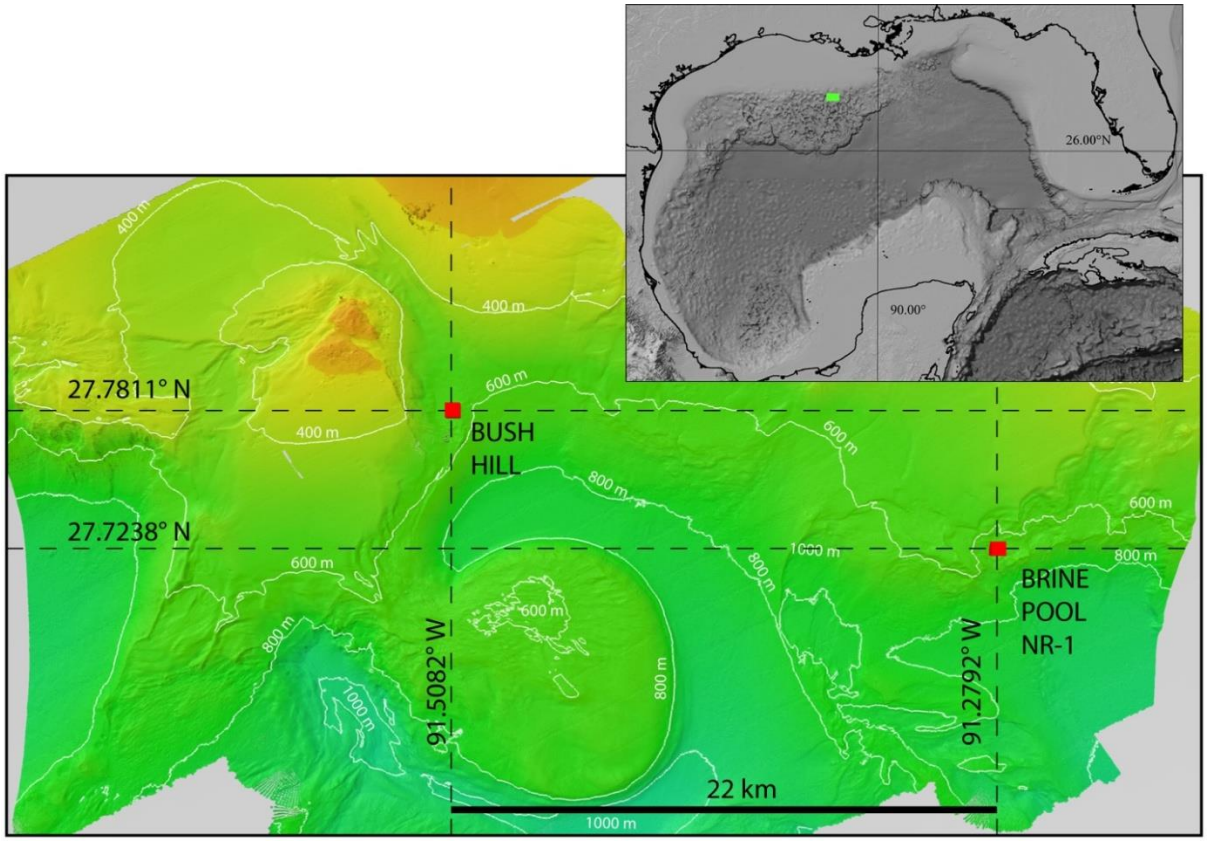

Figure 1. Position of Bush Hill and Brine Pool NR-1 relative to the complex slope geomorphology in the Gulf of Mexico.

2.1.2. Bush Hill Green Canyon 184/85

Bush Hill (Long -91.5082º Lat 27.7811º) is a topographic high rising to a depth of ~445 m, located on the western flank of a shelf canyon that appears to be a large slope failure feature (Figure 1). Figure 1 is a 20m resolution digital terrain model courtesy of the Coastal and Ocean Mapping Center at the University of New Hampshire and the NOAA Ocean Exploration Program. It lies over a secondary fracture to a large growth fault associated with salt tectonics (MacDonald et al. 2003). Laser line scanning of ~28,000 m^2 of the top of the feature found *Bathymodiolus childressi* mussel clumps to cover 72 m^2, exposed carbonates 610 m^2 and tubeworm bushes 332m^2 (MacDonald et al. 2003). As is typical of many seep features, the living chemosynthetic fauna covers a relatively small area. In this case ~2% of the mound. Bush Hill was the first seep site directly observed by submersible on the Louisiana upper slope in 1986. It lies at the juncture of Green Canyon Blocks 184 and 185 and is in the Jolliet development field.

2.1.3. Brine Pool NR-1 Green Canyon 233

Brine Pool NR-1 (Long -91.2792º Lat.27.7238º) was found during seafloor surveying with the nuclear research submarine NR-1 of the U.S. Navy (MacDonald et al. 1990). It has been interpreted as a brine-filled low relief expulsion feature in a larger mud volcano (MacDonald and Peccini 2009). Newer bathymetry shows the pool is on the eastern flank of a 9 m high hill in a region marked by many slope-failure scars (Figure 1). The pool is ringed by a mat of the mussel *Bathymodiolus childressi*. Neither tubeworms nor exposed carbonates occur in the immediate area of the pool but they have been encountered in the surrounding terrain.

2.2. THE SEEP MUSSEL *BATHYMODIOLUS CHILDRESSI*

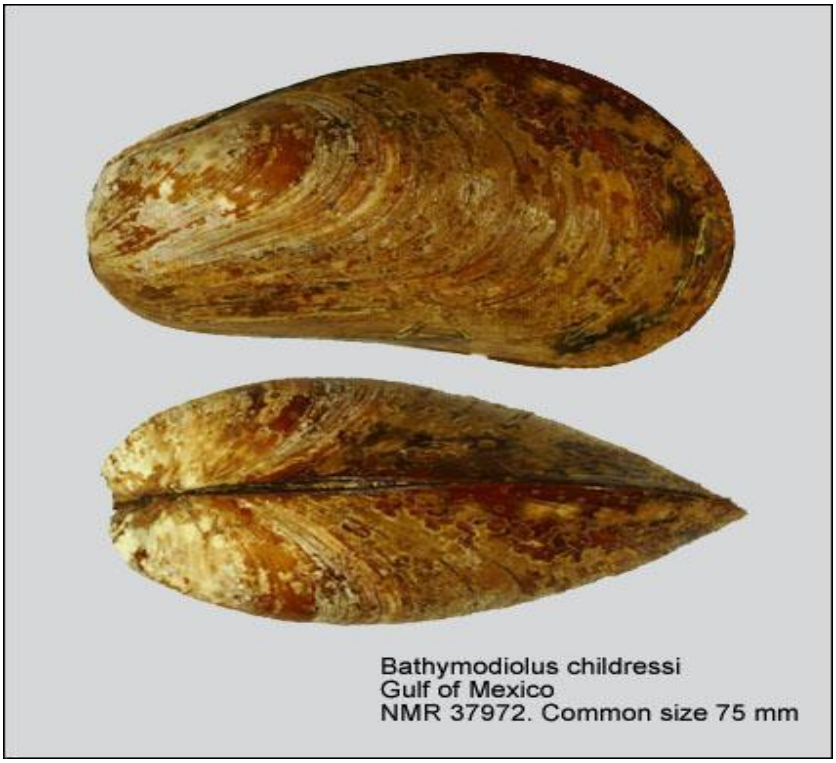

Figure 2. *Bathymodiolus childressi* is a dominant and foundation species at upper slope seep communities in the Gulf of Mexico.

Bathymodiolus childressi Gustafson et al. (1998) (Figure 2; Image courtesy of the Chemosynthetic Ecosystems Project, Natural History Museum of Rotterdam. J. Trausel and F. Slieker photographers) is the dominant mussel at upper-slope seep sites in the Gulf where it is sufficiently abundant to be a foundation species providing physical substrate as well as a food source for other biota. Based on molecular evidence there is a single interbreeding population in the Gulf (Carney et al. 2006). As of 2010, 51 species in the genus *Bathymodiolus* have been described strictly from hydrothermal and cold seep environments (Saeter et al. 2010). Molecular phylogenetics suggests that the genus is probably polyphyletic and is in a genus/species cluster with *Adipicola* (nine species) and *Idas* (30 species) which are associated with organic falls such as wood and whale carcasses (Duperron 2010; Lorion 2010). *B. childressi* is a distinct species in this larger complex

which includes Atlantic and Pacific members. The distributions of Atlantic members delineate the Atlantic Equatorial Belt (AEB) Cold Seep Faunas (Olu et al. 2009). *B. maritanius* is a closely related species found at West African seeps on the Nigerian and Moroccan continental slopes (Genio et al. 2008). Initially considered a Gulf endemic distributed between 450 and 2200m depth, the species has been reported from the submarine canyons of the U.S. northeast slope (Demopoulos et al., 2014; Wagner et al., 2014) and additional range extensions are likely.

Though most species of *Bathymodiolus* harbor thiotrophic microbes in enlarged gill tissue, *B. childressi* is nearly unique in harboring only methanotrophic symbionts as far as the current level of sampling has demonstrated (Duperron et al. 2007; Duperron 2010, Kellermann et al. 2012). The metabolism of methane-oxidizing symbiotic microbes places a high oxygen demand on the host mussel (Childress and Girguis 2011), which is met by maintaining a flow of oxygen-rich ambient seawater across the gills. In effect, all species of *Bathymodiolus* are capable of filter feeding even though the in situ ecology of the genus appears to be primarily chemoautotrophic (Page et al. 1990, Young and Pile 1999).

2.2.1 Mussel Shell Deposition

An answer to the question of whether mussel shells record the history of methane seepage experienced by *Bathymodiolus childressi* depends upon the details of shell deposition. When $CaCO_3$ shells are deposited by aquatic invertebrates, the main source of CO_2 in carbonate is dissolved inorganic carbon (DIC) in the water. This is in stark contrast to air breathing animals in which the main source of CO_2 for deposited hard parts (bone, shell) is respiratory carbon, which causes these hard parts to be isotopically representative of the $\delta^{13}C$ of their diet. This difference in respiratory carbon input between air breathing and aquatic animals is a result of atmospheric CO_2/O_2, ratios being about 30 times lower than those in aquatic settings, resulting in air breathing animals having significantly less availability of CO_2 from the air as they take up O_2 (McConnaughey et al. 1997) .

Aquatic animals do, however, incorporate some respiratory carbon into shell material; hard parts range from 7–15% in mollusks and as much as ~30% in fish otoliths. Although the molluscan amount is small, detection of that small amount in chemosynthetic mussels is facilitated by the fact that the methane-derived respiratory carbon is so very distinctive (-40–80‰ $\delta^{13}C$). Successful detection, however, requires adjustment for kinetic isotope fractionations that exist as the different crystalline forms of $CaCO_3$ (calcite or aragonite) develop in the shell. Romanek and colleagues (1992) used two different fractionations for aragonite and calcite of 2.7‰ and 1‰, respectively, for the enrichment that occurs as $CO_3^{=}$ becomes incorporated into the shell from the extra pallial fluid (EPF) (Figure 3; from McConnaughey and Gillikin 2008).

In this study, we have used an estimate of 90% calcite and 10% aragonite composition of the shells that agrees with the findings of Demina and colleagues (2012) about the range of calcite compared with aragonite composition of shells found in vent related *Bathymodiolus* species and results in a fractionation factor of 1.17‰. The EPF undergoes an alkalization through the use of an ion transporter called Ca^{2+} Atlases, which pumps protons away from the site of calcification. This process creates an increase in pH of 0.5 units, resulting in a shift in the predominant species of CO_2 from HCO_3^- to $CO_3^{=}$ as a result of deprotonation. This process results in a 4–6 fold increase in the concentration of $CO_3^{=}$ in the extra pallial fluid (EPF) compared with the surrounding tissues (McConnaughey and Gillikin 2008). This results in a depletion of HCO_3 in the surrounding tissues

and sets up a diffusion gradient by which CO_2 is constantly entering the tissues and the EPF to maintain the super saturation of $CO_3^{=}$ set up by the slight alkalizination of the EPF.

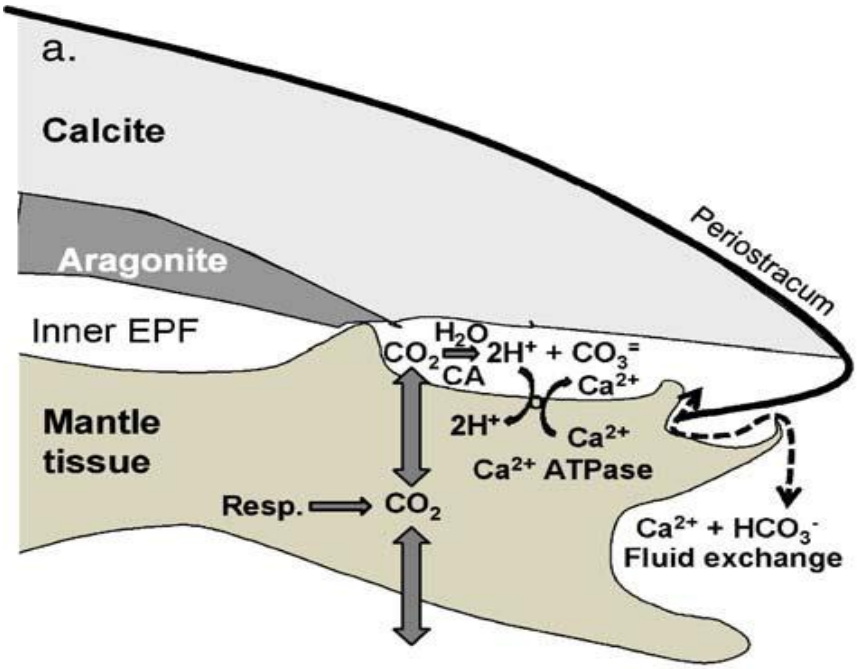

Figure 3. Deposition of mussel shell.

2.3. THE SEEP SNAIL *BATHYNERITA NATICOIDEA*

Bathynerita naticoidea is an abundant heterotrophic snail at upper slope seeps that has a tight but poorly understood connection with the mussels (Figure 4; image courtesy of Life Desk E. Thomas photographer). It is closely associated with *Bathymodiolus childressi* and feeds with its scraping radula on the shell of the mussel consuming any mucus films, microbes and detrital material. It is a heterotroph but not a mussel predator. Rather, it can be seen as an integrator of all particulate food sources present within the mussels beds. Thus its isotopic values may reflect mussel production through a mucus food source, free-living microbes and photosynthetically-derived detritus.

Figure 4. *Bathynerita naticoidea* is a small gastropod typically 1.2 cm in greatest dimension.

Gastropods are common members of hydrothermal vent and seep communities ; 218 species are recognized in the most recent compilation (Sasaki et al. 2010). Upper-slope seeps in the Gulf are occupied by *Bathynerita naticoidea*. Initially described from Bush Hill specimens by AH. Clarke (1989). The snail appears to be restricted to the upper-slope seeps and is always in association with *Bathymodiolus childressi*. *Naticoidea* is the only species in the genus. The animal is abundant, survives collection, and stays alive in cold aquaria at ambient pressure. As a result, it has received modest research attention and has been the subject of two theses (Zande 1994; Van Gaest 2006).

