Effects of substrate on essential fatty acids produced by phytobenthos in an austral temperate river system

Tatenda Dalu[1,3], Aaron W. E. Galloway[2,4], Nicole B. Richoux[1,5], and P. William Froneman[1,6]

[1]Department of Zoology and Entomology, Rhodes University, P.O. Box 94, Grahamstown 6140 South Africa
[2]Oregon Institute of Marine Biology, University of Oregon, P.O. Box 5389, Charleston, Oregon 97420 USA

Abstract: Aquatic and riparian habitats increasingly are affected by anthropogenic stressors, but the effects of these stressors on the nutritional quality of primary producers are often unknown. We compared essential fatty acids (EFAs) in the phytobenthos (benthic algae) growing on different substrate types (bricks, clay tiles, rocks, macrophytes, and sediments) at 2 river sites subject to differing anthropogenic stressors (using nutrient concentration as a proxy) in a temperate southern hemisphere location. We hypothesized that the fatty acid (FA) content of phytobenthos changes in response to shifts in local nutrient availability but not substrate type. EFA content (18 : 2ω6, 18 : 3ω3, 20 : 4ω6, 20 : 5ω3, and 22 : 6ω3) in the phytobenthos differed overall among substrates, sites, and seasons and was generally greater in summer than in autumn and winter. EFA content was significantly greater on artificial than natural substrates and was greater at the nutrient-enriched downstream site than at the upstream site. The response of EFA content at the downstream site suggests that land use affected the synthesis of EFAs by phytobenthos and, hence, food quality for aquatic consumers. These findings indicate a potential link between physical factors, such as substrate availability and land management, and the quality of basal food resources available to primary consumers in aquatic food webs.
Key words: essential fatty acids, phytobenthos, South Africa, substratum, nutrients

Aquatic habitats are affected increasingly and globally by climate shifts and anthropogenic activities that cause changes in land use, habitat and water availability, and pollution levels (Chen et al. 2011, Larson et al. 2013, Mead et al. 2013, van Vliet et al. 2013, Mantyka-Pringle et al. 2014, Sternberg et al. 2014). These modifications affect primary production rates and foodweb structure in these systems (Mead et al. 2013, Mantyka-Pringle et al. 2014). Changes in primary productivity affect secondary productivity (Kim and Montagna 2012), diversity (Cardinale et al. 2004), and trophic interactions (Kratina et al. 2012) throughout the food web.

The phytobenthos includes primary producers from diverse groups that play important roles in C and nutrient dynamics (Schletter et al. 2011, Spitale et al. 2014) and is an important basal energy source in aquatic food webs (Evrard et al. 2010, Law 2011). Phytobenthic communities occur on various habitats: surfaces of stone (epilithon), aquatic plants (epiphyton), sand (epipsammon), and wood (epixylon) and in interstitial spaces among deposited inorganic and organic sediment particles (epipelon; Sabater et al. 1998, Bate et al. 2002). The distribution and composition of phytobenthos in any river reflect a complex series of interactions among hydrology, water chemistry, substrate, and biotic factors (Bate et al. 2004, Schletter et al. 2011). However, the relative importance of these factors in determining the phytobenthic community structure varies spatially and temporally. This variability is exacerbated by human activities because phytobenthos can become particularly abundant in water systems that are affected by nutrient enrichment or flow modifications (Bate et al. 2004, Piirsoo et al. 2008).

Fatty acids (FAs) are important components of some lipids and are critical biochemical constituents of all biota. FAs often are used as biomarkers to trace trophic relationships (Brett et al. 2006, Allan et al. 2010, Boëchat et al. 2014). Moreover, FAs are linked inextricably with many key behavioral, physiological, and biochemical processes and various ecological interactions and, therefore, are integral to ecosystem functioning (Arts et al. 2001, Larson et al. 2013). Larson et al. (2013) documented that an increase in seston FA content was related to an increase in nutrient loading. Müller-Navarra et al. (2004) and Cashman et al. (2013) highlighted that increased nutrients led to decreases in sestonic eicosapentaenoic acid (20 : 5ω3) and docosahexaenoic acid (22 : 6ω3) but led to increased ω3 : ω6 ratios and overall FA content via changes in algal species composition. Different

E-mail addresses: [3]dalutatenda@yahoo.co.uk; [4]agallow3@uoregon.edu; [5]n.richoux@ru.ac.za; [6]w.froneman@ru.ac.za

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types of primary producers have distinct FA profiles (Dalsgaard et al. 2003, Taipale et al. 2013, Galloway and Winder 2015), which cause food quality to vary greatly among aquatic habitats (Arts et al. 2001, Brett et al. 2006, Larson et al. 2013). The biochemical composition of phytobenthos can vary among streams in association with changes in land use and nutrient inputs (Cashman et al. 2013, Larson et al. 2013).

We define essential fatty acids (EFAs) as those that most animals need to meet physiological requirements but must obtain from their diet because they cannot synthesize them de novo. Plants and algae are capable of biosynthesizing ω3 and ω6 polyunsaturated fatty acids (PUFAs) de novo (Dalsgaard et al. 2003). Therefore, these producers are important in food webs as the primary source of these critical molecules. EFAs subsequently are transferred from primary producers to heterotrophic organisms at higher trophic levels and may be highly conserved in aquatic systems (Arts et al. 2001, Kainz et al. 2006). EFAs are required as an energy source and for biological processes in diverse animal consumers. For example, ω3 FAs influence gene expression regulating eicosanoids and hormone synthesis. Some aquatic animals can convert these 18-C EFAs to other important EFAs, e.g., 18 : 2ω6 to arachidonic acid (20 : 4ω6) and 18 : 3ω3 to 20 : 5ω3 and 22 : 6ω3 (Stanley-Samuelson 1994, Arts et al. 2001, von Elert 2002). In freshwater and marine environments, indications are strong that 20 : 5ω3 and 22 : 6ω3 are key nutrients that limit zooplankton productivity, i.e., somatic growth and reproduction (Klein Breteler et al. 1999, Budge et al. 2014, Kainz et al. 2014).

Phytobenthos nutritional content is influenced by a variety of physicochemical variables, e.g., nutrients and light, that in turn can affect planktonic and benthic invertebrate growth and community dynamics (e.g., Sterner and Elser 2002, Cashman et al. 2013). In addition, substrate type can influence phytobenthic community structure and EFA content at a microenvironmental scale (Burkholder 1996, de Souza and Ferragut 2012). We compared EFA content in phytobenthos across experimental substrate types and land-use attributes (measured using nutrient concentrations) to assess the implications of substrate type on FA synthesis by primary producers in a freshwater ecosystem. An experimental approach based on sampling artificial and natural substrates across 3 seasons was used in the shallow littoral habitats of an austral river system. Two regions (up- and downstream) with differing landuse characteristics and, hence ambient nutrient levels, were targeted. Our hypothesis was that EFA content in phytobenthos communities differs among seasons, substrates, and regions. Our predictions were that: 1) EFA content would be greater in the downstream (eutrophic) habitat, 2) phytobenthos would have higher EFA content in summer because of increased overall productivity, and 3) phytobenthos growing on artificial and natural substrates would not differ with respect to EFA. Understanding differences in EFA content as a result of substrate and habitat characteristics is important for predicting potential consequences to ecosystem functioning from increasing human alteration of freshwater habitats because the nutrient content of basal resources, and therefore food quality, is an essential determinant for secondary production.

