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Interactions Between Phytoplankton and Bacteria in the Uptake of Organic Compounds

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INTERACTIONS BETWEEN PHYTOPLANKTON AND BACTERIA IN THE UPTAKE OF ORGANIC COMPOUNDS

by

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ABSTRACT

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The most common methods for estimating bacterial productivity are [^{3}H]-leucine and [^{3}H]-thymidine incorporation. Uptake of these compounds has been attributed primarily to bacteria; however, because dissolved organic nitrogen may be an important N source for some phytoplankton, the use of these compounds to estimate bacterial productivity needs to be reexamined. In order to ascertain whether phytoplankton could compete with bacteria on relevant timescales and thereby bias bacterial productivity estimates in estuaries, I examined the ability of cultured phytoplankton and size-fractionated natural populations to take up leucine and thymidine in systems seasonally dominated by phytoplankton mixotrophs. In addition, grain density autoradiography was used to verify the incorporation of labeled substrates into individual phytoplankton cells. Results demonstrate that a variety of phytoplankton species can take up both leucine and thymidine at low concentrations during short incubations. The presence of silver grains in autoradiographs of phytoplankton cells after incubations of natural whole water samples with radio-labeled leucine and thymidine confirms that phytoplankton are capable of incorporating both substrates.

This thesis is dedicated to my parents and my sister for their unending support.

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CHAPTER 1

INTRODUCTION

Over the last two decades, advances in microbial techniques have led to a better understanding of microbial processes, the cycling of nutrients in aquatic systems, and interactions among various microorganisms for nutrient substrates. Though much progress has been made, many questions regarding heterotrophic processes and competition among microorganisms remain. At the same time, it has been recognized that uptake of organic nutrients, particularly organic nitrogen, may play an important role in developing and sustaining algal blooms in coastal waters and estuaries (Paerl 1988; Smayda 1989; Lewitus et al. 1999; Glibert et al. 2001; Anderson et al. 2002). Although the ability of phytoplankton to take up organic substrates, such as amino acids, has been previously demonstrated (North and Stephen 1972; Hellebust and Lewin 1977; Wheeler 1977; Rivkin and Putt 1987; Antia et al. 1991; Mulholland et al. 2002, 2003), the ability of phytoplankton to compete with bacteria for organic substrates in eutrophic systems and its impacts on microbial food webs is still unclear.

Dissolved Organic Matter (DOM) and Marine Phytoplankton

Despite evidence that phytoplankton can incorporate organic compounds to supplement inorganic nutrient acquisition, it was earlier believed that phytoplankton did not significantly contribute to the overall incorporation of organic substrates in nature and certainly could not compete with bacteria for these substrates. Culture studies evaluating

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uptake of nutrients often used unrealistically high concentrations of organic compounds, such as amino acids, resulting in biased kinetic parameters (Antia et al. 1991). Early studies by North and Stephens (1972), Hellebust and Lewin (1977), and Wheeler et al. (1977) demonstrated the capacity of natural assemblages of phytoplankton to take up organic compounds. Although the studies focused mainly on diatoms, results showed that some species were capable of incorporating amino acids and glucose at ambient concentrations to support their growth. Rivkin and Putt (1987) examined the ability of marine phytoplankton to take up organic compounds in light and dark incubations. Results demonstrated that diatoms were capable of using amino acid C when light was limited. The ability of phytoplankton to use organic C to supplement autotrophic C fixation may not only give mixotrophic phytoplankton (those capable of both heterotrophy and autotrophy) an advantage over strict autotrophs (Rivkin and Putt 1987; Klaveness 1988; Mulholland et al. 2002), but put them in competition with bacterial heterotrophs.

Traditionally, uptake of DOM has been attributed to bacteria, which take up organic substrates and incorporate them either directly into their biomass or use them for respiration (Fuhrman and Azam 1982; Azam et al. 1983; Williams 1991). Based on the greater surface area to volume ratio of bacteria as compared to phytoplankton, direct uptake of DOM by phytoplankton in nature, where nutrient concentrations are generally low, has been considered minor (Bronk, 2002). Although bacteria are still considered the main users of DOM, there is increasing evidence that phytoplankton may be able to directly compete with bacteria for organic compounds, including amino acids (Sherr 1988, Antia et al. 1991; Paerl 1991; Bronk 2002; Mulholland et al. 2002; Mulholland and

Lomas in prep.). In fact, studies have shown that a variety of phytoplankton are capable of assimilating nitrogenous DOM, referred to as dissolved organic nitrogen (DON), and associated carbon (C) or both, to meet their nutritional needs in nature (Wheeler et al. 1977; Antia et al. 1991; Lewitus and Caron, 1991; Berg et al. 1997). In estuaries and coastal waters, phytoplankton have been shown to take up amino acid N and C at high rates during short incubations even when they are present at low concentrations (Mulholland et al. 2002, 2003; Mulholland and Lomas, accepted).

In addition to incorporating small labile organic compounds, many phytoplankton species, including dinoflagellate mixotrophs, have been shown to degrade high molecular weight DOM (e.g. proteins and peptides). Extracellular enzymatic activity, such as leucine aminopeptidase (LAP) and peptide hydrolysis (PH), appears to be common in a wide variety of phytoplankton species, including those associated with harmful algal blooms (Mulholland et al. 2002, 2003; Stoecker and Gustafson, 2003; Dyhrman 2005). As a result, amino acids, such as leucine, and other small labile compounds may be more widely available for uptake by bacteria or phytoplankton, particularly in highly productive or eutrophic systems. In estuaries, where amino acid concentrations can be high, bacteria and phytoplankton may compete for organic compounds. The ability of phytoplankton to take up amino acids (such as leucine) and other organic compounds (such as thymidine) to supplement their nutritional needs may have serious implications for estimates of bacterial productivity made using these substrates in estuaries.

Estimates of Bacterial Production

Bacteria are considered key players in aquatic food webs, contributing to the decomposition and remineralization of organic matter and the recycling of nutrients (Ducklow et al. 1986; Robarts and Zohary 1993). Studies examining the link between bacterial biomass and cycling of organic matter are critical to our understanding of aquatic systems. As a result, several methods have been developed to estimate bacterial productivity and the conversion of organic matter into bacterial biomass (Ducklow and Shiah 1992; Robarts and Zohary 1993).

The most common methods for estimating bacterial productivity are the tritiated thymidine (Fuhrman and Azam 1980) and leucine (Kirchman et al. 1985; Bell 1993; Kirchman 1993; Kirchman and Ducklow 1993) incorporation methods. These methods measure incorporation of thymidine and leucine into DNA and protein, respectively. While both methods are highly sensitive and substrates are quickly taken up by heterotrophic bacteria, there are several limitations associated with the methods. Limitations include: 1) intracellular isotope dilution as a result of *de novo* synthesis of leucine (2) incorporation of labeled substrates into macromolecules other than DNA and protein, (3) variation in conversion factors between various systems for the [^{3}H]-thymidine method, (4) the potential for extracellular isotope dilution in highly eutrophic waters where organic compounds may be enriched, and (5) variation in microbial activities among different bacterial groups (Simon and Azam 1989; Bell 1993; Kirchman 1993; Ducklow 2000; Cottrell and Kirchman 2002; Hietanen et al. 2002).

An assumption inherent in the leucine and thymidine incorporation methods is that the labeled substrates ([^{3}H]-leucine or [^{3}H] - thymidine) are only incorporated by

actively growing heterotrophic bacterial cells (Fuhrman and Azam 1980; Robarts and Zohary 1993; Ducklow 2000). Initial surveys testing this assumption found that many bacteria either do not incorporate leucine and thymidine (Johnstone and Jones 1989; Gilmour et al. 1990) or do not take up these substrates at concentrations or on timescales relevant in bacterioplankton studies (Fuhrman and Azam 1982; Bern 1985; Rivkin 1986). The inability to incorporate thymidine is generally thought to be due to the lack of thymidine kinase, an enzyme required for incorporating thymidine into DNA (Robarts and Zohary 1993). Phytoplankton such as marine diatoms, although found capable of incorporating leucine and thymidine (Rivkin 1986), were not considered strong competitors with heterotrophic bacteria because incorporation of these compounds by diatoms was detected only when substrates were added at high concentrations (75 to 100 nmol L^{-1}) and incubation times were long (12 to 30 hours). In natural systems, it is generally accepted that bacteria out-compete phytoplankton for leucine or thymidine because they have a higher affinity for DOM and higher surface area to volume ratios (Lewitus 2006).

In recent years the specificity of leucine uptake to heterotrophic bacteria has been called into question. Many bloom-forming dinoflagellates and pelagophytes have been shown to take up amino acids at high rates in estuaries (Mulholland et al. 2002, 2003, 2004). In addition, studies with prokaryotes have demonstrated that axenic cultures of the cyanobacteria, *Microcystis aeruginosa* and *Nodularia* sp., are capable of incorporating leucine (Kamjunke and Jahnichen 2000; Hietanen et al. 2002). Although these studies examined incorporation of leucine by cyanobacteria, it is still unclear to what extent cyanobacteria take up leucine in natural systems and how they compete with

heterotrophic bacteria in nature. Although there appears to be compelling evidence suggesting a variety of phytoplankton and cyanobacteria are capable of taking up amino acids including leucine, it is unclear to what extent they do so in nature in the presence of heterotrophic bacteria.

In this study some of the outstanding questions regarding uptake of specific compounds by marine and estuarine phytoplankton are addressed. The hypotheses for this study are: 1) phytoplankton, particularly those that can use DON are capable of uptake of leucine and thymidine, and (2) bacterial productivity is overestimated by the radiolabeled leucine and thymidine incorporation methods when phytoplankton competing for similar resources are present in the same environment. Because they have been important in evaluating bacterial productivity, incorporation of [^{3}H] - leucine and [^{3}H] - thymidine by phytoplankton was examined in cultures and natural systems. In Chapter 2, three phytoplankton cultures were surveyed to determine the ability of phytoplankton to incorporate leucine and thymidine into protein and DNA, respectively. Results indicate that at least some dinoflagellates and diatoms are capable of incorporating both substrates. In Chapter 3, incorporation of leucine and thymidine was measured in size-fractionated populations collected from the Lafayette River, VA, a eutrophic estuary where the resident population contains known phytoplankton mixotrophs. The ability of phytoplankton to take up leucine and thymidine on timescales relevant to bacterioplankton studies and to compete with bacteria for similar resources in nature was evaluated. Results from this study indicate that phytoplankton are capable of both leucine and thymidine incorporation in organically enriched estuaries.

CHAPTER 2

INCORPORAITON OF LEUCINE AND THYMIDINE BY PHYTOPLANKTON

Introduction

Bacteria are considered primary components in aquatic food webs where they contribute to the decomposition and remineralization of organic matter. To quantify their productivity in the environment, several techniques have been developed (Hagström 1984; Kirchman 2000). These techniques include: 1) measuring the uptake and incorporation of radiolabeled (^{14}C and ^{3}H) substrates such as amino acids, purines, and pyrimidines; 2) quantifying changes in bacterial cell numbers over time; 3) quantifying changes in dissolved organic carbon concentrations over time; and 4) measuring rates of respiration (Ducklow and Shiah 1992; Bell 1993; Kirchman 1993; Carlson et al. 1999). Currently, the most commonly employed methods for estimating bacterial productivity are the thymidine (Fuhrman and Azam 1980, 1982) and leucine incorporation methods (Kirchman et al. 1985; Bell 1993; Kirchman 1993; Kirchman and Ducklow 1993). These methods measure incorporation of thymidine and leucine into DNA and protein, respectively.

Assumptions inherent in these methods are that labeled substrates (e.g., ^{3}H-thymidine or ^{3}H-leucine) are taken up by growing bacterial cells and that heterotrophic bacteria are the only microorganisms that incorporate the substrates directly into the cellular macromolecules (Fuhrman and Azam 1982; Kirchman et al. 1985; Robarts and Zohary 1993). Studies that examined the incorporation of leucine (Kirchman et al. 1985) and thymidine (Fuhrman and Azam 1982; Pollard and Moriarty 1984) by microorganisms

suggested that these two substrates were specific to heterotrophic bacteria. Although, some eukaryotic phytoplankton were capable of incorporating thymidine during DNA synthesis (Pollard and Moriarty 1984; Rivkin 1986), it was concluded that because bacterial productivity methods typically employ short incubations (< 2 hours) and low substrate additions (nmolar levels), incorporation by phytoplankton in the environment would be insignificant. In addition, fungi were not capable of taking up (Grivell and Jackson 1968) [^{3}H]-thymidine into DNA. The inability to incorporate thymidine was attributed to the lack of a thymidine kinase, an enzyme required for incorporation into DNA (Robarts and Zohary 1993).

In contrast, some recent studies have shown that picophytoplankton, such as cyanobacteria, are capable of incorporating leucine at high rates (Kamjunke and Jahnichen 2000; Hietanen et al. 2002) even at low concentrations (30 to 100 nmol L^{-1}), and are competitive with heterotrophic bacteria (Kirchman et al. 1985; Riemann and Azam 1992; Kamjunke and Jahnichen 2000). Phototrophs shown to incorporate leucine include *Microcystis aeruginosa* (Kamjunke and Jahnichen 2000), *Nodularia* spp. (Hietanen et al. 2002), and *Prochlorococcus* spp. (Zubkov et al. 2003, 2005).

