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Update of "Analysis of ^{15}N incorporation into D-alanine: A new method for tracing nitrogen uptake by bacteria" (Veuger et al. 2005, Limnol. Oceanogr. Methods 3:230-240)

Bart Veuger, Jack J. Middelburg, Henricus T. S. Boschker, and Marco Houtekamer
Centre for Estuarine and Marine Ecology, Netherlands Institute of Ecology (NIOO-KNAW), P.O. Box 140, 4400 AC Yerseke, The Netherlands

Introduction

After publication of the original manuscript, the presented method has been applied in various ^{15}N- and ^{13}C-labeling studies to trace incorporation of ^{15}N and/or ^{13}C into bacterial and total microbial biomass in different coastal sediments (Veuger et al. 2006, Veuger et al. 2007, Veuger and Middelburg 2007). These applications and analysis of the D-Ala content of some algal and cyanobacterial cultures yielded valuable information regarding the interpretation of ^{15}N and ^{13}C incorporation into D-Ala and other hydrolysable amino acids (HAAs), which is presented in this update.

Calculation of D/L-Ala ratio

In the original publication, the abundance of D-Ala was expressed relative to that of L-Ala as D/L-Ala ratio (%) that was calculated as D/L × 100. However, in most papers, the abundance of D- versus L-AAs is presented as D/L (i.e., not %) or as %D (D/(D + L) × 100) (e.g., McCarthy et al. 1998, Dittmar et al. 2001, Amon et al. 2001, Kaiser and Benner 2005). To prevent unnecessary confusion and for consistency, we now also use D/L.

Hydrolysis-induced racemization

In the original publication, it was assumed that formation of D-Ala from L-Ala by racemization during acid hydrolysis of the samples was negligible compared to the amount of D-Ala from bacteria. However, it is now clear that hydrolysis-induced racemization yields substantial amounts of D-Ala and hence should be corrected for. Recently, Kaiser and Benner (2005) showed that liquid-phase hydrolysis of proteins and algal biomass in 6 M HCl for 20 h at 110°C (same settings as used in present method) yielded D/L-Ala ratios between 0.017 and 0.019. These values are very similar to those measured for our axenic algal cultures (0.015–0.018, Table 4). Moreover, in all ^{15}N- and ^{13}C-labeling experiments so far, excess ^{15}N and ^{13}C D/L-Ala ratios were never <0.015, even for incubations of surface sediment slurries in the light in which algal biomass was an order of magnitude higher than bacterial biomass (Veuger and Middelburg 2007). Altogether, these results indicate that racemization during hydrolysis of microbial biomass (using hydrolysis conditions as used in the present method) results in D/L-Ala ratios of 0.015 to 0.02 (i.e., ~1.5% to 2% of L-Ala is converted to D-Ala). Following Kaiser and Benner (2005), we used these values to empirically correct our D-Ala data. Measured D-Ala concentrations and excess label in D-Ala can be corrected for hydrolysis-induced racemization as follows:

$$x \text{ bacterial D-Ala} = [\text{measured } x \text{ D-Ala}] - (0.017 \times [\text{measured } x \text{ L-Ala}])$$

where x is the concentration of excess ^{15}N or ^{13}C; 0.017 is used as the average of the 0.015–0.02 range for hydrolysis-induced racemization. When results are presented as D/L-Ala ratios (concentrations of excess ^{15}N or ^{13}C), the most straightforward way is to present measured (i.e., uncorrected) ratios and to indicate the racemization background of 0.015–0.02 graphically (see Fig. 7).

