

## Stoichiometric theory extended to micronutrients: Comparison of the roles of essential fatty acids, carbon, and nitrogen in the nutrition of marine copepods

**Abstract**—Conventional stoichiometric theory, which is used to study the limitation of zooplankton production by C, N, and other elements, is extended to include the essential polyunsaturated fatty acids (PUFAs) 20:5(*n*-3) and 22:6(*n*-3). Using typical biochemical compositions of consumer (*Calanus helgolandicus*) and algal food (hypothetical diatom-dinoflagellate mixtures), the analysis shows that PUFAs or macronutrients can be important in limiting zooplankton production, depending on the biochemical compositions of consumer and food and the efficiencies with which dietary components are used. Predicted limitation by fatty acids is strongest when zooplankton use a monospecific diet, indicating that such limitation may be of particular significance in laboratory zooplankton, which are often fed phytoplankton monocultures. The analysis illustrates several factors that operate to minimize the limiting potential of fatty acids to the extent that limitation by C or N could occur: selective grazing to obtain a nutritionally balanced diet, plasticity in consumer biochemical composition, and high C requirements for respiration. The possibility of macronutrient limitation is increased further if zooplankton are able to actively synthesize essential PUFAs, although this is not thought to be the case in most aquatic systems. The work highlights the need for complete data sets incorporating fatty acids and bulk elemental properties of consumers and prey, as well as an improved understanding of the roles and cycling of essential fatty acids, if we are to be able to provide a unified view of zooplankton nutrition.

Limitation of zooplankton production by the elements C, N, and P has been extensively studied using stoichiometric equations. Ratios of C and nutrient elements in consumer and prey are compared and, taking into consideration utilization efficiencies, the element in shortest supply relative to demand is invoked as limiting. A strong case for P limitation of zooplankton in freshwater environments has been made in this way (e.g., Hessen 1992). Egg production in marine copepods has been correlated with food N (Checkley 1980; Kiørboe 1989), which would suggest limitation by that element. However, Anderson and Hessen (1995) postulated that, despite this strong correlation, limitation by N is not consistent with stoichiometric theory. Two lines of evidence support their claim. First, relatively high ( $>0.1$ ) N:C ratios in seston, coupled with low consumer carbon gross growth efficiency ( $K_C$ ), would indicate that N limitation of marine zooplankton should be uncommon. The second argument arises from the stoichiometric axiom that as a nutrient element becomes limiting its excretion by the consumer tends to zero, in which case assimilated material would be exclusively used for new biomass (Anderson and Hessen 1995). As an example, daphnids fed P-deficient diets exhibit high  $K_P$  ( $> 0.8$ ; DeMott et al. 1998). Yet the copepods in the Checkley (1980) and Kiørboe (1989) laboratory experiments produced eggs with a low  $K_N$  of  $\sim 0.4$ , even when food N:C was as low as 0.03. Nitrogen assimilation efficiency is typ-

ically 0.7; this apparently unnecessary waste of N when grazing N-deficient prey led Anderson and Hessen (1995) to conclude that something other than elemental N, but which covaries with N, may limit production.

A number of recent studies have demonstrated correlations between zooplankton production and minor dietary constituents—micronutrients—such as certain amino and fatty acids (Kleppel et al. 1999). Particularly marked relationships have been found between zooplankton growth (including egg production) and intake of the polyunsaturated fatty acids 20:5(*n*-3) (eicosapentaenoic acid, EPA) and 22:6(*n*-3) (docosahexaenoic acid, DHA) (Jónasdóttir 1994; Müller-Navarra et al. 2000). These fatty acids are closely associated with biomembranes and eicosanoids, a class of compounds involved in a wide range of physiological processes (Sargent et al. 1989). Correlative evidence, although useful, does not provide proof of causal limitation. To date there has been little progress in combining this evidence with stoichiometric arguments to provide a unified view of zooplankton nutrition. Theoretical approaches to biochemical constraints are virtually nonexistent (Gulati and DeMott 1997; Tang and Dam 1999).

