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The effects of spatial variance, temperature and pH on bacterial community composition in the sea anemone, *Anemonia viridis*.

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Abstract

Marine organisms are well known to have associations with microorganisms and little is known about the specific nature of the microorganism host interactions. This study investigates the bacterial community composition in the sea anemone *Anemonia viridis* using Denaturing Gradient Gel Electrophoresis; bacterial community composition was assessed for spatial variance. The results show that there was no significant variation within a location or between the locations. These results are concordant with previous studies and suggest that bacterial populations are stable in *Anemonia viridis*.

Introduction

Marine invertebrates are ubiquitous throughout the world, as are the microorganisms that live symbiotically within them. Marine organisms are well known to have specific relationships with numerous microorganisms (Thakur & Müller, 2005) and little is known about the microbial relationship and diversity associated with such marine organisms (Menezes *et al*, 2010). What is known however, is that in many ecosystems these complex microbial communities are extremely important components (Ainsworth, Thurber, & Gates, 2010; Menezes *et al*, 2010). Even though interactions between bacteria and invertebrates are common in the marine environment, the bacterial mode of association (mutualism, commensalism or parasitism) and particular physiological functions are often unidentified (Schuett & Doepke, 2009).

Sea anemones, corals, jellyfish and hydras are all part of the phylum Cnidaria (Sabourault *et al*, 2009), Cnidaria can be found globally and in a multitude of depths and environmental conditions. The characteristics of Cnidaria include a sac-like body with a single oral opening surrounded by tentacles (Sabourault *et al*, 2009). Apart from the distinct characteristics of their anatomy they all exhibit a mutual relationship with numerous microorganisms. It has been suggested that functions of microorganisms to the host involve autotrophy, nitrogen fixation, nitrification, nutrient cycling in a modified version of the marine microbial loop and the participation in the host chemical defence system against predators and epibionts (Thakur & Müller 2005; Kellogg, 2004).

Investigations by Barott *et al*, (2011); Olson & Kellogg, (2010) and Ritchie, (2006) have suggested that microorganisms in corals are host-specific, and Wegley *et al*, (2007) hypothesised that organisms can adapt to differing ecological niches by 'switching' their microbial associates. Rohwer *et al*, (2002) describe in their investigation how they found that the microorganisms are not only host-specific in corals but also stable and would be maintained over space and time. Hentschel *et al*, (2006) confirmed this in sponges and described temporal and biogeographical variation to be minor.

Sea anemones possess endobiotic bacteria which harbour within the column, tentacles and the mucus surface layer (Schuett & Doepke, 2009). Investigations into the diversity of microorganisms found within sea anemones have reported a wide variety of heterotrophic bacteria (Du *et al*, 2010). Schuett & Doepke (2009) conducted an investigation where their aim was to find the pathogenic potential of endobiotic bacteria, and they found that eighty one percent of the species or groups were host-specific.

With the search for new sources of natural products increasing (Larsen *et al*, 2005; Menezes *et al*, 2010), it is vital to fully understand how these microorganisms vary within the organisms and between geographical location, how stable they are with environmental changes and the roles they exhibit to the host organisms. In this investigation the bacterial community composition was analysed in *Anemonia viridis*. Variation within and between different geographical/ spatial destinations were related to the following hypothesis; bacterial community composition will not vary within a site or between spatial destinations.

Materials & Methods

Sample Locations

There were five sample locations all within the British Isles, UK. Three were on the south coast (one Cornwall & two Devon) and two sites were on the north coast of Cornwall as shown in Fig. 1

