

**GROWTH RATES OF ABUNDANT MARINE BACTERIAL CLADES
IN PURE CULTURES AND IN THE DELAWARE ESTUARY**

by

Thomas St Julien Lankiewicz

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Marine Studies

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ABSTRACT

Ratios of 16S rRNA to 16S rRNA genes (rRNA:rDNA) have been used to assess the contribution of bacterial taxa to total community growth and carbon cycling. However, interpretations of rRNA:rDNA ratios is based upon a limited number of studies with rapidly growing bacteria. The most abundant bacteria in the oceans probably grow more slowly than those bacteria whose rRNA:rDNA versus growth rate relationships are known. To understand how rRNA:rDNA varies in abundant marine bacteria, I used quantitative PCR and reverse transcriptase quantitative PCR to measure rRNA:rDNA in bacteria known to be abundant in coastal Delaware waters and elsewhere. Four marine isolates were examined including *Ca. Pelagibacter ubique* HTCC1062, a coastal isolate of SAR11, the most abundant bacterial clade in the oceans. In culture, there were significant relationships between rRNA:rDNA and growth rate for some strains but not for others. The rRNA:rDNA ratios determined along a transect in the Delaware estuary suggested that oligotrophic bacteria grew up to ten-fold faster than copiotrophic bacteria in the same communities. I find that rRNA:rDNA ratios can be useful for estimating growth rates in some bacterial taxa and that knowledge of the rRNA:rDNA versus growth rate relationship for a given taxon can enhance interpretations of rRNA:rDNA data from natural communities.

Chapter 1

INTRODUCTION

Growth rates of bacteria and other microbes are important to understanding marine food web processes and biogeochemical cycles. The abundance and activity of bacterial taxonomic groups depend on the growth rate as well as top-down control by grazing and viral lysis. Survival strategies using rapid growth might co-exist with strategies of slow growth due to trade offs between growth rate and defensive specialization (Våge *et al.*, 2013, 2014). Bacterial taxonomic groups that grow rapidly likely transfer more carbon up the food chain than other slow growing taxa (Azam *et al.*, 1983). These rapidly growing bacteria may also respire more rapidly, contributing more to atmospheric CO₂ flux than slow growers (del Giorgio & Cole, 1998).

There are few estimates of growth rates of specific taxonomic groups in natural communities, although average rates for the entire community have been explored more extensively. Growth rates of entire natural communities are estimated to range from 0.05 d⁻¹ in oligotrophic systems to 0.3 d⁻¹ in coastal waters, as calculated from leucine incorporation and cellular abundance (Kirchman *et al.*, 1982; Ducklow, 2000). These rates are unlikely to represent rates for individual taxa due to the broad diversity of bacterial survival strategies among bacteria, leading to various combinations of

activity and abundance in natural communities (Jones & Lennon, 2010; Campbell *et al.*, 2011; Lennon & Jones, 2011). Estimates of growth rates of individual taxonomic groups in natural communities range from 0.13-0.73 d⁻¹ in the North Atlantic and as high as 6.1 d⁻¹ in communities from estuarine environments (Malmstrom *et al.*, 2004; Yokokawa *et al.*, 2004). These estimates are based on leucine incorporation detected by microautoradiography coupled with fluorescent *in situ* hybridization (FISH) or by net changes in abundance detected by FISH in incubations that exclude grazers. Both methods rely on incubations, which probably alter the natural state of the community. Additionally, the phylogenetic resolution of these estimates is limited because the oligonucleotide probes used for FISH in previous studies recognize broad phylogenetic groups at the class or even phylum level (Amann *et al.*, 1995).

Ratios of rRNA:rDNA in bacterial taxa is one method that potentially could yield growth rates estimates at higher phylogenetic resolution (Campbell *et al.*, 2009, 2011; Campbell & Kirchman, 2013). Since ribosome content is positively correlated with growth rate for many bacteria, and because 16S rDNA sequence similarity is used to define taxonomic groups (DeLong *et al.*, 1989), rRNA:rDNA could be used to link an estimate of growth to specific bacterial taxa (Kerkhof & Ward, 1993). Early studies on ribosome content and growth focused on model enteric bacteria such as *E. coli* and *Salmonella typhimurium* (Kjeldgaard *et al.*, 1958; Schaechter *et al.*, 1958; Harvey, 1970; Bremer & Dennis, 1987), while later studies examined more diverse bacteria such as *Desulfovibrio vulgris* and *Rickettsia prowazekii* (Poulsen *et al.*, 1993; Pang & Winkler, 1994). These bacteria often grow at rates more than 100-fold

faster than estimates of the mean growth rates of natural bacterial communities in the oceans (Ducklow, 2000). Some studies have linked rRNA content of marine bacteria to growth rate in culture, but several of these isolates are unidentified, and others are not closely related to the most abundant types of marine bacteria (Kemp *et al.*, 1993; Kerkhof & Ward, 1993; Fegatella *et al.*, 1998). The growth rates of these marine bacteria are also high compared to the estimated growth rates of most natural bacterial communities (Ducklow, 2000).

Kemp *et al.* (1993) found that the relationship between rRNA versus growth rate varied among four unidentified isolates, which has implications for interpreting cellular rRNA content in natural communities. Many studies interpret rRNA per cell as a measure of bacterial activity without knowledge of rRNA content versus growth rate relationships of community members (Blazewicz *et al.*, 2013). Some of these studies provide valuable information on relative activity of taxonomic groups over time or space (Campbell *et al.*, 2009), but they were unable to report actual growth rates. Calibration of rRNA:rDNA levels to specific growth rates could enable interpretation of rRNA:rDNA ratio data as actual growth rates and allow for more robust comparisons of ratios between taxonomic groups in natural communities.

The goal of this study was to examine rRNA:rDNA of bacterial isolates in culture and then to apply the determined rRNA:rDNA to growth rate relationships in a series of natural communities. I chose four taxa that are abundant in coastal Delaware and elsewhere, are genetically diverse, and are thought to use contrasting survival strategies. *Pelagibacter* is a representative of the SAR11 clade of

Alphaproteobacteria, the most abundant bacterial taxon in the oceans (Morris *et al.*, 2002; Rappé *et al.*, 2002; Brown *et al.*, 2012). Some studies have described SAR11 as relatively slow growing in nature, while others suggest that it grows no more slowly than the average community growth rate (Malmstrom *et al.*, 2004; Teira *et al.*, 2009; Campbell *et al.*, 2011). *P. ubiquus* HTCC1062 grows more slowly than other bacterial isolates in culture and has a streamlined genome (1.3 Mbp), characteristic of an oligotroph (Rappé *et al.*, 2002; Giovannoni *et al.*, 2005, 2014). Another oligotroph, the gammaproteobacterium SAR92 HTCC2207, has the second smallest genome of the four isolates (2.0 Mbp), and grows slowly relative to most isolates, but faster than *P. ubiquus* (Cho & Giovannoni, 2004). *Ruegeria pomeroyi* DSS-3 is a copiotroph in the Alphaproteobacteria class with a relatively large genome (4.1 Mbp) and one megaplasmid (0.5 Mbp) (Moran *et al.*, 2004). *Ruegeria* has genes for rapid growth, attachment to particles, and several other metabolic strategies that suggest it is a copiotroph (Gonzalez *et al.*, 2003; Moran *et al.*, 2004). *Polaribacter* sp. MED152 is a coastal Flavobacterium, a class that has been implicated in the degradation of high molecular weight organic matter (Cottrell & Kirchman, 2000). The genome (3.0 Mbp) of MED152 predicts copiotrophic attributes as it encodes for genes involved in attachment to particles and polymer degradation (Gonzalez *et al.*, 2008). My results indicate that the rRNA content versus growth rate relationship was variable for each of the four bacteria.

Chapter 2

METHODS

Batch Culture Growth Conditions and Sampling

Batch cultures were grown in acid washed polycarbonate bottles, in triplicate, in the dark, and at 18 °C. To monitor cellular abundance, cells stained for 1 h with 5x SYBR Green I were counted using a FACSCaliber flow cytometer (BD Biosciences). Samples for DNA and RNA were preserved in equal volumes of RNA Later, filtered onto 0.22 µm GVWP membranes (Millipore), and stored in Buffer RLT (Qiagen) at -80 °C. I extracted nucleic acids using the All Prep DNA and RNA extraction kit (Qiagen).

Ca. Pelagibacter *ubique* and SAR92 were grown on variations of the AMS1 media described by Carini et al. (2013). The medium for *Pelagibacter* contained low concentration (100 µM) bicarbonate with a 10 mM HEPES buffer addition. SAR92 was grown on AMS1 salts with the organic carbon and vitamin additions described by Steindler et al. (2011). *Pelagibacter* and SAR92 were started from glycerol stocks, and after repeated growth to high density, each experimental culture was started with 300 mL of exponentially growing cells. For the experiment, the volume for *Pelagibacter* and SAR92 cultures was 20 L and mixed by slow bubbling with 0.22

μ m-filtered air. Nucleic acid sample volumes (1 L) were also identical for *Pelagibacter* and SAR92.

Liquid cultures of *Polaribacter* were started from colonies on an agar plate, and *Ruegeria* was rehydrated from a freeze-dried stock in YTSS medium. After transferring *Ruegeria* several times, 3 L cultures on YTSS were started with 100 mL of exponentially growing cells. Volumes for *Ruegeria* nucleic acid samples ranged from 3-50 mL depending upon the density of the culture at time of sampling.

Ruegeria and MED152 cultures were shook continuously at 100 rpm for aeration and mixing. Difco Marine Broth 2216 was combined with AMS1 salts as liquid media for MED152 (Gonzalez *et al.*, 2008; Carini *et al.*, 2013). The volume of cultures for starting experiments was 50 mL for MED152. Experimental cultures were 1.5 L, and volumes for MED152 nucleic acid samples ranged from 10-100 mL.

