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HORIZONS

Advancing interpretations of ^{14}C-uptake measurements in the context of phytoplankton physiology and ecology

ALLEN J. MILLIGAN[1], KIMBERLY H. HALSEY[2] AND MICHAEL J. BEHRENFELD[1]*

[1]DEPARTMENT OF BOTANY AND PLANT PATHOLOGY, OREGON STATE UNIVERSITY, CORVALLIS, OR, USA AND [2]DEPARTMENT OF MICROBIOLOGY, OREGON STATE UNIVERSITY, CORVALLIS, OR, USA

*CORRESPONDING AUTHOR: mjb@science.oregonstate.edu

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The ^{14}C-uptake method is the most common approach employed for estimating primary production in the ocean. Normalizing ^{14}C-uptake to chlorophyll *a* and time yields a value termed the assimilation number, which is thought to reflect phytoplankton physiology. It is often assumed that the measured rate of ^{14}C-uptake is between net and gross primary production, depending on the time scale of the incubation. Recent studies employing multiple oxygen and carbon isotopic methods to measure photosynthesis of phytoplankton grown over a range of steady-state division rates have provided mechanistic insights on the relationship between ^{14}C-uptake and gross-to-net primary production. Results from these studies show that short-term (<12 h) "photosynthesis-irradiance" measurements are not a reliable means of estimating net production, gross production or nutrient limitation, but can provide important information on the photoacclimation state of the phytoplankton. Long-term (24 h) incubations yield assimilation numbers that are in good agreement with net production rates, but are independent of nutrient-limited division rates. Despite complications in interpreting ^{14}C-uptake data, we suggest that these measurements are important for understanding phytoplankton physiology and carbon cycles while, at the same time, efforts are needed to establish new incubation-free methods for measuring phytoplankton division rate and biomass.

KEYWORDS: ^{14}C method; primary production; gross production

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On the occasion of the 50 years anniversary of Steemann Nielsen's (Steemann Nielsen, 1952) seminal ^{14}C primary production paper, Banse (Banse, 2002) suggested that the measurement of phytoplankton primary production tells us very little about the controls on phytoplankton biomass. He made the assumption that ^{14}C is useful for measuring net primary production but warned that "The concentrations of phytoplankton and the rate of change cannot be understood just from resource-controlled cell division rates, let alone photosynthesis". He ended with the line ". . .think about what you are going to do with the data before you gather them!"

While Banse's main concern was predicting phytoplankton abundance and temporal change, the main arguments in response to his article defended the continued use of ^{14}C primary production estimates for two reasons: (i) the assimilation number [^{14}C fixation normalized to chlorophyll *a* (Chl *a*)] provides an index of phytoplankton physiology through changes in light and nutrient availability (Parsons, 2002) and (ii) making routine ^{14}C measurements provides a long-term record of change (Sherr and Sherr, 2002). Neither Banse's criticism nor the subsequent responses assess the critical assumptions that go into measuring carbon fixation with ^{14}C. Recently, Halsey *et al*. have been exploring some of the common assumptions that go into measuring primary production. Using multiple carbon and oxygen isotopic methods they found that short-term ^{14}C fixation does not reliably measure net or gross production (Halsey *et al.*, 2010, 2011, 2013). For an extensive review of photosynthetic energy allocation as well as the metabolic pathways affecting ^{14}C-uptake, see Halsey and Jones (Halsey and Jones, 2015). While awareness of the problems associated with ^{14}C measurements is not new, these studies illuminate the underlying physiological processes that drive variability in observed ^{14}C measurements; a topic that has occupied biological oceanographers for decades (Marra, 2002; Barber and Hiking, 2007).

The ^{14}C technique is thought to measure something between net and gross carbon fixation depending on the length of the incubation (Marra, 2002). We define net carbon fixation as carbon fixed (gross carbon fixation) minus carbon respiratory losses and light-dependent losses due to photorespiration and light-enhanced mitochondrial respiration. Gross carbon fixation is the total carbon fixed without correction for losses. Gross carbon fixation is roughly 3 folds greater than net, but can range from 1.2 to 7 (Marra, 2009; Halsey and Jones, 2015). Long incubations (12−24 h) are an estimate of net carbon fixation and short incubations (20 min to 2 h) approach gross carbon fixation. Extensive field data support the view that long incubations are very near net carbon fixation (Marra, 2009), but there is poor evidence that short incubations reliably measure gross carbon fixation. The ease of incubations using the photosynthetron method, however, has led to an increased reliance on short incubations. A re-evaluation of the "short is gross" assumption is therefore important.

