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Overestimation of prokaryotic production by leucine incorporation—and how to avoid it

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Abstract

Prokaryotes play a central role in aquatic ecosystems by consuming approximately half of the organic matter produced by aquatic primary production, of which a fraction is used for growth. Accurately measuring this prokaryotic biomass production is key to understanding aquatic carbon and nutrient cycles, since it is instrumental in driving biogeochemical processes that control parameters such as atmospheric carbon content. Aquatic prokaryotic biomass production is typically estimated from incorporation rates of the amino acid leucine during radiotracer experiments—a method widely used since the 1980s. Here we evaluate the underlying assumptions of the method with a focus on the associated conversion factors and review them in the context of empirical data. We demonstrate that the commonly used theoretical conversion factors fail to account for leucine's use as precursor for de novo protein synthesis and its respiration. As a consequence, prokaryotic biomass production is likely considerably overestimated when applying the standard conversion factors. Most severely affected are open-ocean, mesopelagic and benthic environments, where 25% of the estimates are likely to be overestimated by at least a factor of 6.1, 4.9, and 6.5, respectively. We propose a refined carbon-to-leucine conversion factor and make recommendations for improving and selecting appropriate experimental protocols.

Prokaryotic productivity in the oceans

Aquatic prokaryotes, comprising the Bacteria and the Archaea, play a central role in the carbon cycle by consuming approximately half of the organic matter produced during aquatic primary production (Williams 1981; Cole et al. 1988). This consumed matter is incorporated into their biomass, a process which is termed prokaryotic heterotrophic production (PHP), or respired to carbon dioxide (CO_2) (Ducklow 2000). Hence, the term PHP describes the growth of prokaryotic communities. Accurately measuring it is key to understanding the role of aquatic ecosystems in the carbon and nutrient cycles, since it is instrumental in driving fundamental biogeochemical processes, which control parameters such as atmospheric carbon content.

As first described by Pomeroy (1974), the microbial loop is the prokaryotic consumption of dissolved organic matter, which is typically unavailable to most other marine organisms. Thereby this matter is re-incorporated into the cellular pool and, via bacterivory, made available to higher trophic levels. Within the concept of the microbial loop, PHP describes the magnitude of the flux of matter channeled from the dissolved to the particulate pool. Prokaryotic metabolism is also fundamental to the concept of the Biological Carbon Pump, which encompasses the ecological processes that determine carbon sequestration in the ocean's interior. Specifically prokaryotic respiration, and how it changes with depth, is a key term controlling the attenuation of organic matter flux in the ocean (Steinberg et al. 2008; Giering et al. 2014). Given that measuring prokaryotic respiration in the water column is challenging, PHP is frequently used as a proxy to derive respiration rates (Ducklow et al. 2000). A more recent concept in ocean biogeochemistry is the Microbial Carbon Pump, which postulates that prokaryotes convert labile dissolved organic matter into recalcitrant dissolved organic matter, most likely through successive rounds of metabolic processing (Jiao et al. 2010). Rates of PHP measured in concert with dissolved organic carbon concentrations provide an indication of the bioavailability of the organic matter pool at a given point in the ocean (Obernosterer et al. 1999). Furthermore, rates of PHP are likely a key factor and, therefore, indicator of the strength of the Microbial Carbon Pump.

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Measuring prokaryotic heterotrophic production and associated challenges

Prokaryotic growth has been measured by observing population changes over a set time period using metrics such as cell numbers (cells mL^{-1}) or biovolume (μm^3 mL^{-1}). However, growth itself can cause changes in cell characteristics, often rendering estimates of PHP made using cell-based metrics inaccurate. For example, Simon and Azam (1989) showed that the molecular composition of prokaryotic cells (protein contents, cell walls, cell membranes, DNA, RNA, dry weight, and carbon content) changed non-linearly with increasing cell volume. It is therefore difficult to infer organic carbon production rates and changes in, for example, DNA from changes in cell size. Conversely, the ratios between protein and dry weight (Pro : DW) and carbon and dry weight (C : DW) have been shown to be constant in coastal pelagic prokaryotes at 63 ± 1% and 54 ± 1%, respectively (Simon and Azam 1989). These consistent ratios enable a direct calculation of prokaryotic carbon content from prokaryotic protein content. Taking this a step further, Simon and Azam (1989) concluded that an increase in prokaryotic protein (i.e., prokaryotic protein production, PPP) can be used to estimate PHP without knowledge of cell carbon or cell volume.

The calculation of PHP from new protein production based on the assumption of constant mass ratios is theoretically straightforward. Proteins are made of amino acids, some of which make up a relatively constant proportion of the cell's protein. One amino acid that occurs in relatively constant proportions in mixed assemblages of coastal, pelagic prokaryotes is leucine ($C_7H_{13}NO_2$; $CH(CH3)_2$—CH_3—$CH(NH_2)$—COOH), which makes up 7.3 ± 1.9 mol% of total protein amino acids (Simon and Azam 1989). Tracking the incorporation of leucine (or any other "constant" cellular constituent such as thymidine incorporation into DNA) can hence be used as a tracer of new production. Leucine is particularly useful as an indicator of PHP as it is taken up almost exclusively by prokaryotes (Kirchman et al. 1985). Moreover, leucine incorporation rates are typically high (10-fold higher than thymidine incorporation rates) making leucine a convenient tracer for unproductive ecosystems (Simon and Azam 1989).

The assay to measure leucine incorporation by prokaryotes (Kirchman et al. 1985) is relatively uncomplicated, which makes it an attractive method to determine PHP. Terms commonly associated with method are defined in Table 1. In brief, radiolabeled leucine (*see* Supplementary Fig. S1 for types of

Table 1. Terms commonly associated with the leucine incorporation method.

Term	Definition
Absorption	Synonymous with *uptake*
Assimilation	Synonymous with *incorporation*
Break-down	Synonymous with *degradation*
Degradation	Decomposition of a compounds into elements or simpler compounds
Incorporation	Molecules that are integrated into cell structures such as organelles and membranes. For the leucine incorporation method, this process typically refers specifically to the integration into protein measured as the cellular material that is insoluble in trichloroacetic acid (TCA)
Isotope dilution	Unlabeled leucine that "dilutes" the signal of labeled leucine. The level of isotope dilution is corrected for when converting leucine incorporation into PHP using an "isotope dilution factor," which is defined as the ratio between total (labeled + unlabeled) leucine and labeled leucine. Extracellular dilution refers to the dilution of free labeled leucine in seawater by leucine already present in the seawater, while intracellular dilution refers to the dilution of labeled leucine incorporated into protein by unlabeled leucine taken up from the environment or produced by the cell de novo (Forsdyke 1968; Moriarty and Pollard 1981)
Leucine conversion	Degradation of leucine and subsequent synthesis of other amino acids from its degradation products
Leucine incorporation	*See* incorporation
Leucine respiration	Production of CO_2 from leucine molecules via leucine degradation. The respiration of ^{14}C-leucine produces $^{14}CO_2$
Prokaryotes	Bacteria and archaea. In early literature describing the leucine incorporation method, the term "bacteria" was used synonymously
Radiolabel	Substitution of a stable atom within a compound with a radioactive atom whose decay can be measured using sensitive radiation detectors
Respiration	Production of CO_2 during metabolic processes. This process is not specific to leucine, and CO_2 may be produced from any other compound in the cell
Tracer	Labeled atom in leucine, commonly ^{14}C, ^3H, or ^{15}N (*see* Fig. 1). Can be both stable or radioactive. Typically, the radioactive ^3H is used.
Uptake	Transport of compound into the cell. The compound may be present in the cell within the cytosol or incorporated into cell structures

