

Addressing uncertainties in the assessment of phytoplankton lysis rates in the sea

Recent reports of high phytoplankton lysis rates in the Mediterranean Sea (Baldi et al. 1997; Agustí et al. 1998; Agustí and Duarte 2000a) have focused attention on the possible uncertainties involved in the estimation of this important process. Riegman et al. (2002) identify a number of potential sources of error in the dissolved esterase method, which was originally proposed by van Boekel et al. (1992) as a semiquantitative approach to estimate phytoplankton lysis rates. Modifications to the assay protocol and tests of key, untested assumptions (e.g., Agustí et al. 1998) have introduced improvements that allow the method to be used quantitatively. Riegman et al. (2002) appear to erroneously assign these modifications to Agustí and Duarte (2000) and, in doing so, overlook some of the tests provided in Agustí et al. (1998).

Riegman et al. (2002) identify three possible sources of error in the determination of the components needed to calculate phytoplankton lysis rate: (1) dissolved esterase activity (DEA) in seawater, (2) the decay rate of DEA, and (3) phytoplankton particulate esterase activity (PEA). The measurement of these three components is based on the quantification of the hydrolysis of fluorescein diacetate (FDA) to yield fluorescein, mediated by unspecific esterases. The arguments raised by Riegman and coworkers (2002) are rendered complex because the tests they report do not correspond to any previously published procedure (van Boekel et al. 1992; Brussard et al. 1995, 1996; Agustí et al. 1998; Agustí and Duarte 2000a). Their modifications, involving an increased assay temperature (from 20°C to 25°C) and other changes, introduce uncertainties in the comparisons drawn. For instance, Riegman et al. (2002, p. 916) state that the increased temperature results in “only a twofold difference in the estimations of lysis rates.”

Riegman et al. (2002) also noted a discrepancy between the original assays (van Boekel et al. 1992) and that which we used. This derives, however, from an error in the final concentrations of edetic acid (EDTA) and FDA, which were 0.2 and 0.02 mM, respectively, rather than 0.02 and 0.2 mM, respectively, as indicated in Agustí et al. (1998) and Agustí and Duarte (2000a). However, we have confirmed that this error had no consequences on the estimates.

Riegman et al. (2002) noted that FDA hydrolysis rates must be corrected for nonenzymatic FDA hydrolysis before DEA calculations, a requirements that was overlooked in most previous uses of the method (van Boekel et al. 1992; Brussard et al. 1995, 1996; Agustí et al. 1998; Agustí and Duarte 2000a). Riegman et al. (2002) estimated nonenzymatic FDA hydrolysis would proceed at an average rate of 15 ± 2 nmol fluorescein $L^{-1} h^{-1}$ in the North Sea; this is similar to the values found in the rate of FDA hydrolysis in deep (500 m) subtropical Atlantic Ocean waters, averaging 15.2 ± 0.6 nmol fluorescein $L^{-1} h^{-1}$ (mean \pm SE, $N = 40$, data from Agustí et al. 2001).

Failure to account for nonenzymatic FDA hydrolysis

should result in an overestimation of lysis rates, as indicated by Riegman et al. (2002). This bias should be small for waters with high FDA hydrolysis rates, such as in the Mediterranean Sea. Indeed, this source of error was found to be negligible, yielding an average (\pm SE) bias of 0.0072 ± 0.0041 d^{-1} , which is only 4% of the rates reported for the Mediterranean littoral by Agustí and Duarte (2000a). In contrast, this bias should lead to significant overestimation of lysis rates in waters where nonenzymatic FDA hydrolysis is large relative to DEA, such as the North Sea (e.g., Riegman et al. 2002), Antarctic (Agustí and Duarte 2000b), and the Mediterranean in winter (Agustí and Duarte 2000a). The rates published for the North Sea (van Boekel et al. 1992; Brussard et al. 1995, 1996) would be significantly lower if corrected for this effect. Hence, we concur with Riegman et al. (2002) that future applications of the method should account for nonenzymatic FDA hydrolysis, particularly where lysis rates are likely to be low.

Riegman et al. (2002) question the use of commercial porcine liver esterases to assess the decay rate of esterases at different ambient temperatures in seawater on the basis of possible differences with phytoplankton-derived esterases. However, the null hypothesis that the decay rate of porcine liver esterases is similar to that of phytoplankton-derived esterases could not be rejected experimentally in our study (see p. 1,842 in Agustí et al. 1998) or in that of Riegman et al. (2002), indicating that there is no basis for this criticism.

