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Methane as a carbon source for the food web in raised bog pools

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Abstract. Raised bog pools are extremely nutrient poor and rich in humic substances, and these features limit primary production. To assess the base of the invertebrate food web in bog pools we measured the stable-isotopic signatures of primary producers, dead organic matter, and invertebrates, and the composition and stable-C-isotope ratio of their phospholipid-derived fatty acids (PLFAs). The stable-isotopic signatures showed the presence of multiple trophic levels and differential use of basal food sources by the invertebrates among and within species, individuals, and size classes. Carnivorous and omnivorous invertebrates assimilated polyunsaturated fatty acids (PUFAs) derived from algae, and possibly macrophytes, and fatty acids that are specific for methane-oxidizing bacteria (MOB). Part of the bacterial biomass conveyed to higher trophic levels in the bog pools originated from MOB. Pelagic zooplankton appeared to rely more on bacteria, whereas insects relied more on algae. Periphyton, a primary algal food source, was the basal food source most depleted in ^{13}C and was inferred to sustain $\geq$½ the invertebrate food web. The relatively depleted $\delta^{13}C$ values of PUFAs in invertebrates suggest a role for methane-derived C. We argue that the CO_2 assimilated by the algae could be derived from MOB. Therefore, depleted $\delta^{13}C$ values of invertebrates do not necessarily indicate a direct pathway between MOB and these invertebrates because algae may form an intermediate level.

Key words: peatland, algae, methane-oxidizing bacteria, zooplankton, insects, stable isotopes, fatty acids.

Heterotrophic organisms are sustained by living or dead biomass. This organic matter can be locally produced or imported from elsewhere. In pristine raised bogs, primary production is strongly nutrient limited, and the nutrient content of the dominant *Sphagnum* mosses and vascular plants is extremely low (Aerts et al. 1999). Pools are a significant feature of raised bogs (Belyea and Lancaster 2002) and harbor a large diversity of aquatic macroinvertebrates (Desrochers and van Duinen 2006, Verberk et al. 2006). In these pools, humic water and low levels of light further constrain the primary production by submerged macrophytes and algae (Karlsson et al. 2009). Decomposition rates of dead organic matter also are

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very low because of the acidic conditions and the low nutrient content of living and dead organic matter, which consists mostly of mosses and vascular plants (Belyea 1996, Smolders et al. 2002). The limited primary production and low nutritional value of living and dead plants give rise to the question: What basal food sources sustain the food web in raised bog pools?

Runoff water containing organic C sources could potentially provide an additional basal food source to sustain the food web in raised bog pools. In lakes, the relative importance of these allochthonous organic C sources to the food web increases with decreasing lake trophy and decreasing phytoplankton production (Grey et al. 2000, Pace et al. 2007). Unlike lakes and streams, raised bog pools are isolated from other water bodies and do not have a large catchment area that could supplement the food web with allochthonous organic C and other nutrients. Bog pools are embedded in peat, which constantly releases humic substances. Rydin and Jeglum (2006) suggested that bacteria feeding on these dissolved humic substances might be a 2nd basal food source (in addition to photosynthesis) in bog pools. Jones (1992) described humic substances as an important C source in planktonic food chains in lakes in which primary production of algae is limited by oligotrophy or humic substances. Humic substances are highly recalcitrant to microbial degradation, but Tranvik (1988) found that lakes with a high content of humic substances could support a higher bacterial biomass than clearwater lakes because of their larger pools of dissolved organic C (DOC). Biogenic methane (CH_4) could be a 3rd basal C source. In bogs, CH_4 is produced during the decomposition of peat (Raghoebarsing et al. 2005). CH_4-derived C could contribute to the food web via methanotrophic bacteria, which are ingested by zooplankton (Bastviken et al. 2003, Taipale et al. 2007), chironomid larvae (Jones et al. 2008), and caddisfly larvae grazing their own cases (Trimmer et al. 2009). So in total, 3 potential basal C sources exist in bog pools: primary producers, bacteria feeding on humic substances, and CH_4-derived C.