leucine tracers) is added to seawater and incubated in darkness at in situ temperature, typically for a few hours. The protein is then extracted from the seawater and the radioactivity it contains is measured (Simon and Azam 1989). Leucine incorporation is derived from the radioactivity incorporated in combination with the specific activity of the tracer, and PHP is then calculated via the application of a leucine-to-carbon conversion factor (LeuCF). Empirical LeuCFs ($LeuCF_{emp}$) are derived by measuring the change in cell abundance relative to leucine incorporation over several days (Kirchman et al. 1982, 1986). However, most studies do not directly determine a $LeuCF_{emp}$ and instead use what is commonly referred to as the "theoretical LeuCF" ($LeuCF_{Theo}$). The two most commonly used $LeuCF_{Theo}$, varying slightly in their assumptions (as discussed below), are 1.55 and 3.1 kg C [mol Leu]$^{-1}$ (hereafter $LeuCF_{1.55}$ and $LeuCF_{3.1}$, respectively; Simon and Azam 1989; Knap et al. 1994). Few $LeuCF_{emp}$ have been determined relative to the high frequency with which the leucine incorporation assay has been used to determine PHP. Furthermore, there is a lack of understanding regarding the variability of LeuCF over spatial scales and environmental gradients. Thus, researchers must select what they consider to be the most appropriate LeuCF, thereby introducing subjectivity and uncertainty into estimates of PHP (Burd et al. 2010; Giering et al. 2014).

Implications for estimating production rates

We now demonstrate that the choice of $LeuCF_{Theo}$ may not always be appropriate. To explore potential implications for PHP estimates, we investigated the range of published $LeuCF_{emp}$. We identified 54 studies that measured $LeuCF_{emp}$ (Supplementary Table S1), typically following the methods by Kirchman et al. (1982, 1986). Briefly, $LeuCF_{emp}$ is measured by incubating natural samples diluted with filtered seawater over several days (up to 8 d). Subsamples for prokaryotic abundance and leucine incorporation rates are taken periodically (e.g., every 12–24 h). Leucine incorporation rates are measured by adding a leucine tracer at considerably higher concentrations than the ambient pool (typically 5–160 nM final concentration). $LeuCF_{emp}$ (in kg C [mol Leu]$^{-1}$) is calculated by comparing changes in prokaryotic abundance (ΔPA in number of cells L^{-1} [incubation time]$^{-1}$) and leucine incorporation (Leu_{inc} in mol L^{-1} [incubation time]$^{-1}$):

$$LeuCF_{emp} = \left(\Delta PA \times Leu_{inc}{}^{-1}\right) \times CC \qquad (1)$$

where ($\Delta PA \times Leu_{inc}{}^{-1}$) is the leucine-to-cell conversion factor, and CC is the cell carbon content (fg C cell^{-1}). Different protocols have been used to calculate ΔPA and Leu_{inc}, including the derivative method (Kirchman et al. 1982), the integrative method (Riemann et al. 1987), and the cumulative method (Bjornsen and Kuparinen 1991). The integrative and cumulative methods produce similar conversion factors (Pedrós-Alió et al. 2002; Alonso-Sáez et al. 2008), while $LeuCF_{emp}$ calculated using the derivative method can be much higher (Kirchman and Hoch 1988; Calvo-Díaz and Morán 2009).

A complication in the method is its reliance on knowing the cell carbon content. Only few of the reviewed studies measured the cell carbon content, and most used published values (ranging from 10 to 120 fg C cell^{-1}) or calculated cell carbon content from measured cell volume using published regressions. Depending on which regression is used, the resulting

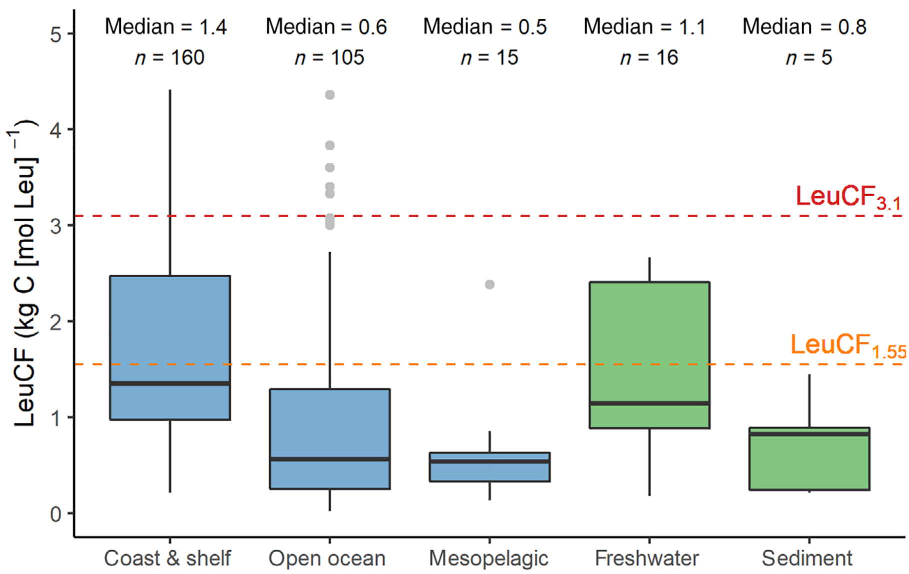

Fig. 1. Empirical leucine conversion factors ($LeuCF_{emp}$) published in the literature. Based on 54 publications and 296 published values. Reported $LeuCF_{emp}$ range from 0.02 to 36.4 kg C [mol Leu]$^{-1}$ with a median of 1.14 kg C [mol Leu]$^{-1}$ (first and third quantile: 0.53 and 2.03, respectively). Orange and red dotted lines show respectively the theoretical $LeuCF_{1.55}$ and $LeuCF_{3.1}$.

cell carbon content can vary widely (Khachikyan et al. 2019). For this review, we extracted both the published $LeuCF_{emp}$ (using the cell carbon content suggested by the authors) and the published leucine-to-cell conversion factors (recalculated, if needed, using the cell carbon content provided by the authors). If necessary, data from figures were extracted using PlotDigitizer (v2.6.3). Study sites were categorized as "coast & shelf" (including continental slopes and estuaries), "open ocean" (sites typically with a depth > 1000 m), "mesopelagic" (> 200 m depth at open ocean sites), "freshwater" (lakes, freshwater swamp, and rivers), and "sediment" (soil and freshwater sediments).