The estimation of PEA is a critical component of the method and carries the most uncertainty because the values published by van Boekel et al. (1992) are >10-fold lower than those we calculated (Agustí et al. 1998). For instance, applying the PEA/chlorophyll *a* (Chl *a*) ratios reported by Riegman et al. (2002, table 2) of 2.2 nmol fluorescein $L^{-1} h^{-1}$ (μg Chl *a*) $^{-1}$ increases average phytoplankton lysis rates for Blanes Bay, corrected for nonenzymatic hydrolysis, from 0.17 d^{-1} (Agustí and Duarte 2000a) to 16 d^{-1} (range: 1.28–108 d^{-1}). These values are clearly impossible; they are 100-fold greater than the values (range: 0.01–0.4 d^{-1}) inferred using an independent method by Garcés and Masó (2001) in a Mediterranean coastal location near that studied in Agustí and Duarte (2000a). In contrast, the independent estimates by Garcés and Masó (2001) were within the range of values reported by the latter.

Riegman et al. (2002) argue that failure to correct for nonenzymatic hydrolysis may have led to overestimated PEA/Chl *a* ratios in Agustí et al. (1998). However, the use of a blank did account for this effect (Agustí et al. 1998), and the measured PEA was always far greater (>200 nmol fluorescein $L^{-1} h^{-1}$) than the 15 nmol fluorescein $L^{-1} h^{-1}$ corresponding to nonenzymatic hydrolysis. However, failure to correct for the fluorescence of the lysozyme extraction medium did indeed lead to overestimated PEA values in Agustí et al. (1998), as correctly suggested by Riegman et al. (2002). Subtraction of the last blank resulted in an average

PEA/Chl *a* ratio for various phytoplankton cultures of 201.3 ± 90 nmol fluorescein $L^{-1} h^{-1}$ (μg Chl *a*) $^{-1}$, compared to the average PEA/Chl *a* ratio of 331 ± 43 nmol fluorescein $L^{-1} h^{-1}$ (μg Chl *a*) $^{-1}$ reported by Agustí et al. (1998). These differences are important, albeit not statistically significant (*t*-test, $P > 0.05$). Yet fluorescence derived from high Tris-HCl + EDTA and lysozyme additions are not large enough to account for the >10-fold discrepancy between the PEA/Chl *a* values in van Boeckel et al. (1992) and Riegman et al. (2002) and those in Agustí et al. (1998).

Riegman et al. (2002) also suggest that the lysozyme extraction of PEA we proposed (Agustí et al. 1998) is a possible source of discrepancy between the PEA/Chl *a* values. We tested this by using an independent extraction method, based on the disruption of the cells in a mixer mill on a 0.2 M sucrose medium buffered to pH 8.0 with Tris. The estimates of PEA/Chl *a* ratios derived using this independent extraction method were in very close agreement with those from the lysozyme extraction ($R^2 = 0.96$, $P < 0.0001$, $N = 8$), providing no evidence for inflated PEA/Chl *a* ratios derived using the lysozyme extraction method.

Riegman et al. (2002, p. 917) point to sonication, the method used by van Boeckel et al. (1992) to extract PEA, as a source of underestimation of PEA/Chl *a* ratios because sonication "lead[s] to an instantaneous and highly variable loss of esterase activity," consistent with our own observations and those for freshwater phytoplankton species (D. F. Bird unpubl. data). Attempts by Riegman et al. (2002) to bypass this severe problem by assaying PEA on undisturbed cells resulted in PEA values even lower than those obtained using sonication extraction (Riegman et al. 2002). This low efficiency must result from the obvious package effect caused by assessing fluorescence inside cells. Indeed, comparison of PEA estimates on intact cell suspensions with estimates derived using either lysozyme or the alternative extraction by cell rupture on sucrose-buffered medium yielded PEAs four- to sevenfold higher than those obtained using intact cell suspensions, providing evidence of an important underestimation associated with a package effect in measurements on undisturbed cells.

Cell size has been shown to be an important determinant of PEA (Agustí et al. 1998). Hence, a substantial part of the discrepancy between PEA/Chl *a* ratios may derive from Riegman et al. (2002) and van Boeckel et al. (1992) not including autotrophic prokaryotes, which are relatively unimportant in the North Sea but are very important in the Mediterranean and the oligotrophic ocean (Agawin et al. 2000), in their assessment of PEA/Chl *a* ratios. *Synechococcus* and *Prochlorococcus* have higher PEA/Chl *a* ratios (623 and 590 nmol fluorescein $L^{-1} h^{-1}$ [μg Chl *a*] $^{-1}$, respectively) than eukaryotic algae (average 43.7 ± 15 nmol fluorescein $L^{-1} h^{-1}$ [μg Chl *a*] $^{-1}$). In addition to cell size, species-specific differences in Chl *a* extraction efficiency may add to among-species variance in PEA/Chl *a* ratios. This suggests that different PEA/Chl *a* ratios must be used to estimate lysis rates in phytoplankton assemblages, in contrast to those in the Mediterranean and the North Sea, supporting the recommendation by Riegman et al. (2002) to use different PEA/Chl *a* ratios for contrasting phytoplankton communities. Use of season-specific PEA/Chl *a* ratios may also improve esti-

mates somewhat; varying PEA/Chl *a* introduced an uncertainty of only 13% about the mean lysis rates in the Bay of Blanes (Agustí and Duarte 2000a).