In addition to cyanobacteria, a variety of other phytoplankton can take up and assimilate dissolved organic nitrogen (DON) to meet their nutritional needs in nature (Antia et al. 1991; Lewitus and Caron 1991; Berg et al. 1997; Glibert et al. 2001; Mulholland et al. 2002; Berman and Bronk 2003). In particular, a variety of phytoplankton in natural waters has been shown to take up amino acids, a component of the DON pool (Wheeler et al. 1977; Rivkin and Putt 1987; Paerl 1988; Antia et al. 1991; Lewitus et al. 1999; Mulholland e4t al. 2002). Phytoplankton mixotrophs, capable of

both heterotrophic and autotrophic metabolisms, are common in organically enriched estuaries (Paerl 1988; Stoecker 1998; Lewitus et al. 1999) and have been shown to take up DON as a nitrogen source (Glibert et al. 2001; Berman and Bronk 2003). DON has been implicated as a nutrient source that may preferentially stimulate harmful algal blooms (HABs) (Graneli et al. 1999; Anderson et al. 2002). *Aureococcus anophagefferens*, the brown tide pelagophyte, has been shown to take up amino acid N and C at high rates even in short incubations (< 15 minutes) and using tracer additions (30 nmol L^{-1}) (Mulholland et al. 2002; Mulholland 2004). In estuaries, where dissolved free amino acid (DFAA) concentrations can be high and dissolved inorganic nitrogen (DIN) limiting, bacteria and phytoplankton may compete for organic compounds, including those thought to be primarily bacterial substrates.

The ability of some cyanobacteria and phytoplankton to take up DON compounds in competition with heterotrophic bacteria in nature is now becoming more widely recognized. Incorporation of nitrogenous organic compounds, such as amino acids and thymidine, by phytoplankton to supplement their nutritional needs may result in overestimates of bacterial productivity using thymidine and leucine incorporation methods. In order to determine whether this phenomenon might be a problem, thymidine and leucine incorporation were measured in cultures of three common, bloom-forming phytoplankton.

Materials and Methods

A series of experiments was conducted to ascertain whether cultured phytoplankton could incorporate [^{3}H]-leucine and [^{3}H]-thymidine under conditions

commonly employed in bacterial productivity bioassays (short incubations and low substrate additions). Species were selected based on what was known about their ability to take up organic nutrients and included a diatom species that characteristically occurs in spring bloom populations as well as two dinoflagellate species that bloom in the spring and summer in the Chesapeake Bay and its tributaries. Because it was impossible to maintain axenic cultures at realistic growth rates, a number of methods were evaluated to distinguish between phytoplankton and bacterial contributions to thymidine and leucine incorporation. These are described below.

Phytoplankton cultures – Cultures of *Nitzschia* sp. and *Prorocentrum minimum* were obtained from Moss Landing Marine Laboratories and from Dr. Diane Stoecker at Horn Point Laboratory, Maryland, respectively. *Akashiwo sanguinea* was isolated from the Lafayette River, Virginia, by Dr. Yingzhong Tang at Old Dominion University. *Nitzschia* and *A. sanguinea* were maintained in sterile filtered artificial seawater with f/2 nutrients, vitamins, and trace metals (Guillard and Ryther 1962). *P. minimum* was maintained in sterile filtered artificial seawater (salinity = 15) with L-1 nutrients, vitamins, and trace metals (Guillard and Hargraves 1993). All cultures were grown at 18°C, on a 12:12 h light: dark cycle until cells were adapted (at least three generations), then grown up to volume for experimental treatments.

Leucine and Thymidine Incorporation – Experimental incubations to measure the incorporation of [^{3}H]-leucine and [^{3}H]-thymidine were carried out as described by Kirchman (1993; leucine), Fuhrman and Azam (1982; thymidine), and Bell (1993; thymidine). All assays were done in triplicate in 12 mL disposable centrifuge tubes and for each experiment, there were triplicate trichloroacetic acid (TCA)-killed control (5%

final concentration) incubations. To estimate leucine and thymidine incorporation in the cultures, triplicate, 12 mL aliquots of whole culture and trichloroacetic acid (TCA)-killed controls (5% final concentration) were placed in disposable centrifuge tubes. Assays were initiated by adding 8μCi of ^{3}H-leucine or ^{3}H-thymidine (Sigma, 10 nmol L^{-1} and 12 nmol L^{-1} final concentrations of leucine and thymidine, respectively). Assays were terminated either by adding cold TCA (final concentration 5%) directly to the assay tube or by filtration after a 15 minute incubation period. For assays terminated by adding cold TCA, the extracted sample was then filtered on 0.2 μm nitrocellulose filters to collect extracted macromolecules. For filtered samples, cells were collected onto 0.2 or 5.0 μm nitrocellulose filters, and then rinsed three times with 5% cold TCA and 80% cold ethanol to precipitate and purify DNA (thymidine) and protein (leucine) (Fuhrman and Azam 1980; Wicks and Robarts 1988; Kirchman 1993). Filters were dried and stored in scintillation vials in a ventilation hood for 24 hrs, then dissolved with ethyl acetate, and 10 mL scintillation fluid was added to each sample. Samples were stored in the dark until they were counted for one minute using a Packard TRI-CARB 2300TR scintillation counter. Raw counts were adjusted for background to yield disintegrations per minute. A blank vial with scintillation fluid was counted for five minutes prior to the samples to determine background counts.

To calculate leucine and thymidine incorporation rates, samples were corrected for disintegrations per minute (DPM) counted in the killed controls and DPM converted to nmol leucine or thymidine using the activity of the substrate and theoretical DPM of 2.22×10^{12} DPM Ci^{-1}. Incorporation 'observed' in the killed controls was < 3% for leucine and < 15% for thymidine. The activity of leucine and thymidine was 161 Ci

$mmol^{-1}$ and 46 Ci $mmol^{-1}$, respectively. Incorporation rates of leucine and thymidine are reported as nmol leucine (Leu) or thymidine (TdR) L^{-1} h^{-1}.

Appropriate incubation times in the linear response range were determined by doing time course experiments (Fig. 1). Time course experiments were conducted using Lafayette River water. All subsequent incorporation experiments in this study were conducted using 15 minute incubation times. Preliminary experiments included both light and dark incubations. Because there were no significant differences in leucine and thymidine incorporation in light and dark incubations (Fig. 2), all subsequent incubations, and all those reported here, were conducted at ambient light levels in the lab.

To determine whether the bacteria and phytoplankton fraction could be adequately separated by simple filtration prior to the extraction of macromolecules, a series of experiments were done to determine: 1) whether there were significant differences in estimates of incorporation when macromolecules were extracted directly in the assay tubes versus on filters after filtration, 2) whether bacteria were effectively passed through 5.0 μm filters. To compare leucine and thymidine incorporation in the phytoplankton versus bacteria fraction, triplicate bioassays of whole culture samples were filtered onto 0.2 μm and 5.0 μm nitrocellulose filters using gentle filtration (<5 psi). To quantify bacterial retention on filters, samples were also collected on both 0.2 and 5.0 μm filters to enumerate bacterial abundance (see below).

Antibiotic treatments – Experiments were conducted to evaluate leucine and thymidine incorporation in cultures treated with antibiotics two hours prior to experiment (Middelburg and Nieuwenhuize 2000). Incorporation of leucine and thymidine by Gram-positive and Gram-negative bacteria was reduced using broad-spectrum antibiotics

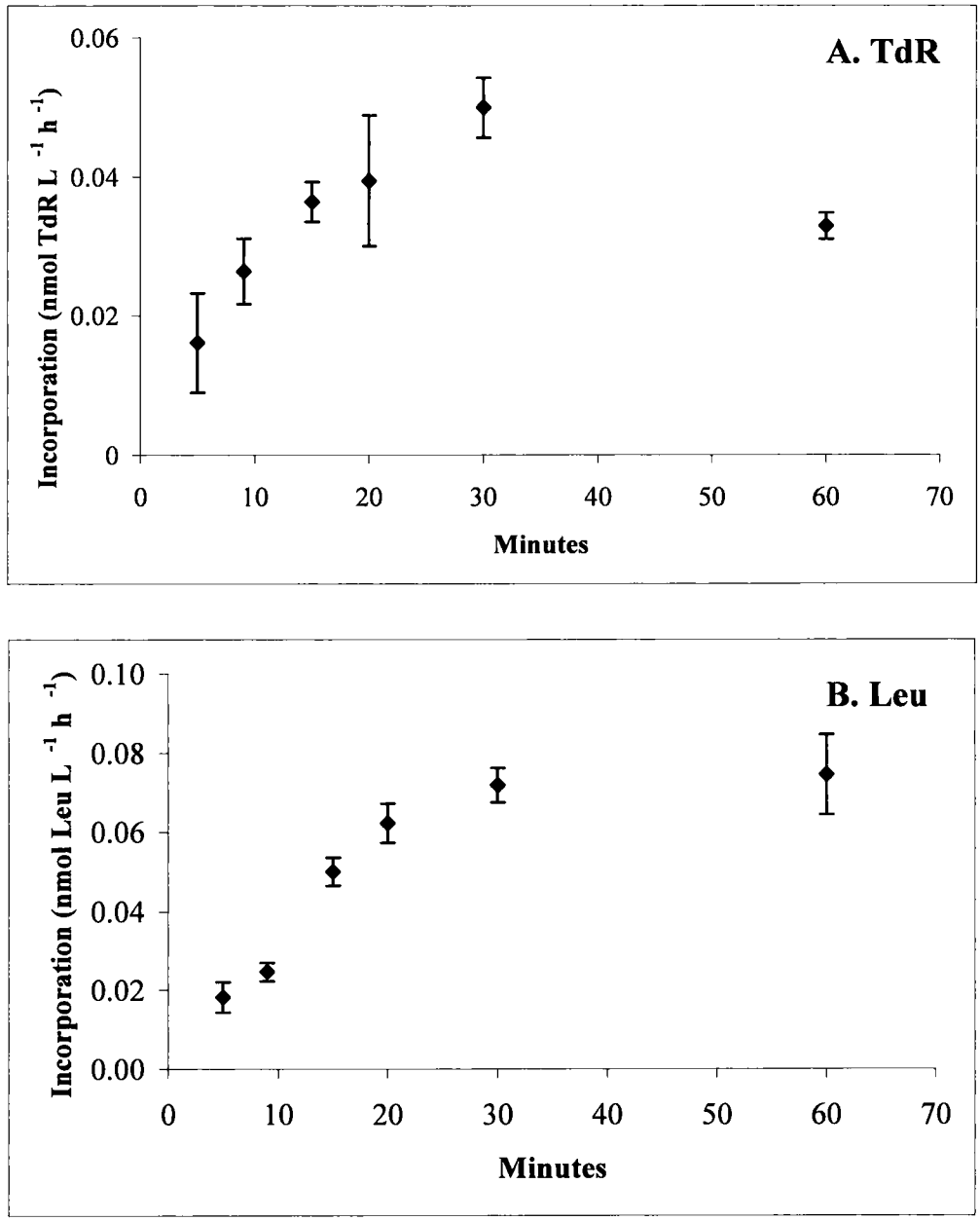

Figure 1. Linear response range for thymidine (A) and leucine (B) incorporation from preliminary experiments in the Lafayette River. Error bars are the standard deviations of triplicate incubations.

(Penicillin G, Streptomycin sulfate, Neomycin; Sigma) (Oremland and Capone 1988; Middelburg and Nieuwenhuize 2000; Wang et al. 2004) applied as described by

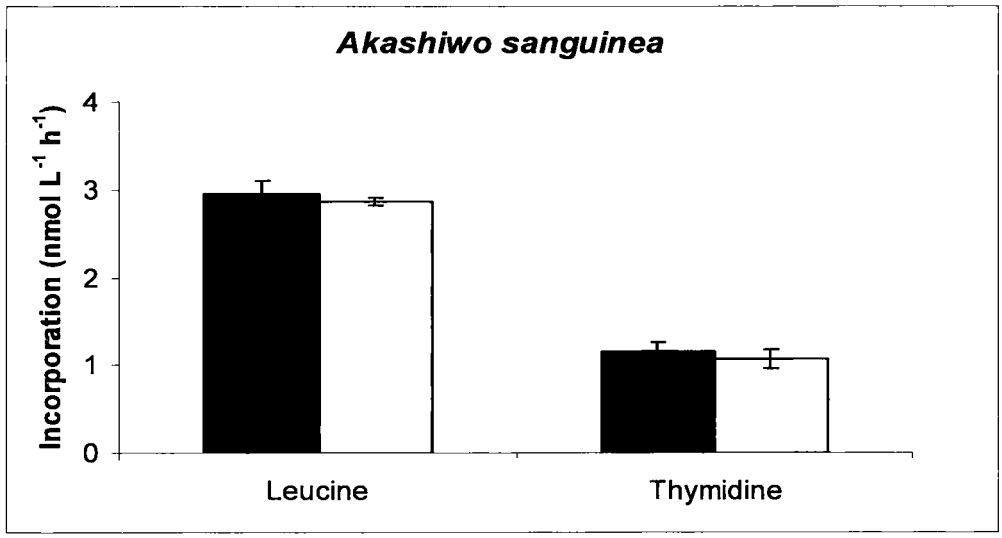

Figure 2. Incorporation rates of leucine and thymidine by *Akashiwo sanguinea* cultures in light incubations (black bars) and dark incubations (white bars). Error bars are the standard deviations of triplicate incubations.

Middelburg and Nieuwenhuize (2000). Antibiotic stock solutions were added to 25 mL culture flasks (10 mg L^{-1} final concentration) and incubated for two hours under ambient laboratory temperature and light conditions. Triplicate culture flasks without antibiotic additions served as controls. Incorporation of leucine and thymidine was then measured in control and antibiotic-treated whole culture samples.

To independently test whether the antibiotic treatments affected phytoplankton growth, incorporation of ^{14}C-bicarbonate was measured to evaluate its photosynthetic efficiency in antibiotic-treated and control cultures. Triplicate 25 mL aliquots from treated and untreated phytoplankton cultures were incubated (light and dark) with 25 µl ^{14}C-bicarbonate (0.5 µCi per sample, Sigma) for six hours. Incubations were terminated by filtration onto 0.2 µm filters and filters were placed into scintillation vials. Vials were stored in the dark until samples were counted for one minute on a Packard TRI-CARB

2300TR scintillation counter. Raw counts were adjusted for background to yield disintegrations per minute. A blank vial with scintillation fluid was counted for five minutes prior to the samples to verify counter accuracy.