Dual stable-isotope analysis ($\delta^{13}C$ and $\delta^{15}N$) of producers and consumers is a powerful tool for distinguishing among potential food sources and describing the configuration of food webs. This approach is based on a predictable change in the stable-isotopic composition between trophic levels (DeNiro and Epstein 1978, Minagawa and Wada 1984) and has been applied in a wide range of ecosystems. However, the clarity with which foodweb relationships can be discerned depends greatly on the variation and distinctness of the isotopic signatures of the food sources. In addition, the isotopic signature of a consumer could result from consumption of a single food source, but more realistically usually reflects consumption of a mixture of ≥ 2 food sources. One way to gain a better understanding of the relative importance of basal food sources in food webs is to combine analysis of stable-isotopic composition with analysis of phospholipid-derived fatty acid (PLFA) composition (Kharlamenko et al. 2001, Perga et al. 2006, Van den Meersche et al. 2009). The PLFA approach is based on the specific PLFA composition of bacteria and algae and on the inability of animals to synthesize specific PLFAs and essential polyunsaturated fatty acids (PUFAs; Kharlamenko et al. 2001).

To our knowledge, only Kato et al. (2010) and van Duinen et al. (2006b) have done foodweb studies in temperate bogs by applying stable-isotope analyses. Kato et al. (2010) focused on a hummock-hollow complex rather than raised bog pools. Both studies highlighted a missing basal C source but did not agree on the isotopic composition of that source. Kato et al. (2010) identified dead leaf stalks of a dominant vascular plant (*Menyanthes trifoliata*) and benthic particulate organic matter as the most likely potential food sources for aquatic and terrestrial detritivores, but aquatic predators seemed to rely on another unknown basal food source, enriched in ^{13}C compared to benthic particulate organic matter. In our previous study in raised bog pools (van Duinen et al. 2006b), we inferred that the missing basal C source should be more depleted in ^{13}C than the living macrophytes, filamentous algae, and dead organic matter present in these pools, but we were unable to verify its identity. This depleted food source could be based on CH_4, which is the only component carrying a very negative $\delta^{13}C$ value (Boschker and Middelburg 2002). The role of CH_4 in freshwater food webs has recently attracted much attention (Jones and Grey 2011).

Here, we revisit the enigma of the missing basal C source and investigate the food web of 3 pools in the raised bog Nigula, Southwest Estonia. We analyzed both stable isotopes and PLFAs to assess whether this food web is sustained by the primary producers that dominate the plant biomass in these pools (macrophytes, *Sphagnum* mosses, and vascular plants), their dead organic matter, and dissolved organic substances, or whether algae or methanotrophic bacteria also contribute to the food web. Specifically, we address the following questions: 1) Do the isotopic signatures of the aquatic invertebrates of different trophic levels indicate use of macrophytes, algae, dead organic matter, dissolved organic substances, or other basal

