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Net gain of long-chain polyunsaturated fatty acids (PUFA) in a lugworm *Arenicola marina* bioturbated mesocosm

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ABSTRACT: A net gain in long-chain polyunsaturated fatty acids (PUFA) occurs in *Arenicola marina* bioturbated mesocosms in the light and dark when brewery yeast is the only food. Fatty acid profiles were determined by GC-MS and quantified for system units. Bacterial fatty acid biosynthesis is indicated by accumulation of the bacterial fatty acids anteiso 14:0, iso 15:0, iso 16:0, anteiso 16:0, iso 17:0, C16:1n-7 and C18:1n-7 (*cis*-vaccenic acid). De novo production of longer chain PUFA within the worm tissues is implicated by a chain elongation converting linoleic acid (C18:2n-6) to C20:2n-6 and subsequent desaturation, resulting in a gain of arachidonic acid (C20:4n-6) and eicosapentaenoic acid (C20:5n-3) via the intermediary C20:3n-6. To test for the presence of PUFA-synthesising bacteria in system units, 495 cloned 16S rDNA fragments were compared with the databases and degenerate PCR primers were designed for the ketoacyl acyl carrier protein synthase gene highly conserved in the polyketide synthase region of known PUFA-synthesising *Gammaproteobacteria*. *Gammaproteobacteria* related to known PUFA producers were not abundant (<1% of clones) and PCR primers, whose specificity was confirmed by conspecific amplification of DNA product from genomic DNA of *Shewenella frigidimarina* 12253^T and *Colwellia psychrethraea* 8813^T, did not amplify product from mesocosm DNA samples. PUFA production is therefore primarily due to processes within the lugworm *A. marina*. Biosynthesis by invertebrates may be significant in benthic ecosystems and their culture has the potential to contribute to non-fishery sources of essential fatty acids required for aquaculture feeds.

KEY WORDS: Fatty acid biosynthesis · Elongase · PUFA · Polychaeta · Marine food web · *Arenicola marina* · Aquaculture nutrition · Aquaculture feeds

INTRODUCTION

The caudate lugworms *Arenicola marina* (L.) and *A. defodiens* Cadman and Nelson-Smith, 1993 are frequently the biomass-dominant species on the intertidal beaches of western Europe (Cadée 1976, Beukema & de Vlas 1979, Olive 1993). The basic feeding method is well established, but the precise nature of their dietary structure remains problematical. Lugworms irrigate a J-shaped burrow driving oxygenated water into the deeper sediment layers (Riisgård & Banta 1998, Meysman et al. 2005), where they ingest sand at a feeding pocket, egesting it at frequent intervals at the sand surface to form characteristic casts and a faecal mound. Organic material (detritus, diatoms and bacteria) may move down a narrow column of sand (the

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head shaft) lying above the feeding pocket and contribute to the diet, but algae are not utilised effectively (Rijken 1979) and the presence of cellulases has not been conclusively demonstrated (Longbottom 1970, nor since). On the other hand feeding on bacteria associated with enriched sand is considered likely. The short residence time of the ingested sand in the gut (Hüttel 1990, Chen & Mayer 1999) makes it unlikely that internal fermentation (as in ruminants) occurs; rather, the lugworm-bioturbated burrow/sand-substrate system may act as an external rumen in which bacteria are cultivated, ingested and digested—a process of food enhancement referred to as gardening (Plante & Mayer 1994, 1996). Feeding at depth on anaerobic sediment is most characteristic of *A. defodiens* (Cadman & Nelson-Smith 1993) but it is also observed in *A. marina* when feeding on sediments rich in organic material in nature (Cadée 1976) and in the laboratory (Rijken 1979), and is routinely observed in commercially farmed populations[1] (S. Craig unpubl. data, P. J. W. Olive pers. obs.). Lugworms are well equipped for the digestion of bacteria, having blind-ending caecae, the oesophageal glands that release bacteriolytic surfactants leading to differential digestion of the bacteria associated with the ingested sediment (Plante & Mayer 1996, Plante 2000).

Arenicola marina contain a wide variety of fatty acids (Olive et al. 2002) as do all other polychaetes and relatives investigated to date (e.g. Taghon 1988, Fidalgo e Costa et al. 2000, Copeman & Parrish 2003, García-Alonso et al. 2008). The origin of the fatty acids in benthonic, easily cultured polychaete worms, such as Nereidae and Arenicolidae, has assumed an economic importance because their fatty acid content, coupled with their high palatability, has lead to their inclusion as a component of aquaculture brood stock diets (Izquierdo et al. 2001).

A frequently accepted paradigm is that, in marine ecosystems, polyunsaturated fatty acids (PUFA) are mainly derived from biosynthesis by diatoms in the photic zone and recycled through the marine food web (Copeman & Parrish 2003, Bergé & Barnathan 2005). The relative abundance of long-chain PUFA derived from diatom biosynthesis in marine ecosystems has indeed been thought to have allowed marine animals to evolve a dietary dependence on them without loss of function (see discussion in Sargent et al. 2002). Accordingly, the occurrence of long-chain PUFA in marine invertebrates is thought to be indicative of a direct trophic link to the photic zone, even in the case of deep-sea organisms, where such a linkage would appear tenuous and counter-intuitive (Bühring & Christiansen 2001, Pond et al. 2002, Phleger et al. 2005). Such an analysis, however, underplays the importance of (1) the presence of bacteria able to synthesize PUFA (predominantly eicosapentaenoic and docosahexaenoic acids, EPA and DHA, respectively) in the marine benthos (Russell & Nichols 1999, Nichols & McMeekin 2002, Nichols 2003), (2) invertebrate–bacteria symbioses (Dubilier et al. 2008) and (3) possible chain elongation and desaturation resulting in de novo production of PUFA by invertebrates (Pereira et al. 2003). This is best known for the terrestrial nematode *Caenorhabditis elegans*, where the existence of the complete genome sequence has facilitated genetic dissection of the pathways involved (Watts & Browse 2002), but it cannot be excluded for other invertebrates. In the present study, we adopted a mesocosm approach to determine the extent to which the lugworm is dependent on external sources for the many PUFA that accumulate in its lipids. We quantified all fatty acid inputs to system units maintained in both the light and the dark, and the fatty acid content of the various system compartments after a period of bioturbation and worm growth.

MATERIALS AND METHODS

Mesocosm construction and function. ***Open systems:*** Plastic boxes (23 × 37 × 27 cm, width × length × height) were prepared by the addition of 3 l of marine sand to which had been added an equal volume of either (1) spent brewer's yeast (food type BY, an abundant by-product of the brewing industry; Federation Brewery), (2) a mixture of the same spent yeast and a fish farm slurry (food type BYFW; Blue Water Flatfish Farms) providing bacterial enrichment or (3) marine sand without the addition of food (unfed negative controls). Further unenriched beach sand was then added to each box to a depth of around 15 cm. Two boxes of each type were placed in concrete tanks in natural daylight, during April–May when the photophase exceeded 12 h, and 2 boxes of each type were placed in concrete tanks from which light was excluded by means of a double layer of black plastic sheeting. For each replicate pair of boxes, one was not stocked with worms (no worms negative control); the other received 30 individually weighed juvenile *Arenicola marina* (mean weight = 0.02 g) supplied from cultures reared by Seabait Ltd. Each tank was then supplied with running seawater maintained at a temperature of 16 ± 2.5°C using a mixture of power station-heated and natural seawater (all operations with the permission of RioTintoAlcan, Lynemouth Power Station).

The juvenile lugworms entered the unenriched overlying sand substrate and commenced bioturbation

[1]GB patent number GB2383934, USA patent application 10/483314, European Patent application 02745561.7

activities as evidenced by the onset of casting activity. The animals were allowed to grow in the system units without the addition of further food for 42 d, after which time worms were removed for analysis. The experimental design included a double negative control (unfed, no worms) that made it possible to test for the influence of both worms and the added food on the generation of fatty acids within system units.

Closed systems: A closed system was designed using multiple plastic boxes (20.5 × 31.5 × 15 cm) supplied with recirculated artificial seawater (Tropical Marine Centre) made from deionised tap water in a closed laboratory environment. The experimental protocol was based on a simplified nested factorial design with the treatments: dark (D) and light (L), each with spent brewer's yeast (BY) and without food (C) with and without the addition of worms. Spent brewer's yeast, aged in seawater for a period of 2 to 3 wk, was mixed with clean sand (low water neap tide level, Cullercoats) at a dosage of 0.0155 ml cm^{-3} (150 ml per box). A small quantity (20 g) of used sand, in which lugworms had previously been grown, was added as a source of suitable bacteria. Further clean sand was then added to a depth of 8 cm. Light was excluded from dark mesocosms using double thickness black plastic; light treatments were exposed to a 16 h light:8 h dark cycle (Sangamo Suntracker lighting system) using Phillips 56W fluorescent tubes. The temperature was kept at 17 to 18°C, salinity at 32 to 33 ppt, pH at 8.0 to 8.3 and dissolved oxygen at 7.5 to 8.5 mg l^{-1}. After 2 d, 216 individually weighed juvenile lugworms, previously depurated to empty their guts for 24 h, were selected at random and 18 individuals placed in each box (except for the no worms negative controls).

Initial samples of beach sand, used sand, brewer's yeast and whole lugworms were flushed with oxygen-free nitrogen and kept at –20°C. After 42 d, further sand samples were taken from the deep yeast layer (DYL), new casts (NC), old casts (OC) and the mid-sand layer (MSL) at a depth of approximately 6 cm using a plastic core. Boxes were then emptied; surviving worms were counted, depurated for 24 h, weighed and placed in vials, flushed with nitrogen and stored at –20°C for analysis.

Worm growth: The number of surviving worms was determined and their wet weight measured after damp drying on absorbent paper. Survival rate (%), specific growth rate (SGR), percentage weight gain (PWG) and absolute growth rate (AGR) were computed. In order to make allowances for mortality in subsequent calculations, the number of worms added to the system was assumed to be equal to the number of worms eventually harvested. This procedure underestimates slightly the net gain in any fatty acid per system unit, but in the closed systems the underestimate was small because of the high survival rate (>97%) and the uniform size and low body mass (~0.02 g) of the juvenile worms added.

ANOVA, followed by *a posteriori* multiple comparisons among means (Tukey's HSD), was used to compare SGR, PWG, AGR and survival rates between treatments. All experimental protocols were conducted in accordance with institutional, national and international guidelines concerning the use of animals in research and were in compliance with published guidelines for applied ethological research (Sherwin et al. 2003).

