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Dynamics of microbial community in the marine sponge *Holichondria* sp.

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Abstract

Marine sponges often harbor communities of symbiotic microorganisms that fulfill necessary functions for the well being of their hosts. Microbial communities are susceptible to environmental pollution and have previously been used as sensitive markers for anthropogenic stress in aquatic ecosystems. Previous work done on dynamics of the microbial community in sponges exposed to different copper concentrations have shown a significant reduction in the total density of bacteria and diversity. A combined strategy incorporating quantitative and qualitative techniques was used to monitor changes in the microbial diversity in sponge during transition into polluted environment.

Introduction

Sponges are known to be associated with large amounts of bacteria that can amount to 40% of the biomass of the sponge. Various microorganisms have evolved to reside in sponges, including cyanobacteria, diverse heterotrophic bacteria, unicellular algae and zoochlorellae(Webster et al., 2001b). Since sponges are filter feeders, a certain amount of transient bacteria are trapped within the vascular system or attached to the sponge surface.

Microbial communities are susceptible to different environmentral pollution agents and have previously been used as sensitive markers for anthropogenic stress in aquatic ecosystems(Webster et al., 2001a). It is possible that shifts in symbiont community composition may result from pollution stress, and these shifts may, in turn, have detrimental effects on the host sponge. The breakdown of symbiotic relationships is a common indicator of sublethal stress in marine organisms. Perhaps the most well known example of symbiont-host response to temperature and pollution stress is the 'bleaching' effect in corals, where symbiotic pigmented dinoflagellates (zooxanthellae) are expelled from coral polyps, leaving only the transparent coral tissue covering a white calcareous skeleton.

The introduction of molecular approaches has identified the diversity of such microbes, and these techniques have allowed the recovery of whole communities of environmental microbes, including some that are unculturable. Previous studies have used 16S rDNA sequences to clarify the symbiotic relationships between eubacteria and *Halichondria*.

One sponge can harbor diverse symbionts, including heterotrophic bacteria, cyanobacteria, green sulfur bacteria, and archaea.

The current study aimed to survey and compare the microbial community structure associated with *Halochondria* sp., and to characterize the dynamic changes in the microbial symbiont population due to the effect of pollution.