Photosynthetic rates were calculated using the difference in productivity measured in light and dark bottle incubations (mg C L^{-1} h^{-1}) and dissolved inorganic carbon (DIC; mg C $m^{3\ -1}$) estimates (Nielsen 1952; Hobson et al. 1976). Productivity rates for light and dark incubation bottles were calculated from the following formula:

$$\text{Productivity (mg C L}^{-1}\text{ h}^{-1}\text{)} = \frac{\text{disintegration per minute (DPM) x DIC}}{\text{theoretical isotope activity x incubation time}}$$

Productivity was calculated using a theoretical isotope activity of 1.11×10^7. Salinity was used to determine total alkalinity, carbonate alkalinity, and total CO_2 (meq L^{-1}) (Wong 1979). DIC concentrations were estimated based on total CO_2 (meq L^{-1}). Photosynthetic rates were determined by taking the difference between productivity in light and dark incubation bottles.

Abundance – Phytoplankton cells were enumerated microscopically in triplicate in samples preserved with acid Lugol's. Bacterial cells were quantified using DAPI (4'6-Diamindino-2-phenylindole dihyrochlororide) fluorescent staining (Porter and Feig 1980). Samples for bacteria counts were preserved by adding 1-2 mL of 10% glutaraldehyde (1% final concentration). Triplicate slides were prepared for each size fraction (0.2 μm and 5.0 μm) using DAPI (1 mg mL^{-1}) stain. Cells were counted from 10 fields using an Olympus epifluorescent microscope.

Results

Size-fractionation studies – Estimates of bacterial abundance in all three phytoplankton cultures tested were about two orders of magnitude lower when cultures were collected on 5.0 μm filters versus on 0.2 μm filters (Table 1). This suggests that most bacteria were not attached to algal cells since they passed through the 5.0 μm filter. Observations during bacterial counts indicated that bacteria attached to particles, other than algal cells, were either few or not present in both size fractions. This indicates that filtration was a viable way to remove most bacteria to evaluate the respective contributions of bacteria and large phytoplankton to leucine and thymidine incorporation, at least in some circumstances.

Experiments evaluating the differences in estimates of incorporation in cells extracted directly in the assay tube versus after filtration on the filter showed no significant difference in thymidine (Fig. 3A) or leucine (Fig. 3B) incorporation rates between the two extraction methods, indicating that the timing of macromolecule extraction does not bias rate estimates.

Size-fractionation results indicate that virtually all of the leucine and thymidine incorporation in cultures of *Nitzschia* sp. and *A. sanguinea* was due to the > 5.0 μm siz e fraction (Fig. 4). This size fraction contained phytoplankton cells but only 1.5% and 1.8%,respectively, of the bacterial population (Table 1). This result suggests that the phytoplankton fraction was responsible for the bulk of the leucine and thymidine incorporation. In contrast, there was little incorporation of leucine or thymidine by the > 5.0 μm size fraction in the cultures of *P. minimum*, suggesting most of the incorporation was by bacteria in these cultures.

Table 1. Phytoplankton and bacterial cell densities in cultures. Bacterial cells were enumerated on 0.2 and 5.0 μm filters. Standard deviations are in parentheses (n = 3).

Phytoplankton Culture	**Phytoplankton (x 10^4) (cell mL^{-1})**	**Bacterial Abundance (x 10^6) (cell mL^{-1})**	
		0.2 μm	**5.0 μm**
Akashiwo sanguinea	2.0	5.1 (0.63)	0.09 (0.04)
Prorocentrum minimum	1.5	5.2 (0.81)	0.02 (0.02)
Nitzschia sp.	0.33	7.3 (1.02)	0.11 (0.10)

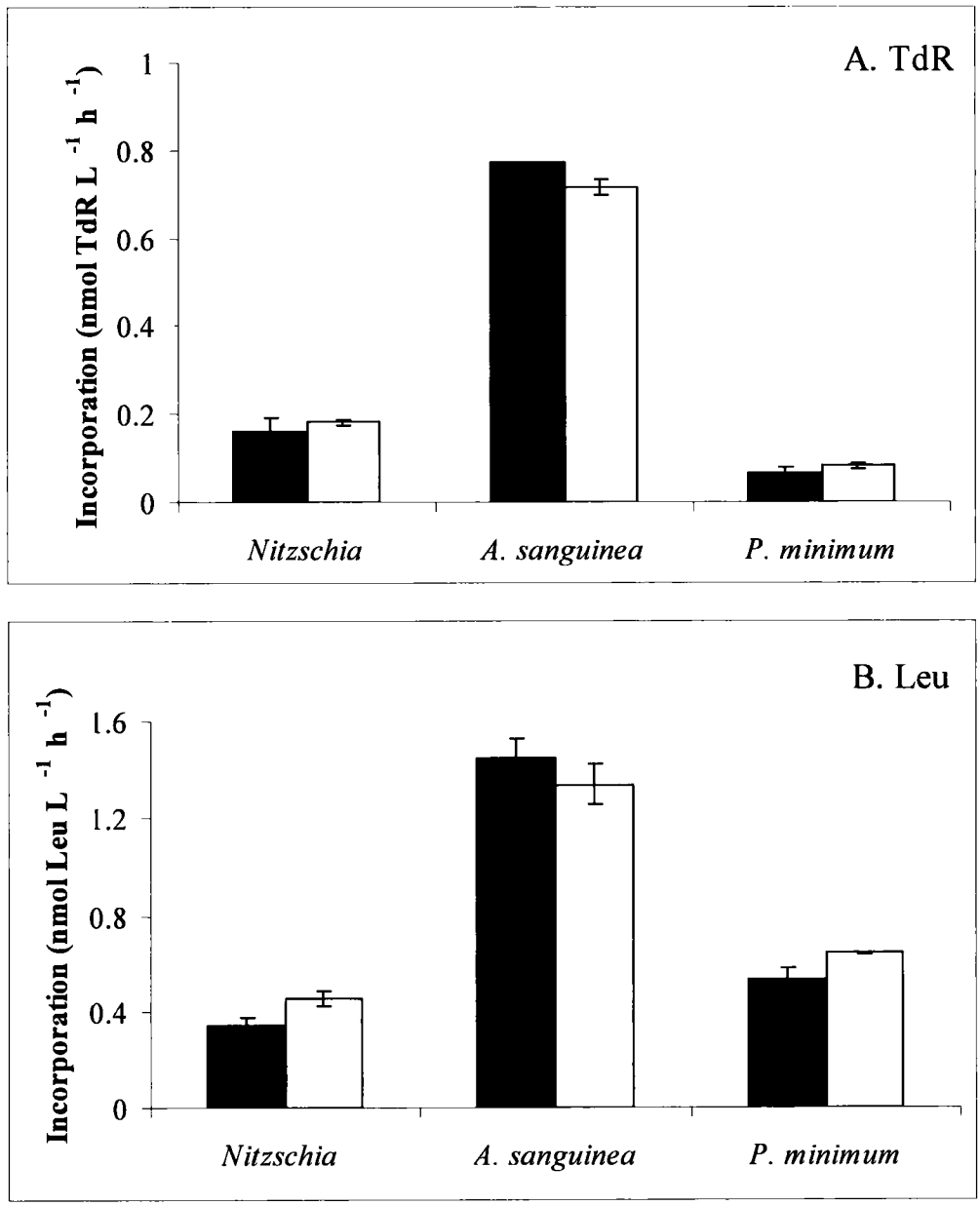

Figure 3. Incorporation of thymidine (A) and leucine (B) in samples extracted in assay tubes and then filtered onto 0.2 μm nitrocellulose filters (black bars) or after filtration onto 0.2 μm nitrocellulose filters (white bars). Error bars are the standard deviations of triplicate incubations.

Antibiotic treatments – The use of broad-spectrum antibiotics did not negatively affect phytoplankton photosynthesis during the leucine and thymidine incorporation

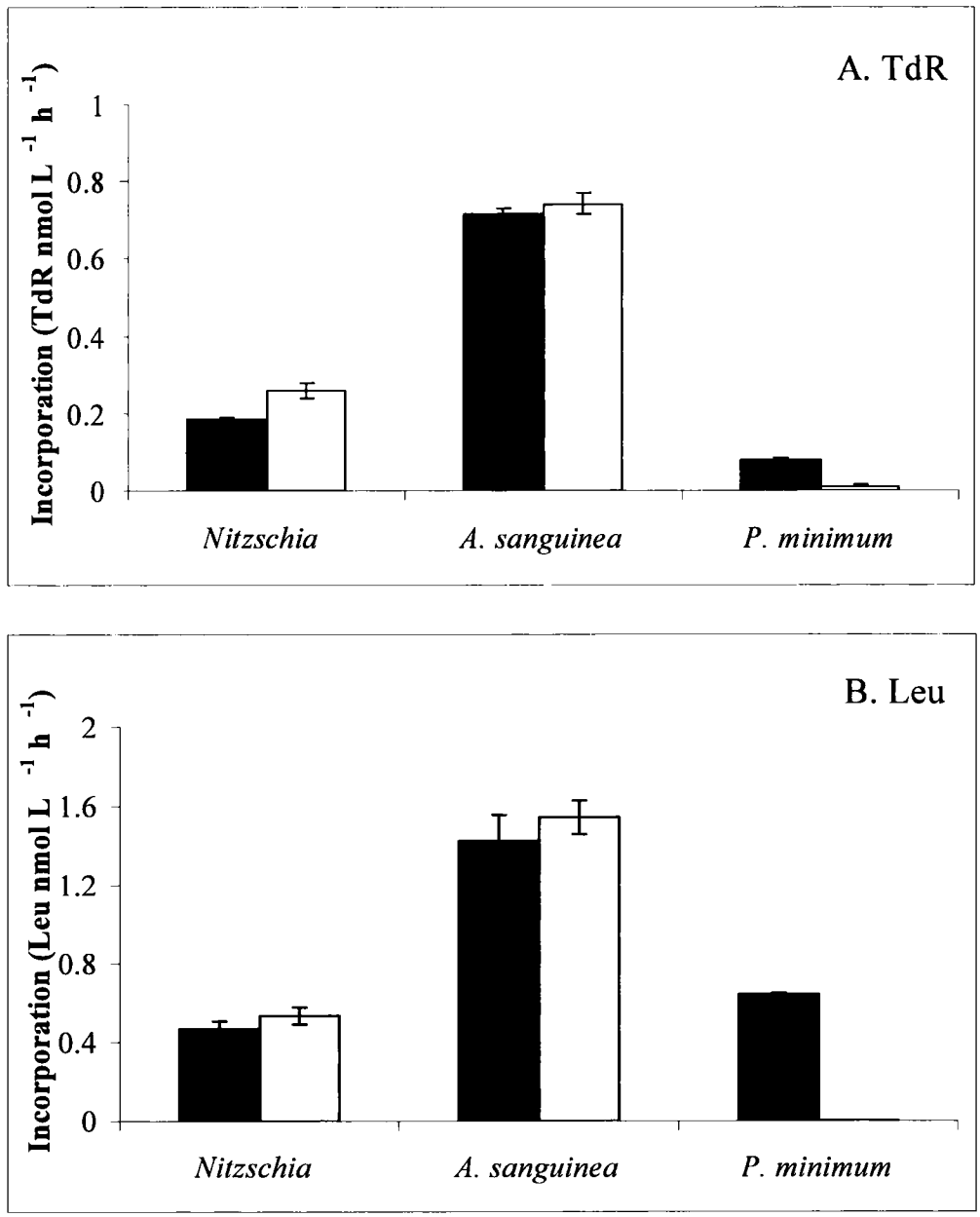

Figure 4. Incorporation rates of thymidine (A) and leucine (B) in phytoplankton cultures. Black bars are from whole culture incubations filtered onto 0.2 μm nitrocellulose filters and then extracted with TCA and white bars are from incubations filtered onto 5.0 μm nitrocellulose filters and then extracted with TCA. Error bars are the standard deviations of triplicate incubations.

bioassays (Table 2). The only significant difference was an increase in photosynthetic carbon assimilation by *A. sanguinea* after treatment with antibiotics (p-value = 0.04, n = 3, one –way ANOVA).

A comparison of leucine and thymidine incorporation rates for incubations with and without antibiotic treatments suggests that bacteria contributed substantially to thymidine (Fig. 5A) and leucine (Fig. 5B) incorporation in cultures of *Nitzschia* sp. and less to thymidine incorporation in cultures of *A. sanguinea.* Leucine and thymidine incorporation rates in *Nitzschia* sp. cultures were reduced after antibiotic treatments. In contrast, leucine and thymidine incorporation rates in cultures of *A. sanguinea* and *P. minimum* were not greatly reduced when antibiotics were added to the phytoplankton cultures. In fact, leucine incorporation rates were higher in cultures of *A. sanguinea* that had received antibiotic treatments and were virtually the same in treated and untreated *P. minimum* cultures.

Because antibiotic treatments did not appear to decrease the photosynthetic capability (Table 2) by phytoplankton and the antibiotic treatments at least partially reduced leucine and thymidine incorporation, we surmise that bacteria were at least partially inhibited while phytoplankton were not in all of the cultures. However, leucine and thymidine incorporation rates were comparable in both treated and untreated dinoflagellate cult res. When all these points are combined, it suggests that the phytoplankton fraction was at least partially responsible for incorporating these compounds.

Table 2. Photosynthetic carbon uptake by phytoplankton cultures incubated with and without broad-spectrum antibiotics. To reduce Gram-positive and Gram-negative bacteria, 10 mg L^{-1} of Penicillin G, Streptomycin sulfate, and Neomycin was added to each phytoplankton culture. Standard deviations are in parentheses (n = 3). The asterisks (*) indicate a significant difference between cultures with and without antibiotic additions, as determined using one-way ANOVA.

