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Novel method for measuring aquatic bacterial productivity using D_{10}-leucine based on protein synthesis rate

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ABSTRACT: The most widely used method for measuring bacterial production is tritium-labeled leucine (^{3}H-Leu). Although this method provides methodological simplicity and high sensitivity, the employment of radioactive isotopes is often restricted by regulations, particularly in field settings. In this study, we developed a non-radioactive method for measuring bacterial productivity based on the protein synthesis rate, using deuterium-labeled leucine ($(CD_3)_2CDCD_2CD(NH_2)COOH$; D_{10}-Leu); the proposed method was then compared and verified with the ^{3}H-Leu method. The procedures of the proposed method are (1) incorporation of D_{10}-Leu by bacteria, (2) acid hydrolysis (HCl) to amino acids and (3) quantification of D_{10}-Leu (*m/z* 142.10) by liquid chromatography mass spectrometry (LC-MS/MS). In the LC-MS/MS analysis, we detected a larger amount of D_9-Leu (*m/z* 141.10) and D_8-Leu (*m/z* 140.10) than that of D_{10}-Leu, suggesting that incorporated D_{10}-Leu was rapidly metabolized such as in deamination and aminotransferase reactions. The incorporation rates of D_{10}-Leu, D_{10}-Leu + D_9-Leu (D_{10}+D_9-Leu) and D_{10}-Leu + D_9-Leu + D_8-Leu (D_{10}+D_9+D_8-Leu) were significantly positively correlated to that of ^{3}H-Leu, confirming the validity of the proposed method. Since D_7-Leu (*m/z* 139.10) could not be detected, the amount of exogenous leucine incorporated into protein can be accurately estimated through D_{10}+D_9+D_8-Leu measurement. The new compound-based quantification method using stable isotope-labeled leucine can be a powerful tool to estimate pure protein synthesis rate for measuring bacterial production.

KEY WORDS: Bacterial production · Protein synthesis · Leucine · Deoxyadenosine · Stable isotopes · Liquid chromatography mass spectrometry

1. INTRODUCTION

Heterotrophic bacteria play an important role in energy and material cycling in aquatic environments (Azam 1998). Thus, it is essential to measure bacterial production in order to estimate the flux of energy and materials through the microbial loop. The most widely used methods for measuring bacterial production are tritium-labeled thymidine (^{3}H-TdR; Fuhrman & Azam 1980, 1982) and leucine (^{3}H-Leu; Kirchman et al. 1985, Simon & Azam 1989). The ^{3}H-TdR and ^{3}H-Leu incorporation rates are considered to reflect DNA and protein synthesis rates, and bacterial production is estimated by applying appropriate conversion factors to the synthesis rates (Riemann & Bell 1990). Although these methods provide methodological simplicity and high sensitivity, the employment of radioactive isotopes is often restricted by regulations, particularly in field settings.

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In 1999, a non-radioactive alternative method of DNA synthesis rate was developed, which proposed the use of the TdR analog 5-bromo-2′-deoxyuridine (BrdU; Steward & Azam 1999). The incorporation of BrdU can be quantified by chemiluminescence intensity through binding an antibody with BrdU in an immunochemical reaction. Further, the BrdU incorporation rate was significantly correlated to ^{3}H-TdR incorporation rates (Steward & Azam 1999). More recently, another non-radioactive method for measuring DNA synthesis rate was developed, which proposed the use of stable isotope ^{15}N-labeled deoxyadenosine (^{15}N-dA method; Tsuchiya et al. 2015). After the incorporation of ^{15}N-dA by bacteria, the samples are processed by DNA extraction, enzymatic hydrolysis to nucleosides and quantification of the ^{15}N-dA amount by liquid chromatography mass spectrometry (LC-MS). In this method, the problem of non-specific labeling, which often causes overestimation of ^{3}H-TdR incorporation rates (Robarts 1998), is avoided because the target compound (^{15}N-dA) is quantified directly by LC-MS. This allows researchers to quantify pure DNA synthesis rates. The ^{15}N-dA incorporation rate is also significantly correlated to ^{3}H-TdR and ^{3}H-Leu incorporation rates (Tsuchiya et al. 2015, 2019, 2020a). Despite this progress in developing non-radioactive methods for measuring DNA synthesis rates, no non-radioactive methods for measuring protein synthesis rates are currently available.

In the present study, we developed and tested a non-radioactive method for measuring bacterial protein synthesis rates in aquatic environments based on the incorporation of stable isotope deuterium (D)-labeled leucine followed by quantification using liquid chromatography mass spectrometry (LC-MS/MS). The accuracy of the proposed method was verified with ^{3}H-Leu and ^{15}N-dA methods.

