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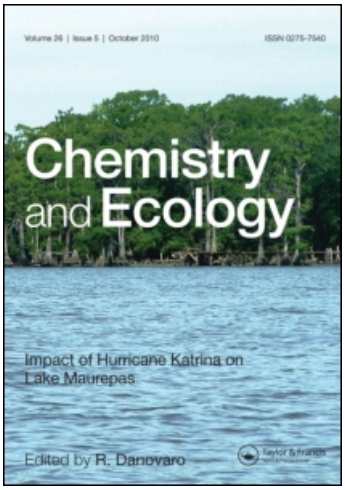

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Nutrient Limitation of Marine Microbial Production: Fact or Artefact?

K. J. Flynn[a]
[a] Biochemistry Research Group, School of Biological Sciences, University College Swansea, Singleton Park, Swansea

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NUTRIENT LIMITATION OF MARINE MICROBIAL PRODUCTION: FACT OR ARTEFACT?

K. J. FLYNN

Biochemistry Research Group, School of Biological Sciences, University College Swansea, Singleton Park, Swansea SA2 8PP

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The popular view of marine phototrophic ("primary") production being limited by nutrient availability is questioned because of inbuilt faults in experiment design and possible errors in interpretation. There appears to be a preoccupation with high net rates of $^{14}CO_2$-fixation and incorporation of inorganic nutrients, especially of NO_3^-. These events are typical of immature ecosystems in which nutrient cycling is poorly developed. Microbial loop production is tightly coupled with average zero net production and low levels, but high fluxes, of key nutrients. Moreover, experimental methods may interfere with microbial processes creating artefacts and resulting in a diagnosis of nutrient deprivation.

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INTRODUCTION

The routine use of methods of sea water analysis often show the presence of very low concentrations of inorganic N and P. In analogy with terrestrial production, it is often concluded that marine "primary production" (by which phototrophic production is commonly referred) in such waters is nutrient limited. Claims of nutrient limitation of microbial production often appear in the literature (e.g. Fogg, 1982, Carpenter and Capone, 1983; Goldman, 1984; Lancelot and Billen, 1985), including works which question other beliefs about marine ecology (e.g. Smetacek and Pollehne, 1986). Paerl *et al.* (1987) refer to production being "chronically limited by nitrogen availability."

The concept of nutrient limitation is an important one to question because it is at the heart of the wider question of the involvement of physical processes in the control of biotic events. If microbial production is indeed nutrient limited then it could be argued (and often is) that the presence or absence of physical events such as upwellings invariably play a dominant role in the control of marine production. The realization that bacteria play an important role in marine ecology adds a new twist to the problem because these organisms, and heterotrophic microbes in general, may compete with the phototrophs for common nutrients. Both phototrophic and heterotrophic microbes produce biomass at the expense of dissolved compounds and may occupy similar niches as food organisms for those that feed on particulate matter (Goldman and Caron 1985). However, only phototrophic activity results in an increase in chemical energy within the water column. It seems appropriate to consider nutrient limitation of both algal and

bacterial production, thus taking the broad definition of "primary production" as the formation of organic particulate material at the base of the food web (Unesco 1973) as a basis, rather than the more common concern with the process of $^{14}CO_2$-fixation.

In the euphotic zone, phototrophic production may conceivably be limited by light, temperature, predation or by the absence of any of the atomic constituents for particulate organic matter (POM). Of the latter, concentrations of P and especially of N are widely considered to be the most likely limiting nutrients (Smith 1984, Smith *et al.* 1986). For heterotrophic production, the availability of energy as organic C is also a possible limiting factor (Keller *et al.* 1982).

1. CHEMICAL EVIDENCE

The detection of low concentrations of inorganic nutrients is probably the most common "evidence" of nutrient limitation. Despite the development of methods for detecting a wide range of nutrients, often nitrate and phosphate are the only ones measured routinely. More often than not levels of organic nutrients are not determined at all; organic nutrients (other than urea) are not widely considered to be of importance for algal growth (see Flynn and Butler, 1986). There is also a repeated failure to recognise that the flux of a nutrient is at least as important as concentration. Oligotrophic waters, which by definition contain low concentrations of (inorganic) nutrients, may support growth of nutrient-replete organisms (Goldman *et al.*, 1979) provided that the flux of nutrients from regenerator to consumer is rapid enough. However, such interactions may be destroyed by commonly employed experimental methods.