Conversion factors vary widely within each hydrographic regime (Fig. 1). For the marine environment, $LeuCF_{emp}$ tend to be higher in coastal and shelf regions (median 1.4 kg C [mol Leu]$^{-1}$) and lower at open ocean sites (median 0.6 kg C [mol Leu]$^{-1}$) and in the mesopelagic zone (median 0.5 kg C [mol Leu]$^{-1}$). The range of $LeuCF_{emp}$ is larger than the range of leucine-to-cell conversion factors (Supplementary Fig. S2), highlighting the additional uncertainties introduced by assuming cell carbon content. For the open ocean, mesopelagic and sediment regimes, published $LeuCF_{emp}$ are significantly lower than the $LeuCF_{1.55}$ ($p < 0.05$; one-sample Wilcoxon test). For coastal-and-shelf regions and freshwater sites, published $LeuCF_{emp}$ are significantly lower than the $LeuCF_{3.1}$ ($p < 0.05$; one-sample Wilcoxon test), though they are not significantly lower than the $LeuCF_{1.55}$). Overall, 66% of all reported $LeuCF_{emp}$ are lower than the $LeuCF_{1.55}$, and 85% below the $LeuCF_{3.1}$.

To illustrate the effect that the choice of LeuCF has on understanding ocean productivity, we calculated the factor by which PHP would have been over- or underestimated if the theoretical $LeuCF_{Theo}$ rather than the empirical $LeuCF_{emp}$ would have been applied (Fig. 2). We found that in all environments PHP is likely to be overestimated if a $LeuCF_{Theo}$ is applied. Most severely affected are open-ocean, mesopelagic and benthic environments, where 25% of the estimates are likely to be overestimated by at least a factor of 6.1, 4.9, and 6.5, respectively (assuming $LeuCF_{1.55}$).

Evaluating the validity of the assumptions underlying PHP determination from leucine incorporation

The validity of the $LeuCF_{Theo}$ is dependent on a series of assumptions (Fig. 3): (1) the ambient extracellular and intracellular leucine concentrations ("isotope dilution factors") are negligible owing to the design of the assay, (2) the proportion of leucine in the target prokaryotic community's protein is identical to that assumed by the $LeuCF_{Theo}$ (as determined by Simon and Azam (1989)), and (3) that all the leucine taken up by the prokaryotes is incorporated as leucine into protein, as opposed to being channeled into other metabolic pathways. Evidence suggests one or more of these assumptions may be incorrect as measured $LeuCF_{emp}$ are often below the minimum $LeuCF_{Theo}$ (1.55 kg C [mol Leu^{-1}]; Fig. 4). For example, 93% of the published $LeuCF_{emp}$ measured in the mesopelagic zone (200–1000 m depth) are < 1.55 kg C [mol Leu^{-1}], with a median of 0.54 kg C [mol Leu]$^{-1}$ (Table 2).

Concentration of leucine tracer and associated uptake rates

Quantitative methods employing tracers must determine and account for competition by non-tracers that follow the same metabolic pathway. In the case of leucine incorporation, this competition is the incorporation of ambient, unlabeled leucine into protein. The extracellular pool is the naturally occurring leucine present in seawater (Fig. 3a) (Suttle et al. 1991). The intracellular pool (Fig. 3b) consists of leucine that has either been taken up from the extracellular pool or that has been synthesized de novo within the cell (Fig. 3c). When converting leucine incorporation into PHP, the incorporation of non-tracer leucine (i.e., the level of isotope

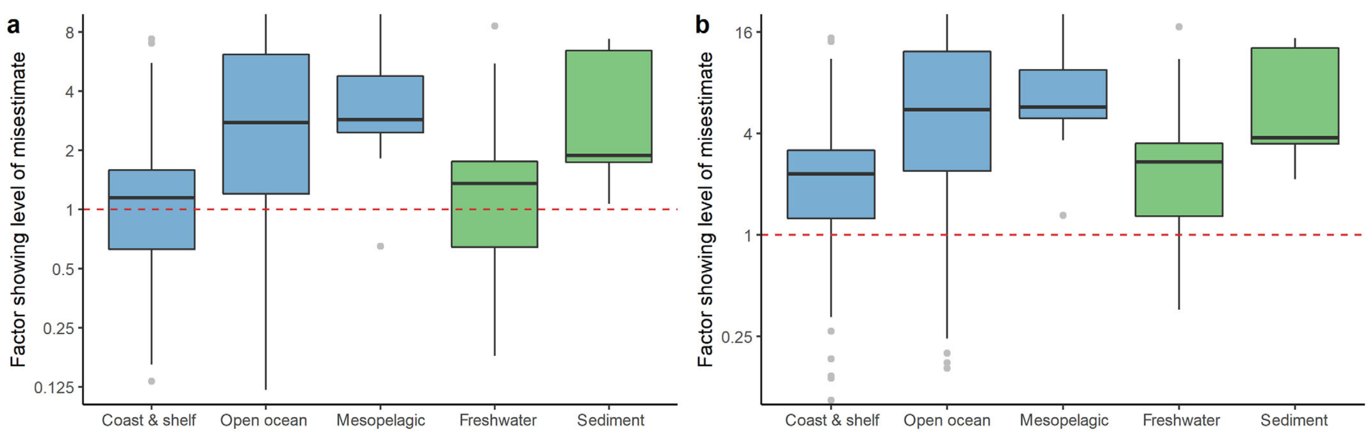

Fig. 2. Factor by which PHP could have been overestimated if a theoretical $LeuCF_{Theo}$ of (**a**) 1.55 kg C [mol Leu]$^{-1}$ and (**b**) 3.10 kg C [mol Leu]$^{-1}$ had been applied rather than the empirically determined $LeuCF_{emp}$. Red dashed lines indicate a match between empirical and theoretical LeuCFs. Note log scale of y-axis.