Phytoplankton Cultures	Salinity	Primary production (mg C l^{-1} h^{-1}) Without Antibiotics	With Antibiotics
Akashiwo sanguinea	30	709 (64) *	925 (41) *
Prorocentrum minimum	15	558 (149)	632 (105)
Nitzschia sp.	30	5747 (115)	5240 (1267)

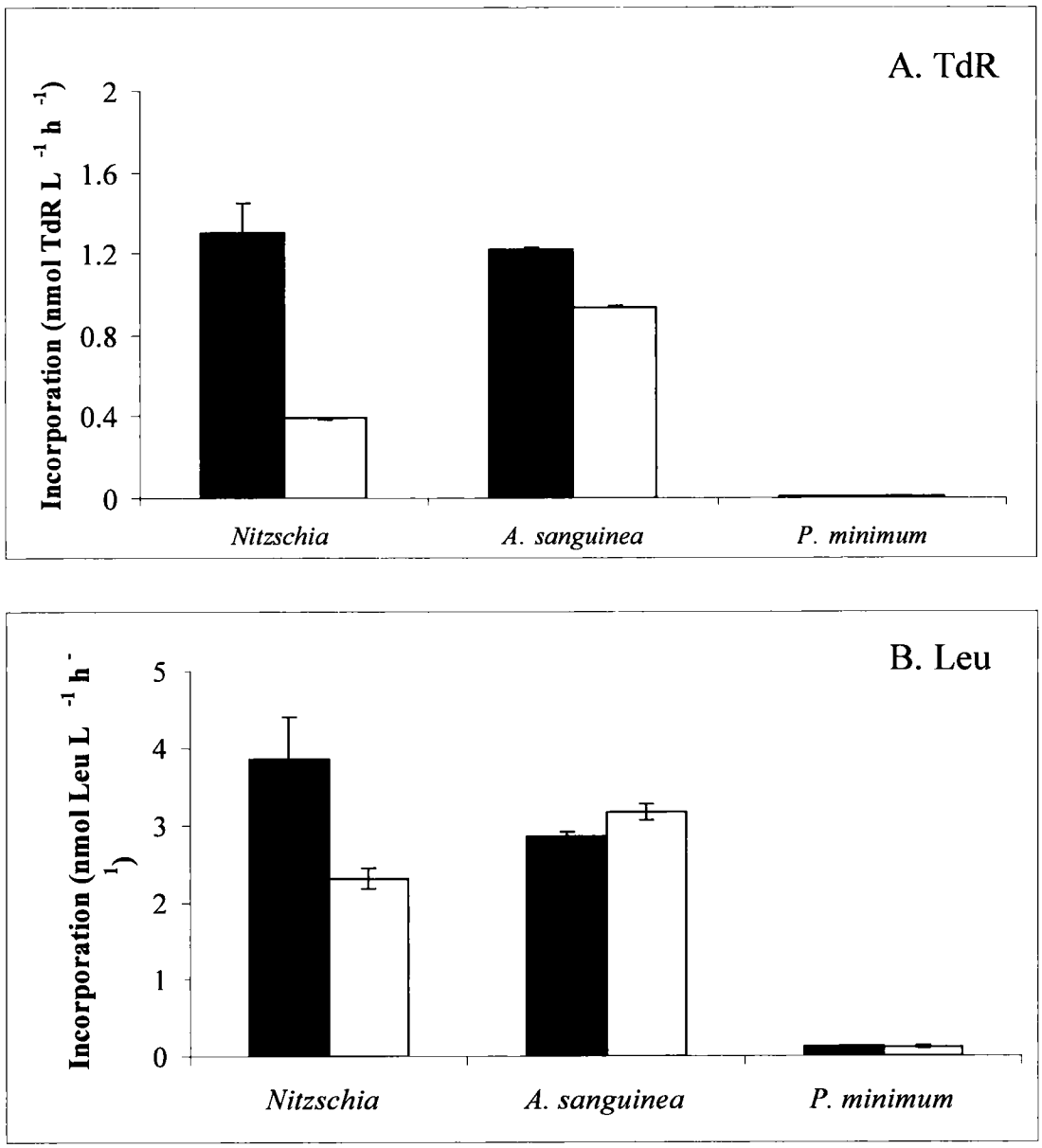

Figure 5. Thymidine (A) and leucine (B) incorporation rates in phytoplankton cultures incubated with (white bars) and without broad-spectrum antibiotics (black bars). Error bars are the standard deviations of triplicate incubations

Discussion

The ability of phytoplankton to incorporate leucine and thymidine poses serious concerns for interpreting estimates of bacterial productivity made in whole water samples

using the [^{3}H] - leucine and [^{3}H] – thymidine incorporation methods. In this study, results suggest that all three phytoplankton tested were capable of incorporating leucine and thymidine to at least some degree on 15 minute timescales and at 10-12 nmol L^{-1} substrate additions (conditions employed in bacterial productivity bioassays) even in nutrient replete cultures. This is contrary to previous studies, which concluded it was unlikely that phytoplankton could significantly contribute to leucine or thymidine incorporation, particularly if incubation times are short (e.g., < 2 hrs) (Furhman and Azam 1980; Pollard and Moriarty 1984; Kirchman et al. 1985; Rivkin 1986). Further, these results are environmentally significant because the phytoplankton examined during this study are common residents in marine and estuarine communities and are known to form phytoplankton blooms.

Size fractionation studies have been criticized because it is impossible to exclude attached bacteria from the larger size fractions. In this study, the incorporation of leucine and thymidine by attached bacteria cannot be excluded, but because less than 2% of bacteria were retained on the 5.0 μm filters, yet incorporation rates remained nearly unchanged, it seems implausible that attached bacteria were responsible for all of the incorporation in cultures.

Antibiotics have been used in microbial studies to evaluate the contribution of heterotrophic bacteria and phytoplankton in the uptake of nutrients (Middelburg and Nieuwenhuize 2000; Veuger et al. 2004; Wang et al. 2004). Wang et al. (2004) used an antibiotic mixture (Penicillin G and Streptomycin) to inhibit bacterial growth in algal cultures of *Alexandrium tamarense* C101 and examine the effects of antibiotics on *A. tamarense*. When antibiotics were added at high concentrations (> 300 mg L^{-1} Penicillin

G; > 668 mg L^{-1} Streptomycin), bacterial abundance was reduced to undetectable levels within 2 hours. Penicillin G and Streptomycin concentrations in the present study were significantly lower than those used by Wang et al. (2004).

A. sanguinea and *Nitzschia* sp. were able to incorporate both leucine and thymidine at high rates under conditions similar to those used in bacterial productivity studies and under nutrient replete culture conditions. In contrast, incorporation of these compounds in nutrient replete *P. minimum* cultures was low. *A. sanguinea* and *P. minimum* are bloom-forming, mixotrophic dinoflagellates found in coastal and estuarine waters around the world. Both are known to consume prey when dissolved inorganic nutrients are low (Bockstahler and Coats 1993; Stoecker et al. 1997, Li et al. 2000a, b; Jeong et al. 2005) and take up organic N and C to supplement inorganic nutrient uptake (Fan et al. 2003; Mulholland 2004). In this study, incorporation of leucine and thymidine by cultured phytoplankton was investigated only under nutrient replete conditions and this may bias results because organic nutrient uptake and extracellular hydrolysis is thought to be favored when inorganic nutrients are low (Glibert et al. 2001; Anderson et al. 2002; Mulholland et al. 2004; Mulholland and Lee in prep.).

The ability of *A. sanguinea* and possibly other common phytoplankton to incorporate leucine and thymidine in competition with bacteria when inorganic nutrients are limiting may have serious implications for bacterial productivity estimates when phytoplankton competing for similar resources are present in high concentrations.

Nitzschia are common in coastal and oceanic communities and they also incorporate leucine and thymidine at high rates in the laboratory. Although some diatoms, including *Nitzschia*, have been shown to take up amino acids (North and

Stephens 1972; Wheeler et al. 1974; Rivkin and Putt 1987; Antia et al. 1991), it was not expected that *Nitzschia* would significantly contribute to leucine incorporation in this study because of the short incubation times used. This study is the first to show that *Nitzschia* may be capable of incorporating thymidine in short incubations in the > 5.0 μm size fraction.

In this study, incubations with and without broad-spectrum antibiotics indicated that bacteria were the primary determinants of thymidine and leucine incorporation. On the contrary, thymidine and leucine incorporation rates in the size-fractionation studies suggested that *Nitzschia* contributed up to 30 - 50% of thymidine (Fig. 4A) and leucine incorporation (Fig 4B). Due to conflicting results, replication of the size-fractionation experiments, using high concentrations of broad-spectrum antibiotics (e.g. > 300 mg L^{-1}), are necessary to accurately determine the contribution of *Nitzschia* to leucine and thymidine incorporation.

Although mixotrophic phytoplankton are often associated with nutrient-enriched estuaries, some oceanic diatoms (including *Nitzschia*) (North and Stephens 1972; Wheeler et al. 1974; Antia et al. 1991) and coastal and oceanic cyanobacteria (Kamjunke and Jahnichen 2000; Hietanen et al. 2002; Zubkov et al. 2003; Zubkov and Tarran 2005; Moore et al. 1995) are capable of taking up organic N compounds. In oceanic waters, the contribution of cyanobacteria (and phytoplankton) to DON uptake had previously been considered negligible compared to other bacterioplankton. However, recently, it was reported that *Prochlorococcus* and *Synechococcus*, two dominant cyanobacterial genera, were capable of taking up and competing with bacterioplankton for amino acids in

oceanic gyres and subtropical regions (Zubkov et al. 2003, 2004; Zubkov and Tarran 2005).

The ability of phytoplankton to incorporate organic compounds, including thymidine and leucine, needs to be carefully examined across aquatic environments. Based on accumulating evidence that uptake and mobilization of organic compounds is more widespread than previously thought, more taxa and group-specific methods need to be developed to better understand nutrient and carbon biogeochemistry in the context of community structure in aquatic systems.

CHAPTER 3

INCORPORATION OF THYMIDINE AND LEUCINE BY PHYTOPLANKTON IN AN ESTUARINE SYSTEM

Introduction

Heterotrophic bacteria have traditionally been considered to be the main consumers of dissolved organic matter (DOM) in marine systems (Azam and Hodson 1977; Fuhrman and Azam 1982; Azam et al. 1983; Carlson 2002). Because of their role in recycling and remineralizing organic matter, measurements of bacterial productivity have become an integral part in quantifying carbon cycling in aquatic systems. The two most common methods for estimating bacterial production in aquatic systems are incorporation of two organic compounds, [^{3}H]-leucine (Kirchman et al. 1985; Kirchman 1993; Kirchman and Ducklow 1993) and [^{3}H]-thymidine (Fuhrman and Azam 1980, 1982; Bell 1993).

Incorporation of thymidine and leucine are thought to be specific to heterotrophic bacteria and it has been assumed that only actively growing bacterial cells incorporate the substrates into DNA (thymidine) or protein (leucine) (Fuhrman and Azam 1980; Fuhrman and Azam 1982; Bern 1985; Kirchman 1985; Robarts and Wicks 1989). Further, even though early studies evaluating leucine and thymidine incorporation by microorganisms other than bacteria found that some phytoplankton were capable of incorporating thymidine (Fuhrman and Azam 1982; Pollard and Moriarty 1984; Bern 1985; Rivkin 1986) during long incubations (>12 hrs), it was concluded that because bacterial productivity methods typically employ short incubations (< 2 hrs) and low

substrate additions (nmol L^{-1} level), there should be no significant incorporation by phytoplankton in nature. Consistently, it was demonstrated that cyanobacteria were unable to incorporate leucine (Kirchman et al. 1985; Torreton and Dufour 1996) or thymidine (Bern 1985). So, although collective evidence suggested that leucine and thymidine were specific to heterotrophic bacteria, many of the phytoplankton studies used to support this conjecture employed unrealistically high concentrations of organic nutrients (e.g., amino acids) and used long incubation times (Antia et al. 1991; Lewitus 2006), which were not relevant to bacterial thymidine and leucine incorporation assays performed in natural systems.

In contrast, more recent evidence has demonstrated that microorganisms other than heterotrophic bacteria are capable of incorporating leucine or thymidine or both at significant rates (Kamjunke and Jahnichen 2000; Hietanen et al. 2002; Zubkov and Tarran 2005; Chapter 2) during short incubations and at low concentrations (< 100 nmol L^{-1}) (Kirchman et al. 1985; Riemann and Azam 1992). In particular, Kamjunke and Jahnichen (2000) found that *Microcystis aeruginosa* (strain PCC 7806), a colony-forming cyanobacterium, incorporated leucine at high rates when it was supplied at nanomolar concentrations. Subsequent studies of other cyanobacterial genera have demonstrated that *Nodularia* and *Prochlorococcus* are capable of incorporating amino acids including leucine (Hietanen et al. 2002; Zubkov and Tarran 2005). The ability of cyanobacteria to take up leucine in a variety of environments, including the open ocean, has serious implications for interpreting estimates of bacterial productivity made using the leucine incorporation method across systems.

In addition to cyanobacteria, leucine and thymidine incorporation were

demonstrated in cultures of a diatom, *Nitzschia* sp. and two dinoflagellates cultures (*Akashiwo sanguinea* and *Prorocentrum minimum*) incubated under nutrient replete conditions (Chapter 2), indicating that incorporation of these substrates by phytoplankton may be more widespread than previously thought. Although bacteria are considered the main users of organic matter in nature, studies are increasingly demonstrating that phytoplankton are capable of utilizing many nitrogenous DOM compounds in marine environments (Antia et al. 1975; Wheeler et al. 1977; Rivkin and Putt 1987; Mulholland et al. 2002, 2003; Berman and Bronk 2003). In particular, it has been recently observed that a variety of bloom-forming phytoplankton are capable of taking up and assimilating organic nitrogen (N) to meet their nutritional needs (Berg et al. 1997; Mulholland et al. 2002; Berman and Bronk 2003; Fan et al. 2003) and that organic enrichment and this capability may indeed promote the formation of harmful algal blooms (Graneli et al. 1999; Glibert et al. 2001; Anderson et al. 2002).