2. MATERIALS AND METHODS

2.1. Protocol

The newly developed method for measuring bacterial productivity presented in this study was implemented via a 5-step procedure: (1) incubation, (2) filtration, (3) hydrolysis, (4) dry and dissolve and (5) quantification of D_{10}-Leu incorporation.

Step 1: Water samples (20–40 ml in the present study) were incubated with D_{10}-L-leucine (chemical formula: $(CD_3)_2CDCD_2CD(NH_2)COOH$, DLM-567, Cambridge Isotope Laboratories; hereafter D_{10}-Leu) dissolved in Milli Q water for several hours with an appropriate final concentration of D_{10}-Leu (usually ≤50 nM). Incubations were terminated by the addition of cold trichloroacetic acid (TCA, 5% final concentration).

Step 2: Samples were filtered onto a 0.2 µm pore size polytetrafluoroethylene (PTFE) membrane filter (Omnipore, Millipore), rinsed twice with 1 ml of ice-cold 5% TCA and then twice with 1 ml of ice-cold 80% ethanol. The filters were then stored at −20°C until further analysis.

Step 3: The filters were hydrolyzed with 500 µl of 6 mol l^{-1} HCl at 110°C for 20 h in 10 ml centrifuge glass test tubes with screw-caps on a heat block (AL500, Scinics). The recovery rate of leucine through the acid hydrolyzation process was considered to be 100% (Damm et al. 2010).

Step 4: After hydrolysis, samples were dried using a rotary evaporator (Rotavapor R-200, Büchi) to remove HCl. In the present study, 6 samples were dried simultaneously using a rotary evaporator equipped with a custom adaptor for increased efficiency (Fig. 1). The samples were then dissolved in 200 µl of 0.3 mol l^{-1} HCl including 20 µl of 1 µg ml^{-1} $^{13}C_6$-L-leucine (CLM-2262-H, Cambridge Isotope Laboratories; $^{13}C_6$-Leu) as a surrogate (internal standard) for proceeding with the LC-MS/MS analysis.

Step 5: The samples were filtered by using a polyvinylidene fluoride (PVDF) filter vial (0.45 µm pore size; 35541 or 25541, Thomson Instrument), and 10 µl of the filtrate were injected into the LC-MS/MS.

2.2. LC-MS/MS analysis

LC-MS/MS analysis was performed using an LCMS-8040 liquid chromatography mass spectrometer (Shimadzu) with a Nexera X2 liquid chromatograph system (Shimadzu). The analytical conditions were as follows: column, Intrada Amino Acid, 3 µm, 2 × 100 mm (WAA24, Imtakt); solvent A, 100 mM ammonium formate (aqueous solution); solvent B, acetonitrile in 0.3% formic acid; column oven temperature, 40°C; flow rate, 0.3 ml min^{-1}. The amino acids were separated using a linear gradient from 88 to 0% solvent B from 8.5 to 9.5 min, 0% solvent B for 4 min, a linear gradient from 0 to 88% solvent B from 13.5 to 14 min, and then 88% solvent B until the end of analysis (20 min). The leucine peak was eluted around 7.0 min. The method assumed that D_9-, D_8- and D_7-Leu could be detected and quantified in addition to incorporated D_{10}-Leu due to the metabolism pathway within the cell (Fig. 2). Leucine, D_{10}-Leu, D_9-Leu, D_8-Leu, D_7-Leu and $^{13}C_6$-Leu were detected

Fig. 1. Rotary evaporator equipped with a custom adaptor for drying 6 samples simultaneously

