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Estimation of bacterial use of dissolved organic nitrogen compounds in aquatic ecosystems using Biolog plates

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ABSTRACT: We demonstrated that bacterioplankton utilization of N-containing substrates, in relation to overall substrate utilization, can be assessed by calculating a nitrogen use (NUSE) index from Biolog-ECO microplate readings. The NUSE index was positively correlated with bacterial-specific aminopeptidase activity in coastal plankton samples, and it decreased after ammonia or amino acid addition to seawater cultures; this indicated that the index was sensitive to changes in N concentrations in natural samples. The index provides valuable information on dissolved organic nitrogen processing by natural bacterial assemblages, and thereby substantially expands the utility of the Biolog-ECO microplates.

KEY WORDS: Biolog · Bacterioplankton · Substrate utilization · Dissolved organic nitrogen · DON · Nutrients · Amino acids

INTRODUCTION

Biolog-ECO microplates are designed to estimate the functional diversity of bacterial assemblages, by measuring the relative utilization of various ecologically relevant organic substrates. They have allowed comparisons of community-level physiological profiles of different bacterial assemblages in aquatic environments (Grover & Chrzanowski 2000, Schultz & Ducklow 2000, Sala et al. 2005a). For example, Sala et al. (2005b) showed that changes in the composition of bacterioplankton assemblages were associated with changes in physiological profiles in the NW Mediterranean. However, certain aspects of the Biolog approach are controversial, such as how representative the substrates in the plates are of substrates in nature, and the fact that the method involves culturing (Haak et al. 1995, Smalla et al. 1998, Preston-Mafham et al. 2002). The aim of the present study was to test if the Biolog approach could be applied to estimate bacterial use of dissolved organic nutrient (DON) compounds and, thereby, to indirectly evaluate the use of the Biolog approach when applied to seawater samples.

Biolog-ECO microplates hold 31 substrates: 10 substrates contain both C and N (e.g. 6 different amino acids), 2 contain both C and P, and the remaining substrates contain only C. We tested whether Biolog-ECO microplates could be used to calculate a nitrogen use (NUSE) index, expressed as the proportion of utilization of substrates containing both C and N relative to total substrate utilization, to assess the utilization of low molecular weight (LMW) DON components by natural bacterial communities.

The DON pool is increasingly recognized as a dynamic component of N and C cycles in marine systems, with N concentrations often substantially greater than those in the dissolved inorganic nitrogen pool. The latter is considered to supply most of the N required by planktonic organisms (Berman & Bronk 2003). Consumption of LMW DON components is generally investigated in experiments where the release of ammonia or amino acid uptake are monitored (Berman

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et al. 1999, Rosenstock & Simon 2003). This approach has shown that the majority of DON in aquatic environments is consumed by heterotrophic bacteria (Bronk 2002).

Considering the different elemental composition of substrates in the Biolog-ECO microplates, and the large dependence of bacterial growth on nutrient availability, we compared the utilization of N-containing substrates in the plates to overall utilization of substrates by calculating a NUSE index. If applicable to natural aquatic samples, we hypothesized that the NUSE index should show a positive correlation with *in situ* aminopeptidase activity, a commonly used indicator of peptide hydrolysis and N limitation, and that the index would be sensitive to enrichment by N sources (e.g. ammonia or amino acids).

MATERIALS AND METHODS

Water was collected from contrasting marine environments in the NW Mediterranean Sea and in Antarctica. Sampling of surface water (0.5 m depth) in the oligotrophic coastal area of Blanes Bay, Spain, was conducted monthly between January 2003 and December 2004. Surface water samples were also taken from Barcelona Harbour (14 occasions between June 2001 and October 2002), Tarragona Harbour (ca. 100 km south of Barcelona, sampled 4 times in June 2001), and from Arenys de Mar Harbour (ca. 40 km north of Barcelona, sampled 4 times in January-February 2002). Sampling in Antarctic waters was carried out in areas of the Bransfield Strait, Gerlache Strait, and Bellingshausen Sea during the TEMPANO cruise in the austral summer of 2002. Water was collected from 2 to 6 depths (above 150 m) from 6 stations. On each sampling occasion, Biolog-ECO microplates were prepared and total inorganic nitrogen (TIN) was measured. In addition to Biolog plate preparation and TIN concentration measurements, aminopeptidase activity (AMA) was determined at the Blanes Bay site.

