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DOI

[10.1111/j.1365-2427.2007.01868.x](https://doi.org/10.1111/j.1365-2427.2007.01868.x)

Publication date

2008

Published in

Freshwater Biology

[Link to publication](#)

Citation for published version (APA):

Caramujo, M. J., Boschker, H. T. S., & Admiraal, W. (2008). Fatty acid profiles of algae mark the development and composition of harpacticoid copepods. *Freshwater Biology*, 53(1), 77-90. <https://doi.org/10.1111/j.1365-2427.2007.01868.x>

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Fatty acid profiles of algae mark the development and composition of harpacticoid copepods

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SUMMARY

1. The value of algal fatty acids (FA) as diet biomarkers for benthic harpacticoid copepods was investigated. A high proportion of 18:1 ω 9 and 18:2 ω 6 FA was observed in the lipid reserve fraction of copepods fed with cyanobacteria. In contrast, a high proportion of 16:1 ω 7 and ω 3 FA (including eicosapentaenoic) was present in the lipid reserve fraction of copepods grown on diatoms.

2. Copepods that were grown on cyanobacteria showed reduced survival and took 26% more time to develop from the first copepodid stage to adult than copepods that were grown on diatoms. Copepods feeding on the cyanobacteria showed reduced FA content when compared with animals fed with diatoms. This reduction in FA content was more pronounced in the apolar lipid fraction (mainly reserve lipids) than in the polar (mainly structural) lipid fraction.

3. The FA profiles of algae were used to calculate a function discriminating between diatoms and cyanobacteria. This function was applied to the FA profiles in the reserve lipid fraction of copepods and correctly classified copepod diet. 16:1 ω 7, 18:2 ω 6 and 20:5 ω 3 were the most important FA in the discriminant function. The suitability of this chemometric method to infer copepod diet was further tested by using algal class FA data from literature to derive the discriminant functions. The correct classification of the diet when the functions were applied to FA composition of the copepod reserve lipids suggests that this method may be employed in trophic web studies. 18:3 ω 3, 18:1 ω 9 and 16:1 ω 7 were the most important FA in the functions discriminating diatoms, cyanobacteria and green algae. The identification and quantification of the whole suit of 16:1 ω 7, 18:1 ω 9, 18:2 ω 6, 18:3 ω 3 and 20:5 ω 3 in trophic web studies is therefore of paramount importance to infer diet origin of aquatic herbivores.

4. The FA profile of copepod polar lipids did not reflect that of the diet. The presence of long chain polyunsaturated FAs in the polar lipid fraction of copepods feeding on the cyanobacterium suggests that C18 FAs from the diet may be elongated and desaturated by the copepod. The ability to elongate and desaturate FAs may reduce the importance of some FAs as diet biomarkers while it may turn the copepods into valuable trophic intermediaries in transferring organic matter from microorganisms to higher trophic levels.

Keywords: *Attheyella trispinosa*, cyanobacteria, diatoms, fatty acids, gas chromatography-mass spectrometry

Introduction

Fatty acids (FA) are chemical compounds present in all bacteria and eukaryotic organisms that have great structural diversity with substantial taxonomic specificity. When FA show some stability during transfer in the food web, their analysis may disclose dietary relationships as well as temporal changes in food sources (Sargent *et al.*, 1987; Fraser *et al.*, 1989; Dalsgaard *et al.*, 2003 and references therein). Odd-chain length and branched FA appear to be good indicators of zooplankton grazing on bacteria; FA of the $\omega 6$ series indicate grazing on ciliates when they are detected in the microcrustaceans triacylglycerols (TAG) and, a high content of palmitoleic acid (16:1 $\omega 7$) is observed in animals feeding on diatoms (Kaneda, 1991; Ederington, McManus & Harvey, 1995; Desvillettes *et al.*, 1997a; Hirche *et al.*, 2003). Graeve, Kattner & Hagen (1994) demonstrated that diet may induce changes in the FA pattern of neutral lipids in calanoid copepods and, the FA profiles of TAG (part of the apolar lipid fraction) have been observed to be close to those of the dietary algae (Henderson & Tocher, 1987; Bourdier & Amblard, 1989). Contrasting to the influence of the diet in the FA pattern of neutral lipids, the FA composition of phospholipids (PL) is probably under metabolic control and thus weakly influenced by dietary FAs (*in Desvillettes et al.*, 1997a). Additionally, it has been shown that the FA composition of PL may also reflect a thermal acclimation response that would contribute to the maintenance of membrane fluidity, i.e. decrease of saturated FA proportion with decreasing temperature (Henderson & Tocher, 1987; Nanton & Castell, 1999; Jobling & Bendiksen, 2003).

The value of FA as diet biomarkers is nevertheless affected by the extent of the transformations that the consumers may perform on ingested FA. According to Brett & Müller-Navarra (1997), all herbivores convert α -linolenic acid (18:3 $\omega 3$) to eicosapentaenoic (EPA, 20:5 $\omega 3$) and docosahexaenoic (DHA, 22:6 $\omega 3$) acids albeit with different efficiency. Zooplankton have the ability to convert linoleic (18:2 $\omega 6$) and α -linolenic (18:3 $\omega 3$) acids to arachidonic acid (20:4 $\omega 6$) and EPA, respectively, although several studies suggest that this phenomenon is more important in copepods than in cladocerans (Goulden & Place, 1990; Desvillettes *et al.*, 1994; Desvillettes, Bourdier & Breton, 1997b). Cyclopoid copepods are also potentially able to incorporate

dietary 18:3 $\omega 3$ originating from microalgae into the PL and bioconvert it into DHA (Farkas, Kariko & Csengeri, 1981; Desvillettes *et al.*, 1997b). The ability exhibited by some copepods to bioconvert 18:3 $\omega 3$ to EPA and DHA may not be shared by all taxa as calanoids seem to be unable to elongate and desaturate 18:3 $\omega 3$ to produce significant amounts of longer-chain polyunsaturated fatty acid (PUFA) (Lee, Hirota & Barnett, 1971; Støttrup & Jensen, 1990; Jónasdóttir, Fields & Pantoja, 1995; Bell *et al.*, 2007). It is considered that harpacticoid copepods are able to bioconvert dietary 18:3 $\omega 3$ to both EPA and DHA (Watanabe *et al.*, 1978; Norsker & Støttrup, 1994; Nanton & Castell, 1998, 1999). Anderson & Pond (2000) presented an interesting speculation regarding the apparent lack of enzymes to undertake PUFA synthesis in calanoids because they may be commonly limited by macronutrients in the plankton environment while the opposite is observed for the generally benthic copepods where abundant detritus deficient in essential FA is available. In fact, data on seasonal variations in food quality available for invertebrates in the pelagic and benthic environments of a lake has hinted that during the summer the pelagic grazer production was limited by food quantity whereas benthic grazer production was limited by food quality (Ahlgren *et al.*, 1997).