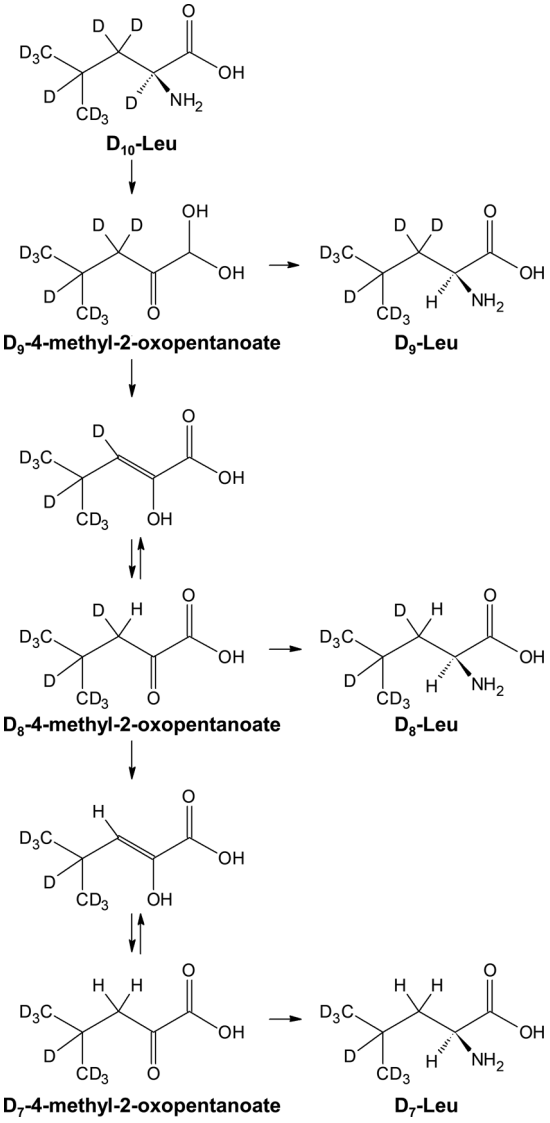

Fig. 2. Presumed reaction pathways following uptake of D_{10}-Leu

in the positive multiple reaction monitoring (MRM) mode using *m/z* 132.20 > 86.10, 142.10 > 96.10, 141.10 > 95.10, 140.10 > 94.10, 139.10 > 93.10 and 138.00 > 91.10, respectively. In a comparison experiment conducted in July (described in Section 2.3), D_8-Leu and D_7-Leu were detected in the selected ion monitoring (SIM) mode using *m/z* 140.10 and 139.10, respectively.

2.3. Comparison experiment among D_{10}-Leu, ^{3}H-Leu and ^{15}N-dA methods

Incorporation rates of D_{10}-Leu were compared with those of ^{3}H-Leu and ^{15}N-dA in laboratory experiments conducted in June 2018, July 2018 and May 2020 (hereafter the June, July and May experiments, respectively). In the June experiment, water samples were collected at a pelagic survey station (Stn 12B; 35° 11′ 39″ N, 135° 59′ 39″ E, maximum depth ~60 m) in the north basin of Lake Biwa, Japan, using a Niskin water sampler. Water was collected from 5 depths (5, 10, 15, 20 and 40 m) on 4 June 2018 from aboard RV 'Biwakaze' of the Lake Biwa Environmental Research Institute, Shiga Prefecture, Japan. The samples were brought to the National Institute for Environmental Studies in a cooler box kept at 4°C, and comparison experiments were conducted there, as we had to use radioisotope (^{3}H-Leu) under controlled (registered) conditions in the national laboratory.

The lake water samples were filtered through 1 µm pore size polycarbonate membrane filters (Whatman Nuclepore, GE Healthcare Life Sciences) using gentle vacuum. Aliquots of lake water (40 ml for D_{10}-Leu and ^{15}N-dA methods; 1 ml for the ^{3}H-Leu method) from each depth were incubated with a 50 nM final concentration of D_{10}-Leu, ^{15}N-dA (NLM-3895-PK, Cambridge Isotope Laboratories) and ^{3}H-Leu (L-[4,5-^{3}H(N)]-, NET135H, Perkin Elmer), respectively. Each sample was incubated at 8, 16 and 26°C for 1–4 h. ^{3}H-Leu blanks were prepared by adding TCA (final concentration 5%) to the samples collected from each depth at the start of the incubation at 16°C.

At the end of the incubation, the samples with D_{10}-Leu and ^{3}H-Leu were quenched by adding ice-cold TCA (final concentration 5%). The samples of D_{10}-Leu were processed as described above. In the June experiment, D_8-Leu and D_7-Leu were not quantified.

The samples with ^{3}H-Leu were filtered onto a 0.22 µm mixed cellulose ester membrane filter (GSWP, Millipore) and rinsed twice with 1 ml cold 5% TCA and then twice with 1 ml cold 80% ethanol. The filter was dissolved in a glass scintillation vial by adding 1 ml of ethyl acetate, and then 1 ml of scintillation cocktail (UltimaGold, Perkin Elmer) was added to the vial. After vortexing the vial, radioactivity was determined by a liquid scintillation counter (Wallac 1414, Perkin Elmer), and ^{3}H-Leu incorporation rates were calculated. The samples with ^{15}N-dA were quenched by adding 99.5% ethanol (final concentration, >20%) at the end of incubation, filtered onto 0.2 µm PTFE membrane filters and then stored at −80°C until further analysis. The ^{15}N-dA incorporation rates were quantified according to the procedure described by Tsuchiya et al. (2015, 2020a).