Fatty acid analysis. Lipid was extracted from sand and animal samples in a mixture of 2:1 v/v chloroform and methanol (Folch et al. 1957). Sand samples (1 to 2 g wet weight) were extracted with 10 ml per g of sand. Weighed samples of worm tissue (0.5 to 1.5 g wet weight) were homogenized in chloroform/methanol (2:1 v/v, 30 ml g^{-1} wet weight), extracted overnight, vacuum-filtered (Whatman 2E filter paper) and 25% of the starting volume of 0.88% KCl added. After separation, the upper layer was discarded and solvent removed by evaporation under oxygen-free dry nitrogen at 37°C until dry. Lipids were resuspended in 1 ml of toluene and 2 ml 1% sulphuric acid in methanol, flushed with nitrogen and incubated at 80°C for 1 h. Fatty acid methyl esters (FAME) were then resuspended with hexane (with 0.01% butylated hydroxytoluene) and kept in amber GC vials under oxygen-free nitrogen at –20°C. For each analysed tissue, 3 similar aliquots were weighed, lyophilised overnight to constant weight and the average weight loss used to estimate the equivalent dry weight of the analysed tissue sample. FAME were then expressed as mg g^{-1} dry weight.

FAME were analysed using capillary gas chromatography-flame ionization detection (GC-FID) on a Hewlett-Packard 5890 Series 2 chromatograph equipped with a DB-23 J&W column (30 m × 0.25 mm internal diameter, 0.25 µm film thickness), using hydrogen as the carrier gas at constant pressure (50 kPa) giving a flow rate of approximately 2 ml min^{-1}. For optimum separation, the temperature profile was programmed at 60°C for 1 min and then increased by 20°C min^{-1} to 140°C, held for 3 min, increased by 10°C min^{-1} to 190°C, held for 3 min, and then increased by 10°C min^{-1} to 220°C and held for 8 min.

The preliminary identity of individual peaks was established using a selection of external standards: (1) single nonadecanoic acid methyl ester (C19:0); (2) Supelco cod liver oil; (3) Supelco FAME mix; (4) Supelco 37 component FAME mix; and (5) GLC-538 (Nu-Chek prep standards). Confirmation and characterisation of fatty acids, including those not present in the external standards, was carried out by GC-MS performed on an Agilent 6890 GC split/splitless injector (260C) linked to a Hewlett-Packard 5973MSD. The

acquisition was controlled by a HP Kayak xa pc chemstation computer, initially in full scan mode (50 to 550 amu s^{-1}) or in selected ion mode for greater sensitivity. The sample (1 ul) was injected by an HP7683 auto sampler and the split opened after 1 min. After the solvent peak had passed, the GC temperature programme and data acquisition commenced. Separation was performed on a fused silica capillary column (30 m × 0.25 mm internal diameter) coated with 0.25 µm DB-23 J&W phase. The same temperature programme as GC was adopted (see above). The total running time was about 30 min with helium as the carrier gas. The acquired data was stored on DVD for later data processing, integration and printing.

Each fatty acid peak was examined and identified by comparing the mass spectra of the fatty acid with the spectra from the GC-MS library programme NIST 98. Machine calibration and fatty acid concentrations were carried out by comparison of the peak area with that of the C19:0 internal standard; calibration curves for C19:0 showed that for each GC run, the column response was linear (r^2 = 0.98) in the concentration ranges used and no further adjustment was necessary.

Each treatment was replicated 3 times. In order to test for differences in fatty acid concentration between treatments, the lugworm tissues from each treatment were kept separate and were pooled (i.e. within-treatment replicates) prior to homogenisation. It was not deemed feasible to estimate individual variation between worms within treatments. Two sample runs for each replicate were carried out. Two-level nested ANOVAs were carried out to test for significant differences among runs within treatments and single classification ANOVA was carried out subsequently to test for differences among treatments. Such differences were further analysed using Tukey's multiple comparison test (Tukey's HSD) within SPSS version 12. Statistical tests were carried out for each fatty acid identified and, where significant differences were detected, the same lower case letter is used in tabulation of data to identify means that did not differ significantly ($p > 0.05$); different lower case letters among means indicate significant differences ($p < 0.05$). Tabulated values show mean (±SE) concentrations (mg g^{-1} dry weight).

Quantification of system inputs and outputs. ***Fatty acid accumulation in lugworm tissues:*** The quantity of each fatty acid (mg) in the worm tissues at the end of the experiment was calculated as the product of fatty acid concentration (mg g^{-1} dry weight) and worm biomass (g dry weight). Start values were determined from the measured wet weight and adjusted to allow for mortalities during the course of the experiment (initial number assumed to be equal to the final number of worms recovered). The net gain or loss was then determined for all fatty acids.

System inputs and outputs: Fatty acid inputs to system units were calculated individually for each fatty acid as the sum of (1) the fatty acid content of the added beach sand and used sand added, (2) the fatty acid content of the added food yeast (or yeast + fish farm waste mixture) and (3) the concentration of fatty acids in the whole worm tissue multiplied by the initial worm biomass (zero in no worm controls). The fatty acid output from the system units was calculated as the sum of the average fatty acid content of sand samples from the DYL, NC, OC and MSL multiplied by the total sand volume and the concentration of fatty acids in the whole worm tissue multiplied by the final worm biomass (zero in no worm controls).

A mean net gain or net loss (mg) of each fatty acid present was then calculated for system units. The term 'biosynthesis' is used here to indicate a net increase in the amount of any fatty acid in any one of the closed system units during the course of the experiment.

Screening for EPA and DHA-synthesising bacteria using universal and specific primers. Samples of sand from the DYL, NC, OC and MSL were taken from mesocosms cultured in the absence of light to investigate bacterial biodiversity. Total genomic DNA was extracted using a sodium dodecyl sulphate (SDS)-based extraction method (Zhou et al. 1996), purified to remove small DNA fractions (BIO-RAD Micro Bio-Spin 6 column, Tris) with the addition of 100 µl of 10% polyvinylpolypyrrolidone (PVPP) to the top of the column to aid humic acid removal (Berthelet et al. 1996). The 16S rRNA genes were amplified using universal bacterial primers 27F and 1525R (Weisburg et al. 1991) by PCR (Peltier thermal cycler, DNA Engine DYAD) with the following reaction mix: 100 to 200 ng sediment DNA, 10 µM (0.4 µl) of each primer, 0.4 µl 10 mM dNTP, 0.6 µl non-aselletated BSA (0.5 mg ml^{-1}), 2 µl 10× buffer (Biolab), 0.5 µl Biolab standard *Taq* and Milli-Q water to a final volume of 20 µl. Thermal cycling protocol was 95°C for 2 min, 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 2 min and a final step at 72°C for 15 min. Clone libraries were constructed from the 16S genes (TOPO TA cloning kit) and inserted fragments amplified via plasmid amplification (TempliPhi 100 amplification kit). Approximately 200 clones from each site were sequenced by the IRES Sequencing Service at Newcastle University, UK (full details in Ashforth 2008). Sequence contigs were aligned and new DNA fragments formed in Sequencher™ (Gene Codes). Chimeras originating from more than one target sequence during PCR were detected using MALLARD (www.bioinformatics-toolkit.org). The sequences were classified into phylogenetic groups using RDP Classifier (http://rdp.cme.msu.edu/classifier/classifier.jsp). These sequences were aligned in CLUSTAL (www.ebi.ac.uk/Tools/clustalw2/index.html) and neighbour-joining trees were created in TREECON. The anaerobic path-

way for the biosynthesis of polyunsaturated fatty acids in bacteria is known to use large multidomain proteins in a novel polyketide synthases (PKS) system. PKS sequences belonging to known PUFA producing strains of *Shewanella*, *Colwellia* and *Moritella* were collected and aligned in MEGA 4 and were blasted (Altschul et al. 1997) against the non-redundant (nr) database at the National Center for Biotechnology Information (NCBI). Genera were aligned separately and together in CLUSTAL X. Most of the *Shewanella* sequences aligned together as did the *Moritella* with the *Colwellia*. Consensus sequences were made from the alignments and primers for the ketoacyl ACP synthase gene located on the pfaA section of the PUFA-biosynthetic gene cluster for *Shewanella* and for *Colwellia–Moritella* were recommended using the online program GeneFisher (http://bibiserve.techfak.unibiefeld.de/genefisher2/).

Based on each consensus alignment for *Shewanella* and *Moritella–Colwellia*, GeneFisher produced 8 possible primer pairs. Primers were blasted against the database to check their specificity. Of those 8, the most compatible primer pair, in terms of melting temperature, primer length and blast specificity (Table 1), was selected and ordered from Invitrogen.

Two strains obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen — *Shewanella frigidimarina* 12253^T (supplied as dried culture) and *Colwellia psychrerythraea* 8813^T (American Type Culture Collection; ATCC 27364; supplied as actively growing on agar) — were used as positive controls to test the primers and optimise conditions. *S. frigidimarina* 12253^T was grown overnight at 20°C in Bacto marine broth and then spread onto Bacto Marine agar plates. Colonies were seen after 2 d incubation at 20°C. *C. psychrerythraea* 8813^T was spread directly onto Bacto Marine agar plates and incubated at 10°C for 14 d. Cultures from both strains were checked for confirmation of genera and purity in terms of colony homology, Gram stain (both negative) and cell shape (both rod). DNA was extracted from each strain using the Sigma genelute bacterial genomic DNA kit.

Gradient PCR was carried out for *Shewanella frigidimarina* 12253^T (with *Shewanella* PKS primer pair) and *Colwellia psychrerythraea* 8813^T (with *Moritella–Colwellia* PKS primer pair). The initial PCR reaction mix included 100 ng genomic DNA, 0.4 µl 10 µM primer, 0.4 µl 10 mM dNTP, 0.6 µl non-acetylated BSA (0.5 mg ml^{-1}), 2 µl of 10× buffer Biolab, 0.5 µl Biolab standard *Taq* and Milli-Q water to a final volume of 20 µl. The thermal cycling protocol was 95°C for 2 min, 30 cycles of 95°C for 30 s (gradient 15°C, lowest 50°C for 30 s), 72°C for 2 min and a final step at 72°C for 4 min. It was observed that the *Shewanella* PKS primer pair's optimum annealing temperature was 56.7°C and for the *Colwellia–Moritella* PKS primer pair it was 60.4°C. Sensitivity of the primers was determined by PCR of 100, 10, 1 and 0.1 ng of genomic DNA using the same reaction mix and cycling protocol for each primer set. As a negative control, the PCR reaction was repeated using genomic DNA of *C. psychroreythria* 8813^T in the reaction mix with the forward and reverse *Shewenella* primer pair and *S. frigidimarina* 12253^T as genomic DNA in the reaction mix with the forward and reverse *Colwellia–Moritella* primer pair.