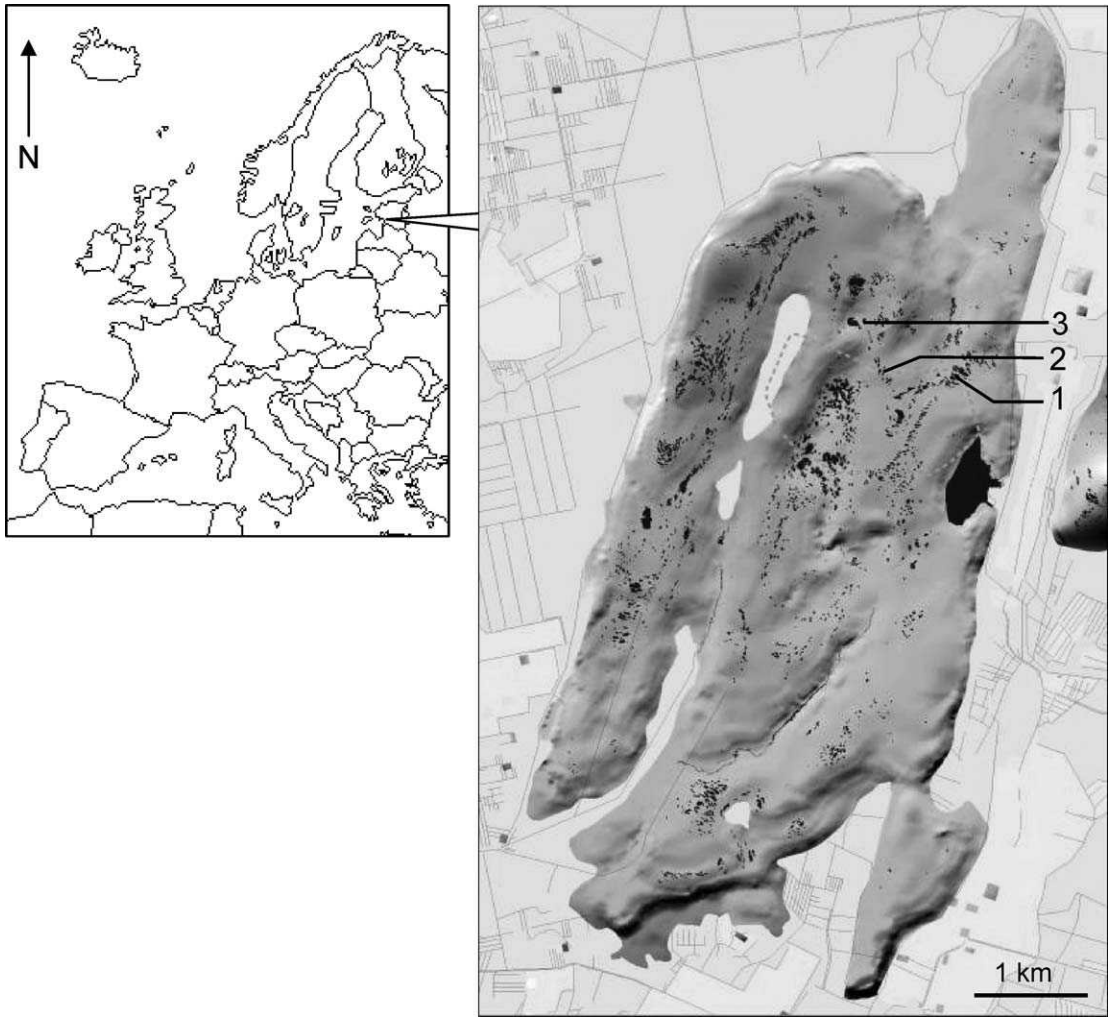

FIG. 1. Geographical location of the study sites in Nigula Nature Reserve, Southwest Estonia. The 3 raised bog pools studied are indicated on a contour map of the raised bog. Bog pools are shown in black.

food sources? 2) Can the PLFA composition of aquatic invertebrates be used to infer the trophic pathways in the food web in raised bog pools?

Methods

Study area

The 3 bog pools (N1, N2, and N3) are situated in the pristine raised-bog massif of Nigula Nature Reserve, southwest Estonia (Fig. 1). At each of the 3 pools, samples of surface water, sediment pore water, and benthic organic matter (BOM) were collected in May 2001 and September 2002 to assess pH and the concentrations of nutrients and other components. For further details about the methods used for analyses see van Duinen et al. (2003, 2006a). The concentration of DOC in surface water was analyzed in samples collected in December 2006 and July 2007. For each pool, nutrient content and other background data are presented as averages of the 2 sampling periods (Table 1).

Sampling and analyses of stable-isotope ratios

Samples of invertebrates and potential food sources for analysis of stable-isotope ratios were collected at the 3 pools in late summer 2002, 2006, and 2007 (Table S1; available online from: http://dx.doi.org/10.1899/12-121.1.s1). Most of these samples were collected in September 2002. Additional samples of DOC, periphyton, and invertebrates flying and walking around the bog pools were collected in late summer of 2006 and 2007. The aquatic larvae collected in September 2002 and the adults of the same taxa flying around the bog pools in August 2006 had stable-isotope ratios in the same range (Tables S2, S3; available online from: http://dx.doi.org/10.1899/12-121.1.s1), a result indi-

TABLE 1. Mean (±1 SD) water-quality data for surface water, sediment pore water, and benthic organic matter at the sampling sites. n = 2 sampling periods. o-PO_4 = orthophosphate, DIC = dissolved inorganic C, DOC = dissolved organic C, DM = dry mass.

Site	Nigula 1	Nigula 2	Nigula 3
Surface water			
pH	3.9 ± 0.1	3.9 ± 0.2	4.0 ± 0.1
o-PO_4 (μmol/L)	0.23 ± 0.10	0.17 ± 0.16	0.28 ± 0.01
NO_3+NH_4 (μmol/L)	4.8 ± 2.5	9.3 ± 4.0	10.7 ± 9.0
Ca (μmol/L)	17.2 ± 8.9	23.3 ± 12.0	25.3 ± 9.9
Cl (μmol/L)	58.7 ± 5.3	90.5 ± 31.3	71.3 ± 5.9
DIC (μmol/L)	22.6 ± 31.9	44.7 ± 25.5	32.6 ± 40.2
DOC (μmol/L)	1654 ± 98	1925 ± 59	1671 ± 457
Sediment pore water			
pH	4.7 ± 0.7	4.6 ± 0.6	4.5 ± 0.2
o-PO_4 (μmol/L)	0.18 ± 0.06	0.16 ± 0.11	0.47 ± 0.65
NO_3+NH_4 (μmol/L)	2.2 ± 2.6	12.7 ± 14.5	26.6 ± 17.6
Ca (μmol/L)	58.2 ± 44.5	55.1 ± 37.7	51.0 ± 26.7
Cl (μmol/L)	55.2 ± 8.9	58.5 ± 6.9	63.0 ± 18.3
DIC (μmol/L)	42.3 ± 14.9	38.9 ± 14.9	54.2 ± 29.1
Benthic organic matter			
C:P (g/g)	1293 ± 636	1309 ± 403	652 ± 347
C:N (g/g)	29.8 ± 17.7	24.7 ± 15.1	17.2 ± 5.7
Ca (μmol/g DM)	72.9 ± 25.1	51.3 ± 10.0	66.4 ± 14.3