A nutrient limitation experiment (NUT) was performed in July 2003 with water from Blanes Bay. Unfiltered seawater samples (250 ml) were enriched with ammonia (NH_4Cl: 4.2 µM N final concentration), phosphate (Na_2HPO_4: 0.6 µM P final concentration), or glucose (20 µM C final concentration). A control bottle received no nutrients. The samples were incubated for 24 h in the dark at *in situ* temperature, and then Biolog-ECO microplates were prepared from each treatment.

Seawater culture experiments (ORG) were carried out on 5 sampling occasions in Blanes Bay. We inoculated 1.9 l of sterile filtered seawater (0.2 µm pore size, Sterivex, Millipore) with 100 ml of natural bacterioplankton, 0.8 µm pore size filtered seawater (polycarbonate filter, Nuclepore). Cultures were enriched with amino acids (24 µM C final concentration; from a stock of 0.4 mM C each of arginine, glutamine, leucine, lysine, and tyrosine) or glucose (24 µM C final concentration). After incubation for 4 d in the dark at *in situ* temperature, a Biolog plate was prepared from each seawater culture.

Table 1. Substrates present in the Biolog-ECO microplates divided into categories according to the nutrients they provide

Compound	Category	Nutrients
L-arginine	Amino acid	C+N
L-asparagine	Amino acid	C+N
L-phenylalanine	Amino acid	C+N
L-serine	Amino acid	C+N
Glycyl-L-glutamic acid	Amino acid	C+N
L-threonine	Amino acid	C+N
Phenylethylamine	Amine	C+N
Putrescine	Amine	C+N
N-acetyl-D-glucosamine	Carbohydrate	C+N
D-glucosaminic acid	Carboxylic acid	C+N
Glucose-1-phosphate	Carbohydrate	C+P
D,L-α-glycerol phosphate	Carbohydrate	C+P
β-methyl-D-glucoside	Carbohydrate	C
D-galactonic acid γ-lactone	Carbohydrate	C
Piruvic acid methyl ester	Carboxylic acid	C
D-xylose	Carbohydrate	C
D-galacturonic acid	Carboxylic acid	C
Tween 40	Polymer	C
i-erythritol	Carbohydrate	C
2-hydroxy benzoic acid	Phenolic compound	C
Tween 80	Polymer	C
D-mannitol	Carbohydrate	C
4-hydroxy benzoic acid	Phenolic compound	C
α-cyclodextrin	Polymer	C
γ-hydroxybutyric acid	Carboxylic acid	C
Glycogen	Polymer	C
Itaconic acid	Carboxylic acid	C
D-cellobiose	Carbohydrate	C
Ketobutyric acid	Carboxylic acid	C
α-D-lactose	Carbohydrate	C
D-malic acid	Carboxylic acid	C

Concentrations of nitrate, nitrite and ammonia were measured according to Grasshoff et al. (1983). TIN was calculated as the sum of the concentrations of NO_3, NO_2 and NH_4.

Samples for AMA were prefiltered (1 µm pore size) to attribute activity to bacteria. AMA was determined spectrofluorometrically after addition of the fluorogenic substrate leucine-7-amido-4-methylcoumarin as described in Sala et al. (2001). Bacterial abundance was determined by epifluorescence microscopy of 4′,6-diamidino-2-phenylindole (DAPI) stained samples (Porter & Feig 1980). Cell-specific AMA was calculated by dividing AMA by bacterial abundance.

Biolog-ECO microplates (Biolog) were inoculated with 150 µl of sample per well and incubated for 6 d at room temperature. After incubation, plates were stored at –20°C until absorbance was measured on a microplate reader (590 nm wavelength, ELX800 BIOTEK Instruments). Absorbance in the blank wells was subtracted from the average of the triplicate wells for each substrate in the plate. Substrates were classified according to their content of C, N, and P (Table 1). Absorbances were added arithmetically and the NUSE index was calculated as the proportion (expressed as a percentage) of the summed absorbances of C + N substrates over total absorbance measured in each plate.

RESULTS AND DISCUSSION

Estimating the potential for substrate utilization by aquatic bacterial assemblages in relation to resource availability and nutrient limitation is of primary importance to understanding bacterial processing of dissolved organic matter. In surface water samples from the NW Mediterranean Sea, we found a significant positive correlation between the NUSE index and bacterial-specific AMA (n = 22, r^2 = 0.49) (Fig. 1). Aminopeptidase plays a critical role in the acquisition of DON, through its hydrolysis of macromolecules (such as proteins) into monomers (e.g. amino acids), which are readily taken up by bacteria (Antia et al. 1991). This enzyme activity has been shown to be a good estimator of N deficiency in planktonic communities, since a higher N demand leads to a higher activity of the enzyme (Sala et al. 2001). Therefore, the positive correlation between the NUSE index and AMA supported the notion that the use of N-containing substrates in the Biolog-ECO microplates reflected the demand for nitrogen by the bacterial assemblage. Thus, calculating the NUSE index from the utilization of substrates in the Biolog plates yielded insight into the physiological DON demand of marine bacterioplankton.