In the July experiment (26 July 2018), water samples were collected from 7 depths (0, 5, 10, 15, 20, 40 and 60 m) at the same station as the June experiment. Water samples were filtered through 1.0 µm pore size polycarbonate filters. We then incubated 1.5 ml (^{3}H-Leu method) and 40 ml (D_{10}-Leu and ^{15}N-dA methods) of the filtrate with a 50 nM final concentration of ^{3}H-Leu, D_{10}-Leu and ^{15}N-dA, respectively. Each sample was incubated at 8, 16 and 28°C for 2–5 h. After the incubation and quenching with the addition of ice-cold TCA (final concentration 5%), ^{3}H-Leu samples were centrifuged at 14 000 × *g* (10 min at room temperature) according to Smith & Azam (1992). The supernatant was removed by suction, and 1 ml of ice-cold 5% TCA was added to the tube. The centrifuging step was repeated, and the supernatant was then removed. One ml of ice-cold 80% ethanol was added to the tube, the centrifuging step was repeated, and the ethanol was then similarly removed. The tube was left to dry overnight at room temperature. Scintillation cocktail (1 ml) was added directly to the microfuge tube, and radioactivity was determined as described above. The D_{10}-Leu and ^{15}N-dA samples were processed as described above.

In the May experiment, surface-water samples were collected on 13 May 2020 at 5 stations (Stns 1, 3, 7, 9 and 12) in Lake Kasumigaura, Japan, using a 2 m vertical column sampler. Water samples were filtered through pre-combusted (450°C for 4 h) GF/F glass fiber filters (Whatman), and 1.5 ml (^{3}H-Leu method) and 20 ml (D_{10}-Leu method) of the filtrate were then incubated with a 50 nM final concentration of ^{3}H-Leu and D_{10}-Leu, respectively. Each sample was incubated at 8, 16 and 24°C for 2–4 h. The ^{3}H-Leu samples were processed according to Smith & Azam (1992), and radioactivity was determined as described above. The D_{10}-Leu samples were processed as described above.

Relationships among incorporation rates of D_{10}-Leu, D_{10}-Leu + D_9-Leu (D_{10}+D_9-Leu), D_{10}-Leu + D_9-Leu + D_8-Leu (D_{10}+D_9+D_8-Leu), ^{3}H-Leu and ^{15}N-dA were analyzed using a standard major axis (SMA) Model II linear regression (Legendre 2001).

3. RESULTS AND DISCUSSION

In the June experiment, D_{10}-Leu incorporation rates ranged from 23.4–114 pmol l^{-1} h^{-1}. D_9-Leu was also detected, and the incorporation rate (57.3–523 pmol l^{-1} h^{-1}) was larger than that of D_{10}-Leu in all samples. The ratios of D_{10}-Leu to D_{10}+D_9-Leu incorporation rates ranged from 0.148–0.290. In the July experiment, D_{10}-Leu and D_9-Leu incorporation rates were 2.83–78.7 and 25.4–314 pmol l^{-1} h^{-1}, respectively. Incorporation rate of D_9-Leu was also higher than that of D_{10}-Leu, similar to the June experiment, and the ratio of D_{10}-Leu to D_{10}+D_9-Leu was 0.0727–0.214. Although D_7-Leu was not detected, D_8-Leu was detected in all samples, ranging from 12.1–364 pmol l^{-1} h^{-1}, similar to that of D_9-Leu. The ratio of D_8-Leu to D_{10}+D_9-Leu fluctuated by an order of magnitude, ranging from 0.109–1.02. In the May experiment, D_{10}-Leu, D_9-Leu and D_8-Leu incorporation rates were 19.1–90.4, 71.0–331 and 0–45.0 pmol l^{-1} h^{-1}, respectively. Again, D_7-Leu was not detected in all samples. The ratio of D_8-Leu to D_{10}+D_9-Leu ranged from 0–0.139.

Regarding the metabolism of leucine after uptake of D_{10}-Leu, there are 2 pathways: (1) incorporation to protein synthesis directly and (2) degradation to 4-methyl-2-oxopentanoate by the deamination reaction (Rudman & Meister 1953, Sanwal & Zink 1961). In the case of (1), the leucine used directly for protein synthesis can be detected as D_{10}-Leu. In the case of (2), the deamination reaction to 4-methyl-2-oxopentanoate caused loss of a deuterium at the C-2 of leucine (Fig. 3). When the degraded D_{10}-Leu is used for protein synthesis again, 4-methyl-2-oxopentanoate becomes leucine by the aminotransferase reaction, and non-labeled H is then added to the C-2 of leucine. Thus, the leucine used for protein synthesis can be detected as D_9-Leu.