The stoichiometric approach is developed here to include the fatty acids EPA and DHA, thereby permitting a direct comparison of the roles of macronutrients and micronutrients in zooplankton nutrition. Two key assumptions of the method must be explored in order to advance the theory: (1) substrates are used conservatively for growth and are solely of dietary origin, and (2) grazers are homeostatic, i.e., have fixed biochemical ratios in their biomass that determine dietary requirements. Elements such as C, N, and P are immutable and must be of dietary origin. In contrast EPA and DHA can be synthesized by elongating and desaturating linolenic acid, although it is generally believed that the enzymes involved are slow and inefficient in most zooplankton. High zooplankton growth rates are therefore usually only attainable when direct dietary sources of these essential fatty acids are available (Brett and Müller-Navarra 1997). In this context “essential” compounds are defined as those that cannot be synthesized or are synthesized in inadequate amounts to sustain growth and survival and must therefore be obtained at least in part, but not necessarily wholly, from the diet (Spector 1999). Although active elongation may be uncommon, retroconversion of DHA to shorter chain EPA has been demonstrated in animals such as brine shrimp (Navarro et al. 1999). The extent to which this process occurs in other organisms is not well known.

Whereas most crustacean zooplankton have a rather rigid elemental stoichiometry (Andersen and Hessen 1991), thereby justifying the assumption of consumer homeostasis, fatty acid composition is variable often reflecting the composition in the diet (Brett and Müller-Navarra 1997). Aquaculturalists

have exploited this plasticity by introducing a variety of enrichment diets to increase the polyunsaturated fatty acid (PUFA) content of crustaceans prior to feeding to fish larvae (Han et al. 2000). This accumulation in body tissues can occur if PUFAs are supplied to the consumer in quantities in excess to requirements, in which case by definition the compounds are nonlimiting. However, storage reserves of PUFAs are unlikely to significantly contribute to sustained provision of these fatty acids for egg production in natural zooplankton populations. The PUFA composition of eggs then sets demands for substrates rather than merely reflecting composition of the diet. Little is known about the extent to which EPA and DHA can be lowered in the diet without impairing their functional roles. The degree to which the two fatty acids can be substituted for each other is also unknown in zooplankton. An example of very specific functionality is provided by experimental fish biology where the development of neural and visual functions in larvae has been strongly linked to dietary availability of DHA (Bell et al. 1995).

Existing stoichiometric theory is modified here to take into account the dual origin of fatty acids (diet, synthesis) and to permit simultaneous intercomparison of any number of dietary components. Let  $F_{i,j}$  and  $E_{i,j}$  be the  $i:j$  ratios of components  $i$  and  $j$  in food and consumer, where  $i$  and  $j$  can assume any of C, N, EPA, or DHA. Each substrate can be potentially used with a maximum gross growth efficiency (GGE)  $K_i^*$ , although in practice only the limiting component will be used thus. Note that the concept of GGE, which is the fraction of ingested material that is allocated to growth, is best applied to substrates that are solely of dietary origin, in this case C and N. Parameters  $K_{EPA}^*$  and  $K_{DHA}^*$  are defined here as the utilization efficiencies of ingested fatty acids for growth, ignoring extra production due to synthesis by zooplankton. In order to take into account this synthesized EPA and DHA, a new parameter,  $\phi_i$ , is introduced, which is the fraction of demand for constituent  $i$  which is met directly by dietary intake (the remainder being synthesized). The food C:N ratio that defines the transition between  $i$ - and  $j$ -limited growth, at which point both  $i$  and  $j$  are used with their maximum efficiencies and the supply of  $i$  and  $j$  equals their demand for production, is then  $K_i^* F_{i,j} / \phi_i = K_j^* E_{i,j} / \phi_j$ . Component  $i$  will limit relative to  $j$  if demand exceeds supply, and so the strength of limitation of  $i$  relative to  $j$ ,  $S_{i,j}$  is

$$S_{i,j} = \frac{\phi_i K_j^* E_{i,j}}{\phi_j K_i^* F_{i,j}} \quad (1)$$

Note that interconversion between components is not permitted using this method, i.e., synthesis of one component at the expense of another. In this way the relative limitation of any pair of components can be compared. The most severely limiting component in the diet, denoted  $w$ , is component  $i$  corresponding to the maximum calculated  $S_{i,j}$  when  $i$  is set in turn to each currency, for any chosen  $j$ . Now a food item composed of  $F_{i,w}$  moles  $i$  will be used to produce  $K_w^* E_{i,w} \phi_i / \phi_w$  moles  $i$ , so the realized GGE of  $i$ ,  $K_i$ , is