During the temperate summer, levels of dissolved organic matter (DOM) increase (Butler *et al.*, 1979). This high proportion of organic over inorganic nutrients is also seen in mid ocean waters and is characteristic of mature ecosystems. Because of the nature of the marine ecosystem, a significant input of new nutrients will inevitably perturb the system because it involves mass transport of water. A population in the euphotic zone cannot be "fertilized" by upwelling water or other turbulence because this will displace the population from that geographic location promoting the development of a new ecosystem. The presence of excess NO_3^- may be indicative of an immature ecosystem but its absence, or the absence of an abundance of other inorganic nutrients, need not be indicative of nutrient limitation so much as of the operation of the nutrient cycle in a mature ecosystem. In such a system most atomic constituents exist as living matter or as refractory compounds.

Less than one third of DOM has been characterized but much appears refractory; Williams and Druffel (1987) report that the apparent age of dissolved organic C (DOC) in surface waters of the central North Pacific ocean is 1300 yr. The C/N/P ratio of DOM has an excess of C in comparison with that of living organisms (Jackson and Williams 1985) suggesting, perhaps, that the components are less labile. Billen (1984) suggests the possible rate limitation of exoenzymatic decay of macromolecular bipolymers as a factor leading to the long half-life of components of DOM. Newell *et al.* (1981) report that DOC released from algal debris is used 10 times quicker than algal POC. We could conclude that the availability of chemical energy as DOC may not be a limiting factor in the

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euphotic zone because otherwise surely more organisms would have evolved methods to utilize it. Williams (1984) suggests that the need to conserve nutrients may be more important than to conserve energy.

In studies of the bacterial utilization of NH_4^+ (Laws *et al.*, 1985) and dissolved free amino acids (DFAA-Wheeler and Kirchman, 1986), a source of C was not added for the heterotrophs suggesting a sufficiency of DOC in the water column (but see Ferguson *et al.*, 1984). However, even for a source of dissolved organic N (DON), such as DFAA, bacteria may need an additional source of energy to maximize growth. Keller *et al.* (1982) report that the bacterial uptake of DFAA appeared to depend more on DOC production by phototrophs than on the concentration of DFAA. Other evidence for a close coupling of bacterial and phototrophic activities comes from the work of Lancelot and Billen (1984). A lowered rate of bacterial growth at night (Azam and Fuhrman 1984) is also consistent with a need for new DOC from phytoplankton.

Butler *et al.* (1979) suggested that, as the temperate year proceeds successions of algae emerge which are able to utilize the organic nutrients that accumulate in the water column and that as a consequence the expected nutrient limitation suggested by low levels of dissolved inorganic N (DIN) and P (DIP) may not occur. A role of organics in the nutrition of phytoplankton, in order to lessen nutrient deficiency when inorganics are unavailable, may be difficult to demonstrate if $^{14}CO_2$-fixation is used as a measure of production because, although algal growth may continue, $^{14}CO_2$ fixation may not increase in proportion. Flynn and Butler (1986) discuss the role of DFAA, a significant component of DON, in the nutrition of phytoplankton, commenting that experiments appear typically to have been optimized for the detection of DIN assimilation. However, the existence of organics in the water column is indicative of the presence of chemical energy and that a phototrophic ability may not confer a competitive advantage. We must be careful not to assign an exclusive role for bacteria in areas of microheterotrophy. There is nothing sacrosanct about the phototrophic abilities of algae which prevents them from having evolved a heterotrophic ability if this confers a competitive advantage.

As a final point, Redfield (1958) argued that over geological time any short-fall in N-requirements could be met by N_2-fixation. Even if this were so, such a process could not prevent N-limitation from ever occurring, but it does beg the question that if N-limitation is common then why is N_2-fixation not a more common feature of marine ecology? Carpenter *et al.* (1987) report that the low rates of N_2-fixation estimated by experimentation are not artefacts. Paerl and Carlton (1988) suggest the low rates of fixation are in part due to the low availability of colonizable surfaces in the euphotic zone (specifically at the sea surface) the presence of which promote lower O_2 tensions which are conducive to N_2-fixation.

2. STUDIES OF THE BIOTA

There are two approaches to determine the nutrient status of a population; an experimental approach and one using biomarkers as indicators of physiological state.