Material and methods

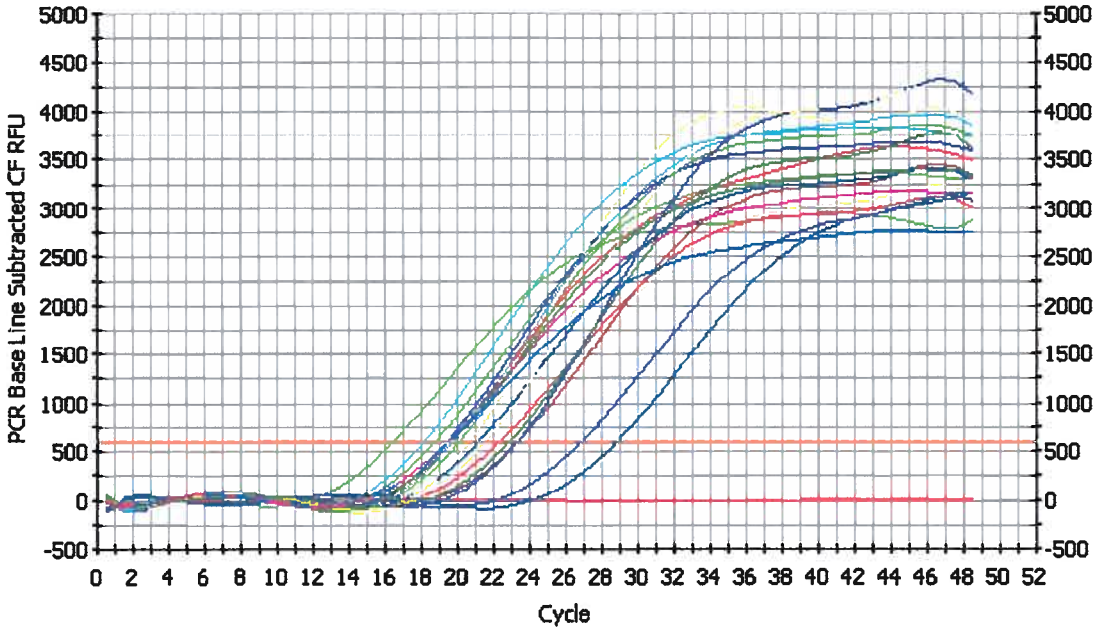
Sponges and DNA extraction

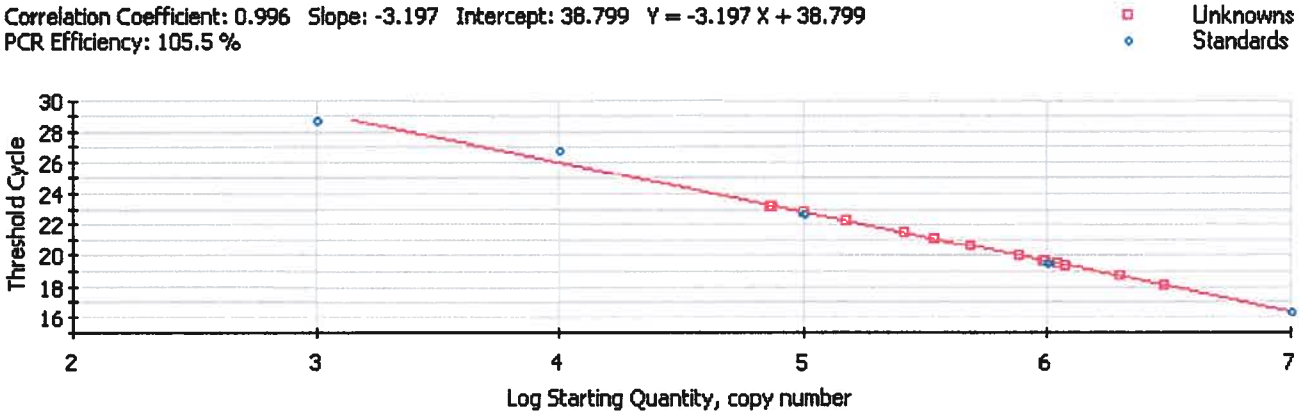
Halichondria sp. was collected by the marine resource center. The sponge was transferred to the laboratory on ice divided into two and stored at marine running seawater and Eel pond seawater. DNA was extracted from 1 g (wet weight) of each section.

Determination of total bacteria

The sponge tissue was weighed, minced with a razor blade and homogenized using a homogenizer. The tissue homogenates were diluted with sterile seawater from 10^{-1} to 10^{-5}. A 100-μl aliquot of each dilution was plated in triplicate onto SWC agar and colonies were counted after 1-week incubation at room temperature. To determine the total number of bacteria, a defined volume of the tissue homogenate was fixed with an equal volume of 8% paraformaldehyde/phosphate-buffered saline (PBS) and stored at 4°C until use. The tissue homogenate was centrifuged for 5 min at 13000 rpm, washed two times with PBS and fixed in an ethanol (70%). DAPI (4,6-diamidino-2-phenylindole) was added to a final concentration of 5 μg ml^{-1}. A volume of 1 ml was filtered onto a black, 25-mm-diameter, 0.2-μm polycarbonate membrane, which was supported by a 0.45-μm cellulose nitrate filter. Vacuum was applied with a hand pump. The filters were washed two times with sterile seawater. Bacterial numbers were determined following microscopical inspection. Three independent samples were processed per sponge. For each sample an average bacterial number from 10 different counting fields was determined.

Quantitative-PCR (Q-PCR) was performed using a Bio-Rad iCycler iQ real time detection system(Bio-Rad, Hercules CA). 25µl reactions were prepared in 96 well plates using Bio-Rad iQ SYBR green supermix, eubacterial primers at 1µM, and 1-5µl template DNA. primers selected to amplify a small fragment 100-200bp in length. For all experiments a hot start 2-step amplification reaction was run with 20 sec denaturation at 95°C, 60sec annealing and extension at 60°C cycled 50 times. All samples run in duplicate including standard curve, which ranged from $5x10^7$ targets/reaction to $1x10^7$.