Quantifying rDNA and rRNA by qPCR and RT-qPCR

Quantitative PCR (qPCR) and reverse transcriptase quantitative PCR (RT-qPCR) were used to quantify copies of genes and transcripts of 16S rRNA. I measured template DNA and RNA concentrations using PicoGreen and RiboGreen assays, and template was then diluted to below 500 pg/ μ L and re-quantified. All qPCR reactions were done on a RotorGene 6000 (Corbett Robotics), using SYBR Green qPCR and RT-qPCR reaction kits (Qiagen). Reactions were 12.5 μ L and

completed in duplicate. Standards were prepared linearized plasmids from clones of known sequence. Reaction conditions for thermal cycles were 55 °C for 10 min (RT-qPCR only), 95 °C for 10 min, 95 °C for 15 sec, X °C for 15 sec, 72 °C for 15 sec, followed by 72 °C for 10 min, and a melt analysis ramping from 72-100 °C. The 15 sec steps were repeated for 45 cycles, and X varied by primer set. For *Pelagibacter* X=62 °C, SAR92 X=60 °C, *Ruegeria* X=61 °C, and MED152 X=60 °C. Reaction efficiencies ranged from 93 to 102%, and reaction efficiencies and y-intercepts did not vary between corresponding qPCR and RT-qPCR reactions.

Primer design and specificity

Taxon-specific primers were designed for the 16S rRNA gene sequences of *Ca. Pelagibacter ubique* HTCC1062 (NR_074224.1), SAR92 HTCC2207 (AY386335.1), *Ruegeria pomeroyi* DSS-3 (NR_028727.1), and *Polaribacter* sp. MED152 (DQ481463.1) (Table 1). I designed primers in Oligo 7 using the GreenGenes database to minimize amplifying 16S rRNA gene sequences of other bacteria (DeSantis & Hugenholtz, 2006). Specificity was confirmed *in silico* using ARB-SILVA TestPrime (Klindworth *et al.*, 2013) and tested using DNA extracted from a mixed community in the Delaware estuary as template. The single product resulting from each PCR reaction was sequenced. BLAST analysis of the cloned sequences indicated that each primer set retrieved its intended 16S rRNA target. The

mean percent identity of the amplicons to their target sequence was 98.9% for *Pelagibacter* (number of clones (n)=12), 98.5% for SAR92 (n=12), 93.7% for *Ruegeria* (n=36), and 97.4% for MED152 (n=36).

Table 1 Sequences of primers for qPCR and RT-qPCR, listed 5' to 3'.

	<u>Forward</u>	<u>Reverse</u>
<i>Pelagibacter ubiquus</i>	GGCCTGGAATAACACGAGGAA	GGGCTCATCCAATGGTGCATA
SAR92 HTCC2207	GCGGCCACCTGGACTAAT	TGCGCCACTAAGAGATCAAGT
<i>Polaribacter</i> MED152	GCGGATTAGAAAGTTAGGGGTG	TCGCCACTGGTGTCTCTCC
<i>Ruegeria pomeroyi</i>	TGGGCAATGGAGGTAACTCT	AGCCGGTCCTTATTCTTACAG
All bacteria*	CGGTCCAGACTCCTACGGG	TTACCGCGGCTGCTGGCAC

*Sequences from Lee *et al.*, (1996) and Delbes *et al.*, (2000).

Conversion of RNA:DNA Data from Previous Studies to rRNA:rDNA

To compare data from my culture experiments to previous measurements of cellular RNA content, I converted RNA:DNA values in the literature to rRNA:rDNA. Most data in previous studies was reported as RNA:DNA versus growth rate, as determined by various methods including mass, calorimetric assays, and fluorometric assays. I assumed that 90% of the total cellular RNA was rRNA for all bacteria tested

(Chen & Duan, 2011). In addition, I assumed that the fraction of rDNA in the total DNA pool was equal to the number of base pairs in a bacterial 16S rRNA gene, multiplied by the copy number of the 16S rRNA gene, then divided by the total base pairs in the genome. For bacteria that had unknown 16S rRNA copy number or genome size, I assumed a copy number or genome size based on close phylogenetic relatives of the isolates (Herbert, 1961; Kemp *et al.*, 1993).

The data for the following bacterial taxa were analyzed to calculate rRNA:rDNA: *E. coli* (Rosset *et al.*, 1966; Harvey, 1970; Bremer & Dennis, 1987), *Salmonella typhimurium* (Kjeldgaard & Kurland, 1963; Rosset *et al.*, 1966; Bremer & Dennis, 1987), *Aerobacter aerogenes* (Caldwell *et al.*, 1950; Neidhardt & Magasanik, 1960; Rosset *et al.*, 1966; Sykes & Young, 1968), *Delsulfovibrio vulgaris* (Poulsen *et al.*, 1993), *Rickettsia prowazekii* (Pang & Winkler, 1994), *Sphingomonas* sp. strain RB2256 (Fegatella *et al.*, 1998), *Pseudomonas stutzeri* Zobell (Kerkhof & Ward, 1993), and several unidentified marine isolates (Kemp *et al.*, 1993).

Transect of the Delaware Bay

The Delaware Bay was sampled November 18-22, 2013 (PAPI 6). I sampled 24 stations with salinities ranging from 0.3 to 31.5 PSU. At each station environmental data, as well as DNA and RNA samples, were collected. Samples for nucleic acids were collected by filtering whole seawater onto 0.22 μm GVWP membranes (Millipore) and storing these in 1 mL CTAB buffer at -80 °C. Extractions

of DNA and RNA were performed as described by Dempster et al. (1999). Total prokaryotic abundance was estimated using epifluorescence microscopy with 4', 6-diamidino-2-phenylindole (DAPI)(Porter & Feig, 1980). Bacterial production was estimated using ^3H -leucine incorporation (Kirchman *et al.*, 1985; Kirchman, 2001) and the centrifugation protocol described by Smith and Azam (1992). A conversion factor of $3.1 \text{ kg C mol}^{-1}$ was used to convert ^3H -leucine incorporation into bacterial production (Simon & Azam, 1989). I estimated the growth rates of natural bacterial communities by dividing bacterial production by total bacterial biomass (Kirchman, 2001). Bacterial biomass was estimated from abundance and assuming 15 fg cell^{-1} (Fukuda *et al.*, 1998).

Chapter 3

RESULTS

rRNA:rDNA Ratios in Culture

Four cultured representatives of abundant bacterial taxa were grown in batch cultures in which cell abundance and rRNA content were followed over time (Figure 1). All growth curves were divided into exponential phase and near-stationary phase. Oligotrophs *Pelagibacter* and SAR92 grew more slowly than copiotrophs *Ruegeria* and MED152. The maximum growth rate in exponential phase was 0.4 d^{-1} for *Pelagibacter* and 1.0 d^{-1} for SAR92, significantly lower than rates for *Ruegeria* (2.1 d^{-1}), and MED152 (6.2 d^{-1}). Growth rates in near-stationary phase were also variable between isolates. The growth rate in this phase for *Ruegeria* was 0.3 d^{-1} and 1.5 d^{-1} for MED152, while the slow growth rates of oligotrophs *Pelagibacter* (0.06 d^{-1}) and SAR92 (0.2 d^{-1}) were even lower.

Ratios of 16S rRNA to rDNA (rRNA:rDNA) varied between isolates and also varied over time within experiments (Figure 1). The rRNA:rDNA ratios in fast growing *Ruegeria* and all MED152, regardless of growth rate, were 12-fold higher than the rRNA:rDNA ratios in *Pelagibacter*, SAR92, and near-stationary *Ruegeria*. The sharpest contrast between rRNA:rDNA ratios over time was seen for *Ruegeria*.

Ratios of rRNA to rDNA in exponentially growing *Ruegeria* were 5.5-fold greater than in near-stationary phase *Ruegeria*. Ratios in SAR92 decreased from 20 to 16 (20%) when growth slowed from 1.0 d^{-1} to 0.2 d^{-1} . The rRNA:rDNA ratios in *Pelagibacter* approximately doubled (from 22 to 45) when transitioning from exponential growth to near-stationary phase growth. There was no significant change in rRNA:rDNA in MED152 over the course of batch growth. One MED152 culture did not grow at all; the rRNA:rDNA in this culture was significantly lower than in growing MED152 cultures (Student's t-test, $p < 0.05$, Appendix B).

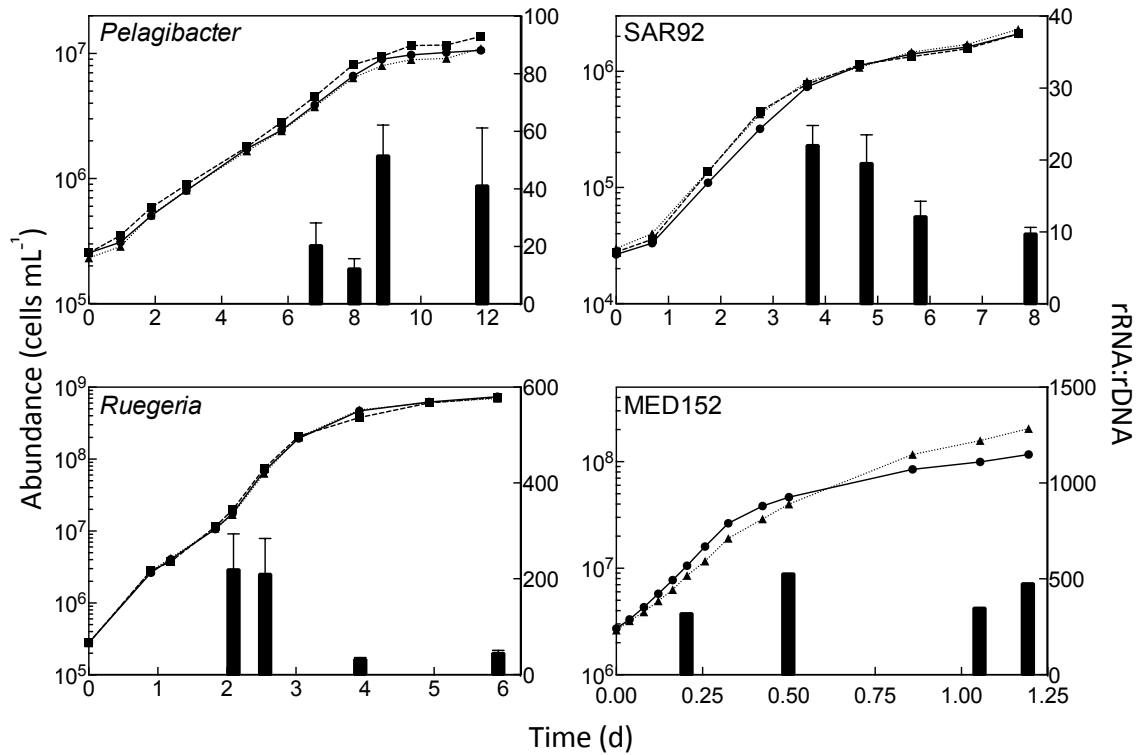


Figure 1 Cellular abundance and rRNA:rDNA ratios for *Pelagibacter ubique* HTCC1062, SAR92 HTCC2207, *Ruegeria pomeroyi* DSS-3, and *Polaribacter* sp. MED152 over time in triplicate batch cultures. The points are the abundance data and bars are rRNA:rDNA ratio data. The error bars are SD of biological replicates (n=3).