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(a) *Experimental approach.* The experimental approach usually involves "spiking" samples with nutrients in order to test for enhanced production or incorporation. This may stress the population. Typically algae are separated from any predators which may be regenerating nutrients and maintaining a population density, and enclosed in the artificial environment of a bottle or flask under different illumination in the presence of the more rapidly growing bacteria which may be competing for the same nutrients. Le Fèvre (1986) says of *in situ* bottle experiments that they "can hardly be regarded a satisfactory model of phytoplankton in nature with respect to conditions experienced and therefore physiological responses." The same can be said of experiments using deck incubators.

The fact that under such conditions nutrients may be shown to be limiting may indicate little other than the inevitability of such limitation for organisms maintained for a significant proportion (10–50%) of a generation time in the absence of nutrient regeneration and of predator pressure. Such experiments are complicated further because production is estimated by $^{14}CO_2$-fixation which, apart from the fact that this estimates photosynthesis and not production of biomass, is not ideal because the presence of different nutrients affects C-fixation rates. This is evidently true of NH_4^+ uptake (Collos, 1986) which may be taken up very rapidly irrespective of the nutrient status of the cells (McCarthy and Goldman, 1979). Goldman *et al.* (1981) mention the need to use artifically high substrate concentrations else the time course for C-fixation may not be linear over the incubation period.

The use of estimates of dark C-fixation have been proposed as a method of detecting N-deprivation (Yentsch *et al.*, 1977, Goldman and Dennett 1986, Dixon and Syrett 1988, Dixon and Holligan in preparation). This technique depends on the flow of C and N along certain biochemical pathways. Dixon and Syrett (1988) suggest that the method may be of use as an indicator of the form (oxidized or reduced) of inorganic N being used. Urea may not give an enhancement (Goldman and Dennett, 1986).

The processes of nutrient uptake, C-fixation, amino acid synthesis and protein synthesis do not appear to occur in synchrony in algae. Collos (1986) discusses the aphasic relationship between algal nutrient uptake and growth. When starved of N, algae continue to fix CO_2 resulting in the formation of starch, lipid or oil, and in the release of dissolved organic carbon (DOC) (Lancelot and Billen, 1985). These processes continue to a greater or lesser extent during nutrient-replete growth depending on rates of the individual metabolic processes. For example, excess amino acid synthesis may result in a release of dissolved free amino acids (Flynn and Butler, 1986; Flynn and Al-Amoudi, 1988). The measurement of $^{14}CO_2$-fixation, typically used to estimate production, does not appear to be a robust method for use in determining nutrient status.

Measurements of protein synthesis (Lancelot *et al.*, 1986; Lohrenz and Taylor, 1987) appear to be a more suitable method of measuring production, not least because it is probably less sensitive than the individual processes (nutrient uptake, C-fixation and incorporation) to perturbation and because, unlike C-fixation, it is a fundamental component of biomass production. Species may differ in their ability to synthesize protein in darkness (Cuhel *et al.*, 1984) because the pathways of amino acid synthesis may require new photosynthetic products (Hipkin *et al.*, 1982; Flynn and Syrett, 1986b) so that the importance of the "previous light history" (Lancelot *et al.* 1986) may depend on the species in

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question. In theory, measurements of protein synthesis could also provide a method for differentiating between eukaryote and prokaryote production by the use of inhibitors of protein synthesis (Wheeler and Kirchman, 1986) and antibiotics. Unfortunately we know that some eukaryotes are adversely affected by the presence of antibiotics (Jensen, 1983), and that cycloheximide (an inhibitor of protein synthesis in eukaryotes but not in prokaryotes) may not affect short term uptake by eukaryotes (e.g. Flynn and Syrett, 1986a). Furthermore, concentrations affecting some species may have no effect on others (Walls and Gallon in preparation).

A major problem would appear to be that factors measured using methods requiring intervention in the habitat (nutrient uptake, $^{14}CO_2$-fixation, filter fractionation and enclosure in flasks or bottles) are too sensitive to the stress which we inevitably apply to the organisms during the experiments. We have the problem of identifying responses to natural stress from those incurred as a result of our tests. A point made by, amongst others, Goldman (1984) and Harris (1984) is that the spatial and temporal sampling that is used in field experiments is incompatible with the process being measured. Thus phytoplankton in oligotrophic waters may not show the nutrient deprivation expected from analysis of bulk volumes of sea water (Goldman *et al.*, 1979) because the processes of nutrient regeneration and subsequent uptake occur on scales of seconds and minutes and in microlitre dimensions and not the hours and litres used in our experiments. Goldman *et al.* (1981) refer to the "severe incompatibilities between choosing an incubation period based solely on analytical requirements from one based on the best representation of the time scale of the physiological responses of phytoplankton." The problem is worse for studies of bacterial activity.