METHODS

Study area

The Kowie River system is situated in the temperate Eastern Cape province of South Africa and has its headwaters in the Grahamstown hills, from whence it flows in a southeasterly direction (Whitfield et al. 1994) (Fig. 1). The Kowie River is ~90 km long and drains a relatively small catchment area of ~800 km^2. Mean annual rainfall is 650 mm, and rainfall occurs mainly during spring and autumn (Heydorn and Grindley 1982). Phytobenthos was sampled once at the end of each of 3 seasons (summer: 13 November–13 December 2012, autumn: 25 April–31 May 2013, and winter: 10 July–9 August 2013) at 2 sites along the Kowie River. The dominant phytobenthic community groups (mostly diatoms) and physicochemical attributes were reported by Dalu et al. (2014b).

Two study sites 25 km apart were selected, one up- (lat 33°20′59.2″S, long 026°33′37.6″E) and one downstream (lat 33°30′16.0″S, long 026°44′40.9″E). The upstream site was situated in a minimally disturbed forested area, whereas the downstream site was situated near intensive livestock and irrigated crop farms and was polluted as a result of inputs from sewage discharge and agricultural activities. The upstream site had a mean (±SD) water depth of 0.35 ± 0.1 m, channel width of 1.8 ± 0.3 m, water velocity of 0.4 m/s, and 75% riparian vegetation cover. The downstream site had a mean water depth of 0.7 ± 0.2 m, channel width of 7.5 ± 0.2 m, water velocity of 0.18 m/s, and 20% riparian vegetation cover (Dalu et al. 2014b). The mean discharge for the Kowie River in the vicinity of the downstream site was 1.25 m^3/s (2009–2011) and 10.7 m^3/s (2012; DWA 2013). The upstream site was nutrient poor, with mean PO_4^{3-} and NO_3^- concentrations of 0.6 and 5.4 mg/L, respectively, whereas the downstream site had higher nutrient concentrations, with mean PO_4^{3-} and NO_3^- concentrations of 2.1 and 7.9 mg/L, respectively.

Phytobenthos sampling and preparation

Phytobenthos was collected from natural (sediments, macrophytes, and rocks) and artificial (bricks, grey and brown tiles) substrates according to sampling methods described by Taylor et al. (2005). At each site, 18 (9 brown and 9 grey) tiles measuring ~22 × 10 × 0.7 cm were placed vertically in each of 6 support structures (30 × 18 cm) punctured with holes to allow the free flow of water around the tiles. The structures were placed randomly in the river, anchored at 20- to 40-cm water depth with a rope tied to riparian

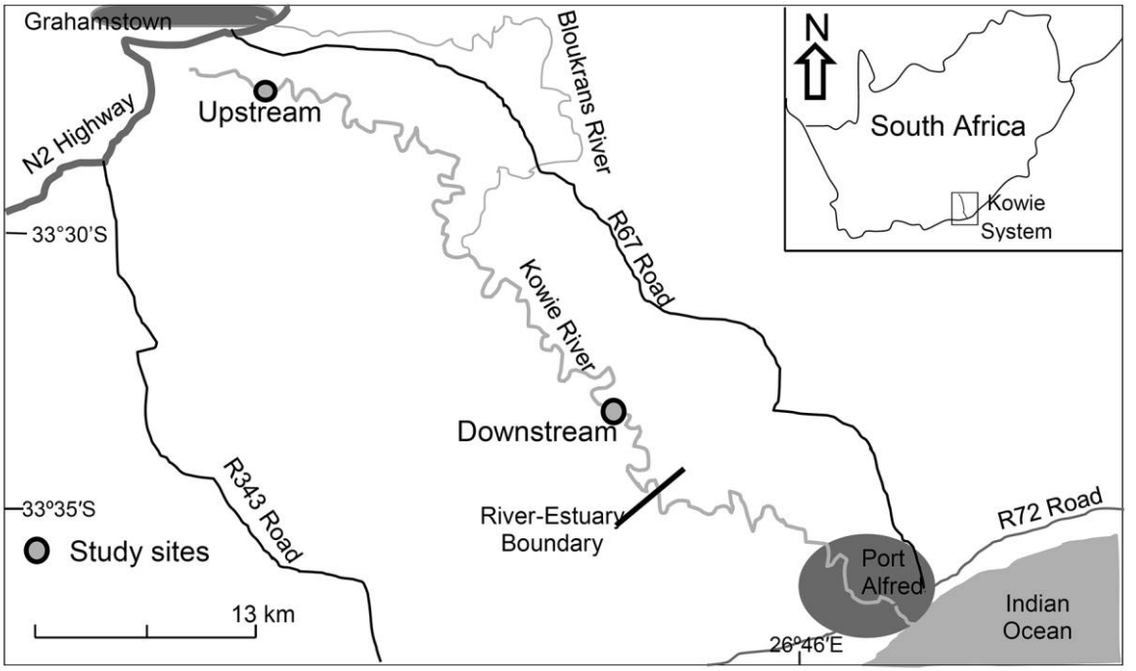

Figure 1. Location of the 2 study sites on the Kowie River, South Africa.

vegetation. Three bricks were placed randomly at water depths of 20 to 40 cm at each of the 2 sites to act as an additional artificial substrate. Tiles and bricks were collected 30 d after deployment. All substrates were shaken gently in stream water to remove any loosely attached sediment. The tile structures were lifted gently from the stream, and all tiles were removed carefully. A toothbrush was used to brush phytobenthos from each brick (n = 3) or tile (n = 3; 3 tiles = 1 sample) into containers filled with distilled water. The resulting suspensions were stored on ice and processed immediately (within ~8 h of collection) upon arrival in the laboratory.

Natural substrates (plants, sand, and rocks) were sampled on the day the artificial substrates were collected. Three replicates of each substrate type were collected at each site. Epiphytic phytobenthos was sampled from randomly selected *Cyperus* sp. within a 5- × 5-m area. Each replicate consisted of the phytobenthos on ≥5 whole stalks comprising stems and leaves. For each replicate, stalks were cut carefully and removed from the stream. The phytobenthos was removed by brushing the stalks with a toothbrush into a container filled with distilled water. Epipsammic phytobenthos was sampled at 3 haphazardly chosen locations at each site by drawing sand into a syringe (depth ~0.5–1 cm) at water depths of 10 to 20 cm (upstream) or 20 to 40 cm (downstream) (Taylor et al. 2005, Dalu et al. 2014a, b). The contents of the syringe were emptied into a container. Each replicate of epilithic phytobenthos consisted of material brushed from ≥10 pebbles/cobbles ranging from 64–256 mm in diameter collected from each site. Phytobenthos was removed by brushing material from the pebbles making up a replicate into a container filled with distilled water.

In the laboratory, all visible foreign particles (e.g., insects) were removed from the samples by prefiltering through a 63-μm-mesh sieve. The material that passed through the sieve was filtered onto precombusted (5 h at 500°C) Whatman glass-fiber filters (GF/F, pore size = 0.7 μm) for FA analysis. The GF/F filters were observed under a dissecting microscope and any large particles that passed through the 63-μm mesh were removed manually. The phytobenthos samples on GF/F filters were freeze-dried with a VirTis benchtop 2K (SP Industries, Warminster, Pennsylvania) for ≥24 h. Freeze-dried material was scraped from the filters and ground to a fine, homogeneous powder. Aliquots (35–70 mg) from each replicate were used for FA analysis (described below). Samples for biomass assessment were analyzed according to Bahls (1993).