$$K_i = \frac{\phi_i K_w^* E_{i,w}}{\phi_w F_{i,w}} \quad (2)$$

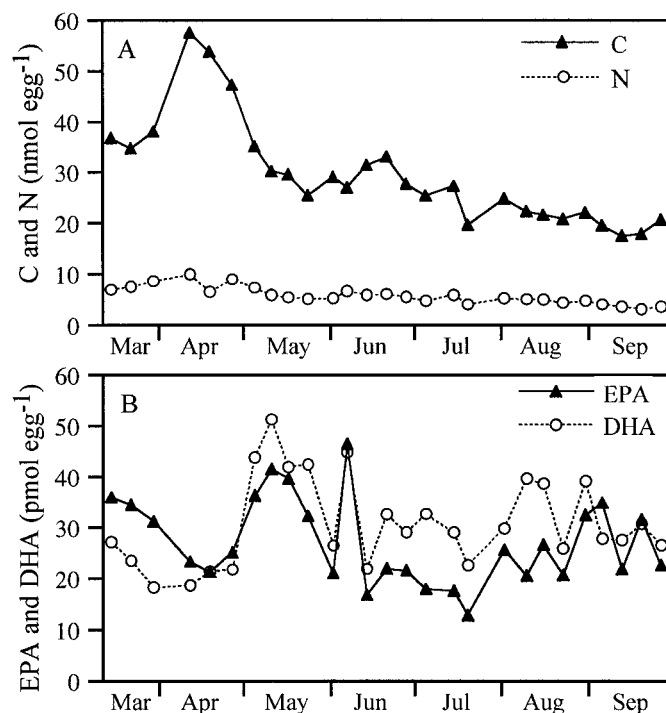


Fig. 1. Seasonal progression of (A) C and N, and (B) EPA and DHA in *Calanus helgolandicus* eggs in the English Channel; data from Pond et al. (1996).

The comparison of dietary components is most easily made using  $w$  as the common currency. The “limiting potential” of  $i$ ,  $L_i$  ( $0 \leq L_i \leq 1$ ) is then  $S_{i,w}$ , which gives a direct measure of realized relative to maximum GGE:

$$L_i = \frac{\phi_i K_w^* E_{i,w}}{\phi_w K_i^* F_{i,w}} = \frac{K_i}{K_i^*} \quad (3)$$

The limiting potential of the substrate that actually limits production,  $w$ , is always 1.0.

Data sets for biochemical composition of consumer and prey, which incorporate C, N, and essential fatty acids in comparable units, are scarce. Although numerous studies have documented fatty acids in zooplankton and algae, measurements are usually expressed as percentage total fatty acid without reference to bulk C or N. Zooplankton nutrition studies frequently use eggs as a production measure. We use the data of Pond et al. (1996), which describe the seasonal progression of biochemical composition of *Calanus helgolandicus* eggs in the English Channel during 1994 (Fig. 1). Egg composition varied as diatoms, dinoflagellates, flagellates, and ciliates dominated at different times, although strong correlations between egg composition and the presence of different groups were not apparent (Pond et al. 1996). Despite declines in C and N during the year (Fig. 1A), no obvious decrease was apparent in EPA and DHA (Fig. 1B), which suggests a minimum level of PUFA per egg was needed in order to sustain production. Ratios of egg constituents are shown in Fig. 2. We use a constant egg N:C of 0.20 for the analysis, which is very similar to the fitted major axis regression (Fig. 2A) and which obviates the need to separately relate C and N to fatty acids in eggs. In order