During the June experiment, we hypothesized that total leucine incorporation rates could be estimated by measuring D_{10}-Leu and D_9-Leu based on the pathway explained above. When we measured D_8-Leu and D_7-Leu additionally in the July and May experi-

ments, D_7-Leu was not detected in any samples; however, a large amount of D_8-Leu was detected, suggesting that additional reactions to D_8-4-methyl-2-oxopentanoate occurred (Fig. 2). In the July and May experiments, incorporations of D_9+D_8-Leu were 10 ± 3 (mean ± SD) and 4.5 ± 1.0 times higher than that of D_{10}-Leu, respectively, suggesting that most of the leucine incorporated into protein was leucine which had been metabolized via the pathways mentioned above (i.e. detected as D_9-Leu and D_8-Leu) and a smaller faction was directly incorporated into protein (i.e. detected as D_{10}-Leu). Since expression levels of the enzymes of the branched-chain amino acid pathway are regulated depending on the cellular concentration of each of the branched chain amino acids (Soini et al. 2008, Amorim Franco & Blanchard 2017), the cellular leucine concentration should be properly controlled. Thus, exogenous leucine excessively taken up by bacteria may be pooled as 4-methyl-2-oxopentanoate, and the leucine can be incorporated into protein after the salvaging from 4-methyl-2-oxopentanoate, as necessary. According to the leucine degradation pathway map of KEGG (00280 Valine, leucine and isoleucine degradation; Kanehisa et al. 2019), the pathway returning to 4-methyl-2-oxopentanoate from the metabolite substances after 4-methyl-2-oxopentanoate is not shown, suggesting that it is possible to estimate the incorporation amount of extracellular D_{10}-Leu to bacterial proteins by quantifying D_{10}-Leu, D_9-Leu and D_8-Leu incorporation rates.

Incorporation rates of ^{3}H-Leu and ^{15}N-dA were significantly positively correlated with those of D_{10}-Leu, D_{10}+D_9-Leu and D_{10}+D_9+D_8-Leu in both the June and July 2018 experiments (Table 1, Fig. 3). In the third experiment in May 2020, incorporation rates of ^{3}H-Leu were also significantly correlated to those of D_{10}-Leu, D_{10}+D_9-Leu and D_{10}+D_9+D_8-Leu (Fig. 4). The results demonstrate the validity of the proposed method. In both cases of ^{3}H-Leu and ^{15}N-dA, the coefficients of determination, r^2, of D_{10}+D_9+D_8-Leu correlations were higher than those of D_{10}-Leu and D_{10}+D_9-Leu correlations (Table 1, Figs. 3 & 4).

Since no D_7-Leu could be detected, the amount of exogenous leucine incorporated into protein can be estimated through quantification of D_{10}+D_9+D_8-Leu without excess or deficiency. Moreover, the relationship is supported by the slope of the linear regression between incorporation rates of ^{3}H-Leu and D_{10}+D_9+D_8-Leu (0.954 with 95% confidence interval of 0.841 and 1.08; Table 1, Fig. 5). Although the potential for overestimation of ^{3}H-Leu incorporation rate exists, due to non-specific ^{3}H incorporation in an oligotrophic system (e.g. Alonso-Sáez et al. 2007), the result of the present study suggests that no overestimation of ^{3}H-Leu incorporation rates occurred in the studied system (Lake Biwa and Lake Kasumigaura). The result agrees with a previous study showing that small amounts of ^{3}H in any amino acid other than leucine were found after ^{3}H-Leu incorporation (Simon & Azam 1989). Thus, the compound-based quanti-

Table 1. Summary of standard major axis model II linear regressions between incorporation rates of ^{3}H-leucine (Leu), ^{15}N-deoxyadenosine (dA), D_{10}-Leu, D_{10}+D_9-Leu and D_{10}+D_9+D_8-Leu in June, July and May experiments (Figs. 3, 4 & 5)

Objective variable	Explanatory variable	Slope	Intercept	n	r^2	p
June experiment (Fig. 3)						
^{3}H-Leu	D_{10}-Leu	10.6	−11.2	15	0.746	<0.001
	D_{10}+D_9-Leu	1.80	20.9	15	0.825	<0.001
^{15}N-dA	D_{10}-Leu	0.155	−0.0647	15	0.711	<0.001
	D_{10}+D_9-Leu	0.0262	0.403	15	0.816	<0.001
July experiment (Fig. 3)						
^{3}H-Leu	D_{10}-Leu	9.14	−16.1	21	0.422	<0.01
	D_{10}+D_9-Leu	1.51	−23.5	21	0.661	<0.001
	D_{10}+D_9+D_8-Leu	0.954	−2.70	21	0.932	<0.001
^{15}N-dA	D_{10}-Leu	0.226	−1.13	20	0.454	<0.01
	D_{10}+D_9-Leu	0.0376	−2.19	20	0.462	<0.001
	D_{10}+D_9+D_8-Leu	0.0236	−1.59	20	0.736	<0.001
May experiment (Fig. 4)						
^{3}H-Leu	D_{10}-Leu	4.79	−28.0	15	0.751	<0.001
	D_{10}+D_9-Leu	0.991	−32.6	15	0.972	<0.001
	D_{10}+D_9+D_8-Leu	0.870	−25.9	15	0.977	<0.001
July + May experiments (Fig. 5)						
^{3}H-Leu	D_{10}+D_9+D_8-Leu	0.954	−20.4	36	0.868	<0.001