FA analysis

Aliquots of phytobenthos dry biomass from each sample (10–20 mg) were processed by means of a modified 1-step method (Indarti et al. 2005) following modifications provided by Bergamino et al. (2014). Aliquots were added to lipid-cleaned 15-mL test tubes containing 2 mL chloroform with 0.01% butylated hydroxytoluene. About 0.06 mL of an internal standard (nonadecanoic acid 19:0; 6.2–10 mg standard in 10 mL chloroform) was added to each sample to permit quantification of the FA methyl esters

Table 1. Permutational analysis of variance (PERMANOVA) based on Euclidian distances of essential fatty acid (EFA) content (18 : 2ω6, 18 : 3ω3, 20 : 4ω6, 20 : 5ω3, and 22 : 6ω3) in phytobenthos and subsequent post hoc tests to identify differences within each season. df = degrees of freedom, MS = mean squares, p(MC) = Monte Carlo probability, bold indicates $p < 0.05$.

PERMANOVA	Source of variation	df	MS	Pseudo-F	p(MC)
Total	Season	2	66.621	17.571	**0.0001**
	Site	1	47.088	12.419	**0.0009**
	Substrate type	5	65.148	17.183	**0.0001**
	Season × site	2	37.658	9.932	**0.0002**
	Season × substrate type	8	53.304	14.059	**0.0001**
	Substrate type × site	5	78.928	20.817	**0.0001**
	Season × substrate type × site	8	58.822	15.514	**0.0001**
	Residual	62	3.792		
Summer	Site	1	129.29	12.505	**0.0013**
	Substrate type	4	173.62	16.794	**0.0001**
	Site × substrate type	4	210.42	20.353	**0.0001**
	Residual	20	10.338		
Autumn	Site	1	5.0557	4.7848	**0.0263**
	Substrate type	5	13.576	12.849	**0.0001**
	Site × substrate type	5	11.317	10.711	**0.0001**
	Residual	26	1.0566		
Winter	Site	1	8.0703	50.564	**0.0001**
	Substrate type	5	5.5524	34.788	**0.0001**
	Site × substrate type	5	3.2246	20.204	**0.0001**
	Residual	24	0.160		

(FAMEs). Sodium sulfate-dried methanol/sulfuric acid solution (2 mL) was added to each sample. The samples were topped with N and sealed with Teflon tape before being placed at 100°C for 30 min. After cooling, 1 mL Milli-Q® water (Millipore, Billerica, Massachusetts) was added to each tube before centrifugation at 2500 rpm for 3 min. After centrifugation, the top aqueous layer was discarded and the lower FAME layer was transferred through a column of anhydrous sodium sulfate into a clean tube, followed by 3 rinses with chloroform. The final FAME mixture was evaporated to dryness and topped with 0.5 mL hexane. Gas chromatographic analyses of FAMEs suspended in hexane were performed with a 7890A GC (Agilent, Beijing, China) fitted with a Zebron WAXplus 320 column (Phenomenex, Torrance, California) and with He as the carrier gas. Aliquots (1 μL) of the samples were injected (250°C inlet temperature) with a G7683 auto-injector (Agilent). The initial oven temperature was 150°C, and it was raised to 225°C at 2.5°C/min after 5 min (detector temperature set at 280°C).

Peaks were detected with a flame ionization detector, integrated with ChemStation software (version B.04.02; Agilent), and identified with external standards (37 component FAMEs standard and marine PUFA no. 1; Sigma Aldrich, St Louis, Missouri) and an Agilent Triple Quadrupole QQQ mass spectrometer with MassHunter software (version 5.00; Agilent) coupled with a National Institute of Standards and Technology 08 MS library. Each FA was reported quantitatively as μg FA/mg dry biomass of each sample in the shorthand format, x : aωb, where x is the number of C atoms in the acyl chain, a is the number of double bonds, and b is the 1st double-bond position from the methyl end of the molecule. Sums of PUFA (ΣPUFA) content (i.e., sum of all ω3, ω4, ω5, and ω6 FAs) were calculated, and the following FAs were considered to be EFAs: 18 : 2ω6, 18 : 3ω3, 20 : 4ω6, 20 : 5ω3, and 22 : 6ω3. In addition, 3 combinations of PUFAs were calculated: Σω3 (sum of all ω3 FAs) and Σω6 (sum of all ω6 FAs) and the ω3 : ω6 ratios (calculated from Σω3 relative to Σω6 FAs) as indicators for nutritional quality (Arts et al. 2001, Larson et al. 2013, Masclaux et al. 2014).

Data analysis

Biomass-specific content (μg FA/mg dry biomass) of individual EFAs in phytobenthos was compared between study sites, among substrate types, and among and within seasons. Distance-based Permutational Analysis of Variance (PERMANOVA; Anderson 2001, McArdle and Anderson 2001) based on Euclidean distance dissimilarities was used to analyze the multivariate EFA data. Each term in the analysis was tested with 9999 permutations of the relevant permutable

Table 2. Mean (±SD, n = 3) essential fatty acid (EFA) content in phytobenthos (μg FA/mg dry biomass) on different substrates in the Kowie River. Macro = macrophytes, T. = clay tiles, – = no phytobenthos were collected, TFA = total fatty acids.