PCR products were purified using the Gen-elute PCR clean-up kit and sequenced by the IRES Sequencing Service of Newcastle University. The DNA contigs were aligned in Sequencher^{TM} and the resulting DNA fragments were blasted against the NCBI database. Both the *Shewanella* and *Moritella–Colwellia* DNA fragments matched their target sequences, i.e. the *S. frigidimarina* 12253^T and *C. psychrerythraea* 8813^T PKS genes, respectively.

RESULTS

Open system units

Growth and survival

The growth and survival data are summarised in Table 2. Comparisons between treatments were carried out by ANOVA, which confirmed that there were no significant differences among the initial mean weights of the groups of juvenile lugworms introduced to the different system units ($p > 0.05$), but there were significant differences among treatment mean weights after

Table 1. *Shewanella* and *Moritella*. Data for the forward and reverse primers selected for the ketoacyl ACP synthase gene in the polyketide synthesis (PKS) gene cluster region that is highly conserved in PUFA-biosynthesising bacteria. Primers were selected from a set of 8 potential primers suggested by the GeneFisher programme based on their melting temperature, primer length and blasted against the NCBI nr database to check specificity, prior to manufacture

Primer pair	Sequence (5′-3′)	
	Forward	Reverse
Shewanella PKS	RGAYCAATATRTHCACTGGGAA	GGYAABACYTTTTGRTGCA
Moritella–Colwellia PKS	ATCGGYTGGGAAGARAACTCA	CATCTTCACGTGSCATCCA

Table 2. *Arenicola marina*. Open mesocosms. Initial and final weight (means ± SE), specific growth rate (SGR), absolute growth rate (AGR) and survival of *A. marina* grown in system units. C: negative unfed control; BY: brewer's yeast; BYFW: brewer's yeast plus fish waste. Where ANOVA revealed significant differences among mean weights, Tukey's HSD is summarised by assigning a different lowercase letter to indicate significant differences ($p < 0.05$)

	Weight (g)		SGR	AGR	Survival
	Initial	Final	(% d^{-1})	(mg d^{-1})	(%)
Light					
C	0.01 ± 0.00^{a}	0.31 ± 0.02^{b}	6.95	6.88	37
BY	0.02 ± 0.00^{a}	0.73 ± 0.06^{cd}	8.72	16.95	20
BYFW	0.02 ± 0.00^{a}	0.79 ± 0.03^{d}	8.54	18.18	30
Dark					
C	0.02 ± 0.00^{a}	0.17 ± 0.01^{a}	5.15	3.56	37
BY	0.02 ± 0.00^{a}	0.63 ± 0.09^{c}	8.13	14.55	30
BYFW	0.03 ± 0.00^{a}	0.78 ± 0.05^{d}	8.21	17.88	57

42 d ($p < 0.01$). *A posteriori* tests for differences among the treatment group means showed that all treatments provided with brewer's yeast substrate increased in weight to a greater degree than the unfed controls ($p < 0.01$) and the highest growth rates were observed in 3 treatments, BYFW(L), BYFW(D) and BY(L), among which there were no significant differences. Nevertheless, significant growth, albeit at lower values than in fed treatments, was observed in the unfed negative controls in comparison with their starting values ($p < 0.01$). This indicates that there was an extraneous source of nutrition in these open systems. This could have been (1) organic matter already associated with the initial sediment, (2) organic matter associated with entrapped silt entering the system with the seawater supplied, (3) photosynthesis within the box units kept in the light, (4) anaerobic bacterial chemolithoautotrophy within the anaerobic sulphidic layer of the mesocosms or (5) a combination of these. However, the weight gain of the worms harvested from the control treatments, C(L) and C(D), was much less than in all others, indicating that the added food substrates fuelled the major part of the growth observed in fed mesocosm units. Among the unfed negative controls, worm growth was significantly greater in the light than in the dark ($p < 0.01$), but significant differences were not observed between light and dark treatments when an enriched food substrate was provided. Presumably, the lightdependent component of the food resource which gave rise to the limited growth in the negative controls was trivial in comparison with that of the given food in the fed treatments. Since there were differences in survival among the mesocosm units, Pearson's product moment correlation coefficient was calculated to test for a possible effect of the survival rate on growth rate, but was not significant ($p > 0.05$).

Fatty acid accumulation

The fatty acid inputs to the system units comprised the fatty acid constituents of the juvenile worms, the sand substrate and the added food. The biomass of the juvenile worms was small (individual weights ≈ 0.02 g); nevertheless, the small addition of long-chain fatty acids to the systems by this route was calculated. The main fatty acid components of the yeast substrate were C16:0 (0.07 mg g^{-1}), C16:1n-7 (0.03 mg g^{-1}), C18:0 (0.02 mg g^{-1}), C18:1n-9 (0.01 mg g^{-1}) and C18:2n-6 (0.02 mg g^{-1}), which together comprised >95% of the fatty acids added. In addition, C14:0, C18:3n-3 and

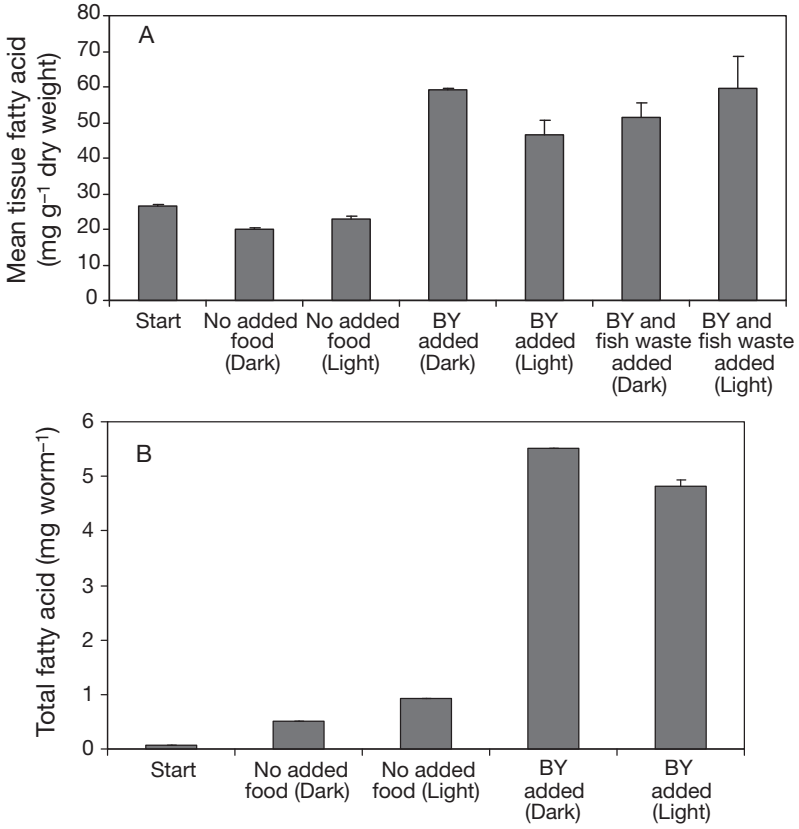

Fig. 1. *Arenicola marina*. Open mesocosm. (A) Mean tissue fatty acid content (mg g^{-1} dry weight) in worm tissues at Day 0 (start) and after 42 d growth in the various treatments (with and without added food in the light and dark). (B) Total fatty acid content of an average worm at the start and after 42 d growth with and without the addition of brewery yeast (BY) in the light and dark

C20:3n-3 were detected but only in trace amounts (<0.01 mg g^{-1}), and no others were present.

The concentration of each fatty acid in the worm tissues at the start and after 42 d of growth is shown in Table 3. A total of 32 different fatty acids were detected in the worm tissues including several branched-chain fatty acids (iso 15:0, iso 16:0, anteiso 16:0 and iso 17:0), indicative of uptake of fatty acids of bacterial origin. The relative abundance of the different fatty acids was similar in all the samples, C16:0, C16:1n-7, C18:0, C18:1n-9, C18:1n-7, C18:2n-6, C20:1n-9, C20:2n-6, C20:4n-6 (AA) and C20:5n-3 (EPA) being prominent. In order to test for differences among treatments, the 42 d tissue concentrations were compared by ANOVA and, when significant, Tukey's HSD was used to compare means. The results of the *a posteriori* tests are summarised in Table 3. The concentrations of C16:0, C18:0, C18:1n-9, C18:2n-6, C20:2n-6 and C20:4n-6 were all notably higher in fed worms (BY and BYFW) than in the unfed negative controls.