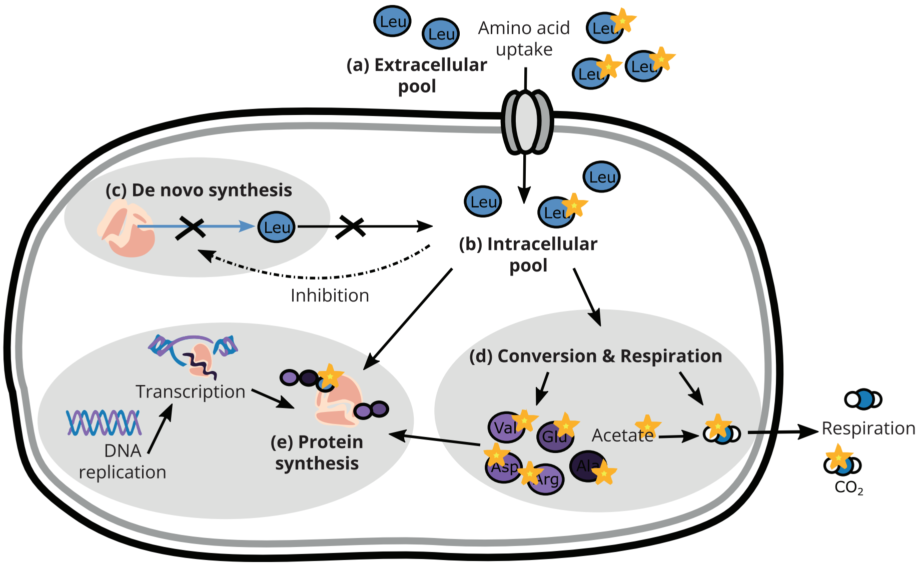

Fig. 3. Pathway of radiolabeled leucine through the cell. (a) Labeled leucine (i.e. added leucine with known specific activity) is added to the extracellular pool, and (b) taken up by the cell into the intracellular pool. In the cell, labeled leucine (c) inhibits de novo synthesis of leucine, (d) can be converted into other amino acids or respired, and (e) is used for protein synthesis.

dilution) is accounted for using an "isotope dilution factor." The isotope dilution factor for the respective pools is the ratio between total leucine concentrations (labeled + unlabeled) and labeled leucine concentrations, with extracellular dilution referring to the free leucine in seawater and intracellular dilution referring to leucine in the cell.

The intracellular isotope dilution factor is usually > 1 as prokaryotes produce some leucine de novo (Simon and Azam 1989). Simon and Azam (1989) used two independent methods to measure intracellular isotope dilution (at final concentrations of 0.5 and 10 nM Leu) and found that it stayed fairly constant at ~ 2 over 22 h and was always < 3. Other reported intracellular isotope dilution factors range from 1.1 to 11.8 (1.1 at > 40 nM, Jorgensen 1992; 2–3 at 2 nM, Simon and Rosenstock 1992; 2.2 at 10 nM, and 11.8 at 0.5 nM, Simon 1991). While Kirchman et al. (1985) did not directly measure isotope dilution, they observed that de novo synthesis is generally negatively correlated with the amount of leucine added to the extracellular pool (Kirchman et al. 1985), with a decrease in de novo synthesis of up to 60% (Monheimer 1979; Kirchman et al. 1985, 1986). All these findings point to a low intracellular isotope dilution when the leucine tracer is added at considerably elevated concentrations compared to the ambient pool. In order to minimize isotope dilution, the standard leucine incorporation assay therefore employs labeled leucine concentrations well above the ambient leucine concentrations found in seawater, typically over a magnitude higher. When calculating PHP, intracellular isotope dilution is then assumed to be either 1 ("no isotope dilution"; e.g., Tanaka and Rassoulzadegan 2004; Arístegui 2005; Alonso-Saez et al. 2007; Obernosterer et al. 2008; Baltar et al. 2009; Calvo-Díaz and Morán 2009; Kirchman et al. 2009) or 2 ("50% uptake, 50% de novo synthesis"; e.g., Reinthaler et al. 2006).

The addition of labeled leucine at high concentrations relative to those found in seawater has the added advantage of increasing the relative contribution of the tracer to the total extracellular leucine pool and therefore reducing the extracellular dilution factor. Kirchman et al. (1986) recommended the addition of ≥ 10 nM of labeled leucine to "swamp" the ambient leucine pool, which is typically ~ 1 nM. For open ocean sites, the recommended target final leucine concentration depends on the in situ leucine concentrations and typically varies between 20 and 40 nM (e.g., Kirchman 2001; Alonso-Saez et al. 2007; Gasol et al. 2009). The extracellular dilution factor is therefore often assumed to be 1 (no extracellular isotope dilution), but can be calculated when both ambient leucine concentration and final tracer concentration in the incubation medium are known.

Several studies have suggested that "swamping" the ambient leucine pool may alter prokaryotic metabolism. In oligotrophic regions, leucine concentration was found to influence the proportion of leucine-active cells (Kirchman et al. 1985). The proportions of cells that took up leucine during 4-h incubations were 30% at 0.5 nM tracer addition and 63% at 10 nM (Kirchman et al. 1985). More recently, Hill et al. (2013) showed that exposure to saturating concentrations (~ 20 nM) led to an overestimation of leucine incorporation rates (compared to rates determined using the dilution bioassay, which allows derivation of in situ leucine uptake rates at in situ leucine concentrations) in oligotrophic regions and an underestimation in production regions. Thus, the attempt to reduce isotope dilution factors by adding leucine at saturating