Bacterial D/L-Ala ratios

In the original manuscript, we adopted the common assumption that aquatic bacterial communities are dominated by Gram-negative (G–) bacteria and hence that the D/L-Ala ratio of these bacterial communities is similar to that for Gram negatives that appear to have a relative uniform D/L-Ala ratio of ~0.05 (previously called 5%) (Table 2). However, some of the recent ^{15}N- and ^{13}C-labeling studies in sediments yielded excess ^{15}N and ^{13}C D/L-Ala ratios up to ~0.1 (Veuger et al. 2006, Veuger et al. 2007, Veuger and Middelburg 2007). These relatively high D/L-Ala ratios appear to be due to substantial label incorporation by Gram-positive (G+) bacteria and/or cyanobacteria, since these are characterized by a thicker peptidoglycan layer (Madigan et al. 2000) and a corresponding higher D-Ala content and D/L-Ala ratio. Although Gram negatives indeed appear to dominate bacterial communities in the water column, Gram positives have been reported to contribute up to 30% of the total bacterial community in anaerobic subsurface sediment (Moriarty and Hayward 1982). This relatively high G+ abundance in deeper sediment is consistent with increasing relative abundance of phospholipid-derived fatty acids (PLFAs) characteristic for Gram positives with

Table 4. Measured D/L-Ala ratios for biomass from axenic cultures of four algae and two cyanobacteria. Samples were processed and analyzed following the protocol presented in the original manuscript. Concentrations were derived from GC-c-IRMS analyses.

		D/L-Ala
Amphora	diatom	0.016
Odontella	diatom	0.016
Thalassiosira	diatom	0.018
Chlorella	green alga	0.015
Leptolyngbya	cyanobacterium	0.07
Synechococcus	cyanobacterium	0.11

increasing depth in soils (Fierer et al. 2003) and estuarine sediments (Boschker, unpublished data; van Oevelen, unpublished data). When assuming a D/L-Ala ratio of ~0.2 for G+ bacteria (Sonesson et al. 1988), a G+ contribution of ~30% of the total bacterial community (i.e., ~70% is G–) results in a D/L-Ala ratio of ~0.1 for the total bacterial community (see Fig. 7). This indicates that the D/L-Ala ratio for natural (heterotrophic) bacterial communities ranges between 0.05 (100% G–) and 0.1 (70% G– and 30% G+) (Fig. 7). Another potential cause for D/L-Ala ratios >0.05 is the presence of cyanobacteria. Although cyanobacteria are classified as G–, their peptidoglycan layer is thicker than that of other Gram negatives and has a higher degree of cross-linking between the sugar strands (i.e., relatively rich in cross bridges containing D-Ala) (Hoiczyk and Hansel 2000). The resulting relatively high D/L-Ala ratio is confirmed by the measured D/L-Ala ratios for our cyanobacterial cultures (0.07–0.11, Table 4) which is within the 0.05–0.1 range of D/L-Ala ratio for natural bacterial communities (Fig. 7).

Interpretation of D-Ala results

In the ^{15}N- and ^{13}C-labeling studies in which the method has been applied so far, the best indication of the bacterial contribution to total (microbial) label incorporation was provided by the ratio between excess ^{15}N (or ^{13}C) in D-Ala versus that in L-Ala (the excess ^{15}N D/L-Ala ratio). The bacterial contribution to total ^{15}N incorporation can be estimated as follows:

$$\text{Bacterial contribution (\%)} = [\text{excess } ^{15}\text{N D/L-Ala} - 0.017] / [\text{bacterial D/L-Ala} - 0.017] \times 100$$

where 0.017 represents the average racemization background (see above). Using bacterial D/L-Ala ratios of 0.05 and 0.1 provides a maximal and minimal estimate of the bacterial contribution, respectively. Although this approach gives a min-max range rather than a precise value, this theoretical range can in practice often be narrowed down. For example, a measured excess ^{15}N D/L-Ala ratio of 0.08 indicates that the D/L-Ala ratio for the bacterial community involved in the ^{15}N incorporation was at least 0.08. This narrows down the corresponding min-max range considerably and therefore yields a rather precise estimate. Moreover, the min-max range can often be narrowed down further using additional data such as concentrations and/or label incorporation into other biomarkers (e.g., pigments, PLFAs) and in ^{15}N studies by incubating samples with ^{13}C-glucose to obtain an (excess ^{13}C) D/L-Ala ratio for the active bacterial community (see original manuscript).