The ability to bioconvert FA and survive on poor diets turns cyclopoid and harpacticoid copepods into an important link in aquatic food webs because of the enhancement of the nutritional value of food available for larger predators that include fish (e.g. Rundle & Hildrew, 1992), amphibians (Blaustein, Friedman & Fahima, 1996; Wilbur, 1997) and other invertebrates (Schmid-Araya & Schmid, 2000). Biochemical enhancement of bacterial or algal nutrients such as FA has been reported for some species of heterotrophic protozoans that bridge the gap between the microbial loop and higher trophic levels (Klein-Breteler *et al.*, 1999; Veloza, Chu & Tang, 2006). Despite their sporadic use in aquaculture, copepods are known to be nutritionally beneficial to optimize growth, survival and metamorphosis of fish larvae and to reduce the incidence of disease (Watanabe *et al.*, 1978; Norsker & Støttrup, 1994; Nanton & Castell, 1998, 1999; Shields *et al.*, 1999; Støttrup, 2000). There is evidence that the superior efficacy of copepods relative to enriched *Artemia* may be ascribed to the digestibility and availability of highly

unsaturated FA supplied as preformed PL (Kanazawa, Teshima & Sakamoto, 1985).

In this study, we have investigated the suitability of using FA content and composition in the reserve lipids of harpacticoid copepods as diet indicators. We propose to test the applicability of a chemometric method to discriminate algal diets based on their FA content and to classify the diet of herbivores using the FA composition of their reserve lipids. We supply complete FA profiles of freshwater benthic algae and a harpacticoid copepod that are generally absent from literature. Additionally, we have studied the suitability of diatoms and cyanobacteria to sustain the development of a freshwater, meiobenthic copepod and the potential of the copepod to transfer essential FA from phototrophs to higher trophic levels.

Methods

Algae and copepod cultures

The benthic diatoms *Achnanthes lanceolata* [(Brébisson) Grunow 1880] and *Nitzschia perminuta* [(Grunow) M. Peragallo 1903] and the benthic cyanobacteria *Leptolyngbya foveolarum* [(Rabenhorst ex. Gomont) Anagnostidis et Komárek 1988] and *Cylindrospermum stagnale* (Kutzing) Rippka *et al.* 1979 were obtained from non-axenic cultures kept in the laboratory (for details, see Van Der Grinten *et al.*, 2004). The non-axenic cultures were kept in 500 mL Erlenmeyer flasks with 150–200 mL sterile modified Woods Hole medium (Guillard & Lorenzen, 1972). The cultures were kept at 20 °C under a light intensity of 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 16:00 : 08:00 hours light : dark regime. Algae were harvested near the end of the exponential growth phase, and concentrated fresh algae were fed to the copepod cultures after centrifuging. Aliquots of the algal cultures were collected on glass microfibre filters and stored at –80 °C until lipid extraction. The carbon content of the algae at the harvest point was measured in aliquots using a T.O.C. Analyzer (Model 700; O.I. Co., Houston, TX, U.S.A.).

The freshwater harpacticoid copepod *Attheyella trispinosa* (Brady 1880) had been cultured in the laboratory for several generations in M4 Elendt medium (Elendt, 1990), after collection from experimental ponds at the university campus. Throughout the experiments, copepods were grown in M4 Elendt medium and were fed with algae at a concentration of

$1.7 \times 10^7 \mu\text{m}^3 \text{cm}^{-2}$. The cultures were kept at 20 °C under dim light and a 16:00 : 08:00 hours light : dark regime. To obtain nauplii for development on each of the algal diets, ovigerous females or mating pairs of *A. trispinosa* were transferred from the stock cultures and individually placed into wells (dm = 2.0 cm, 12 well polystyrene clusters, Corning® and Costar®; Sigma–Aldrich Inc., St. Louis, MO, U.S.A.) filled with 4 mL of medium and a mixture of *N. perminuta* and *A. lanceolata* (1 : 1). Previous observations have shown that 24–48 h nauplii endure handling better than younger nauplii and therefore manipulation of nauplii was performed only 24–48 h after hatching. In the first experiment, nauplii from 48 ovigerous females were grown in glass vials (dm = 7.0 cm) already containing each of the four algal diets and 120 mL of medium. Every 2 days, half of the medium was replaced, most of the sediment was removed with a pipette and fresh food was added. When copepodids reached the adult stage, sets of 50–150 newly moulted females grown on each of the algal diets (six replicate cultures) were placed on microfibre glass filters, immersed in liquid N₂ and stored at –80 °C before lipid extraction.

In the second experiment, sets of 25 nauplii were placed in triplicate wells containing 4 mL medium and each of the algal diets to assess the influence of diet in the post-embryonic development time and survival (well dm = 22.1 mm, Corning® and Costar®; Sigma–Aldrich Inc.). When nauplii reached the first copepodid stage, the copepodids were transferred to larger wells (dm = 34.8 mm) containing 10 mL of medium and each of the algal diets. Medium was replaced every 2 days when food was also added. The post-embryonic development time of a set of nauplii was established as the time taken by more than half the total number of surviving individuals to reach the adult stage. Survival rate was assessed as the proportion of nauplii reaching the adult stage. Adult females were allowed to remain under the experimental conditions for an additional week to inspect oocyte maturation and deposition in the oviducts.

FA extraction and analysis

Lipids were extracted with a modified Bligh and Dyer method according to Findlay, King & Watling (1989). For each algal culture $n = 8$ except for *C. stagnale* where $n = 4$ and for each copepod culture $n = 6$.