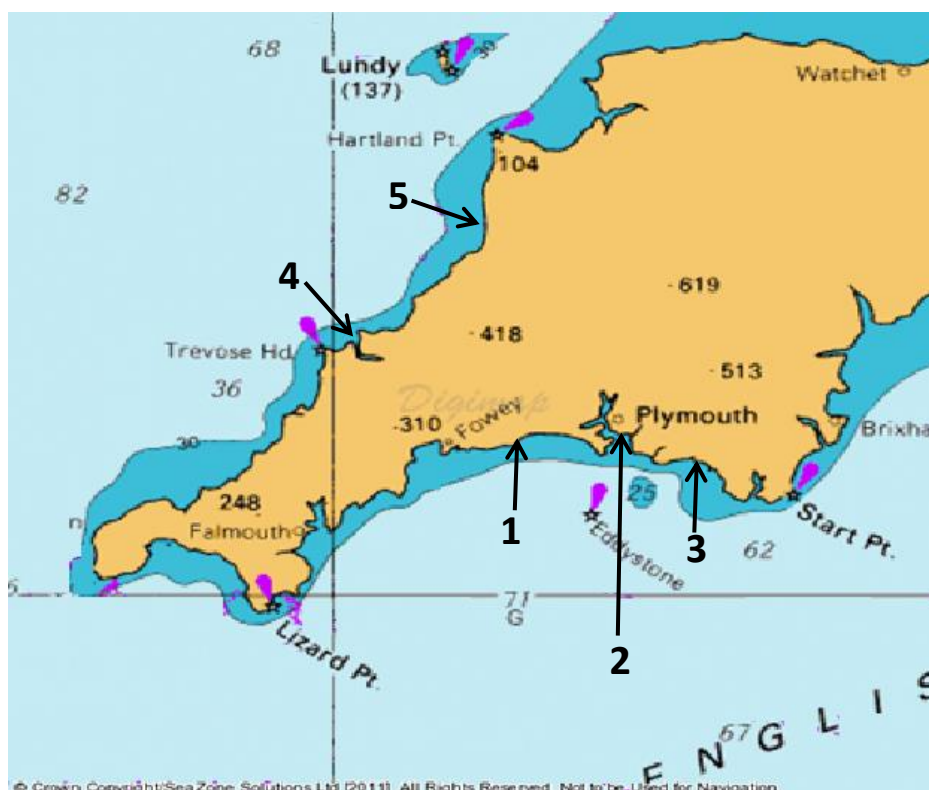


Fig. 1: The five sample site destinations - 1: Hannaford Point, Looe ($50^{\circ}20'39.5''N$, $4^{\circ}27'20.23''W$) 2: Mount Batten Bay, Plymouth ($50^{\circ}21'33.0''N$, $4^{\circ}7'53.0''W$) 3: Bigbury-On-Sea ($50^{\circ}16'53.1''N$, $3^{\circ}53'56.72''W$) 4: Hayle Bay, New Polzeath ($50^{\circ}34'47.41''N$, $4^{\circ}55'18.50''W$) 5: Bude Bay, Bude ($50^{\circ}50'15.83''N$, $4^{\circ}33'22.9''W$) (<http://digimap.edina.ac.uk/marineclassic/map.action?scale=general>).

Collection and process of samples

Before collection all of the sampling equipment (forceps, scissors and Micro centrifuge tubes (MCC), an example of a set for one collection site) was sterilised in an autoclave at $121^{\circ}C$ for 15 min at 15 psi. All sampling was conducted during September – October 2011 and samples were taken from a lower shore transect across the length of the beach, from different rock pools ranging in size 0.5 – 1.0 m. From each site three samples of *A. viridis* were taken. Only brown morphs of the species were collected from the sample locations. Five tentacles from each of the sample organisms were cut and placed into sterile, labelled MCC tubes. The samples were kept on ice until returning to the laboratory (within four hours of collection), where they were flash frozen in liquid nitrogen and stored at $-20^{\circ}C$. Temperature, salinity, light intensity and O_2 were all measured from each of the rock pools where samples were collected, the weather remained the same and the tidal range varied within 0.5 m at low tide on all of the collection days and times.

DNA Extraction

DNA was extracted using QIAGEN's DNeasy blood & tissue kit (QIAGEN, UK). The protocols for animal tissues and all chemicals used were issued by the supplier and followed precisely with one exception (the proteinase K incubation period was extended by 13 h). The samples were fully defrosted to room temperature. Once fully defrosted 20-25 mg of the tentacles were cut from each sample and weighed on a balance using sterile scissors. Each of the cut samples were placed in to a new sterile MCC tube and 180 µl of buffer ATL was added. The samples were then homogenised using a pencil homogeniser in their individual MCC tubes, 20 µl of proteinase K was added to each and mixed by vortex. The samples were then incubated at 56°C for 16 h (overnight, until they were fully lysed) whilst occasionally being vortexed. At the end of the 16 h incubation period each of the samples were vortexed for 15 s, 200 µl of buffer AL was added, vortexed thoroughly then 200 µl of ethanol (96-100%) was added and mixed thoroughly again. The mixtures were pipetted into a DNeasy mini spin column inside a 2 ml collection tube then centrifuged at 6,000 x g for 1 min, the flow-through and collection tubes was discarded after centrifugation. Each of the spin columns were placed in to new 2 ml collection tubes and 500 µl of buffer AW1 was added then each of the samples were centrifuged for 1 min at 6,000 x g. Again the flow-through and collection tubes were discarded. The spin columns were placed into new 2 ml collection tubes and 500 µl of buffer AW2 was added and then centrifuged for 3 min at 20,000 x g. The flow-through and collection tubes were discarded carefully so the spin column did not come into contact with the flow-through. The spin columns were transferred to new 1.5 ml MCC tubes and 200 µl of buffer AE was added for elution and incubated at room temperature for 1 min. They were then centrifuged for 1 min at 6,000 x g, a final elution and centrifugation was repeated for maximum yield. The DNA yield was measured on NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. USA). All DNA extractions were then stored at -20°C until required.