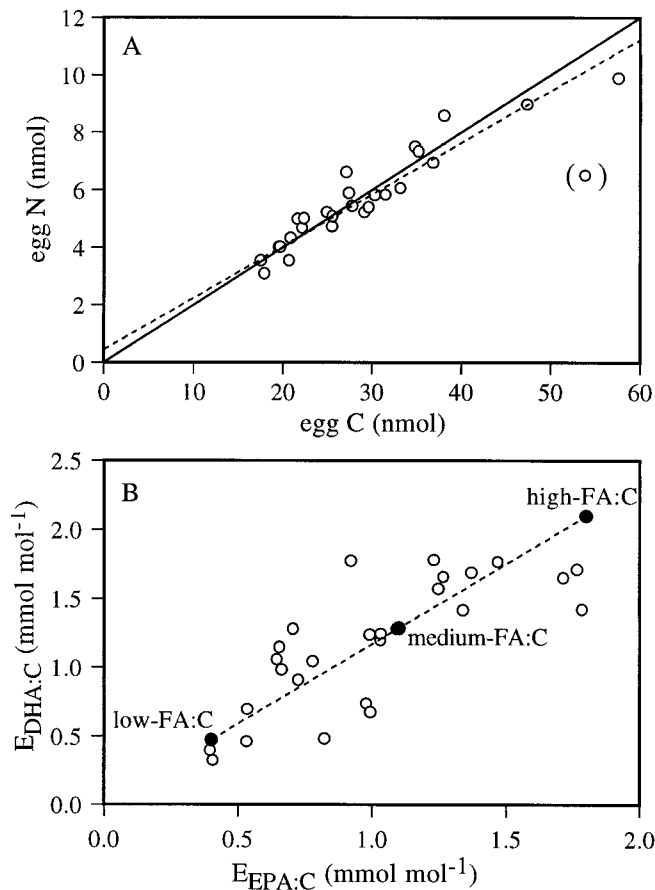


Fig. 2. Relationships between biochemical constituents in *C. helgolandicus* eggs. (A) N vs. C; dashed line:  $y = 0.18x + 0.44$  (reduced major axis regression, bracketed point excluded); solid line:  $y = 0.20x$ . (B) DHA:C vs. EPA:C (open circles);  $y = 1.16x + 0.01$  (reduced major axis regression); solid circles denote the three egg types derived for the stoichiometric analysis.

to relate fatty acids (FAs) to C (and hence N), the data in Fig. 2B are normalized to carbon. From the fitted major axis regression for DHA versus EPA we define three contrasting egg types, which reflect the range of variability in biochemical composition encountered: low FA:C, medium FA:C, and high FA:C (Fig. 2B, Table 1). In each case it is assumed that egg composition sets the demand for substrates, rather than being a consequence of diet, so that, for example, limitation by PUFAs is more likely to be encountered in high-FA:C eggs than low-FA:C eggs.

Ideally, we would wish to use concurrent field data for phytoplankton biochemical composition to undertake the stoichiometric analysis. However, copepods are selective feeders (Atkinson 1996), and it is therefore necessary to know the food types actually ingested rather than simply those available. It is difficult if not impossible to quantify the former in the case of the Pond et al. (1996) data because of large fluctuations in algal species composition and significant detrital presence. Instead we decided to study factors limiting zooplankton production using Pond et al.'s data to define the biochemical requirements of the consumer, in con-

Table 1. Biochemical composition of *C. helgolandicus* eggs and phytoplankton species as used in the stoichiometric analysis. N:C are  $\text{mol mol}^{-1}$ , FA:C ratios are  $\text{mmol mol}^{-1}$ .

	N:C	EPA:C	DHA:C
Copepod eggs			
Low FA:C	0.20	0.400	0.474
Medium FA:C	0.20	1.100	1.286
High FA:C	0.20	1.800	2.098
Phytoplankton			
<i>T. weissflogii</i>	0.150	0.722	0.160
<i>P. minimum</i>	0.145	0.065	0.481

junction with hypothetical prey—mixtures of the diatom *Thalassiosira weissflogii* and the dinoflagellate *Prorocentrum minimum*—using biochemical compositions from the batch cultures of Jónasdóttir (1994). Both groups are commonly grazed by copepods. The algal ratios (Table 1) are averages of measurements for each species made in different cultures and at different culture stages and exemplify each group: diatoms are typically rich in EPA but deficient in DHA, whereas dinoflagellates are characterized by high DHA and low EPA. The analysis presented is not intended to make a definitive statement about the limitation of *C. helgolandicus* in the Pond et al. study. Our aims are to present a new stoichiometric method and to use it to gain insight into the potential for limitation of marine zooplankton by macronutrients and micronutrients using typical consumer and prey compositions. We hope the work will encourage others to make the necessary measurements to provide further assessments of this kind.