Three replicate samples of total lipid extract of copepods feeding on each algal culture were separated on a silicic acid column (Merck, Darmstadt, Germany) by sequential elution with chloroform, acetone and methanol to obtain two lipid fractions: neutral lipids containing the TAG reserve lipids in the chloroform fraction and, polar lipids containing mainly membrane lipids in the combined acetone and methanol fractions. Fatty acid methyl esters (FAME) were obtained from total lipid extracts and apolar lipid fractions using derivatization with H₂SO₄-methanol or by mild alkaline transmethylation of the polar lipid fractions (Guckert *et al.*, 1985). FAME of the total copepod extracts ($n = 3$) and two replicate algal extracts were analysed by capillary gas chromatography with flame ionization detection using a Varian 3400 gas chromatograph (for details see Boschker *et al.*, 2001). Identification of FAME was based on retention time data of known standards, later confirmed by mass spectrometry (Hewlett-Packard Mass Selective detector, Santa Clara, CA, U.S.A.). Quantification was accomplished by using FAME 19:0 as internal standard. We have used a FA shorthand notation of the form $A:B\omega X$, where A represents the number of carbon atoms, B gives the number of double bonds and X gives the position of the double bond closest to the terminal methyl group (Guckert *et al.*, 1985).

Calculations and statistical analyses

ANOVA were applied to FA weight data of both algae and copepods. Data were square-root transformed to meet the requirement of homoscedasticity when group variances were proportional to the means. Unplanned comparisons were made using Tukey's B test ($\alpha = 0.05$) or Hochberg's GT2 tests (because of unequal sample size) to identify homogeneous subsets. When homoscedasticity could not be assumed, Tamhane's T2 test was applied to enable multiple comparisons.

Fatty acid ratios were calculated to inspect if characteristic algal class ratios were maintained in the lipid extracts of copepods thus reflecting their diet. The ratio of 16:1 ω 7/16:0 above one was considered an indicator of diatoms (e.g. Dunstan *et al.*, 1994). The C₁₈-PUFA content in cyanobacteria was used to classify the cyanobacterial strains according to the Kenyon–Murata system (Kenyon,

Rippka & Stanier, 1972; Murata, Wada & Gombos, 1992).

Discriminant analysis (DA, SPSS; SPSS Inc., Chicago, IL, U.S.A.) was applied to the FA algal data set to test if the FA composition of the apolar (mainly reserve) lipid fraction of copepods may be used to discriminate the origin of the diet. Reserve lipid fraction was chosen to minimize the effect of possible selective retention or bioconversion of FA that are incorporated into membrane lipids (or polar lipids). FA acid data were entered in DA as individual FA weight percentage in the lipid extract. DA is a method that recognizes patterns and enables the separation of groups established *a priori* using data provided by a certain number of independent variables (i.e. algal FA content). The independent variables were used to produce a set of linear functions (orthogonal to each other) that maximize the differences between the values of the dependent variable (algal group). The discriminant functions can be represented graphically in two dimensional plots and the correlation of the original FA variables with these functions (i.e. considered as factor loadings) may be projected on the new ordination planes as vectors. The length of the vector of each original variable (i.e. FA) is directly proportional to the degree of correlation of the variable with the ordination plane. The correlation degree between variables can be considered directly proportional to the cosine of the angle between their vectors. The discriminant function coefficients were applied to FA content of the apolar lipid fraction of copepods feeding on known diets to test if the diet of copepods were rightly classified from their FA profiles. Independent variables were entered together into the model after discarding variables that had equal group means, were absent from one algal class or were significantly correlated with other variables. The selection of which variable to discard in a correlated pair was performed according to the results of exploratory DA (not shown). The less important variable in explaining the observed variation in the dependent variable was discarded from the analysis. DA has been used in several studies to separate species according to their diet (e.g. Navarro *et al.*, 1995; Guisande *et al.*, 2003). The canonical discriminant functions were derived from (i) the FA composition of cyanobacteria and diatoms in the present study and (ii) from published FA data for cyanobacteria, diatoms and Chlorophyceae (green algae).

The use of published data tested the suitability of DA to classify algal classes based on their FA composition and of using the discriminant functions to identify copepod diet when more than two algal classes are potentially available as food. Concomitantly, we tested the accuracy of diet classification when the discriminant functions are derived from species different from those offered as food to copepods. The use of published algal FA data was restricted to diatoms, cyanobacteria and green algae because these algal classes are the most important benthic algae in freshwater systems. Literature sources of algal FA contents are: Bourdier & Amblard (1989); von Elert & Stampfl (2000); Poerschmann, Spijkerman & Langer (2004) and Weers, Siewertsen & Gulati (1997) for freshwater green algae ($n = 4$); Zhukova & Aizdaicher (1994) for marine green algae ($n = 4$); Ahlgren, Gustafsson & Boberg (1992); Walsh, Jones & Dunstan (1997); Vargas *et al.* (1998) and Gugger *et al.* (2002) for freshwater, non-toxic cyanobacteria of the Kenyon–Murata group II ($n = 15$); Léveillé, Amblard

& Bourdier (1997) for a freshwater diatom ($n = 1$); Dunstan *et al.* (1994); Budge & Parrish (1999) and Ederington *et al.* (1995) for marine diatoms ($n = 16$). The inclusion of marine species in the analysis resulted from the scarcity of detailed FA profiles of freshwater diatoms in literature.

Results

The total FA content of diatoms was two- to threefold higher than the FA content of cyanobacteria (Fig. 1, *post hoc* Tamhane T2 $P < 0.001$). Both total and saturated FA contents of species within each algal group were similar (Tamhane T2 $P > 0.162$) although the diatom *A. lanceolata* had a slightly higher total FA content than *N. perminuta*. Diatoms had trace amounts of C₁₈ PUFA, which were the most abundant PUFA in cyanobacteria ($F_{3,27} = 87.03$, $P < 0.001$, Figs 1 & 2). In *L. foveolarum*, the most abundant PUFA was 18:2 ω 6, while in *C. stagnale* 18:3 ω 3 FA was the major PUFA (Fig. 2). EPA (20:5 ω 3) was abundant in diatoms and