Bacterial rDNA amplification and terminal-restriction fragment length polymorphism (T-RFLP) analysis

Bacterial 16S rDNA was partially amplified with 21F, and labeled at the 5' end with FEM. The PCR reaction was performed in a thermal cycler with an initial denaturation step of 95 °C for 5 min followed by 30 cycles with the temperature profile, 95 °C for 1 min, 55 °C for 1 min, 72 °C for 30 sec, ending with a final extension step of 72 °C for 7 min. Each amplicon was digested by a four-cutter restriction enzyme *Hha*I and analyzed with an automated sequencer (ABI 3100).

Fluorescence in situ hybridization (FISH)

A tissue core was punched out from the center of the sponge with an ethanol-sterilized scalpel blade. For hybridization sections were fixed with 4% formamide for 12h In situ hybridizations were performed as described previously(Preston et al., 1996).
Oligonucleotide probes were used at a concentration of 50 ng of labelled probe μl^{-1} hybridization buffer. The slides were rinsed, air-dried and mounted in Citifluor. Examination was done with a Zeiss microscope. Digital image processing was performed using the software Adobe Photoshop.

Denaturing gradient gel electrophoresis (DGGE)

Genomic DNA was extracted using the Fast DNA Spin Kit for plant according to the manufacturer's instructions and stored at 4°C. The universal primers 349F with the GC-clamp and 519R were used for 16S rDNA amplification. PCR was performed as follows: one initial denaturation step for 5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 55°C, 30sec at 72°C; and one final elongation step for 7 min at 72°C. DGGE was performed using a Bio-Rad DCode Universal Mutation Detection System on a 10% (w/v) polyacrylamide gel in 0.5×TAE and using a 20–80% denaturing gradient. Electrophoresis was performed for 6 h at 120 V and 60°C. Gels were stained for 30 min in aqueous ethidium bromide solution (0.5 $\mu g\ ml^{-1}$) and photographed with a GelDoc system (Gel Doc 2000, Bio-Rad).

Results and discussion

Quantitative analysis

Counting of bacterial numbers tissue extracts revealed $5x10^7$ bacteria g^{-1} sponge tissue. Only 0.1% of the bacterial population was culturable on SWC agar plates. These numbers are consistent with estimates that >99% of the microorganisms in the environment cannot be cultivated on laboratory media. The total bacterial numbers and culturable bacterial units (CFUs) remained unchanged over the time course of the experiment irrespective of the maintenance conditions.

TABLE 1.

	Sea water	Eel pond water
CFU/ml	960(11)	910(2)
Direct counting	$5x10^7$	$2x10^7$
Quantitative PCR	$8.08x10^7$–$6.84x10^7$	$8.08x10^7$–$6.29x10^7$

DGGE and T-RFLP analysis

Because the same specimen can be sampled repeatedly without visible injury or reduction in pumping activity, it is possible to monitor the changes of an individual sponge. Two independent molecular approaches were employed to describe microbial community changes in sponges over time and with respect to maintenance conditions. Bacterial community structures were assessed using a T-RFLP analysis. Profiles were generated from the digestion of fluorescent-labeled PCR fragments using the restriction enzyme *Hha*I. tRFLP analysis confirmed that *Holichondaria* sp. hosts a highly diverse microbial community with different operational taxonomic units The DGGE analysis from EPW-maintained sponges resulted in complex profile. When the banding pattern of freshly collected sponges was compared, it is noticeable that sponge fragments shared more similarities than compared to the two other samples, which was taken 3 days later. When the sponges were maintained in regular seawater, the DGGE pattern remained largely unchanged. When maintained in Eal pond water, there was a noticeable appearance of additional bands at *t*=3 days which coincided with the tRFLP. Molecular techniques used previously to assess the effects of pollution on microbial communities have revealed both decreases and increases in diversity. In this research a significant increase in the microbial community diversity was detected after incubation both in sea and Eel pond water for 3 days.

