Quantifying Changes in Bacterial Growth Efficiency (BGE): A New Tool for Assessing Ecological Function in Natural and Restored Salt Marshes

A Methods Guide

Introduction

Throughout North America there is increasing emphasis on the restoration of degraded wetlands, as evidenced by the passage of the ambitious Wetlands Restoration and Improvement Act. Although clearly the goal of such projects is restoration of the functional properties of marsh and wetland ecosystems there is still no scientifically robust procedure that can be used to ascertain if ecological function has indeed been restored. We present here methodology we have developed to quantify bacterial growth efficiency (BGE). This is defined as the fraction of organic carbon consumed by bacteria that is incorporated into biomass, which is a key parameter that characterizes the function and ecological role of natural aquatic bacteria (for review see del Giorgio and Cole 1998).

We have shown as part of our CICEET funded research that immediately after tidal flow was restored as part of the extensive restoration of two separate derelict salt marsh hay sites in Delaware Bay, NJ, (www.pseg.com/companies/nuclear/estuary/introduction/program_features.html) and before typical salt marsh vegetation was reestablished, bacterial growth efficiencies (BGE) were low in comparison to adjacent pristine reference salt marshes. In the 5 y subsequent to restoration we have documented how the BGE at these two major restoration sites has progressively increased although they are still not yet quite at the same level as the reference marshes. Yet, vegetation characteristic of salt marshes in this region became well established after 3 y, which although visually indicating restoration success, did not translate into complete restoration of fundamental bacterial processes. These results indicate that BGE may serve managers and permitting authorities as a dynamic indicator of ecosystem function and integrity. This index provides more fundamental information than current measures of plant and animal abundance because BGE is clearly highly influenced by changes in the sources and magnitude of organic carbon inputs induced by either marsh degradation or restoration.

Our approach is based on the fact that the flow of organic matter in aquatic ecosystems is largely mediated by microbial assemblages, composed of heterotrophic bacteria and a host of microheterotrophic organisms (Ducklow and

Carlson 1992, Pomeroy and Wiebe 1993). These microbial assemblages react faster than any other biotic component to ecosystem change and thus provide a highly sensitive index of ecosystem condition and recovery. Bacteria are primarily responsible for the transformation of detrital organic matter in aquatic ecosystems, and the carbon that is converted into protoplasm is then available to be transferred via the microbial food web up to higher trophic levels (Kreeger and Newell 2000). The degree to which bacteria influence overall secondary production of aquatic ecosystem, however, is heavily dependent upon the efficiency with which bacteria themselves convert dissolved organic matter into biomass (defined here as growth efficiency). BGE is regulated by the supply and quality of the organic substrates available to bacteria, as well as by the availability of nutrients, particularly N and P in their various organic and inorganic forms (del Giorgio and Cole 1998). BGE is thus a measure that integrates fundamental properties of aquatic ecosystems, such as organic matter and nutrient availability.