Although a variety of phytoplankton species, particularly mixotrophic phytoplankton, can use DOM to meet their N needs (Paerl 1988; Berg et al. 1997; Lewitus et al. 1999; Glibert et al. 2001; Mulholland et al. 2002), it is still unclear whether these species can take up leucine and thymidine in nature. Competition between bacteria and phytoplankton for thymidine and leucine during bacterial productivity bioassays would complicate interpretation of bacterial productivity estimates made using the leucine and thymidine incorporation methods in whole water samples from environments where phytoplankton mixotrophs are common. The goal of this study was to evaluate the ability of natural populations of phytoplankton to incorporate [^{3}H]-leucine and [^{3}H]-thymidine. Incorporation rates in size-fractionated populations were measured over two

seasons in the Lafayette River, VA, where blooms of mixotrophic phytoplankton are known to occur, to determine whether resident phytoplankton were incorporating leucine and thymidine during incubations designed for assaying bacterial productivity and containing active bacterial populations. Therefore, phytoplankton incorporation of leucine and thymidine was assessed in incubations characteristically used for bacterial productivity assays; leucine and thymidine were added at nanomolar concentrations (< 20 nmol L^{-1}) and incubation times were short (< 1 hr). The working hypothesis was that if phytoplankton are significant competitors for these substrates in short bottle incubations, bacterial productivity could be overestimated by the radiolabeled leucine and thymidine incorporation methods, at least in some environments.

Materials and Methods

Size-fractionated incorporation of leucine and thymidine was examined in natural systems seasonally dominated by phytoplankton mixotrophs. To further distinguish bacterial and phytoplankton incorporation of leucine and thymidine, antibiotic treatments were used to reduce bacterial activity in some incubations and grain density autoradiography was used to visualize the incorporation of labeled substrates into individual phytoplankton cells.

Sampling site and sample collection – The Lafayette River is a subtributary of the lower Chesapeake Bay (Figure 6). Over the years, phytoplankton populations in the Chesapeake Bay tributaries have been increasingly dominated by dinoflagellates (Marshall and Alden, 1997; Marshall et al., 1998; Tango et al., 2005). In the Lafayette River estuary, dinoflagellates are an important component of the phytoplankton

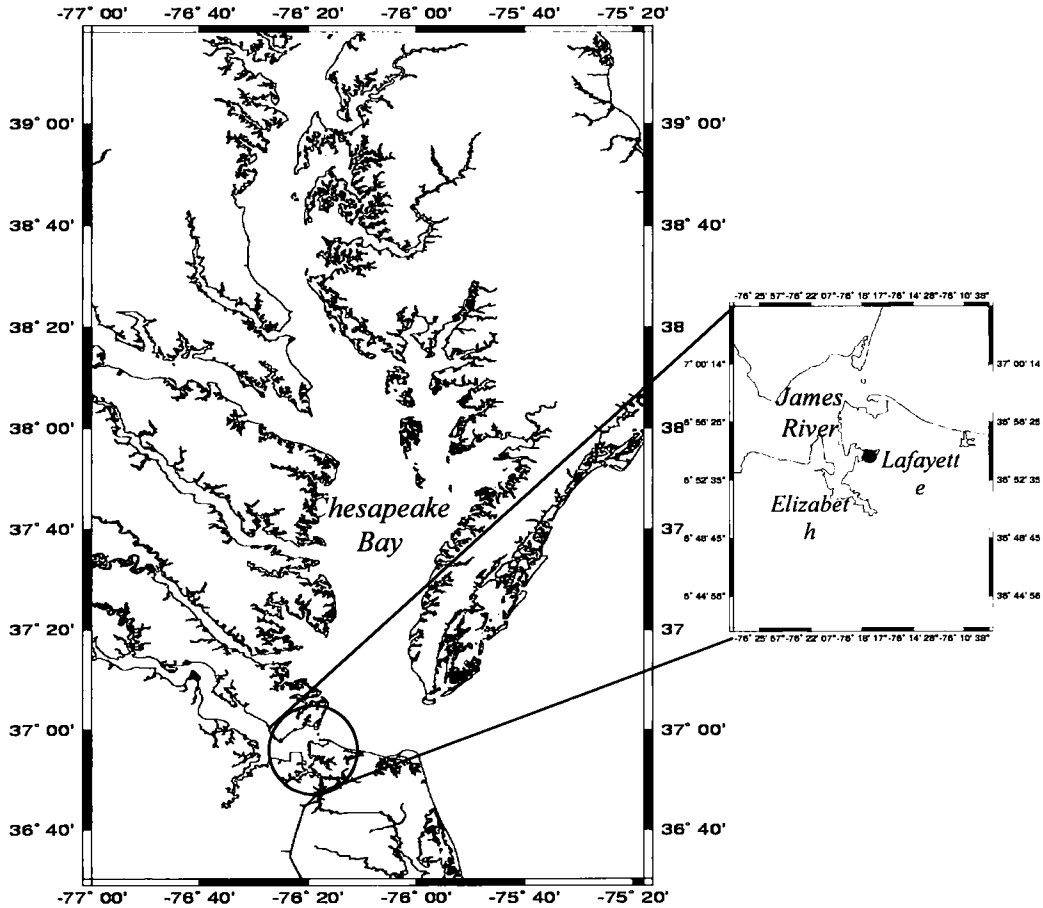

Figure 6. Map and inset showing the Lafayette River, a sub-tributary of the Chesapeake Bay. The sampling site in Norfolk, Virginia, is marked with a dot.

community and different species bloom seasonally (Marshall et al. 1998; Marshall et al. 2005). Phytoplankton assemblages include species that form seasonal blooms such as *P. minimum*, which blooms in the spring, and *A. sanguinea* and *Scrippsiella* spp., which bloom during the summer and fall.

Leucine and thymidine incorporation rates were measured in size-fractionated samples from the Lafayette River twice a month during 2003, and once a month during

2005, during seasons when blooms of dinoflagellates are common. Water samples were collected from the surface using a clean bucket. Water was placed into acid-cleaned carboys and transported to the laboratory. All sampling equipment was acid-cleaned using 10% HCL between sampling dates. Experiments were initiated in the laboratory within 30 minutes of sample collection.

Leucine and Thymidine Incorporation –Tritiated leucine and thymidine incorporation was measured in whole water and size-fractionated water samples (Fuhrman and Azam 1982; Simon and Azam 1989; Bell 1993; Kirchman 1993). Triplicate whole water, water filtered through 5.0 μm filters (< 5.0 μm water), and trichloroacetic acid (TCA) killed controls (5% final concentration) were incubated with 8 μCi of leucine or thymidine (Sigma, 10 nmol L^{-1} and 12 nmol L^{-1} final concentration, respectively) for 15 minutes. Incubation times were chosen based on time-course studies of leucine and thymidine incorporation (Fig. 1). Preliminary experiments demonstrated no significant difference between light and dark incubations (Fig. 2); therefore, experiments were conducted at ambient laboratory light levels.

To estimate leucine and thymidine incorporation by large phytoplankton versus bacteria, whole water incubations were filtered onto 0.2 μm and 5.0 μm nitrocellulose filters using gentle filtration (< 5 psi) prior to their extraction. It was previously determined that this effectively separated large, bloom-forming dinoflagellates from most bacteria, and that rate estimates made in assays wherein macromolecules were extracted directly on filters after filtration onto 0.2 μm nitrocellulose filters were comparable to those made when macromolecules were extracted directly in the assay tube and then filtered onto 0.2 μm filters (Chapter 2).

Filtered samples were rinsed three times with 5% TCA and 80% ethanol to precipitate and purify DNA (thymidine) or protein (leucine) (Fuhrman and Azam 1980; Wicks and Robarts 1988; Kirchman 1993). Filters were dried and stored in scintillation vials in a ventilation hood for 24 hrs then dissolved with ethyl acetate and 10 mL scintillation fluid added to each sample. Samples were stored in the dark until they were counted for one minute using a Packard TRI-CARB 2300TR scintillation counter. Leucine and thymidine incorporation rates are reported as nmol leucine (Leu) or thymidine (TdR) L^{-1} h^{-1} (Bell 1993; Kirchman 1993). Raw counts were adjusted for background to yield disintegrations per minute. A blank vial with scintillation fluid was counted for five minutes prior to the samples to determine background counts.

To calculate leucine and thymidine incorporation rates, samples were corrected for disintegrations per minute (DPM) counted in the killed controls and DPM converted to nmol leucine or thymidine using the activity of the substrate and theoretical DPM of 2.22×10^{12} DPM Ci^{-1}. Incorporation 'observed' in the killed controls was generally <15% for leucine and < 25% for thymidine. The activity of leucine and thymidine was 161 Ci $mmol^{-1}$ and 46 Ci $mmol^{-1}$, respectively. Incorporation rates of leucine and thymidine are reported as nmol leucine (Leu) or thymidine (TdR) L^{-1} h^{-1}.

Abundance estimates – Direct counts of phytoplankton and bacterial abundance were done microscopically. Phytoplankton cells were counted in triplicate samples preserved with acid Lugol's. Samples for bacterial cell abundance were preserved by adding 1-2 mL of 10% glutaraldehyde (1% final concentration). Bacteria were enumerated after DAPI (4'6-diamidino-2-phenylindole dihydrochloride) (1mg mL^{-1}) staining (Porter and Feig, 1980). Triplicate slides were prepared and counted for each

size fraction in 2005 samples and 10 fields from each slide were counted.

Antibiotic treatments - To verify that phytoplankton were incorporating leucine and thymidine in natural populations, broad-spectrum antibiotics (Penicillin G, Streptomycin sulfate, Neomycin; Sigma) were used to reduce incorporation of leucine and thymidine by Gram-positive and Gram-negative bacteria (Oremland and Capone, 1988; Middelburg and Nieuwenhuize, 2000). Antibiotic stock solutions were added to 25 mL flasks containing whole water or water filtered through 5.0 μm filters (< 5.0 μm water samples) (10 mg L^{-1} final concentration) and incubated for two hours at 18 –22°C under near-ambient light conditions. Parallel incubations without antibiotic additions served as controls. Incorporation of leucine and thymidine was then measured in control and antibiotic-treated whole water and < 5.0 μm water incubations.

Microautoradiography – Grain density microautoradiography (MA) was used to directly determine whether phytoplankton were incorporating [^{3}H] - leucine and [^{3}H]-thymidine (Knoechel and Kalff 1976; Rogers 1979; Burkholder et al. 1990; Burkholder 1992; Carman 1993; Cottrell and Kirchman 2003) in bacterial bioassays of Lafayette River water. Duplicate 12 mL whole water samples were incubated with 8 μCi of leucine or thymidine (Sigma, 10 nmol L^{-1} and 12 nmol L^{-1} final concentrations, respectively) for 15 minutes under ambient light conditions and at room temperature. Parallel incubations without added ^{3}H -radiolabel served as negative controls. Incubations were terminated by additions of 1-2 drops of acid Lugol's solution (Knoechel and Kalff 1976). Samples were filtered immediately onto 5.0 μm nitrocellulose filters using gentle filtration (< 5 psi) and rinsed three times with sterile-filtered artificial seawater. Filters were placed

face down for approximately one minute before gently pulling the filter up. Slides were stored horizontally in a desiccator for approximately 24 hours.

Slides were prepared for MA using techniques modified from Burkholder et al. (1990), Carman (1993), and Cottrell and Kirchman (2003). To prepare for track MA, slides were dipped in molten Kodak NTB-3 emulsion (34°C) and immediately placed on an ice-cold metal tray for 15-20 minutes. Slides prepared for grain-density MA were dipped in NTB-3 emulsion and diluted into two parts emulsion, one part de-ionized water (42°C). Once the emulsion was solidified, the slides were carefully transferred into slide boxes and stored at room temperature (< 24°C) until dry. Exposure time for the slides ranged from 24-36 hours. The emulsion was developed using Kodak D-19 developer, 1% acetic acid stop bath, 30% and 10% Kodak fixateur (sodium thiosulfate), and de-ionized water. The slides were dried and stored at room temperature in a desiccator. Prior to viewing, 30% glycerol was added to rehydrate the slide and a cover slip mounted. MA slides were photographed using a DP-70 digital camera and Olympus BX-50 compound microscope. To determine if silver grains were present MA slides were photographed using either light or dark field. Due to variations and possible errors during development of the slides (e.g. clarity of development and position of phytoplankton cells), images were photographed using different exposure times.

Results

Community Composition – The microbial community composition between April and August, 2003, and June and September, 2005, changed in the following ways: there were changes in the dominant species, changes in bacterial abundance, and changes in chlorophyll *a* (chl *a*) concentrations (Tables 3 and 4). Dinoflagellates usually dominated

Table 3. Average leucine and thymidine incorporation rates, bacterial abundance, chlorophyll *a* concentrations, and dominant phytoplankton taxa from the Lafayette River in April to August 2003. Standard deviations are in parentheses (n = 3).

Date	Incorporation Rates: Leucine (nmol Leu $L^{-1}h^{-1}$)	Incorporation Rates: Thymidine (nmol Tdr $L^{-1}h^{-1}$)	Bacterial abundance (x 10^6) cells mL^{-1}	Chlorophyll *a* $\mu g\ L^{-1}$	Dominant taxa
4/11/03	1.37 (0.21)	0.07 (0.01)	81	14.0 (13.1)	*Prorocentrum minimum*, diatoms, *Heterocapsa triquetra*
4/28/03	0.32 (0.03)	0.07 (0.01)	3.6	23.2 (0.9)	*Prorocentrum minimum*, mixed Dinoflagellates, *Cryptomonas* spp.
4/30/03	0.95 (0.004)	0.15 (0.01)	32.9	23.2 (0.6)	*Prorocentrum minimum*
5/06/03	0.51 (0.09)	0.05 (0.01)	149	9.50 (1.5)	*Prorocentrum minimum*
5/08/03	0.50 (0.03)	0.08 (0.004)	95.6	12.8 (1.8)	*Prorocentrum minimum*
5/28/03	0.36 (0.05)	0.02 (0.005)	112	2.0 (0.1)	Diatoms, mixed dinoflagellates, *Euglena* spp.
6/10/03	0.24 (0.10)	0.03 (0.001)	31.6	4.9 (0.1)	*Prorocentrum minimum, Pyramimonas* spp. *Cryptomonas* spp., mixed diatoms, *Pyramimonas* spp., mixed dinoflagellates
6/24/03	0.67 (0.07)	0.70 (0.14)	55.5	149.7 (15.5)	*Cryptomonas* spp., *Gymnodinium splendens, Prorocentrum minimum, Scrippsiella* sp.