Studies of nutrient limitation in bacterial communities are complicated not only by factors affecting algal studies, but by the difficulty in detecting a change in the structure of the population. Periods of incubation of 4–9 h (e.g. Wheeler and Kirchman, 1986) may allow a significant increase of bacterial numbers; bacterial generation times may be as short as 6 h (Hagström *et al.* 1984). Ferguson *et al.* (1984) report changes in community structure during such incubations, suggesting that confinement selects for bacteria which are culturable in complex media, perhaps as a consequence of the release of organics from larger organisms by rupture during filter fractionation. In the sixties, enrichment experiments were shown to exert selective pressures on phytoplankton (Menzel *et al.*, 1963). Although it is suggested that picoplankton are ubiquitous (Fogg, 1986) it is not known whether clonal differences are common in time and space. Microphytoplankton are often identified to species level (although strain differences may be more common than expected—Yamada *et al.*, 1983), but with picoplankton perhaps some form of genetic identification is needed, or a more frequent use of electron microscopy. Sargent *et al.* (1987) discuss the use of lipid biomarkers for identification of microbes.

An associated problem with studies of bacteria is that of determining the metabolic status of the cells. Williams (1984) suggests an average figure of 20% of bacteria as being active at any one time but what the term "active" means in reality is subject to debate. Kjelleberg and Hermansson (1987) suggest that many "inactive" cells are not dormant with respect to substrate capture. They also discuss influences of nutrient status and attachment on size. It is apparent that the size of bacteria present in nutrient replete conditions is larger (some species of

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algae show similar variations—Harris *et al.* 1987), but it is not known if the extremely small size of some marine bacteria is indicative of nutrient deprivation (and if so of which nutrient) or if the larger individuals are more efficiently predated (Pomeroy, 1984; Wiebe, 1984), which is most likely in either circumstance. It is important that we know with greater certainty whether the circumstantial evidence of bacterial cell size and the results from thymidine uptake studies are truly indicative of nutrient status. Karl and Winn (1986) and Fuhrman *et al.* (1986) discuss the use of adenine incorporation as a measure of total microbial production; the latter argue that it is of little use because of the differential use by target organisms. This problem applies to all spiking experiments. However, it is quite likely that many microbes are not growing under optimal conditions because conditions could never be ideal for all components of a population at any instant in time unless only one clone was present.

In order to escape the problems associated with the experimental approach, other indicators of nutrient status can be used.

(b) *Biomarkers.* The most commonly used biomarker is C/N/P ratios which are usually compared to the Redfield (1958) ratios. Examination of C/N/P ratios of algae may be expected to give some information about the nutrient status in the recent past. However, it is apparent that, because of phasing of uptake and assimilation (Collos, 1986), C/N ratios may increase during the day (when most experiments are conducted) and decrease at night (Lancelot and Billen, 1985). Fluctuations in C/N/P may also be more common in *r*-select species whose rapid growth may be more likely to result in imbalances. Phytoplankton in oligotrophic waters may have C/N/P ratios close to the optimum (Goldman *et al.*, 1979; Harris, 1984; Goldman, 1986), perhaps as a consequence of their slower growth and better physiological "house keeping" (see Collos, 1986). Bacteria are suspected to maintain an efficient control of their metabolism, stopping and starting growth in response to rapid fluctuations in nutrient availability (van Gemerder and Kuenen, 1984; Williams, 1984; Kirchman and Hodson, 1986) such that changes in C/N/P may be relatively minor. The principal problem with the use of C/N/P ratios, other than those of the analysis itself, where often measurements are made back in the laboratory rather than at sea, is the problem of differentiating between dead and living POC. This problem may be overcome by flow cytometry using suitable criteria to differentiate between products of active metabolism and decay, although these may be of limited use with attached bacteria.