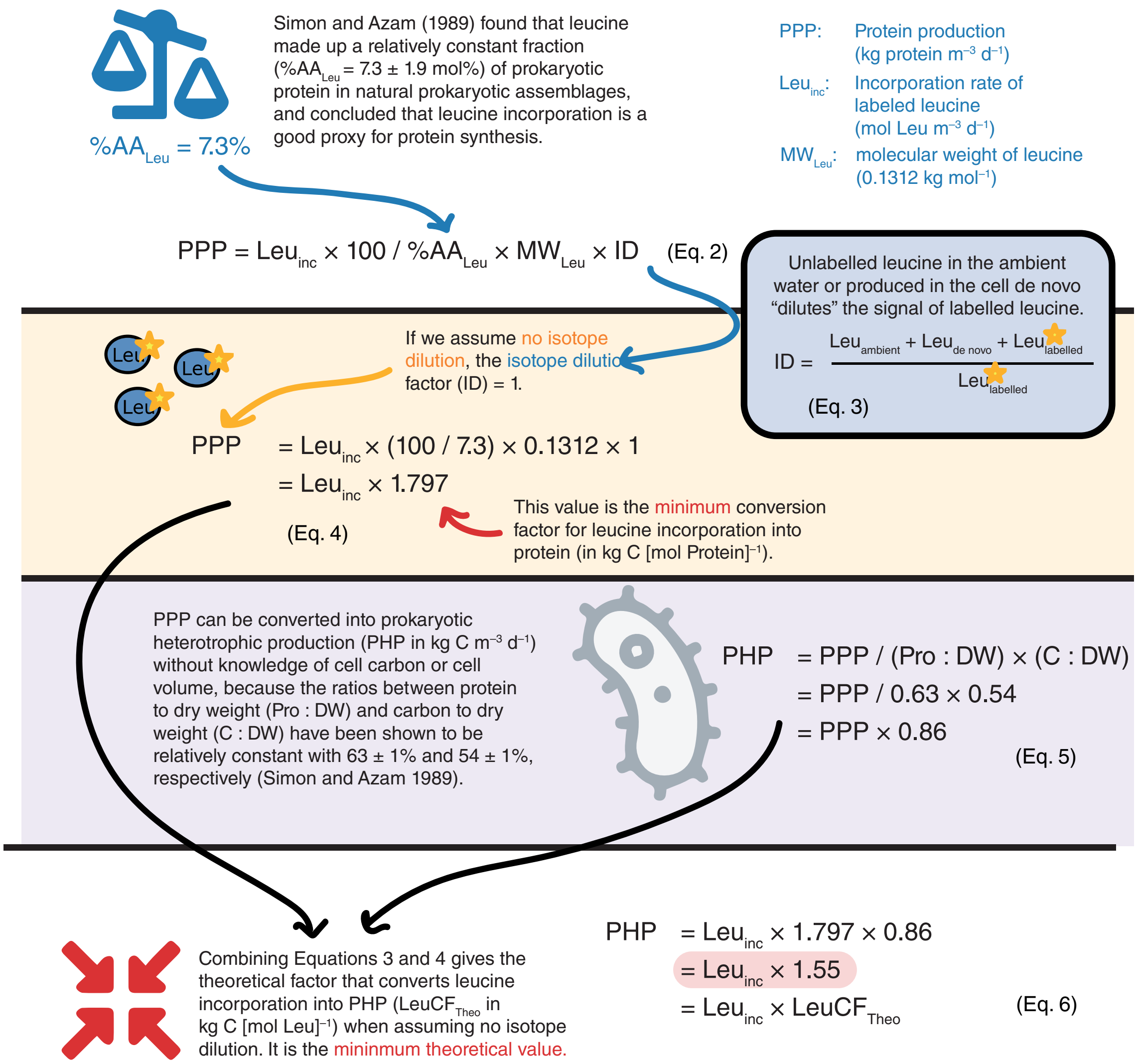

Fig. 4. Derivation of the theoretical leucine-to-carbon conversion factor (LeuCF$_{Theo}$). ‘Labeled’ refers here to added leucine with known specific activity. Equation 3 shows the combined intra- and extracellular isotope dilution (ID).

Table 2. Summary of published LeuCF$_{emp}$ (in kg C [mol Leu]$^{-1}$). Based on 54 publications and 296 published values (*see* supplementary material for details).

Hydrographic setting	Min	First Qu.	Median	Third Qu.	Max	*n*
Coast and shelf	0.21	0.98	1.35	2.47	36.40	160
Open ocean	0.02	0.25	0.56	1.29	19.20	105
Mesopelagic	0.13	0.33	0.54	0.63	2.38	15
All marine	**0.02**	**0.52**	**1.14**	**2.00**	**36.40**	**280**
Freshwater	0.18	0.88	1.15	2.41	8.60	16
Sediment	0.21	0.24	0.82	0.89	1.45	5
All environments	**0.02**	**0.53**	**1.14**	**2.03**	**36.40**	**296**

concentrations changes leucine incorporation rates by prokaryotes, and thus renders the leucine incorporation assay an inaccurate way to determine PHP. Furthermore, exposure to unnaturally high resources may trigger changes in prokaryotic metabolism beyond the incorporation rate (*see* “Leucine incorporation and conversion”).

Leucine incorporation and conversion

The theoretical $LeuCF_{Theo}$ allows the conversion of leucine incorporation rates into rates of carbon production. It is calculated using the average proportion of leucine in amino acids, the molecular weight of leucine and the isotope dilution factor (Fig. 4). Two of the major assumptions for the calculation of the $LeuCF_{Theo}$ are (1) that leucine makes up a constant fraction (7.3 ± 1.9%mol) of total protein in prokaryotes (Simon and Azam 1989) and (2) that labeled leucine is not converted to other compounds that are subsequently incorporated into protein or, in case protein is not extracted, prokaryotic biomass (Fig. 3e) (Kirchman et al. 1985). Originally, the method called for extraction with hot trichloroacetic acid (TCA) to retrieve incorporation into protein only (Kirchman et al. 1985). Since then, likely owing to the complexity of the hot TCA extraction, two methods have become common: extraction with cold TCA, which also includes nucleic acids and other macromolecules (Chin-Leo and Kirchman 1988; Kirchman 1992; Jorgensen 1992), or simple filtration that includes all cell components (Zubkov et al. 1998). With the typically applied isotope dilution of 1, the $LeuCF_{Theo}$ based on these assumptions is 1.55 kg C [mol Leu]$^{-1}$ (i.e., $LeuCF_{1.55}$), which is the minimum possible value. While the original values used for calculating the $LeuCF_{Theo}$ are based on empirical data (Kirchman et al. 1985; Simon and Azam 1989), the method now assumes that these values are applicable to any aquatic environment. Resulting PHP estimates are hence considered to be at the lower end of likely rates. Alternatively, several protocols—including the JGOFS protocols (Knap et al. 1994)—assume an isotope dilution of 2 and hence apply a conversion factor of 3.1 kg C [mol Leu]$^{-1}$ (i.e., $LeuCF_{3.1}$).

In the open ocean, empirically determined $LeuCF_{emp}$ are markedly lower than the $LeuCF_{1.55}$, with a median of 0.60 kg C [mol Leu]$^{-1}$ (quartile range: 0.28–1.70; Table 2). This discrepancy indicates that incorporation of labeled tracer is much higher than assumed by the method assumptions (i.e., >7.3% mol of protein).

Leucine conversion into other amino acids

Several studies have observed that leucine can be converted into other amino acids such as valine (Monticello and Costilow 1982; Kirchman et al. 1985), alanine (Simon and Azam 1989) and, to a lesser extent, aspartate and glutamate (Monticello and Costilow 1982). Monticello and Costilow (1982) showed that the anaerobic bacterium *Clostridium sporogenes* converted 4.7% of the added leucine (10 mM final concentration) into valine, 1.5% into glutamate, and 0.9% into aspartate. These conversion rates of leucine are small in relative terms, and experiments carried out in relatively productive ecosystems report that little (0–20%) of the leucine taken up was converted into other amino acids before incorporation into protein (Kirchman et al. 1986). However, prokaryotes in oligotrophic and mesopelagic systems are likely to convert a higher fraction of added leucine into other amino acids due to the inherent shortage of resources. This behavior was shown during a study in an oligotrophic system in the Bahamas, where the fraction of added leucine (concentrations 0.5–30 nM) converted to other amino acids was up to 70% (Kirchman et al. 1985). Kirchman et al. (1985) investigated this pathway across a range of marine environments and found that, across all incubations ($n = 35$), 24–97% of the tracer recovered in protein was in the form of leucine. In oligotrophic environments, up to 76% of the labeled leucine was converted into other amino acid (i.e., only 24% of the leucine-derived tracer was recovered as leucine). These results indicate that the proportion of leucine-derived tracer within protein could potentially be larger than expected. Indeed, based on the protein composition values reported by Simon and Azam (1989), amino acids that could be derived from leucine make up 47.8 mol% of prokaryotic protein (Table 3), which implies that up to 55.1%mol of prokaryotic protein could contain leucine-derived tracers. These high values are, however, based on observations from oligotrophic systems; in non-oligotrophic systems, leucine-derived amino acids are likely much lower.