The total tissue fatty acid content (mg g^{-1}) of specimens at the start of the experiment and after 42 d growth in the different treatments is shown in Fig. 1A and the fatty acid content per worm is shown in

Table 3. *Arenicola marina*. Open mesocosms. Fatty acid content (mg g^{-1} dry weight [DW] ± SE, n = 3) in tissue samples of *A. marina* at the start (Day 0) and after 42 d growth. C: negative unfed control; BY: brewer's yeast; BYFW: brewer's yeast plus fish waste; L: light; D: dark. Where ANOVA revealed significant differences among mean values, Tukey's HSD is summarised by assigning a different lowercase letter to indicate significant differences ($p < 0.05$). ND: not determined; FA: fatty acids; SAT: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; BCFA: branched-chain fatty acids

Fatty acid	Day 0	Day 42					
	Start sample mg g^{-1} DW	C(L) mg g^{-1} DW	C(D) mg g^{-1} DW	BY(L) mg g^{-1} DW	BY(D) mg g^{-1} DW	BYFW(L) mg g^{-1} DW	BYFW(D) mg g^{-1} DW
C14:0	0.41 ± 0.01	0.23 ± 0.01	0.16 ± 0.01	1.34 ± 0.14	1.74 ± 0.03	2.53 ± 0.21	2.57 ± 0.09
C15:0	0.34 ± 0.01	0.34 ± 0.01	0.24 ± 0.01	0.77 ± 0.07	0.93 ± 0.03	0.76 ± 0.13	0.87 ± 0.06
iso 15:0	ND	ND	ND	1.02 ± 0.09	0.98 ± 0.10	1.15 ± 0.14	1.06 ± 0.05
C16:0	3.99 ± 0.04	2.52 ± 0.75	3.09 ± 0.12	8.81 ± 0.88	10.37 ± 0.34	12.00 ± 3.27	11.64 ± 3.24
C16:1n-7	1.10 ± 0.04	0.86 ± 0.02	0.90 ± 0.03	3.21 ± 0.34	5.96 ± 0.17	3.70 ± 0.33	2.32 ± 0.78
C16:1n-5	0.11 ± 0.00	0.11 ± 0.00	0.11 ± 0.00	0.35 ± 0.03	0.56 ± 0.00	0.28 ± 0.03	0.34 ± 0.02
iso 16:0	0.53± 0.02	0.55 ± 0.02	0.52 ± 0.02	1.96 ± 0.18	3.57 ± 0.16	2.21 ± 0.27	2.69 ± 0.15
anteiso 16:0	0.12 ± 0.01	0.12 ± 0.01	0.10 ± 0.00	0.88 ± 0.10	1.52 ± 0.18	1.01 ± 0.15	1.11 ± 0.04
iso 17:0	0.13 ± 0.01	0.34 ± 0.02	0.41 ± 0.01	0.42 ± 0.03	0.99 ± 0.08	0.29 ± 0.05	0.26 ± 0.02
C16:2n-4	0.05 ± 0.00	0.08 ± 0.01	0.09 ± 0.01	0.17 ± 0.02	0.26 ± 0.02	ND	ND
C17:0	0.53 ± 0.01	0.58 ± 0.03	0.49 ± 0.02	0.59 ± 0.04	0.71 ± 0.04	0.47 ± 0.07	0.41 ± 0.04
C18:0	0.94 ± 0.02	0.74 ± 0.03	0.64 ± 0.03	0.78 ± 0.16	1.51 ± 0.10	3.20 ± 0.53	3.23 ± 0.33
C18:1n-11	0.16 ± 0.01	0.18 ± 0.03	0.11 ± 0.01	0.66 ± 0.08	1.02 ± 0.03	ND	ND
C18:1n-9	0.73 ± 0.05^{a}	0.75 ± 0.05^{a}	0.60 ± 0.02^{a}	3.80 ± 0.38^{b}	3.72 ± 0.23^{b}	10.87 ± 1.28^{c}	9.01 ± 0.27^{c}
C18:1n-7	3.37 ± 0.03	3.57 ± 0.09	3.02 ± 0.11	7.14 ± 0.46	8.78 ± 0.13	5.49 ± 0.44	5.50 ± 0.25
C18:2n-6	0.27 ± 0.01^{a}	0.82 ± 0.05^{a}	0.87 ± 0.03^{a}	1.87 ± 0.27^{b}	2.56 ± 0.06^{bc}	2.91 ± 0.50^{c}	1.80 ± 0.10^{b}
C19:1n-9	ND	0.10 ± 0.04	ND	0.11 ± 0.01	0.10 ± 0.01	0.03 ± 0.01	0.01 ± 0.01
C18:3n-6	0.12 ± 0.00^{ad}	0.13 ± 0.05^{ad}	0.03 ± 0.03^{a}	0.30 ± 0.02^{b}	0.28 ± 0.02^{b}	0.03 ± 0.02^{ac}	0.20 ± 0.01^{bd}
C18:3n-3	0.15 ± 0.02	0.31 ± 0.04^{ab}	0.31 ± 0.01^{a}	0.71 ± 0.08^{d}	1.01 ± 0.01^{c}	0.91 ± 0.12^{cd}	0.33 ± 0.02^{ab}
C20:0	ND	0.06 ± 0.05	ND	0.09 ± 0.04	ND	ND	ND
C20:1n-9	2.02 ± 0.04	2.10 ± 0.10	1.96 ± 0.06	2.42 ± 0.07	2.67 ± 0.05	3.02 ± 0.33	3.11 ± 0.09
C20:1n-7	0.36 ± 0.00	0.53 ± 0.03	0.52 ± 0.01	0.62 ± 0.02	0.75 ± 0.04	0.64 ± 0.12	0.52 ± 0.06
C20:2n-6	0.20 ± 0.00	0.34 ± 0.03	0.29 ± 0.01	1.26 ± 0.18	1.68 ± 0.13	2.11 ± 0.33	1.51 ± 0.13
C20:3n-6	0.13 ± 0.00	0.12 ± 0.01	0.11 ± 0.00	0.49 ± 0.08	0.47 ± 0.05	0.52 ± 0.05	0.31 ± 0.03
C20:4n-6 (AA)	1.60 ± 0.02^{d}	0.82 ± 0.03^{ab}	0.62 ± 0.02^{a}	1.19 ± 0.18^{b}	1.33 ± 0.08^{cd}	1.24 ± 0.17^{cd}	0.41 ± 0.04^{a}
C20:3n-3	ND	0.06 ± 0.01	0.03 ± 0.03	0.13 ± 0.02	0.20 ± 0.01	0.17 ± 0.04	0.10 ± 0.02
C20:4n-3	ND	0.09 ± 0.02	0.11 ± 0.01	0.23 ± 0.04	0.25 ± 0.04	0.20 ± 0.03	0.09 ± 0.01
C20:5n-3 (EPA)	5.32 ± 0.02^{d}	3.87 ± 0.04^{b}	2.85 ± 0.10^{a}	2.97 ± 0.31^{a}	3.14 ± 0.09^{ab}	2.38 ± 0.45^{a}	0.67 ± 0.06^{c}
C22:1n-9	0.09 ± 0.00	0.12 ± 0.00	0.07 ± 0.03	0.14 ± 0.00	0.14 ± 0.01	0.18 ± 0.02	0.19 ± 0.00
C22:4n-6	1.36 ± 0.02	0.40 ± 0.02	0.26 ± 0.01	0.42 ± 0.04	0.37 ± 0.04	0.36 ± 0.03	0.17 ± 0.03
C22:5n-3	2.21 ± 0.02	1.37 ± 0.12	0.84 ± 0.04	1.03 ± 0.08	0.75 ± 0.05	0.54 ± 0.02	0.28 ± 0.04
C22:6n-3 (DHA)	0.38 ± 0.03^{d}	0.48 ± 0.01^{a}	0.43 ± 0.02^{ad}	0.24 ± 0.03^{b}	0.26 ± 0.01^{b}	0.22 ± 0.04^{b}	0.01 ± 0.01^{c}
Total FA	26.71 ± 0.07^{a}	22.72 ± 0.96^{a}	19.80 ± 0.65^{a}	46.16 ± 4.16^{b}	58.58 ± 0.78^{b}	59.41 ± 8.85^{b}	50.74 ± 3.97^{b}
Total SAT	6.20 ± 0.04^{a}	4.48 ± 0.77^{a}	4.62 ± 0.18^{a}	12.39 ± 1.11^{ab}	15.26 ± 0.47^{b}	18.95 ± 4.14^{b}	18.72 ± 3.71^{b}
Total MUFA	7.93 ± 0.13^{a}	8.33 ± 0.29^{a}	7.30 ± 0.20^{a}	18.46 ± 1.34^{c}	23.71 ± 0.48^{b}	24.20 ± 2.51^{b}	21.01 ± 0.19^{bc}
Total PUFA	11.80 ± 0.06^{de}	8.89 ± 0.13^{ad}	6.85 ± 0.23^{a}	11.04 ± 1.32^{cd}	12.55 ± 0.44^{ce}	11.59 ± 1.77^{cd}	5.89 ± 0.12^{a}
Total BCFA	0.78 ± 0.04^{a}	1.02 ± 0.05^{a}	1.03 ± 0.04^{a}	4.28 ± 0.39^{d}	7.06 ± 0.47^{c}	4.67 ± 0.61^{d}	5.12 ± 0.23^{d}
Total n-6	3.69 ± 0.02^{abd}	2.63 ± 0.16^{ab}	2.19 ± 0.07^{a}	5.55 ± 0.77^{cd}	6.68 ± 0.33^{c}	7.16 ± 1.09^{c}	4.40 ± 0.18^{bd}
Total n-3	8.06 ± 0.06^{d}	6.18 ± 0.03^{b}	4.57 ± 0.18^{a}	5.32 ± 0.54^{ab}	5.61 ± 0.13^{ab}	4.43 ± 0.68^{a}	1.49 ± 0.09^{c}

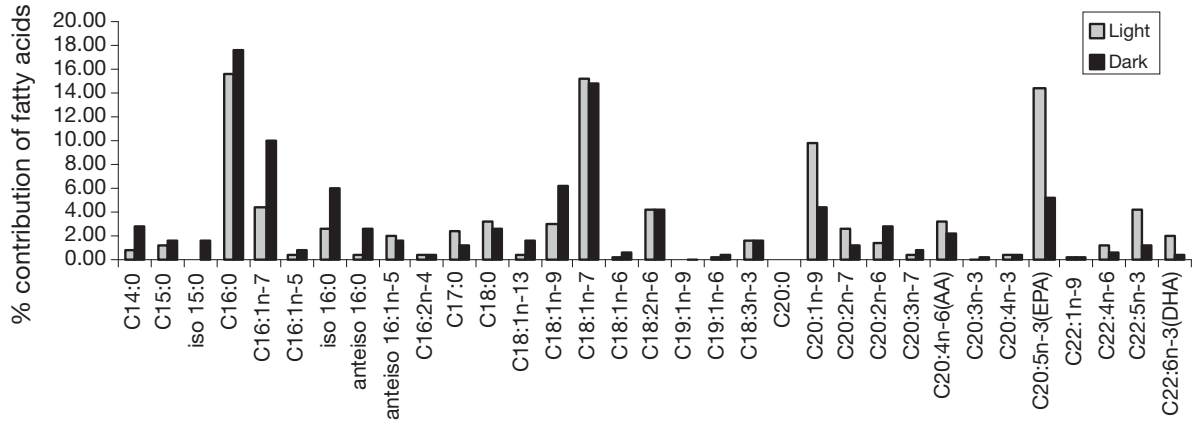

Fig. 2. *Arenicola marina*. Open mesocosm. Percentage contribution of individual fatty acids to the total lipid of *A. marina* tissues from worms grown on brewery yeast in open systems for 42 d in the light and dark

Fig. 1B. Although the tissue fatty acid concentration of the unfed worms decreased during the course of the experiment (Table 3 C(L) and C(D)), a small increase in fatty acid content per worm was observed (Fig. 1B). However, this small gain was trivial compared to the gain in the worms provided with the brewer's yeast, and clearly the given food fuelled by far the greater part of the observed gain in fatty acids, the extent of which was similar both in the light and the dark ($p > 0.05$).

