ABSTRACT: Consideration of the trophic interactions that link species requires study of the decomposition process to reveal different pathways for nutrient regeneration, elemental recycling, and microbial production in the water column. Presently there is scarce literature on tunicate decomposition due to the difficulties in identifying and quantifying zooplankton carcasses in the natural environment. Nevertheless, the presence and activity of Gammaproteobacteria, Alphaproteobacteria and Sphingo-Flavobacteria are being assessed during decomposition of the filter-feeding tunicate, Dolioletta gegenbauri. Gammaproteobacteria often increase in micro- and mesocosms, Alphaproteobacteria are more abundant in increased salinity, and Sphingo-Flavobacteria increase with levels of high molecular weight. Culture-independent techniques are powerful tools to analyze the shifting population dynamics of bacterial communities during degradation of benthic and pelagic tunicates. Combined, the data obtained from these bacterial decomposition studies can contribute insights into the nutrient pathways between filter-feeding zooplankton and microbial communities following tunicate and plankton blooms.

KEY WORDS: Bacteria, Tunicates, Decomposition

INTRODUCTION

Phytoplankton are microscopic single-celled plants (Smith and Johnson 1997) that dominate the epipelagic region (Smithsonian 2009) and grow abundantly in the oceans. They are the foundation of the marine food web and a source of oxygen for other oceanic life. As phytoplankton die they are colonized and decomposed by bacteria releasing hydrocarbons into the water. Continual degradation results in oxygen depletion which is often fatal to marine organisms. According to research by the US Environmental Protection Agency, algal production is correlated to the levels and ratios of nitrogen and phosphorus in the water; concentrations of 0.03 to 0.1 mg/L phosphate or higher will likely trigger blooms (NOAA/EPA 1988). Excessive blooms can result in problems for other organisms and degrade water quality (Levinton 2001), especially the accumulation of harmful toxins. Ecological balances must be maintained in order to control recurrent population explosions.

Zooplankton are free floating or weakly swimming animals that rely on currents for distant travels (MESA 1999). Zooplankton range from microscopic copepods to larger jellies and colonial salps (Corey and Beutel 2009). They are predators of phytoplankton and have a wide range of feeding habits which include filter feeding, predation and symbiosis with autotrophic phytoplankton (MESA 1999). After assimilation, zooplankton release fecal matter containing phytoplankton detritus into the water. Conover (1964) reported this percentage of
assimilation by zooplankton to range from an estimated 6% to 99%. Phytoplankton are able to convert carbon dioxide or bicarbonate and inorganic nitrogen and phosphorus into the organic elements of their cells (Pomeroy et al., 2007) and can have concentration factors surpassing $10^5$ for certain metals (IAEA 1985). Zooplankton consumption of these organisms and fecal release can influence the cycling of many elements (Fisher et al. 1991, Reinfelder and Fisher 1991), hence contributing immensely to the microbial loop.

**Microbial Loop**

The microbial loop is a subtle addition to the traditional food web. In the loop, energy and carbon are channeled via bacteria to protozoa, larger zooplankton, fishes and cetaceans (Pomeroy et al. 2007). This loop involves the circulation of energy and matter among marine microbes based on dissolved organic matter-DOM (Odum and Barrett 2005). Several autochthonous contributions of marine DOM are derived from direct algal exudation, zooplankton sloppy feeding and excretion, passive leakage, viral lysis, bacterial release and solubilization of particulate organic matter-POM (Siuda and Chróst 2002; Azam and Malfatti 2007; Suksomjit et al. 2009).

**Tunicates**

Tunicates, gelatinous zooplankton, are classified in the Phylum Chordata and Subphylum Urochordata. They are filter feeders that are capable of clearing large volumes of water daily, ingesting a wide range of sizes and phytoplankton assemblages. Doliolids, pelagic tunicates, are ecologically important. Their decomposition contributes to the DOM pool, thus transporting a considerable portion of productivity to the marine microbial food web through the regeneration of nutrients and recycling of elements (Tang et al., 2009). As the tunicate carcass decomposes, shifts transpire within the bacterial population.

![Image 1. Picture of a Dolioletta gegenbauri nurse.](image)

**Bacteria**

Aquatic heterotrophic bacteria are the main users of DOM. They are minute with ~$12fgC$ ($1.2 \times 10^{-14}$ g C) per cell; however, because of their large surface area-to-volume ratios, they are able to dominate DOM assimilation in the oceans (Kirchman 2008). Oxic ocean surface waters are dominated by heterotrophic bacteria groups such as Proteobacteria and Bacteriodetes (Kirchman et al. 2003). The objective of this research was to assess the presence and activity of two Proteobacteria groups—alpha and gamma, and one complex group in the Bacteriodetes—the Sphingo-Flavobacteria. Gammaproteobacteria often increase in micro- and mesocosms, while Alphaproteobacteria are more abundant in high salinity waters. SAR11, a subgroup of the $\alpha$-proteobacteria, often account for large fractions of prokaryotes in coastal and open oceans, and Sphingo-Flavobacteria abundance correlates with levels of high organic molecular weight. The goal of this study was to evaluate the abundance of Alphaproteobacteria, Gammaproteobacteria and Sphingo-Flavobacteria during doliolid decomposition.
METHODS