Dortch *et al.* (1985) suggest a range of other indices to characterize the nutrient status of a population which involve the measurement of labile chemicals, such as ratios of free amino acids/protein, protein/DNA or RNA/DNA, in order to detect N-sufficiency. The suitability of these methods, both for consistency of results and for use at sea, are yet to be tested, but there are clear differences between N-replete and N-deplete cells with respect to such indices. Another possible index is the composition of the intracellular amino acid pool suggested to be used as a "metabolic finger print" (Admiraal *et al.*, 1986). Work by Flynn and Al-Amoudi (1988) and Al-Amoudi and Flynn (1988) suggest that this may not be a particularly good indicator because the composition also reflects the past metabolic history of the cells such as the effect of periods of darkness. This problem is also likely to affect the reliability of the indices suggested by Dortch *et*

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al. (1985). A common problem with laboratory studies is that algae are often grown in continuous illumination and the lack of phasing in cell division may mask cyclic changes in levels of RNA/DNA, amino acid content etc.

The ratio of glutamine/glutamate (GLN/GLU) appears to be of use as an indicator of N-deprivation. Flynn *et al.* (1988), using algae, and Hipkin and Flynn (in preparation), using yeast, have shown that elevated ratios of GLN/GLU are indicative of N-replete cells. Whereas the C/N/P ratios tell us something about the nutrient status of cells in the recent past, GLN/GLU appears to indicate the current status of N-assimilation in a fashion comparable to measurements of nutrient uptake and $^{14}CO_2$-fixation, except that the use of this biomarker does not carry the risk of artefacts generated by prolonged incubations in artificial conditions.

The situation is complicated further because many field studies are conducted in conditions described as blooms, patches or outbursts of algal growth because of the difficulties in measuring $^{14}CO_2$-fixation in populations of low density. However one describes them (see Le Fèvre, 1986), such outbursts can only occur in the absence of limiting predator pressure. Such sampling is extremely biased. For every unit of time that production may be found to be nutrient limited, how many units have not, or have been, predator limited but went undetected? Futhermore, most studies are conducted in daylight whilst predators often migrate into the euphotic zone at night not only reducing prey numbers but regenerating nutrients. If we usually examine algal production under conditions which are conducive to nutrient limitation then it is hardly suprising if we sometimes diagnose such limitation. Certainly several indices should be used in concert in attempting a diagnosis of nutrient deficiency.

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3. ALGAL-BACTERIAL INTERACTION

For C there will be no competition between phototrophs and heterotrophs (except in darkness when some of the former may become facultatively heterotrophic) but for other nutrients such as N and P competition is to be expected. By present understanding, picoplankton are more likely to obtain nutrients successfully at very much lower concentration than larger organisms, if only because of the more favourable surface area/volume ratio. Bacteria appear to be very efficient at energy and nutrient conservation (typically 60–80%—Azam *et al.*, 1983; Palumbo *et al.*, 1983; Carlucci *et al.*, 1984; Wiebe 1984) and to be able to remove nutrients from very low concentrations (Hagström *et al.*, 1984; Fuhrman, 1987) using binding proteins (Morita, 1984). Many can probably use components of dissolved organic N (DON) and P (DOP), which are unavailable to algae, for example by the use of extracellular enzymes (allowing the use of dissolved combined amino acids—Amano *et al.*, 1982; Hollibaugh and Azam, 1983). The data of Laws *et al.* (1985), Wheeler and Kirchman (1986) and Currie and Kalff (1984) suggest that bacteria may be able to outcompete phytoplankton for DIN, DON and DIP.

Nutrient limitation of algal growth does not mean that production of new biomass is limited unless it can be shown that bacterial production is also halted. The situation is complicated by the increased rate of DOC release by N-deprived algae (Azam *et al.*, 1983; Lancelot and Billen, 1985), potentially stimulating more

bacterial growth and hence more competition. Bacteria also have a lower C/N ratio than algae resulting in a greater need for N for a given biomass. However, if bacterial competition for nutrients results in the death of the phototrophs then the process of CO_2-fixation, a major source of DOC release, will halt.