Table 3. Amino acids in prokaryotic protein (%mol). Based on Simon and Azam (1989).

Leucine and its conversion products	Contribution in amino acids (%mol)
Leucine	7.3 ± 1.9
Valine	8.0 ± 2.6
Glutamate	11.5 ± 5.7
Aspartate	15.5 ± 3.8
Alanine + arginine	12.8 ± 5.5
Total	*55.1*

Leucine respiration

Studies to determine whether leucine fuels aquatic prokaryotic respiration, using ^{14}C leucine tracers, consistently report the generation of labeled CO_2 (Hobbie and Crawford 1969; Suttle et al. 1991; Jorgensen 1992; Alonso-Saez et al. 2007; del Giorgio et al. 2011; Hill et al. 2013). When added tracer concentrations are close to ambient concentrations, lower proportions of the leucine taken up have been observed to be respired (< 10% in the Sargasso Sea (Suttle et al. 1991)). Leucine respiration is consistent with reports that, in addition to conversion to other amino acids (Monticello and Costilow 1982; Kirchman et al. 1985; Simon and Azam 1989), leucine may be catabolized to non-proteinaceous compounds (Massey et al. 1976) in the process of energy production (Supplementary Fig. S3).

However, although the respiration of leucine tracer will lead to the production of labeled compounds within the cell, given that they form the metabolic pathways of energy production, it is likely that they will have a high flux. Furthermore, as they pass into the Krebs cycle they will be converted to glucogenic amino acids and compounds that are not precipitated when extracting protein. Thus, the labeled components and end products of the respiration pathway are unlikely to be retained in the analysis, or likely to be short-lived within the cell, and, therefore, they will be far less impacting in perturbing the accuracy of LeuCFs relative to the impact of leucine's conversion to other amino acids. Exposure to saturating concentrations of leucine (i.e. "swamping") causes a much greater stimulation in respiration than in production (Hill et al. 2013). Since respiration will rapidly divert the label out of the cell, the leucine saturation method provides relative rates of prokaryotic production to one another, which—despite the associated limitations—support its utility for continued use.

Refining the $LeuCF_{Theo}$

Given that the $LeuCF_{Theo}$ is derived from the isotope dilution factor and the proportion of leucine-derived labeled amino acids in protein, the accuracy of PHP estimates is contingent on the values selected for these terms being representative of the target environment. We next assess how ranges of values observed for these two factors affect the $LeuCF_{Theo}$. Furthermore, we employ empirical data to derive the most probable $LeuCF_{Theo}$ and compare this to the commonly used $LeuCF_{1.55}$ and $LeuCF_{3.1}$.

Effect of variability in isotope dilution and amino acid metabolism

Following the reported range of observed isotope dilutions (Simon and Azam 1989; Simon 1991) (*see* "Concentration of leucine tracer and associated uptake rates"), we simulated a right-skewed distribution of isotope dilution factors that ranges from 1 to ~ 10 with most of the observations having a value of 2–3 ($X \sim N(1.2^{1/3}, 0.2)^3 + 1$; Fig. 5c). For the fraction of leucine-derived label that is incorporated into protein (% AA), we are aware of only one relevant study that looked into the probability with which these conversions may occur (Kirchman et al. 1985). These data include observations from a salt marsh estuary, the continental shelf of the United States, the western boundary of the Gulf Stream, and oligotrophic waters off the Bahamas. If we assume that the observations by Kirchman et al. (1985) are a fair representation of the natural variability, we can use their observed distribution, calculating the probability density function using kernel density estimates. We assumed that 100% recovery of leucine-tracer as leucine (Kirchman et al. 1985) is equivalent to 7% leucine in protein (Simon and Azam 1989). Conversely, when the labeled leucine is converted and incorporated into all possible other amino acids (making up 55 %mol AA; Table 3), only 13% of the leucine-tracer would have been recovered as leucine (13% = 7 %mol Leu/55 %mol AA). We recalculated LeuCF following the equations in Fig. 4 using the Monte Carlo method with 100,000 randomly sampled values

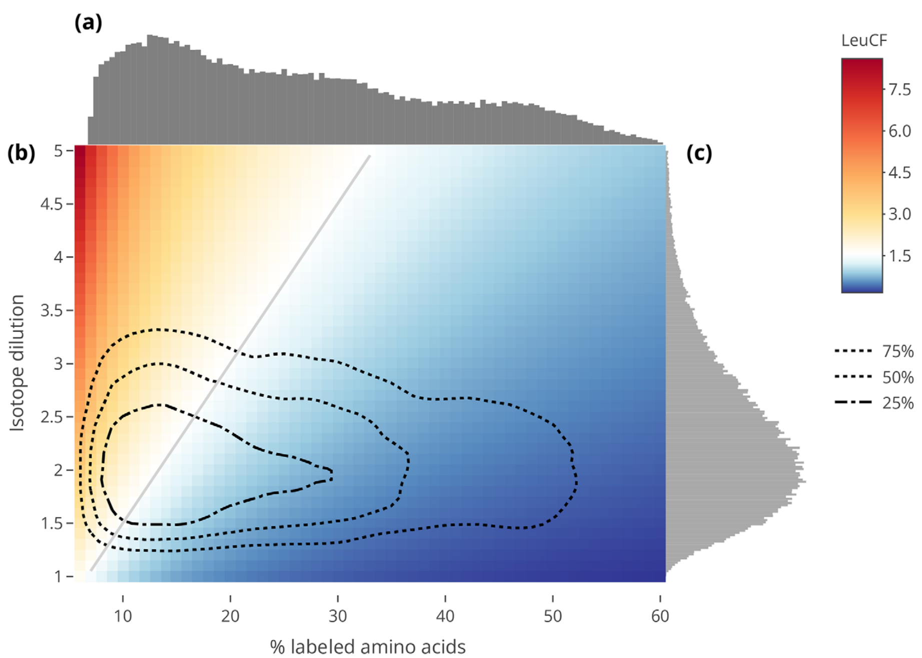

Fig. 5. (**a**) Simulated probability distribution of observed % leucine-derived labeled amino acids. (**b**) Contour plot showing the combined effect of various combinations of proportions of labeled amino acids in protein and isotope dilution on LeuCF. Red and blue colors identify LeuCF above and below the $LeuCF_{1.55}$, respectively. (**c**) Simulated probability distribution of observed isotope dilution. Dashed and dotted lines show the top 25%, 50%, and 75% of most likely combinations and resulting LeuCF, calculated using two-dimensional kernel density estimation on the 100,000 LeuCFs from the Monte Carlo simulation. Gray diagonal line identifies the $LeuCF_{1.55}$.

for isotope dilution and %AA from the above distributions. We assumed an average molecular weight of 120 g mol^{-1} for amino acids in protein (based on Table 2 by Simon and Azam 1989).