Finally, Riegman et al. (2002) point to the problem of the contribution of heterotrophs to DEA in natural waters. Indeed, the uncertainty associated to the possible contribution of heterotrophs to DEA was the main reason for their conclusion that the esterase method should be taken as semi-quantitative (van Boeckel et al. 1992). Subsequent evidence that heterotrophs appeared to have much lower specific esterase activity than autotrophs and that their contribution to DEA was negligible compared to that of autotrophs in Mediterranean waters allowed the method to be used quantitatively there (Agustí et al. 1998; Agustí and Duarte 2000a). Riegman et al.'s (2002, p. 916) assertion that "the presence of EA can be attributed to the lysis of phytoplankton cells if the biomass of the phytoplankton is greater than that of heterotrophs" needs, however, be qualified, for it is not the biomass of the organisms, but their production and, particularly, the fraction of their production that can be lost via cell lysis (i.e., that not removed by processes other than cell lysis) that constrains their possible contribution to DEA. Indeed, the biomass of bacteria is high in the Mediterranean, but most of bacterial production is removed by phagotrophy, which does not release EA to the medium (cf. Agustí et al. 1998). Hence, Agustí et al. (1998) calculated that heterotrophs contributed <5% of the dissolved esterase activity in the Mediterranean waters studied.

Riegman et al. (2002) have suggested procedures that will lead to improved accuracy in the estimation of phytoplankton lysis rates using the dissolved esterase method. They conclude that failure to apply those improvements render our published estimates of seasonal lysis rates in Blanes Bay (Agustí and Duarte 2000a) inaccurate, but provide no indication of the size of the error involved. We, therefore, recalculated phytoplankton lysis rates of Blanes Bay by correcting the estimates (Agustí and Duarte 2000a) for nonenzymatic hydrolysis of FDA and using the PEA/Chl *a* ratios corrected for blank fluorescence. The recalculated phytoplankton lysis rates ranged from $0.006 d^{-1}$ to $1.6 d^{-1}$, with an average of $0.19 \pm 0.022 d^{-1}$ (mean \pm SE, Fig. 1); these are somewhat greater, but not significantly so, than our published mean rate of $0.17 d^{-1}$ (range $0.0083 d^{-1}$ to $1.47 d^{-1}$, Agustí and Duarte 2000a). The recalculated values were strongly correlated with the previously reported values ($r = 0.98$, $P < 0.001$), and followed a similar seasonal pattern, with low rates in winter and high rates in summer (Fig. 1). Hence, the conclusions by Agustí and Duarte (2000a) are robust, although the lysis rates in summer were higher than the conservative values reported, which underestimated lysis rates by about 10%, on average, due to the different sources of error identified by Riegman et al. (2002).

Demographic analyses of phytoplankton populations require knowledge of the balance between birth and death rates (Reynolds 1984). Although estimates of birth (i.e., growth) and predation abound, phytoplankton lysis has been a neglected process. Earlier estimates assumed this process to occur at an exponential rate (Jassby and Goldman 1974; Knoechel and Kalff 1978; Reynolds 1984; Lampert and Sommer 1997). In contrast, Riegman et al. (2002) assumed