Nutrition is strictly heterotrophic and no symbiotic microbes have been found. The snail browses on the surface of *B. childressi* with its scarping radual. It may consume exuvia, byssal fibers and periostracum from the mussel and any detrital material on the shell. That detritus can be both bacterial and algal (Zande and Carney 2001). Specimens from Bush Hill had a significantly lower average size that those at the Brine Pool. Most animals are ~1.2 cm in largest dimension. In trials carried out by Zande (1994), eggs contained within cases underwent full development to the swimming veliger stage without the case opening. Van Gaest (2006) repeated the experiment but found that the case did open and development took place in the plankton; this gives the snail the potential for long-distance dispersion (Young et al. 2012). The relationship between *B. childressi* and *B. naticoidea* remains poorly understood. In simple behavioral trials, the snails showed significant preference for experimental habitats containing water from *B. childressi* aquaria (Dattagupta et al. 2007). This was not a response to a simple stimulant, such as methane.

2.4. Stable Isotopes of Biomass as an Analytical Tool

2.4.1. Basic Carbon, Nitrogen and Sulfur Isotope Analysis

Stable isotopes are those element forms that exist in the natural environment and are not radioactive in nature. They are commonly referred to as HCNOS isotopes in biological studies indicating they are the isotopic forms of hydrogen, carbon, nitrogen, oxygen, and sulfur and are commonly used to identify biological processes in natural systems (Fry 2006). Stable isotopes of carbon and nitrogen have been used in many ways: to delineate food webs and decipher who is eating whom (Peterson and Fry 1987) and when coupled with sulfur the stable isotopes of carbon and nitrogen have identified the relative transience of fish in estuaries (Fry and Chumchal 2011). There are many current applications for these tools, because they allow for the relatively cheap tracing of elements through both biological and physical processes without the need for expensive or radioactive tracers. However, this study addresses only the use of naturally occurring carbon, nitrogen, and sulfur.

Stable isotope measurements are reported in δ (del) notation. That is, they are reported in standard notation as: $\delta = (({}^{H}F/{}^{L}F)\text{sample}) / (({}^{H}F/{}^{L}F)\text{standard})-1)) * 1000$ the fractional abundance (${}^{H}F/{}^{L}F$) of heavy and light isotope of both the sample and the standard. The del notation is then in the permil (‰) basis, that is, parts per thousand, which allows for the amplification of very small differences into a more familiar and manageable scale. The standards that samples are measured against are: for carbon, PeeDee Belemnite (PDB) and for nitrogen, air, and sulfur, Canyon Diablo Troilite (CDT)(Fry 2006). These standards have been meticulously established to provide lab to lab continuity and quality assurance for stable isotope analysis and are currently maintained through the International Atomic Energy Agency.

2.4.2. Trophic Mixing Estimation from Three Isotopes

The cause of scatter in stable isotope data is most often considered to be the mixing of multiple food sources and metabolic isotope fractionations. For this reason, mixing models are often developed that allow estimation of the trophic resources being used. When the isotopic content of a specimen is considered to be the sum of fractional contributions from multiple sources, calculation of those contributions provides an ecologically useful means of data analysis and interpretation (Phillips 2012). The computational requirements are that the isotopic values are modeled as a series of linear equation, as below.

$$A \times \delta^{13}C_{source1} + B \times \delta^{13}C_{source2} + C \times \delta^{13}C_{source3} + D \times \delta^{13}C_{source4} = \delta^{13}C_{measured}$$

$$A \times \delta^{15}N_{source1} + B \times \delta^{15}N_{source2} + C \times \delta^{15}N_{source3} + D \times \delta^{15}N_{source4} = \delta^{15}N_{measured}$$

$$A \times \delta^{34}S_{source1} + B \times \delta^{34}S_{source2} + C \times \delta^{34}S_{source3} + D \times \delta^{34}S_{source4} = \delta^{34}S_{measured}$$

And, the proportional contribution of resources sum to 1.0.

$$A + B + C + D = 1.0$$

Unique solutions can be determined for the contributions, so long as the number of sources is not greater than one more than the number of variables (isotopes). Obtaining those solutions using linear algebra requires first that a square matrix of isotope (CNS) composition of the sources be established, inverted, and multiplied by a rectangular matrix of sample isotope values. The calculations were carried out in Microsoft© Excel© , using the built in array functions MINVERSE and MMULT.

There are various approaches for establishment of the basal sources. In this study, the approach of Wissel and Fry (2005) was used; it considered prior knowledge of highly probable sources in the two seep systems and sources consistent with the pattern of isotope values and the ecology of *B. childressi*. Spatially, the four basal sources define the vertices of a tetrahedron in a Cartesian space with the axes $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. All samples that consist of a mixture of these four sources must lie within that tetrahedron. This improves on the estimation of sources and requires adjusting initial vertices until all sample points are corralled. This trial and error process of source adjustment began with 2-dimension examination of each isotope pair and then refining the tetrahedron in a 3-dimension view. The steps were carried out using OriginPro® 8.6 using X, Y, Z scatter plots and trajectory graphing functions.

2.5. Condition Index as an Analytical Tool

A condition index (CI) is intended to be a measure of the health of organisms. In an environmental management context, it can be used as a tool in the assessment of the sensitivity of a population to impact and as an indication of an impact. Numerous CI have been developed and can be generally categorized as either morphological or physiological (Hayes and Shonkwiler 2001). The former have the potential of being non-invasive and non-lethal. The latter require tissue sampling but may be better linked to the processes actually determining health. The CIs are often a simple ratio or a more complex statistical value. The use of shell-to-soft tissue comparisons is a common CI for bivalves that is suggested by the variable water content of the flesh of bivalves such as oysters. A flesh:shell mass ratio attributed to Freeman (1974) is a common CI in bivalve aquaculture (Alunno-Bruscia et al. 2001).

The assessment of the individual condition of chemosynthetic mussels and determination of the spatial variability of that condition were pioneered by Smith (1985) at the initial hydrothermal discovery site in the Galapagos Rift. It was established that the ratio of soft tissue dry mass to internal shell volume was greater within dense central patches than small peripheral patches of the vent mussel *Bathymodiolus thermophilus*. Smith's method of measuring condition and spatial

contrasts has been widely applied with some modification to other chemosynthetic systems including those in the Gulf .

Condition of the seep mussel *Bathymodiolus childressi* has been assessed at Bush Hill (Nix et al. 1995) and the Brine Pool (Smith et al. 2000) following the overall approach of Smith (1985) but using ash-free dry weight rather than simple dry weight as the tissue measure. Those results are presented in Table 1. The condition was maximum at the inner edge of the Brine Pool mussels (Smith et al. 2000) and decreased slightly at the outer edge. The lower condition at the outer edge was still markedly higher than found at Bush Hill (Nix et al. 1995). Two additional sites had conditions between those of Bush Hill and the Brine Pool and were attributed to site-specific differences in the supply of methane. These determinations provide a basis of comparison with the isotope and total carbon, nitrogen and sulfur measurements obtained in the current study.

Table 1. Mean and standard deviation of condition index and glycogen content found for Bush Hill

Site	Date of sample	Condition Index Ash-free dry mass/shell volume	Glycogen % Of Wet Mass
Bush Hill	1991	0.04 ± 0.01	0.88 ± 0.63
	1992	0.05 ± 0.02	1.13 ± 0.88
	1993	0.05± 0.01	0.73 ± 0.44
Brine Pool Inner	1993	0.12 ± 0.02	3.1 ± 0.9
	1994	0.13 ± 0.02	2.8 ± 1.0
	1995	0.10 ± 0.3	1.7 ± 0.6
Brine Pool Middle 1	1993	0.10 ± 0.01	3.2 ± 0.4
	1994	0.10 ± 0.03	1.9 ± 0.04
	1995	0.09 ± 0.02	1.6 ± 0/8
Brine Pool Middle 2	1994	0.12 ± 0.02	2.8 ± 0/9
Brine Pool Outer	1993	0.11 ± 0.02	3.0 ± 0.7
	1994	0.10 ± 0.02	2.2 ± 0.8
	1995	0.06 ± 0.02	0.9 ± 1.9

3. DESIGN AND METHODS

3.1. SAMPLING

The specimens analyzed in this study were collected and archived primarily between 1989 and 1992 as parts of "Chemosynthetic Ecosystems Study" or Chemo-I study (MacDonald et al. 1995) and "Stability and Change in Gulf of Mexico Chemosynthetic Communities" (MacDonald 2002). Faunal composition was being examined, and the proposed sampling design for those studies was to have had replicated collections partitioned by substrate (mud or rock) and position (edge or inter portion of a mussel bed) and nested within four seeps. In the field, the mussels did not cooperate. Rock predominated as a substrate and some sites had too few mussel beds to allow for the desired replication. A simpler comparison among sites and among locations, however, proved very informative. Compositional monotony was encountered at the four sites (Carney 1994). The spatial and temporal design that could be examined from the archived samples is presented in Table 2.

Table 2. Position and dates sampling design based on archived collection of mussels

SEEP	Feature	Position	Year	Specimens	*JSL* Dive
Bush Hill	Bed 1	Edge	1991	17	3139
			1992	14	3269
				10	3269
		Interior	1991	21	3139
			1992	8	3269
				3	3269
	Bed 2	Edge	1992	6	3274
		Interior	1992	6	3274
				6	3274
				3	3274
	Bed 1A	Edge	1991	15	3139
		Interior	1991	13	3139
				3	3139
			Bush Hill Subtotal =	125	
Brine Pool	North	Interior	1989	20	2598
			1991	14	3145
		Edge	1989	13	2598
			1991	5	3145
		Scattered	2006	11	3521
	South	Interior	1989	13	2598
			1991	8	3145
		Edge	1989	9	2598
		Scattered	2006	35	3522
			Brine Pool Subtotal =	128	
			Total Specimens =	253	

At Bush Hill, three mussel beds were sampled and designated Bed 1, 2, and 1A (close proximity to 1 and possibly an extension of it). At each bed, fauna was collected at an outer edge and an inner portion. Only Bed 1 was sampled over two successive years. The Brine Pool has essentially a single large mussel bed or ring. It was sampled at its northern and southern ends. At each end, collections were made at an Inner area extending over the brine and an Outer edge over mud. The Brine Pool sampling was repeated over a two-year interval. The Brine Pool was resampled during a NOAA-supported program in 2006 and provided a single longer-term set of samples. That 2006 sampling did take place at the northern and southern ends of the Brine Pool but was scattered rather than targeted the outer edge and inner portion. Snails were analyzed from Bush Hill and Brine Pool along with two sites, GC-272 and GC-234, which had also been studied in earlier investigations.

In an ideal sampling design seeking spatial and temporal effects, there should be a large number of replicates for each factor. Separate seeps, mussel beds within seeps, position within those beds, and mussel specimens should be heavily replicated. Time series sampling should sample the same number of replicates over several years. Unfortunately, deep seeps do not lend themselves to ideal sampling. The composition, size, number, and persistence of beds are highly different at each site. Much opportunistic sampling must take place. Replication must be low, and it may not be practical to resample the same bed over time. Indeed, because sampling removes animals, the systems are modified through time. Spatial and temporal trends from such sampling should be interpreted with caution.

All megafauna sampling used the grab of the *Johnson Sea Link* (*JSL*) submersible. The *JSL* grab is an integral part of the hydraulic manipulator on *JSL*. It consisted of two semi-cylindrical jaws 19.2 cm long and 17.8 cm wide when open, which rotate closed about a common axis. The bite of the *JSL* grab samples 341 cm^2 square meters of bottom. Each sample consisted of two scoops of the grab. Samples were sorted at sea and stored in 5% formaldehyde. Upon completion of the Chemo-I and Chemo-II studies, specimens in 5% formaldehyde were archived at Louisiana State University.

3.2. Stable Isotope Analysis

In 2010, the archived samples from Bush Hill and Brine Pool NR-1 were opened, washed in a deionized water (DI) bath for a period that did not exceed 24 hours, dissected and dried at 60°C for a minimum of 12 hours until completely dry. Mussels were separated into three parts: mantle, abductor muscle, and an outer portion of shell. The adductor muscle portions were processed using a WIG-L-BUG (Dentsply International) into a fine powder and then loaded with vanadium pentoxide catalyst into the sampler for CNS analysis. The shell portion of the sample was dried, periostracum removed, then ground into a fine powder, treated with bleach for 12 hours to remove organic material, rinsed copiously with DI, then dried again and loaded with vanadium pentoxide catalyst in the sampler for analysis.

Mussel and snail size was quantified by caliper measurements of maximum shell length and shell height to the nearest millimeter. Mussel half shell volume was measured using small glass beads to completely fill half of the shell and then weighed. Glass bead weight was then graphed versus shell height and the resulting function y = 0.7953e^(0.1835 * shell height (mm)) with an R^2 value of 0.9842 was used to calculate volume for the half shell of each individual. A subsample of the specimens was used to establish this relationship.

Mussel and snail tissue samples were processed for CNS isotope analysis using the methods described by Fry (2007), reporting $\delta^{13}C$ ‰ compared with PeeDee Belemnite (PDB), $\delta^{15}N$ ‰ compared with atmospheric nitrogen (AIR), and $\delta^{34}S$ ‰ compared with Canyon Diablo Triolite (CDT). The analytical confidence levels are 0.1‰ for both $\delta^{13}C$ and $\delta^{15}N$, and 0.4‰ for $\delta^{34}S$. Laboratory check standards were interspersed within samples in a range of weights within each run in order to ensure consistency within and between the sample runs. Shell samples were run in the same manner as tissue samples, but only carbon isotope values were kept, because the sulfur and nitrogen isotope values were highly variable. These fluctuations in data were an effect of the extremely small sample size that remained after the bleach treatment. A check for the effects of formaldehyde on CNS isotopes were performed using fresh commercial *Mytilus sp.* obtained locally by performing analysis on preserved and unpreserved portions of each individual (n=6). Due to the extremely depleted nature of $\delta^{13}C$ in cold seep animals that are dependent on methane

as a carbon source, this effect is likely an enrichment and likely to have a much smaller effect on the overall (‰) values due to the large range in observed $\delta^{13}C$ values.

3.3. ESTIMATION OF MUSSEL SHELL SEEP CARBON INCORPORATION

The formula used to assess methane-derived respiratory carbon shells in this study is:

$$F(\delta^{13}C_{Tissue}) + (1-F)(\delta^{13}C_{DIC}) = \delta^{13}C_{Shell} - \Delta.$$

- Where F is equal to the rate of incorporation of respiratory carbon,
- $\delta^{13}C_{Tissue}$ is the value of the tissue of each mussel.
- (1-F) is the remainder of the carbon not provided through respiration,
- $\delta^{13}C_{DIC}$ is the value of seawater DIC (0.69‰; Fu 1998).
- $\delta^{13}C_{Shell}$ is the value of the shell of each mussel.
- Δ is a fractionation factor associated with the kinetic isotope effects between the crystalline structures of calcite and aragonite.

3.4. GENERAL DATA ANALYSIS

The eight variables for mussel and snail tissue consisted of shell length, dry tissue sample mass, $\delta^{13}C$, $\delta^{15}N$, $\delta^{34}S$, total C mass, total N mass, and total S mass. For mussel shells, there were two parameters: shell and $\delta^{13}C$. Exploratory data analysis consisted of plots of values and examination of correlation matrices. Data tables are provided in Appendix I and statistical analyses contained in Appendix II. Simple univariate parametric tests were carried out, when appropriate, to test the significance of an apparent relationship. Due to the large sample size some relationships are statistically significant but explain only a minor amount of data variance.