Polymerase Chain Reaction (PCR)

The process for PCR and the primers used were taken from Muyzer *et al*, (1993). PCR uses enzymes to amplify the variable V3 region of 16S rDNA (Mühling *et al*, 2008). The nucleotide sequences were as follows, the reverse primer 1, 5'-ATTACCGCGGCTGCTGG-3' and the forward primer 2, 5'CGCCCGCCGCGCGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGA GGCAGCAG-3'. Primer 2 contains a 40-nucleotide GC-rich sequence (GC clamp). Both primers were used as a combination to amplify the 16S rDNA regions in the various bacterial species which correspond to positions 341 to 534 in *E.coli* (Muyzer *et al*, 1993). A single PCR reaction volume was 30 µl consisting of; 15 µl 2x DNA Taq ready-mix (Bioline Reagents Ltd), 1 µl reverse primer 1, 1 µl forward primer 2, 3 µl Template DNA and 10 µl of Mol.Bio grade H₂O and were all vortexed for 5 s. Once the PCR reactions were made they were processed using a PCR amplification machine/ thermal cycler (GeneAmp PCR system 9700) with the following protocols; the temperature was raised to 65°C (10°C above the expected annealing temperature) for 1 min. This temperature was then decreased by 1°C every second cycle until it reached 55°C where five additional cycles then occurred, each cycle first denatures the DNA (creating single stranded DNA) and then anneals the complementary sequence by pairing hydrogen bonds to produce a polynucleotide of the required DNA. This procedure reduces spurious by-products forming which can

occur during the amplification process (Muyzer *et al*, 1993). Primer extension was carried out at 72°C for 3 min.

Amplified products were analysed on a 10cm 1.5% agarose gel by electrophoresis. The agarose gel was made by mixing 90 ml of 1xTAE buffer and 1 g of agarose then boiling the solution (no crystalline structures seen in the solution). The solution was then left in a bath at 45°C for 30 min. Nancy-520 stain (9 µl) (Sigma-Aldrich, USA) was added just before pouring and then the gel was left to set for 30 min. Once the PCR thermal cycler had finished its process 6 µl of 6x concentration loading buffer (4 ml glycerol, 6 ml 1x TE and 25 mg bromophenol blue) was added to each of the PCR reactions and vortexed for 1 s. The agarose gel was added to the buffer (1x TAE, enough to cover the gel) in the electrophoresis machine. Each of the PCR reactions were pipetted into the agarose gel wells (8 µl), the gel was then electrophoresed at 90 V for 45 min. The gel was then analysed for the presence/absence of bands using Quantity one analysis software V4.6.3 (Bio-Rad laboratories, CA, USA).

Denaturing Gradient Gel Electrophoresis (DGGE)*

The Denaturing Gradient Gel Electrophoresis protocols were based on Muyzer *et al*, (1993). Before the gels could be poured all of the reagents and equipment were prepared for an 8% gradient (enough for two gels). The 6x concentration loading buffer (4 ml glycerol, 6 ml 1x TE and 25 mg bromophenol blue) and the 50x TAE buffer (242.3 g of 2M tris base, 18.6 g of 50 mM EDTA di-sodium salt, approx. 57 ml glacial acetic acid and pH to 7.8 with additional glacial acetic acid) were made. The running buffer (150 ml 50x TAE and 7 ltr of MilliQ H₂O) was added to the running tank and pre-heated to 60°C.