cating that stable-isotopic signatures in these non-dynamic bog pools do not differ considerably between years. BOM was collected from the peat bottom by means of a plankton net with mesh size of 45 μm. Zooplankton was collected from the open water by means of a plankton net with mesh size of 115 μm and light traps. As an additional potential source to aquatic invertebrates, periphyton (mainly consisting of algae) was collected by scraping from plastic sheets after rinsing with demineralized water. These sheets (30 × 25 cm) hung vertically in the water bodies for 1 mo (August–September 2007) with their upper end close to the water surface. Fishes do not occur in these pools, and amphibians are rare. Gut contents were not removed from invertebrates because trials with several species showed that they did not empty their guts within ≥2 d of living in filtered bog-pool surface water. Invertebrates were sorted, washed with demineralized water, and kept in a refrigerator until they were identified to species or genus level. Identified material was dried for 24 h at 70°C and subsequently ground using liquid N. Large macroinvertebrates were analyzed individually, whereas smaller individuals were pooled by species. C and N isotopic composition of each sample was determined in duplicate or triplicate with a Carlo Erba NA 1500 elemental analyzer (Carlo Erba Instruments, Milano, Italy) coupled online via a Finnigan ConFlo III interface with a Thermo Finnigan DeltaPlus mass-spectrometer (Thermo-Fisher Scientific, Bremen, Germany).

Surface-water samples for analysis of the $\delta^{13}C$ value of DOC were collected in December 2006 and July 2007 by filtering surface water through a filter with mesh size of 0.2 μm (Schleicher & Schuell FP 030/3; Schleicher & Schuell, Dassel, Germany) and adding 100 μL of 50% H_3PO_4 to a 40-mL water sample. The C isotopic composition of DOC was measured on a high-performance liquid chromatograph (Thermo Electron, Bremen, Germany) coupled via a LC-IsoLink interface to an isotope-ratio mass spectrometer (IRMS; Delta V Advantage; Thermo Electron, Bremen, Germany). The technique of the IsoLink interface is based on the wet oxidation of organic analytes with peroxodisulfate under acidic conditions. The CO_2 produced is subsequently separated from the mobile phase in a capillary gas exchanger flushed with He gas that is dried before introduction into the IRMS (Boschker et al. 2008).

Stable-isotope data are presented as the relative difference between the ratios of the sample and the standards with the formula:

$$\delta R = \left(\left[R_{sample}/R_{standard}\right] - 1\right) \times 100$$

where R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$, $\delta^{13}C$ or $\delta^{15}N$ is the per mille (‰) deviation of the sample from their isotope standards (Vienna PeeDee belemnite for $\delta^{13}C$, atmospheric N_2 for $\delta^{15}N$). High reproducibility was achieved (<0.2‰) based on replicate measurements of samples and the internal standards, sucrose (IAEA-CH-6) for $\delta^{13}C$ and ammonium sulfate (IAEA-N-2) for $\delta^{15}N$.

Estimation of the contribution of basal C sources

We estimated the feasible contributions of potential basal C sources (benthic organic matter [BOM], DOC, submerged *Sphagnum*, vascular plants, filamentous green algae, and periphyton) for each trophic group of invertebrates with isotope-mixing models for $\delta^{13}C$ to get an indication of the contribution of these C sources to the higher trophic levels. Invertebrates were classified in trophic groups according to Nilsson (1996, 1997) and references therein, Vallenduuk and Moller Pillot (2007), Moller Pillot (2009), and Higler (2005). The mean $\delta^{13}C$ value was calculated for each group of basal C sources and each trophic group of invertebrates (carnivores, omnivores, and herbidetritivores [species classified as herbivores, detritivores, and herbidetritivores]) collected in the 3 bog pools and used as input to the mixing model. We used IsoSource (version 1.3.1; Phillips and Gregg 2003), creating all possible combinations of proportions of the 6 potential basal C sources, with increments of these proportions set at 1%. Combinations that summed to the average $\delta^{13}C$ value of the trophic group within a tolerance of 0.1‰ were considered feasible solutions. We assumed trophic fractionation was negligible.