Much of this release of DOC, sometimes termed extracellular organic C, is of unknown composition except that the C/N ratio is high (Lancelot and Billen, 1985). Although it is difficult to decide if mucilage constitutes a dissolved substance, the term DOC will be used. The release of DOC can amount to almost 30% of total fixed C (Mague *et al.*, 1980). More typically, release by nutrient replete algae is more likely to be 10–20% (Williams and Yentsch, 1976; Jensen, 1983; Joint *et al.*, 1986). Fogg (1986) suggests that the leakage of organics from picophytoplankton may be relatively greater per cell than from microphytoplankton. Although the magnitude of this activity is disputed by Sharp (1984), and some estimates are artefacts of bad filtration practice (Goldman and Dennett 1985), there is little doubt that a release of DOC into the water column does occur. Furthermore, most of the algal biomass may end up as DOC (Cadeé, 1986). The environment is loaded with a form of chemical energy which is utilized by heterotrophs, especially bacteria. Moving offshore the relative importance DOC contributions changes from terrestrial input (Ducklow and Kirchman 1983), to microphytoplankton, down to picophytoplankton (Fogg, 1986; Williams and Druffel, 1987). DOC is an important source of energy for bacteria. Jensen (1983) reports that an amount of extracellular DOC from phototrophs equivalent to 3–30% of C-fixation is taken up by bacteria, of which 50–80% is incoporated, figures similar to those of Wolter (1982). An instantaneous uptake of DOC released by phytoplankton may occur (Larsson and Hagström 1979).

When phytoplankton are growing exponentially, free-living bacteria predominate (these are the forms most likely to use DOC—Sieburth *et al.*, 1978), but numbers of attached bacteria increase in senescent populations (Albright *et al.*, 1986). One can conceive of a cyclic succession of phytoplankton plus free bacteria (using DOC) competing for common nutrients (Laws *et al.*, 1985; Wheeler and Kirchman, 1986), followed by attached bacteria consuming detritus, with predators of both groups regenerating nutrients (Goldman, 1984). Newell and Linley (1984) suggest that the rapid decline and dissipation of temperate summer blooms of phytoplankton is performed by bacteria and protozoans utilizing both the products and biomass of living and dead algae with the regeneration of 60–70% of N in non-stratified waters (see Probyn, 1987). In stratified offshore waters, N regeneration by protozoa and bacteria appears less important (Newell and Linley, 1984), a result not inconsistent with that of the laboratory studies of Goldman *et al.* (1987), showing that nutrient regeneration by protozoa is less when feeding on N-deprived algae. Phototrophs may only regain predominance when levels of organics are lowered confering a competitive advantage for autotrophy. As Sharp (1984) suggests, death may be a common occurrence in phytoplankton populations with a good proportion of bacterial nutrition coming not from DOC release by active algae but from their death.

In an established, mature, marine ecosystem, in which the food web is developed, phototrophic activity may function in an analogous fashion to an anaplerotic biochemical reaction, topping up the system with organic chemical energy when levels become low enough to enable phototrophs to compete favourably with heterotrophs for common nutrient requirements. Photosynthesis

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results in the conversion of radiant to chemical energy. It is appropriate when considering nutrient limitation of algal production, especially when estimated by $^{14}CO_2$-fixation), to ask if there is evidence for a shortage of chemical energy (organic compounds such as components of DOC) in the environment which may favour phototrophic rather than heterotrophic activity.

CONCLUSIONS

Much of the evidence for the nutrient deprivation of "primary production" comes from the detection of low levels of a few, at some times relatively minor, components of dissolved atomic constituents, and from studies of algal physiology using questionable experimental techniques. Studies of bacterial production are adversely affected by ignorance of identification, physiology and position in food webs. It could be argued that methods involving filter-fractionation and incubation in containers may at best adversely affect bacteria less than phytoplankton, and at worst actually stimulate bacterial activity. There appears to be a need for the development of techniques using biomarkers for the determination of nutrient status and of short term experimental methods (say <30 minutes incubation) which would be less likely to generate artefactual data.

Algal and bacterial production interact in a mature ecosystem such that between them the bulk of atomic constituents are maintained as microbial POM with phototrophic activity maintaining a level of chemical energy commensurate with the concentration of atomic constituents present. In such a system, the detection of low nutrient concentrations by chemical analysis of bulk volumes of sea water may have little bearing on the nutrient status of the microbes.

The importance of physical processes is in the redistribution of nutrients and in conditioning the water column for the development of a new ecosystem. Production is the gross increase in biomass, but our methods are not adequate to enable the determination of this in mature ecosystems dominated by components of the microbial loop and we therefore measure net production. In a mature ecosystem net production will be zero, assimilation being balanced by respiration. Is the diagnosis of nutrient-deprived population in waters depleted of inorganic nutrients merely the result of a failure to recognize the existence of a mature ecosystem which will be unbalanced by the artificial addition of substrates?

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