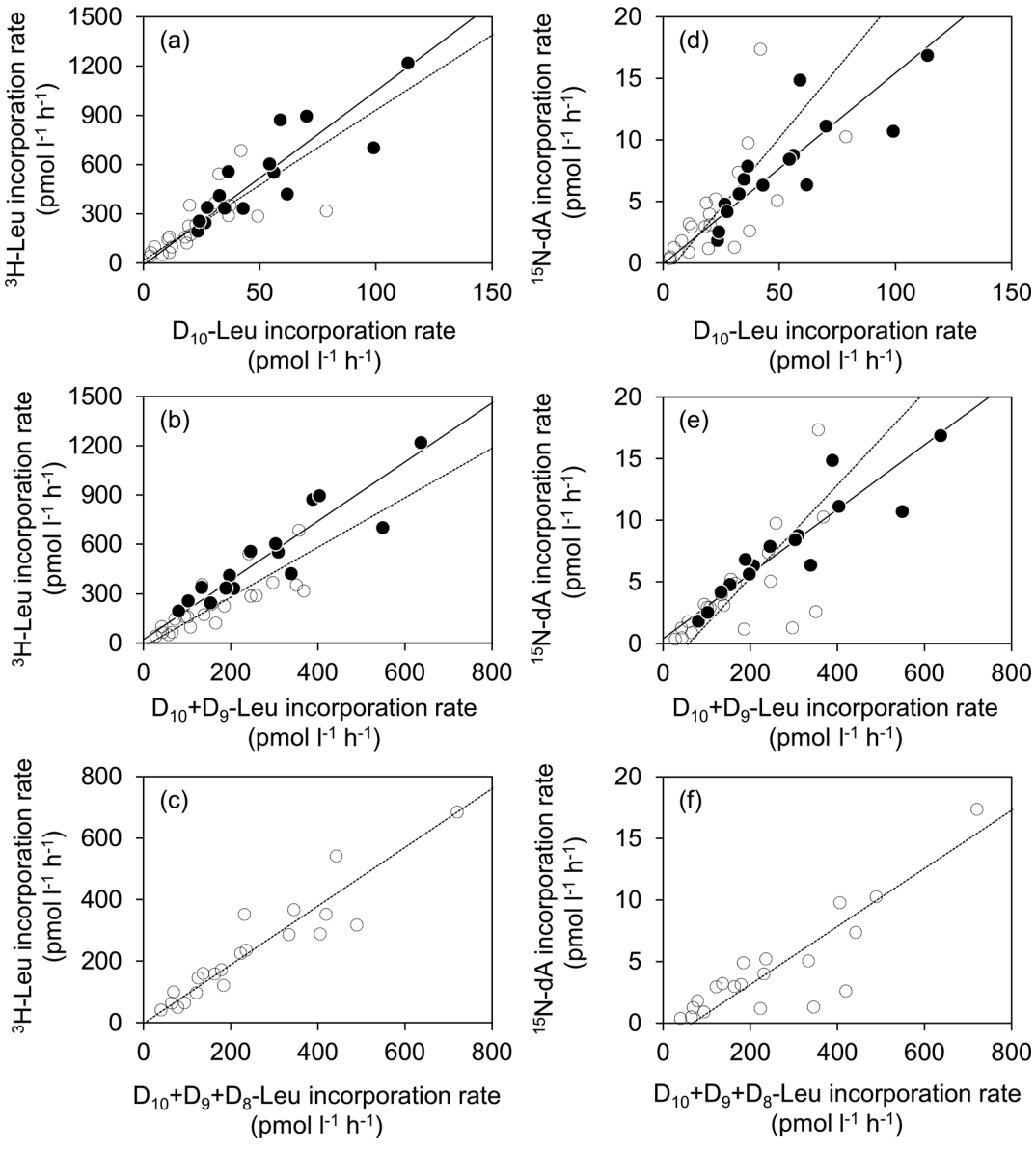

Fig. 3. Relationships among incorporation rates of D_{10}-Leu, D_{10}+D_9-Leu, D_{10}+D_9+D_8-Leu, ^{3}H-Leu and ^{15}N-dA. Closed and open circles represent samples in the June and July experiment, respectively. The solid (June) and dashed (July) lines indicate standard major axis model II linear regressions. The regression results are shown in Table 1

fication method of stable isotope-labeled leucine can be a powerful tool to estimate pure protein synthesis rates for measuring bacterial production without any biases derived from non-specific incorporation.