The individual fatty acid content of worms grown in the light and the dark was strikingly similar (Fig. 2), the only fatty acid being significantly more abundant in the tissues of worms grown in the light being EPA. There was a net gain of fatty acids in the system units, and since the tissue fatty acid profiles were largely unchanged in the worms grown on the yeast diet, those gains were not restricted to fatty acids present in the yeast food. To establish with certainty that a net synthesis of any specific fatty acid occurred within system units (distinct from tissue accumulation due to yeast plus extraneous food sources), we designed a closed system in which all extraneous sources of fatty acids other than the given yeast food were, to the best of our ability, excluded.

Table 4. *Arenicola marina*. Closed mesocosms. Initial and final weight (mean ± SE) of the specimens, specific growth rate (SGR), absolute growth rate (AGR) and survival of *A. marina* grown in system units. C: negative unfed control; BY: brewer's yeast; L: light; D: dark. Where ANOVA revealed significant differences among means, Tukey's HSD is summarised by assigning a different lowercase letter to indicate significant differences ($p < 0.05$)

	Weight (g)		SGR	AGR	Survival
	Initial	Final	(% d^{-1})	(mg d^{-1})	(%)
Light					
BY	0.14 ± 0.01^{a}	0.31 ± 0.03^{a}	1.83 ± 0.21	3.93 ± 0.60	96.30 ± 1.51
C	0.13 ± 0.00^{a}	0.11 ± 0.00^{b}	-0.52 ± 0.06	-0.62 ± 0.06	100
Dark					
BY	0.16 ± 0.01^{a}	0.37 ± 0.02^{a}	2.07 ± 0.09	5.17 ± 0.21	88.89 ± 5.24
C	0.12 ± 0.01^{a}	0.09 ± 0.00^{b}	-0.59 ± 0.12	-0.61 ± 0.12	100

Closed systems

Growth and survival

The growth and survival of worms in the closed system units is summarised in Table 4. Random allocation of specimens to system units was tested and there were no significant differences between boxes at the start of the experiment ($p > 0.5$). After 42 d, a significant loss of weight had occurred in the unfed negative controls ($p < 0.01$) and significant growth in the fed treatments ($p < 0.01$). This confirms that in the closed systems there were no extraneous sources of food sufficient for worm growth. Growth was less than in the outdoor open systems (compare data in Tables 2 & 4), but survival was improved.

Fatty acid accumulation and net biosynthesis

System inputs and outputs were calculated as before. The main constituents (92% of total fatty acids added) of the added brewer's yeast were: C16:0 (33.9%), C16:1n-7 (18.1%), C18:0 (10.6%), C18:1n-9 (9.3%), C18:1n7 (3.6%) and C18:2n-6 (16.4%). The fatty acids C14:0 and 2 branched-chain fatty acids (br14:0 and anteiso 14:0) were present in lower quantities, and a trace amount (<0.01 mg g^{-1} dry weight) of

C18:3n-3 was detected. The beach sand contained only trace amounts (~0.01 mg g^{-1} dry weight) of fatty acids in total. A small amount (20 g) of used sand was added to the system units, primarily to seed the system with an appropriate mix of bacteria from lugworm-bioturbated sand, which contained slightly more fatty acid (0.07 mg g^{-1} dry weight) comprised only of br14:0, C16:0 and trace quantities of C16:1n-7 and C18:0. By the end of the experiment, the fatty acid content of the combined sand samples was less than 0.002 mg g^{-1} dry weight, indicating that the fatty acids added with the yeast had either been utilised by the worms or flushed from the system units.

The fatty acid content of the worms harvested from the system units contained a complex mixture of 25 fatty acids including substantial quantities of fatty acids not present in the given diet (Table 5). The predominant fatty acids present in the tissues of the fed worms at the end of the experiment were C16:0 (19%), C16:1n-7 (7%), iso 16:0 (2.4%); anteiso 16:0 (1%), C18:1n-9 (10%), C18:1n-7 (9%), C18:2n-6 (8%) and C20:2n-6 (5%), where percent values represent mean values for tissues of fed worms. The total fatty acid concentration in the lugworm tissues at the end of the experiment was significantly greater in worms given the added yeast (BY treatments) than in unfed negative controls (C treatments) ($p < 0.01$); and, as in the closed mesocosm experiment, the total fatty acid content of worms cultured in the dark and in the light did not differ significantly ($p > 0.05$). Tissues of unfed worms became noticeably depleted in C18:2n-6, C18:3n-3 and C20:2n-6, which could no longer be

Table 5. *Arenicola marina*. Closed mesocosms. Fatty acid content (mg g^{-1} dry weight [DW] ± SE, n = 3) in samples of *A. marina* tissue at the start (Day 0) and after 42 d growth. See Table 3 for abbreviations. Where ANOVA revealed significant differences among means, Tukey's HSD is summarised by assigning a different lowercase letter to indicate significant differences ($p < 0.05$). br: branched chain fatty acid with unidentified branch position

FA	Day 0 Start sample mg g^{-1} DW	Day 42 BY(L) mg g^{-1} DW	Day 42 BY(D) mg g^{-1} DW	Day 42 C(L) mg g^{-1} DW	Day 42 C(D) mg g^{-1} DW
C14:0	0.89 ± 0.14	0.72 ± 0.09	1.08 ± 0.01	ND	ND
br 14:0	1.05 ± 0.08	1.06 ± 0.04	1.59 ± 0.22	9.10 ± 1.35	5.75 ± 0.41
anteiso 14:0	0.38 ± 0.07	0.70 ± 0.03	0.89 ± 0.07	ND	ND
C15:0	0.74 ± 0.06	0.62 ± 0.01	0.75 ± 0.02	0.03 ± 0.03	0.03 ± 0.02
iso 15:0	0.32 ± 0.04	0.41 ± 0.05	0.48 ± 0.02	0.03 ± 0.02	ND
C16:0	6.01 ± 0.42	8.85 ± 0.08	9.98 ± 0.44	3.20 ± 0.02	2.52 ± 0.06
C16:1n-7	1.78 ± 0.26	3.40 ± 0.04	3.51 ± 0.15	0.21 ± 0.17	ND
iso 16:0	4.12 ± 2.56	1.10 ± 0.15	1.26 ± 0.04	0.68 ± 0.05	0.66 ± 0.14
anteiso 16:0	0.50 ± 0.06	0.67 ± 0.09	0.77 ± 0.05	0.21 ± 0.09	0.11 ± 0.05
C17:0	0.83 ± 0.09	0.48 ± 0.02	0.53 ± 0.01	0.24 ± 0.14	0.03 ± 0.02
iso 17:0	0.39 ± 0.03	0.29 ± 0.03	0.28 ± 0.01	0.41 ± 0.07	0.41 ± 0.04
C18:0	1.29 ± 0.14	2.50 ± 0.13	2.75 ± 0.10	0.99 ± 0.02	0.84 ± 0.03
C18:1n-11	0.62 ± 0.05	1.81 ± 0.16	2.24 ± 0.14	0.06 ± 0.05	ND
C18:1n-9	1.89 ± 0.03^{a}	5.02 ± 0.16^{b}	5.15 ± 0.18^{b}	0.88 ± 0.02^{a}	0.60 ± 0.03^{a}
C18:1n-7	4.92 ± 0.19^{b}	4.30 ± 0.07^{bc}	4.79 ± 0.21^{b}	2.73 ± 0.08^{ac}	2.07 ± 0.02^{a}
C18:2n-6	0.64 ± 0.04^{b}	3.85 ± 0.12^{a}	3.63 ± 0.25^{a}	ND	ND
C19:1n-9	0.23 ± 0.02	0.17 ± 0.00	0.19 ± 0.02	0.29 ± 0.24	ND
C18:3n-3	0.14 ± 0.01^{b}	0.57 ± 0.04^{a}	0.61 ± 0.07^{a}	ND	ND
C20:1n-9	2.46 ± 0.08	2.51 ± 0.12	2.56 ± 0.11	2.50 ± 0.08	1.90 ± 0.08
C20:2n-7	0.24 ± 0.01	0.24 ± 0.02	0.15 ± 0.01	ND	ND
C20:2n-6	0.87 ± 0.04	2.48 ± 0.04	2.63 ± 0.23	ND	ND
C20:3n-6	0.09 ± 0.01	0.60 ± 0.00	0.56 ± 0.07	ND	ND
C20:4n-6 (AA)	1.80 ± 0.10^{a}	1.58 ± 0.09^{a}	1.33 ± 0.15^{ac}	1.10 ± 0.05^{ac}	0.64 ± 0.18^{c}
C20:5n-3 (EPA)	4.26 ± 0.42^{b}	2.61 ± 0.07^{a}	2.56 ± 0.12^{a}	3.06 ± 0.05^{a}	2.18 ± 0.09^{a}
C22:6n-3 (DHA)	0.43 ± 0.07^{a}	0.27 ± 0.02^{ac}	0.31 ± 0.05^{ab}	0.03 ± 0.03^{cd}	ND
Total FA	36.91 ± 3.21^{ab}	46.85 ± 0.24^{ab}	50.56 ± 1.55^{b}	25.74 ± 0.77^{ac}	17.75 ± 0.52^{c}
Total SAT	9.77 ± 0.83^{a}	13.18 ± 0.33^{ab}	15.09 ± 0.51^{b}	4.46 ± 0.18^{c}	3.41 ± 0.06^{c}
Total MUFA	11.89 ± 0.41^{ac}	17.22 ± 0.02^{ab}	18.44 ± 0.66^{b}	6.67 ± 0.30^{cd}	4.57 ± 0.11^{d}
Total PUFA	8.48 ± 0.41^{a}	12.22 ± 0.35^{a}	11.78 ± 0.85^{a}	4.19 ± 0.13^{b}	2.83 ± 0.23^{b}
Total BCFA	6.77 ± 2.55^{a}	4.23 ± 0.24^{a}	5.26 ± 0.29^{a}	10.43 ± 1.34^{a}	6.94 ± 0.49^{a}
Total n-11	0.62 ± 0.05	1.81 ± 0.16	2.24 ± 0.14	0.06 ± 0.05	ND
Total n-9	2.69 ± 0.07	2.68 ± 0.12	2.75 ± 0.09	2.79 ± 0.17	1.90 ± 0.08
Total n-7	6.94 ± 0.44	7.94 ± 0.13	8.45 ± 0.37	2.94 ± 0.25	2.07 ± 0.02
Total n-6	3.40 ± 0.06^{b}	8.52 ± 0.25^{a}	8.14 ± 0.66^{a}	1.10 ± 0.05^{b}	0.64 ± 0.18^{b}
Total n-3	4.84 ± 0.48^{a}	3.46 ± 0.09^{b}	3.48 ± 0.21^{ab}	3.09 ± 0.07^{b}	2.18 ± 0.09^{b}