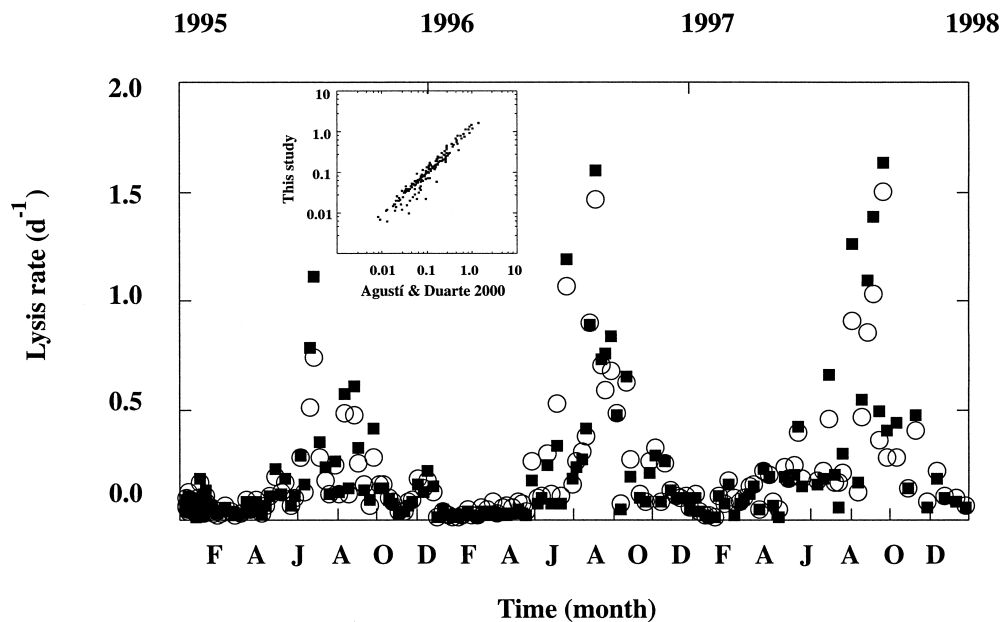


Fig. 1. The time series of phytoplankton lysis rates in Blanes Bay reported in Agustí and Duarte (2000a), open circles, compared to that derived by recalculating the lysis rates following the recommendations of Riegman et al. (2002) for blank corrections, solid squares. The insert shows the relationship between the published estimates (Agustí and Duarte 2000a) and the recalculated lysis rates in Blanes Bay.

this to be a linear process, although an explicit declaration of this assumption is not found in their earlier papers (van Boekel et al. 1992; Brussard et al. 1995), and they question the use of an exponential decay rate to represent lysis rates. Riegman et al. (2002) identify a discrepancy in rate calculations as used in Agustí et al. (1998) and Duarte and Agustí (2000) that derived from an erratum on the latter, where the correct equations applied were those in Agustí et al. (1998).

Linear and exponential rates have different interpretations, involve different assumptions, and cannot be compared directly. Linear rates are relative values, equivalent to a turnover rate, whereas exponential rates are specific values (Hunt 1990). Linear rates, or turnover rates, are adequate when the size of the pool, in this case the esterase activity within phytoplankton cells, is in steady state within the time interval involved in the rate calculation (i.e., daily). Hence, it should not be used to estimate loss rates during, for instance, bloom collapses, when phytoplankton biomass, and thereby the esterase activity they contain, changes rapidly over time. Population rate processes, whether birth or death, reflect the compound effect of per capita events and are, therefore, best represented as exponential, specific processes. Hence, mortality has been represented as an exponential process in general demographic analyses in biology (Caswell 1989), marine (Duarte et al. 1994) and land (Sarukhán et al. 1985) plants, industrial quality control (Cox and Oakes 1984), and atomic physics. There is also widespread evidence that phytoplankton mortality is also adequately represented as an exponential process, such as the exponential decline characterizing the collapse of phytoplankton blooms (Reynolds 1984; Lampert and Sommer 1997), including that of the North Sea *Phaeocystis* bloom reported by Brussard et al. (1995). Inter-

estingly, Brussard et al. (1995) used an exponential model to calculate the decline rate of this bloom, which they found to be primarily attributable to cell lysis. Use of an exponential model to calculate phytoplankton lysis rates has the additional advantage of allowing direct comparison with growth rates, which are calculated as exponential, specific rates.

Riegman et al. (2002) call for the development of alternative methods to estimate phytoplankton processes. We support this. Alternative methods are already becoming available, such as the release of particulate polysaccharides assessed by lipid biomarkers and molecular probes (Baldi et al. 1997) and the estimation of cell lysis from cell cycle analysis (Agustí et al. 1998; Garcés and Masó 2001). Indeed, the comparison between phytoplankton lysis rates estimated using the dissolved esterase method and cell cycle analysis provided confidence on the suitability of the method to quantitatively estimate phytoplankton lysis rates (Agustí et al. 1998), although there is a need for additional validation of the rates. Despite the important improvements to this method (e.g., Agustí et al. 1998; Riegman et al. 2002; analysis given by us here) since it was originally proposed by van Boekel et al. (1992), further efforts are needed to increase its precision and accuracy because sources of uncertainty remain, as they do for any other method used to examine phytoplankton processes (e.g., the ^{14}C method for estimating phytoplankton production, Williams 1993). The resulting improvements should enhance the utility of the dissolved esterase method, which is noninvasive, requiring no incubation of organisms. As additional methods and more reliable estimates become available, attention should shift from debate about the methods to the controls of phytoplankton

lysis in the sea, the prediction of lysis rates in the different oceanic provinces, and the incorporation of this process into conceptual and mathematical models of carbon flow and food web function in the ocean.

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Received: 20 May 2001

Accepted: 7 November 2001

Amended: 19 December 2001

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