With the observed natural variability in isotope dilution and %AAs, LeuCF likely ranges from 0.18 to 9.38 kg C [mol Leu]$^{-1}$ with a median for 0.98 kg C [mol Leu]$^{-1}$ (quantile range: 0.29–1.60 kg C [mol Leu]$^{-1}$) (Fig. 5). This value is considerably lower than both the $LeuCF_{1.55}$ and $LeuCF_{3.1}$. Our simulated data, however, match published $LeuCF_{emp}$ for the marine environment well (median 1.21 kg C [mol Leu]$^{-1}$; range: 0.02–36.4 kg C [mol Leu]$^{-1}$; Table 2). Our simulation demonstrated that empirical $LeuCF_{emp}$ below the $LeuCF_{1.55}$ can be explained by prokaryotes using leucine as substrate for the synthesis of other amino acids.

Linking metabolic state with $LeuCF_{Theo}$

As "swamping" prokaryote with leucine likely triggers them to use it for the synthesis of other amino acids and energy production (*see* "Leucine incorporation and conversion"), it is logical to conclude that these processes may be linked. Specifically that the incorporation of tracer into protein (the actual LeuCF) and rates of leucine respiration are related (e.g., Alonso-Saez et al. 2007). To test this hypothesis, we interrogated the results of two studies that measured both leucine respiration (using ^{14}C-leucine) and $LeuCF_{emp}$ (using ^{3}H-leucine) at saturating leucine concentrations (20–40 nM). The measurements were made on the upper-ocean communities in the eastern North Atlantic (Alonso-Saez et al. 2007) and eastern North Pacific (del Giorgio et al. 2011). It is noteworthy that the tracers ^{14}C- and ^{3}H- may follow different pathways during leucine metabolism (Supplementary Fig. S1), and the final estimates (^{14}C-respiration vs. ^{3}H-incorporation) should be compared with caution.

We found a significant negative exponential relationship between the proportion of respired leucine (relative to leucine uptake) and $LeuCF_{emp}$ ($p < 0.01$, $R^2 = 0.34$, $n = 24$) (Fig. 7). In other words, when a large fraction of the leucine is respired, the yield of carbon biomass per incorporated leucine is lowest. Highest leucine respiration rates (and thus lowest $LeuCF_{emp}$) occurred in offshore regions, which agrees well with the suggestion that open-ocean prokaryotes are substrate limited and use the excess leucine for other metabolic processes.

To further explore the link between the synthesis of other amino acids and energy production (i.e., respiration), we developed a simple theoretical model using the biochemical relationships within the cell (Fig. 4). We assume that leucine respiration and leucine conversion to other amino acids are directly proportional (%AA = 100% − %Leu respiration); hence, when no tracer is respired (%Leu respiration = 0%), all labeled leucine is incorporated into protein in its original form. When labeled leucine is respired, an equal amount of labeled leucine is converted to other amino acids. We calculated LeuCF using the equations in Fig. 4, assuming an average molecular weight of 120 g mol^{-1} for amino acids in protein (MW_{AA}) (Simon and Azam 1989) and an isotope dilution of 2.

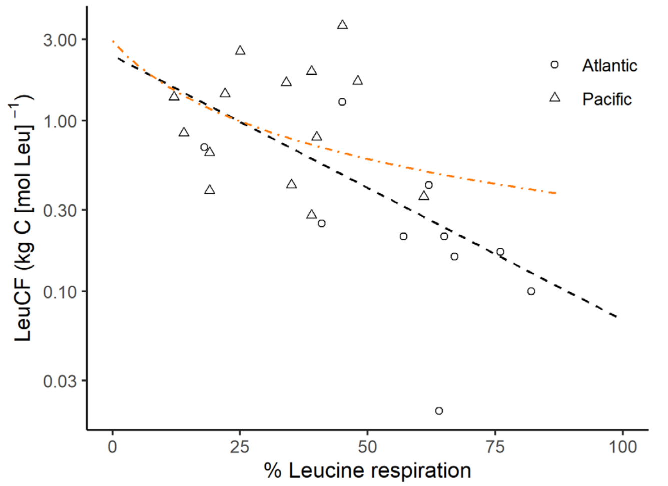

Fig. 6. Relationship between leucine-to-carbon conversion factor (LeuCF; kg C [mol Leu]$^{-1}$) and fraction leucine respired (% of total leucine incorporation + respiration). Data are empirically determined rates from different regions (offshore, shelf break and upwelling) in the Atlantic (Alonso-Saez et al. 2007) and the Pacific (del Giorgio et al. 2011). Black dashed line shows regression fit ($p < 0.01$, $R^2 = 0.34$, $n = 24$). Orange dotted line shows the model fit. Symbols show study region: Atlantic (triangles) and Pacific (crosses). Note the log scale on the y-axis.

The model outputs match the observations reasonably well when leucine respiration is < 50% (Fig. 6), supporting a direct link between leucine respiration and conversion. The model overestimates LeuCF when leucine respiration is > 50% (Fig. 6), indicating that the conversion of leucine to other amino acids is not linearly proportional to leucine respiration. Rather, prokaryotes appear to produce disproportionately less carbon biomass per incorporated amino acid when leucine respiration is very high. The model illustrates that there is a tangible link between leucine respiration and measured $LeuCF_{emp}$, and that we can recreate this trend when we assume that leucine respiration and leucine conversion to other amino acids are linked. This insight further strengthens the hypothesis that the exposure of open ocean prokaryote assemblages to saturating leucine concentrations may be more representative of nutrient addition experiments, rather than an indication of in situ microbial metabolism (Hill et al. 2013).

Recommendations and conclusion

The power of the leucine incorporation assay to determine PHP lies in its relative speed, simplicity, and economy, given the few disposable resources it requires (Kirchman et al. 1985). As the standard protocol to determine aquatic PHP since 1993 (Kirchman 1993; Knap et al. 1994), its application has the advantage of a large number of existing measurements (as of writing > 750 citations for Kirchman et al. 1985) against which to synthesize new data. Such datasets have tremendous power in monitoring the oceans and in establishing changes in microbial functioning over time. Hence we endorse the continued application of this method but recommend additional considerations when applying it for the determination of PHP (Fig. 7).