Ratios of protein to DNA synthesis rates, or ^{3}H-Leu and ^{3}H-TdR incorporation ratio (Leu:TdR), can vary depending on the physiological state of bacterial assemblages and/or environmental conditions (e.g. Chin-Leo et al. 1990, Tibbles 1996, Shiah & Ducklow 1997). In the June experiment, the D_{10}+D_9-Leu:^{15}N-dA molar incorporation ratio showed a relatively constant value (36.7 ± 7.7; see Fig. A1 in the Appendix), whereas, although the D_{10}+D_9+D_8-Leu:^{15}N-dA molar incorporation ratio was 49.7 ± 8.9 at 0–20 m depth, the ratio was relatively high (162 ± 61) at 40 and 60 m depths (Fig. A1) in the July experiment, suggesting the ratios were affected by physiological states and/or substrate availability. The D_{10}+D_9+D_8-Leu:^{15}N-dA ratio (37.7–269) was relatively higher compared to previously reported Leu:TdR values, i.e. 2–77 in Chesapeake Bay, Mid-Atlantic Bight and Delaware Bay (Chin-Leo & Kirchman 1990), 7.8–22.2 in the subarctic Pacific (Kirchman 1992), 5.6–29.5 in the Horn Point salt marsh tidal creek (Shiah & Duck-

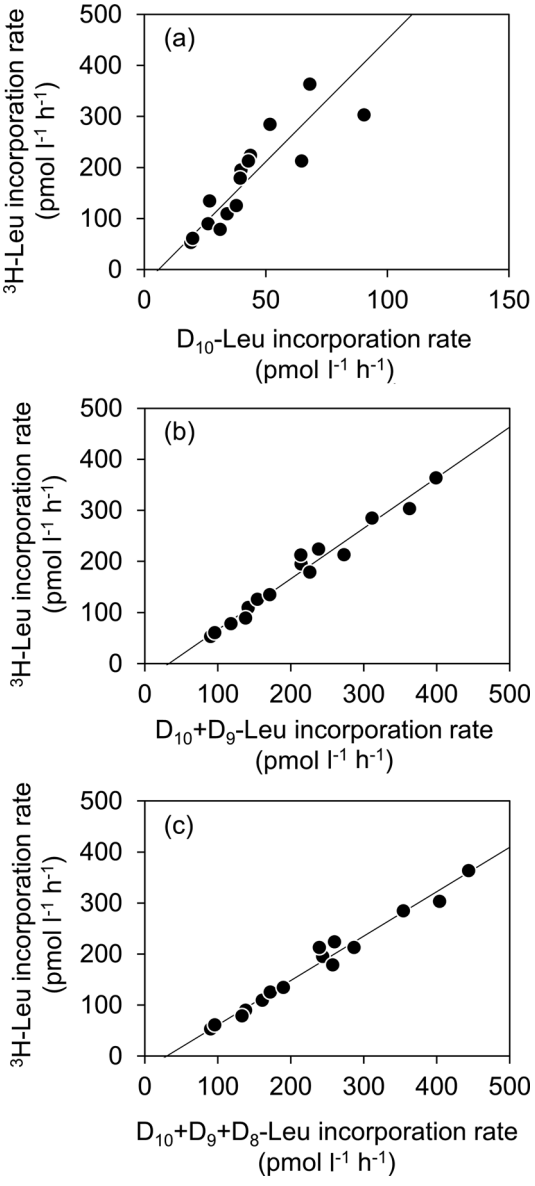

Fig. 4. Relationships among incorporation rates of D_{10}-Leu, D_{10}+D_9-Leu, D_{10}+D_9+D_8-Leu and ^{3}H-Leu in the May experiment. Fitted lines represent standard major axis model II linear regressions. The regression results are shown in Table 1

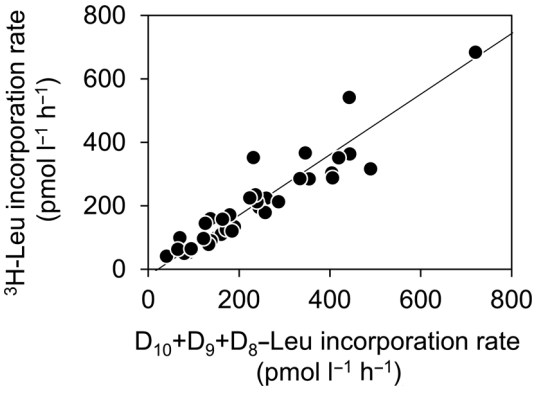

Fig. 5. Relationship between incorporation rates of D_{10}+D_9+D_8-Leu and ^{3}H-Leu in the July and May experiments (combined). Fitted line represents standard major axis model II linear regression. The regression results are shown in Table 1

low 1997) and 2.6–116.3 in the North Atlantic and equatorial Pacific (Ducklow 2000). The plausible reason why the ratio was relatively high in the present study is that the ^{15}N-dA incorporation rate is significantly lower than that of ^{3}H-TdR (0.28 in Sagami Bay, 0.55 in Lake Kasumigaura and 0.61 in Lake Biwa; Tsuchiya et al. 2015, 2020a) as a result of non-specific incorporation of ^{3}H. In future studies, compound-specific quantification methods both for deoxyribonucleosides (Tsuchiya et al. 2015, 2020b) and leucine will more accurately estimate Leu:TdR and Leu:dA molar incorporation ratios, which will advance our understanding of aquatic microbial ecology.