To determine the relationship between rRNA:rDNA and growth, I plotted rRNA:rDNA ratios against growth rate for each experiment (Figure 2). Growth rates for this analysis were calculated through the three time points immediately prior to a sampling point for nucleic acids. The rRNA:rDNA versus growth rate relationships of *Pelagibacter*, SAR92, and *Ruegeria* regression analyses are all significantly non-zero (Figure 2). The linear relationships between rRNA:rDNA and growth rate were positive for *Ruegeria* ($R^2=0.77$, $p<0.001$) and SAR92 ($R^2=0.32$, $p<0.01$). The *Pelagibacter* rRNA:rDNA versus growth rate relationship was negative ($R^2=0.46$, $p<0.001$). There was no significant relationship for MED152 ($p>0.05$).

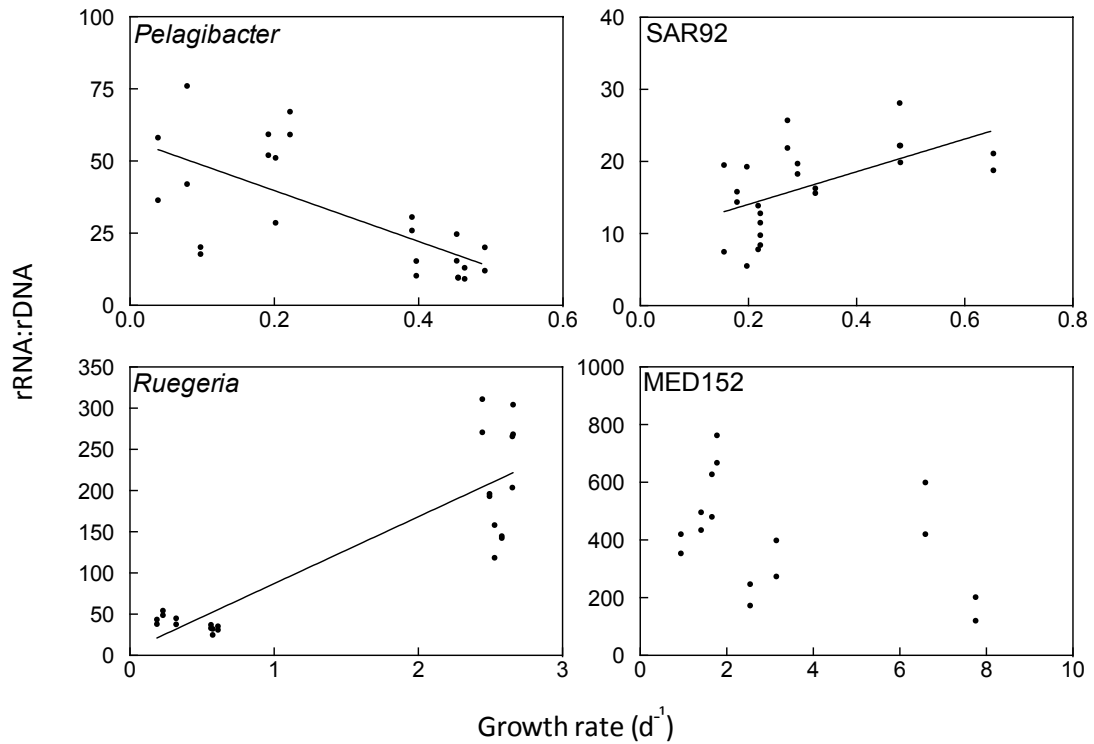


Figure 2 Ratios of rRNA:rDNA vs. growth rate for four bacterial taxa. Lines were calculated from model II linear regression analyses ($p < 0.05$). The slopes of all three lines are different from one another (Student's t-test, $p < 0.05$). For each sample two sets of matching technical replicates are plotted.

rRNA:rDNA Ratios in this Study Versus a Historical Dataset

I compared my rRNA:rDNA data from bacterial cultures of this study with a historical dataset of rRNA:rDNA in other cultured bacteria (Figure 3). Ratios from MED152 and *Ruegeria* in exponential phase fell within the rRNA:rDNA range of the historical dataset (rRNA:rDNA>100), while ratios in near-stationary *Ruegeria* were below the historical data (rRNA:rDNA<100). Ratios and growth rates from *Pelagibacter* and SAR92 cultures were below the historical dataset regardless of growth phase (rRNA:rDNA<100). Model II linear regression analysis of all data indicated that the rRNA:rDNA versus growth rate relationship was significant (slope=0.75±0.05, R²=0.61, p<0.001) (Figure 3).

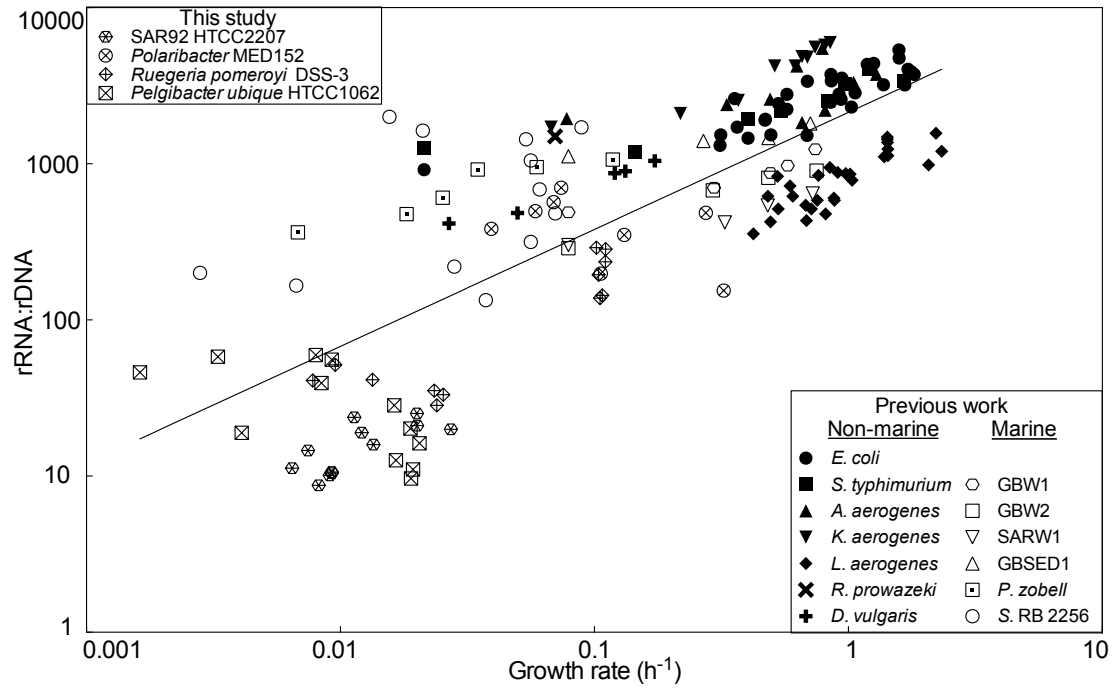


Figure 3 Ratios of rRNA:rDNA vs. growth rate for the four isolates in this study and for the historical data set. The line was calculated from model II linear regression analysis of all data ($R^2=0.61$, $p<0.001$).

rRNA:rDNA Ratios for a Transect of the Delaware Estuary

I examined rRNA:rDNA of the four chosen taxa, as well as biological environmental parameters during a cruise of the Delaware estuary in November 2013. Ratios of rRNA:rDNA were similar to ratios from cultures for some taxa but not for others. Values of rRNA:rDNA in the Delaware estuary were within 10-fold of the values found in cultures, but were generally lower than the maximum ratios observed in culture experiments (Table 2). Ratios for *Ruegeria* and MED152 were significantly lower in the estuary than in culture, regardless of the culture growth phase (Student's t-test, $p < 0.05$, Appendix B). SAR92 ratios in the transect were not significantly different from ratios in cultures of this bacterium. SAR92 was the least abundant of the groups examined in the Delaware estuary and was only present in 4 of 24 transect samples (Appendix A). Ratios for *Pelagibacter* during the transect were not significantly different from exponentially growing *Pelagibacter* in culture. However, transect ratios were significantly different from ratios for *Pelagibacter* in near-stationary phase (Student's t-test, $p < 0.05$, Appendix B). The mean community growth rate across the estuary, calculated from bacterial production and cellular abundance, was $0.2 \pm 0.3 \text{ d}^{-1}$ (SD, $n=23$).

Table 2 Ratios of rRNA:rDNA for taxonomic groups in this study. Culture samples were divided into two periods of growth. Transect samples were from the Delaware estuary. * Differed significantly from transect ratios (Student's t-test, $p < 0.05$). Errors are SD.