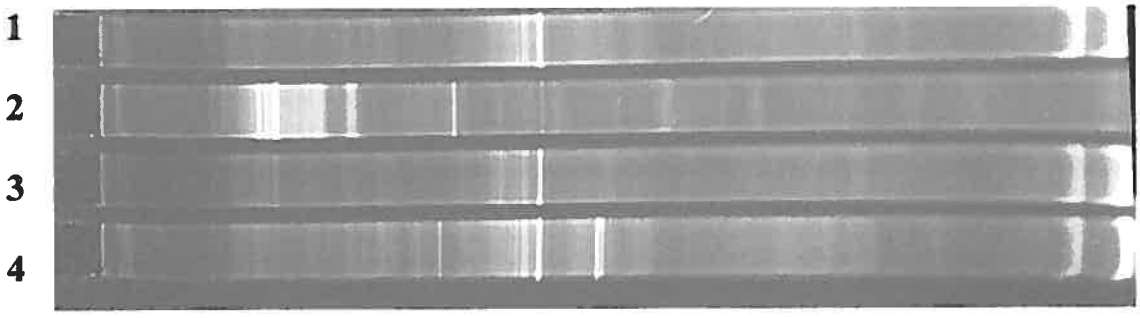


FIG. 3. DGGE fingerprints of bacterial assemblages obtained from *Holichondria* sp. at different times (1, t=0; 2, t=3 incubation in eel pond water, 3,t=0; 4, t=3 incubation in sea water).

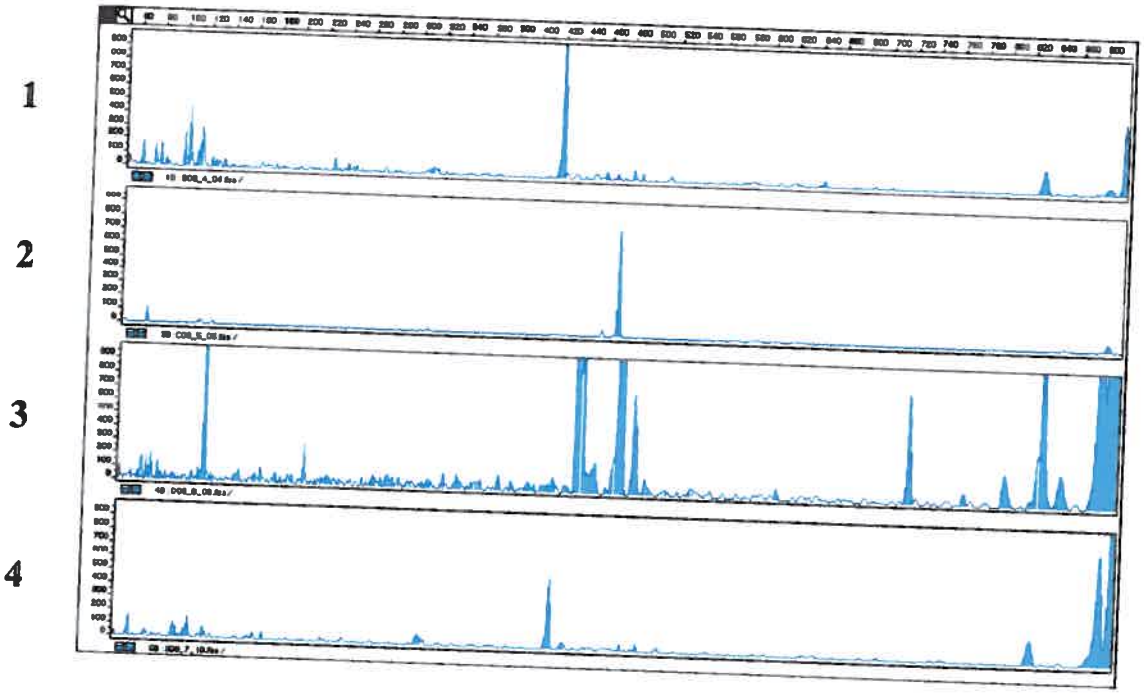


FIG. 4. TRFLP profiles of *Hae*I-digested 16S rDNAs amplified from *Holichondria* sp. at different time points (1, t=0; 2, t=3 incubation in eel pond water, 3,t=0; 4, t=3 incubation in sea water).

FISH analysis

FISH has been widely used to confirm the presence of particular organisms in specific habitats and to describe microbial diversity of natural samples. Autofluorescence of the sponge tissue (possibly related to the large number of microscopic collagen fibrils in this species) hampered precise cell enumeration, especially with the bacteria-specific probe (EUB338), which revealed an extremely high density of cells in uncontaminated sample . However the application of the EUB338 FISH probe clearly demonstrated an abundant signal both in t=0 and t=3 both in seawater and Eal pond water. In contrast the application of the ARCH915 probe did not result in highly signal at time 0. However samples taken from the EPW treatment at t=0 detected with the general EUB338 probe showed a large fraction of non-detected cells. It is tempting to speculate that these cells may represent a fraction of the newly encountered archeal or eukaryote population.