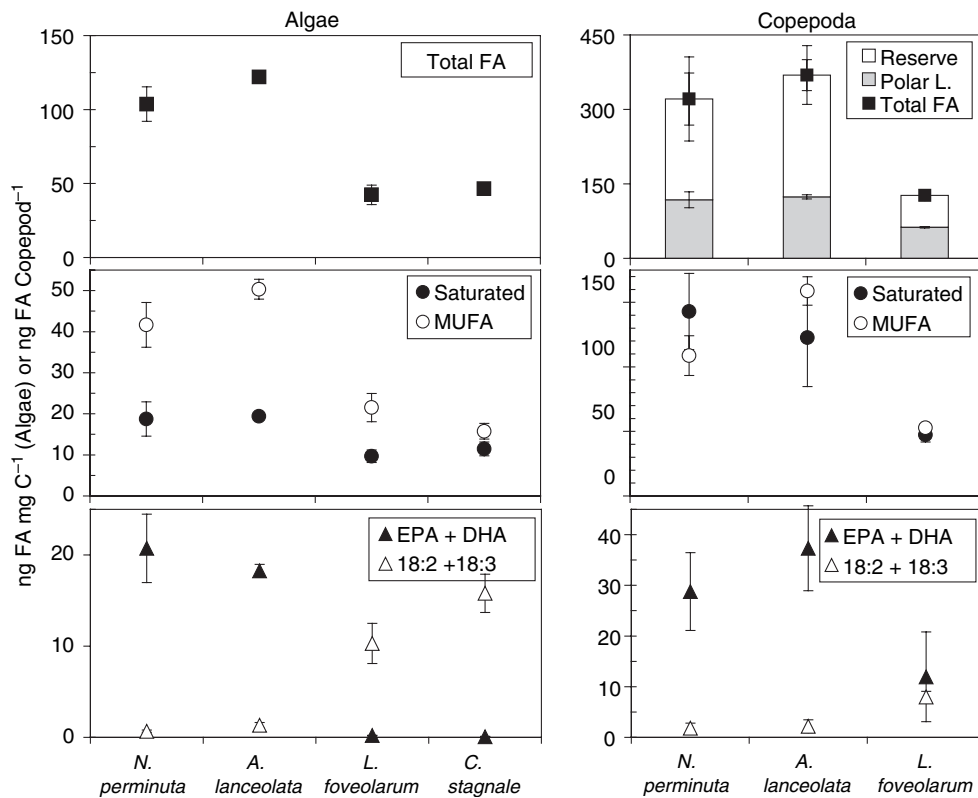


Fig. 1 Carbon specific fatty acid (FA) content of algae and FA content of the adult female copepod *Attheyella trispinosa*. FA content of copepods is also given as separate content in reserve (reserve) and polar (PLFA) lipid fractions. Values are averages from replicate cultures ± 1 SD (error bars).

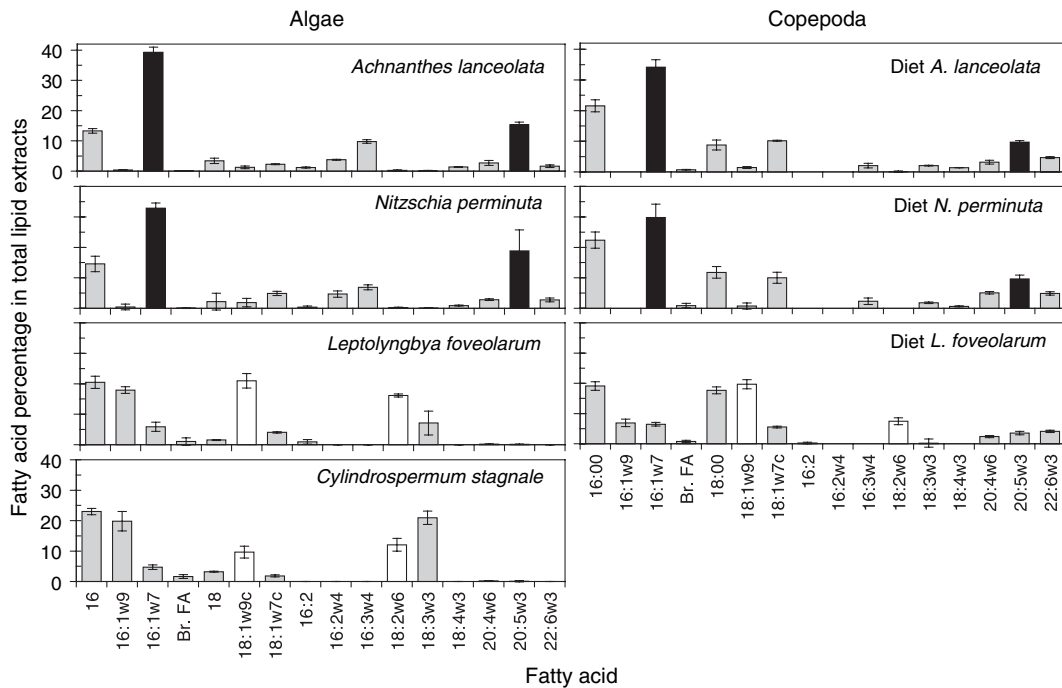


Fig. 2 Average fatty acid (FA) percentage of total FA weight for diet algae and copepods. Black columns indicate abundant FA in diatoms and white bars indicate abundant FA in cyanobacteria. Error bars indicate ± 1 SD. Copepods did not grow on a diet of *Cyldrospermum stagnale*.

present in trace amounts in the cyanobacterium *L. foveolarum* (Fig. 2). DHA (22:6 ω 3) was present in small amounts in diatoms and was undetected in both cyanobacteria. Diatom species had a similar monoene fatty acid (MUFA) content while the cyanobacterium *L. foveolarum* had a higher MUFA content than *C. stagnale* (Tamhane T2 $P < 0.01$; Fig. 1). The most abundant MUFA in diatoms was 16:1 ω 7 (Fig. 1). In cyanobacteria, 16:1 ω 7 was present in small amounts and 16:1 ω 9 and 18:1 ω 9 were the major MUFA (Fig. 2). The ratio of 16:1 ω 7/16:0 was roughly 2 for diatoms and <0.5 for cyanobacteria (Fig. 3). Diatoms had higher contents of C₁₆ FA than C₁₈ FA while cyanobacteria had similar amounts of C₁₆ and C₁₈ FA (Fig. 3). Both diatoms and the cyanobacterium *C. stagnale* had more ω 3 unsaturated FA than ω 6 unsaturated FA while the opposite was observed for the cyanobacterium *L. foveolarum* (Fig. 3). The ratio of PUFA to total FA was similar among diatoms and *C. stagnale* while the cyanobacterium *L. foveolarum* had the lowest ratio of the four algae ($F_{3,27} = 6.62$, $P < 0.05$; Tamhane $P < 0.05$, Fig. 3).