The most algebraically complex task was assessing the percentage of respiratory carbon incorporated into the shell (F). It is calculated using the formula from McConnaughey and colleagues (1997), which calculates F as a function of the respective contributions from respiratory sources (obtained using the $\delta^{13}C$ mussel abductor tissue values) and the dissolved inorganic carbon pool. The formula

$$\delta^{13}C \text{ shell} - \Delta = (\delta^{13}C_{tissue} * F) + (\delta^{13}C_{DIC} * (1 - F))$$

- Where $\delta^{13}C_{tissue}$ is the measured abductor tissue value for the individual mussel and serves as the end member value of the respiratory carbon incorporated into the shell.
- $\delta^{13}C_{shell}$ is the measured value of the shell for the individual mussel.
- $\delta^{13}C_{DIC}$ is 0.69 ‰ of the surrounding dissolved inorganic carbon pool and is the predominant end member for carbon incorporated into the shell.
- Δ is 1.17‰ as a fractionation factor correction for the difference in carbon fractionation between the formation of the two predominate crystalline forms of $CaCO_3$, calcite and aragonite. This correction assumes a 90% composition of calcite in the shell with a 10% composition of aragonite and is in line with previous measurements in mytilid

mussels (Demina et al. 2012) and utilizes fractionation factors associated with the formation of aragonite and calcite (Romanek et al. 1992).

4. RESULTS

The data used in the analyses presented below are contained in nine linked Microsoft© Excel© files in Appendix I. Three tables for the Brine Pool present stable isotope content, trophic source proportional mixing, and biomass elemental mass proportion for C, N, and S (Appendix I; Tables 1, 2, and 3). Similar values for Bush Hill mussel tissue are also provided (Appendix I; Tables 4, 5, and 6). Shell respiratory carbon incorporation is provided in Appendix I Table 7. Stable isotope values for the snail *Bathynerites naticoidea* Brine Pool and Bush Hill are in Appendix I Table 8 and Appendix I Table 9, respectively. All files are in the general form of a data frame for analysis in the R statistical package. In addition to the measured variables factors there are also two position factors. Position factor 1 is beds at Bush Hill and North or South end at the Brine Pool. Position factor 2 is Inner and Edge. At the Brine Pool, Inner is adjacent to the brine.

Each specimen analyzed has an Archive ID based on the dive number of either *JSL* I or II. The following number from 1 to 12 is the sample storage chamber on the submersible. Any other following number refers to an actual storage container in the Louisiana State University specimen archive. At the time of collection the chamber number for each sampling location was recorded in a cruise log.

4.1. *BATHYMODIOLUS CHILDRESSI* TISSUE

Results for shell length, δ^{13}C‰, δ^{15}N‰, δ^{34}S‰, C%, N%, and S% (element mass percent) are presented as density plots (Bowman and Azzalini 2003) in Figure 5 and in Tables 3 and 4 with descriptive statistics. The percent values are based on molar percent of the dried tissue samples.

4.1.1. C, N, and S % Mass Differences

At Bush Hill C, N, and S comprised 47.34± 2.91, 12.88± 0.94, and 1.28±0.14 molar percent of the dried tissue leaving an ash component of 38.5%. During the first two samplings at the Brine Pool, the C, N, and S values were 48.70± 2.48, 12.46± 0.92, and 1.33± 0.10. Samples collected in 2006 had respective values of 47.75± 3.79, 12.94± 1.06, and 1.53± 0.18. The ash component at the Brine Pool in 1989–1991 was 37.5% in 2006. Inspection of density plots (Figure 5) confirm that there are overlapping ranges but differences in the modal value being most obvious in the case of sulfur.

In spite of the relatively similar values, the molar percent of the tissue sample contributed by total nitrogen, carbon, and sulfur (Tables 3 and 4) were significantly different ($\alpha = 0.05$) between the two seeps during the earlier 1989–1992 samplings. When the older and 2006 samplings at the Brine Pool were compared, only the sulfur percentage had changed significantly; it was higher at the more recent time.

4.1.2. Stable Isotope Differences

With respect to between-seep differences, the stable isotope results confirm the previous studies that were based on smaller sample sizes (MacAvoy et al. 2003). Bush Hill and Brine Pool NR-1 represent widely separate situations primarily reflective of the underlying methane source (Figure 5B). Isotopic carbon results were consistent with a biogenic methane source at the Brine Pool and a thermogenic methane source at Bush Hill. The δ^{13}C values at Bush Hill occur over a range of -36.2‰ to -46.2‰ with a mean of -38.65‰ . δ^{15}N has a range of 6.4‰ to -0.42‰ with a

mean of 4.34‰ and $\delta^{34}S$ a range of 9.0‰ to -12.28‰, mean 1.28‰ (Figure 5D and 5F). At Brine Pool NR-1 the ranges are $\delta^{13}C$ -49.9‰ to -75.0‰ mean of -60.1, $\delta^{15}N$ 4.4‰ to -18.0‰ Mean of and $\delta^{34}S$ 13.9‰ to -1.1‰ mean of. There is no overlap in $\delta^{13}C$ values at each seep although the two ranges approach each other at their extremes. The $\delta^{15}N$ values have minimal overlap and are largely distinct at each seep. Five specimens collected at the northern outer edge of the Brine pool were outliers with respect to $\delta^{15}N$; they had values similar to Brine Pool mussels.

Table 3. Brine Pool mussels separated into older and newer sampling

Statistic	Length cm	$\delta^{15}N$	$\delta^{13}C$	$\delta^{34}S$	%N	%C	%S
Brine Pool 1991-1992 n=82							
Mean	8.86	-14.06	-60.10	10.01	12.46	48.70	1.33
StDev	2.49	4.82	4.92	3.47	0.92	2.48	0.10
Skewness	-0.45	3.12	-0.62	-1.48	-0.55	0.34	-0.45
Kurtosis	-0.93	8.99	-0.45	1.49	-0.32	6.71	0.85
Minimum	3.0	-17.82	-75.01	-1.12	10.2	38.3	1.0
Maximum	12.9	4.42	-49.88	13.92	14.1	59.9	1.6
Range	9.9	22.24	25.12	15.04	3.9	21.6	0.6
Brine Pool 2006 n=46							
Mean	8.65	-16.08	-62.88	10.82	12.94	47.75	1.53
StDev	2.82	1.02	4.54	1.19	1.06	3.79	0.18
Skewness	-0.26	0.78	0.10	-1.22	-0.52	-0.91	-1.50
Kurtosis	-0.98	0.62	-1.13	1.30	0.03	1.26	2.95
Minimum	2.9	-17.99	-70.73	7.24	10.0	36.2	0.9
Maximum	13.1	-12.94	-54.30	12.61	14.8	54.6	1.9
Range	10.2	5.05	16.43	5.37	4.8	18.4	1.0

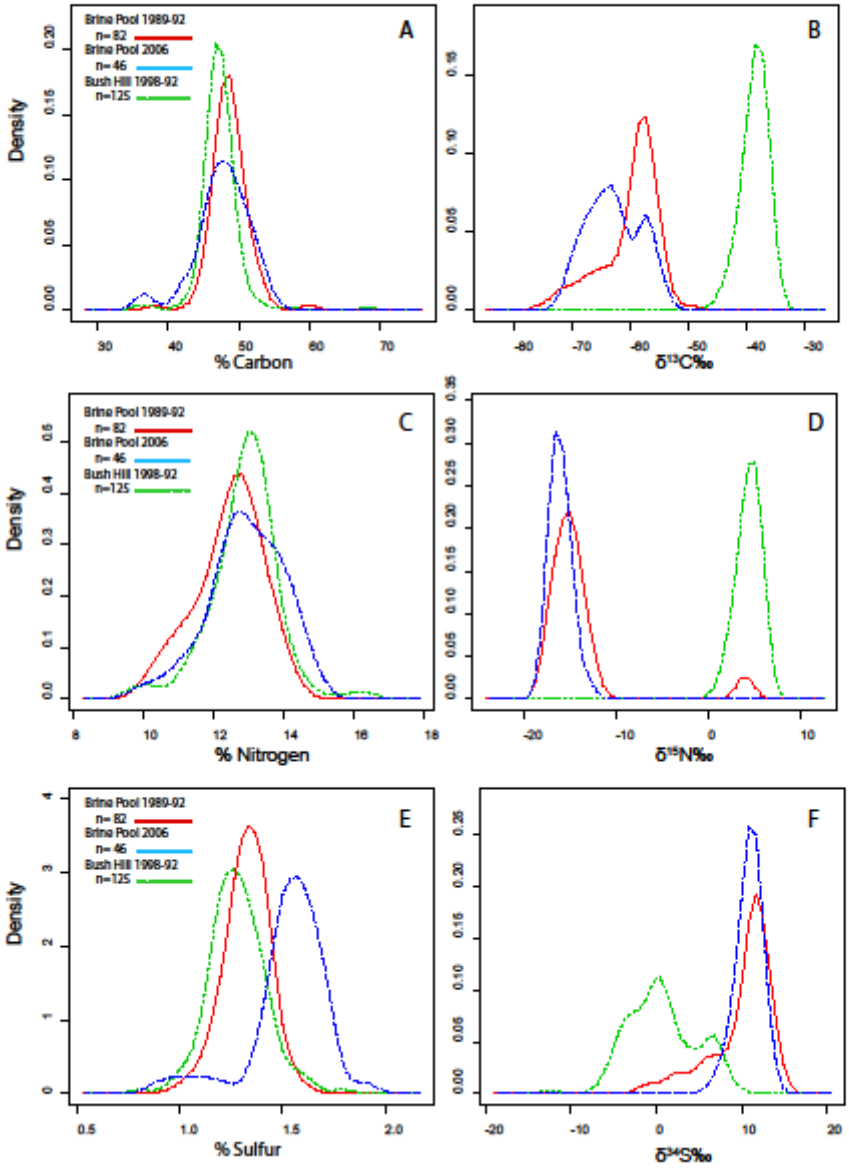

Figure 5. *Bathymodiolus childressi* adductor mussel mass proportions and isotope value density plots for carbon (A & B), nitrogen (C & D), and sulfur (E & F), showing contrasts between Brine Pool and Bush Hill.

Descriptive statistics for the seven parameters measured are presented in Table 3 for the Brine Pool and Table 4 for Bush Hill. The isotope values echo the distinctiveness found between the density plots of the two seeps (Figure 5).

Table 4. Bush Hill mussels parameters

Statistic	Length cm	$\delta^{15}N$	$\delta^{13}C$	$\delta^{34}S$	%N	%C	%S
Bush Hill 1989-1992 **N=125**							
Mean	6.81	4.34	-38.65	0.69	12.88	47.34	1.28
StDev	1.65	1.17	1.91	3.94	0.94	2.91	0.14
Skewness	-0.33	-0.66	-1.16	0.05	-0.31	2.41	0.27
Kurtosis	-0.56	0.10	1.29	-0.21	3.00	22.33	2.07
Minimum	2.7	0.42	-46.24	-12.38	9.9	36.2	0.8
Maximum	9.8	6.42	-36.22	9.00	16.3	68.1	1.8
Range	7.1	6.0	10.02	21.38	6.40	31.9	1.0

Table 5. Major Comparisons t-Tests between Brine Pool and Bush Hill

	Length cm	$\delta^{15}N$	$\delta^{13}C$	$\delta^{34}S$	%N	%C	%S
Test of Equal Means between Brine Pool versus Bush Hill							
t	6.5938	33.9449	37.6748	17.89	3.2344	3.5751	2.7226
P value	<0.00001	<0.00001	<0.00001	<0.00000	0.00146	0.000441	0.007043
Test of Equal Means Older Brine Pool versus 2006 Brine Pool							
t	0.4368	3.651	3.2226	1.9379	2.5809	1.5266	7.0011
P value	0.6633	.0004304	.001717	0.0552	0.01161	0.1315	<0.00001

Significant differences α=.05 highlighted in yellow

Correlations among length, the three isotopes and the three elemental percentages are presented in Tables 5, 6, and 7. These show a combination of similarities and differences. Half-shell volume (HSV) has a relatively large positive correlation, $\delta^{13}C$ only at the Brine Pool where much larger animals were included in the archive. At Bush Hill, HSV showed no significant correlations. The correlations among the three isotopes were all significant and of the same sign at both seeps, although the magnitude varied. Correlations among elemental percentages were always positive; this indicates that these three elements increase and decrease together.

Table 6. Brine Pool mussel parameter correlations

	Length	$\delta^{15}N$	$\delta^{13}C$	$\delta^{34}S$	%N	%C	%S
HSV	1.0000	0.0656	0.4818	0.1346	-0.2696	-0.1538	-0.0479
$\delta^{15}N$		1.0000	0.5011	-0.7218	0.0294	-0.0194	-0.3308
$\delta^{13}C$			1.0000	-0.3248	-0.2444	-0.0824	-0.2663
$\delta^{34}S$				1.0000	0.0493	0.1331	0.0949
%N					1.0000	0.6936	0.3896
%C						1.0000	0.1434
%S							1.0000

(significant differences α=.05 highlighted in yellow).

Table 7. Bush Hill mussel parameter correlations

	Length	$\delta^{15}N$	$\delta^{13}C$	$\delta^{34}S$	%N	%C	%S
HSV	1.0000	0.2461	0.0365	0.1518	-0.0649	-0.0732	-0.0461
$\delta^{15}N$		1.0000	0.7713	-0.5468	0.1339	-0.0124	0.2994
$\delta^{13}C$			1.0000	-0.7462	0.2560	0.0788	0.4086
$\delta^{34}S$				1.0000	-0.2620	-0.0844	-0.3153
%N					1.0000	0.6507	0.6903
%C						1.0000	0.4594
%mgS							1.0000

(significant differences α=.05 highlighted in yellow).

4.1.2.1. Isotope Patterns Within Seeps

The presence of isotope spatial patterns within the simple design of sampling at the Brine Pool was examined using an unbalanced Factorial Analysis of Variance carried out for each isotope separately on pooled specimens from 1989 and 1991 (Table 8). For all three isotopes, the means were significantly different between the Northern and Southern end of the pool (position factor 1). There were also significant differences between means of all isotopes collected at the inner portion of the mussel ring and the outer edge (position factor 2) . Interactions between the two positional factors, however, were also significant and indicated that the effects of the main factors was inconsistent. Spatial differences exist, but in the form of simple spatial gradients. The inconsistency of the spatial pattern was confirmed with post-hoc Tukey Honest Significant Distance (TukeyHSD) analysis (Appendix II; Table 1).

The effects on isotope values from year of sampling and the pool end position (position factor 1) along with the interaction of year of sampling and pool end position was also examined by unbalanced factorial analysis (Table 9). The isotopes varied different from one another. $\delta^{13}C$ means were significantly different at different ends and at different years, but interactions were

also significant indicating inconsistent year and position effects. $\delta^{15}N$ also showed significant year effects, lacking position differences and also showed inconsistent results as indicated by significant interactions. Values of $\delta^{34}S$ show no significant year effects but did have different means between North and South ends of the pool. No significant interactions were found for $\delta^{34}S$. Post hoc TukeyHSD analyses reinforced the finding of interactions for $\delta^{13}C$ and $\delta^{15}N$ (Appendix II; Table 2).