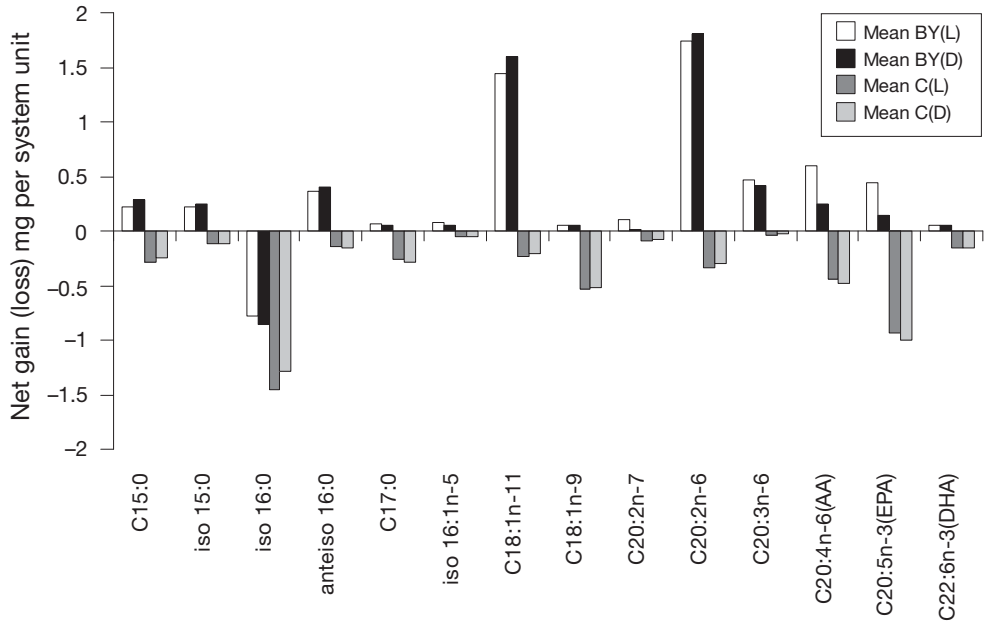

Fig. 3. *Arenicola marina*. Closed mesocosm. Net gain (loss) in the total quantity of individual fatty acids (mg) present within system units during the growth experiments (42 d bioturbation) in the light (L) and dark (D) with the addition of brewer's yeast (BY) and in negative unfed controls (C) without the addition of yeast. Fatty acids that were present in the yeast food have been omitted for simplicity

detected at the end of the experiment. Tissue concentrations of arachidonic acid (AA) also declined to a significant extent, but EPA concentrations were maintained, even in the unfed worm tissues. The total amount of this fatty acid present in the unfed system units was, however, substantially lower than at the start because of the weight loss of the unfed worms (Table 4). Similarly, DHA could not be detected in the tissues of worms cultured in the dark without food, and only traces were found (0.03 mg g^{-1}) in the tissues of the unfed specimens maintained in the light.

In contrast to the unfed controls, worms provided with yeast food increased in weight substantially (Table 4), and the tissue concentration of most fatty acids also increased (Table 5). Since most of the fatty acids were not present in the yeast food provided, there was a net gain in many of the constituent fatty acids of the worm tissues and the gain was observed equally in the light and dark treatments. To determine if there was a net gain of each fatty acid as opposed to a transfer between system compartments, the net gain (or loss) per system unit of each fatty acid during the course of the 42 d experimental treatment was quantified. This revealed a substantial net gain (expressed as mg per system unit) of virtually all those fatty acids not present in the given food in all system units to which yeast was added (Fig. 3). In contrast, losses occurred in the unfed negative controls. ANOVA did not reveal any significant differences between light and dark treatments ($p > 0.05$). The net gains of C18:1n-11, C20:2n-6, C20:3n-6, C20:4n-6 (AA) and C20:5n-3 (EPA) were particularly prominent, whereas the net gain of C22:6n-3 (DHA), though positive, was relatively small. The fatty acids accumulating in the system units must have been either produced in the sediment and ingested by the worms or synthesised by the worms themselves. To test for the former, we searched for known PUFA-synthesising bacteria phylotypes in the sand samples using a 16S rDNA approach and attempted to amplify conserved regions of the PUFA-synthetic gene cluster from sediment samples.

Presence and abundance of EPA- and DHA-producing bacteria

Ashforth (2008) has compiled 16S DNA libraries for 495 clones comprising 213 unique phylotypes isolated from the mesocosm sand samples and here we report on the extent to which these included potentially PUFA-synthesising phylotypes. Comparison of clone sequences with GenBank revealed the presence of only a few clones similar to known EPA- and DHA-producing bacteria. One of these, *Shewanella affinis* KMM 3586, a known EPA-producing species, was the top hit for a clone (accession no. FJ717202, clone H7 10.1_2 in Ashforth 2008) (maximum identity 100%, coverage 98%), while DHA-producing *Colwellia psychrethryraea* ATCC27364 was the top match to another clone (acccession no. FJ717208, clone F12

10.2_1 in Ashforth 2008) (97% maximum id, 100% coverage). Other clones (accession nos. FJ717227, FJ717239, FJ717229 and FJ717223) also blasted closely to (but not as the top match) *Colwellia psychrethryraea* 34H which, although not proven, is also expected to produce PUFA (Okuyama et al. 2007). The *Gammaproteobacteria*-like clones (which would include known EPA- and DHA-producing phylotypes), however, were not among the most abundant species in any of the mesocosm samples and comprised <2% of the total number of clones isolated.

The presence of, albeit in very low numbers, potentially EPA- and DHA-producing phylotypes in the mesocosm samples suggested that we should search, using degenerate primers, for genes that are highly conserved and characteristic of PUFA-synthesising bacteria. Primers were designed for the amplification of the highly conserved ketoacyl ACP synthase gene located in the PKS protein region of known PUFA-synthesising bacteria. The specificity of the primers was determined using both positive and negative controls. Reacting the primers using genomic DNA from *Shewanella frigidimarina* 12253^T and *Colwellia psychrerythraea* 8813^T generated products of the correct size; these were then sequenced and both the *Shewanella* and *Colwellia–Moritella* designed primers amplified DNA fragments that matched *Shewanella* and *Colwellia–Moritella* PKS genes, though the *Shewanella* fragment matched more closely to *Shewanella* SCRC-2738 (another PUFA producer) than *S. frigidimarina* 12253^T, the 9th best hit with a coverage value of 99% and a maximum identity of 80%. Negative controls, in which the *Shewenella* PCR primers were cross-reacted with *Colwellia* DNA and vice versa, did not amplify any DNA fragments. The sensitivity of the primers was determined by reacting with 100, 10, 1 and 0.1 ng of species DNA which indicated that they were sensitive to 10 ng of specific DNA in the reaction mix, but did not detect quantities as low as 1 ng DNA.

Environmental DNA from the sediments of the *Arenicola marina* culture mesocosm was analysed by PCR using the *Shewanella* and *Colwellia–Moritella* primers under the optimum conditions with the appropriate annealing temperature for each primer pair using 100 ng of environmental DNA in the reaction mix. No bands were observed for the tubes containing the mesocosm DNA template or for aqueous negative controls, whereas activity was confirmed for positive controls. This negative result was confirmed by replicate PCR testing including replicates where the quantity of environmental DNA was increased to 900 ng in the reaction mix.

Failure to amplify DNA fragments using the PCR primers for the PKS cluster ketoacyl ACP synthase gene could be caused in 3 ways: (1) the presence of relatively few PUFA-producing organisms in the sediment samples from the mesocosm; (2) PUFA-producing organisms present in the sediment being sufficiently genetically different that the degenerate primers could not bind to the target region of their PKS gene; or (3) the presence of inhibitors in the environmental DNA which prevented PCR. We mitigated against the first by repeated assays and by increasing the amount of extracted DNA in the reaction mix, and against the second by designing different degenerate primers for *Shewanella* and *Moritella–Colwellia* which blasted closely to the PKS genes of other PUFA-synthesising species in the database within the 2 genera.

Adopting a parsimonious approach, it is necessary to conclude the PUFA-synthesising organisms were not sufficiently numerous in the sand samples to be detected by the degenerate primers for a gene characteristic of PUFA-synthesising species. We conclude therefore that if PKS genes containing phylotypes were present, they were present only in relatively low numbers and did not comprise a major food element for the lugworm. It is therefore highly unlikely that such organisms were responsible for the substantial gains in PUFA in those mesocosm units provided with the yeast food.

DISCUSSION

Net gain of specific fatty acids

The present study has confirmed that a diverse range of fatty acids accumulate in the tissues of the marine polychaete *Arenicola marina* (L.) when cultured in a sand substrate enriched only with spent brewer's yeast. The relative frequency of individual fatty acids of the lugworm tissues remained more or less unchanged during growth experiments, despite the fact that most of the fatty acids found in the worm tissues were not present in the restricted diets used. We carried out experiments in open systems in which relatively small amounts of other food sources could have been present, but also in closed systems in which they were eliminated. The total number of fatty acids present (25 in total) was lower in the worms from the closed systems than in those taken from the open systems (32 in total); the fatty acids C18:3n-6, C20:3n-3, C20:4n-3, C22:1n-9, C22:4n-6 and C22:5n-3 were present in small but measurable amounts in the tissues from the open systems (see Table 2) but were not detectable in lugworms grown on the yeast substrate when all extraneous sources of fatty acids were excluded. We conclude that *A. marina* does not synthesise these fatty acids de novo. Also noticeable (see Fig. 2) was the greater amount of EPA present in worm

tissues grown in the open systems in the light than in the dark ($p < 0.05$). We conclude that if this fatty acid is available in the diet, it is taken up by the lugworm; nevertheless, *A. marina* was found to be able to accumulate substantial quantities of EPA in its tissues even when grown in the dark and when EPA was not present in the given food.