Naupliar stages feeding on the cyanobacterium *C. stagnale* failed to develop beyond the first copepodid

stage and development experiments using *C. stagnale* as food were terminated after 20 days when all individuals had died. Individuals feeding on the cyanobacterium *L. foveolarum* had a lower survival rate ($F_{2,11} = 600.44$, $P < 0.001$; Tukey's B $P < 0.05$) and developed slower ($F_{2,11} = 28.13$, $P < 0.001$; Tukey's B $P < 0.05$) compared with individuals feeding on diatoms (Fig. 4). Females feeding on diatoms developed mature oocytes and their deposition in the oviducts was visible as dark longitudinal bands in the body. Matured oocytes were never observed in the oviducts of females feeding on the cyanobacterium *L. foveolarum*.

The total FA content of copepods growing on the cyanobacterium was roughly a third of the FA content of copepods growing on a diatom diet ($F_{2,17} = 61.51$, $P < 0.001$, *post hoc* Hochberg $P = 0.314$, Fig. 1). The depressed FA content of copepods feeding on *L. foveolarum* relative to those feeding on diatoms was more severe in the apolar lipid fraction, i.e. mainly reserve FA ($\approx 70\%$ less, Fig. 3) than for the polar lipid fraction, i.e. mainly structural FA ($\approx 50\%$ less; significant $F_{2,8} = 90.19$, $P < 0.001$; Tukey's B $P < 0.05$). This trend was observed for all major FA classes with the

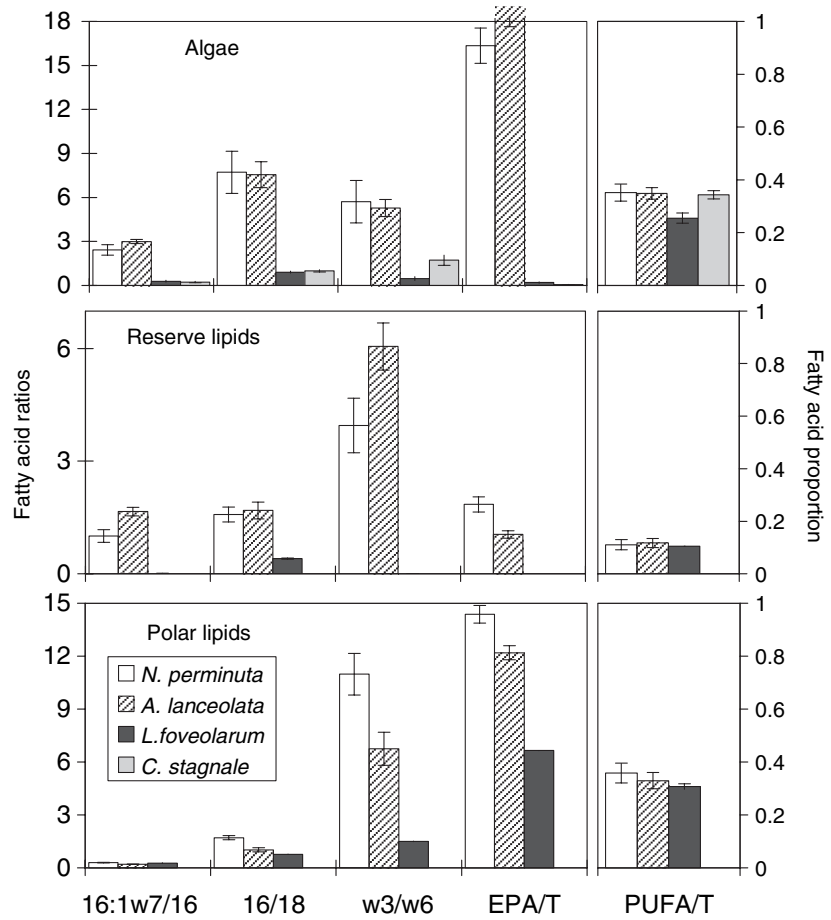


Fig. 3 Average fatty acid weight ratios of algae, and apolar (reserve) and polar (PLFA) lipid fractions of copepods grown on the different algae. Error bars indicate ± 1 SD of the mean. Copepods did not grow on a *Cylindrospermum stagnale* diet.

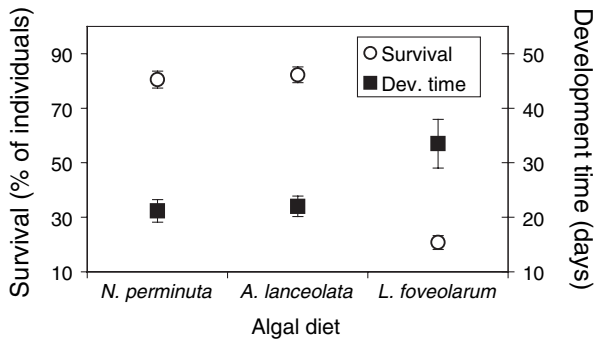


Fig. 4 Average survival percentage and development time of copepods grown on different algal diets ($n = 3$). Error bars indicate ± 1 SD.

exception of C_{18} FA (Fig. 1). Copepods feeding on *L. foveolarum* had significantly higher content of C_{18} PUFA than copepods feeding on diatoms ($F_{2,17} = 38.20, P < 0.001$; Tukey's B $P < 0.05$, Fig. 2). Copepods feeding on diatoms had more 16:1 ω 7 and EPA and less 18:1 ω 9 and 18:2 ω 6 FA than copepods feeding on

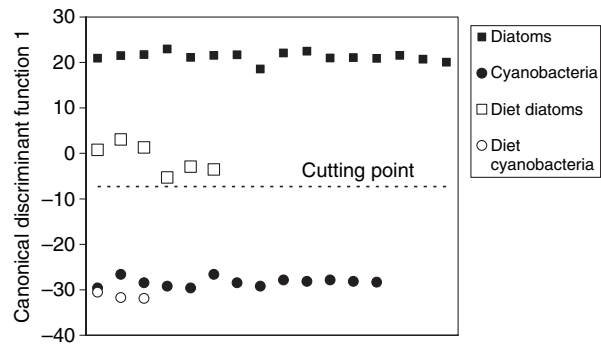


Fig. 5 Distribution map of algae (closed symbols) and copepods feeding on diatoms (open squares) or cyanobacteria (open circles) according to the discriminant function calculated from algal fatty acid profiles. Function 1 = $-42.035 + 0.271(16:0) + 1.070(16:1\omega 7) - 0.230(18:1\omega 9) + 0.359(18:2\omega 6) + 0.699(20:4\omega 6) + 0.911(20:5\omega 3)$, Wilks' $\lambda = 0.020, \chi^2 = 148.928, P < 0.001$.