	Exponential	Near-stationary	Transect
<i>Pelagibacter ubique</i>	22 ± 16	45 ± 17*	27 ± 17
SAR92 HTCC2207	20 ± 2	16 ± 6	9 ± 6
<i>Ruegeria pomeroyi</i>	215 ± 67*	39 ± 8*	4 ± 3
MED152 sp. MED152	330 ± 167*	470 ± 191*	15 ± 14
All bacteria	--	--	40 ± 20

Chapter 4

DISCUSSION

Ratios of 16S rRNA to 16S rRNA genes (rRNA:rDNA) is one method of linking identity and growth activity in mixed bacterial communities (Campbell *et al.*, 2009, 2011; Campbell & Kirchman, 2013). There is a positive relationship between rRNA:rDNA and growth rate in many bacteria, but the bacteria for which these relationships are known are not representative of dominant members of marine bacterial communities (Kemp *et al.*, 1993; Kerkhof & Ward, 1993; Fegatella *et al.*, 1998). In addition isolates of the most abundant group of organisms in the oceans (SAR11) grow at slower rates in culture than bacteria with known rRNA:rDNA versus growth rate relationships (Rappé *et al.*, 2002). I determined the rRNA:rDNA ratio versus growth rate relationship in culture for four diverse marine bacteria that are representative of abundant clades in the Delaware Bay estuary and elsewhere. I then determined rRNA:rDNA ratios for taxonomic groups closely related to the cultured isolates, in the Delaware Bay.

I found the anticipated positive relationship between rRNA:rDNA and growth rate for *Ruegeria* and SAR92, but not for *Pelagibacter* and MED152. The rRNA:rDNA ratio is expected to depend on growth rate because increased ribosome content enables more protein synthesis and allows for faster growth (Kjeldgaard *et al.*,

1958; Schaechter *et al.*, 1958; Neidhardt & Magasanik, 1960; Rosset *et al.*, 1966; Sykes & Young, 1968). In spite of the lack of a positive relationship for *Pelagibacter* and MED152, the positive relationship still held when all strains were compared together. Oligotrophs SAR92 and *Pelagibacter* grew more slowly than copiotrophs MED152 and *Ruegeria*, and the rRNA:rDNA ratios for the oligotrophic taxa were correspondingly lower than ratios for copiotrophic taxa.

The rRNA:rDNA to growth rate relationship varied among the isolates, even for those with the expected positive relationship. Kemp *et al.* (1993) also found varying rRNA:rDNA versus growth rate relationships for the four isolates they examined. A possible physiological explanation for this variation is that protein translation is more efficient in some taxa than in others due to codon bias (Andersson & Kurland, 1990; Novoa & Ribas de Pouplana, 2012). Differences in translational efficiencies lead to difference in ribosome load (Klumpp *et al.*, 2012) and could explain variance among rRNA:rDNA growth rate relationships. But these possible explanations do not address why some rRNA:rDNA versus growth rate relationships are not positive. In contrast to my finding no rRNA:rDNA versus growth rate relationship in *Pelagibacter*, Salter *et al.* (2014) recently found a positive correlation between leucine incorporation and rRNA:rDNA for SAR11 in the Mediterranean Sea.

I found no positive relationship between rRNA:rDNA and growth rate for neither *Pelagibacter ubique* nor MED152. That rRNA content is not positively correlated with growth rate suggests transcriptional regulation of rRNA is not an important regulatory mechanism in *Pelagibacter*. *Pelagibacter* may regulate gene

expression at a post-transcriptional level with riboswitches (Tripp *et al.*, 2009), as proposed by other studies (Smith *et al.*, 2010, 2013). MED152, like *Pelagibacter*, also does not decrease transcription of rRNA in response to decreases in growth rate in batch culture. Perhaps types of regulation beyond transcriptional controls could also be relevant in MED152.

I applied the rRNA:rDNA ratios measured in the culture experiment to rRNA:rDNA ratios from bacterial communities along a transect in the Delaware estuary. I used a linear model ($Y=23X+9.6$) to estimate growth rates (X) for SAR92 from rRNA:rDNA ratios (Y) in the estuary. Since *Pelagibacter* and MED152 did not have positive rRNA:rDNA relationships, I used the mean rRNA:rDNA ratios and growth rates for these two taxa to estimate their growth rates in the Delaware estuary. Using this approach, I estimate that growth rates of SAR92 varied from 0.0 d^{-1} to 0.36 d^{-1} in the Delaware. Values for rRNA:rDNA ratios for *Ruegeria* in the Delaware were 50-fold lower than ratios from fast growing *Ruegeria* in culture and 10-fold lower than growing *Ruegeria* in culture. These ratios indicate that *Ruegeria* in natural communities grew far slower than the slowest *Ruegeria* growth rate measured in culture (0.3 d^{-1}). Ratios for MED152 in Delaware were 27-fold lower than the mean ratio of MED152 cells in culture where growth rates ranged from $1.5\text{-}6.2 \text{ d}^{-1}$, suggesting that growth rates of MED152 in the Delaware were much lower than 1.5 d^{-1} . In fact, MED152 ratios in the Delaware were similar to the ratios from the experiment in which MED152 did not grow (Student's t-test, $p>0.05$, Appendix B, Figure 12). In contrast, ratios for *Pelagibacter* in the Delaware were similar to ratios

from cells growing at 0.4 d^{-1} , but not similar to ratios in cultured *Pelagibacter* growing at 0.06 d^{-1} (Student's t-test, $p < 0.05$). These data indicate that *Pelagibacter* grows rapidly in natural communities at least in the Delaware estuary in late November.

The fast growth rate of *Pelagibacter* (0.4 d^{-1}) just calculated for the Delaware is similar to growth rates of SAR11 determined by Malmstrom et al. (2004) in the North Atlantic ($0.13\text{-}0.72 \text{ d}^{-1}$), Teira et al. (2009) in coastal mesocosm experiments ($0.46\text{-}0.59 \text{ d}^{-1}$), and Ferrera et al. (2011) in the NW Mediterranean Sea ($0.1\text{-}1.8 \text{ d}^{-1}$). Many studies say that SAR11 is a slow growing clade of bacteria. However, my results suggest that *Pelagibacter* grows faster than the mean growth rate of the total community (0.2 d^{-1}) in the Delaware estuary, calculated from bacterial production and abundance (Appendix A, Figure 5). My results provide an additional estimate of *Pelagibacter* growth rates that can contribute to the discussion of SAR11 survival strategy (Zhao et al., 2013; Våge et al., 2013, 2014). These data suggest that the high abundance of SAR11 is because it is a superior competitor rather than a defensive strategist.

The other bacteria grew more slowly than *Pelagibacter* in natural communities and also more slowly than previous measurements of growth rates for bacteria in related phylogenetic groups. Maximum growth rates of SAR92 were 3 to 10-fold lower than previous measurements of growth rates of the entire gammaproteobacterial class, which ranged from 0.9 d^{-1} to 3.8 d^{-1} in the Delaware Bay, North Atlantic Ocean and Mediterranean Sea (Yokokawa et al. 2004; Teira et al. 2009; Ferrera et al. 2011). This SAR92 isolate is part of the oligotrophic marine Gammaproteobacteria (Cho &

Giovanoni, 2004), which may explain why it grows more slowly in the Delaware estuary than other Gammaproteobacteria (Yokokawa *et al.*, 2004). Maximum growth rates of *Ruegeria* in the Delaware were 0.06 d^{-1} , low even compared with the minimum growth rates of *Ruegeria* in culture (0.4 d^{-1}). Bacteria in the broader phylogenetic group *Roseobacter*, which contains *Ruegeria* (Gonzalez *et al.*, 2003), grow more rapidly; growth rates of *Roseobacter* range from $0.3\text{-}1.5 \text{ d}^{-1}$ in the Delaware estuary and Mediterranean Sea (Yokokawa *et al.* 2004; Teira *et al.* 2009). Ratios for MED152 in natural communities were more than 10-fold lower than ratios of MED152 growing in culture, suggesting slow growth in natural communities. Previous measurements of *Flavobacteria* growth rates indicate that some members of this taxonomic group can grow at rates from 0.5 d^{-1} to 5.1 d^{-1} in coastal and estuarine environments (Yokokawa *et al.* 2004; Ferrera *et al.* 2011), much faster than the rates we estimate for the MED152 subgroup.

Calibrating rRNA:rDNA levels to specific growth rates in culture can provide useful interpretations of rRNA:rDNA data from the environment. However, the differences between measurements of rRNA:rDNA ratios in culture and natural systems could potentially lead to difficulties in interpretation of ratios for bacterial taxa in natural communities. The primers used in this study likely amplify more diverse 16S rRNA sequences in natural communities than just the isolates examined in culture (See Appendix A). Additionally, extraction efficiencies and PCR amplification efficiencies could differ between samples. These issues could be especially pertinent to samples from the natural environment, which may be more

difficult to extract and more difficult to amplify with PCR due to additional contaminating compounds in the extract. I found that I was able to efficiently extract acceptable yields of DNA and RNA from all samples used in my analysis. All qPCR and RT-qPCR fluorescence curves were monitored to ensure that there was no inhibition of reactions due to contaminants, and any inhibited reactions were not used for analysis.

There may be issues with interpretation of batch cultures as having two growth rates as we have in these results. The physiological limitations that cause slow growth in the exponential phase of batch culture are different from the limitations that are responsible for slow growth in near-stationary phase. The most common response of bacteria entering stationary phase, regardless of the type of nutrient limitation experienced, is to degrade cellular rRNA (Deutscher, 2003). However, there is evidence that some marine *Vibrio*'s may or may not immediately degrade their rRNA during the onset of starvation depending on the conditions under which stationary phase is induced (Kramer & Singleton, 1992). That some bacteria may not decrease their rRNA under starvation conditions could help to explain the lack of a positive relationship between rRNA:rDNA and growth rate for *Pelagibacter* and MED152.

Finally, growth conditions such as temperature and organic carbon concentration in lab experiments may differ from those experienced by natural communities. I did not test how temperature might affect rRNA:rDNA ratios in my experiments, but in faster growing organisms there is no effect of temperature on cellular rRNA content at 12 °C below the organism's optimal growth rate (Schaechter

et al., 1958). Temperature on the PAPI 6 cruise ranged from 9.2 °C to 11.2 °C, which is less than 10 °C cooler than my batch cultures (18 °C). Carbon sources used to grow bacteria in culture are likely more defined and freely available than those in the environment, which could lead to differences in active metabolisms between bacteria in cultures and *in situ*. However, Schaechter *et al.* (1958) did not find an effect of differing carbon sources on rRNA content that could be separated from the effect of growth rate on rRNA content.