	Upstream						Downstream					
	Natural			Artificial			Natural			Artificial		
FA	Sediment	Macro	Rocks	Brown T.	Grey T.	Brick	Sediment	Macro	Rocks	Brown T.	Grey T.	Brick
Summer												
18 : 2ω6	0.04 ± 0.04	0.91 ± 0.32	0.15 ± 0.03	2.03 ± 0.61	0.32 ± 0.03	–	0.16 ± 0.01	0.56 ± 0.18	0.25 ± 0.25	0.53 ± 0.36	6.00 ± 1.09	–
18 : 3ω3	0.01 ± 0.01	0.08 ± 0.03	0.03 ± 0.02	0.60 ± 0.02	0.12 ± 0.01	–	0.06 ± 0.03	0.01 ± 0.02		0.12 ± 0.08	1.40 ± 0.23	–
20 : 4ω6	0.02 ± 0.30	0.17 ± 0.05	0.05 ± 0.01	1.23 ± 0.79	0.13 ± 0.02	–	0.12 ± 0.01	0.11 ± 0.13		0.22 ± 0.11	2.86 ± 1.06	–
20 : 5ω3	0.11 ± 0.12	2.65 ± 1.00	0.28 ± 0.22	4.90 ± 2.14	0.65 ± 0.09	–	0.81 ± 0.59	0.16 ± 0.16	0.34 ± 0.38	2.36 ± 1.9	22.83 ± 9.33	–
22 : 6ω3	0.03 ± 0.01	0.18 ± 0.17	0.02 ± 0.03	0.69 ± 0.08	0.15 ± 0.03	–	0.04 ± 0.03	0.73 ± 0.49	0.30 ± 0.04	0.22 ± 0.19	2.14 ± 0.93	–
Σω3	0.25 ± 0.23	3.22 ± 1.00	0.48 ± 0.34	7.63 ± 2.64	1.10 ± 0.15	–	1.04 ± 0.67	1.16 ± 0.60	0.70 ± 0.46	4.36 ± 3.98	39.95 ± 18.6	–
Σω6	0.09 ± 0.08	1.48 ± 0.68	0.24 ± 0.06	4.49 ± 1.71	0.68 ± 0.07	–	0.39 ± 0.03	0.98 ± 0.33	0.26 ± 0.24	1.27 ± 0.49	12.59 ± 2.28	–
ω3 : ω6	2.74 ± 0.08	2.28 ± 0.34	1.88 ± 1.21	1.71 ± 0.06	1.61 ± 0.05	–	2.63 ± 0.90	1.12 ± 0.30	3.20 ± 1.13	3.06 ± 1.95	3.19 ± 1.42	–
TFA	7.01 ± 1.90	16.83 ± 4.95	6.13 ± 2.40	15.36 ± 2.29	79.07 ± 12.06		1.96 ± 1.54	28.57 ± 9.57	5.84 ± 1.65	21.79 ± 15.06	226.05 ± 50.33	
Autumn												
18 : 2ω6	0.06 ± 0.05	0.64 ± 0.37	0.11 ± 0.12	0.08 ± 0.04	0.12 ± 0.02	0.04 ± 0.02	0.01 ± 0.02	0.59 ± 0.18	0.18 ± 0.05	0.12 ± 0.03	0.17 ± 0.04	0.02 ± 0.02
18 : 3ω3	0.87 ± 1.28	0.23 ± 0.08	0.15 ± 0.05	0.17 ± 0.09	0.16 ± 0.02	0.10 ± 0.06	0.01 ± 0.01	0.45 ± 0.24	0.24 ± 0.06	0.20 ± 0.04	0.31 ± 0.06	0.04 ± 0.01
20 : 4ω6	0.17 ± 0.23	0.57 ± 0.36	0.08 ± 0.04	0.05 ± 0.02	0.15 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.25 ± 0.04	0.27 ± 0.08	0.28 ± 0.04	0.38 ± 0.05	0.03 ± 0.02
20 : 5ω3	0.56 ± 0.79	6.26 ± 4.15	0.45 ± 0.28	0.28 ± 0.14	0.63 ± 0.09	0.17 ± 0.05	0.25 ± 0.02	2.28 ± 0.62	1.67 ± 0.43	3.47 ± 0.37	3.96 ± 0.52	1.28 ± 1.46
22 : 6ω3	0.04 ± 0.06	0.23 ± 0.08	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.22 ± 0.04	0.03	0.08 ± 0.02	0.12 ± 0.04	0.01 ± 0.01
Σω3	2.05 ± 3.10	1.29 ± 0.53	0.51 ± 0.14	0.64 ± 0.34	1.00 ± 0.15	0.36 ± 0.06	0.34 ± 0.03	5.25 ± 3.66	2.16 ± 0.53	4.15 ± 0.45	4.88 ± 0.72	1.57 ± 1.74
Σω6	0.30 ± 0.36	0.29 ± 0.19	0.17 ± 0.07	0.16 ± 0.07	0.33 ± 0.04	0.09 ± 0.02	0.12 ± 0.02	1.28 ± 0.40	0.71 ± 0.12	0.61 ± 0.04	0.74 ± 0.20	0.30 ± 0.02
ω3 : ω6	4.54 ± 2.83	5.89 ± 2.93	3.17 ± 0.51	3.95 ± 0.51	2.99 ± 0.21	4.05 ± 0.17	2.97 ± 0.78	3.85 ± 1.49	3.14 ± 1.27	6.73 ± 0.28	6.75 ± 0.88	5.23 ± 0.81
TFA	5.08 ± 0.15	27.56 ± 13.11	7.73 ± 0.81	13.84 ± 2.12	17.5 ± 3.80	2.69 ± 1.71	1.29 ± 0.09	13.56 ± 4.11	14.34 ± 1.50	12.86 ± 1.41	6.38 ± 0.76	1.47 ± 0.30
Winter												
18 : 2ω6	0.06 ± 0.05	0.64 ± 0.37	0.11 ± 0.12	0.08 ± 0.04	0.12 ± 0.02	0.04 ± 0.02	0.01 ± 0.02	0.59 ± 0.18	0.18 ± 0.05	0.12 ± 0.03	0.17 ± 0.04	0.02 ± 0.02
18 : 3ω3	0.87 ± 1.28	0.23 ± 0.08	0.15 ± 0.05	0.17 ± 0.09	0.16 ± 0.02	0.10 ± 0.06	0.01 ± 0.01	0.45 ± 0.24	0.24 ± 0.06	0.20 ± 0.04	0.31 ± 0.06	0.04 ± 0.01
20 : 4ω6	0.17 ± 0.23	0.57 ± 0.36	0.08 ± 0.04	0.05 ± 0.02	0.15 ± 0.02	0.03 ± 0.01	0.02 ± 0.01	0.25 ± 0.04	0.27 ± 0.08	0.28 ± 0.04	0.38 ± 0.05	0.03 ± 0.02
20 : 5ω3	0.56 ± 0.79	6.26 ± 4.15	0.45 ± 0.28	0.28 ± 0.14	0.63 ± 0.09	0.17 ± 0.05	0.25 ± 0.02	2.28 ± 0.62	1.67 ± 0.43	3.47 ± 0.37	3.96 ± 0.52	1.28 ± 1.46
22 : 6ω3	0.04 ± 0.06	0.23 ± 0.08	0.01 ± 0.01	0.02 ± 0.01	0.03 ± 0.02	0.01 ± 0.01	0.01 ± 0.01	0.22 ± 0.04	0.03	0.08 ± 0.02	0.12 ± 0.04	0.01 ± 0.01
Σω3	0.24 ± 0.12	0.15 ± 0.08	6.11 ± 6.05	1.32 ± 1.24	1.44 ± 0.03	0.48 ± 0.08	1.26 ± 1.27	2.66 ± 1.07	4.77 ± 1.20	6.97 ± 1.35	5.16 ± 0.14	1.62 ± 0.11
Σω6	0.09 ± 0.04	0.08 ± 0.04	3.37 ± 2.87	0.62 ± 0.59	0.37 ± 0.07	0.10 ± 0.02	0.23 ± 0.23	1.48 ± 0.80	0.62 ± 0.21	1.25 ± 0.26	0.92 ± 0.36	0.30 ± 0.02
ω3 : ω6	2.62 ± 0.19	2.02 ± 0.11	2.42 ± 1.31	3.19 ± 1.98	3.85 ± 0.08	5.10 ± 0.59	5.56 ± 0.07	2.33 ± 1.98	8.76 ± 4.75	5.59 ± 0.19	6.11 ± 1.88	5.33 ± 0.09
TFA	25.91 ± 9.85	15.53 ± 2.48	22.31 ± 5.22	21.3 ± 4.86	24.31 ± 5.93	19.41 ± 4.34	20.00 ± 2.61	18.4 ± 1.81	14.41 ± 3.42	18.6 ± 3.17	21.3 ± 3.52	19.6 ± 3.45

units (Anderson and ter Braak 2003), and significant terms were investigated with a posteriori pairwise comparisons with the PERMANOVA *t* statistic (PERMANOVA+ for PRIMER version 6; Anderson et al. 2008). Separate 2- and 3-way analyses of variance (ANOVA) were used to assess the differences in biomass, ΣPUFAs, ΣEFAs, Σω3 and Σω6, and ω3:ω6 in phytobenthos between the 2 study sites and between 2 main substrate types (artificial or natural) among seasons and within each season (SPSS version 16.0; SPSS, Chicago, Illinois). Tukey's Honestly Significant Difference (HSD) post hoc analysis was used to assess significant differences indicated by ANOVA.

Principal components analysis (PCA) in R (version 3.1.1; *prcomp* function, *varimax rotation*; R Project for Statistical Computing, Vienna, Austria) was used with log(x + 1)-transformed data from each season to visualize multivariate EFA patterns between habitats and substrate types within each season.

Casewise multiple univariate regression analysis was carried out (SPSS version 16.0) to search for any relationships among the physicochemical variables and EFA contents in phytobenthos growing on different substrates. The physicochemical data (except pH and temperature; Dalu et al. 2014b) were log(x + 1)-transformed.