L. foveolarum. These differences in FA content of copepods according to their diets were more pronounced in the reserve lipid fraction than in the polar

fraction (Fig. 3). The ratio of 16:1 ω 7 : 16:0 was higher in the apolar lipid fraction of copepods feeding on diatoms than in copepods feeding on *L. foveolarum* ($F_{2,8} = 19045.8$, $P < 0.001$). The 16:1 ω 7 : 16:0 ratio was low in the copepod polar lipid fraction regardless of their diet. ω 3 FA were virtually absent from the apolar lipid fraction of copepods feeding on *L. foveolarum* while they were abundant in the polar lipid fraction of copepods irrespectively of their diet ($F_{2,8} = 2941.2$, $P < 0.001$). Diatom algae had a higher PUFA ratio than the cyanobacterium *L. foveolarum* yet, in copepods, the amount of PUFA was proportional to the total FA content ($F_{2,8} = 3.4$, $P > 0.05$).

Discriminant analysis correctly predicted algal group membership from the FA data set measured in this study. Algae were classified by one discriminant function with 100% cross-validation accuracy (Eigenvalue = 647.8, Canonical correlation = 0.99; Fig. 5). Cross-validation was made by a leave-one-out classification, i.e. each case was classified using a discriminant function based on all cases except the given case. The most important FA predicting group membership were 16:1 ω 7 and 20:5 ω 3 which presented the highest standardized canonical discriminant function coefficients (1.07 and 0.911, respectively). 16:1 ω 7 and 18:2 ω 6 had the highest correlations (i.e. loadings) with the discriminant function, contributing to the discriminant function in opposite directions (Fig. 6). FA that had equal group means (16:2, 18:0 and 18:1 ω 7) or were highly correlated with other FA (16:1 ω 9

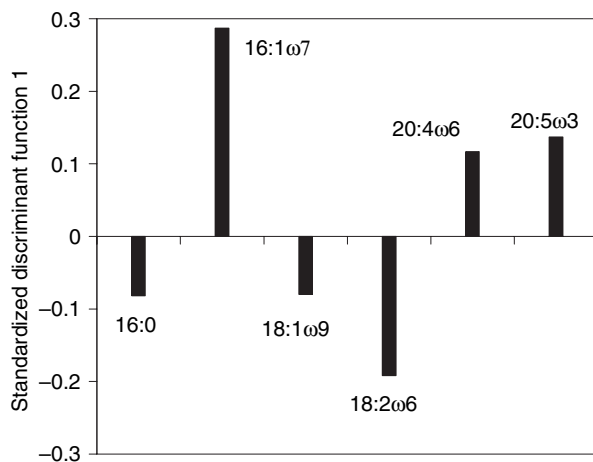


Fig. 6 Within-group correlations between discriminating variables (fatty acid, FA) and the standardized canonical discriminant function enabling the discrimination of diatoms and cyanobacteria based on their FA profiles.

inversely correlated with 16:1 ω 7, 18:3 ω 3 correlated with 18:2 ω 6) or were present in only one algal class (e.g. 16:2 ω 4, 16:3 ω 4, 18:4 ω 3 and DHA in diatoms) were removed from the analysis. The diet of copepods feeding on diatoms or cyanobacteria was accurately identified by the application of the discriminant function to the FA content of copepod reserve lipids (Fig. 5). DA therefore confirmed the observed trends in FA composition of copepods and their diet.

When a different FA data set that included the composition of diatoms, cyanobacteria and green algae was analysed (published data, see Methods), DA enabled correct membership classification among the three algal groups (89.4% cross-validation accuracy, Fig. 7). Two discriminant functions were obtained from the analysis: function one contained 95.3% of total variance (Eigenvalue = 43.9; Canonical correlation = 0.98) and, function two contained the remaining 4.7% variance (Eigenvalue = 2.5; Canonical correlation = 0.83). The most important FA in predicting group membership were 18:3 ω 3 for function 1 and 18:1 ω 9 for function 2 with the standardized canonical discriminant function coefficients of 1.16 and 0.61 respectively. 16:1 ω 7 and 18:3 ω 3 had the largest correlations with the discriminant function 1, contributing in opposite directions (Fig. 8). 18:3 ω 3

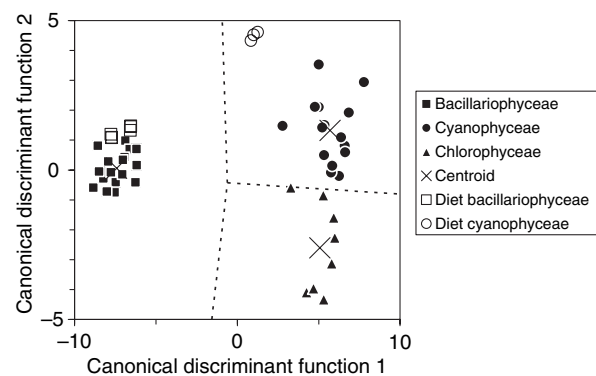


Fig. 7 Distribution map of algae (closed symbols) and copepods feeding on diatoms (open squares) or cyanobacteria (open circles) according to the discriminant functions calculated from algal fatty acid (FA) profiles. Algal FA data from literature (see Methods). Large asterisks indicate group centroids. Copepod positions were determined from their reserve FA content. Function 1 = $-6.750 - 0.012 (14:0) + 0.098 (16:0) - 0.087 (16:1\omega7) + 0.228 (18:1\omega9) - 0.077 (18:1\omega7) + 0.197 (18:2\omega6) + 0.232 (18:3\omega3)$, Wilks' $\lambda = 0.070$, $\chi^2 = 168.780$, $P < 0.001$; function 2 = $-1.929 + 0.040 (14:0) + 0.059 (16:0) + 0.017 (16:1\omega7) + 0.243 (18:1\omega9) + 0.108 (18:1\omega7) + 0.040 (18:2\omega6) - 0.091 (18:3\omega3)$, Wilks' $\lambda = 0.314$, $\chi^2 = 39.356$, $P < 0.001$.