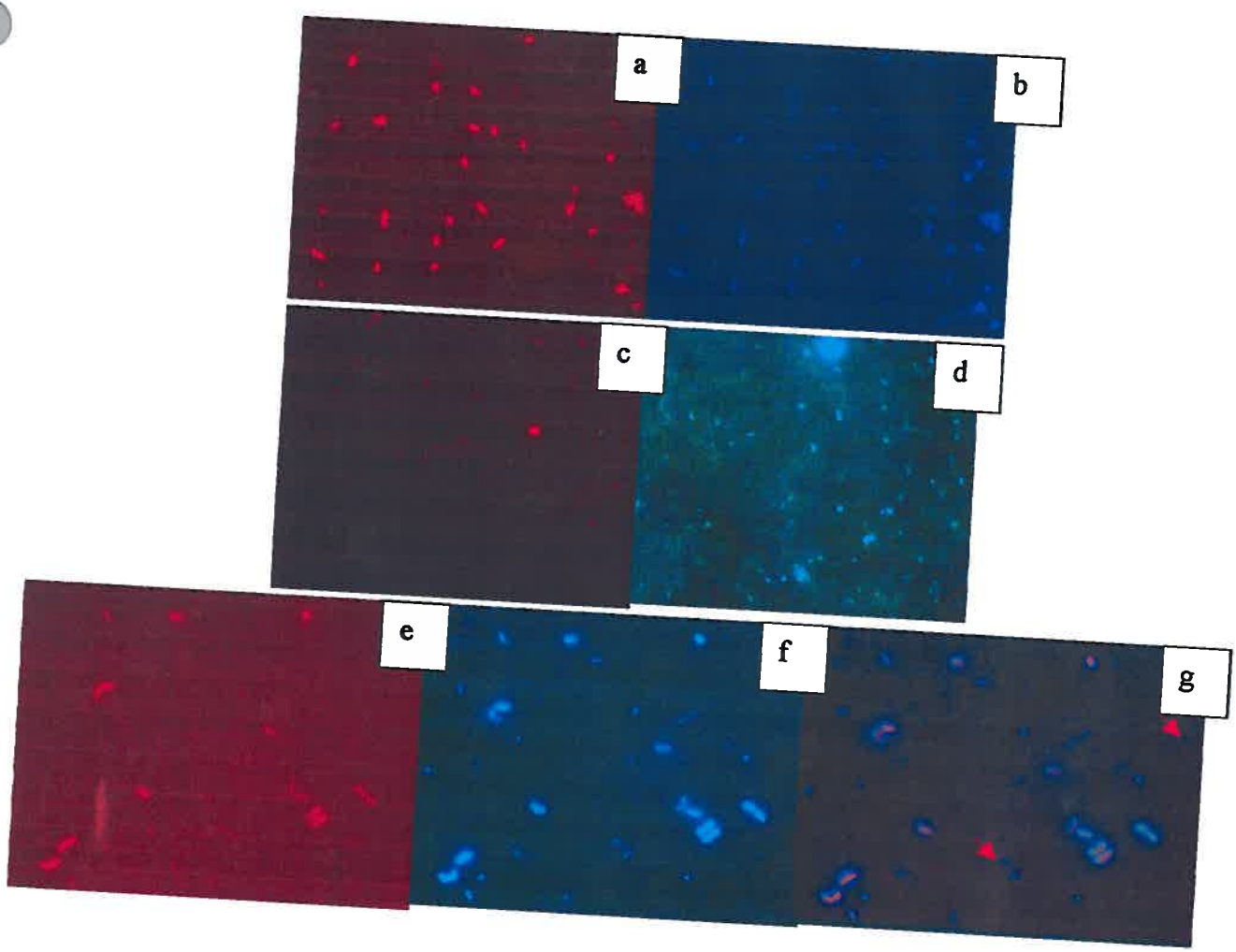


FIG. 6. Photomicrographs of FISH-stained marine bacteria and archaea. Each panel depicts DAPI staining in blue (right) and probe staining in red (left). a,e FISH with the general bacterial probe EUB338. c, FISH with the archaea probe ARCH915. g , superimposed figure of e and f, red dotes shows cells that did not stained with EUB338.

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