Table 3. Continued.

Dates	Incorporation Rates Leucine (nmol Leu $L^{-1}h^{-1}$)	Thymidine (nmol Tdr $L^{-1}h^{-1}$)	Bacterial abundance (x 10^6) cells mL^{-1}	Chlorophyll *a* $\mu g\ L^{-1}$	Dominant taxa
7/17/03	1.98 (0.12)	0.29 (0.01)	37.7	49.6 (1.7)	*Gymnodinium splendens, Cochlodinium heterolobatum, Scrippsiella trochoidea*
7/22/03	1.38 (0.03)	0.13 (0.006)	70.2	33.7 (0.1)	*Gymnodinium splendens,* mixed diatoms, *Cryptomonas* spp.
8/05/03	1.19 (0.12)	0.35 (0.07)	54	21.7 (2.4)	Diatoms, *Cryptomonas* spp., *Scrippsiella trochoidea, Gymnodinium splendens, Prorocentrum minimum*

Table 4. Bacterial abundance, chlorophyll *a* concentrations, and dominant phytoplankton taxa in water samples collected from the Lafayette River in 2005. Bacteria were enumerated on both 0.2 and 5.0 μm filters. Standard deviations are in parentheses (n = 3)

	Bacterial abundance (x 10^6) cells mL^{-1}		Chlorophyll *a*	
Date	0.2 μm	5.0 μm	μg L^{-1}	Dominant taxa
6/24/05	54.8 (26.4)	0.10 (0.01)	15.72 (0.79)	*Akashiwo sanguinea, Polykrikos* spp.
7/11/05	87.7 (28.6)	41.73 (7.15)	41.73 (7.15)	*Akashiwo sanguinea, Scrippsiella* sp., *Cochlodinium* spp.
8/16/05	74.2 (15.7)	0.03 (0.01)	35.27 (5.59)	*Gymnodinium* sp., *Cochlodinium* spp.
9/20/05	65.4 (7.3)	0.03 (0)	11.43 (0.42)	*Gymnodinium* sp., *Gyrodinium uncatenum, Akashiwo sanguniea*

the phytoplankton community during sampling events, although cryptophytes and diatoms were often abundant. Chl *a* concentrations during sampling events ranged from 2.0 to 149.7 μg L^{-1} during 2003, and from 11.4 to 41.7 μg L^{-1} during 2005 (Table 4). During a *P. minimum* bloom on 30 April 2003, chl *a* concentrations and *P. minimum* abundance in the surface water both varied by nearly 2 orders of magnitude (5.2 to 187. 7 μg L^{-1} and 682 to 238,000 cells mL^{-1}, respectively) over a diel cycle suggesting that blooms are dynamic events. During 2003, the Lafayette River experienced a monospecific *P. minimum* bloom in April that lasted approximately 10 days, and a mixed dinoflagellate bloom dominated by *Gymnodinium splendens* during July that lasted one week. Bacterial abundance during 2003 varied, but was lowest in April and ranged from 2.2 to 149 x 10^6 cells mL^{-1} (Table 3). In 2005, bacterial abundances were comparable to those observed between June and August in 2003, ranging from 54.8 to 87.7 x 10^6 cells mL^{-1} (Table 4). During 2005, there was a mixed dinoflagellate bloom (dominated by *A. sanguinea* with abundant *Scrippsiella* sp.) that lasted from June until mid-July. A second mixed dinoflagellate bloom, dominated by *Gymnodinium* sp. developed in August and September. No correlation between bacterial abundance and chlorophyll *a* was observed in 2003. In 2005, however, there was a strong correlation between the two (R = 0.8779) (Fig. 7).

During 2005, bacteria were enumerated on both 0.2 and 5.0 μm filters. Bacterial abundances were more than 2 or 3 orders of magnitude lower in material collected on 5.0 μm filters than on 0.2 μm filters (Table 4). In addition, observations during bacterial counts indicated that bacteria attached to particles, other than algal cells, were either few or not present in both size fractions.

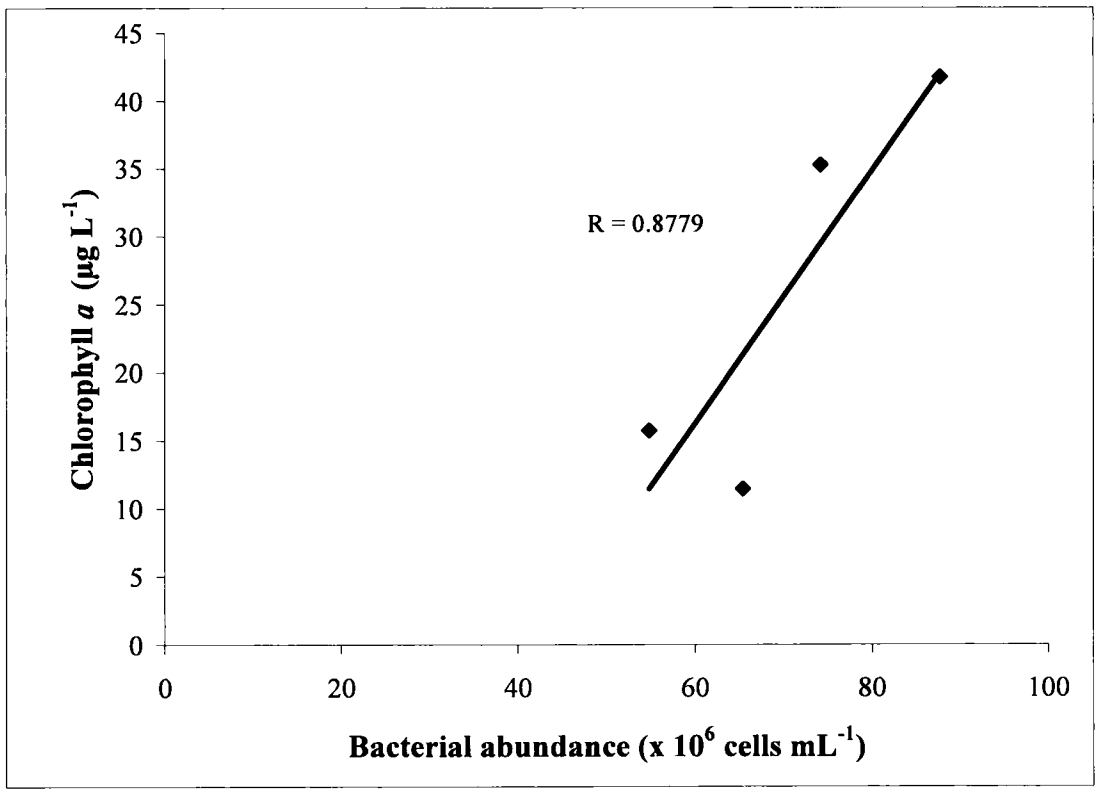

Figure 7. Relationship between bacterial abundance and chlorophyll *a* from samples collected in the Lafayette River in 2005.

Leucine and Thymidine Incorporation – Leucine and thymidine incorporation in the Lafayette River water varied over time and with the dominant phytoplankton taxa during 2003 (Table 3) and 2005 (Figs. 8 and 9). In 2003, leucine incorporation rates increased by an order of magnitude during late July and early August compared to late May and early June, when *G. splendens* was present in high concentrations (Table 3). Phytoplankton versus bacterial leucine and thymidine incorporation rates were estimated by comparing leucine and thymidine incorporation in incubations filtered onto 0.2 µm and 5.0 µm filters. There were significant differences in leucine and thymidine incorporation in the > 0.2 µm size fraction and the > 5.0 µm size fractions during 2005

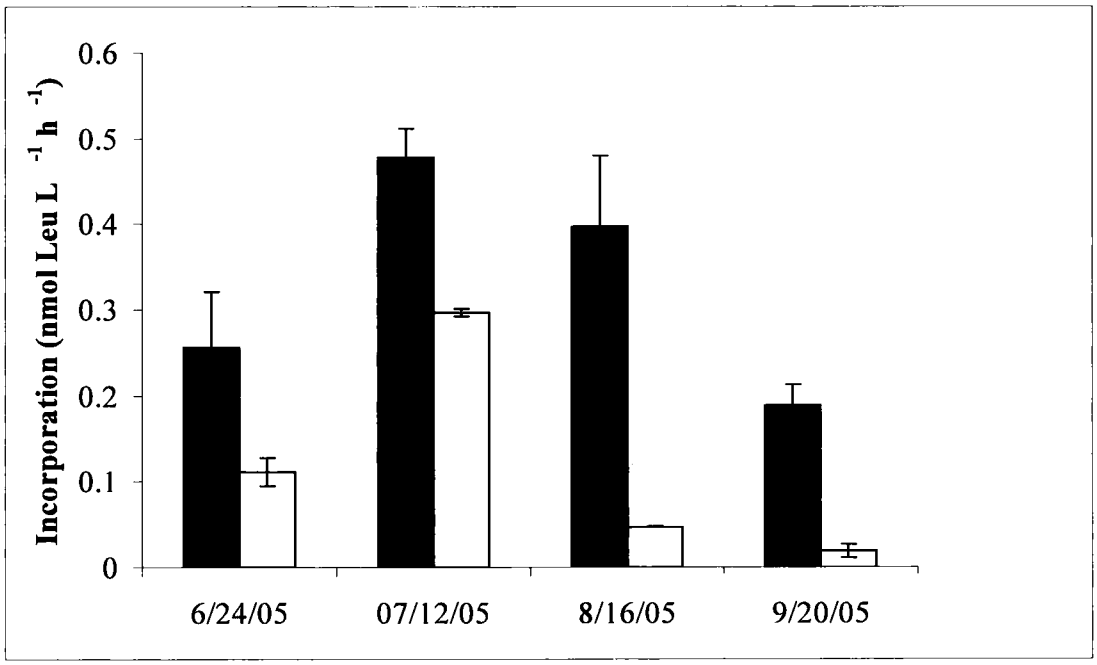

Figure 8. Leucine incorporation rates in whole water samples collected from the Lafayette River between June and September 2005. Comparisons are made between leucine incorporation rates by cells collected onto 0.2 μm nitrocellulose filters (black bars) or onto 5.0 μm nitrocellulose filters (white bars). Error bars are the standard deviations of triplicate incubations.

(Figs. 8 and 9). Incorporation rates into the > 5.0 μm size fraction during 2005 represented 10 to 50% of total leucine incorporation and 15 to 50% of the total thymidine incorporation, even though bacterial abundance on 5.0 μm filters was only between 0.04 and 0.18% of that collected on 0.2 μm filters. Incorporation of these substrates into larger cells was highest in 2005, during the mixed dinoflagellate bloom dominated by *A. sanguinea* and *Scrippsiella* sp. in June and July. Although no size-fractionated leucine and thymidine incorporation rates are available for 2003, incorporation of these compounds was higher during July and August when the mixed dinoflagellate bloom was

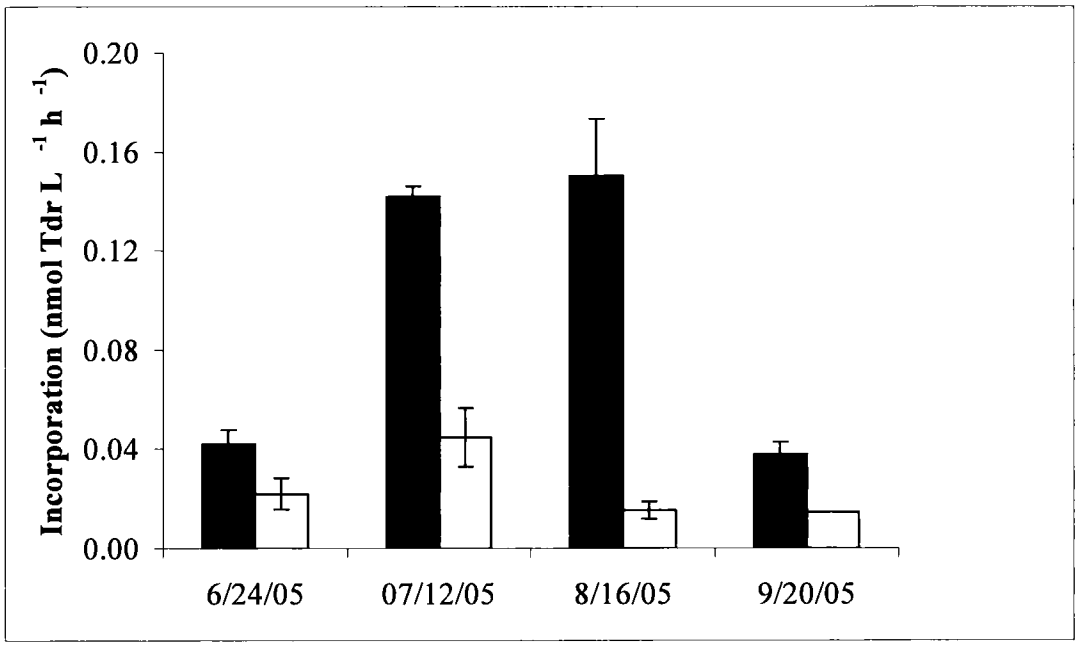

Figure 9. Thymidine incorporation rates in whole water samples collected from the Lafayette River between June and September 2005. Comparisons are made between thymidine incorporation rates by cells collected onto 0.2 μm nitrocellulose filters (black bars) or onto 5.0 μm nitrocellulose filters (white bars). Error bars are the standard deviations of triplicate incubations.

dominated by *Gymnodinium splendens*.