From either short- or long-term ^{14}C incubations, an assimilation number is retrieved, meaning the carbon fixed per unit Chl *a* per time. Assimilation numbers have long been viewed in biological oceanography as measures of phytoplankton physiology and indicative of light and nutrient conditions for growth. The impact of light is straightforward. Photoacclimation to low light intensities impacts the light-saturated assimilation number because increases in pigment are not matched by changes in carbon fixation capacity (Macintyre *et al.*, 2002). Additionally, the packaging effect decreases absorption per unit pigment (Bricaud *et al.*, 1995). During photoacclimation, cells can also alter the concentration of accessory pigments relative to Chl *a* and influence carbon fixation or oxygen evolution rates through increased absorption per unit Chl *a*. This effect of variable accessory pigments can be accounted for by normalizing carbon fixation to absorbed light rather than Chl *a* (Macintyre *et al.*, 2002).

The view that nutrient limitation also influences assimilation number carries with it an implicit assumption that phytoplankton produce photosystems (light harvesting pigments and reaction centers) that are less efficient when nutrients are limiting. The natural question arises, why, after roughly 2 billion years of evolutionary optimization to low nutrient environments, would nutrient-limited phytoplankton produce inefficient photosynthetic reaction centers? Why not make fewer, but fully functional reaction centers? Despite some evidence that assimilation number varies with nutrient stress (Curl and Small, 1965; Thomas and Dodson, 1972; Kolber *et al.*, 1988; Herzig and Falkowski, 1989), there is strong evidence that under carefully controlled, steady-state conditions, assimilation efficiencies do not reflect macro-nutrient limitation (Caperon and Meyer, 1972; Laws and Wong, 1978; Laws and Bannister, 1980; Osborne and Geider, 1986; Sakshaug *et al.*, 1989; Halsey *et al.*, 2010, 2011, 2013). Iron limitation is likely to be an exception, as discussed below.

In the series of publications by Halsey *et al.* (Halsey *et al.*, 2010, 2011, 2013, 2014), N-limited continuous phytoplankton cultures were used to quantify primary production using multiple techniques. Specifically, ^{14}C incorporation was measured over a range of time scales (from minutes to 24 h), and gross and net O_2 production was measured using O_2 isotopes and membrane inlet mass spectrometry. Oxygen isotopes provide the ability to distinguish between oxygen generated from photosynthesis ($^{16}O_2$) and consumed (tracer levels of $^{18}O_2$) while cells are exposed to light. From these measurements, a gross oxygen production rate is obtained

that differs from the gross carbon fixation rate because of oxygen losses due to light-dependent oxygen consumption (i.e. photorespiration, light-enhanced mitochondrial respiration, Mehler activity, chlororespiration) and the direct reductant use for nitrogen and sulfur reduction. Gross carbon fixation is then calculated as the gross oxygen production minus light-dependent oxygen consumption, divided by the fraction of oxygen equivalents that are used for nitrogen and sulfur assimilation. Net primary production was also assessed as the product of specific division rate (μ) and phytoplankton carbon (C_{phyto}). The studies show that assimilation numbers for gross and net production are not dependent on nutrient-limited growth rate (Fig. 1). Variable fluorescence was also invariant with nutrient-limited division rate, in agreement with Parkhill *et al.* (2001) and Kruskopf and Flynn (2006). However, the measured property showing a strong dependence on nutrient limitation was ^{14}C-uptake during short incubations (20 min to 2 h). These observations demonstrate that net and gross assimilation numbers do not register nutrient limitation [consistent with the data of Laws and Bannister (1980) and Laws and Wong (1978)], while short-term ^{14}C-uptake varies linearly with division rate. Halsey *et al.* (Halsey *et al.*, 2011, 2013) showed that the relationship between ^{14}C and division is due to the time-dependent physiological processes impacting carbon metabolism and linked to the cell cycle.