My findings indicate that even given the complications there is value in using rRNA:rDNA data to address questions of growth rates in natural communities. High rRNA:rDNA is an indicator of high growth rate across taxa both in the data from this study and in the compiled historical dataset. The distribution of high ratios and faster growth rates in copiotrophs and low rRNA:rDNA and slower growth rates in oligotrophs contribute to the shape of this relationship. Within the larger dataset, individual taxa vary in rRNA:rDNA versus growth rate relationships, regardless of being labeled as a copiotroph or oligotroph. This study suggests a scale at which we can determine if organisms are growing at different rates; it seems that differences in an order of magnitude in rRNA:rDNA ratios can safely be interpreted as differences in growth rate. The results highlight the importance of laying a basis for the interpretation of taxa specific data due to the diversity of natural communities in the oceans (Giovannoni & Stingl, 2007). Given these findings, future work is needed to examine the taxonomic level at which rRNA:rDNA versus growth rate relationships are conserved. Using ratios of rRNA:rDNA will help to define the distribution of

taxonomic growth rates in the oceans which in turn will help understanding of the contribution of these organisms to biogeochemical cycling.

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Appendix A

SUPPLEMENTAL DATA FROM TRANSECT OF THE DELAWARE ESTUARY

The cruise dataset includes environmental properties, relative rRNA gene abundance, and rRNA:rDNA data not included in the main text. I measured total prokaryotes (DAPI) bacterial production, as described in the main text, for 24 stations ranging in salinity from 0.5 to 31.5 PSU. Photosynthetically active radiation (PAR) over depth was measured using a Profiling Natural Fluorescence Radiometer (Biosperical Instruments Inc.), and this data was used to estimate the coefficient of light attenuation. Chlorophyll *a* (Chl *a*) concentrations were measured as described by Pennock and Sharp (1986). I also measured the abundance of 16S rRNA and rRNA genes for all bacteria (Lee *et al.*, 1996; Delbes *et al.*, 2000), and for *Pelagibacter ubiquus* HTCC1062, SAR92 HTCC2207, *Ruegeria pomeroyi* DSS-3, and *Polaribacter* sp. MED152. All qPCR and RT-qPCR reactions were completed in duplicate as described in the main text. Additionally, I developed taxa specific primers for the *rpoB* gene of *Ca. Pelagibacter ubiquus*, to compare rRNA:rDNA ratios with an independent measure of *Pelagibacter* abundance.

The transect data conformed to many expectations, but rRNA:rDNA ratios of the four taxa of interest did not have clear trends along the estuarine salinity gradient. Total prokaryotes increased toward the mouth of the estuary from 1.3×10^6 cells mL⁻¹

to 2.5×10^6 cells mL^{-1} , while light attenuation decreased from the low salinity end to the mouth of the estuary ($5.4\text{-}0.57 \text{ m}^{-1}$) as expected (Figure 4). Bacterial production did not vary consistently along the estuary ($14.6\text{-}93.3 \text{ pmol L}^{-1} \text{ h}^{-1}$), while maxima of chl *a* were near the freshwater end ($4.48 \text{ } \mu\text{g L}^{-1}$) and the mouth of the estuary ($4.39 \text{ } \mu\text{g L}^{-1}$). The abundance of total bacterial 16S rRNA genes ranged from 1.25×10^5 copies mL^{-1} to 1.25×10^6 copies mL^{-1} , which was within expectations given extraction losses (Figure 5). Ratios of 16S rRNA:rDNA for all bacteria did not correlate with salinity or any of the other environmental parameters measured (Figure 5). All four taxa increased in relative abundance (taxon rDNA/all bacteria rDNA) toward the mouth of the estuary as expected (Figure 6). Each of the four also made up a different fraction of the total community; *Pelagibacter* was from 0.07-18% of the total community, SAR92 0.02-0.93%, *Ruegeria* 0.2-13%, and MED152 0.07-1.2% (Figure 6). These relative abundances and the positive trends with salinity are similar to previous studies of taxonomic groups related to the four taxa of interest in the Delaware estuary (Kirchman *et al.*, 2005; Campbell & Kirchman, 2013). Independent measurements of *rpoB* genes revealed no relationship between abundance and rRNA:rDNA ratios for *Pelagibacter* ($R^2=0.007$; $n=23$; $p=0.73$, Figure 7).

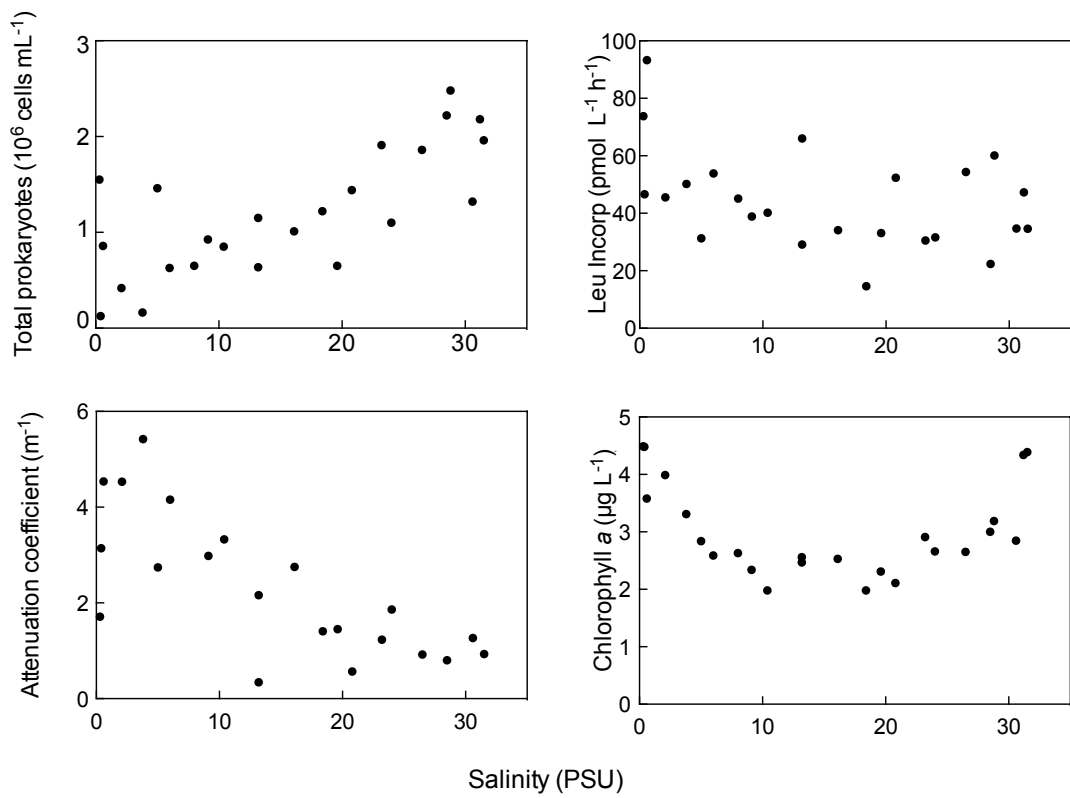


Figure 4 Environmental data for 24 stations in the Delaware estuary.

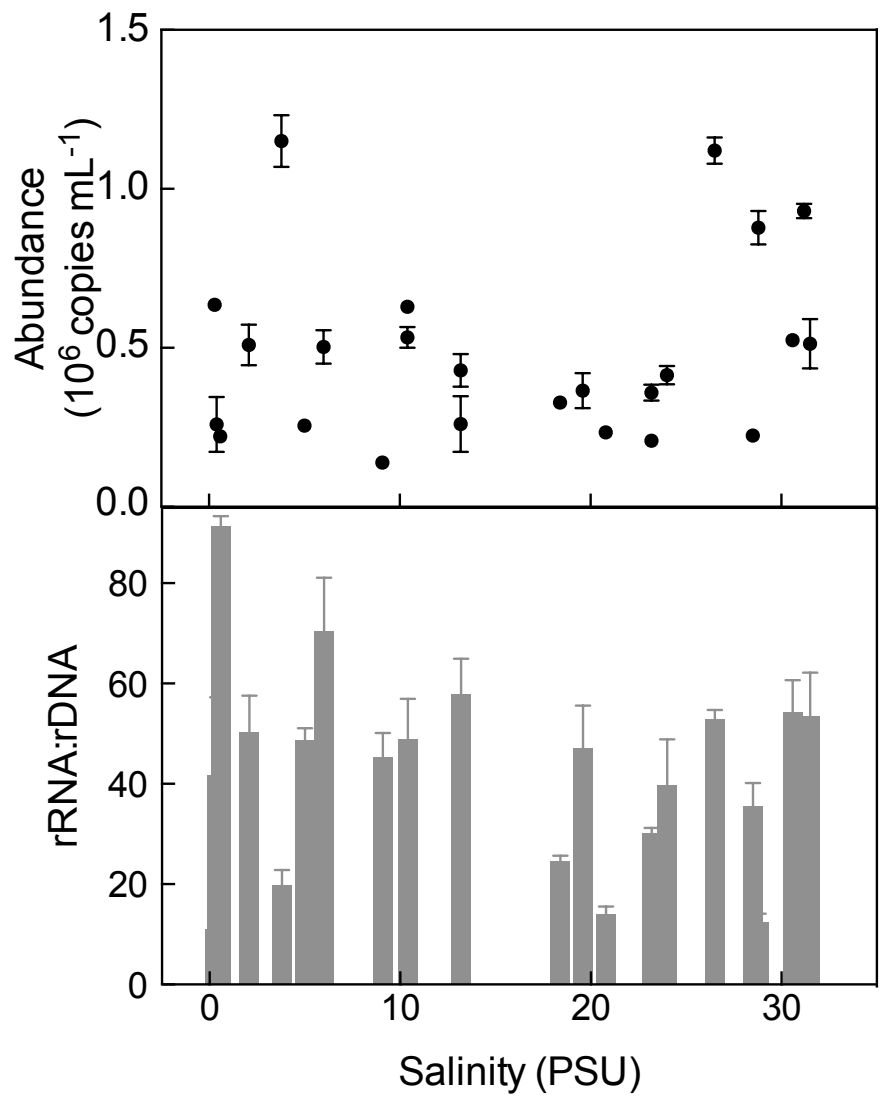


Figure 5 Abundance of total bacterial 16S rRNA genes per mL (top) and rRNA:rDNA (bottom) versus salinity in the Delaware estuary. Error bars are propagated SD of technical replicates for abundance and rRNA:rDNA (n=2).