Antibiotic treatments – Results from experiments comparing antibiotic-treated samples with controls demonstrated that incorporation of leucine and thymidine in whole water (> 0.2 μm) and < 5.0 μm water samples were reduced by up to 90% (leucine) and 47% (thymidine) in incubations receiving antibiotic treatments (Fig. 10). In contrast, thymidine incorporation rates measured in cells collected on 5.0 μm filters (whole water, > 5.0 μm) were not statistically different (p-value = 0.33, n = 5, one-way ANOVA), in antibiotic and control treatments and leucine incorporation rates increased slightly when broad-spectrum antibiotics (Fig. 10) were used (p-value = 0.002, n = 3, one-way ANOVA). This suggests that leucine and thymidine incorporation by cells larger than

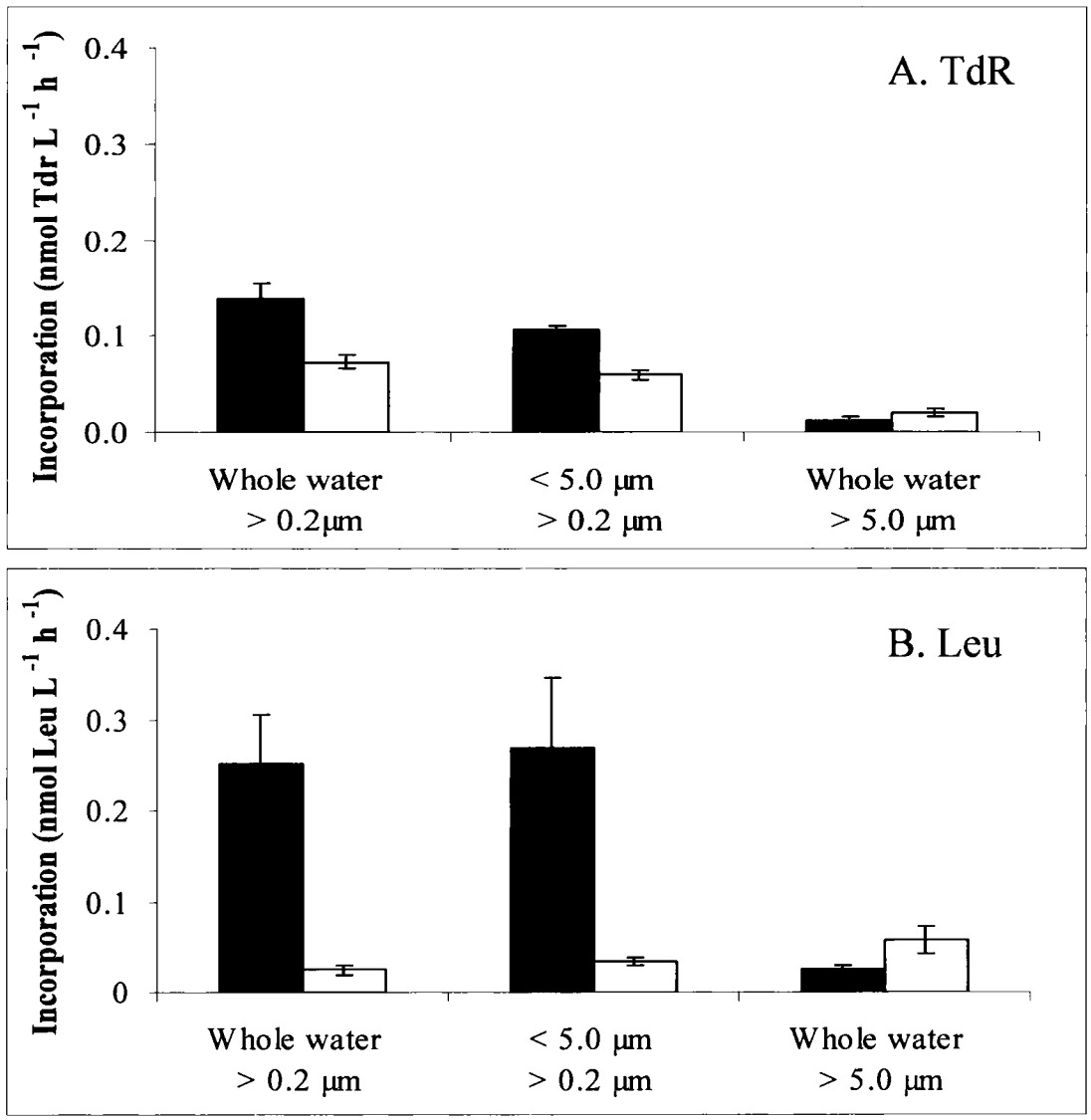

Figure 10. Thymidine (A) and leucine (B) incorporation in size-fractionated samples incubated with (white bars) and without (black bars) broad-spectrum antibiotics. Comparisons are made between thymidine (A) and leucine (B) incorporation rates by whole water and < 5.0 μm water samples filtered onto 0.2 μm nitrocellulose filters and whole water samples collected onto 5.0 μm nitrocellulose filters. Error bars are the standard deviations of triplicate incubations.

5.0 μm, such as phytoplankton (or cells associated with them), were not inhibited by the antibiotic treatment.

Microautoradiography - The presence of silver grains indicates cells were actively incorporating radiolabeled leucine (Fig. 11A) or thymidine (Fig. 11B) in individual species of phytoplankton collected from the Lafayette River. This confirms that phytoplankton incorporated leucine and thymidine in the presence of bacteria during assays designed to measure bacterial productivity in natural aquatic systems. Both cells contained more than 5 silver grains, indicating that the cells were actively incorporating leucine and thymidine. No silver grains were observed in control incubations (no label added) (Fig. 11C).

Discussion

The specificity of leucine and thymidine to heterotrophic bacteria is an important requirement of the [^{3}H]-leucine and [^{3}H] - thymidine incorporation methods for measuring bacterial productivity (Azam and Fuhrman 1984). However, this assumption has recently come under question (Kamjunke and Jahnichen 2000; Hietanen et al. 2002). In this study, at least some phytoplankton incorporated leucine and thymidine during bacterial productivity assays even when incubation times were short (15 minutes) and substrates were added at low (nmol L^{-1}) concentrations. If these results may be generalized, there may be serious bias (more than 50%) in bacterial productivity estimates made using the leucine and thymidine incorporation method in estuaries where phytoplankton mixotrophs that can compete for these substrates in bioassays are abundant.

During 2005, mixed assemblages of dinoflagellates (> 5.0 μm in size) incorporated both leucine and thymidine on short timescales (< 15 min) in whole water

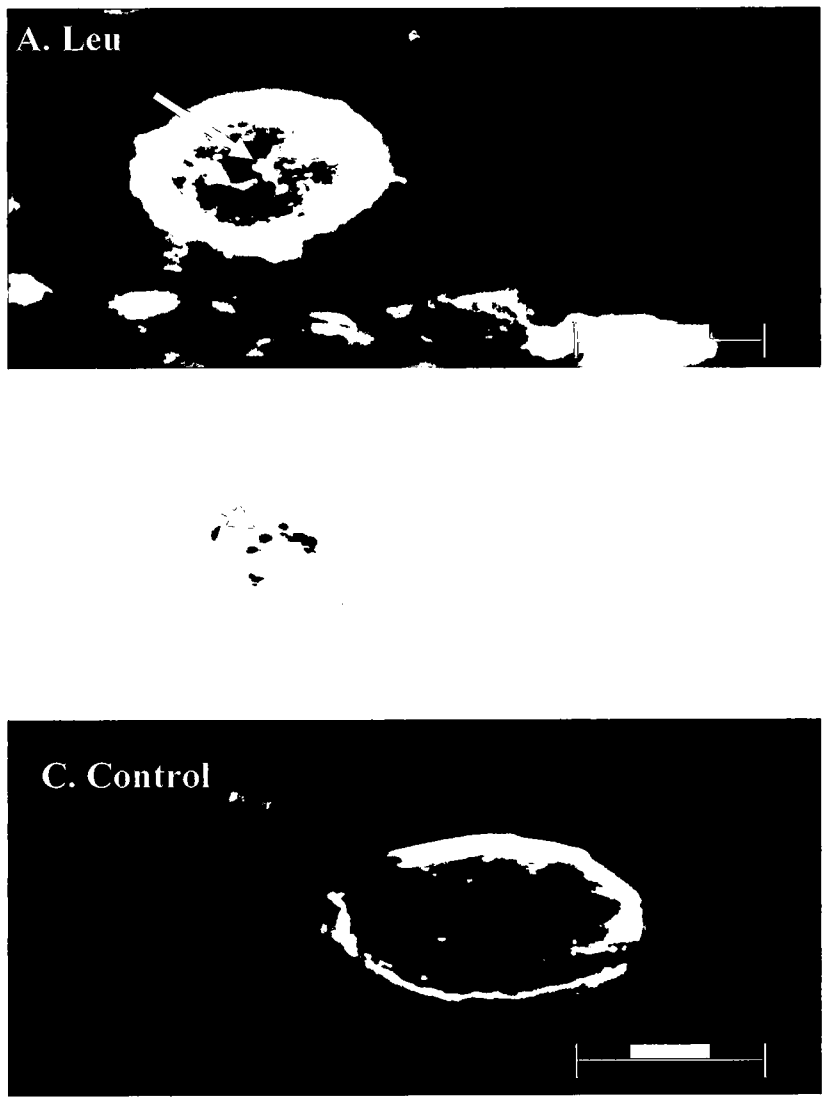

Figure 11. Microautoradiographic images of silver grains in phytoplankton cells from the Lafayette River, Norfolk, VA. Silver grains present in slides indicate incorporation of leucine (slide A) and thymidine (slide B) by individual phytoplankton cells. Slide C (control) does not contain silver grains indicating no incorporation by phytoplankton. Slides were photographed using different exposure times and under light (slide B) and dark (slide A and C) phase. Arrows show location of silver grains in the phytoplankton cell.

samples containing heterotrophic bacteria. Incorporation of these substrates into larger cells was highest during the mixed dinoflagellate bloom dominated by *A. sanguinea* and *Scrippsiella* spp. in June and July, suggesting that these dinoflagellates were able to incorporate leucine and thymidine at high rates, even in short incubations with low substrate concentrations containing bacteria. Phytoplankton assemblages during the months in which studies were conducted consisted primarily of *A. sanguinea, Scrippsiella* spp., *Cochlodinium* spp., and *Gymnodinium* spp., which have been shown to be mixotrophic (Doblin et al. 1999; Jeong et al. 2004; Jeong et al. 2005).

A. sanguinea has been reported not only to consume prey (Bockstahler and Coats 1993; Jeong et al. 2005), but also to take up organic N and C to supplement its nutrition (Mulholland 2004). Up to 50% of thymidine and leucine was incorporated by the > 5.0 μm size fraction when *A. sanguinea* and *Scrippsiella* spp. were present at high densities in the Lafayette River. This result is consistent with the idea that either one or both of these dinoflagellate mixotrophs can take up leucine and thymidine. Cumulative results from culture and field studies suggest that *A. sanguinea* could be partly responsible for the observed leucine incorporation in the Lafayette River.

Studies in the Chesapeake Bay and its tributaries have reported leucine and thymidine incorporation rates ranging 0.06 -0.45 nmol Leu L^{-1} h^{-1} and 0.003-0.258 nmol TdR L^{-1} h^{-1} (Table 5; del Giorgio and Bouvier 2002; Shiah and Ducklow 1994), comparable to leucine and thymidine incorporation rates measured in this study (Table 3, Figs. 8 and 9) and consistent with other studies in coastal bays and estuaries (Table 5).

Throughout these studies, estimates of rates of bacterial C productivity based on thymidine incorporation were much lower than those based on leucine incorporation.

Table 5. Literature values of leucine and thymidine incorporation rates in estuaries and coastal bays. Incorporation rates were converted to nmol thymidine (Tdr) or leucine (Leu) L^{-1} h^{-1} using conversion factors cited in studies.

	Location	Incorporation nmol Tdr and Leu L^{-1} h^{-1}	Reference
Leucine	Delaware Bay	0.134 - 0.197	Cottrell and Kirchman, 2003
	Chincoteague Bay (non-bloom)	0.13 - 1.07	Boniello and Mulholland, unpublished data
	Chincoteague Bay (bloom)	0.15 - 0.35	Boniello and Mulholland, unpublished data
	Choptank River Estuary	0.6 - 0.45	Bouvier and del Giorgio, 2002
	Central Bay, San Francisco	0.121 - 0.667	Murrell et al., 1999
	Suisun Bay, San Francisco	0.143 - 0.528	Murrell et al., 1999
	Sacramento	0.127 - 0.439	Murrell et al., 1999
	Baltic Coastal Seawater	0.65	Hietanen et al., 2002
	Arabian Sea	0.006 - 0.091	Ducklow et al., 2001
	Gulf of Finland	0.09	Heinanen and Kuparinene 1992
	Lafayette River Estuary	0.3 - 1.98	This study
Thymidine			
	Delaware Bay	0.006 - 0.017	Cottrell and Kirchman, 2003
	Chincoteague Bay (non-bloom)	0.01 - 0.04	Boniello and Mulholland, unpublished data
	Chincoteague Bay (bloom)	0.004 - 0.02	Boniello and Mulholland, unpublished data
	Chesapeake Bay	0.003 - 0.258	Shiah and Ducklow, 1994
	Baltic Coastal Seawater	0.175	Hietanen et al., 2002
	Arabian Seawater	0.0014 - 0.026	Ducklow et al., 2001
	Lafayette River Estuary	0.02 - 0.35	This study

The reasons for this discrepancy are unclear, but may be due to phytoplankton preferences for particular organic compounds, something we know little about, or the inability of the microbial community in the Lafayette River to incorporate thymidine, as has been observed elsewhere (Cottrell and Kirchman 2003). Although thymidine incorporation rates were low compared to leucine, there was still a significant fraction of thymidine incorporated into cells collected on 5.0 μm filters, confirming MA results and indicating that at least some phytoplankton were taking up thymidine. Experiments employing antibiotic treatments and MA further demonstrated that phytoplankton larger than 5.0 μm incorporated leucine as well as thymidine at significant rates.