These minor differences apart, the characteristic fatty acid content of the lugworm tissues was maintained in worms maintained on a closely controlled, fatty acid-deficient diet. This provides clear evidence of a dietary independence for many longer chain PUFA which must therefore have been produced within system units. García-Alonso et al. (2008) similarly found that the fatty acid profile of the polychaete *Nereis diversicolor* did not change seasonally and was not noticeably influenced by the fatty acid content of their diets. This implies the existence of mechanisms by which long-chain fatty acids can accumulate in detritivorous polychaete tisses from non-photosynthetic sources.

Since most of the PUFA gained within our closed system units were not present in the given food resource (i.e. seawater-composted spent brewer's yeast), biosynthesis must have occurred within the system units. Such gains were not observed when worms and yeast were not added to the substrate, nor in the unfed negative controls. Therefore, the bioturbation of the sediment–yeast mixture by *Arenicola marina* is a necessary component of the biological processes resulting in the net gain of PUFA. There is substantial interest in the origin of unsaturated fatty acids in marine organisms (Bergé & Barnathan, 2005) because of their importance as a source of essential fatty acids in the diets of humans. A frequently adopted paradigm for marine ecosystems is that PUFA, i.e. long-chain fatty acids of 18 carbons and 2 or more double bonds, are normally obtained by marine animals through their diet, ultimately derived from the synthetic activities of diatoms in the photic zone (Leonard et al. 2004). Although a limitation of our methodology is that net biosynthesis cannot be established with certainty for those fatty acids present in the composted yeast substrate (predominantly C14:0, brC14:0, C16:0, C16:1n7 and traces of C18:1n9 and C18:2n6), we provide positive evidence for net biosynthesis for all those fatty acids present in the lugworm tissues not present in the yeast substrate or present only in trace amounts. That the net gains were observed in both light and dark conditions to virtually the same degree ($p > 0.05$) indicates that the biosynthesis was not a photosynthetically driven process. These fatty acids must have been produced either by bacteria ingested and digested by the worms, or produced within the worms, either by way of an internal symbiosis or synthetic processes of the worms themselves. We have therefore attempted to distinguish between these possibilities.

Potential pathways of PUFA biosynthesis

Bacteriophagy

Bacteriophagy occurs in many marine benthic organisms including polychaetes and is clearly implicated in the feeding of *Arenicola marina* (Plante & Mayer 1994, Retraubun et al. 1996, Grossi et al. 2006). There has been considerable interest in bacterial biosynthesis of PUFA as well as branched-chain fatty acids in bacterial membranes, where they play an important membrane fluidising role (e.g. Russell & Nichols 1999, Valentine & Valentine 2004). The discovery that marine bacteria may have an important role in the production of PUFA (Nichols 2003) therefore establishes a potential pathway from prokaryotic production into the marine food web by way of bacteriophagy and the trophic activities of detritivorous benthic invertebrates such as the lugworm, the importance of which may have been underestimated.

We found significant gains in the lugworm fatty acid profiles of anteiso 14:0 and 16:0, iso 15:0, 16:0 and 17:0, and C18:1n7 in our mesocosm experiments, suggesting that bacterial ingestion and absorption was a significant component of the feeding process within the mesocosms. The presence of the so-called bacterial fatty acids has been used previously to confirm bacteriophagy in *Arenicola marina* (Grossi et al. 2006), *Nereis* (*Hediste*) *diversicolor* (Luis & Passos 1995), *Pectinaria gouldii* and *Nereis* sp. (Copeman & Parrish 2003). Bacteria proliferated in the yeast substrate, causing it to become anaerobic. Ingestion of the anaerobic layer of enriched substrate was shown by the presence of black sediment in the casts and the use of green and blue coloured sand grains in the DYL (Ashforth 2008). Analysis of the bacterial biodiversity in various layers of the mesocosms (see Ashforth 2008 for details) confirms that a gardening process did indeed occur. *Epsilonproteobacteria* phylotypes having 16S rDNA sequence similarities close to *Sulfurimonas denitrificans* were particularly common in the DYL but not present in sand collected from NC; consequently, Ashforth (2008) concluded that chemolithoautotrophic *Epsilonproteobacteria* are likely to have constituted a substantial part of the diet of the lugworm—a classic case of gardening by the worm. However, this class of bacteria, and others abundant in the DYL of the bioturbated sediments, is not PUFA-synthesising and does not therefore represent a potential bacterial source of the C18 and C20 PUFA found to be accumulating in

the lugworm tissues. PUFA biosynthesis occurs predominantly in *Gammaproteobacteria*. For instance, the genus *Shewanella* synthesises EPA while strains belonging to the genera *Colwellia*, *Photobacterium*, *Flexibacter*, *Vibrio* and *Moritella* have been found to produce more than one type of PUFA, e.g. DHA and EPA. In bacteria, PUFA synthesis occurs by way of the PKS pathway (Valentine & Valentine 2004) and we were able to design degenerate primers for a constituent gene in the PKS cluster that is highly conserved in PUFA-synthesising bacteria. The primers worked successfully and generated product from the positive controls, but PCR product was not generated in sufficient quantity for detection in genomic DNA extracted from the mesocosm sand samples. Sensitivity assays established that the primers would react in the PCR mix with quantities as low as 10 ng DNA. We conclude therefore that either (1) the relative abundance of the PKS cluster ketoacyl ACP synthase gene containing phylotypes in the sediment samples was sufficiently low to prevent amplification, or (2) any PUFA-synthesising organisms that were present in the sediment samples were sufficiently different that even degenerate primers could not bind to the target region of their PKS cluster. The former explanation appears to be the more likely given the highly conserved nature of PKS gene systems among PUFA-synthesising bacteria. It is therefore unlikely that PUFA-synthesising bacteria and their subsequent absorption represent the only or indeed the major source of PUFA biosynthesis in the mesocosm systems.

Within-tissue biosynthesis

If bacteria in the sediment are not, as our observations indicate, the source of newly synthesised PUFA accumulating in the system, and since PUFA accumulation occurred in the absence of light, it is necessary to consider the possibility that PUFA are formed within the lugworms, either directly or indirectly; however, we note that there is no evidence for endosymbiosis in lugworms and the rapid transition of ingested sand through the gut (Hüttel 1990, Chen & Mayer 1999) is counter-indicative. Formation of PUFA in animal tissues involves a number of substrate-specific enzymes involved in desaturation processes and chain elongation (Sprecher 2000, Pereira et al. 2003, Leonard et al. 2004). Fig. 4A shows, in annotated diagrammatic form, the known pathways for the biosynthesis of C18 and longer chain unsaturated fatty acids in eukaryotes. We make the assumption that if a pathway is active in the lugworm, each fatty acid involved in that pathway would be present in the tissue lipid, recognising that the relative amounts would be determined in part by the specific substrate and/or reaction constants and other factors. We annotate the diagram to indicate the fatty acids present or absent in the fermented yeast food and in lugworm tissue lipid.

Linoleic acid C18:2n-6 (LA) was relatively abundant in the yeast but the n-3 derivative alpha-linolenic acid C18:3n-3 (ALA) was present only in trace amounts (molar concentration = 0.008 mmol ml^{-1}). In many animals, LA is the substrate for the well-established n-3 and n-6 pathways as shown. All eukaryotes are thought to be able to desaturate C18:0 by Δ9-desaturase to yield C18:1n-9 (oleic acid), but terrestrial animals often lack the Δ12-desaturase necessary to further desaturate this substrate to C18:2n-6 (linoleic acid) and thence to C18:3n-3. The fatty acids C18:2n-6 and C18:3n-3 are therefore essential fatty acids for mammals and some other terrestrial animals which lack Δ12 and Δ15 desaturases. The situation among other animal groups is incompletely known. Marine teleosts, for instance, are reported to lack the ability to convert C18:3n-3 to the downstream fatty acids EPA and DHA in the n-3 pathway and similarly lack the ability to derive AA from the n-6 pathway precursor C18:2n-6, but freshwater species may have a greater repertoire of fatty acid elongases and desaturases (see discussion in Sargent et al. 2002).

The possible fatty acid biosynthesis pathways in lugworms were determined from the presence and absence of specific fatty acids in the given food and lugworm tissues, especially those accumulating in the lugworm tissues. We refer extensively to the excellent reviews of Pereira et al. (2003) and Leonard et al. (2004) and note the following: (1) lugworms not given access to the yeast substrate become depleted in C18:1n9 and the downstream derivatives, C18:2n-6 and C18:3n-3, which were no longer detected in the tissues of unfed worms at the end of the experiment; (2) C18:2n-6 was present in relatively large amounts in the BY(L) and BY(D) lugworm tissues and was one of the fatty acids showing strong bioaccumulation; (3) C18:3n-3 was also present in the fed worms but in much lower amounts; and (4) C18:3n-6; C18:4n-3 and C20:4n-3 were absent in all lugworm tissue samples. The last is of particular interest because these 3 fatty acids are constituents of the n-6 and n-3 PUFA pathways. These findings are strongly indicative of a pathway of fatty acid elongation and desaturation in lugworm tissues in which the formation of C20:2n-6 from the substrate C18:2n-6 plays an important role. The implied pathway of chain elongation by way of elongation of C18:2n-6 to C20:2n-6 and subsequent desaturation by Δ8 desaturase to generate C20:3n-6 (dihomo-γ-linolenic acid DGLA or cis,cis,cis-8,11,14-eicosatrienoic acid) is indicated in Fig. 4A using block arrows. This pathway was first demonstrated among the lower eukaryotes in a euglinid and, in these lower