The two denaturant reagents were made next; stock 80% denaturant polyacrylamide solution consisted of 26.7 ml of 30% acrylamide mix (high purity acrylamide), 2 ml of 50x TAE buffer, 32 ml molecular grade formamide (Sigma-Aldrich, USA), 34 g of 5.6M ultrapure urea (Sigma-Aldrich, USA) and 5.3 ml of MilliQ H₂O. Stock 0% denaturant polyacrylamide solution consisted of 26.7 ml of 30% acrylamide mix (high purity acrylamide), 2 ml of 50x TAE buffer and 71.3 ml MilliQ H₂O. The gel plates were cleaned with acetone and assembled in the clamps. For the making of the gels HI denaturant solution (12 ml of 80% denaturant stock acrylamide, 4 ml of 0% denaturant stock acrylamide and 250 µl of 6x loading buffer) and a LO denaturant solution (8 ml of 80% denaturant stock acrylamide and 8 ml of 0% denaturant stock acrylamide) were made. Prior to pouring 100 µl of fresh 10% ammonium persulphate (electrophoresis grade, Sigma-Aldrich, USA) and 10 µl of TEMED (N,N,N tetramethylethylenediamine) was added to each of the HI and LO denaturant solutions then they were loaded into syringes and mounted to the gradient maker.

The T-shaped tube was attached to each of the syringes and the needle end taped to the gel plates, the gradient maker wheel was turned slowly to avoid the formation of bubbles in the gel, finally the comb was gently inserted and this process was repeated for the second gel. The gels were left for 1.5 h to set. Once the gels had set they were loaded into the running machine where 15 µl of the PCR reactions were pipetted into the corresponding wells, once the gel wells had been loaded the electrophoresis machine ran for 17 h at 65 V at 60°C. After the run time had completed the gels were removed and placed in trays which contained the SYBR Gold staining buffer (200 ml of 1x TAE and 20 µl of SYBR Gold; Invitrogen, UK),

trays were placed onto a shaking platform in a dark incubator at room temperature for 30 min.

*The gels were mixed and poured by the laboratory Technician (Matthew Emery) for health and safety reasons.

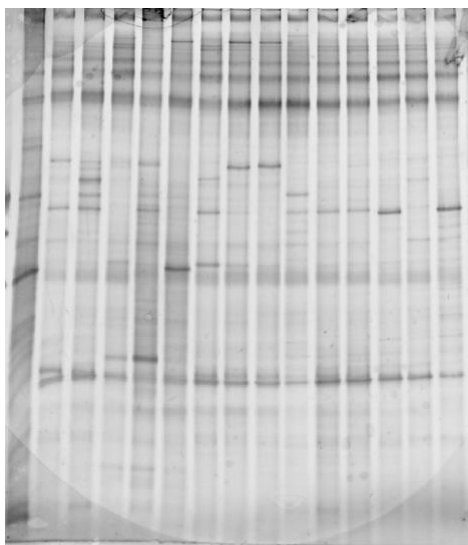
Statistical analysis

DGGE band patterns were transformed into band intensities and a presence/absence matrix for assessment of different sites using Quantity one analysis software V4.6.3 (Bio-Rad laboratories, CA, USA) was constructed. Similarity percentages (SIMPER), a one-way analysis of similarity (ANOSIM) and a Bray-Curtis similarity matrix were used to represent the relative similarities between sites. Margalef's d , and the Shannon–Weaver diversity index were used to calculate species richness and species diversity respectively using Primer 6 software V6.1.13 (Clarke & Warwick 2001). A one-way anova with post hoc LSD was used to test the significance (Merrifield *et al*, 2010) of the total band presence for each site, the peak densities for each site and from the North and South Coast using Minitab 16 V16.1.1 (Minitab Ltd, UK).

Results

Spatial variation analysis of bacterial populations

There were 40 different bacterial phylotypes/ presumed species present in the DGGE analysis which can be seen in Fig. 2, some of which were present throughout the samples while others were not. A Dendrogram and a Multi-Dimensional Scaling analysis (Fig. 3) represent the similarity between the bacterial populations from the PCR-DGGE fingerprints found within each of the 3 samples at each of the 5 site locations. These fingerprints show there is no significant relationship between any of the samples. A summary table representing species similarity (SIMPER) within the sites and pairwise comparisons (ANOSIM) between the sites from the PCR-DGGE fingerprints is shown in Table 1. The SIMPER analysis shows the bacterial profiles to be on average from 55.93% to 82% similar.