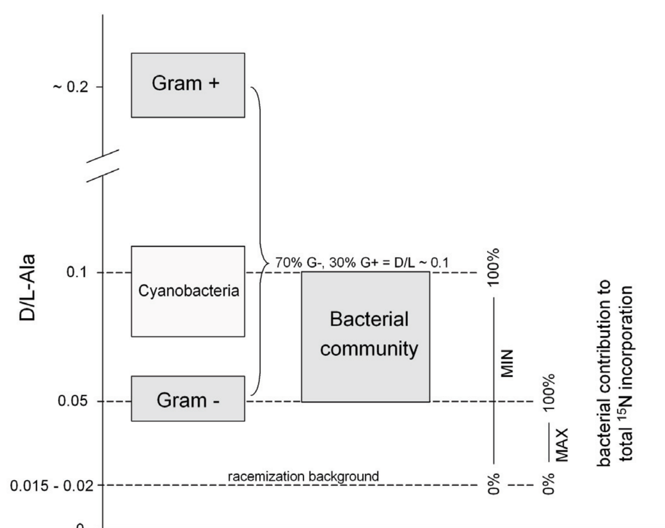

Fig. 7. Schematization of bacterial D/L-Ala ratios and conversion of excess ^{15}N D/L-Ala ratios to the bacterial contribution to total (microbial) ^{15}N incorporation. Note that this also applies for ^{13}C.

Use of label incorporation in other HAAs

Next to analysis of ^{15}N and ^{13}C in D- and L-Ala, analysis of label incorporation into the other L-HAAs also provided very useful information. First, as already mentioned in the original publication, the relative composition of the labeled THAA pool can provide a useful indication of the degree of degradation of the labeled material (e.g., Veuger et al. 2006). Second, as THAAs make up a large fraction of total (microbial) biomass [50% to 60% of bacterial biomass (Simon and Azam 1989, Cowie and Hedges 1992) and 60% to 80% of algal biomass (Cowie and Hedges 1992)], the incorporation of label into THAAs provides a very good indication of label incorporation into total (microbial) biomass. Conversion to total (microbial) label incorporation only requires a very small conversion step (× 1.2 to × 2). This feature proved very useful for both ^{15}N- and ^{13}C-labeling studies and is the only way to quantify total (microbial) ^{15}N incorporation in sediments. Something to take into account here is that when label is traced into HAAs over longer periods of time (days to weeks), the labeled HAA pool can include a contribution by label in fauna (i.e., not only microbial biomass). This also applies to L-Ala, meaning that the excess label D/L-Ala ratio in this case provides a measure of the bacterial contribution to total label incorporation (bacteria + algae + fauna) rather than that to total microbial incorporation (bacteria + algae).

Method evaluation

The two main points addressed in this update are hydrolysis-induced racemization and the variation in bacterial D/L-Ala ratios. Given that D/L-Ala ratios resulting from racemization are consistent and well constrained, we are confident about using the empirical correction for hydrolysis-induced racemization as presented above. The additional information regarding the variation in bacterial D-Ala content and corresponding D/L-Ala ratios introduces some uncertainty to the precise quantification of bacterial ^{15}N incorporation, especially for studies where G+ bacteria and/or cyanobacteria are abundant enough to play a substantial role in total bacterial ^{15}N incorporation. However, this variation is not surprising given the general variation in bacterial cell size and biochemical composition (Madigan et al. 2000), which makes that this kind of variation and associated uncertainty in resulting estimates simply inherent to work with biomarkers in microbial ecology. With this in mind, the range of D/L-Ala ratios for natural bacterial communities between 0.05 and ~0.1 is actually rather well constrained, and the corresponding maximum and minimum estimates therefore provide a robust measure for the bacterial contribution to total microbial ^{15}N incorporation.

Altogether, analysis of ^{15}N incorporation into D-Ala provides a robust method for assessment of the bacterial contribution to total (microbial) ^{15}N incorporation. Combined with very useful information from analysis of ^{15}N in the other HAAs (providing an indication of total label incorporation and the degree of degradation) and the possibility to do the same for ^{13}C, the method provides a very useful, versatile tool for tracing flows of N and C through bacteria and total (microbial) biomass.

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