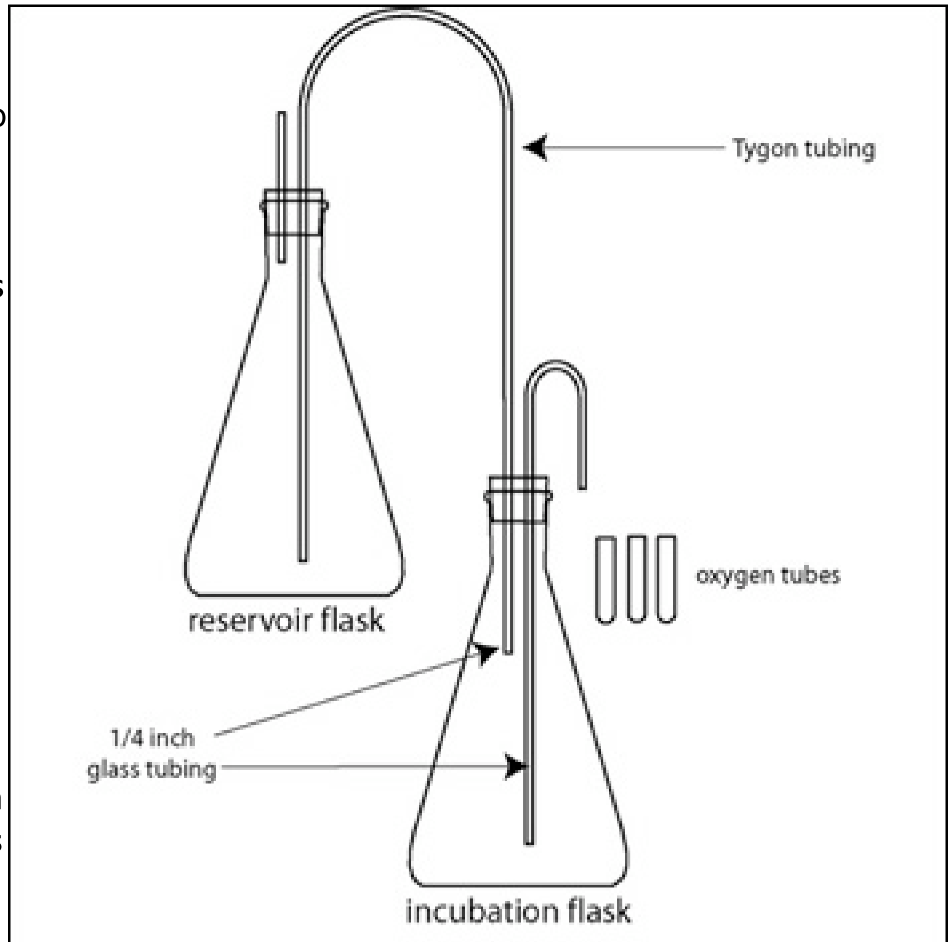
METHODS

Some of the following methodological procedures are also illustrated in a short video that can be viewed at
http://www.coastlive.org/120800_wkshop/1208videos/1208videos.htm.
Specifically, the video with the heading "CICEET Funding" shows the laboratory process underway.

Water Collection and Filtration

Collect approximately 20 L of sub-surface water ~ 2 to 3 h after high tide by immersing 22 L Nalgene HDPE carboys ~0.25 m beneath the surface and transport back to the laboratory for immediate filtration. The water collected on the ebb tide will have been subject to marsh processes for several hours and hence most indicative of changes in fundamental differences in nutrient cycling between natural marshes and those undergoing restoration. Prior to filtration, remove a small sub-sample (~500 ml) from each carboy to determine total bacterial community production and abundance (see below). Gently filter approximately 10 L of water from each site through 142 mm diameter AP15 Millipore filters held in a Millipore filter holder using a peristaltic pump and silicone tubing. This filtration process is necessary to remove protozooplankton grazers and their potential contribution to respiratory processes. In the mesohaline saltmarsh systems where we have applied this method we used flow cytometry as well as a Coulter Multisizer II to count and size the particles present in the water before and after filtration. We found that these filters were effective in reducing the number of > 3 _m particles by over 90%, while allowing over

80% of the free-living bacteria to pass into the filtrate. For new locations such testing should be performed to ensure that the bacterioplankton community is not dominated by bacteria that are attached to particles.



Place filtered water into a flow-through incubation assembly composed of two 4 L glass Erlenmeyer flasks connected by 0.25 inch inner-diameter Tygon tubing (Figure 1). Establish a siphon from the top reservoir flask and out of the sampling tube of the incubation flask Incubate flasks in the dark at in situ field temperatures and sub-sample at 0, 3, and 6 h during summer months and 0, 4, and 8 h during colder months (i.e., when ambient water temperatures falls below 15 C). We have found that these incubation times provide measurable changes in oxygen content. The total volume of water removed at any given sampling time-point should be as small as practical to avoid unnecessary dilution of the incubation flask by replacement water. We routinely use ~ 25 ml for estimating bacterial production and abundance and ~40 ml for oxygen analysis.

Bacterial Respiration

Sub-samples for determining oxygen consumption are collected in triplicate for the initial time-point and in duplicate for each subsequent time-point. The method described below is for use of membrane inlet mass spectrometry to measure oxygen consumption (Kana et al. 1994). This method, based on changes in the atomic ratio of argon and oxygen in the dissolved phase, offers an

extremely precise and rapid assessment of changes in oxygen concentrations. Alternate methods of estimating bacterial respiration have been used with success in other studies (e.g. Winkler titration; Smith and Kemp 2002).

Begin by siphoning water from the lower incubation flask into a 7 ml borosilicate oxygen tube fitted with a ground glass stopper. For this sampling tube use the smallest bore tubing that gives adequate flow (0.125 inch ID Tygon tubing), flushing thoroughly with the end of the Tygon tubing placed at the bottom of the tube and a minimum of 21 ml sample water to ensure complete flushing of water to the bottom of the tube. Special care must be taken to ensure that no small air bubbles are present and that a convex meniscus is visible when tube is full. (This is best achieved by removing the Tygon tubing slowly from the borosilicate tube and arresting flow only after it has been removed completely). Add as a fixative 10 l of half-saturated HgCl2 (i.e. 3.3mg HgCl2 100ml-1), cap firmly with ground glass stoppers, and store vertically and fully immersed under water at in situ temperature. Oxygen concentrations should be determined within one week of sampling. Respiration rates (g C L-1 h1) can then be derived from the slope of the linear regression of incubation time versus oxygen concentration using an RQ value of 1.0.