1a 1b 1c 2a 2b 2c 3a 3b 3c 4a 4b 4c 5a 5b 5c

Fig. 2 PCR-DGGE fingerprints of samples from different locations, numbers represent the sites and letters represent which sample from each site.

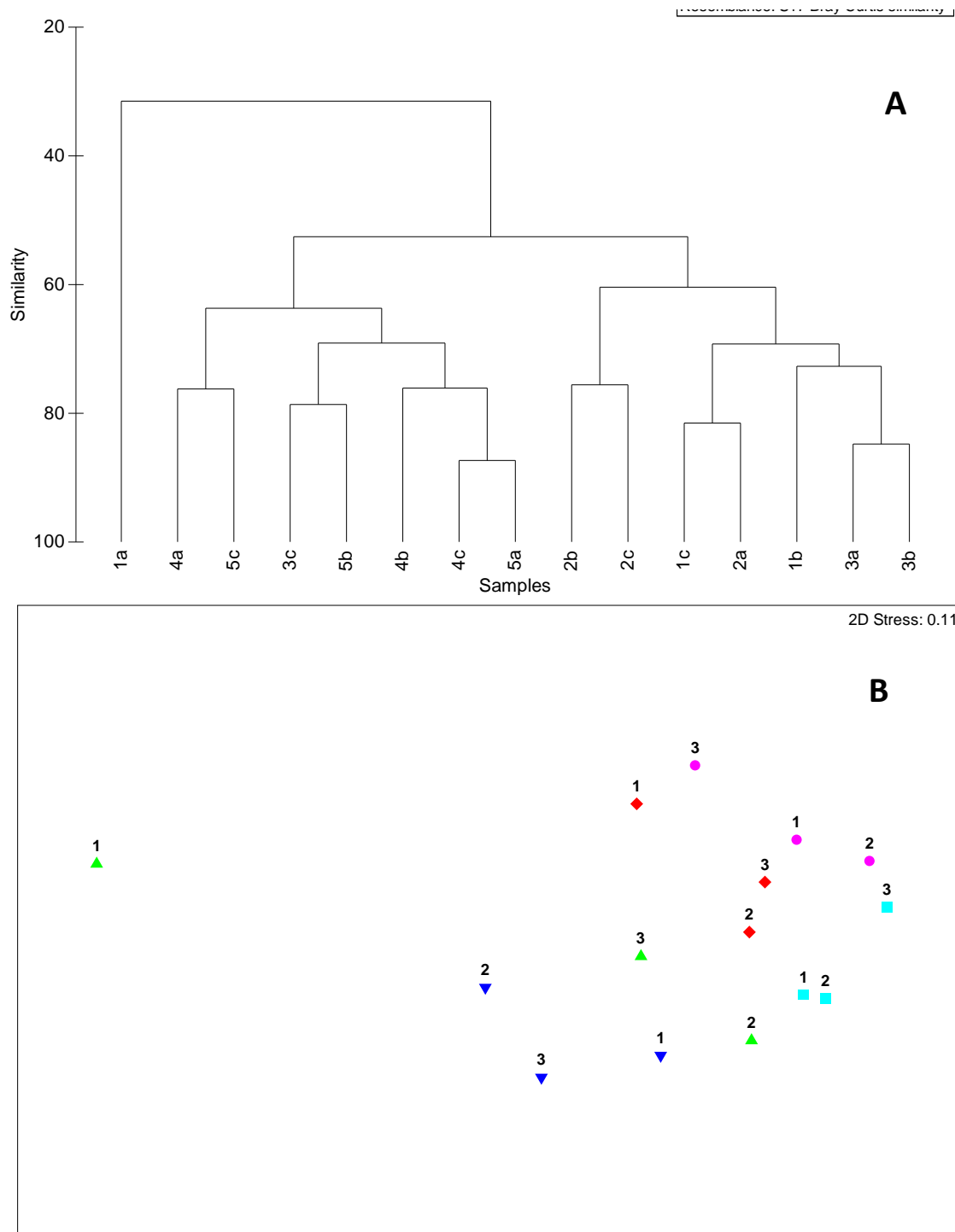


Figure 2: A: Dendrogram of PCR-DGGE fingerprints (Bray Curtis Similarity) showing samples from different locations; numbers represent the sites and letters represent the sample from each site. B: Multi-Dimensional Scaling analysis (Bray Curtis Similarity, 2D Stress: 0.11), ▲ = Site 1, ▼ = Site 2, ■ = Site 3, ◆ = Site 4 & ◆ = Site 5. The numbers on each point represent the sample within the site.