Table 8. Within Brine Pool ANOVA for stable isotope spatial patterns

Response: $\delta^{13}C$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Pool End	216	1	10.238	0.00199	**
Edge:Inner	449	1	21.329	1.500e-05	***
Interaction	443	1	21.055	1.679e-05	***
Residuals	1643	78			Factor
Response: $\delta^{15}N$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Pool End	298.41	1	18.443	4.992e-05	***
Edge:Inner	544.19	1	33.634	1.351e-07	***
Interaction	248.28	1	115.345	0.0001907	***
Residuals	1262.02	78			
Response: $\delta^{34}S$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Pool End	434.57	1	104.302	4.928e-16	***
Edge:Inner	510.48	1	122.522	< 2.2e-16	***
Interaction	360.79	1	86.593	2.759e-14	***
Residuals	324.99 78				
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Spatial patterns at Bush Hill were sought using an unbalanced factorial analysis of variance pooling over the two years of sampling (Table 10). The spatial factors were which bed the samples came from and whether the samples came from the inner portion of the bed or the outer edge. All three isotopes had significantly different means at the three beds 1, 1A, and 2. $\delta^{15}N$ showed the greatest significant difference between edge and inner locations. The interaction of the two position factors indicated an inconsistent effect. $\delta^{13}C$ showed a significant inner:edge effect but only at a lower criteria of α=0.10; again significant interactions indicated an inconsistent spatial pattern. $\delta^{34}S$ lacked a distinct inner:edge different. Due to the significant interaction for all three isotopes, post-hoc TukeyHSD analyses were carried out and confirmed the inconsistency of spatial patterns (Appendix II; Table 3).

Table 9. Within Brine Pool ANOVA for stable isotope pool-end and year effects

Response: $\delta^{13}C$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Year	600	2	16.7914	3.617e-07	***
Pool End	147	1	8.2116	0.004903	**
Interaction	737	2	20.6418	1.899e-08	***
Residuals	2178	122			
Response: $\delta^{15}N$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Year	396.6	2	16.6642	3.996e-07	***
Pool End	0.4	1	0.0348	0.852411	
Interaction	120.0	2	5.0411	0.007878	**
Residuals	1451.9	122			
Response: $\delta^{34}S$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Year	23.43	2	1.5172	0.223427	
Pool End	64.60	1	8.3677	0.004524	**
Interaction	23.77	2	1.5394	0.218649	
Residuals	941.82	122			
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

Temporal analysis at Bush Hill was limited to Bed 1 because it was the only bed sampled in both years (Table 11). $\delta^{13}C$ and $\delta^{15}N$ but not $\delta^{34}S$ showed significant differences in the mean between the two years. $\delta^{13}C$ and $\delta^{15}N$ showed no differences between the inner and edge samples. $\delta^{34}S$ displayed a weak difference between the two positions but one that was inconsistent between the years. As with the previous analysis, post-hoc TukeyHSD analysis confirmed the position and year inconsistency (Appendix II; Table 4).

Due to the many significant interaction terms, simple plots of data taking each pair of isotopes proved to be especially informative about within-seep spatial and temporal variation. At Bush Hill this variation is apparent in data plots for 1991 and 1992 and beds 1, 2, and 1A. Bed 1 was the only bed sampled in both years and tended to have tightly-clustered points in each of the three graphs for both years (Figure 6). The greatest difference found between the years in the centroid of bed 1 data points was a shift of approximately 5‰ in $\delta^{34}S$ (Figure 6 E and F). More conservative between years was $\delta^{13}C$ with a shift of approximately 1.5‰ and a $\delta^{15}N$ shift of approximately 1‰. Contrast between beds within each year was more dramatic. Beds 2 and 1 A had $\delta^{13}C$ values overlapping bed 1, but with an expansion of range that consisted primarily of the addition of more depleted values. $\delta^{15}N$ had a more modest expansion of range, again with more deleted values mixed

into beds 2 and 1A. $\delta^{34}S$ were shifted sufficient between bed 1A and 2 for there to be no overlap (Figure 6 E and F). Bed 2 had a similar shift compared to Bed 1, but a wider range of values caused the two data clusters to overlap (Figure 6 B and C).

Table 10. Bush Hill mussel tissue stable isotope factorial ANOVA for spatial effects

Response: $\delta^{13}C$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Bed	22	2	6.3274	0.002447	**
Inner:Edge	6	1	3.3124	0.071274	.
Interaction	37	2	10.9366	4.363e-05	***
Residuals	202	119			
Response: $\delta^{15}N$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Bed	8.00	2	4.6781	0.011072	*
Inner:Edge	6.29	1	7.3544	0.007681	**
Interaction	7.10	2	4.1553	0.018013	*
Residuals	101.73	119			
Response: $\delta^{34}S$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Bed	491.23	2	42.8340	9.718e-15	***
Inner:Edge	0.27	1	0.0472	0.828308	
Interaction	137.94	2	12.0284	1.747e-05	***
Residuals	682.36	119			
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

At Brine Pool NR-1 the range for $\delta^{13}C$ and $\delta^{15}N$ greatly exceed those found at all Bush Hill sites consistent with mixing of widely different sources (Figure 7). The patterns among bed locations and years (1989, 1992, and 2006) are complex and do not reflect a simple dependence on location with respect to the brine. With the exception of five outlier specimens from the 1991 northern outer portion of the mussel mat, all values for $\delta^{13}C$ and $\delta^{15}N$ fell on a common curvilinear relationship (Figure 7 B, D, and F). In 1989 the curve of $\delta^{13}C$ and $\delta^{15}N$ points was quite wide; northern outer and southern inner samples clustered at the enriched end of the range and those from the northern inner and southern outer samples were spread towards more depleted values (Figure A, C, and E). The pattern of widely scattered points in 1989 and more tightly clustered points in 1991 for $\delta^{13}C$ and $\delta^{15}N$ also held true for $\delta^{34}S$ (Figures 7 C, D, E, and F).

Examination of isotope plots for Brine Pool 2006 values compared with the pooled older 1989 and 1992 values showed relative consistency over the long term (Figure 8; please note scale change between B and F). In some respects, samples from 2006 more closely resembled the widely scattered values found in 1989 than the tightly clustered values of 1991.

Table 11. Inter-Year and With-In Bed ANOVA at Bed 1 mussel tissue stable isotope values

Response: $\delta^{13}C$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Year	63.0	1	137.2755	<2e-16	***
Inner:Edge	0.4	1	0.9375	0.3363	
Interaction	1.0	1	2.1242	0.1495	
Residuals	31.7	69			
Response: $\delta^{15}N$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Year	7.30	1	14.8699	0.0002556	***
Inner:Edge	1.47	1	3.0034	0.0875560	.
Interaction	0.00	1	0.0096	0.9223670	
Residuals	33.86	69			
Response: $\delta^{34}S$					
Factor	Sum Sq	Df	F value	Pr(>F)	
Year					
Inner:Edge	10.359	1	2.7969	0.09897	.
Interaction	137.919	1 37	.2401	5.386e-08	***
Residuals	255.543	69			
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

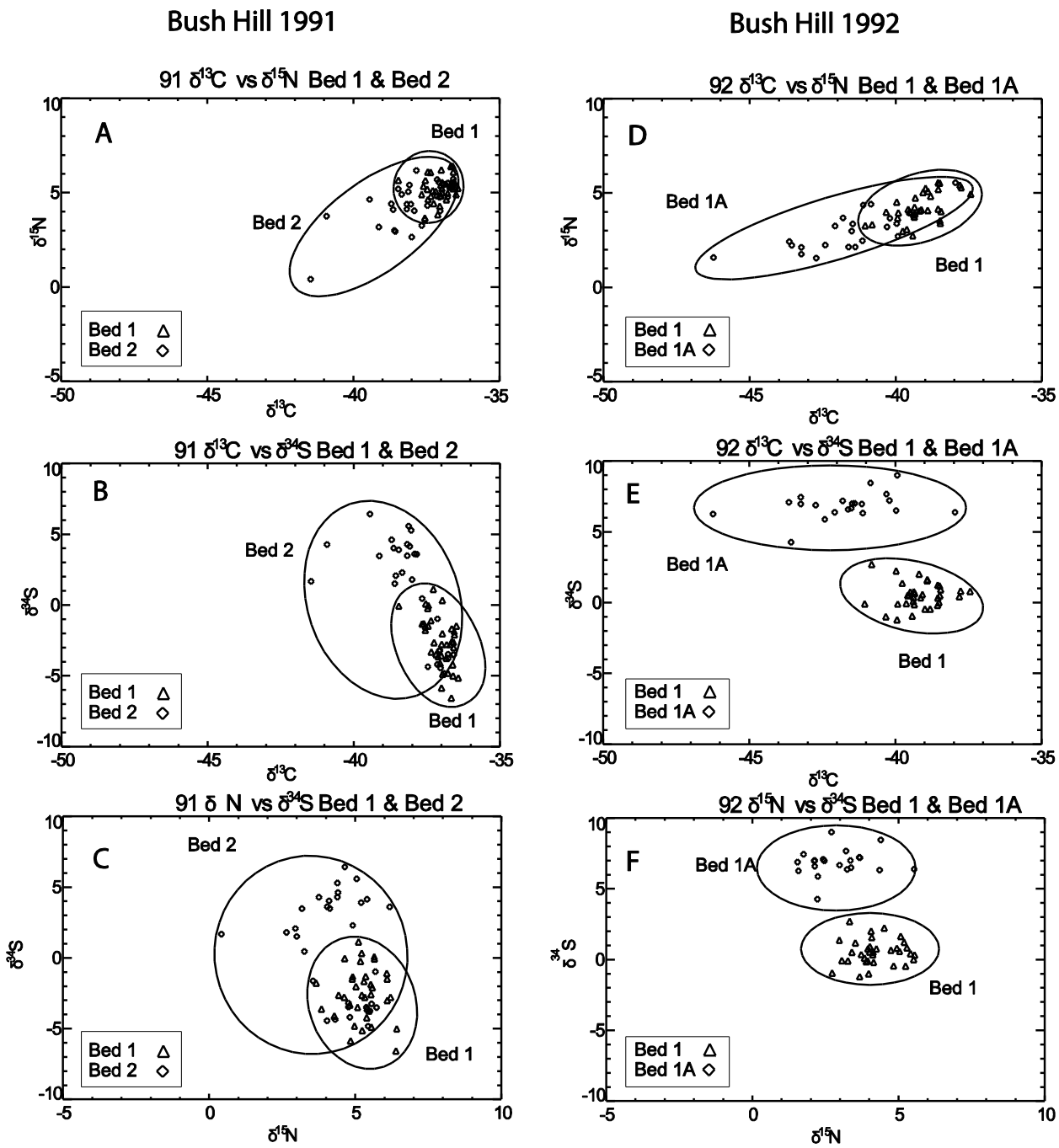

Figure 6. Bush Hill Bed 1 compared to Bed 2 in 1989 and Bed 1A in 1991.

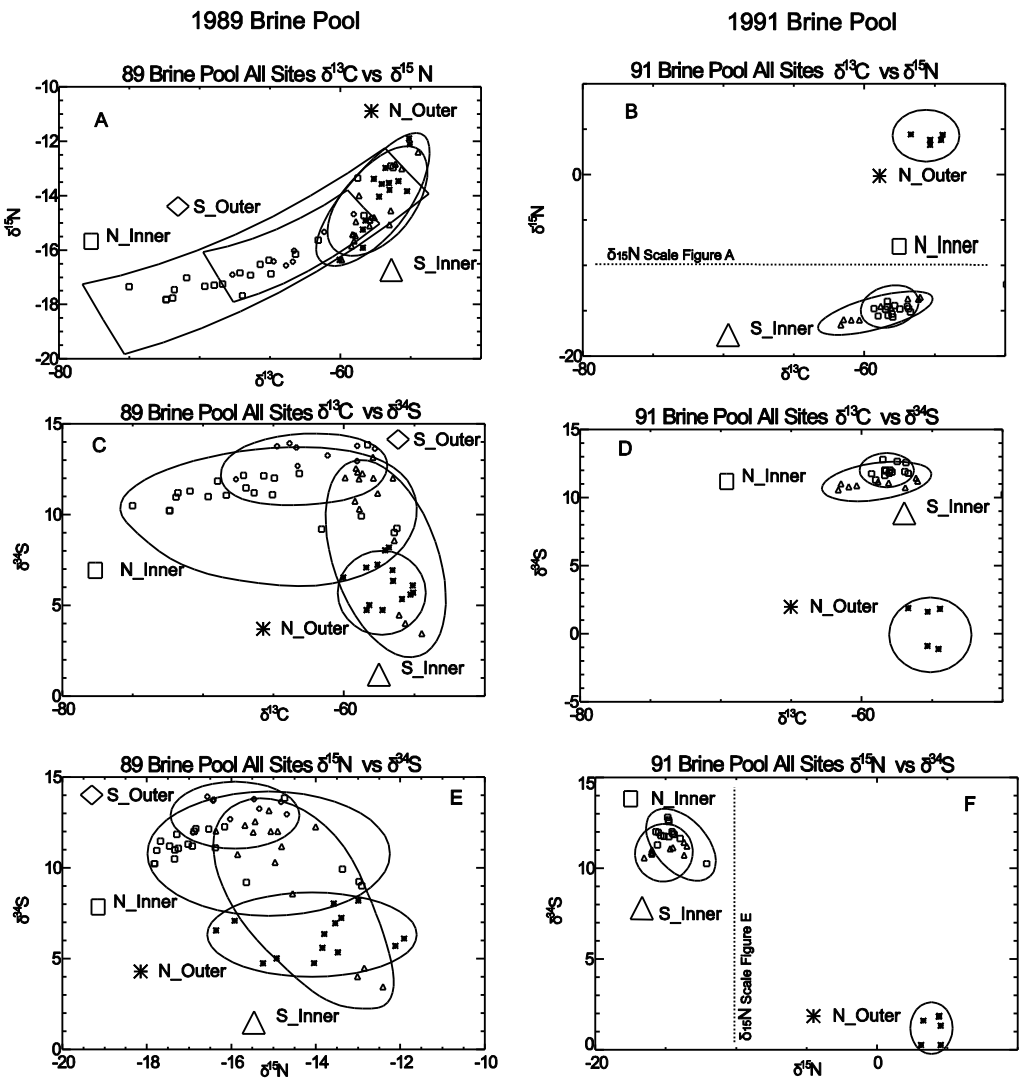

Figure 7. Brine Pool Positions for 1989 and 1991 boundaries added for clarity.