Through exploration of the existing empirical data, we illustrated that the underlying assumption that labeled leucine is only incorporated into cell biomass in the form of leucine is likely often incorrect. We further infer that the characteristics of the ecosystem under investigation likely influence the magnitude at which the label is incorporated as compounds other than leucine. Furthermore, as previously highlighted (Kirchman et al. 1985; Hill et al. 2013), employing significantly elevated concentrations of tracer to overcome isotope dilution, so called swamping, may alter leucine incorporation

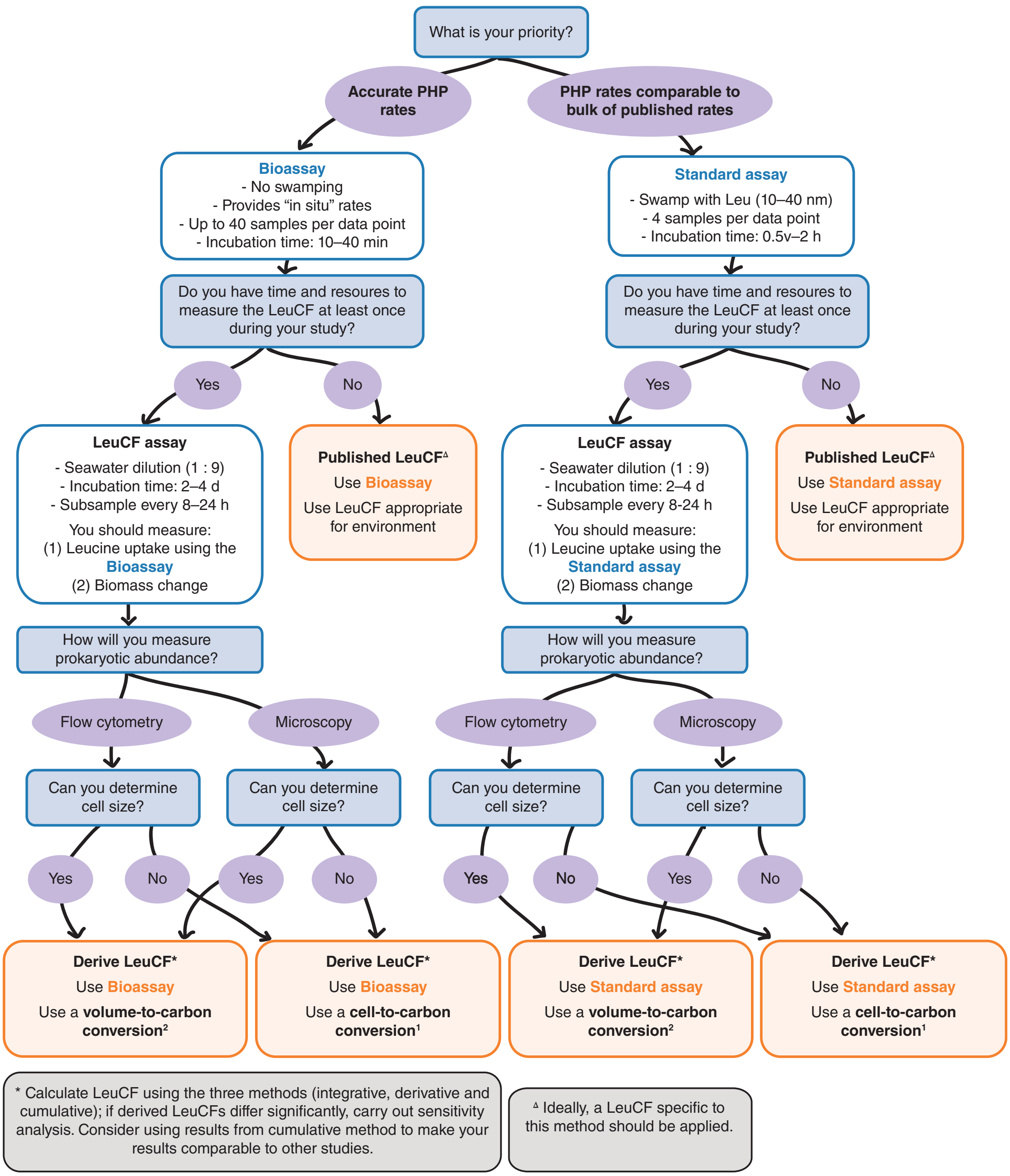

Fig. 7. Suggested workflow for measuring PHP using the leucine incorporation method. [1]For example, Calvo-Diaz and Moran (2006). [2]For example, Norland (1993).

rates according to the nutritional state of the prokaryotic community. PHP accuracy will be improved by deriving and applying an in situ $LeuCF_{emp}$ for the investigated environment. Acquisition of more in situ conversion factors will also build understanding of prokaryotic metabolism according to ecosystem characteristics, helping to refine theoretical conversion factors. To avoid perturbation of microbial leucine metabolism by saturating with leucine, the time-series dilution bioassay approach developed by Wright and Hobbie (1966) and adapted for oceanic amino acid uptake (Fuhrman and Ferguson 1986; Zubkov and Tarran 2005) can be employed. Use of the bioassay technique avoids "swamping" and allows derivation of in situ leucine uptake rates. However, as the dilution bioassay may also alter the intracellular isotope dilution, $LeuCF_{emp}$ specific for this method should be applied.

While the derivation of in situ conversion factors for each study site in combination with use of the dilution bioassay will achieve the most accurate estimates of PHP, this approach incurs greater investment of time and resources, and encompasses a higher degree of complexity. Thus, for identifying appropriate experimental design, it must be determined whether a study's priority is to derive accurate in situ PHP estimates at fewer sites or relative PHP estimates at more sites. In order to improve PHP accuracy without incurring the logistical burden of both the bioassay and the in situ $LeuCF_{emp}$ determination, the latter could be combined with the saturation-based method. In the event that measuring an in situ $LeuCF_{emp}$ is logistically contraindicated, we recommend the selection of a more appropriate theoretical conversion factor, representative of the investigated environment (Fig. 2 and Table 2).

Finally, while we recommend measuring empirical $LeuCF_{emp}$ when possible, the method is subject to the problems common to all experiments or techniques that involve the incubation of natural communities within vessels. When natural microbial communities are incubated for several days, their composition may change (e.g., Teira et al. 2015) with potential implications for the accuracy of the conversion factors determined.

Implications for understanding the ecosystem

Our analysis confirms that the theoretical conversion factors typically used to date overestimate PHP in the majority of cases. If over 25% of the published values have overestimated PHP by a factor of ~ 5 (Fig. 1), this will change our fundamental understanding of the roles of PHP in marine and freshwater systems. While a recent study suggests that PHP rates based on leucine incorporation measurements may be underestimated (Popendorf et al. 2020), our conclusion that PHP rates are likely overestimated when using theoretical conversion factors is consistent with our current understanding of interior carbon flows, particularly mesopelagic carbon budgets (e.g., Giering et al. 2014). There is a clear need for more experimental studies, particularly investigating the amino acid composition and cell carbon content of prokaryotes, metabolism including de novo synthesis of leucine (and other amino acids used for rate measurements), and LeuCF estimated using both saturating and ambient concentrations (ideally in parallel) particularly for oligotrophic and mesopelagic environments. Finally, much can be learned about ecosystem dynamics and aquatic carbon flows by using the well-established leucine incorporation method alongside other modern techniques.

We hope that the synthesis presented here will stimulate the evaluation and refinement of the PHP values derived using the established method in the context of the ecological state of the study site. In addition, this review aims to support the experimental design of future studies so that they achieve their objectives. Ultimately, we hope to facilitate empirically based understanding of the biogeochemical and ecological role of prokaryotes in aquatic systems.

Data availability statement

Data are available in Supplementary Table 1.

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Conflict of interest

The authors declare no conflicts of interest.

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