Table 1: Bacterial community analysis from PCR-DGGE fingerprints of *A. viridis* tentacles from 5 different Sites.

Site	N*	Richness [#]	Diversity ^{**}	Simpser Similarity (%)	ANOSIM		
					R-value	P-value	Dissimilarity (%)
1	20.33 ± 3.79	1.76 ± 0.32	2.99 ± 0.20	55.93			
2	21.67 ± 2.52	1.87 ± 0.21	3.07 ± 0.12	70.61			
3	18.33 ± 2.08	1.59 ± 0.17	2.90 ± 0.12	82.01			
4	20.33 ± 0.58	1.75 ± 0.05	3.01 ± 0.03	75.52			
5	20.67 ± 2.31	1.77 ± 0.19	3.02 ± 0.12	74.05			
Pairwise comparisons							
1, 2					-0.074	0.900	34.79
1, 3					0.407	0.100	39.87
1, 4					0.222	0.200	37.43
1, 5					0.519	0.100	41.62
2, 3					0.852	0.100	36.86
2, 4					0.926	0.100	37.21
2, 5					1.000	0.100	40.62
3, 4					0.667	0.100	29.13
3, 5					0.741	0.100	30.42
4, 5					0.148	0.300	27.11

SIMPER, similarity percentage within sites; ANOSIM, analysis of similarities between sites.

*Average number of bands present/assumed species. Values expressed as means ± standard deviation

[#]Margalef species richness: $d = (S-1)/\log(N)$. Values expressed as means ± standard deviation

^{**}Shannon-wiener diversity index: $H' = -\sum(p_i \ln p_i)$. Values expressed as means ± standard deviation

Discussion

This study examined the relationship between bacterial communities and the host organism *A. viridis*. This is the first time an investigation has tried to link the stability of bacterial communities with this sea anemone.

There have been several studies in recent years which have identified bacteria within host organisms and investigations into corals and sponges have been covered considerably. Investigations into the diversity of bacteria within corals, sponges and anemones have found that mainly heterotrophic bacteria reside within the host organism (Thakur & Müller, 2005; Hentschel *et al*, 2006; Wegley *et al*, 2007; Bourne *et al*, 2009; Schuett & Doepke, 2009; Du *et al*, 2010; Barott *et al*, 2011; Gates & Ainsworth, 2011).

Studies in the past decade have shown that the bacteria which reside within organisms are species specific (Rohwer *et al*, 2002; Hentschel *et al*, 2006; Ritchie, 2006). These investigations have not only shown that they are species specific, but

many have concluded that the bacterial communities were stable in temporal and spatial variability (Rohwer *et al*, 2002; Hentschel *et al*, 2006; Anderson *et al*, 2010).

In this study PCR-DGGE fingerprints were used to survey the bacterial composition within *A. viridis*. The results from the spatial variance analysis showed there were no significant differences within a site; all three samples collected from one location had bacterial communities which resembled each other. The results also indicated no significant difference between the locations, all of the bacterial communities being similar. The DGGE gel did show signs of varying bacteria within some of the samples but this was statistically insignificant.

DGGE is a valid method for identifying species presence but it does have limitations. Sekiguchi *et al*, (2001) found that a single band does not always represent a single bacterial strain in DGGE. The results in this investigation could be affected by this, and sequencing of individual bands present *could* have identified bands where this has occurred. Mühling *et al*, (2008) also suggests using a nested PCR approach and utilising more primers in the application of the PCR process providing a higher resolution genetic fingerprint.

This investigation failed to reject the null hypothesis; the results from this investigation proved positive as they corroborate with previous studies; bacterial communities are not affected by spatial variance and the number of species present is a stable community for *A. viridis*.

To fully understand the extent of these effects, further research is required, such as sequencing, which is required to get a clearer picture of which microorganisms reside within the tentacles of *A. viridis* so that roles of the bacteria can be investigated. It may be useful to combine studies of anemones and corals to evaluate the bacterial species present, whether they are present in both, if so how they contribute to the health of the organism; if not how they might differ. Utilising other methods in conjunction with the DGGE analysis e.g. Terminal Restriction Fragment Length Polymorphism (TRFLP) and Fluorescent In Situ Hybridization (FISH) could produce more quantifiable results.

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