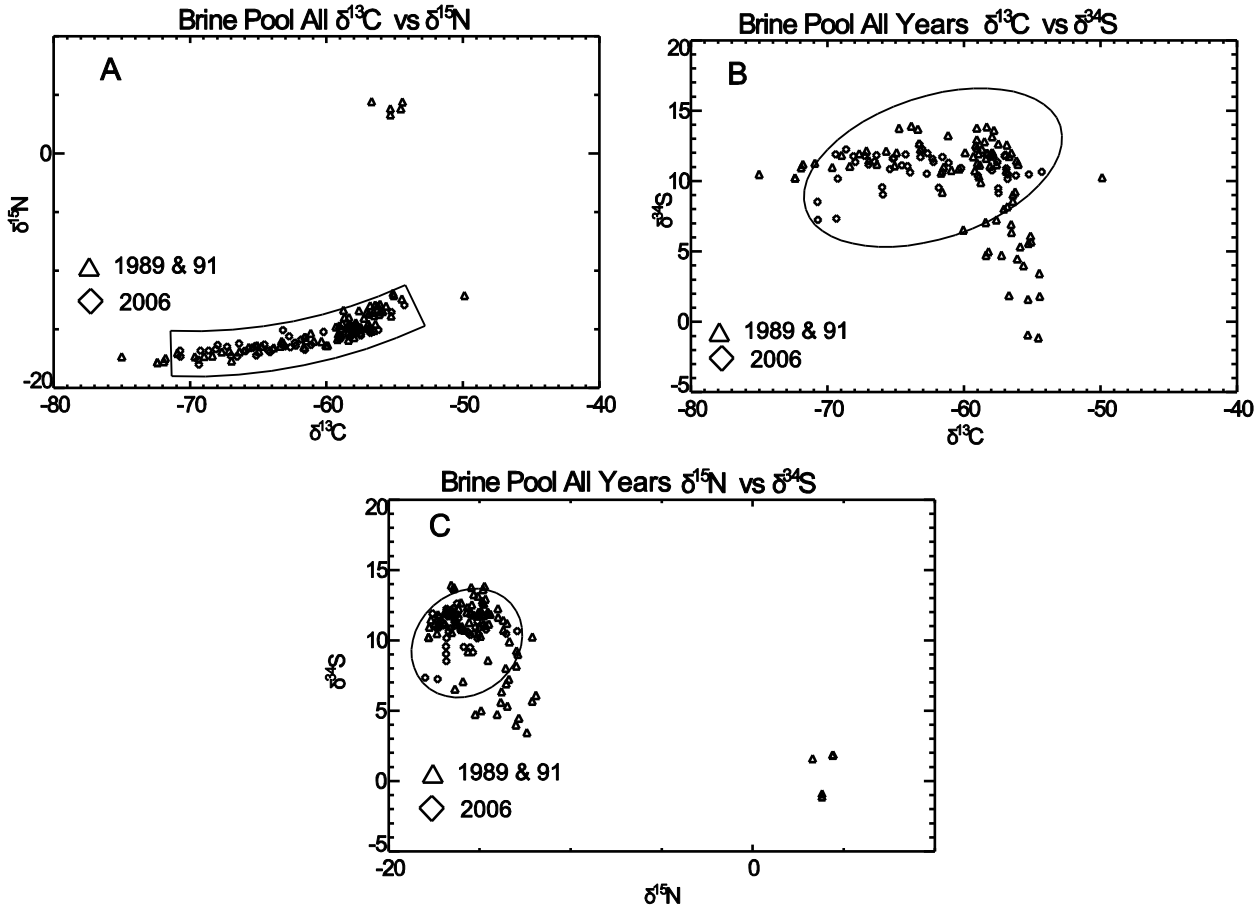

Figure 8. Brine Pool tissue values for1989, 1992, and 2006 with boundaries on 2006 for clarity.

4.1.3. Stable Isotope Estimation of Mixed Use of Four Trophic Sources

The stable isotope signatures of four probable trophic sources were identified for each seep. Phytoplankton detritus (DPHY) was the same at both sites. A total of four methane-pathways sources were identified and designated SM_A_{BH}, SM_B_{BH}, SM_C_{BP}, and SM_C_{BP}; the subscripts denotes the seep. Each seep also had a distinct thiotrophic-pathway source $DSOM_{BH}$ and $DSOM_{BP}$. Table 12 presents the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values for probable trophic sources (vertices) selected for the mixing model. They were chosen for their ecological feasibility in the upper continental slope seep setting and for the degree to which their values allowed for the tightest fit of the mixing tetrahedron around the sample data. The four sources included two different detrital sources and two different symbiotic-pathway sources with both site-specific differences and similarities.

Table 12. Estimated sources (vertices) of a four-source mixing tetrahedron

	Detrital Sources						Symbiotic-Pathway Sources					
	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	$\delta^{34}S$ ‰	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	$\delta^{34}S$ ‰	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	$\delta^{34}S$ ‰	$\delta^{13}C$ ‰	$\delta^{15}N$ ‰	$\delta^{34}S$ ‰
Bush Hill	DPHY Photosynthetic Carbon Seawater Nitrate,& Seawater Sulfate			$DSOM_{BH}$ Chemosynthetic Carbon Seawater Nitrate & Seep Sulfide			SM_A_{BH} Methane Carbon, Seawater Nitrate &Seawater Sulfate			SM_B_{BH} Methane Carbon, Seep Ammonium + Seawater Nitrate & Seep Sulfide + Seawater Sulfate		
	-21.5	7	21	-31	7	-20	-50	7	21	-50	-5	0
Brine Pool	DPHY Photosynthetic Carbon Seawater Nitrate &Seawater Sulfate			$DSOM_{BP}$ Chemosynthetic Carbon Seep Ammonium &Seep Sulfide			SM_C_{BP} Methane Carbon, Seawater Nitrate & Seep Sulfide			SM_D_{BP} Methane Carbon, Seep Ammonium & Seawater Sulfate		
	-21.5	7	21	-31	-25	-20	-65	7	-5	-90	-25	21
	Seawater and Photosynthesis Influenced							Seep Influenced Depletions				

Detritus influx originating from euphotic zone phytoplankton should be the same at the closely-located seeps and relatively labile due to the 500m depth (Biggs et al 2008). Detritus Phytoplankton (DPHY) has a $\delta^{13}C$ value of -21.5‰ and a $\delta^{15}N$ value of 7‰ that is representative of surface particulate organic matter in the Northern Gulf (Rooker et al. 2006; Wells and Rooker 2009) and a $\delta^{34}S$ value of 21‰ representative of seawater sulfate (Rees et al. 1978). A second detrital source consists of free-living sulfur oxidizing microbes (DSOM) on or very close to bottom (Page et al. 1990). The DSOM for both sites ($DSOM_{BH}$ and $DSOM_{BP}$) have the same $\delta^{13}C$ value of -31‰, obtained from thiotrophic *Beggiotoa* mats as a proxy for all free-living chemosynthetic microbes (Demopolous 2010). The DSOM values of $\delta^{34}S$ at both sites is the same extremely depleted -20‰ as the cumulative result of the fractionations during the reduction of seawater sulfate to interstitial seep sulfides (30–70‰ Canfield 2001) and the subsequent oxidation of those sulfides (10–18‰; Dale et al. 2009). Fitting the tetrahedrons to the data requires that the $DSOM_{BH}$ and $DSOM_{BP}$ $\delta^{15}N$ values be different because the values for individuals at both sites have distinctly different ranges. For the Bush Hill $DSOM_{BH,}$ the $\delta^{15}N$ of 7‰ reflects a predominant usage of seawater nitrate (5–7‰) and at the Brine Pool $DSOM_{BP}$ $\delta^{15}N$ of -25‰ reflects the predominant utilization of brine-associated ammonium.

The range and variance at both seeps indicate that two microbial methanotrophic-pathway sources were being used by the populations SM_A_{BH}, SM_B_{BH}, $SM_C_{BP,}$ and SM_C_{BP}. The narrow range and symmetry of $\delta^{13}C$ values (Figure 5B, 5D, &5F) and the fit of the tetrahedron (Figure 11) at Bush Hill are consistent with a single methane source with a $\delta^{13}C$ value of -50‰ shared by two resources. The first resource SM_A_{BH} combines that seep methane carbon with nitrogen from seawater nitrate $\delta^{15}N$ = 7‰ and sulfur from seawater sulfate $\delta^{34}S$ = 20‰. The second resource SM_B_{BH} incorporates seep methane carbon with seep-derived nitrogen and sulfur,

or possibly a mixture of seep-derived ammonium and seawater-derived nitrate $\delta^{15}N$ = -5‰ and sulfate $\delta^{34}S$ = 0‰. It is the relatively minor depletion of these two isotopes that suggests seep and seawater mixing.

At the Brine Pool $\delta^{13}C$ bimodality and asymmetry (Figure 5B, 5D, &5F) and tetrahedral fit (Figure 9; the vertices represent possible trophic sources, and points within each tetrahedron are based on data from the mussels) are best explained by two seep methane-carbon sources, one highly depleted $\delta^{13}C$= -90‰ and another more moderately depleted $\delta^{13}C$= -65‰. The latter carbon combines in SM_B_{BH} with nitrogen from seawater nitrate $\delta^{15}N$ = 7‰ and sulfur from seep sulfide $\delta^{34}S$ = -5‰. The highly depleted carbon combines in SM_C_{BP} with highly depleted nitrogen from seep ammonium $\delta^{15}N$ = -25‰ and sulfur from seawater sulfate $\delta^{34}S$ = 21‰.

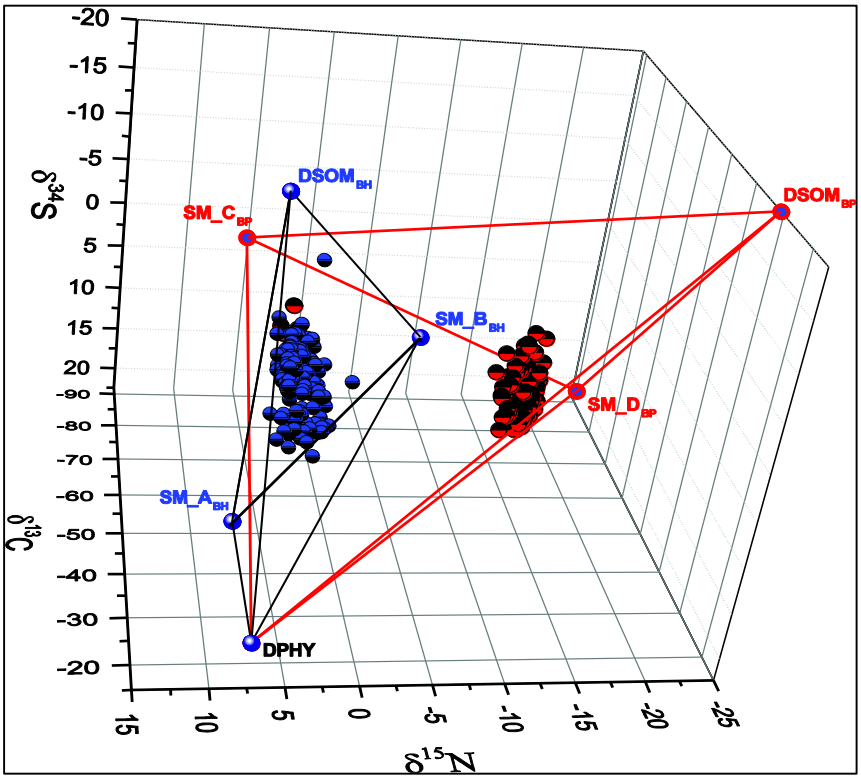

Figure 9. Trophic mixing models as tetrahedrons surrounding Brine Pool (red) and Bush Hill (blue) data points in Cartesian space of the three stable isotopes data.

Because four distinct methane-pathway resources were indicated initial between Brine Pool and Bush Hill comparisons were carried out through separate t-tests for combined methane sources (SM_A_{BH} + SM_B_{BH} compared with SM_C_{BP} + SM_D_{BP}), sulfur microbe sources ($DSOM_{BH}$ vs $DSOM_{BP}$), and DPHY. Subsequent within-seep comparisons were carried out on each of the seep-specific four sources without combinations using an unbalanced Analysis of Variance (ANOVA) and post hoc TukeyHSD analysis.

Paired t-tests between seeps indicated significant differences between the Brine Pool and Bush Hill in average use of the three source categories (Table 13). The mussel population at the Brine Pool made greater use of combined methane-pathway sources (61.1% ± 9.3) predominantly SM_D_{BP} (49.4% ± 11.5) and lower and roughly equal particulate resources, DPHY (20.2% ± 7.0) and $DSOB_{BP}$ (18.7% ± 4.7). At Bush Hill the combined methane-pathways resources were significantly lower in the population (46.2% ± 11.1) and consisted of roughly equal amounts of SM_A_{BH} (24.0% ± 6.6) and SM_B_{BH} (22.2% ± 9.7). Offsetting the lower methane use was increased use of particulate sources; $DSOB_{BH}$ was a major resource (38.2% ± 13) and DPHY was a minor resource (15.6% ± 4.6).

Table 13 Between-Seep t-Test comparisons with methane sources combined.

Site	n	Methane	DPHY	DSOB
Brine Pool	128	61.1 ± 9.3	20.2 ± 7	18.7 ± 4.7
Bush Hill	125	46.2 ± 11.1	15.6 ± 4.6	38.2 ± 13
t		11.6	6.2	-15.9
P_value		<0.001	<0.001	<0.001

(significant values α=.05 highlighted in yellow)

At Bush Hill significant Bed differences were found for all four resources, but Position (Inner compared with Edge) differences were significant only for SM_B_{BH} and $DSOM_{BH}$ (Table 14). Post hoc analysis (Appendix II; Table 5) and box and whisker plots (Figure 10; based on Table 14) revealed that within-bed homogeneity was more prevalent than the ANOVA results might suggest. Beds 1, 1a, and 2 were not significantly different for use of SM_A_{BH} and DPHY. When significant Position effects were found, they were restricted to only a single bed, bed 1 in the case of SM_B_{BH}, bed 2 in the case of $DSOM_{BH}$. Significant Bed:Position interaction for DPHY and $DSOM_{BH}$ indicated inconsistent factor effects.

Table 14. Bush Hill ANOVA for each resource and position factors both years pooled

BUSH HILL					
	Sum Sq	Df	F value	Pr(>F)	
SM_A_{BH}					
Bed	0.0923	2	13.2810	6.218e-06	***
Position	0.0000	1	0.383	0.9748	
Bed:Position	0.0105	2	1.5101	0.2251	
Residuals	0.4133	119			
SM_B_{BH}					
Bed	0.3791	2	31.619	9.71e-12	***

Position	0.0862	1	14.383	0.00236	**
Bed:Position	0.0035	2	0.2878	0.75043	
Residuals	0.7135	119			
$DPHY_{BH}$					
Bed	0.05720	2	19.450	4.91e-08	***
Position	0.00525	1	3.574	0.0611	
Bed:Position	0.02966	2	10.0849	9.000e-05	***
Residuals	0.17497	119			
$DSOM_{BH}$					
Bed	1.0753	2	94.4439	< 2.2e-16	***
Position	0.0977	1	17.1591	6.46e-05	***
Bed:Position	0.1046	2	9.1906	0.0001943	***
Residuals	0.6775	119			

Unlike the relatively homogenous Bush Hill mussels, the Brine Pool sample contained five specimens from a single scoop sample taken in 1991 at the North Edge that were distinct outliers especially with respect to $\delta^{15}N$ values. ANOVA were carried out with the full data set (Table 15) and then with the outliers omitted (Appendix II; Table 6). For the full data set at the brine pool SM_C_{BP} showed End (N vs S) effects and position effects (Inner vs Edge) but with significant interactions (Table 15). Post hoc analysis (Appendix II; Table 7) revealed that the significant results were caused primarily by high values encountered at the North Edge; the three other combinations of End and Position were homogenous. SM_B showed marginally significant End effects, Position effects and an interaction in the analysis of variance, but, in post hoc analysis, the End effect was not significant and differences were due primarily to the low values at the North Edge. Use of DPHY showed significant End effects but insignificant Position effects and no significant interaction. Post hoc analysis revealed the same effects but pairwise comparisons indicated few major differences except for a variable range. DSOM showed no significant End or Position effects although a significant interaction was found. Post hoc analysis indicated a difference in means between Edge and Inner only at the South end. Otherwise, DSOM use was relatively uniform across the sampled locations. Interactions among factors are obvious in box and whisker plots (Figure 11; based on Table 15).

Omission of the outliers (Appendix II; Table 6) had little impact on the conclusion of the analyses; similar conclusions about Bed and Position effects were produced for SM_C_{BP} ,SM_D_{BP} and DPHY. The North End remained the location of significantly greater SM_C_{BP} use, and the maximum found in the North Edge. The major change in results was for DSOM, which showed significant End, Position, and interaction effects. It had the greater use at the North End and the greatest overall at the North Edge.