Newly created photosynthetic carbon products can be separated into two pools: (i) a short-lived, or "transient", pool that is catabolized on the time scale of a cell division and (ii) a long-lived pool that constitutes cell growth and division (i.e. net production). The reason short-term ^{14}C-based assimilation numbers vary with division rate is that the measurement is registering different average lifetimes of the transient carbon pool. Halsey *et al.* (Halsey *et al.*, 2011, 2013) demonstrated this effect by comparing observed time-courses of ^{14}C labeling with the modeled decay of the transient carbon pool. Moreover, Halsey *et al.* (Halsey *et al.*, 2013) used cultures with synchronized division cycles to show that the nutrient-dependent carbon lifetime changes are specifically associated with differing carbon allocation during phases of the cell cycle. At high division rates, a greater fraction of cells are in the division phase, which is when photosynthate is preferentially stored in a longer-lived carbon form and is measurable as fixed ^{14}C. At slower division rates, a greater fraction of cells in a population are in the growth

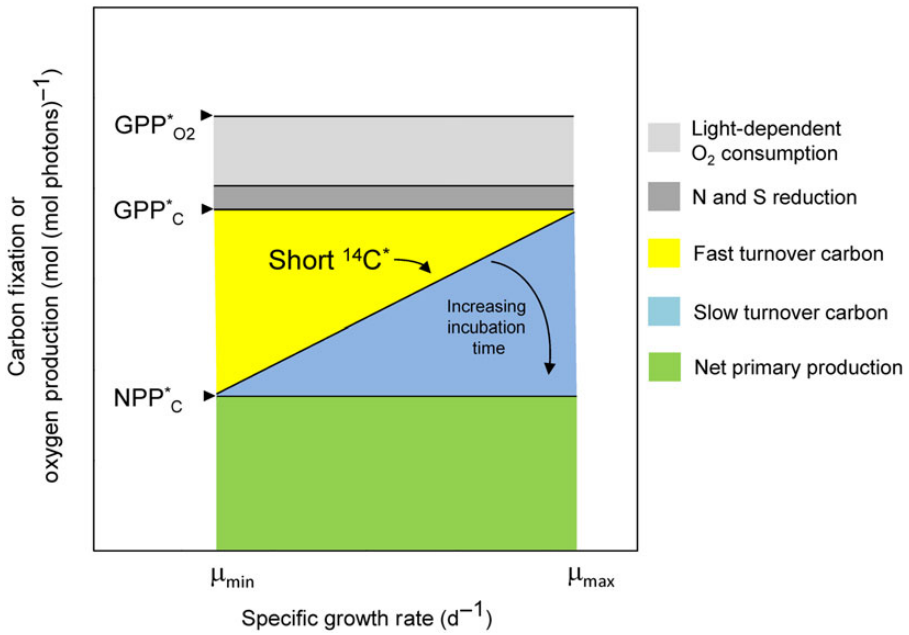

Fig. 1. Production rates normalized to absorbed light across a range of steady-state division rates from nitrogen (NO_3^-) limited chemostats. GPP*$_{O2}$ is gross oxygen production measured using added $^{18}O_2$ and membrane inlet mass spectrometry. Subtracting the light-dependent O_2 consumption (losses due to photorespiration, light-enhanced mitochondrial respiration, Mehler activity and chlororespiration) and the O_2 consumption required for nitrogen and sulfur reduction from GPP*$_{O2}$ yields the gross carbon primary production (GPP*$_C$). Short-term ^{14}C (20 min shown) assimilation varies with division rate and reflects differences in the lifetimes of transient carbon products. For slow growing cells, the turnover of transient carbon pools is extremely fast and measured ^{14}C-uptake matches net production (left side of blue wedge). In fast growing cells, transient carbon turnover is slower so the measured ^{14}C-uptake matches gross carbon fixation (right side of blue wedge). Increasing the incubation duration allows ^{14}C fixed in slow turnover carbon pools to be respired and measured rates approach net carbon fixation (blue wedge arrow). NPP*$_C$ is the calculated net primary production ($\mu \times C_{phyto}$) and the measured ^{14}C in 24 h incubations. For quantitative relationships for different species, see Halsey *et al.* (Halsey *et al.*, 2010, 2011, 2013, 2014).

phase of the cell cycle. In this phase, newly formed carbon is more rapidly respired or used to regenerate reductant pools (NADH, NADPH, etc.), resulting in a significant decline in carbon storage and thus lower ^{14}C labeling during an incubation.