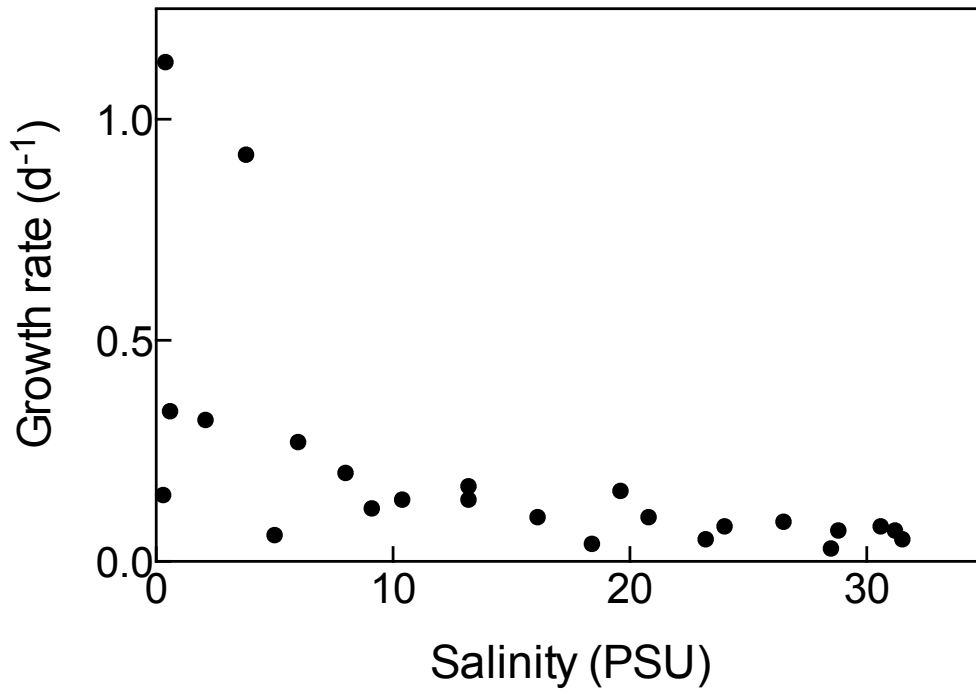


Figure 6 Growth rates versus salinity in the Delaware estuary. Rates are calculated from bacterial production and bacterial abundance.

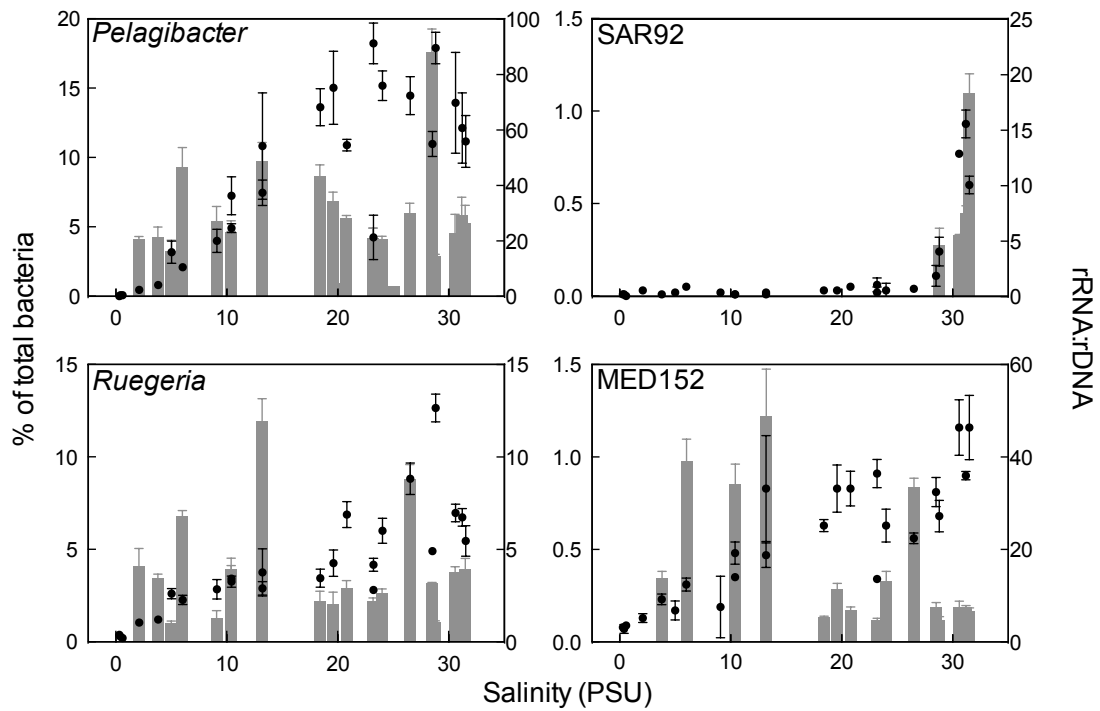


Figure 7 Relative abundance (points) and rRNA:rDNA (bars) versus salinity for the four taxa of interest in the Delaware estuary. Error bars for both relative abundance and rRNA:rDNA represent propagated SD of technical replicates (n=2). Ratios are only given for samples where rDNA > 100 copies mL⁻¹.

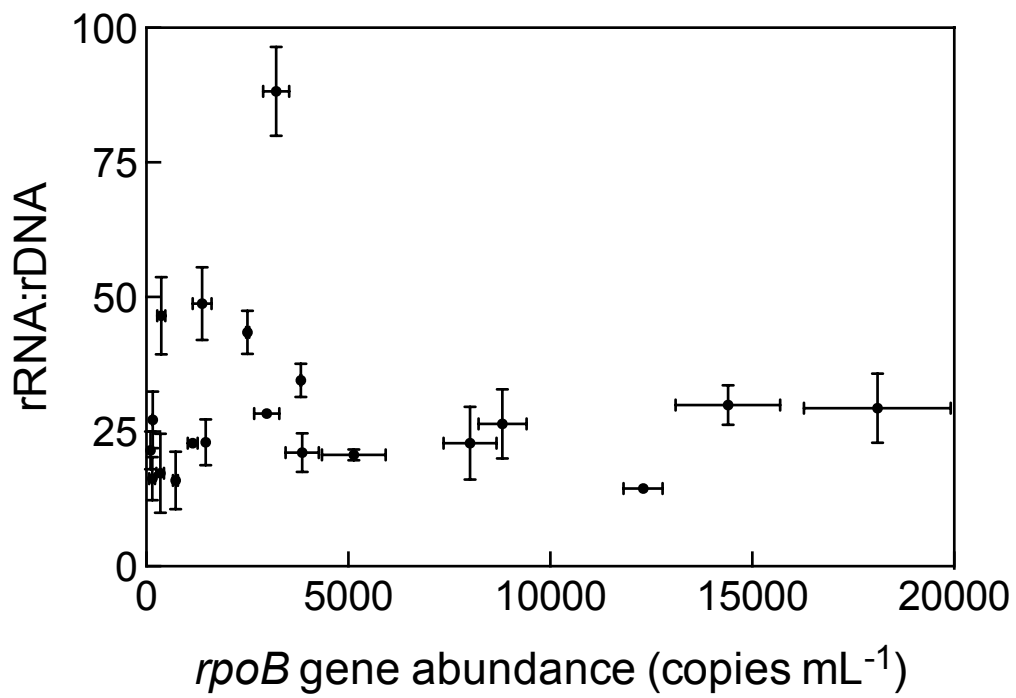


Figure 8 Ratios of rRNA:rDNA versus *rpoB* gene abundance for *Ca. Pelagibacter* ubiquitous HTCC1062 in the Delaware Bay estuary. Error bars represent propagated SD of technical replicates (n=2).

Table 3 Growth rates in the Delaware estuary estimated from rRNA:rDNA vs. growth rate relationships determined in culture. For SAR92 and *Ruegeria* model II linear regressions were used to calculate rates, while *Pelagibacter* and MED152 growth rates were calculated from mean ratios in cultures. Errors are SD.

	Exponential	Near-stationary	Transect
<i>Pelagibacter ubiquus</i>	0.4 ± 0.008	0.06 ± 0.04	0.4 ± 0.008
SAR92 HTCC2207	1.0 ± 0.5	0.2 ± 0.02	0.3 ± 0.09
<i>Ruegeria pomeroyi</i>	2.1 ± 0.03	0.3 ± 0.07	0.06 ± 0.007
MED152 sp. MED152	6.2 ± 0.15	1.5 ± 0.03	$\ll 1.5 \pm 0.03$
All bacteria	--	--	0.2 ± 0.3

Appendix B

MESOCOSM EXPERIMENTS

Two mesocosm incubation experiments were conducted to measure growth of the four taxa over time. For the first mesocosm experiment (Nov. 18-23, 2014) 16 L of 0.22 μm (GE, polycap TC150) filtered seawater was combined with 4 L of 1.0 μm (GE, GFF) filtered seawater in six 20 L acid washed and triple rinsed carboys. Three of these carboys were amended with a final concentration of 10 μM amino acid mixture. The second mesocosm experiment (Jul. 14-19, 2014) also had three carboys containing a 4:1 mixture of 0.22 μm filtered seawater and 1.0 μm filtered seawater; these were not amended and were incubated alongside triplicate carboys of unaltered, whole seawater. Seawater for both experiments was retrieved at the mouth of the Delaware estuary from a salinity of approximately 31 PSU. Both experiments were sampled daily at 24 hour intervals for five days and incubated at 18 °C. Samples were collected for microscopy direct counts (DAPI), flow cytometry, nucleic acids (CTAB), and bacterial production was assayed by the ^3H leucine centrifugation method.