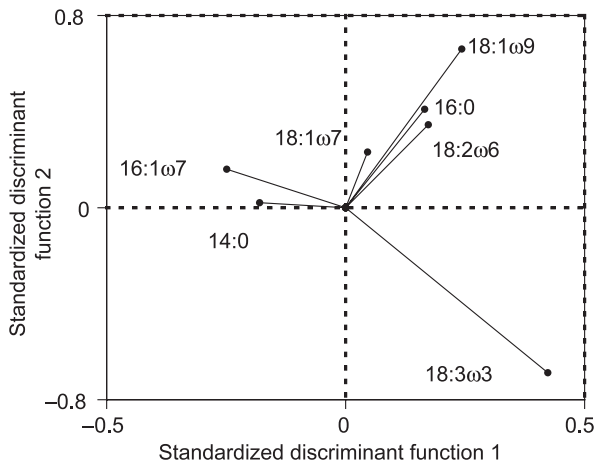


Fig. 8 Within-group correlations between discriminating variables (fatty acid, FA) and the standardized canonical discriminant functions enabling the discrimination of diatoms, green algae and cyanobacteria based on their FA profiles obtained from published data (see Methods).

and 18:1 ω 9 had the largest correlations with the discriminant function 2, contributing in opposite directions. As before, FA that had equal group means (16:2 and 18:0) or were absent from one algal class (16:2 ω 4, 16:3 ω 4, 18:4 ω 3, EPA and DHA) were removed from the analysis. Confirming the results from the first DA, discriminating functions derived from the published algal data when applied to the FA composition of the copepod reserve lipids allowed for the accurate classification of copepod diet (Fig. 7).

Discussion

Harpacticoid copepods could grow and develop on either diatom species and on one species of cyanobacteria. Nevertheless, diatoms were of better food quality than cyanobacteria as indicated by shorter development time and higher survival of copepods. FA composition of the copepod reserve (or neutral) lipids reflected diet FA composition. This relationship may be used to elucidate the use of food sources by copepods in freshwater ecosystems, where benthic algal mats are often a mixture of diatoms and cyanobacteria. The relatively constant FA composition of copepod polar lipids irrespective of the diet suggests the copepod ability to either selectively retain certain FAs from the diet or to synthesize long-chain FAs that are incorporated into the membrane lipids (e.g. EPA and DHA when feeding on cyanobacteria). A pronounced magnification of the

EPA content of *Daphnia* relative to the EPA content of the diet seston has been detected (Brett *et al.*, 2006; Hessen & Leu, 2006). In the present study, we cannot overrule the selective retention of EPA from either the diatom or the cyanobacterial diet by the copepod. However, the presence of DHA in the copepod polar lipids and its absence from the cyanobacterium suggests bioconversion from other dietary FAs.

Fatty acid weight per carbon content in diatoms was at least twice as high as that in cyanobacteria. High FA content in diatoms is a general feature of diatoms relative to cyanobacteria. For example, it has previously been reported that the FA content in diatoms ranges from 11% to 25% dry weight (Dunstan *et al.*, 1994; Tan & Johns, 1996) while in cyanobacteria, the FA content ranges from 1.2% to 5.7% dry weight (Ahlgren *et al.*, 1992; Vargas *et al.*, 1998). The same trend was observed in copepods feeding on each algal group suggesting that diatoms were of higher dietary value than cyanobacteria. The two algal groups exhibited conspicuously different FA profiles. High amounts of 16:1 ω 7 in diatoms and in copepods feeding on diatoms, and a near absence of this FA in cyanobacteria and in copepods feeding on cyanobacteria indicates 16:1 ω 7 has value as a FA trophic marker (e.g. Léveillé *et al.*, 1997). In the copepod reserve lipids, the ratio of 16:1 ω 7/16:0 was above one suggesting that such a ratio characteristic of diatoms also indicates a diatom diet (Dunstan *et al.*, 1994). A high 16:1 ω 7 content has been previously observed in the herbivorous marine genus *Calanus* feeding on phytoplankton with high diatom abundance (Albers, Kattner & Hagen, 1996; Dalsgaard *et al.*, 2003). Copepods feeding on diatoms had a small content of EPA in their reserve lipid fraction and EPA was absent from the reserve lipids of copepods feeding on cyanobacteria. EPA may thus be a indicator of a diatom diet when present in the copepod reserve lipids. However, EPA as single diet marker should not be used when comparing algal diets equally rich in EPA (e.g. diatoms and cryptophytes, see Brett *et al.*, 2006). The opposite pattern was observed for 16:1 ω 9 and 18:2 ω 6 which were abundant in cyanobacteria and nearly absent in diatoms and in copepods feeding on diatoms. 18:2 ω 6 is generally <2% of total FA weight or a minor FA in diatoms (Sargent *et al.*, 1987; Dunstan *et al.*, 1994). However, 18:2 ω 6 FA together with 18:3 ω 3 FA are also commonly abundant in green algae (e.g. Weers *et al.*, 1997) which means 18:2 ω 6

cannot be used alone to differentiate between cyanobacteria and green algae. Additionally, 16:2 ω 4 and 16:3 ω 4 are characteristic of diatoms (e.g. Dunstan *et al.*, 1994; Léveillé *et al.*, 1997, Dijkman & Kromkamp, 2006), but were not observed in the copepod lipids and cannot therefore be used as diet markers. The use of single biomarkers for food source elucidation can therefore be problematic in some systems and, identification of the diet should be based on the analysis of multiple FAs in the reserve lipids.