Calanoid copepods are thought to be unable to synthesize the essential fatty acids EPA and DHA in significant quantities (Nanton and Castell 1998). Parameters  $\phi_{\text{EPA}}$  and  $\phi_{\text{DHA}}$  are therefore set to 1.0, although we will examine the effect of setting lower values. Carbon and nitrogen must be of dietary origin, so  $\phi_{\text{C}}$  and  $\phi_{\text{N}}$  are 1.0. Gross growth efficiency is the product of assimilation and net growth efficiencies. Assimilation efficiency can exceed 0.9 (Urabe and Watanabe 1991; Pond et al. 1995). A fraction of assimilated C is always required to meet respiration costs, so maximum C net growth efficiency is always less than 1.0. This maximum has been estimated for heterotrophs to be around 0.8 (Schroeder 1981). We set  $K_{\text{C}}^*$  equal to 0.7 ( $\approx 0.9 \times 0.8$ ). Other biochemicals of interest can theoretically be used with a net growth efficiency of 1.0 if sufficient C-rich substrates are available for respiration (Anderson and Hessen 1995). Gross growth efficiency then equals assimilation efficiency, and so  $K_{\text{N}}^*$ ,  $K_{\text{EPA}}^*$ , and  $K_{\text{DHA}}^*$  are each set at 0.9.

The predicted limiting potentials of each diet component for the three egg types are shown in Fig. 3. EPA limits egg production when the diatom fraction is low, and DHA when the dinoflagellate fraction is low. Predicted  $K_{\text{N}}^*$  ( $= L_{\text{N}} K_{\text{N}}^*$ ) is 0.41 for low-FA:C eggs produced when the diet is 100% diatoms and lower for the other egg types. Algal monocultures were fed to copepods in the laboratory experiments of Checkley (1980) and Kiørboe (1989). These studies showed copepods using N with a low and constant efficiency ( $\sim 0.4$ ) for food with wide-ranging C:N, which would certainly sug-

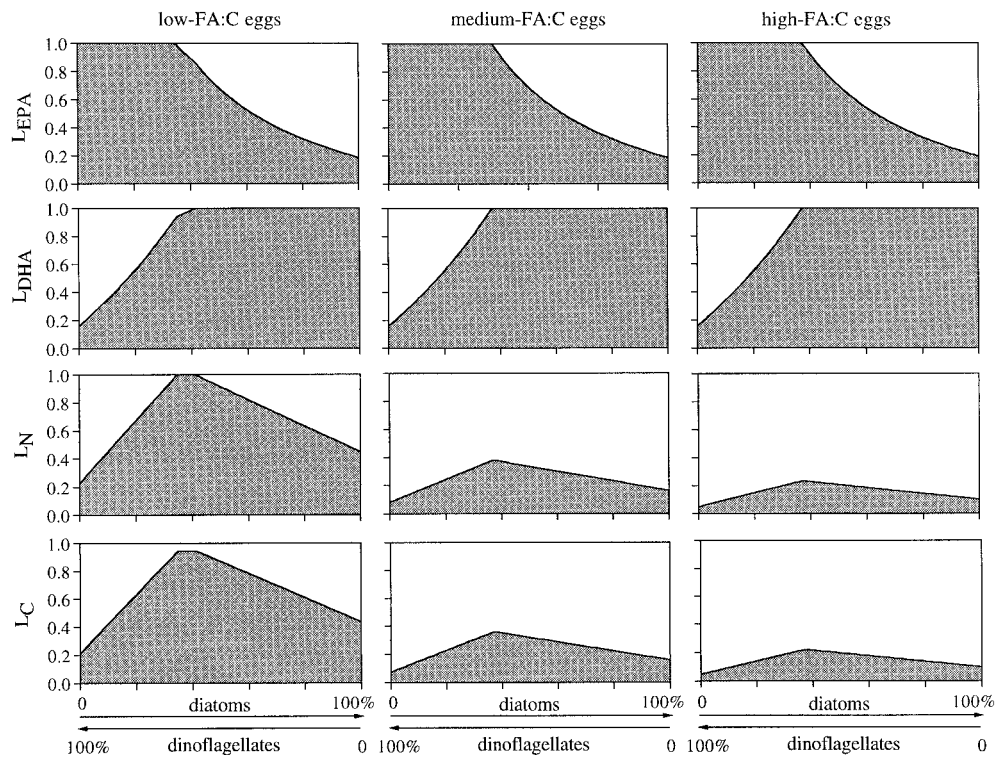


Fig. 3. Calculated limiting potentials of each substrate for each of the three egg types, as influenced by the diatom-dinoflagellate ratio ingested (ratios specified in C units). The limiting potential of the component predicted to limit production is always 1.0.