Doliolid decomposition and Bacteria community

Mortality was induced by gently stabbing the Dolioletta gegenbauri tunicates with a glass pipette. The intact carcasses, 114.5 µg C, were then transferred into a 2 L jar containing pre-filtered seawater. Water samples were collected at 0, 6, 12, 18, 38, 68, and 88 hours. Bacterial abundance and community composition were analyzed by using epifluorescence microscopy with 4’, 6-diamidino-2-phenylindole (DAPI) and specialized for Fluorescent In-Situ Hybridization (FISH). Probes used were Alf968 and SAR11: alphaproteobacteria; Cf319a: Sphingo-Flavobacteria; and Gam42a: gammaproteobacteria.

PCR and Bacteria Identification

Genomic DNA of the bacteria community was prepared using Qiagen DNeasy Blood & Tissue Kit. A segment of 16S rRNA gene was amplified using Qiagen Taq PCR Mastermix. Primers were 27f (5’-AGA-GTTTGATCCTGGCTCAG-3’) and 1492r (5’-TACGGYTACCTTGTTACGACTT-3). Polymerase Chain Reaction (PCR) products were resolved in 1% agarose in 1x Tris-Acetate-EDTA buffer. Bands equivalent to 1465 bp were excised and eluted using the Bio-Rad Freeze and Squeeze Purification Kit. The bands were cloned using the Invitrogen TOPO TA Cloning Kit. Nucleotide sequencing is currently in progress at Functional Biosciences, Inc.

RESULTS

DAPI Counts (4’, 6-diamidino-2-phenylindole)

(Fig 1). At the initiation of the decomposition study, the bacterial abundance was 408 thousand cells/mL. Within 38 hours, the bacteria grew significantly to reach the high peak of 3.89 million cells/mL. The second highest abundance was recorded with 1.79 million cells/mL at 68 hours of decomposition.
Fluorescent In Situ Hybridization

(Fig 2). At the initial stage the Cf group represented 7% of the community composition. 38 hours into the study revealed a slight increase to 10% and a drastic decline down to 1% by 68 hours of decomposition.

(Fig 3). The α-proteobacteria had the smallest abundance initially, characterizing less than 1% of community composition. Its highest abundance was 4% by 38 hours. Similarly to the Cf group, the α-proteobacteria declined by 68 hours of decomposition.
Figure 4. SAR 11 clade % probe positive cells.

(Fig 4). SAR 11, a subclass of the α-proteobacteria, was the dominant bacterial group, initially, representing 36.2% of the community composition. However, this group declined tremendously to less than 1% within the first 38 hours of decomposition. Nonetheless, by 68 hours the bacterial abundance increased slightly to 7.6% of the community.

Figure 5. Gamma-proteobacteria % probe positive cells.

(Fig 5). γ-proteobacteria represented the only bacterial group to demonstrate exponential growth from 7.4% to 15.4% and up to 22.1%.

DISCUSSION

Bacteria demonstrate physiological versatility which is evident in the decomposition results. With nutrients available, such as the 114.5 µgC from the doliolid detritus, bacteria grow. However, once the nutrients are depleted bacteria show a starvation-survival response (González et al. 1993). At the initiation of the study, the SAR 11 clade was the dominant bacterial group. Conversely within the next 38 hours,
the clade drastically declined to less than 1% of the community composition (Figure 4). It is speculated that within the macrocommunity of the jar, the SAR11 group suffered from bacterial starvation due to their minute size (1/2 the size of other bacteria), fewer ribosomes and limited substratum (Malmstrom et al., 2004). The γ-proteobacteria, on the other hand, exhibited a consistent, exponential growth pattern in the detrital waters (Figure 5). These bacterial types are conceivably more efficient decomposers; therefore implying that during the tunicate decomposition, the γ-proteobacteria contributed to the organic matter (DOM) release necessary for growth of SAR11 clade (Figure 4).

The cf cluster and the α-proteobacteria demonstrated a characteristic three-phase growth model (Figures 2 and 3)—the 1st being the Lag phase of slow growth, followed by the Log phase of exponential growth, and finally the 3rd phase termed Logarithmic Decline where the bacterial cells die faster than they are replaced (Fankhauser 2008) Observations of the cf group show enrichment on high particulate organic detritus in marine habitats, while the α-proteobacteria favor low molecular weight compounds such as amino acids or glucose (Cottrell and Kirchman 2000).

In addition to nutrient requirements, the bacterial groups were also subjected to competition and predation. In any natural environment where there is a diverse collection of bacteria, competition over space and resources is expected (Hibbing et al. 2010). In the same natural community, predation is common as well. Nanoplankton flagellates, size range of 2 to 20 µm, are major grazers of bacteria (González et al. 1993). Although for this study the seawater was pre-filtered, it is possible that a few nanoplankton slipped through the pores thus consuming the bacterial cells.

**Future Work**

Experiments for the 88 hour time point are still in progress—PCR, FISH, Cloning and Sequencing. Afterwards, analysis of the bacterial clones and identification of those communities will be conducted.

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