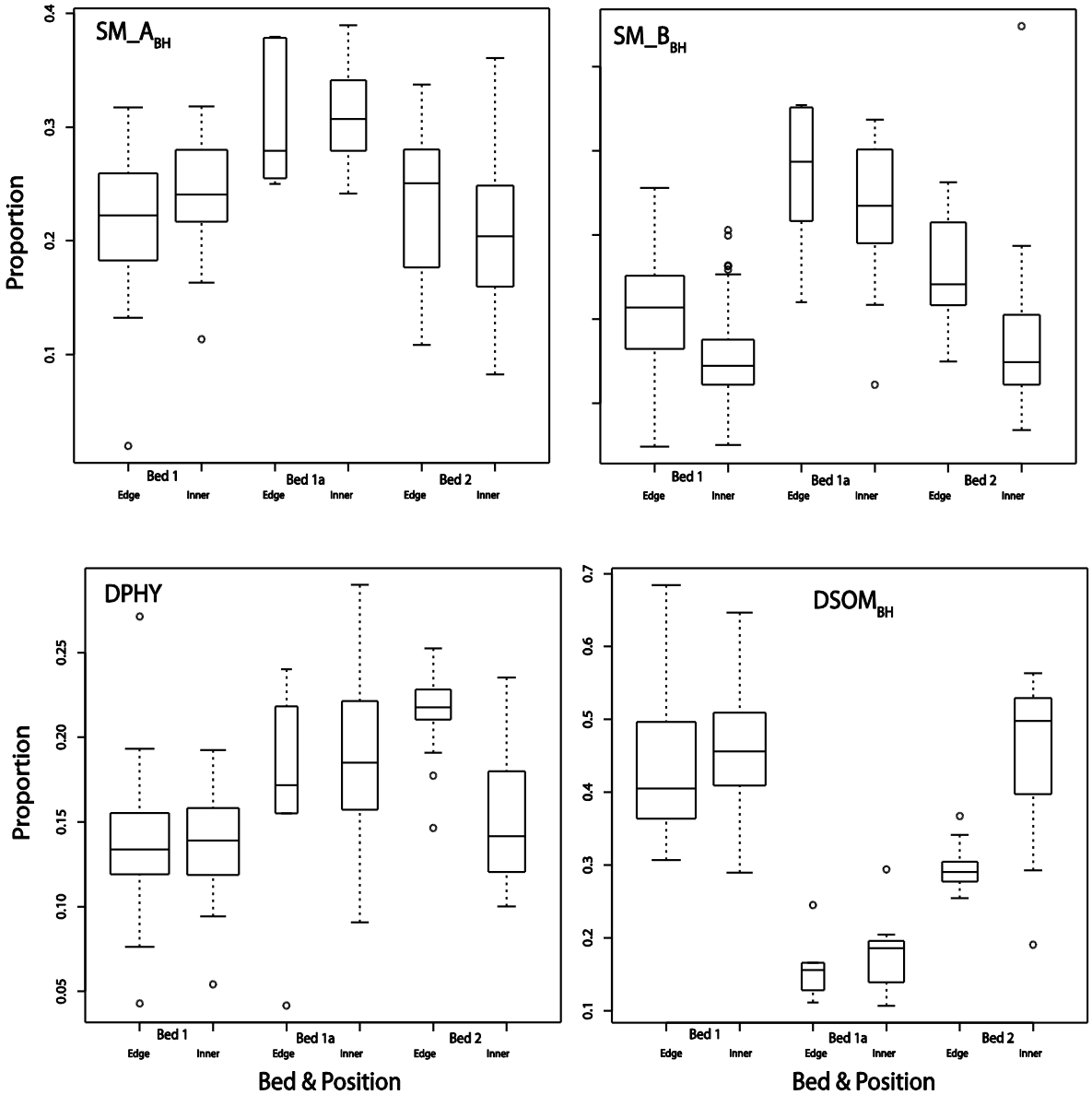

Figure 10. Bush Hill between-bed and with-in bed proportional use of trophic resources presented as box and whisker plots with both sampling years pooled.

Temporal differences at Bush Hill could only be examined for Bed 1 because the other beds were sampled only in a single year (Table 16; Figure 12). DPHY was the only resource to be used homogenously between years and between positions. SM_A_{BH} use increased significantly in 1992 and was consistently lower at the Edge in both years. SM_B_{BH} was similarly higher in 1992 and within years was consistently higher at the Edge. DSOM was used significantly less in 1992 and there were no position effects within the years. Post hoc analyses (Appendix II; Table 7) supported the conclusions of the ANOVA.

Table 15. Brine Pool trophic resource ANOVA for position factors 2006 omitted

BRINE POOL					
	Sum Sq	Df	F value	Pr(>F)	
SM_C_{BP}					
End	0.17956	1	11.22	0.0012450	**
Position	0.33114	1	20.703	1.941e-05	***
End:Position	0.24444	1	15.283	0.0001961	***
Residuals	1.24758	78			
SM_D_{BP}					
End	0.03497	1	3.8889	0.05215	.
Position	0.26860	1	29.8679	5.358e-07	***
End:Position	0.41237	1	45.8540	2.136e-09	***
Residuals	0.70146	78			
DPHY					
End	0.03589	1	8.2427	0.005265	**
Position	0.00113	1	0.2584	0.612662	
End:Position	0.00000	1	0.0009	0.975885	
Residuals	0.33965	78			
$DSOM_{BP}$					
End	0.00022	1	0.0747	0.78540	
Position	0.00416	1	1.4599	0.23060	
End:Position	0.02243	1	7.8711	0.00634	**
Residuals	0.22223	78			

Temporal differences at the Brine Pool were examined with an unbalanced ANOVA with two factors: Year and Pool End North or South (Table 17). The predominant methane-pathway resource SM_D_{BP} and showed no significant differences among the three years and between North and South ends (Figure 13; based on Table 17). In 2006, however, the North and South End mussels had significantly different means; the South used the greater proportion. Thiotrophic

particulate material was similarly homogenous among Years and Ends, except in 2006 the North end. SM_C_{BP} was lowest in 1991 and homogenous between Ends. Use in 1989 and 2006 were not significantly different but Ends differed inconsistently. DPHY showed an inverse pattern with highest use and similar Ends in 1991. Means in 1989 and 2006 were similar and Ends differed inconsistently. Post hoc analyses (Appendix II; Table 8) supported the conclusions of the ANOVA.

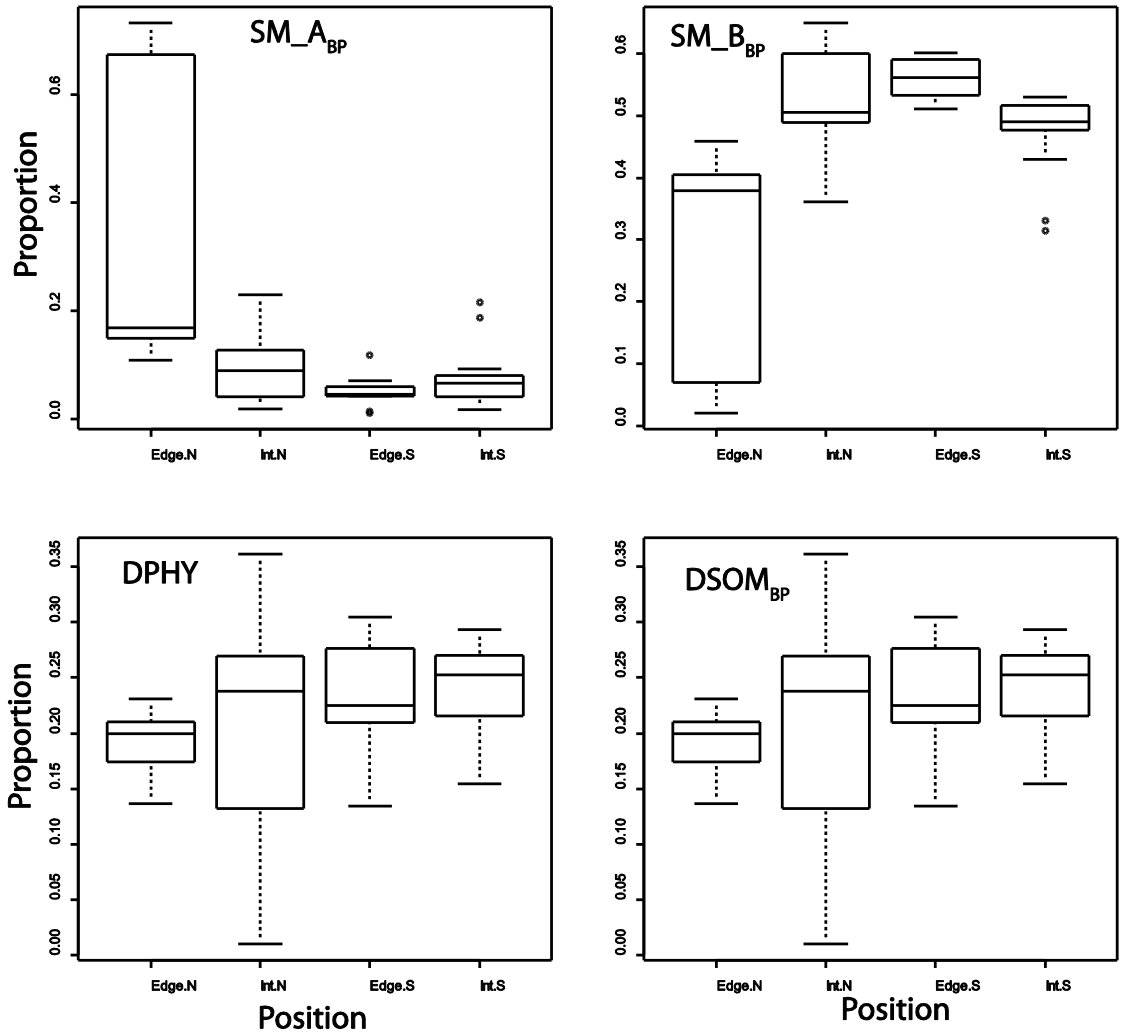

Figure 11. Brine Pool trophic resource proportional use at pool ends and positions displayed as whisker and box plots by position 1991 and 1992 years pooled and 2006 omitted.

Table 16. Bush Hill Bed 1 ANOVA of trophic use between years

Bush Hill Year and Pool-End Effects Bed 1 Only					
	Sum Sq	Df	F value	Pr(>F)	
SM_A_{BH}					
Year	0.0362	1	15.2349	0.0002181	***
Position Bed 1	0.0156	1	6.5690	0.0125615	*
Year:Position	0.0028	1	1.1634	0.2845209	
Residuals	0.1642	69			
SM_B_{BH}					
Year	0.08858	1	25.9948	2.864e-06	***
Position Bed 1	0.01672	1	4.9072	0.03005	*
Year:Position	0.00003	1	0.0096	0.92237	
Residuals	0.23514	69			
DPHY					
Year	0.00001	1	0.0115	0.9151	
Position Bed 1	0.00001	1	0.0081	0.9287	
Year:Position	0.00010	1	0.0828	0.7744	
Residuals	0.08601	69			
$DSOM_{BP}$					
Year	0.2345	1	77.8162	6.311e-13	***
Position Bed 1	0.0000	1	0.0004	0.9836	
Year:Position	0.0013	1	0.4480	0.5055	
Residuals	0.2079	69			
Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1					

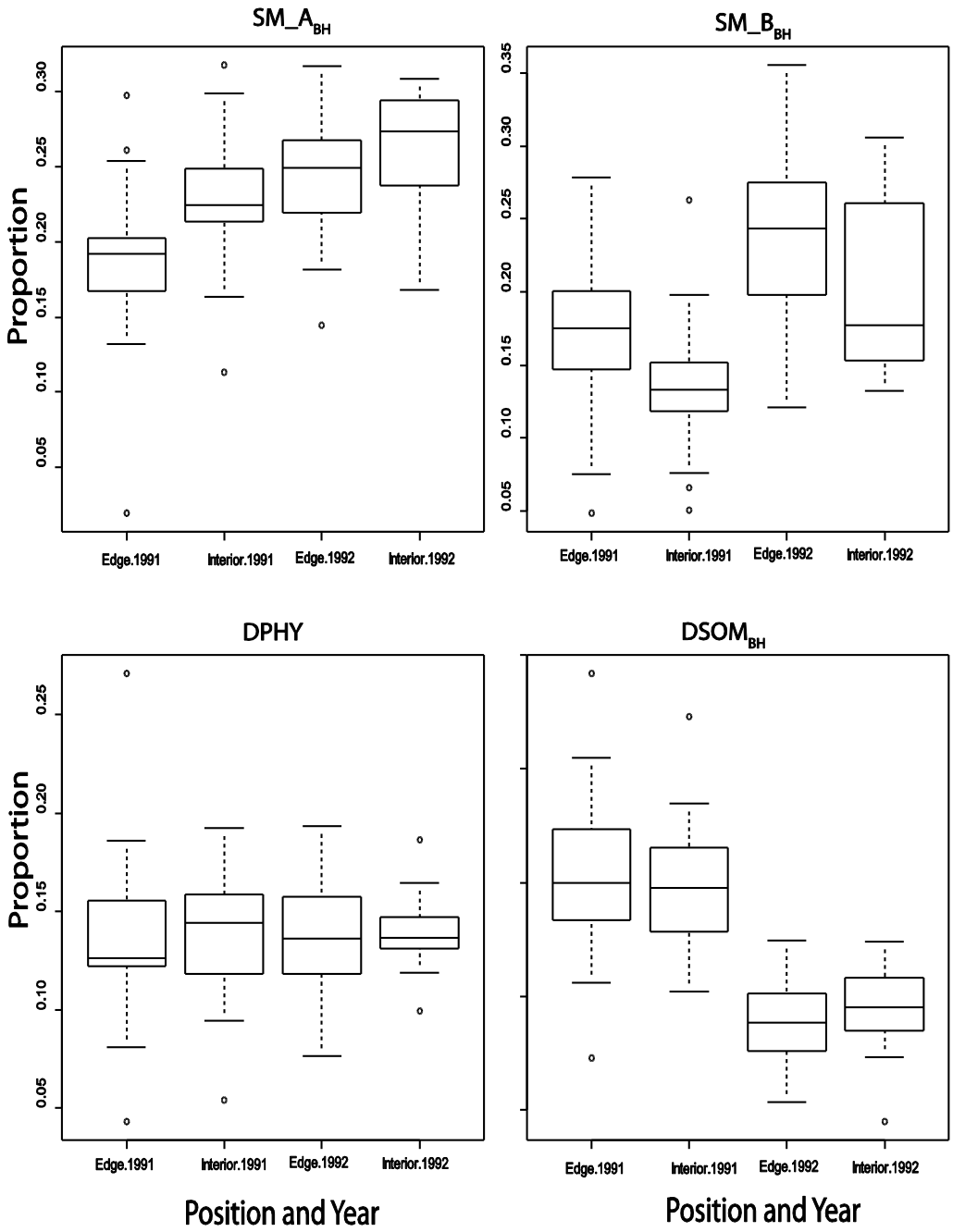

Figure 12. Bush Hill between-year and position within Bed 1 only in trophic resource use displayed as box and whisker plots.