In all mesocosm experiments total prokaryotic abundance and bacterial production increased (Figures 8 and 10), but the rRNA:rDNA ratios for the taxa in all experiments did not change significantly over time (Figures 9 and 11). In many cases rRNA:rDNA ratios in the mesocosm experiments were significantly lower than ratios

from the batch culture experiments (Figure 12). SAR92 transect ratios were statistically higher than ratios in the mesocosms but similar to the mean ratio from the two periods of culture growth (Student's t-test, $p < 0.05$). Ratios for *Ruegeria* during exponential phase are different from all other *Ruegeria* ratios from both mesocosm experiments and transect (Student's t-test, $p < 0.05$). All ratios for MED152 in cultures were higher than MED152 ratios along the transect and in the mesocosms (Student's t-test, $p < 0.05$). *Pelagibacter* ratios during exponential growth are only statistically different from near-stationary ratios, but similar to ratios from all other experiments (Student's t-test, $p < 0.05$). Increases in abundance and production combined with the lack of a response from the four taxonomic groups suggests that some other taxonomic group other than those monitored increased in activity and abundance during the mesocosm experiments.

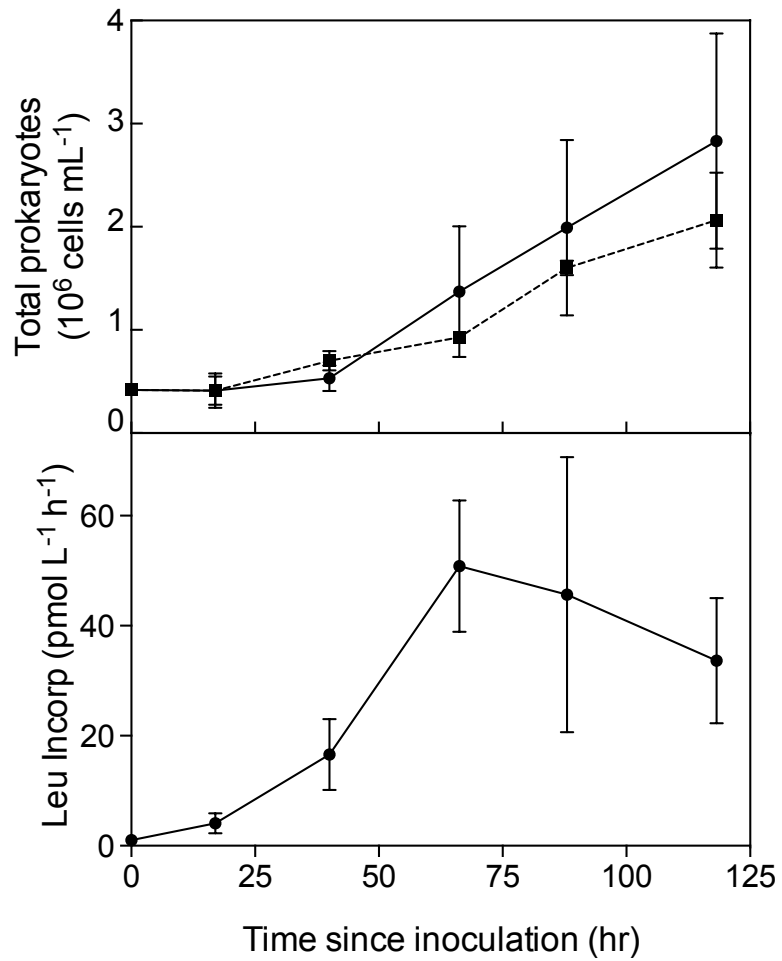


Figure 9 Prokaryotic abundance (top) and bacterial production (bottom) over time in the first mesocosm experiment. Solid lines with circles represent un-amended dilutions while dashed line with squares represent dilutions with added amino acids. Error bars are SD of biological replicates (n=3).

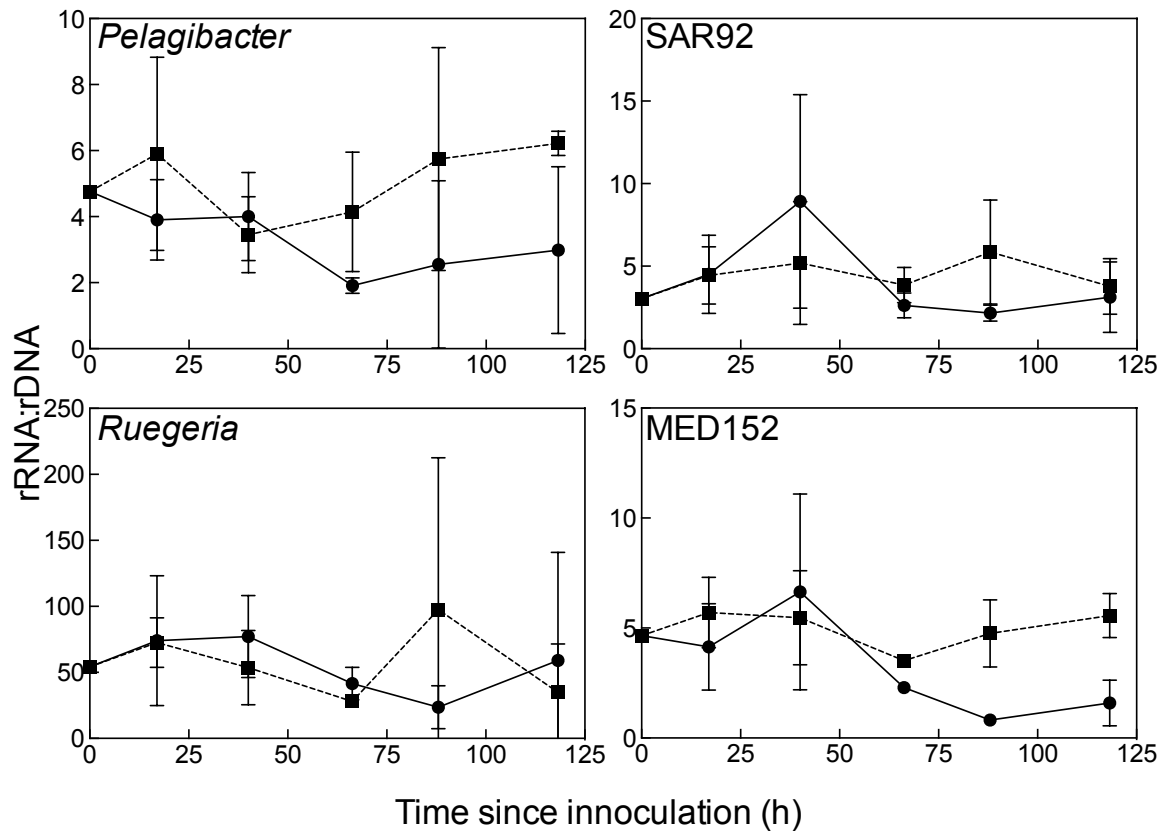


Figure 10 rRNA:rDNA over time in the first mesocosm experiment. Solid lines with circles represent un-amended dilutions while dashed line with squares represent dilutions with added amino acids. Error bars are SD of biological replicates (n=3).

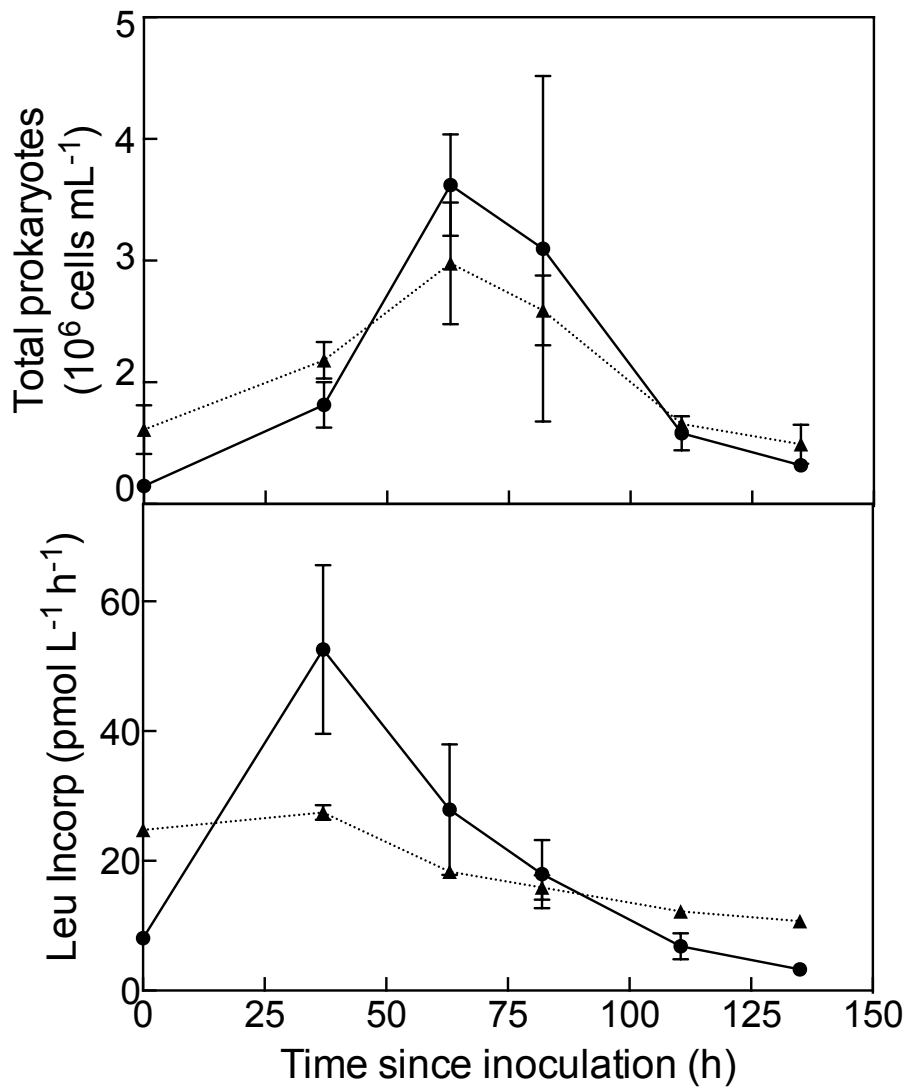


Figure 11 Prokaryotic abundance (top) and bacterial production (bottom) over time in the second mesocosm experiment. Solid lines with circles represent dilutions while dotted line with triangles represent whole seawater. Error bars are SD of biological replicates (n=3).