Physiological profiling using Biolog-Eco microplates has shown that the functional diversity of marine bacterial assemblages differs on both spatial and temporal scales in the Antarctica and in the NW Mediterranean Sea (Sala et al. 2005a,b). We therefore compared variability in the NUSE index against ammonia concentrations in samples from these ocean areas (Fig. 2). The highest values of the NUSE index (37.9 to 32.7) were found in samples from Arenys de Mar and Blanes Bay, where ammonia concentrations were low (0.64 to 0.90 µM). In contrast, low values of the NUSE index (23.1 to 25.9) were found in samples with relatively high ammonia concentrations (13.3 to 3.4 µM) in both Tarragona Harbour and the Antarctic (Fig. 2). When all

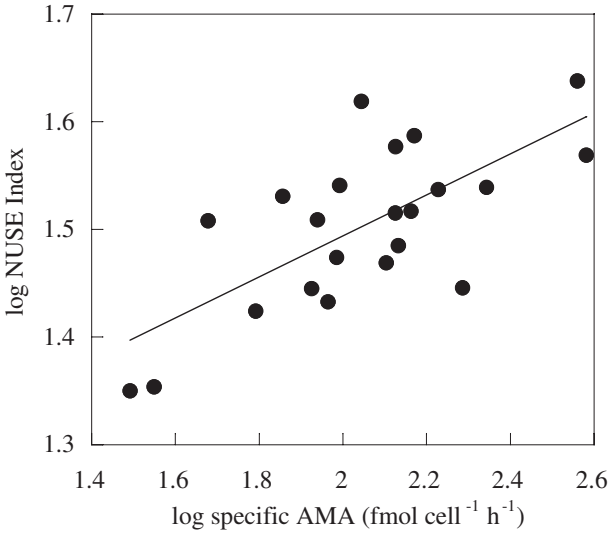

Fig. 1. Relationship between bacterial cell-specific aminopeptidase activity (AMA) and nitrogen use (NUSE) index in surface NW Mediterranean samples

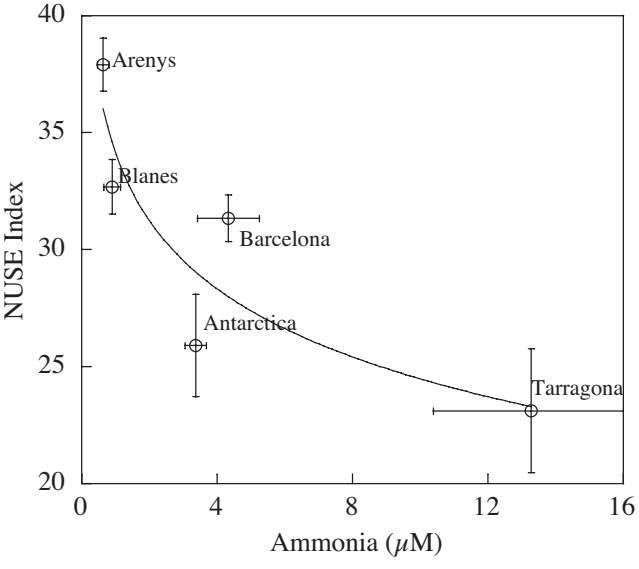

Fig. 2. Relationship between ammonia concentration and NUSE index in NW Mediterranean samples: Arenys de Mar (n = 4), Blanes Bay (n = 21), Barcelona (n = 14), Tarragona (n = 4); and in western Antarctic samples (n = 36). Error bars: SE

sampling sites were combined, there was a significant (p = 0.003) negative logarithmic relationship between ammonia concentrations and the NUSE index (r^2 = 0.79). A similar, but weaker (r^2 = 0.63), negatively logarithmic relationship was also observed between TIN and the NUSE index (data not shown), which indicated that in the studied ecosystems the NUSE index was sensitive to variations in total inorganic nitrogen (TIN) in general, and to changes in ammonia concentrations in particular.