gest limitation by N. The analysis presented here indicates that this low  $K_N$  could be due to limitation by essential micronutrients when copepods graze such monospecific prey, as proposed by Anderson and Hessen (1995). The N- or P-limiting conditions required to induce low N or P to C ratios in phytoplankton tend also to cause deficiencies in essential dietary components such as certain fatty acids (Weers and Gulati 1997), which could explain the experimental correlations between egg production and food N. The predicted limiting potentials of C and N are markedly increased when the diet is a balanced mix of the two algal groups. Indeed N-limitation is predicted to occur ( $L_N = 1$ ) for N-rich eggs (low-FA:C and hence low-FA:N) produced from mixtures with diatom-dinoflagellate ratios between 36:64 and 42:58. A small increase in  $K_N^*$  or decrease in  $K_C^*$  would cause this N-limitation to switch to C-limitation. The predicted higher  $K_C (= L_C K_C^*)$  associated with mixtures agrees with the experimental work of Kleppel and Burkart (1995), who fed the copepod *Acartia tonsa* with the diatom *Thalassiosira weissflogii* and the dinoflagellate *Amphidinium carterae* in monocultures or together. Observed  $K_C$  was highest when the two algae were supplied together. The limiting potential of the fatty acids is lowest relative to C and N in low-FA:C eggs because these have the lowest PUFA demand. Plasticity in consumer fatty acid composition thus provides a means by which the limiting effect of PUFAs is decreased, although the range of plasticity that can occur is unknown.

The sensitivity of the analysis for low-FA:C eggs to selected parameter values is shown in Fig. 4. Decreasing  $\phi_{EPA}$

and  $\phi_{DHA}$  from 1.0 to 0.75, so that a significant amount (25%) of PUFAs are synthesized by the consumer, causes  $L_N$  and  $L_C$  to increase relative to  $L_{EPA}$  and  $L_{DHA}$  (Fig. 4B), widening the range of mixtures in which N-limitation is predicted to occur. The large amounts of EPA and DHA that are required to be biosynthesized to induce C- or N-limitation for most combinations of egg type and food seem improbable. Another important parameter setting is the theoretically high value of 0.7 we assigned to maximum carbon GGE,  $K_C^*$ . Although such high values do occur, the GGE of planktonic crustaceans including copepods is typically between 0.2 and 0.3 (Straile 1997). Note that  $K_C$  predicted using the standard parameter set ( $K_C^* = 0.7$ ) is often low, indicating that low GGE could sometimes be due simply to poor food quality. If, however, zooplankton have a large respiratory requirement for C, this would demand that  $K_C^*$  be lowered accordingly. Halving  $K_C^*$  from 0.7 to 0.35 causes a doubling in  $L_C$  relative to other substrates, such that C-limitation is predicted except in the most imbalanced diatom-dinoflagellate mixtures (Fig. 4C). Limitation by C or N can therefore be easily be produced in the analysis if  $K_C^*$  or  $K_N^*$  is decreased significantly from its initial setting of 0.7 or 0.9. Finally, we show what happens when EPA and DHA are amalgamated into a single diet component and the analysis is reworked (equivalent to allowing interconversion of the two by the consumer before usage, or unlimited substitution of the two in their functional roles). The result is similar to providing the PUFAs in a balanced mixture—limiting potentials of C and N are markedly increased so that limi-