The effectiveness of the antibiotics in inactivating bacteria was not evaluated and so it is possible that not all bacterial incorporation was inhibited. Middelburg and Nieuwenhuize (2001a, b) used broad-spectrum antibiotics (10 mg L^{-1} final concentration) to evaluate the contribution of bacteria to incorporation of inorganic nitrogen compounds. They found uptake of substrates was significantly lowered when broad-spectrumantibiotics were employed. Further, in the present study picoplankton and small phytoplankton and cyanobacteria were not enumerated in the < 5.0 μm size fraction, but these cells are known to be abundant in this system (Marshall et al. 2005). Small picoplankton include organisms such as *Synechococcus* and *Prochlorococcus*, shown to take up amino acids and other organic compounds in oceanic gyres (Zubkov et al. 2003, 2004; Zubkov and Tarran 2005), and *Aureococcus anophagefferens,* shown to take up amino acids in mid-Atlantic coastal bays (Mulholland et al. 2002). Organisms such as these contributed to thymidine and leucine incorporation in the < 5.0 μm size fraction.

Results from incubations in which broad-spectrum antibiotics were administered support the contention that phytoplankton in the Lafayette River estuary incorporate a significant amount of leucine and thymidine during bacterial productivity assays. In the Thames estuary, broad-spectrum antibiotics reduced amino acid and urea uptake in whole water samples by 49% and 86%, respectively, suggesting that bacteria took up significant amounts of urea and non-bacterial cells accounted for at least half of the amino acid uptake (Middelburg and Nieuwenhuize 2000). In the present study, 10 to 50% of the total leucine and thymidine incorporation rates were represented in the > 5.0 μm size fraction. Reasoning that 10 to 50% of the leucine and thymidine incorporation was unlikely to have been accomplished solely by such a small fraction of the bacterial community, this suggests that microorganisms other than bacteria and larger than 5.0 μm (e.g., phytoplankton) were incorporating leucine and thymidine.

Microautoradiography experiments confirm that at least some phytoplankton in the Lafayette River can incorporate [^{3}H]-leucine and [^{3}H]-thymidine in nature in competition with bacteria and in bacterial productivity bioassays. The uptake of organic substrates, including thymidine and amino acids, by diatoms and flagellates has previously been shown using microautoradiography after long (> 12 hr) incubations with radio-labeled substrates (Wheeler et al. 1977). Subsequent studies using microautoradiography revealed that some picophytoplankton (e.g. cyanobacteria), are capable of directly taking up DOM (e.g. amino acids) in the presence of bacteria (Paerl 1991). In these studies, additions (20 nmol L^{-1}) of ^{3}H amino acids were added to isolates of *Synechococcus* spp. (WH7803) and samples incubated for 1 to 6 hours. However, the

results presented here are the first demonstrating thymidine and leucine may be incorporated by phytoplankton during bacterial productivity bioassays.

Incorporation by phytoplankton could result in overestimates of bacterial productivity as great as 50% when using the [^{3}H] - leucine and [^{3}H] - thymidine incorporation methods. In this study, there was significant leucine and thymidine incorporation by phytoplankton in the > 5.0 μm size fraction, even though substrate additions were low and incubations short. Results from this study suggest that post-incubation size fractionation can significantly reduce the bacterial biomass on filters, and allow one to differentiate rate processes due to large phytoplankton from those of bacteria and small phytoplankton and bacteria. However, this method may be appropriate only under a limited set of environmental conditions such as during monospecific blooms of large phytoplankton.

Conclusions

Multiple lines of evidence suggest that some phytoplankton, particularly mixotrophic dinoflagellates, are capable of leucine and thymidine incorporation in organically enriched estuaries, such as the Lafayette River estuary. If true, then uptake of these substrates by phytoplankton may result in serious overestimates of bacterial productivity in systems where phytoplankton capable of incorporating these compounds are present. Estimates of bacterial productivity are important for evaluating heterotrophic microbial processes, such as decomposition and re-mineralization of organic matter (Ducklow et al. 1986; Robarts and Zohary 1993; Ducklow 2000; Cottrell and Kirchman 2003). As such, biases resulting from production estimates made using incorporation of

leucine and thymidine by microorganisms (e.g. phytoplankton) would impact our estimates of carbon and nutrient flow (Ducklow et al. 1986; Ducklow and Shiah 1992; Robarts and Zohary 1993; Kamjunke and Jahnichen 2000; Cottrell and Kirchman 2003) through estuarine communities.

As with bacteria, incorporation of leucine and thymidine by phytoplankton appears to be species specific and environmentally variable (Cottrell and Kirchman 2003). As such, the extent to which phytoplankton are capable of incorporating organic substrates needs to be evaluated. In addition, the nutritional and environmental conditions under which they do so needs to be examined to assess how widespread phytoplankton incorporation of thymidine and leucine is in nature and whether patterns emerge. This will help predict and account for biases during bacterial productivity bioassays. Size fractionation is imperfect, microautoradiography cumbersome, and the application of antibiotics uncertain. Consequently, better techniques for separating group or taxa-specific contributions to particular rate processes are necessary.

CHAPTER 4

CONCLUSIONS

Multiple lines of experimental evidence suggest that natural assemblages and cultures of phytoplankton are able to incorporate leucine and thymidine during [^{3}H] - leucine and [^{3}H] - thymidine incorporation assays designed to estimate bacterial productivity. This is contrary to key assumptions inherent in these assays: that leucine (Kirchman et al. 1985; Hollibaugh 1994) and thymidine (Fuhrman and Azam 1982) are specific to heterotrophic bacteria and incorporation by other microorganisms in the environment under appropriate assay conditions is negligible. The field and laboratory experiments done as part of this study indicate that some phytoplankton may compete with bacteria for leucine and thymidine during short incubations (< 1 hr) and at low substrate additions. Further, these results have important implications for interpreting results from bacterial productivity estimates made using the leucine and thymidine incorporation methods.

Because of the assumption that only bacteria incorporate leucine and thymidine during short incubations and when substrates are supplied at low concentrations, estimates of leucine and thymidine incorporation are often measured on whole water samples that are extracted directly in the incubation tube without any attempt to isolate the bacteria (Bell 1993; Kirchman 1993). However, this technique results in the extraction of DNA and protein from all of the cells in the tube, including bacteria, phytoplankton, and other microorganisms. If non-bacterial cells incorporate leucine and thymidine at significant rates, bacterial incorporation rates will be overestimated. These

studies have demonstrated that results may be at least partially improved by extracting cells directly on filters, particularly if there are large phytoplankton mixotrophs present.

Post-incubation size-fractionation and extraction of macromolecules after filtration onto filters with various pore sizes was used initially to remove most of the bacteria from phytoplankton in monospecific cultures and in blooms of known mixotrophs in the natural environment. This technique worked well because blooms were nearly monospecific and the phytoplankton cells were large and had few attached bacteria. After filtering leucine and thymidine incubations through 5.0 μm filters prior to extraction of macromolecules, bacterial abundances on the filters were less than 0.04 to 0.18% of those present on 0.2 μm filters. Despite the huge reduction in bacterial abundance on 5.0 μm filters, leucine and thymidine incorporation was still 10 to 50% of that observed on 0.2 μm filters. In cultures of *Nitzschia* sp. and *A. sanguinea*, few attached bacteria (< 2%) were caught on 5.0 μm filters as compared to 0.2 μm filters, but 100% of the leucine and thymidine incorporation could be attributed to cells caught on 5.0 μm filters. This would indicate that cells in the phytoplankton fraction (>5.0 μm) and attached bacteria incorporated thymidine and leucine. Although size fractionation results in this study were compelling, in many systems size-fractionation is less than optimal. Due to overlapping of sizes between bacteria and smaller microorganisms such as picoplankton, uptake measurements in size-fractionated populations can be difficult and pose problems for identifying who is actually taking up the substrate (Ducklow 2000). In addition, when attached bacteria are abundant, as is often the case in turbid estuaries, they may contribute significantly to leucine and thymidine incorporation even in larger size fractions (Kirchman and Ducklow 1987; Ducklow 1993; Kirchman 1993).

In addition to these compelling, but indirect lines of evidence, there was significant leucine and thymidine incorporation in incubations of natural populations and cultures in which bacterial incorporation was reduced using broad spectrum antibiotics. This suggests that either antibiotics were not affecting a large portion of the bacteria or that cells other than heterotrophic bacteria were incorporating leucine and thymidine. When the > 5.0 μm size fraction was evaluated, there was little or no effect of antibiotic treatments on leucine and thymidine incorporation. This supports the contention that phytoplankton, the bulk of the biomass on these filters, were competing for leucine and thymidine.

Finally, microautoradiography was used to directly visualize the incorporation of radiolabeled thymidine and leucine by phytoplankton in incorporation bioassays using natural populations. However, this technique is challenging, not always quantitative, and it would be difficult to scale it to use routinely when evaluating bacterial productivity.

Size fractionation is imperfect, antibiotic treatments may have uncertain effects, and microautoradiography is a difficult technique to apply broadly or quantitatively. However, the present study has shown that it is important to re-evaluate competing microbial processes in incubation bottles, particularly in environments where phytoplankton mixotrophs and bacteria may compete for the same nutrient resources. Culture studies can help to determine whether particular organisms may be capable of particular processes, however, culture systems are generally acclimated to unrealistic physical and chemical conditions and lack the biological complexity that affects biogeochemical cycling in the environment. In addition, many organisms, including most mixotrophic dinoflagellates, even those already in culture, do not grow at realistic rates in

the absence of bacteria. This suggests that there may be some mutualistic relationship between these groups in nature.

The use of antibiotics to inhibit incorporation by heterotrophic bacteria, at least on a short-term basis, may be a useful tool for distinguishing phytoplankton and bacterial processes in some situations. However, the effects of antibiotics on the diverse bacterial community and on phytoplankton have not been widely examined and so it is unclear what fraction of the bacteria are inactivated after antibiotic treatments and whether phytoplankton function is at all compromised. Although antibiotics are selective for some bacteria, they may not inhibit all types of bacteria (Oremland and Capone 1988). Therefore, results may be difficult to interpret. For example, in the present study leucine and thymidine incorporation were reduced to different extents in individual cultures and across taxa. It is unclear if this is because different phytoplankton were able to incorporate one substrate more than the other, whether bacterial communities were differentially affected, or whether antibiotics affected the ability of phytoplankton to take up the substrates. For both culture and field samples, antibiotics did not appear to inhibit photosynthetic efficiency or harm phytoplankton cells in any visible way. In fact, when antibiotics were added to *A. sanguinea*, the photosynthetic rate significantly increased compared to incubations without antibiotics. Wang et al. (2004) found that growth was enhanced when phytoplankton cultures were treated with Penicillin G and Streptomycin. However, they noted at higher concentrations (> 60 mg L^{-1} and 134 mg L^{-1} Penicillin G and Streptomycin, respectively) phytoplankton may be inhibited and cells damaged (Wang et al. 2004). Despite variations in antibiotic inhibitors, antibiotics may be a useful tool when examining competition among bacteria and phytoplankton for organic

substrates. The use of antibiotics, coupled with size-fractionated extraction of macromolecules, may also provide an estimate of the contribution of attached bacteria to thymidine and leucine incorporation in whole water assays.

Extracellular Isotope Dilution

Leucine incorporation rates in this study were not corrected for extracellular isotope dilution by dissolved free amino acid (DFAA). In marine systems, DFAA concentrations are thought to be < 1 nmol L^{-1} (Kirchman 1993), thus additions of 10 nmol L^{-1} of [^{3}H]-leucine are considered ample for "swamping" out unlabelled leucine and repressing *de novo* synthesis of leucine (Kirchman 1993). However, Mulholland et al. (2002, 2003) have reported total DFAA concentrations up to 1 μmol L^{-1} in coastal and estuarine environments. In the Lafayette River, DFAA concentrations greater than 100 nmol L^{-1} was measured when phytoplankton biomass was high, such as during bloom events (Watson and Mulholland unpublished data). Leucine concentrations during the same time period were as much as 30 nmol L^{-1} (Watson and Mulholland unpublished). In these experiments, 10 nmol L^{-1} of [^{3}H]-leucine were used to determine incorporation rates. If other amino acids or unlabeled leucine were diluting [^{3}H]-leucine, then this would result in significant isotope dilution. Little is known about leucine uptake and how other amino acids compete with leucine during incorporation experiments. Consequently, the results from leucine incorporation bioassays presented here should be considered conservative. Further, in estuarine systems where amino acid concentrations can be high, extracellular isotope dilution should be considered when conducting leucine incorporation studies.

Implications of Phytoplankton Incorporation

In the Lafayette River, cells present in the phytoplankton size fraction (>5.0 μm) appeared to incorporate thymidine and leucine at high rates during bacterial productivity bioassays, resulting in serious overestimates of bacterial production. This in turn has serious implications for our understanding of carbon and nutrient cycling in marine environments. Although this study examined competition between bacteria and phytoplankton for leucine and thymidine in cultures and an estuarine system, overestimates of bacterial productivity may not be limited to estuaries and coastal systems. Recent studies have shown that some cyanobacteria are capable of taking up and competing with heterotrophic bacteria for amino acid in both freshwater lakes and oceanic gyres (Zubkov et al. 2003; Zubkov and Tarran 2005). Similarly, phytoplankton culture experiments in this study showed that *Nitzschia*, an oceanic diatom, may contribute to the incorporation of both leucine and thymidine at high rates and during short incubations.

Future Directions

Since bacterial productivity measurements were collected during dense phytoplankton blooms, it is uncertain to what extent bacterial productivity estimates may be biased during non-bloom conditions. In order to understand how widespread of a problem leucine and thymidine specificity is in estuarine and oceanic environments, further studies on which phytoplankton species can incorporate these substrates are needed. In addition, new methods for estimating bacterial productivity need to be

developed or traditional methods need to be modified to account for incorporation by other microorganisms, including cyanobacteria and phytoplankton.

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CAREER DEVELOPOMENT

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