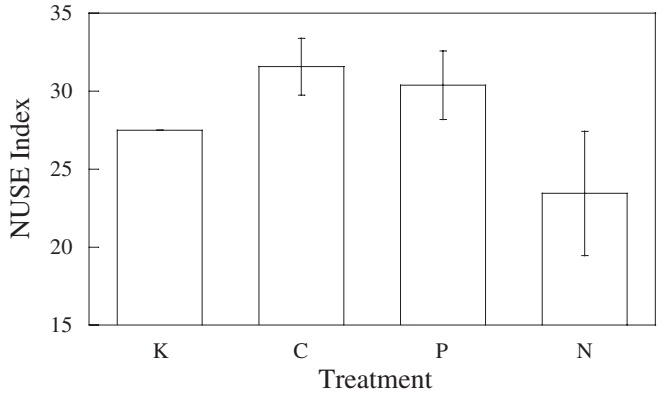

Fig. 3. NUSE index after seawater enrichments with glucose (C), phosphate (P), ammonia (N), or no additions (K). Error bars: SD of 2 replicate treatments for C, P and N

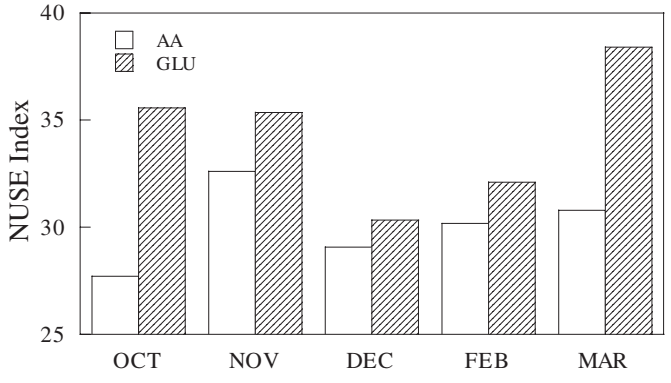

Fig. 4. NUSE index in 5 enrichment experiments with amendments of amino acids (AA) and glucose (GLU)

To further test the robustness of the NUSE index, a first experiment (NUT) was conducted to determine whether bacterial utilization of N-containing substrates was affected by the availability of inorganic nitrogen. Ammonia enrichment resulted in significantly lower values of the NUSE index (*t*-test, n = 2, p = 0.04) compared to pooled data of the C, P and control treatments (Fig. 3). Actually, the NUSE index increased somewhat in the C and P treatments, which could have resulted from an increased N demand once other potentially limiting nutrients were added. The decreased utilization of DON in the ammonia treatments was consistent with observations that heterotrophic bacterioplankton preferentially use ammonia as a N source for growth (Kroer et al. 1994, Veuger et al. 2004). We inferred that the sensitivity of Biolog-ECO microplates to the concentration of inorganic nitrogen in the sample is an asset when investigating substrate utilization in seawater through the NUSE approach.

A second experiment (ORG) was done to measure the effect of increased organic nitrogen concentrations on the use of N substrates in the Biolog plates. The NUSE index significantly decreased in seawater cultures enriched with amino acids compared to cultures enriched with glucose (non-parametric Wilcoxon matched pairs test, $p < 0.05$) (Fig. 4). The decreased NUSE index was largely due to reduced utilization of amino acids present in the Biolog plates. Incidentally, the smallest response in the NUSE index in this series of experiments with water from Blanes Bay occurred in December and February during times characterized by nutrient sufficiency and low specific bacterioplankton AMA. Interestingly, the utilization of N-acetyl-D-glucosamine, an important N-containing bacterial substrate in surface water (Riemann & Azam 2002), was not affected by amino acid enrichment. Nevertheless, the NUSE index was noticeably responsive to the concentration of organic nitrogen in natural samples.

The 2 experiments confirmed the results obtained *in situ*: that the NUSE index gives an estimate of bacterial utilization of N-containing substrates, depending on the bacterial nutritional status (i.e. availability of inorganic and/or organic nitrogen). Thus, although the Biolog approach is associated with a number of methodological concerns (e.g. substrate representativity and culturing), our findings supported its application to make inferences about the nutritional status of (at least parts of) natural bacterioplankton. We conclude that data analysis made from Biolog-ECO microplates results can be expanded substantially to allow not only direct comparisons of functional diversity of physiological profiles of different bacterial assemblages, but also to obtain revealing information concerning the utilization of DON compounds by natural bacterioplankton assemblages.

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