RESULTS

EFAs, Σω3, Σω6, and ω3 : ω6

EFA content differed significantly between sites, among and within seasons, and among substrates (PERMANOVA; Tables 1, 2). ΣEFA content differed among seasons and was highest in summer (71.2 ± 3.8 μg FA/mg dry biomass) and lowest in winter (22.8 ± 4.2 μg FA/mg dry biomass; Tables 1, S1). ΣEFA content differed between sites (2-way ANOVA, $F_{1,62}$ = 4.805, p = 0.030) and was higher at the down- than upstream site in summer and winter, but not in autumn (2-way ANOVA: summer: $F_{1,62}$ = 4.922, p = 0.034; autumn: $F_{1,62}$ = 1.531, p = 0.220; winter: $F_{1,62}$ = 8.007, p = 0.006; Fig. 2A–C). ∑EFA content was higher on artificial than on natural substrates (2-way ANOVA, $F_{1,62}$ = 6.593, p = 0.01), except in autumn (2-way ANOVA, $F_{1,62}$ = 0.208, p = 0.696). The highest ΣEFA content occurred downstream on grey tiles in summer (Fig. 2A). The highest content of an individual EFA (2.65 ± 1 μg FA/mg dry mass) on natural substrate occurred on macrophytes in summer (20 : 5ω3). The 22 : 6ω3 content was lower (<0.8 μg FA/mg dry biomass) than that of any other EFA on all substrates throughout the study period, except on grey tiles at the downstream site in summer (Table 2). All FAs identified by season are included in Tables S2–S4.

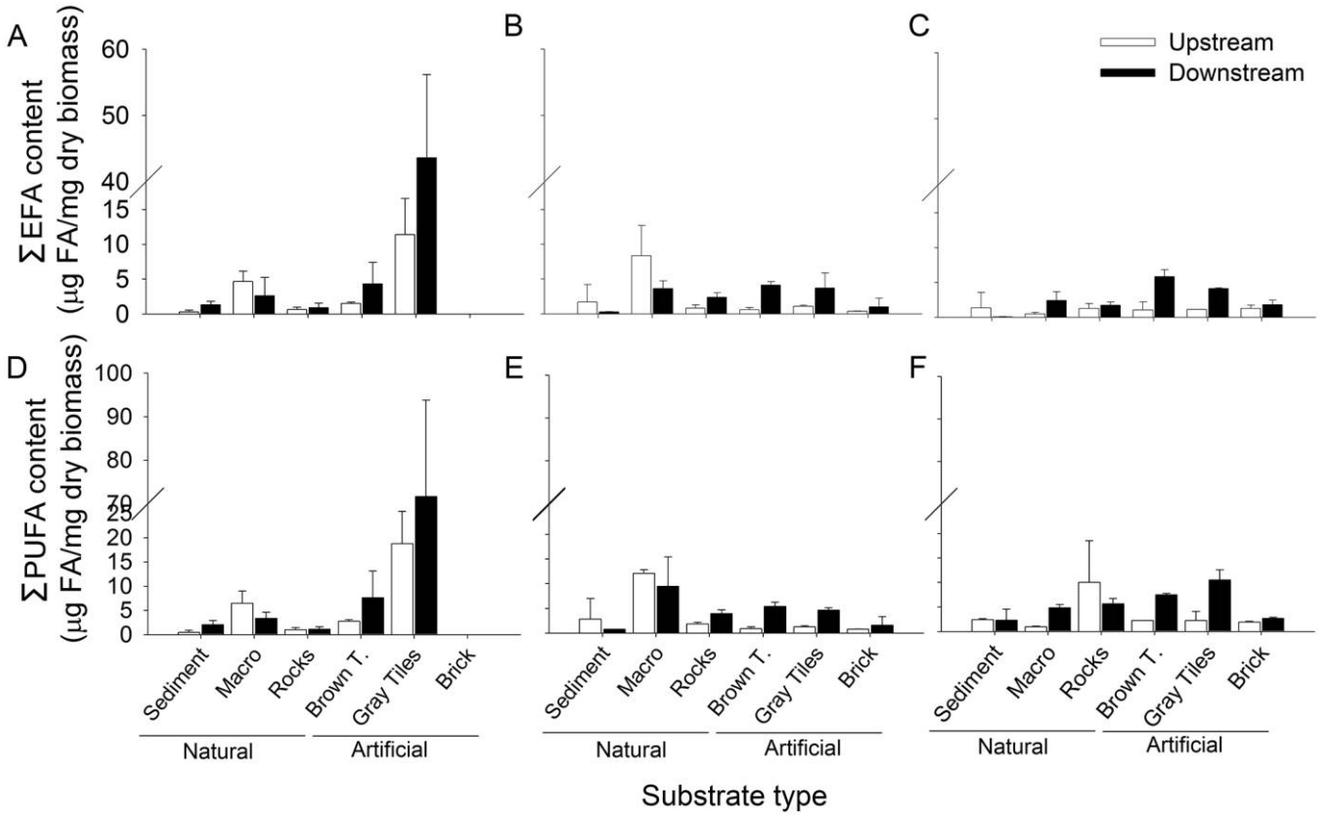

Figure 2. Mean (+SD) sum of essential fatty acids (ΣEFAs) (A, B, C) and sum of polyunsaturated fatty acids (ΣPUFAs) (D, E, F) in phytobenthos growing on different substrates in summer (A, D), autumn (B, E), and winter (C, F). T. = clay tiles, Macro = macrophytes.

Σω3 and Σω6 differed among seasons, sites, and substrate types (3-way ANOVA; Table 3).The ω3 : ω6 ratio was influenced by substrate type, site, and their interaction (3-way ANOVA, Table 3). ω3:ω6 ratios differed between study sites ($p < 0.05$) and were higher at down- than upstream sites in summer and winter, but did not differ in autumn (2-way ANOVA, Table 3).

In summer, Σω3 and Σω6 content differed between sites and among substrate types, and the site × substrate type interaction was significant (summer 2-way ANOVA; Table 3). In autumn, Σω3 and Σω6 content were similar among substrate types and sites (autumn 2-way ANOVA; Table 3). At the upstream site, ω3 : ω6 ratios were higher on artificial than natural substrates (2-way ANOVA, $F_{1,62} = 2.237$, $p = 0.033$), whereas at the downstream site the highest ω3 : ω6 ratio (5.89) occurred on macrophytes (Table 2). In winter, Σω3 and Σω6 content and ω3 : ω6 ratios were higher at the up- than downstream site (winter 2-way ANOVA, Table 3). Phytobenthos had higher ω3 : ω6 ratios on artificial than natural substrates (2-way ANOVA, $F_{1,62} = 4.756$, $p = 0.039$).

Total FA (TFA) content in phytobenthos differed among substrate types, seasons, and between sites (3-way ANOVA; Table 3). On natural substrates up- and downstream, TFA content was highest on macrophytes in summer and autumn and on rocks in winter (2-way ANOVA, $F_{5,58} = 3.211$, $p = 0.038$). TFA content on grey tiles was lower up- (79.07 ± 12.03 μg FA/mg dry biomass) than downstream (226.05 ± 50.33 μg FA/mg dry biomass) in summer (2-way ANOVA, $F_{1,\ 62} = 5.789$, $p = 0.012$). TFA content on sediments and bricks was lower in summer than in autumn and winter at both sites (2-way ANOVA, $F_{1,62} = 4.209$, $p = 0.043$). TFA content did not differ among substrate types at the upstream site in winter (2-way ANOVA, $F_{5,58} = 0.927$, $p = 0.312$) when TFA tended to be highest on sediment (Table 2).

ΣPUFA content on all substrates varied greatly between sites and among seasons (3-way ANOVA; Table 3, Fig. 2D–F). ΣPUFA content was higher at the down- than upstream site (2-way ANOVA, site, $F_{1,62} = 5.512$, $p = 0.001$; Fig. 2D–F). ΣPUFA content differed significantly between artificial and natural substrates (2-way ANOVA, $F_{1,62} = 6.879$, $p = 0.022$). ΣPUFA content was higher on macrophytes than on other natural substrates and higher on grey tiles than on other artificial substrates (Fig. 2D–F).