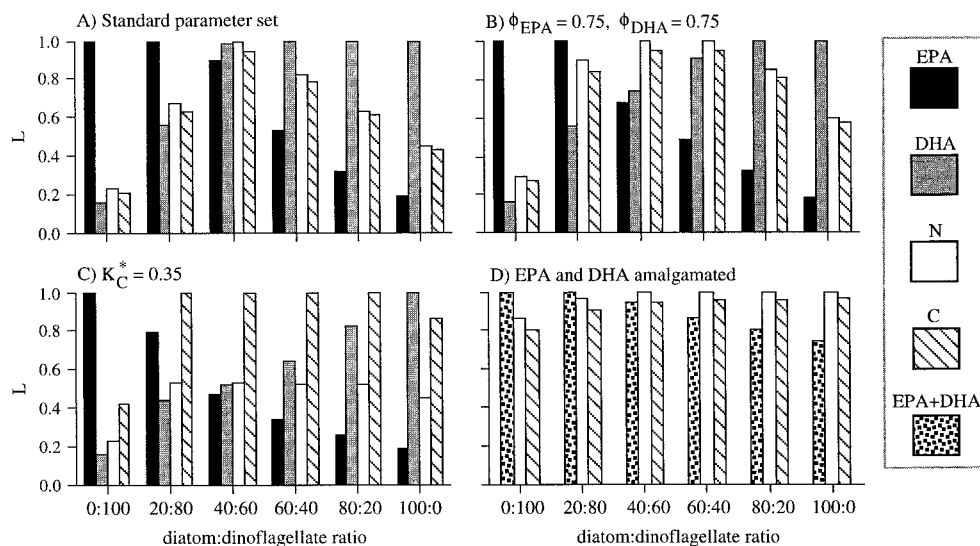


Fig. 4. Sensitivity of predicted limiting potentials for low-FA:C eggs to selected parameter changes.

tation by C or N is much more likely (Fig. 4D). Limitation by fatty acids is then most prevalent when dinoflagellates dominate the diet because these algae have a lower PUFA content relative to C and N than do diatoms.

In conclusion, we have demonstrated that the potential exists for zooplankton production to be limited by essential PUFAs or macronutrients, depending on the biochemical compositions of consumer and diet. Uncertainties regarding the roles of essential fatty acids in zooplankton physiology and the extent to which these compounds are synthesized by the consumer make a definitive statement about what limits production impossible at this time. Several factors may contribute to limitation by C or nutrient elements rather than micronutrients in many instances: zooplankton are selective and can obtain a balanced mix of fatty acids, plasticity in consumer biochemical composition, and high C requirements for respiration. Limitation by essential PUFAs is reduced further if zooplankton actively synthesize them, or if these compounds can substitute for each other in their functional roles. It is interesting to speculate that calanoid copepods and many other zooplankton apparently lack the enzymes to actively undertake PUFA synthesis because they may commonly be limited by macronutrients. An interesting comparison can be drawn with harpacticoid copepods, which characteristically inhabit detritus-rich environments in which food is deficient in essential fatty acids and which do possess the capability to actively synthesize these compounds in significant quantities (Nanton and Castell 1998). On the other hand, correlative evidence linking zooplankton production to dietary PUFAs does suggest an important role for these compounds in nutrition. There may be instances in which food is unusually deficient in fatty acids, such as the presence of large quantities of mucilaginous aggregates (Najdek 1997) or detritus (Coombs et al. 1994). Data sets for grazers and their food that incorporate fatty acids and bulk elements such as C, N, and P in comparable units are few and far between. The analysis presented here highlights the need for such data, as well as an improved understanding of the roles and

cycling of essential fatty acids, if we are to be able to provide a unified view of zooplankton nutrition.

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## Three-dimensional spatial coordinates of individual plankton determined using underwater hologrammetry

**Abstract**—We report the use of a nondestructive metrological technique, hologrammetry, that affords greater scope and higher accuracy for the enumeration, sizing, and spatial distribution of particles. Results are presented on imaging of three living planktonic protists suspended in water using in-line pulsed-laser holography. We demonstrate that a volume of 2,400 ml of water, at 750 mm depth, can be analysed with a resolution everywhere better than 20  $\mu\text{m}$ . High quality images and accurate spatial coordinates of living plankton in subvolumes have been obtained.

Zooplankton play an important role in the functioning of most aquatic ecosystems (Banse 1995). Techniques which enable the study of orientation, motion, local spatial distri-

bution, and predator–prey relationships are vital for a better understanding of species interactions and hence biogeochemical processes. However, until recently these have been studied only by simple netting or optical techniques. Existing techniques for in-situ recording, such as the optical plankton counter (Herman 1988), the plankton video camera (Davis et al. 1992), Schlieren videography (Strickler 1998), or various types of camera to study marine snow aggregates (e.g., Lampitt et al. 1993), do not provide three-dimensional coordinates at the same time as high resolution and a large depth-of-field.

In our work, holograms are recorded of aquatic systems using a pulsed laser and subsequently replayed, in air, in the real (projected) image mode of reconstruction. The images