Bacterial Production

Free-living and total community bacterial production are estimated using 3H-leucine incorporation rates, following modifications of Smith and Azam (1992). Begin by preloading microcentrifuge tubes with 20 l of diluted isotope working stock (40-100 Ci mmol -1; Sigma), such that with the addition of 1.5 ml of sample will result in a final concentration of 40 nM. Add 100 ul 100% TCA (Trichloroacetic Acid) to the tubes which will serve as blanks. Cap and refrigerate until time of sample addition.

Add 1.5 ml of sample water to the blank and three pre-loaded microcentrifuge tubes. Record start time. Repeat for all water samples for which estimates of bacterial production are needed. Vortex each tube for approximately 3 to 5 s on high and place in a water bath in the dark at room temperature to incubate for 1 h. After approximately 55 min., prepare a pipette to dispense 100 l of 100% TCA, remove tubes from the water bath, and remove caps from only the three replicate tubes. After exactly 1 h, add 100 l of 100% TCA to the three open replicate tubes, killing all bacteria and stopping production. Record stop time. Vortex each tube well (including blanks) and centrifuge at 14000 rpm for 10 min. Remove tubes from the centrifuge and gently aspirate the supernatant using a Pasteur pipette and flexible tubing attached to a vacuum pump. It is critical to

avoid aspirating the bacterial pellet, which forms about _ inch up the outside edge of the tube. (It may be helpful to mark the outside rim of each tube prior to loading in the centrifuge to aid in locating the pellet).

After aspirating all samples, add 1.5 ml 5% cold TCA to each tube to precipitate all incorporated leucine. Vortex well and centrifuge at 14000 rpm for 10 min. Remove tubes from centrifuge and aspirate supernatant again (as described above). Add 1.5 ml scintillation cocktail (e.g. UltimaGold) to each tube. Vortex well. Place tubes in uncapped glass 20 ml liquid scintillation vials and count samples in scintillation counter using a protocol specific for tritium and the scintillation cocktail being used. Bacterial production rates are derived from leucine incorporation during the one-hour incubation and a leucine-to-carbon conversion factor. Carbon conversion factors can be determined empirically or derived from literature values (e.g. Ducklow et al. 2002)

Bacterial Growth Efficiency
Growth efficiency is determined by dividing bacterial production by total carbon consumption

$$\frac{BP}{BP+BR}$$

where BR is the estimate of bacterial respiration in g C L-1 hr1, as determined by the 6 or 8 h incubation, and BP the overall mean of bacterial production estimates of subsamples from 0, 3, and 6 (or 0, 4 and 8) hour time-points during the incubation.

Bacterial Abundance
Abundance (cells ml-1) of free-living and whole bacterial communities can be determined by standard flow-cytometric techniques or epifluorescent microscopy coupled with DAPI staining (Porter and Feig 1980). We highly recommend the use of flow-cytometry as a more rapid and reproducible means of enumerating bacterioplankton in natural samples. The following methods refer to the use of a Becton-Dickinson FACSCaliber bench top sheath flow cytometer for enumeration of natural bacterial cells. The nucleic acid stain SYTO-13 (Molecular Probes) is used to determine bacterial abundance and total nucleic acid content of live samples following del Giorgio et al. (1996) and Gasol and del Giorgio (2000), respectively. SYTO-13 is a nucleic acid stain that passively diffuses through the cellular membrane of bacterial cells and binds to both RNA and DNA, fluorescing green when illuminated with UV light. Due to its extremely low intrinsic

fluorescence, unbound SYTO-13 has an extremely low quantum yield and is not visible.

Begin by preparing a 0.5 mM working solution of SYTO-13 by dissolving concentrated stock with DMSO. For use as an internal calibration, prepare a bead stock of 1 m fluorescent microspheres (Molecular Probes) at a concentration of approximately 3000 beads L-1. Pipette 0.5 ml of sample into a 7 mL flow cytometer Falcon tube and add 2 L of 0.5 mM SYTO-13. Vortex well and incubate in the dark for 5 min. Add 10 L of the 1 m bead stock and vortex again. Process samples in the flow-cytometer following del Giorgio et al. (1996) and operational protocols specific to the instrument being used. Bacterial cells are best visualized in a cytogram of side scatter (SSC) versus green fluorescence (FL1). Flow-cytometric analysis should continue until a minimum of 20,000 events are counted to ensure suitable accuracy of abundance estimates. Total cell abundance, as evidenced by the number of intact bacterial nuclei visualized by the flow cytometer, is determined by defining a region encompassing the enumerated heterotrophic bacteria and normalizing this number for with the total number of reference beads counted (del Giorgio et al. 1996). Total nucleic acid content, which serves as an index of bacterial cell size and activity (Gasol and del Giorgio 2000), is determined by identifying regions that represent sub-populations of high and low nucleic acid content cells.

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