PCA

EFA contents were separated among substrate types at the up- and downstream sites in the 3 seasons (Fig. 3A–C).

Table 3. Univariate analysis of variance (ANOVA) based on the summation of essential fatty acids (ΣEFAs), the summation of polyunsaturated fatty acids (ΣPUFAs), Σω3and Σω6, and ω3 : ω6 in phytobenthos to identify differences among treatments. Bold indicates $p < 0.05$.

	ΣEFA		ω3, ω6		ω3:ω6		TFA		ΣPUFA	
Source	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
All seasons										
Site	4.805	**0.03**	13.079	**<0.001**	10.827	**<0.001**	32.399	**<0.001**	14.52	**0.01**
Substrate type	6.593	**0.01**	5.55	**<0.001**	3.881	**0.002**	28.158	**<0.001**	6.879	**0.022**
Season	4.507	**0.006**	5.669	**<0.001**	2.866	**0.005**	39.646	**<0.001**	11.183	**0.031**
Site × substrate type	7.458	**0.045**	4.43	**0.007**	3.881	**0.002**	23.899	**<0.001**	16.098	**0.014**
Substrate type × season	6.033	**0.027**	7.112	**0.002**	20.211	**0.002**	23.631	**<0.001**	4.362	**0.001**
Site × season	4.271	**0.047**	18.636	**<0.001**	13.477	**0.008**	7.254	**0.001**	5.841	**0.044**
Substrate × site × season	3.802	**0.035**	7.265	**0.009**	6.593	**0.002**	32.717	**<0.001**	15.983	**0.021**
Summer										
Site	4.922	**0.034**	6.131	**0.023**	5.603	**0.026**	5.789	**0.012**	15.66	**<0.001**
Substrate type	10.658	**0.003**	7.617	**0.002**	6.131	**0.02**	13.657	**0.002**	10.69	**0.005**
Site × substrate type	5.802	**0.015**	8.606	**<0.001**	8.606	**<0.001**	33.72	**0.032**	17.772	**<0.001**
Autumn										
Site	1.531	0.220	2.243	0.137	0.523	0.092	5.231	**0.031**	1.993	0.118
Substrate type	0.208	0.696	0.863	0.538	2.237	**0.033**	14.166	**<0.001**	8.696	**0.007**
Site × substrate type	0.208	0.705	3.432	0.101	1.095	0.391	3.441	**0.023**	2.122	0.099
Winter										
Site	8.007	**0.006**	6.014	**0.006**	7.225	**0.002**	14.861	**0.001**	4.376	**0.005**
Substrate type	9.201	**0.002**	0.475	0.666	4.756	**0.039**	4.226	**0.008**	128.242	**<0.001**
Site × substrate type	6.138	**0.011**	2.324	0.093	2.384	**0.013**	3.815	**0.012**	3.958	**0.009**

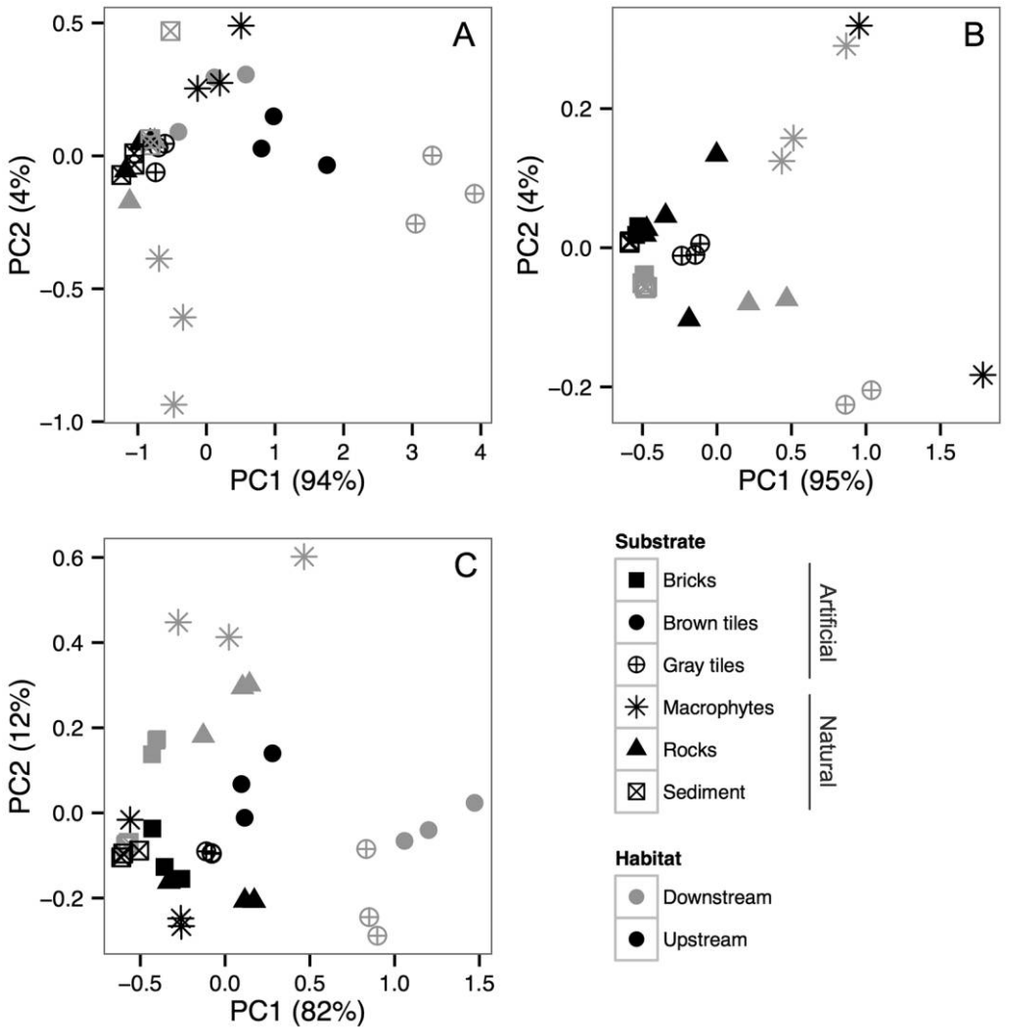

Figure 3. Principal components (PC) analysis of the sum of the content of 5 essential fatty acids in phytobenthos on different substrates in summer (A), autumn (B), and winter (C). An outlier was removed from the analysis in autumn at the upstream site because of excessively high levels of α-linolenic acid (18 : 3ω3) (2.46%) and eicosapentaenoic acid (20:5ω3) (1.46%) relative to all other samples.

Substrate-type differentiation in EFA content was generally more pronounced in the nutrient-enriched downstream site (Fig. 3A–C). During summer at the downstream site, EFA content differed between phytobenthos on grey tiles and all other substrates along the 1st principal component (PC1) axis, whereas EFA content of phytobenthos on macrophytes (i.e., epiphyton) was differentiated from that on brown tiles, grey tiles, and sediments along the PC2 axis (Fig. 3A). In the autumn, the EFA content in phytobenthos on all substrates at the downstream site showed separation along both axes (Fig. 3B), and in winter, phytobenthos on brown and grey tiles downstream were differentiated from others along the PC1 axis (Fig. 3C). Multivariate EFA signatures of phytobenthos from natural and artificial substrate types generally overlapped in the ordination, particularly in the upstream site (Fig. 3A–C).