An important consequence of the above processes is that the average lifetime of the transient carbon pool impacts ^{14}C labeling at all incubation light levels during short-term photosynthesis vs. irradiance (P vs. E) measurements. Consequently, both the initial slope (α^b) and maximum photosynthetic (P^b_{max}) rate covary with division rate. In other words, the resulting P vs. E curves display "E_k independent variability" (Behrenfeld *et al.*, 2008), a term that refers to parallel changes in α^b and P^b_{max} that leave the point of light saturation (E_k) invariant. Without prior knowledge of the division rate of a population, it cannot be determined whether the measured carbon fixation rate is net, gross or a value in between. Thus, while the short-term P vs. E method can reliably measure E_k, observed changes in α^b and P^b_{max} between samples (say along a nutrient gradient) will not indicate light response performance. Furthermore, P vs. E behavior will depend on the time within the diurnal cycle that samples are collected, particularly in populations with synchronized cell cycles. This temporal dependence is caused by shifts in dominant metabolic pathways that are associated with carbon products of different lifetimes, yielding correlated changes in α^b and P^b_{max} over the course of the day (Behrenfeld *et al.*, 2004). Field observations of photoinhibition in P vs. E curves have shown that P^b_{max} declines at noon and does not recover fully after noon (Neale, 1987; Cullen *et al.*, 1992). This observation has been attributed to slow recovery from noontime photoinhibitory irradiance, but this behavior could instead be attributed to changes in cell-cycle-driven carbon fates, as seen in synchronized laboratory cultures (Bruyant *et al.*, 2005).

The above metabolic and cell-cycle processes impacting short-term P vs. E incubations imply that these measures should not be used to quantify depth integrated production nor should they be used to obtain assimilation numbers for the use in satellite-based production models. However, they are helpful in the parameterization of photoacclimation strategies of phytoplankton. The short-term measurements are also helpful in understanding phytoplankton physiology, through measured variability in E_k, but require additional information (e.g. division rate, fate of fixed carbon, or turnover rate) for interpretation.

What can be concluded about short-term P vs. E measures? We can reject the assumption that short incubations reliably measure gross production. In fact, they measure an undefined production value. The short-term ^{14}C data shown in Fig. 1 are correlated to growth rate because carbon allocation is growth rate dependent. It is tempting to conclude that short-term ^{14}C incubations are a predictor of growth rate; however, carbon allocation is dependent on species, and the slope of the response is dependent on incubation time (Halsey *et al.*, 2011, 2013, 2014).

By using multiple approaches where gross and net photosynthesis are directly measured, the Halsey *et al.* studies reveal that variability in short-term ^{14}C fixation reflects cell-cycle-associated changes in carbon flux to metabolic pools of varying lifetimes (Fig. 1). Arriving at this conclusion required an experimental approach that utilized ^{14}C-independent methods to measure gross and net primary production. This combination of "tracers" constrains the range of possible production values and accounts for total photosynthate production and its potential fates (i.e. carbon storage and direct use in synthesis). In the field, independent measures of gross and net production could be used to interpret short-term ^{14}C fixation and reveal insights into phytoplankton carbon metabolism that have not been previously exploited.

What can ^{14}C-uptake reliably measure? Marra (Marra, 2009) concludes that 12–24 h incubations are very near net carbon fixation although Pei and Laws (Pei and Laws, 2014) warn that 24 h incubations can lead to an overestimate in slow growing cells growing under a light–dark cycle. During laboratory time–course studies, Halsey *et al.* (Halsey *et al.*, 2011, 2013) found that incubations as long as 8–12 h are required to reliably quantify net carbon fixation across all division rates. However, these laboratory experiments were performed in continuous saturating light and, under natural light conditions, longer (12–24 h) incubations may be necessary to assess net carbon fixation due to effects of the diel light cycle on measured production rates (Eppley and Sharp, 1975; Marra, 2009). Notably, a requirement for long incubations increases the likelihood of artifacts from so-called "bottle effects". Photoinhibition of carbon fixation, for example, is one artifact that can result from holding bottles at a single light level (Behrenfeld *et al.*, 1998). Also, the α^b in long-term P vs. E incubations reflects the light use efficiency of net production but, phytoplankton in the mixed layer are not subject to long-term low light exposure.

As stated above, assimilation numbers for gross or net production are generally insensitive to nutrient limitation, but phytoplankton growing under iron-limited and high macro-nutrient conditions may be an exception. Under steady-state iron limitation and nitrogen sufficiency, phytoplankton appear to produce chlorophyll that does not contribute to photosynthesis. This excess pigment enhances fluorescence and causes the universally observed drop in variable fluorescence (Behrenfeld *et al.*, 2006; Schrader *et al.*, 2011; Behrenfeld and Milligan, 2013). However, when both nitrogen and iron are in low supply, there is no

excess pigment production and variable fluorescence values remain high. Field observations of variable fluorescence have noted higher than expected values in the iron-limited low-nitrogen equatorial Pacific, in agreement with laboratory observations (Behrenfeld *et al.*, 2006). Why phytoplankton would produce pigment in excess of demand is not known, but could be an evolutionary response to the sporadic nature of atmospheric iron supply to the ocean. Halsey *et al.* (Halsey *et al.*, 2014) observed a similar phenomenon in nitrogen-limited *Micromonas* sp., suggesting that this unique behavior may represent an example of a life strategy evolved by a motile picoplankton species to take advantage of sporadic supply of nutrients other than iron.