Table 17. Brine Pool among-year and pool-end trophic resource use ANOVA

BRINE POOL					
	Sum Sq	Df	F value	Pr(>F)	
SM_C_{BP}					
Year	0.02758	2	8.8690	0.0002592	***
End	0.00031	1	0.2001	0.6554899	
Year:End	0.10165	2	32.6879	5.276e-12	***
Residuals	0.18192	117			
SM_D_{BP}					
Year	0.0061	2	0.7003	0.498488	
End	0.0285	1	6.5408	0.011821	*
Year:End	0.0490	2	5.6207	0.004669	**
Residuals	0.5104	117			
DPHY					
Year	0.0479	2	8.9247	0.000247	***
End	0.0139	1	5.1909	0.024520	*
Year:End	0.2016	2	37.5927	2.462e-13	***
Residuals	0.3137	117			
$DSOM_{BP}$					
Year	0.0001	2	0.0388	0.96198	
End	0.0047	1	3.3771	0.06864	.
Year:End	0.0076	2	2.7166	0.07027	.
Residuals	0.1629				
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1					

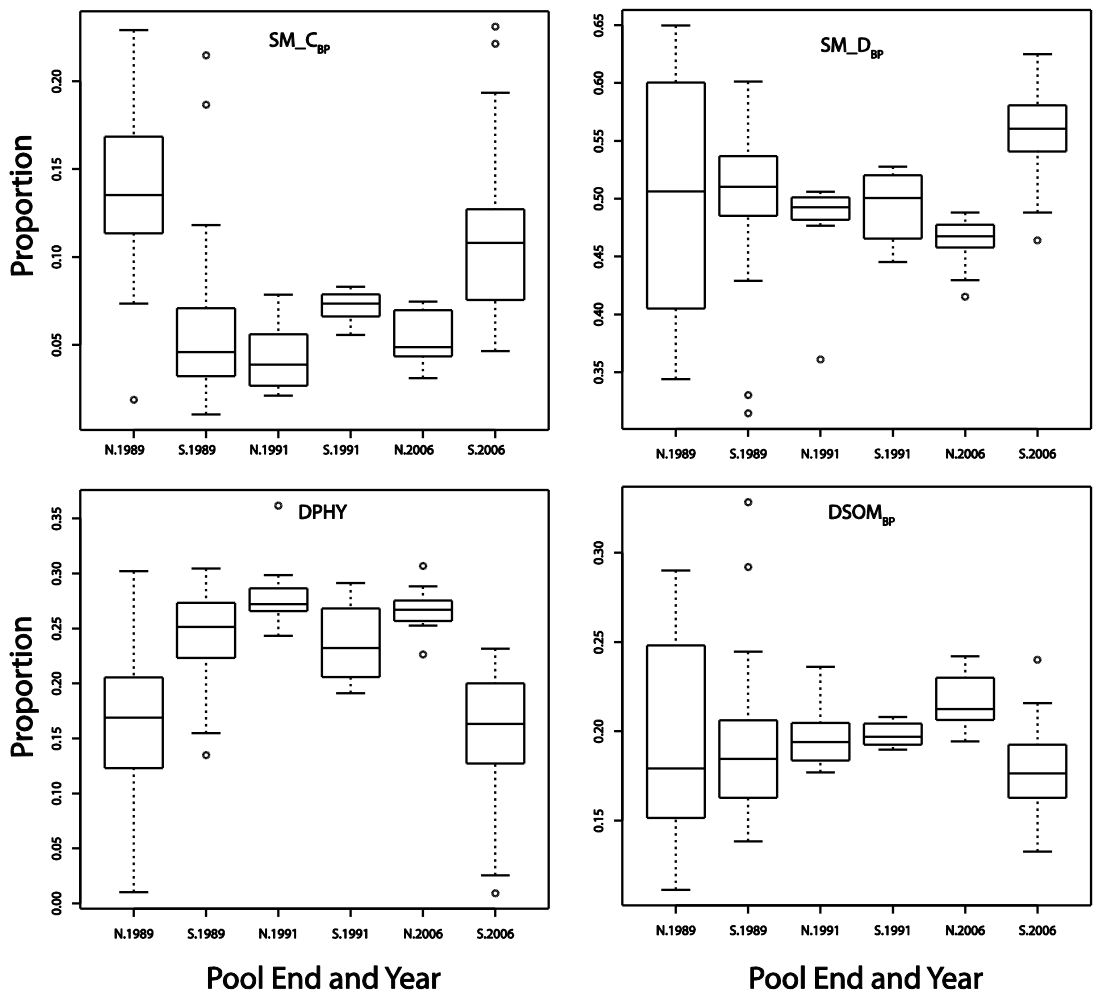

Figure 13. Brine Pool position and year proportional use of trophic resources for all three years presented as box and whisker plots.

4.2. MUSSEL SHELL INCORPORATION OF SEEP RESPIRATORY METHANE

Though mussel shell was found to be composed of predominantly carbon from the seawater inorganic carbon pool, respired carbon from the methane-based tissue was detectable. That amount was distinctly different at the two seeps and indicated a greater incorporation rate at the Brine Pool. Additionally, a size effect was found when HSV was taken as the measure of animal size. Separate regression of C_m compared to HSV at Brine Pool and Bush Hill revealed significant ($p<0.0086$, $p<0.0001$), but relatively small, linear positive relationships between Ln(HSV) and $\%C_m$ at each site that are not significantly different from one another ($p=0.1977$). The intercepts (13.9, 8.1) at the two sites were significantly different ($p<0.0001$). An ANOVA with a Tukey's post hoc test ($\alpha= 0.05$) was performed on the Brine Pool site and on $\%C_m$ for groupings of HSV from 0-10 cm^3, 10–20 cm^3, and >20 cm^3. This resulted in significant differences between the 0-10 cm^3 (14.4%) and the >20 cm^3 (16.4%) groupings. It also resulted in a significant difference between the 10–20 cm^3 (17.8%) and >20 cm^3 (16.4%). A second ANOVA performed on the Bush Hill site resulted in significant differences between 0–10 cm^3 (9.8%) and 10-20 cm^3 (11.6%) groupings and between 10–20 cm^3 (11.6%) and >20 cm^3 (13.1%) groupings.

When these samples were grouped on ranges of HSV and compared between the Brine Pool and Bush Hill sites, there was a significant difference ($p<0.0001$) between the Bush Hill and Brine Pool sites (Figure 12). That is, a mussel with a HSV of 0–10 cm^3 and a mean of 14.4 % ± 3.12 at Brine Pool (n=36) but at Bush Hill (n=40) for a mussel with an HSV of 0–10 cm^3 there is a mean of 9.8 % ± 1.5. For a mussel with a HSV of 10-20 cm^3, there was also a significant difference ($p<0.0001$) between the two sites. The mean at the Brine Pool (n=18) site was 17.8 % ± 2.2 and the mean at the Bush Hill (n=18) site was 11.6% ± 2.4. For a mussel HSV of 20+ cm^3, there was a significant difference ($p<0.004$) between the two sites the mean at the Brine Pool (n=38) site was 16.4 % ± 3 and the mean at the Bush Hill (n=6) site was 12.2 % ± 2.3. There is a statistically significant difference in the incorporation of metabolic carbon between the sites for all size ranges; Brine Pool had significantly increased incorporation of respiratory carbon across the entire range of mussel HSV.

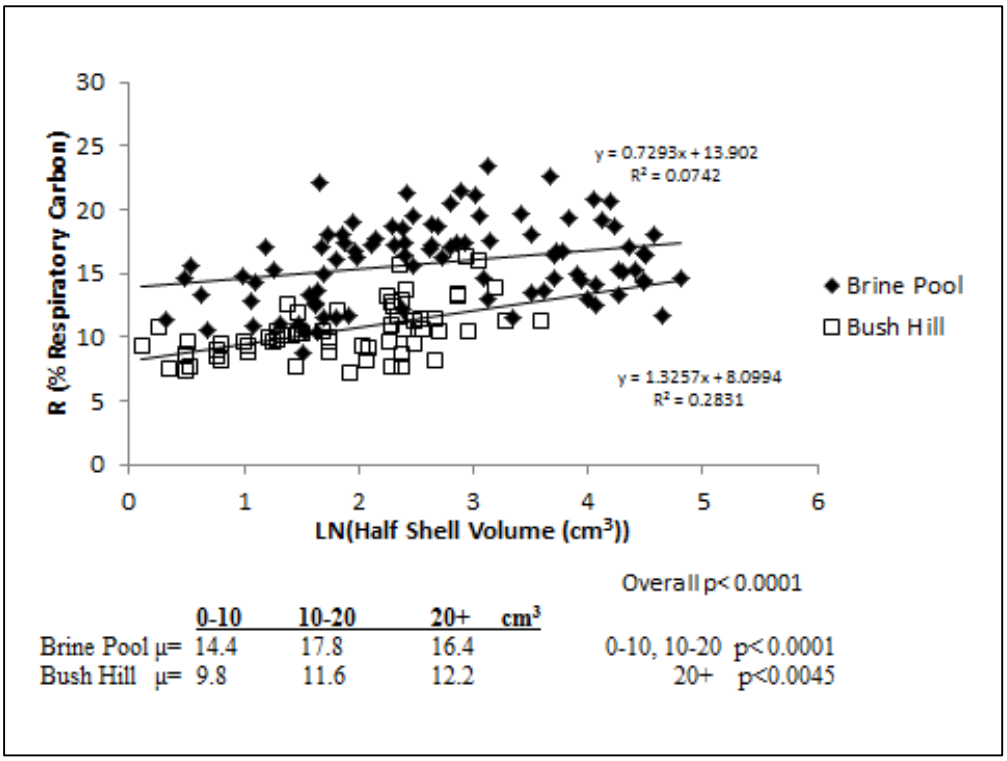

Figure 14. Percent of metabolic carbon in shell compared with size (LnHSV used).

A paired T-test between Bush Hill and Brine Pool mussel $\%C_m$ yields a statistically significant difference ($p<0.0001$) between the two sites (Figure 14). The Brine Pool (n=98) site had a mean of 15.8 % ± 3.1 and Bush Hill (n=64) had a mean of 10.5 % ± 2.1. The T-tests performed on within site differences (North and South) revealed a statistically significant difference ($p< 0.0357$) between the Brine Pool and Bush Hill sites with all sample years pooled. In individual year samplings for North versus South, the 1989 and 1991 samplings were not significant, but the 2006 sampling was significant ($p<0.0048$). There is a statistically significant difference between the two sites for $\%C_m$, but the further detailed sampling design yields few significant results and as such is not useful for the spatial analysis of $\%C_m$.

4.3 *BATHYNERITA NATICOIDEA*

The dominant seep snail, *Bathynerita naticoidea*, on the Louisiana upper slope is a heterotrophic consumer. Figure 15 displays three plots of each pair of isotopes with both mussels and snails presented. At both seeps the snails were enriched in ^{13}C and ^{15}N relative to the substrate-forming mussel population. The extent of that enrichment, however, is greater at the Brine Pool site than at the Bush Hill site. At both seeps the enrichment is not the simple 3 $\delta^{15}N$ and 1 $\delta^{13}C$ associated with source:consumer pairs (Phillips 2012). From this information an alternate interpretation is that the snail populations at each seep are using a differ suite of detrital resources. For $\delta^{34}S$, the snails showed more depleted values indicating that their detrital resource was primarily generated by sulfide microbes of the seep system rather than phytoplankton-based detritus. At Bush Hill this pattern of $\delta^{13}C$ and $\delta^{15}N$ enrichment is consistent with consumption of the same DSOM as consumed by that mussel population. At the Brine Pool it is inconsistent with the estimated detritus resources. The snails are highly mobile and demonstrated no within-seep sample effects.

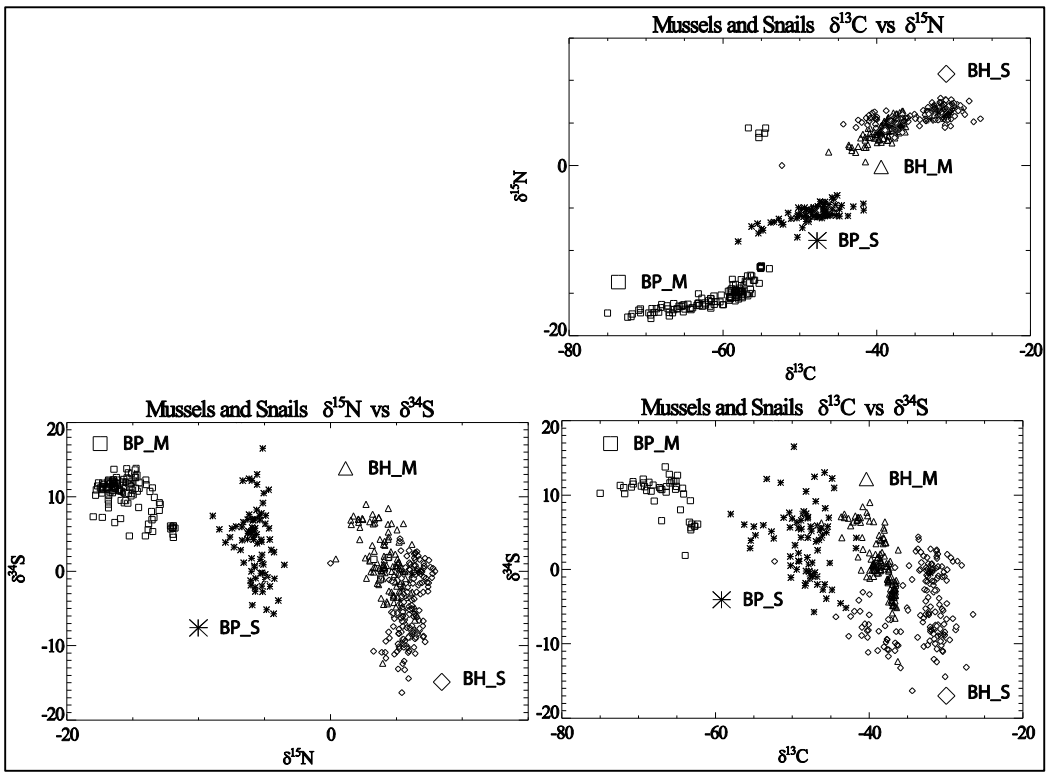

Figure 15. Plots of each pair of isotope values for all mussels (_M) and snails (_S) at Bush Hill (BH) and Brine Pool (BP).

5. DISCUSSION

5.1 ANSWERING THE INITIAL THREE QUESTIONS

In the following subsections the original three questions that this study set out to answer will be considered in light of the above results. Following that, the management relevance of the new findings are considered.

- Does the mass percent of carbon, nitrogen, and sulfur in tissue of the seep mussel *Bathymodiolus childressi* provide a useful tool for assessing the overall condition of populations?
- Does the stable isotope composition of carbon, nitrogen, and sulfur in mussel tissue provide a useful indication of trophic condition of a population that is capable of both chemosynthesis and heterotrophic feeding?
- When trophic condition of a foundation species is determined, is that condition also reflected in a consumer organism (the snail *Bathynerita naticoidea*) in the same system?

Does analysis of mussel shells have the potential of providing information on the trophic condition that might be used to assess past seepage condition?

5.1.1. C, N, & S Mass Proportions in Mussel Populations as a Condition Index

The purpose of a condition index is to provide a simple indication of an organism's overall health which can be extended to a population with appropriately designed sampling. Populations with lower condition can be suspected as being under stress. Methods applied to molluscs were reviewed in section 2.5. Previous finding of higher condition at the Brine Pool and lower condition at Bush Hill were presented in Table 1. The most common methods are best suited for fresh specimens and cannot be applied to archival material.