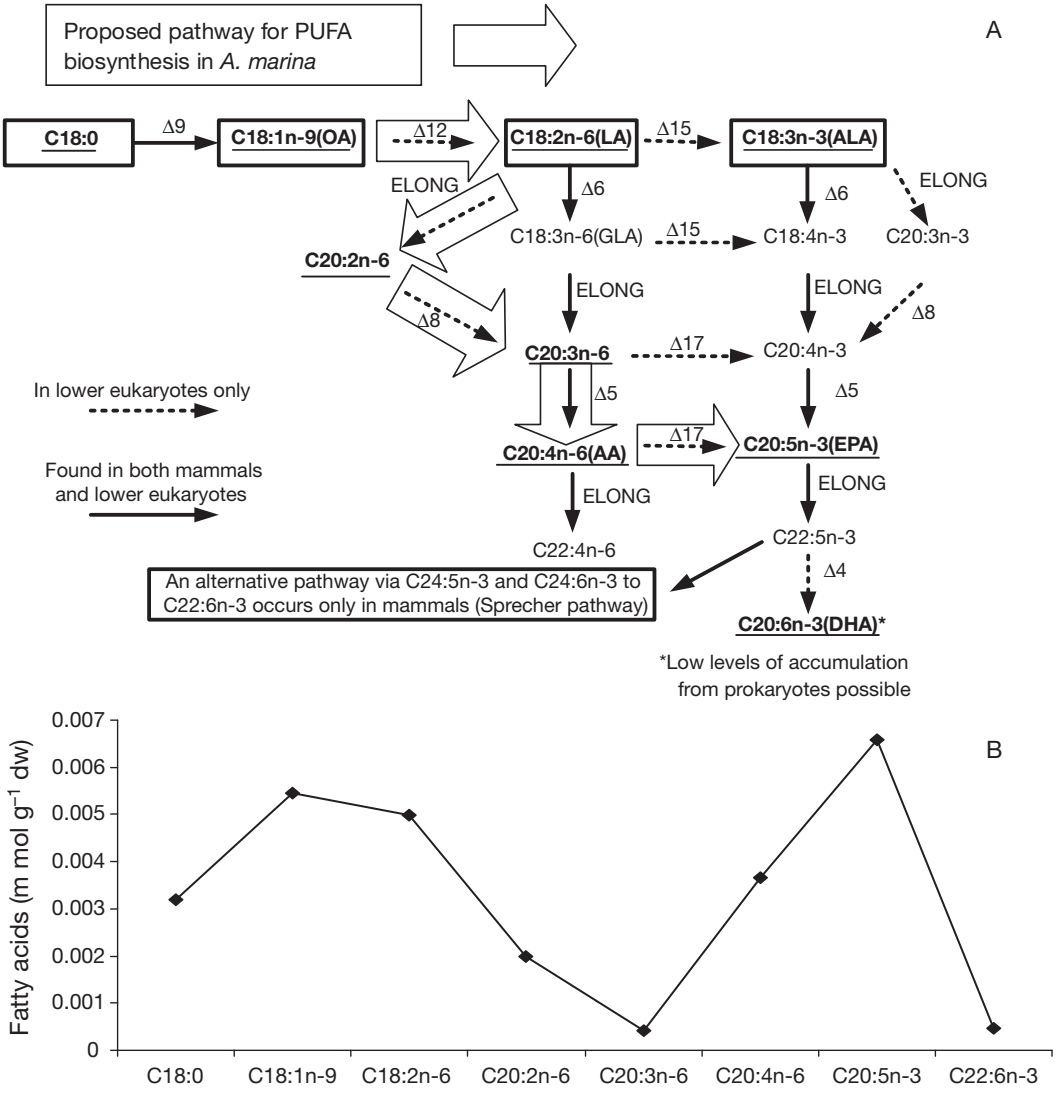

Fig. 4. Potential pathways for de novo synthesis of long-chain fatty acids in *Arenicola marina* (L.) tissues. (A) Annotated diagram of pathways in animal tissues (adapted and annotated from Pereira et al. 2003, Leonard et al. 2004). Underlined **bold** text: fatty acids found in the lugworm tissues; normal text: fatty acids not found in the lugworm tissues; text in rectangular boxes: fatty acids present in the given food (brewery yeast). ELONG: elongation enzymes; Δn: desaturation enzymes inserting a double bond at position Δn, where n represents the carbon position relative to the carboxyl carbon (Δ1). (B) Relative concentration (mmol g^{-1} worm dry weight) of fatty acids associated with the putative pathway involving elongation of C18:2n-6 to C20:2n6 and subsequent desaturation to C20:3n-6, C:204n-6 (AA) and C20:5n-3 (EPA)

eukaryotes, C20:3n-6 is a precursor for AA, as appears also to be the case in *Arenicola marina.* It is of interest that C20:3n-6, a key intermediary in this pathway, has important metabolic functions causing sperm activation and the initiation of spawning in male *A. marina* (Bentley et al. 1990, Pacey & Bentley 1992). Genomic studies have demonstrated that 2 elongase systems and a full range of desaturases are present and active in *Caenorhabditis elegans* as discussed above where Fat-3 mutants of *C. elegans* (Watts & Browse 2002) display an unusual pattern of fatty acid accumulation, in which C20:2n-6 is produced, C22:2n-6 and C22:3n6 are present but other PUFA are absent. Only relatively small amounts of C20:2n-6 are produced in the Fat-3 mutants, however, and the authors conclude that C18 fatty acids with a Δ6 double bond are the preferred substrates for elongation in *C. elegans.*

The conserved nature of the fatty acid elongation–desaturation systems of eukaryotes and the existence of alternative pathways (as shown in Fig. 4A) gives us the confidence to suggest that *Arenicola marina*, and probably other polychaetes, have the abil-

ity to desaturate and elongate fatty acids through a pathway that involves chain elongation of C18:2n-6 to give substantial tissue quantities of C20:2n-6. This step may then allow de novo production of C20:4n-6 (AA) and C20:5n-3 (EPA). The proposed pathway implies the functionality of an elongase enzyme system that converts C18:2n-6 (LA) to C20:2n-6 and a putative *A. marina* Δ8 desaturase which produces dihomo-γ-linolenic acid (DHLA) which is then a substrate for further desaturation by a Δ5 desaturase to AA and by a Δ17 desaturase to EPA. The tissue concentrations (mmol g^{-1} dry weight) of the fatty acids that would be involved in such a pathway are shown in Fig. 4B. We also attach significance to the finding that C22:4n-6 and C22:5n-3 were not present in the lugworms grown in the closed mesocosms. This further implies that the putative *A. marina* elongase system may not utilise AA and EPA as substrates and thus explains the relatively low levels of DHA found in *A. marina* and all other polychaetes studied to date. The so-called Sprecher pathway (also shown in Fig. 4A), in which the very long-chain fatty acid C24:6-n3 is a precursor of C22:6-n3 (DHA) (reviewed in Leonard et al. 2004), is not operative, as we did not detect C24 fatty acids in *A. marina* and they have not been reported in any other polychaete tissues. The source of the relatively small amount of DHA produced within the system units that accumulated in the lugworm tissues remains problematical. We do not exclude a bacterial origin for this, perhaps from the small number of *Shewenella*- or *Colwellia*-like *Gammaproteobacteria* present in the system (Ashforth 2008).

The fatty acid profiles of other polychaetes whose fatty acids have been characterised is consistent with a widespread occurrence of the proposed pathway in the polychaete clade based on evidence reported for *Nereis* (*Hediste*) *diversicolor* (Bradshaw et al. 1990, Luis & Passos 1995, Fidalgo e Costa et al. 2000, García-Alonso et al. 2008), *Nereis virens* (Olive et al. 2002), a number of deep-sea vent worms (Taghon 1988, Pond et al. 2002, Phleger et al. 2005) and cold-water detritivorous species (Copeman & Parrish 2003), all of which show the occurrence and prominence of C20:2n-6 (the elongation product of C18:2n-6 from which C20:3n-6 is derived) and lack or low concentration of fatty acids associated with the conventional n-6 and n-3 pathways. The presence of long-chain unsaturated fatty acids in deep-sea vent worms has been considered problematic because of the general acceptance that these fatty acids are normally derived from the abundant sources present in the photic zone (for discussion see Pond et al. 2002, Phleger et al. 2005). A further feature of all polychaete worms sampled to date is the relatively high tissue concentration of cis-vaccenic acid C18:1n-7. Phleger et al. (2005) suggests desaturation and elongation of bacteria-derived cis-vaccenic acid to gain EPA and DHA is also possible.

The generally held assumption that all long-chain PUFA are ultimately derived from biosynthesis in the photic zone and dissimilated through the marine food chain should now be treated with caution; other sources are certainly possible and their importance in benthic substrates subjected to bioturbation activity may be substantial.

Bioturbation of enriched sediments by marine worms: A potential source of essential fatty acids for aquaculture feeds?

FAO and other reports demonstrate that on current trends there will soon be an inadequate supply of fish meal and more particularly marine fish oil (MFO) to meet the projected growth demand for essential PUFA required for formulated aquaculture feeds (FAO 2006). The demand is such that in recent years the aquaculture sector has taken an increasing share in global fish meal and marine fish oil production and is now estimated to take 87% (783 000 tonnes) of the total marine fish oil production (Jackson 2007). The dependence on dietary sources for long-chain PUFA in some cultured fish species is a major factor in the current concerns about supply of essential fatty acids for aquaculture feed formulations. Fatty acids, obtained from pelagic fisheries, are ultimately derived from photosynthetic organisms in the photic zone and this resource may become limiting to the sustained growth of the aquaculture industry. Caution should be exercised, however, in assuming that marine invertebrates have the same fatty acid dietary dependences as mammals and marine teleosts. The present study shows that production of PUFA previously thought to occur mainly in the photic zone is likely to be a normal feature of benthic production and nutrient recycling, not just in the deep sea, where bacterial symbioses with sulphide-reducing bacteria are implicated, but also in shallow-water benthic environments subject to polychaete-driven bioturbation.

The development of new sources of such fatty acids has been identified as a key requirement for sustainable aquaculture development in the future. These sources could include bioreactors designed to amplify naturally occurring pathways (Bergé & Barthanan 2005). Polychaetes have become well established as dietary components for shrimp and finfish hatcheries (Izquierdo et al. 2001, García-Alonso et al. 2008 for recent discussion), which have in turn resulted in the emergence of a polychaete farming sector in Europe and Southeast Asia. Our small-scale mesocosm study demonstrates that the biosynthesis of long-chain fatty

acids occurs in the absence of light in polychaete-bioturbated marine sediments to which a fermented yeast substrate has been added, and the amplification of naturally occurring ecosystem components in aquaculture production systems, e.g. for the production of benthic organisms such as polychaetes, can clearly result in a net production of essential fatty acids required for aquaculture feed formulations (Sargent et al. 2002, Bell & Sargent 2003). Therefore, large-scale culture of detritivorous polychaetes, such as the lugworm *Arenicola marina*, may contribute to the net supply of important fatty acids.

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