The most straightforward, but technically challenging, method to determine net primary production is to measure phytoplankton carbon biomass (C_{phyto}) and the specific division rate (μ) and multiply the two. There are several methods to determine division rate for subsets of natural phytoplankton assemblages, such as cell-cycle analysis and ^{14}C incorporation into pigments (Mcduff and Chisholm, 1982; Goericke and Welschmeyer, 1993; Laws, 2013). The division rate for the entire phytoplankton population can be determined using the dilution method or ^{14}C incorporation into Chl *a* (Redalje and Laws, 1981; Landry *et al.*, 2008), but each of these methods requires long incubations and will suffer from static light exposure and other "bottle effects". Ideally, incubation-free methods to determine total phytoplankton division rate are needed.

Phytoplankton carbon biomass may be assessed using flow cytometry to estimate biovolume and then converting to carbon using published biovolume–carbon relationships. An advantage of this approach is that flow cytometry requires no incubation, but variability in cell-volume estimates and biovolume–carbon relationships can lead to a 10-fold range in calculated phytoplankton carbon (Dall'Olmo *et al.*, 2011). Alternatively, beam attenuation (c_p) and backscatter (b_{bp}) coefficients have been employed as optical proxies for C_{phyto} (Behrenfeld and Boss, 2003; Martinez-Vicente *et al.*, 2013). More recently, Graff *et al.* (Graff *et al.*, 2012) described a new method for directly measuring phytoplankton carbon whereby phytoplankton cells are isolated using cell sorting flow cytometery and then carbon of the sorted sample determined using total carbon analysis (Graff *et al.*, 2012). Application of this method across large oceanic regions is providing the calibration data necessary to validate flow-cytometric and optical methods (Graff *et al.*, 2015).

The primary concern raised by Banse (Banse, 2002) is that a large fraction of net primary production is rapidly lost to grazers, viral lysis and other loss terms, thus his conclusion was that information on division or production rates alone provides little information on population dynamics and standing stocks. While phytoplankton division and loss rates are far larger terms than net biomass accumulation rates, it is now becoming clear that the rate at which biomass accumulates or declines is, in fact, often related to phytoplankton division. Using a 10-year satellite record of North Atlantic phytoplankton biomass, Behrenfeld (Behrenfeld, 2014) showed that periods of increasing and decreasing phytoplankton biomass reflect a minor disequilibrium between phytoplankton division and loss rates which is driven by relative accelerations and decelerations in division rate. In other words, the absolute magnitude of division rate does not determine changes in phytoplankton concentration, but rather the rate of change of cell division. Thus, a constant increase in growth rate allows phytoplankton to stay ahead of grazing pressure, but when growth rates fail to accelerate, grazing pressure stops the accumulation of phytoplankton biomass even if division rates are still high (Behrenfeld, 2014). This observation thus brings us back to Banse's concern about measurements of production. Indeed, accurate measurements of net primary production are critical for understanding biomass changes in the ocean because of their implications for ecosystem balances.

In addition to understanding the phytoplankton biomass dynamics, field measurements of division rates and carbon standing stocks are also essential for assessing the flow of carbon within and out of the surface photic zone. Knowledge of the net rates of change in biomass, C_{phyto}, and cell division provide the information necessary to calculate mortality (i.e. grazing, viral lysis, sinking), determine the net production rate of a system, and the degree of coupling between grazers and phytoplankton (Behrenfeld *et al.*, 2013). Additionally, through ecosystem modeling, mortality can be partitioned into export and consumption terms (Siegel *et al.*, 2014). Thus, strong arguments remain for continuing field measurements of primary production into the future, but their utility is greatest when conducted alongside assessments of phytoplankton loss processes. Additional studies are also still needed to address physiological processes impacting traditional measurements of assimilation numbers (Halsey and Jones, 2015) and to develop new approaches that provide incubation-free estimates of phytoplankton division rate and biomass.

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