This study suggests that D positioned at C-2 and C-3 of leucine can be changed to H in the D_{10}-Leu metabolic pathway, whereas D positioned at C-4 and C-5 remains throughout the incorporation to protein (Fig. 2). Based on this result, another substrate such as 5,5,5-D_3-L-Leu (DLM-1259, Cambridge Isotope Laboratories) can be used as a tracer of protein synthesis rate as well as D_{10}-Leu. Natural abundance of the same *m/z* of 5,5,5-D_3-L-Leu is 0.03% for SIM mode and <0.01% in MRM mode, suggesting the natural abundance can be ignored in LC-MS/MS analysis. Although future experiments need to verify the 5,5,5-D_1-L-Leu incorporation, 5,5,5-D_3-L-Leu is more affordable and can be an option for an alternative substrate for measuring protein synthesis rates routinely.

In conclusion, we developed a novel method for measuring bacterial production without the use of radioactive isotopes based on protein synthesis rates. In this method, quantification of D_{10}-, D_9- and D_8-Leu by LC-MS/MS allows the estimation of the pure protein synthesis rate. Moreover, the incorporation rate of D_{10}+D_9+D_8-Leu was significantly positively correlated to those of ^{3}H-Leu and ^{15}N-dA methods, and compatibility among the methods were verified. We detected not only D_{10}-Leu but also D_9-Leu and D_8-Leu, suggesting a rapid deamination reaction of leucine in bacterial metabolism. Since no D_7-Leu was detected, the amount of exogenous leucine incorporated into protein can be estimated through the quantification of D_{10}+D_9+D_8-Leu without excess or deficiency. This study, offering a compound-based measurement, clearly de-

monstrates the potential for the D_{10}-Leu method to serve as a practical, non-biased and non-radioactive alternative to the ^{3}H-Leu method for measurement of bacterial productivity. We anticipate that aquatic ecologists needing an alternative to ^{3}H-Leu-based assays of bacterial production, whether due to strict regulations associated with radioisotope use, or to complement the accuracy of conventional methods, will adopt the novel methods developed in the present study.

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Appendix.

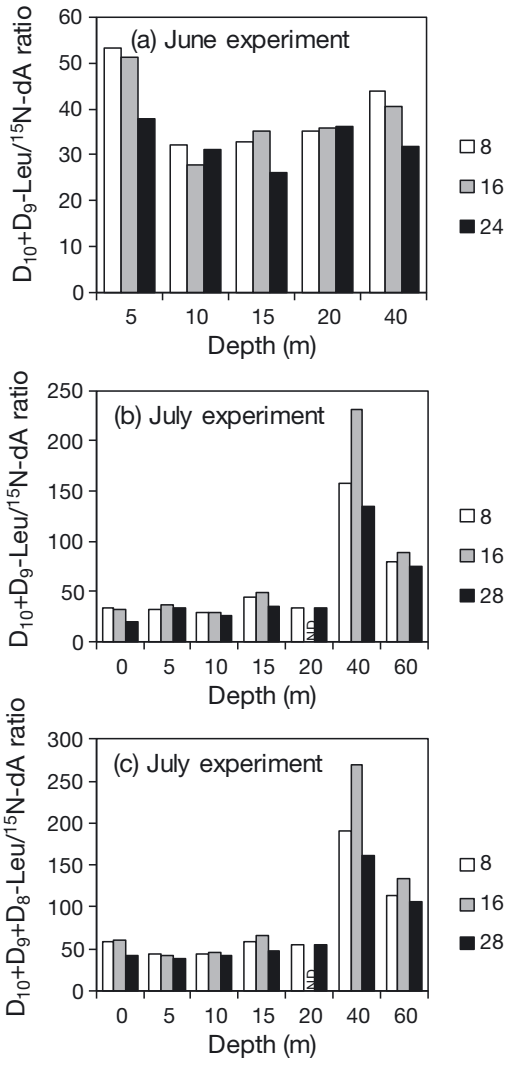

Fig. A1. Ratios of D_{10}+D_9-Leu to ^{15}N-dA and D_{10}+D_9+D_8-Leu to ^{15}N-dA in the June and July experiments. Different shades represent incubation temperatures (°C). ND: no data

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