Physicochemical variables in relation to ΣEFA content in phytobenthos

Regressions between ΣEFA content and physicochemical variables were weak for phytobenthos on sediments, macrophytes, brown tiles, and bricks (e.g., ≤2 significant relationships; Table 4). The ΣEFA content in phytobenthos on grey tile and rock was most strongly related to physicochemical variables. ΣEFA content on macrophytes and brown tiles were related positively with PO_4^{3-} (r = 0.67 and r = 0.50, respectively, both $p < 0.05$), and ΣEFA in phytobenthos on macrophytes was positively related to NO_3^- concentrations (r = 0.48, p = 0.03). ΣEFA content on ≥1 substrate type was related to dissolved O_2, conductivity, total dissolved solids, salinity, temperature, water velocity and depth, or channel width (Table 4).

Table 4. Multiple univariate regression analyses (*r* values) between essential fatty acid (sum of 18 : 2ω6, 18 : 3ω3, 20 : 4ω6, 20 : 5ω3, and 22 : 6ω3 [ΣEFA]) content in phytobenthos growing on different substrates and physicochemical variables along the Kowie River. Macro = macrophytes, T. = clay tiles, bold indicates * = $p < 0.05$ and ** = $p < 0.01$.

	Natural substrate						Artificial substrate					
	Sediment		Macro		Rocks		Brown T.		Gray T.		Bricks	
Variables	$F_{1,16}$	*r*	$F_{1,16}$	*r*	$F_{1,16}$	*r*	$F_{1,16}$	*r*	$F_{1,16}$	*r*	$F_{1,12}$	*r*
Dissolved O_2 (mg/L)	4.52	**−0.47***	0.77	0.21	15.84	**0.71****	0.39	0.15	0.84	−0.22	1.65	0.38
Conductivity (mS/cm)	0.07	0.07	0.01	0.02	8.74	**0.59****	2.39	0.36	28.02	**0.80****	1.73	0.38
Total dissolved solids (mg/L)	0.11	0.08	0.00	0.01	7.58	**0.57***	2.81	0.39	31.4	**0.81****	1.68	0.38
Salinity (ppt)	0.32	0.14	0.18	−0.11	6.62	**0.54***	1.80	0.32	33.29	**0.82****	1.46	0.36
pH	0.13	0.09	1.66	−0.31	12.56	**0.66****	0.62	−0.19	3.14	0.40	1.14	0.32
Temperature (°C)	2.14	0.34	0.05	0.06	1.47	−0.29	3.12	0.40	20.2	**0.75****	1.83	0.39
Water velocity (m/s)	3.06	0.40	1.94	−0.33	0.16	0.10	1.89	0.33	37.41	**0.84****	0.61	0.24
NH_4^+ (mg/L)	1.77	0.32	0.22	0.12	0.05	−0.06	2.62	0.37	2.78	0.38	0.01	−0.03
PO_4^{3-} (mg/L)	2.23	0.35	13.15	**0.67****	1.72	0.31	5.31	**0.50***	1.29	0.27	0.33	0.18
NO_3^- (mg/L)	3.00	0.40	4.69	**0.48***	0.05	−0.05	0.01	0.26	0.52	0.18	0.01	0.02
Water depth (m)	0.65	0.20	0.12	−0.09	2.11	0.34	5.62	**0.51***	39.67	**0.84****	1.57	0.37
Channel width (m)	0.32	0.14	0.15	−0.10	6.83	**0.55***	2.39	0.36	31.33	**0.81****	1.65	0.38

Phytobenthos biomass

Phytobenthos biomass differed among substrate types in all seasons (1-way ANOVA; substrate type, summer: $F_{1,62} = 24.321$, $p < 0.001$; autumn: $F_{1,62} = 8.361$, $p = 0.011$; winter: $F_{1,62} = 4.677$, $p = 0.044$). The highest phytobenthos biomass occurred on grey tiles in summer (Fig. 4A). Phytobenthos biomass generally was higher on artificial substrates (brown and grey tiles) than on natural substrates (Fig. 4A–C), but the difference was not significant in any season (1-way ANOVA; artificial vs natural substrates, summer: $F_{1,62} = 0.193$, $p = 0.670$; autumn: $F_{1,62} = 3.599$, $p = 0.087$; winter: $F_{1,62} = 0.257$, $p = 0.623$). Phytobenthos biomass tended to be higher at down- than upstream sites, but the trend was not significant in any season (1-way ANOVA; site, summer: $F_{1,62} = 0.007$, $p = 0.933$; autumn: $F_{1,62} = 0.068$, $p = 0.800$; winter: $F_{1,62} = 1.160$, $p = 0.307$; Fig. 4A–C).

DISCUSSION

EFA content and composition in phytobenthos varied significantly among substrates and sites, with notably higher EFA quantities in the nutrient-enriched downstream region for most substrates except macrophytes (Table 2). The relationship between ΣEFA and nutrient concentration, the latter of which was used as a proxy of land use in the catchment, resulted mainly from high 20:5ω3 content in phytobenthos at the site situated downstream of intensive landuse practices, including urban centers and farms. Unlike other investigators (e.g., Napolitano et al. 1994, Müller-Navarra et al. 2004, Webb-Robertson et al. 2011, Cashman et al. 2013), who reported decreases in 20:4ω6, 20:5ω3, and other FAs (e.g., 16 : 1ω7, 16 : 2ω6, 20 : 3ω3) at polluted sites, high ΣEFA quantities were observed at our polluted downstream site, as shown by a significant positive regression relationship between nutrients (i.e., NO_3^- and PO_4^{3-}) and ΣEFAs in phytobenthos on macrophytes and brown tiles. Gladyshev et al. (2012) also reported higher EFA quantities in periphyton at downstream polluted than upstream unpolluted sites in the Yenisei River (Krasnoyarsk, Russia).

The increased EFA content in phytobenthos at our downstream site may have arisen from increases and changes in diatom and green algae abundances on the artificial substrates. All phytobenthos types were rich in EFA content, particularly 20 : 5ω3. Green algae are related phylogenetically to vascular plants, and their FA composition differs from that of diatoms (Webb-Robertson et al. 2011, Taipale et al. 2013, Galloway and Winder 2015). The EFA 20 : 5ω3 is typically a dominant component of diatoms (Napolitano et al. 1994), and 18 : 3ω3 is characteristic of some green algae (Ahlgren et al. 1992; 18 : 3ω3 and 18 : 2ω6 also are dominant in higher plants). Thus, high 18 : 3ω3 content suggests higher occurrences of green algae, and 20 : 5ω3 suggests greater diatom occurrence in phytobenthos at different sites, on different substrates, or during different seasons. Thus, the distinctions in the EFA content in phytobenthos growing on different substrates in the Kowie River probably reflected alterations in the taxonomic composition of the phytobenthic communities. Spatial and temporal differences in EFA content in phytobenthos also could be related to shifts in physicochemical variables (Dalu and Froneman 2014, Dalu et al. 2014b). Physicochemical factors affect the growth and development of phytobenthos, and hence their nutritional quality.