The finding that mass percent of C, N, and S are significantly different at the Brine Pool and at Bush Hill is an indication that the element mass method may have utility. Based on random subsamples of the data, significant differences may be tested on samples of as few as 10 specimens per seep site. The full utility of the method, however, will require experimental work to link C, N, and S mass to one of the accepted physiological method. Such experimentation can be carried out on an easily-obtained proxy for seep mussels and then confirmed based upon new sampling of seep specimens.

5.1.2. Tissue Isotopes and Trophic Mixing

Although often included in comprehensive trophic analyses, seep mussels are usually considered a proxy for a chemosynthetic food source to the larger system and their own trophic structure left unanalyzed (MacAvoy et al. 2008, Becker et al. 2010, Becker et al. 2013). The work reported herein approached the task of trophic assessment in two ways. The traditional approach using stable isotope values provided very strong indication that *Bathymodiolus childressi* population are mixotrophic rather than simply chemosynthetic. The ranges and variance of the stable isotope values established from large sample sizes at both seeps are simply two great to be explained by a single trophic pathway. A four-source mixing model was developed taking advantage of the fact that four distinct sources can be resolved given three isotope values.

The most important result of the four-source analysis comes from the estimation of trophic mixing. The data indicate that the mussels are experiencing two symbiotic pathways at each seep based on the local source of methane but different sources of nitrogen and sulfur (SM_A_{BH}, SM_B_{BH}, $SM_C_{BP,}$ and SM_D_{BP}). Previously, a single path for seep methane has been assumed. Also important is the apparent two sources of detrital food at each seep; one source reflects phytoplankton (DPHY) and the other reflects microbial chemosynthesis through thiotrophy ($DSOM_{BH}$ and $DSOM_{BP}$).

Figure 16 provides a schematic representation of the sources and their relationship to the local geochemistry. Detrital sources as circles reflect combinations of seep and water column influences. Thiotrophic detritus at the Brine Pool is greatly influenced by the very high seep ammonium levels found in the pool. Lacking that chemical resource at Bush Hill, thiotrophic microbes use seawater nitrate. Symbiotic pathways as octagons also show a distinct difference between seeps. At Bush Hill, SM_A combines seep methane with water-column sulfur and nitrogen. SM_B uses seep carbon, sulfur, and nitrogen. The difference may simply reflect the distance from the bottom where the mussel siphons. Those very near the sediment surface get the most seep products. Those a few centimeters off bottom receive a mixture. Siphon level may also be a factor at the Brine Pool but a substrate effect may be more important. The SM_A may represent the effects of feeding where the substrate is brine-saturated mud. The SM_B may be feeding in portions of the mat which extend out over brine. The over-brine source reflects the very high ammonium but only modest sulfide in the pool itself. Sulfide emitted by the mud and ammonium emitted by the brine may have such a high flux rate that siphon position has minimal effects.

The greater seepage rate at the Brine Pool is reflected in the 61% of trophic resources coming through methane pathways; detrital input is less important. At Bush Hill, only 36% comes through a methane path, and detritus is more important. Thiotrophic detritus is more important that phytoplankton. These proportions cannot be directly related to seepage rates, but they are consistent with previous conclusions that seepage is higher at the Brine Pool. High dependence on detritus may be a useful Condition Index. Phytoplankton detritus alone, however, may not be able to sustain a methane-limited population. There must be adequate microbial production of detritus via sulfide-based chemosynthesis. The data obtained from the seep snail *Bathynerita naticoidea* confirms that the trophic status of the foundation species is reflected in the food web.

It had been anticipated that simple spatial patterns would be found both within the mussel beds at Bush Hill and the ring of mussels at the Brine Pool. This expectation was based on the assumption that methane seepage must be variable across these areas. One would expect at the minimum that the outer boundaries of mussel populations might reflect the limit of a seepage feature. It was surprising that no such simple relationships were found. Resource use was definitely variable and that variation had a spatial component, but not a spatial effect that could be found, given the scale of sampling.

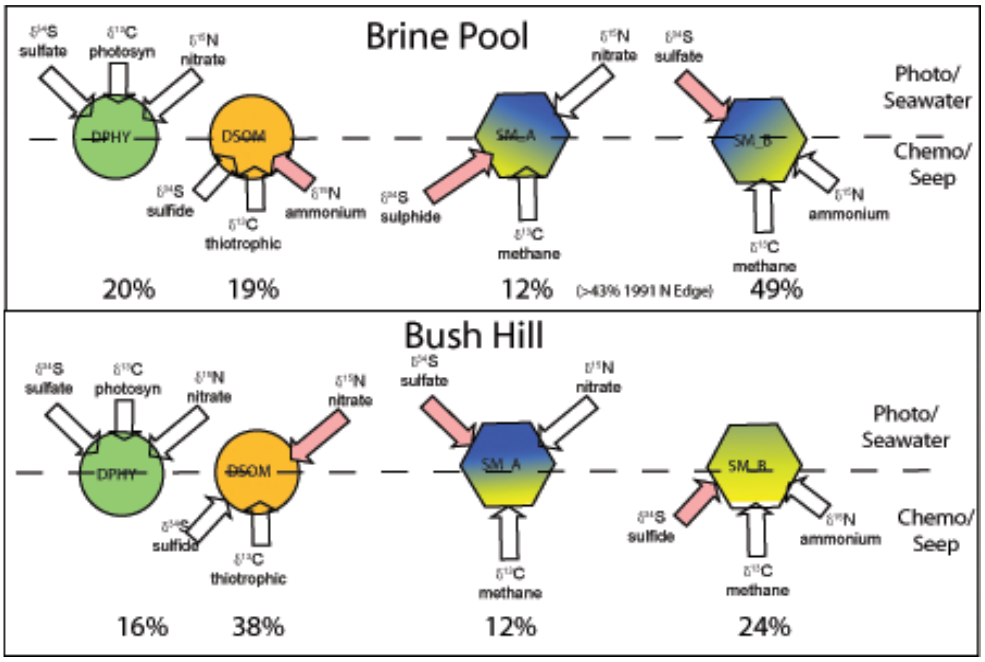

Figure 16. Trophic resources for Brine Pool and Bush Hill representing detrital (circles) and methane resources (hexagons) used by seep mussels.

The temporal analyses were the most tenuous given the limitations of using archival sampling, but the results were informative. There was significant variation over the two years (1991 and 1992) at Bush Hill Bed-1 for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ and at the Brine Pool (1989 and1991) for $\delta^{13}C$, $\delta^{15}N$, and $\delta^{34}S$ (2006 showed no significant variation for $\delta^{34}S$). Though the temporal variation was statistically significant, it is noteworthy that in 2006 the Brine Pool mussel population was still quite similar to 1991. The Brine Pool appears to be a relatively constant feature. A similar long-term comparison at Bush Hill was not possible based on the archive at Louisiana State University.

5.1.3. Shell as a Historical Record of Metabolic Stress

Assessment of status in seep systems is complicated by the fact that seepage passes through a geologically controlled sequence from initiation to termination (Roberts and Carney 1997). Seep communities also pass through stages and end in exposed or sediment-covered authigenic carbonate with no active seepage (Bergquist et al. 2004, 2005). In the search for seeps, it is very common to encounter dead systems; these often consist of a mixture of bivalve shells and authigenic carbonate gravel and there is no obvious evidence of the cause of death (Callender and Powell 1997). Is it possible that the isotopic composition of such shell material contains a record of seepage failure? This study did not carry out such a determination, but it did execute a “proof of method” study to see if respiratory methane could be detected in shells.

The significant between-seeps, between-site, and between-years incorporation of metabolic carbon into the mussel shells indicates that shells can preserve a temporal record of mussel exposure to methane flux. Sampling across shell age on both live and dead specimens may afford good indication of both the history of flux and the flux conditions at time of depth. The current study using only an edge piece of shell proved the feasibility of such an approach.

The observed variation can be interpreted as an indicator of stress. At the Bush Hill site, there is a smaller range of $\%C_m$ across shell volume, and overall mussel size is smaller than at the Brine Pool site. This indicates resource limitation at the site. This limitation and adverse effect of increased volume is observed in the mussels that range from 4–15 cm^3, where a substantial portion of the $\%C_m$ values are in the range of 7–10% and fall well below the regression for this site. The Brine Pool site mussels had a steady availability of seepage resources from 1989 to 1991; there was no change in $\%C_m$ for this time period, but in the 2006 sampling a significantly increased $\%C_m$ indicates an increase in available seep resources for that time period. The Bush Hill site has a significant increase in $\%C_m$ from 1991 to 1992, which indicates a quicker increase in resources in a more resource limited setting. These differences on both a decadal and yearly time scales demonstrate the sporadic nature of methane availability at cold seep sites. Increases and decreases of methane seepage greatly affect the productivity in populations that are reliant on it as a basal resource.

6. CONCLUSIONS AND RECOMMENDATIONS

The primary conclusion is that populations of the foundation species *Bathymodiolus childressi* are mixotrophic rather than purely methanotrophic. Its populations and the community that it supports may be resilient in the face of methane flux decrease. The mussel's varied diet is reflected in the consumers of its system. There is enough respired carbon originating from the mixed diet found in the shells for the cause of dead-mollusc assemblages to be investigated.

Working at only two contrasting seeps, we have reinforced with larger sample sizes that most mussels at a particular seep reflect local average conditions. Contrary to previous interpretation seep-to-seep differences are reflective of different tropic systems and not just different methane sources. On a smaller scale it has also been shown that some distinctly different mixotrophic conditions may exist at spatially restricted places as in the case of the Brine Pool outliers at the Northern edge.

Bathymodiolus childressi is mixotrophic with the trophic plasticity to draw form methane and detrital sources. For the short term, this may provide a degree of resilience should methane seepage decline or become highly variable. Mixotrophy also means that populations may become stressed by natural or manmade alteration of the four sources identified. In a hypothetical case of a manmade impact, offshore development may impact mussel populations via impacts on combinations of trophic sources. A population highly dependent on detritus feeding such as at Bush Hill might be more sensitive to drilling-caused turbidity. It must be noted that no industry impacts to seep systems has been identified or fully explored.

Though lacking a long time range, our observations suggest the need for some refinement of the existing model for seep development. There must be a progression from the fluid-prone initiation phase to mineral-prone methane seepage that provides a window of suitability for *B. childressi* colonization. Suitability for a mixotrophic foundation species requires that there must also be a progression in microbial sulfur geochemistry which provides an adequate thiotrophic detritus food source.

We have also shown that mussel shell retains a record of methane use. This opens up the possibility of documenting methane usage through the lifetime of individuals and reconstructing the final stages of seepage that produced large areas of shell hash in restricted areas of the Gulf.

Living mussel populations and extensive areas of dead shell, including mussels, are stages in a very poorly understood natural succession.

A valuable goal for the management of offshore resources and protection of seep systems at any stage of succession is to continue locating those systems as is now done and then provide an assessment of the status of the major foundation species. Such a status assessment is now only feasible for tubeworms. The work presented herein is a start at assessment of mussel populations and that the cause of death assemblages might be recorded in the shell material.

That succession and its indicators must be known before we can assess the potential impacts of offshore development. In order to gain better understanding, seven recommendations can be made.

1. Confirm the existence and method of feeding on thiotrophic detritus. The existence of an important chemosynthetic detritus as a food source must be tested rather than inferred from the consumers.
2. If confirmed, multiple seeps should be examined to determine the apparent stages of detritus generation.
3. Long-term monitoring of Bush Hill, the Brine Pool. or other suitable sites should be formally organized, initiated. and coordinated. Monitoring or condition and trophic resources should be done without excessive collecting of live material.
4. Both *Bathymodiolus childressi* and *Bathynerita naticoidea* survive collection and rudimentary aquarium conditions. Therefore, use of detritus by chemosynthetic mussels should be carried out under fully-controlled laboratory conditions.
5. The study of death assemblages of chemosynthetic bivalves should be extended and include reconstruction of methane incorporation from shell material.
6. Multi-site surveys should adopt protocols for within-seep spatial sampling as well as species and specimen replication so as to optimize future inter-comparisons.
7. Specimen archives should be maintained for analysis of replicates rather than the more limited archiving of taxonomic voucher specimens.

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APPENDIXES

APPENDIX A: MUSSEL AND SNAIL DATA TABLES

The appendix files consist of Microsoft® Excel® spreadsheets that may be accessed by means of the attachment menu of the final report pdf file.

Appendix A Table 1. Stable Isotope Values for Brine Pool Mussels

Appendix A Table 2. Proportions of four trophic resources for Brine Pool Mussels

Appendix A Table 3. Brine Pool Elemental Mass of Nitrogen, Carbon and Sulfur as Milligrams and Mass Percent

Appendix A Table 4. Stable Isotope Values for Bush Hill Mussels

Appendix A Table 5. Proportions of Four Trophic Resources for Bush Hill Mussels

Appendix A Table 6. Bush Hill Nitrogen, Carbon and Sulfur Mass Percent

Appendix A Table 7. Mussel Shell Half Shell Volume, δ13C Shell, δ13C Tissue (‰) and Percent Metabolic Carbon for Bush Hill and Brine Pool

Appendix A Table 8. Brine Pool *Bathynerites naticoidia* Stable Isotope Data

Appendix A Table 9. Bush Hill *Bathynerites naticoidia* Stable Isotope Data

APPENDIX B: SUPPLEMENTAL DATA ANALYSIS TABLES

The appendix files consist of Microsoft® Excel® spreadsheets that may be accessed by means of the attachment menu of the final report pdf file.

Appendix B Table 1. Brine Pool TukeyHSD post hoc Analyses of Position Effects on Stable Isotope Content of Mussel Tissue

Appendix B Table 2. Brine Pool TukeyHSD post hoc Analyses with Year, Position and Interaction Effects on Stable Isotope Content of Mussel Tissue

Appendix B Table 3. Bush Hill TukeyHSD post hoc Analyses with Position Effects on Stable Isotope Content of Mussel Tissue

Appendix B Table 4. Bush Hill TukeyHSD post hoc Analyses with Position, year and interaction Effects on Stable Isotope Content of Mussel Tissue

Appendix B Table 5. Bush Hill TukeyHSD post hoc Analyses of Four Mussel Tissue Trophic Source Positions with Position Effects

Appendix B Table 6 Brine Pool ANOVA with Position Effects in Stable Isotopes of Mussel Tissue with Five Outliers Excluded

Appendix B Table 7. Brine Pool TukeyHSD post hoc Analyses of four Mussel Tissue Trophic Source Positions with Position Effects

Appendix B Table 8. Brine Pool TukeyHSD post hoc Analyses of four Mussel Tissue Trophic Sources with Year, Position and Interaction Effects

Appendix B Table 9. Bush Hill TukeyHSD post hoc Analyses of four Mussel Tissue Trophic Sources for Bed 1 with Year, Position and Interactions Effects

The Department of the Interior Mission

As the Nation's principal conservation agency, the Department of the Interior has responsibility for most of our nationally owned public lands and natural resources. This includes fostering the sound use of our land and water resources; protecting our fish, wildlife, and biological diversity; preserving the environmental and cultural values of our national parks and historical places; and providing for the enjoyment of life through outdoor recreation. The Department assesses our energy and mineral resources and works to ensure that their development is in the best interests of all our people by encouraging stewardship and citizen participation in their care. The Department also has a major responsibility for American Indian reservation communities and for people who live in island communities.

The Bureau of Ocean Energy Management Mission

The Bureau of Ocean Energy Management (BOEM) works to manage the exploration and development of the nation's offshore resources in a way that appropriately balances economic development, energy independence, and environmental protection through oil and gas leases, renewable energy development and environmental reviews and studies.