Discriminant analysis classifying algae into a dual group system based on their FA content agreed with the direct observations of FA profiles: the content in 16:1 ω 7, 18:2 ω 6 and EPA were the major contributors to the function classifying algae as either diatoms or cyanobacteria. When the DA was applied to a data set including an additional algal group (green algae), the major FA contributors to algal group discrimination became 16:1 ω 7, 18:1 ω 9 and 18:3 ω 3. Green algae and cyanobacteria had abundant 18:3 ω 3 while diatoms had abundant 16:1 ω 7 and these were the main contributors to the first canonical function that discriminated between diatoms and the other two algal groups. The second function discriminated group membership between green algae and cyanobacteria based on the percentage of 18:1 ω 9 and 18:3 ω 3: Cyanobacteria had higher 18:1 ω 9 content relative to green algae and green algae had higher 18:3 ω 3 content relative to cyanobacteria in agreement with several authors (Bourdier & Amblard, 1989; Ahlgren *et al.*, 1992; von Elert & Stampfl, 2000; Léveillé *et al.*, 1997). The need to use a suit of FAs instead of single FA as diet markers is again emphasized by this DA.

The discriminant functions obtained from the analysis of both dietary algae (two algal groups) or published data (three algal groups) correctly classified the diet of copepods based on the FA content of their reserve lipids. The applicability of these discriminant functions to infer copepod diet suggests that the FA content of reserve lipids reflects that of the diet even when some FA bioconversion occurs. The similarity of FA profiles between the diet and the reserve lipids of copepods that bioconvert FAs has been reported for cyclopoid copepods (Desvillettes *et al.*, 1997b). Cyclopoid copepods are potentially able to incorporate dietary 18:3 ω 3 originating from microalgae into the PL and bioconvert it into DHA (Farkas *et al.*, 1981; Desvillettes *et al.*, 1997b). FA bioconversion must have occurred in the present study with *A. trispinosa*. The

cyanobacteria had mostly C₁₆ and C₁₈ FA although *L. foveolarum* may synthesize EPA in trace amounts as previously observed for the cyanobacterial genera *Oscillatoria*, *Microcystis* and *Anabaena* (Ahlgren *et al.*, 1992; Vargas *et al.*, 1998). The two diatoms used in this study were rich in EPA as is characteristic for this group (e.g. Sargent *et al.*, 1987; Dunstan *et al.*, 1994). Notwithstanding the absence or low content of EPA in the diet, *A. trispinosa* had both DHA and EPA. The presence of EPA and DHA in copepods feeding on the cyanobacterium *L. foveolarum* suggests that copepods synthesized these PUFA from other FA, possibly from 18:3 ω 3 as reported for other harpacticoid copepods (Watanabe *et al.*, 1978; Norsker & Støttrup, 1994; Nanton & Castell, 1998, 1999).

Lipids are the primary storage material in diatoms which are therefore of great value as lipid producers, especially PUFA, in aquatic food webs (Dunstan *et al.*, 1994). The concept of FA being transferred conservatively through aquatic food webs was first suggested by Lovern (1935) and later D'Abramo (1979) suggested that the FA content of phytoplankton may determine its quality as food for zooplankton. In both freshwater and marine environments there is mounting evidence that PUFA are essential compounds that can limit zooplankton productivity (Jónasdóttir *et al.*, 1995; Müller-Navarra & Lampert, 1995). Cyanobacteria are generally regarded as poor-quality food (e.g. Gulati & DeMott, 1997). The poor quality of cyanobacteria relatively to that of diatoms and flagellates can be accounted by differences in FA composition (Ahlgren *et al.*, 1990). Recently, the high dietary value of diatoms has been a subject of controversy regarding possible deleterious effects of diatoms on copepod reproduction (see review by Ianora, Poulet & Miralto, 2003). However, this effect impairs hatching success of eggs and, to our knowledge, it does not affect post-embryonic development of copepods, and was never reported for freshwater benthic species. In our study, *A. trispinosa* feeding on diatoms had a development time similar to that of the marine harpacticoids *Tigriopus californicus* growing at 18–20 °C (Powlik, Lewis & Spaeth, 1997) and *Amphiascoides atopus* growing at 23 °C (Sun & Fleeger, 1995). The high nutritive value of diatoms was stressed by the prompt maturation of oocytes in females within 7 days of moulting into the adult stage (Kleppel, Burkart & Houchin, 1998; Hazzard & Kleppel, 2003). The low development rate and high mortality of *A. trispinosa*

growing on *L. foveolarum* confirms the low dietary value of cyanobacteria when compared with diatoms. Both *L. foveolarum* and *C. stagnale* had 18:3 ω 3 in addition to saturated fatty acids and MUFA (group II cyanobacteria according to Kenyon *et al.*, 1972; Murata *et al.*, 1992). 18:3 ω 3 in the cyanobacterium *L. foveolarum* possibly enabled bioconversion by the copepod to obtain the essential EPA and DHA (Farkas *et al.*, 1981). *Cylindrospermum stagnale* had a higher 18:3 ω 3 content than *L. foveolarum* but could not sustain naupliar development to the first copepod stage. It is possible that *C. stagnale* contains some toxins interfering with the copepod digestive enzymes (Juttner & Wessel, 2003). Successful development on a diet of cyanobacteria does not necessarily imply the maintenance of copepod populations on this diet alone. Although copepods grew and developed on *L. foveolarum*, their reproduction may be impaired as females failed to mature oocytes within 7 days after moulting into the adult stage. Nevertheless, the ability to either accumulate long-chain PUFA (EPA) or to bioconvert PUFA (DHA from short-chain PUFA) ascribes great importance to harpacticoids in improving the quality of nutrients that are transferred to higher trophic levels. The higher PUFA content of herbivores relative to the content of their diet was previously reported for *Daphnia* (Brett *et al.*, 2006; Hessen & Leu, 2006; Persson & Vrede, 2006). Brett *et al.* (2006) suggested that the accumulation of PUFA in *Daphnia* would transfer the benefits of consuming ω 3 FA up the food web. The accumulation and potential bioconversion of PUFA in harpacticoids also suggests that these organisms are valuable trophic intermediaries in transferring organic matter in the benthic food web.

Acknowledgments

We thank Elizabeth C. Verbree (Vrije Universiteit Amsterdam, The Netherlands) for her help with fatty acid analysis. This study was made possible by a grant, PRAXIS XXI/BPD/20223/99, awarded to M-JC by Fundação para a Ciência e Tecnologia, Portugal.

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(Manuscript accepted 5 August 2007)