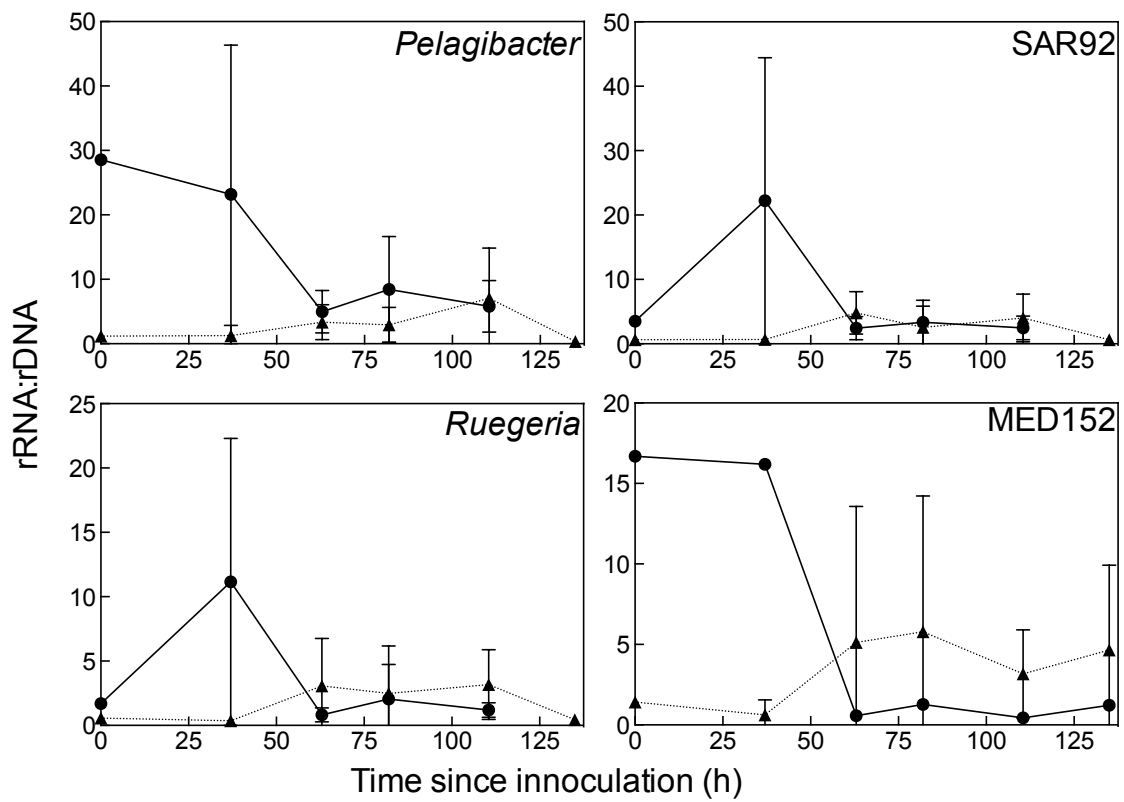


Figure 12 rRNA:rDNA over time in the second mesocosm experiment. Solid lines with circles represent dilutions while dotted line with triangles represent whole seawater. Error bars are SD of biological replicates (n=3).

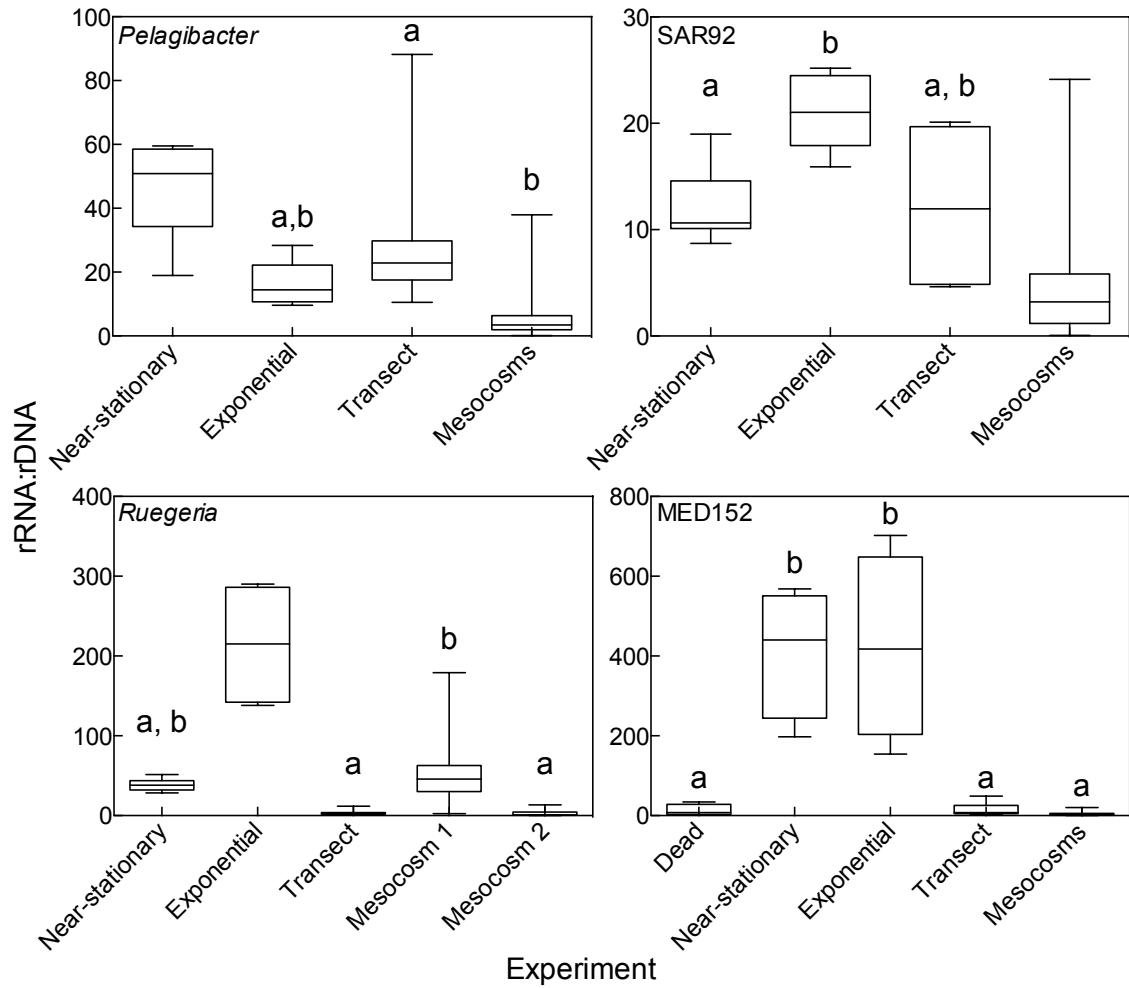


Figure 13 Ratios of rRNA:rDNA in two stages of the batch culture experiments (exponential and near-stationary), the PAPI 6 transect, and mesocosm experiments. Matching letters designate experiments that are not significantly different from each other, while experiments without matching letters are (Student's t-test, $p < 0.05$). Center lines represent means, boxes represent the 25th and 75th percentiles, and whiskers represent minimums and maximums.

Appendix C

MODEL OF COMPILED rRNA:rDNA DATASET

In order to further investigate the relationship between rRNA:rDNA and growth rate for all bacteria, I generated a model dataset from the data in my experiments combined with data from the literature. A model II linear regression was calculated through the compiled data for rRNA:rDNA versus growth rate for all types of bacteria (Figure 3). This regression line and its associated error were used to generate a normal distribution of 1000 data points around the regression line (Figure 3). I then used this model dataset for repeated regression analyses.

A general conclusion from this exercise was that the number of samples needed to determine an *in situ* growth rate increases as the dynamic range of growth for a given organism decreases. This implies that estimating accurate rates for slow growing bacteria could require more samples than the number required to estimate growth rates of fast growing bacteria.

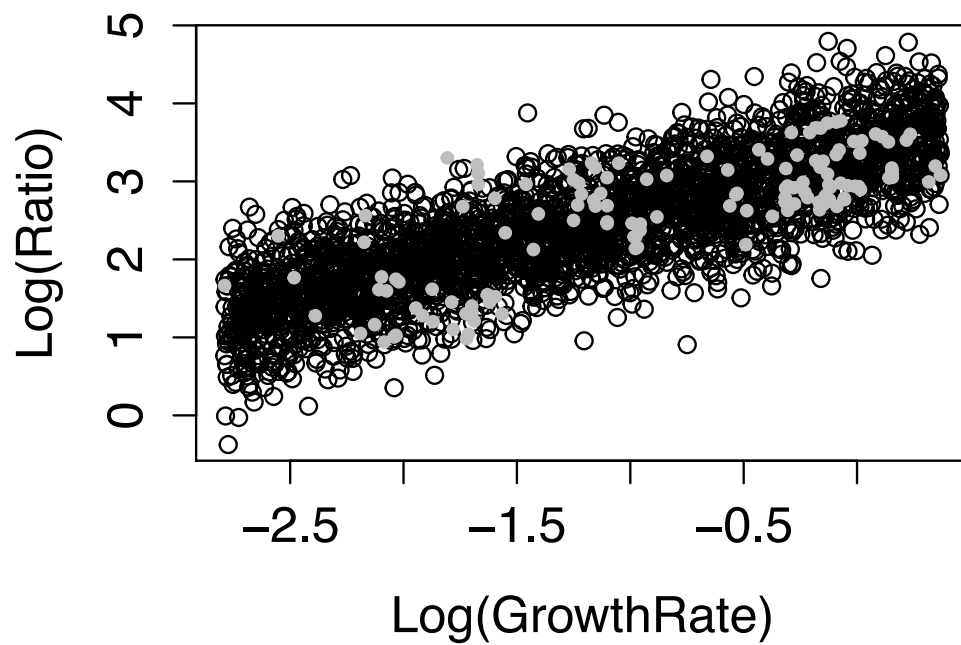


Figure 14 Model data set generated using the bbmle software package in R. Actual data is shown in grey points, while points in black were generated from the model II regression calculated in Figure 3.