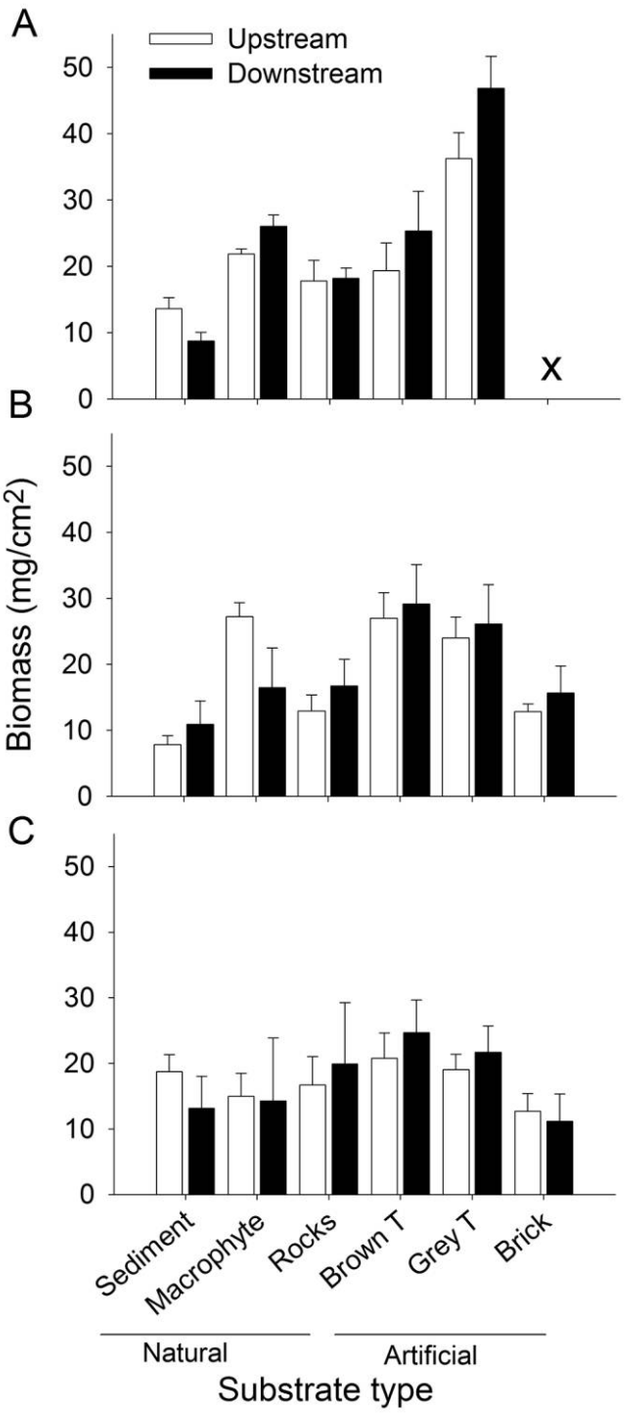

Figure 4. Mean (+SD) phytobenthos dry biomass (mg/cm^2) on 6 substrate types in summer (A), autumn (B), and winter (C). T. = clay tiles. X = no phytobenthos collected.

EFA profiles differed among seasons, sites, and substrate types (Tables S2–S4, Fig. 3A–C). Prowse and Talling (1958) found that phytobenthic development was related to complex environmental changes. Most ΣEFA content on different substrates (except brown tiles) at the upstream site were <1.3 ± 0.3 μg FA/mg dry biomass. In autumn and winter, ΣPUFA content was higher at the down- than upstream site, except for macrophytes (autumn) and rocks (winter) (Fig. 2E, F). The high PUFAs may result from high sewage discharge during the 2 seasons (TD, personal observation).

ΣEFA content on different substrates varied with season. High ΣEFA quantities in all substrates observed downstream were similar to findings by Boëchat et al. (2011) and Larson et al. (2013), who observed increased levels of FAs at the bases of aquatic food webs in response to increasing anthropogenic effects, such as nutrient inputs from fertilizers. In the Kowie River, the phytobenthos exhibited lower nutritional quality, based on the ΣEFA values, at the up- than the downstream site, and 20 : 5ω3 quantities were lower than the saturation threshold (1.3 ± 0.3 μg FA/mg dry biomass) reported by other authors (Ravet et al. 2012, Masclaux et al. 2014). The low ΣEFA content observed at the upstream site (Table 2) could have been a consequence of the high canopy cover, which affects temperature and light regimes and, hence, FA quality (Dalu et al. 2014a, b). For example, Cashman et al. (2013) found that greater light availability and nutrients increased ΣPUFA levels and decreased quantities of several long-chain EFAs (20–22 C) in periphyton.

Among the natural and artificial substrates assessed in the Kowie River, phytobenthos growing on macrophytes and grey tiles had the highest ΣEFA content overall. Phytobenthic biofilms are rich in bacteria and green algae and are often dominated by PUFA-rich diatoms (Hill et al. 2011, Dalu and Froneman 2014). Phytobenthos grew well (based on biomass) on rocks and sediments, but it was nutritionally poor (based on ΣEFA content) compared to phytobenthos growing on macrophytes (Table 1; Dalu et al. 2014a). Relatively high ΣEFA quantities in phytobenthos on macrophytes are consistent with results from other studies (e.g., Rautio and Warwick 2006, Hill et al. 2011, Masclaux et al. 2014), but these studies did not include a variety of substrate types for comparison. Macrophytes can influence the availability of nutrients to phytobenthos by supplying 25 to 60% of the nutrients to their epibiont communities (Díaz-Olarte et al. 2007). Moreover, macrophytes can release labile compounds into the environment, thereby affecting the nutritional quality of the colonizing phytobenthos (Díaz-Olarte et al. 2007, Guariento et al. 2009, Ferragut and de Campos Bicudo 2012). This relationship could have augmented the effect of substrate type on the nutritional quality of phytobenthos on macrophytes at the downstream site of the Kowie River, which is also heavily influenced by nutrient enrichment from sewage discharge and fertilizer input.

The nutritional quality of phytobenthos was higher on macrophytes than other substrate types during autumn, but ΣEFA content on grey tiles was generally highest among the substrate types in summer (Fig. 2A). This result supports an inference that substratum color can influence diatom recruitment in the Kowie River (see also Dobretsov et al. 2013). Moreover, the composition of diatom communities

on grey tiles changed over time. *Achnanthes* spp. and *Pinnularia* spp. dominated in week 1, but *Diploneis* spp., *Gomphonema* spp., and *Staurosira* sp. dominated by week 4 (Dalu et al. 2014b). These changes in community composition should, in turn, have affected ΣEFA content. Higher chlorophyll *a* concentrations were recorded in the biofilms developing on black than on white substrata in Marina Bandar al Rowdha (Muscat, Sea of Oman) (Dobretsov et al. 2013), and phytobenthos prefer darker, less reflective substrata because of their negative phototaxis (Svane and Dolmer 1995). Additional reasons for the differences in communities according to substrate color include variations in albedo and, hence temperature, of the substrata (Dobretsov et al. 2013). The effects of substratum color on phytobenthos community formation are expected to diminish over time as communities stabilize because of increased proportional coverage of growth on the substrate surfaces and eventual elimination of color differences.

Artificial substrates at both sites generally were dominated by young and rapidly growing diatoms (Dalu and Froneman 2014, Dalu et al. 2014a), which have relatively high ΣEFA content and nutritional quality (Galloway and Winder 2015). As anthropogenic development increases, new substrates, such as bricks, tiles, and concrete, are likely to increase in rivers such as the Kowie. The timing of the substrate introductions and the color and nature of the artificial surfaces are likely to affect consumer preferences for phytobenthos on introduced substrates if the nutritional quality of the phytobenthos differs between natural and artificial substrates. Any feeding preferences may affect the trophic structure of the river, which could, in turn, be amplified up the food web to higher levels. Detecting such differences is an important step toward developing an understanding of ecosystem functioning in relation to changes in land use because the nutritional quality of basal resources ultimately determines secondary production (Cashman et al. 2013, Masclaux et al. 2014). The EFA content in phytobenthos growing on different substrates potentially can have an influence on consumer abundances (Masclaux et al. 2014). Future work is needed to identify how higher trophic levels in aquatic food webs respond to changes in anthropogenic factors, i.e., land use, sewage discharge, and climate change, because the effects of shifts in FAs at the base of the food web might affect economically important organisms, such as fishes.

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