Lipid analyses and stable-isotope analysis of PLFAs

BOM, pelagic zooplankton, and 11 mostly carnivorous insect species were collected in the pools in August 2006 and subsequently freeze-dried and ground. Benthic macrofauna was removed from the BOM samples. Lipid analyses and stable-isotope analyses of PLFAs were done as described by Mohanty et al. (2006). Lipids were extracted from 0.5 g of the BOM and 0.1 g of the invertebrate material with a Bligh–Dyer extraction procedure as modified and described by Boschker et al. (1998, 2001). The lipid extract was fractionated on silicic acid into different polarity classes by sequential elution with chloroform, acetone, and methanol. The methanol fraction containing the PLFA was derivatized with mild-alkaline methanolysis to yield fatty acid methylesters (FAMEs). FAME standards of C12:0 and C19:0 were used for calculating retention indices and for FAME quantification. Identification of FAMEs was based on retention-time data with known standards. Additional identification was gained by gas chromatograph mass spectrometry (GC-MS) using a Thermo Finnigan TRACE GC-MS system (Thermo-Fisher Scientific, Bremen, Germany). For identification of methanotroph-specific PLFAs, extracts of cultures of *Methylomonas methanica* S1 NCIMB 11130, *Methylomicrobium album* NCIMB 11123, *Methylobacter luteus* NCIMB 11914, *Methylocystis parvus* NCIMB 11129, *Methylosinus trichosporium* NCIMB 11131, and *Methylosinus sporium* NCIMB 11126 were used as references. PLFA nomenclature used is as described by Guckert et al. (1985). PLFAs are designated by the number of C atoms. The degree of unsaturation is indicated by a number separated from the chain length by a colon. This number is followed by ωxc or ωxt, where x indicates the position of the double bond nearest to the aliphatic end (ω) of the molecule and c and t indicate a cis and trans stereoisomeric position of the double bond on the molecule. The prefixes i and a refer to iso and anteiso branching. The prefix 10Me refers to methyl branching at the 10^{th} C from the carboxyl group. The prefix br indicates an unknown branching. The prefix cy refers to cyclopropyl rings. PLFAs with unknown molecule structure are referred to by using the equivalent chain length (ECL) expressing their retention time relative to those of known straight-chain saturated FAMEs.

FAME concentrations were measured using a gas chromatograph flame ionization detection (GC-FID) system (Thermo Finnigan TRACE GC; Thermo-Fisher Scientific, Bremen, Germany) equipped with a polar capillary column (SGE, BPX-70; 50 m × 0.32 mm × 0.25 μm; SGE Analytical Science, Austin, Texas), using the following oven conditions: initial temperature of 50°C for 1 min, and then the temperature was programmed to 130°C using a ramp of 40°C/min followed by an increase to 230°C with a ramp of 3°C/min.

Stable-C-isotope ratios for individual FAMEs were measured with a Varian 3400 GC (Varian, Walnut Creek, California) equipped with an ATAS Optic 2 programmable direct thermal desorption injection system (ATAS, Veldhoven, The Netherlands). The GC was coupled via a type-II combustion interface to a Finnigan Delta S isotope ratio mass spectrometer (Thermo-Fisher Scientific, Bremen, Germany). The same polar capillary column was used as for FAME identification and quantification on the GC-FID and GC-IRMS systems. The oven temperature for the GC analyses was as follows: initial temperature of 50°C for 4 min, and then the temperature was programmed to 130°C using a ramp of 30°C/min, which was immediately followed by an increase to 200°C using a ramp of 6°C/min, a subsequent increase to 220°C using a ramp of 5°C/min, and a final increase to 250°C using a ramp of 20°C/min. The sample was injected into the direct thermal desorption system at 50°C, after which the temperature was programmed to 260°C with a ramp of 10°C/s.

PLFAs with a relative concentration <0.1% were disregarded. $\delta^{13}C$ values of PLFAs with a relative concentration <1% are regarded as unreliable and not

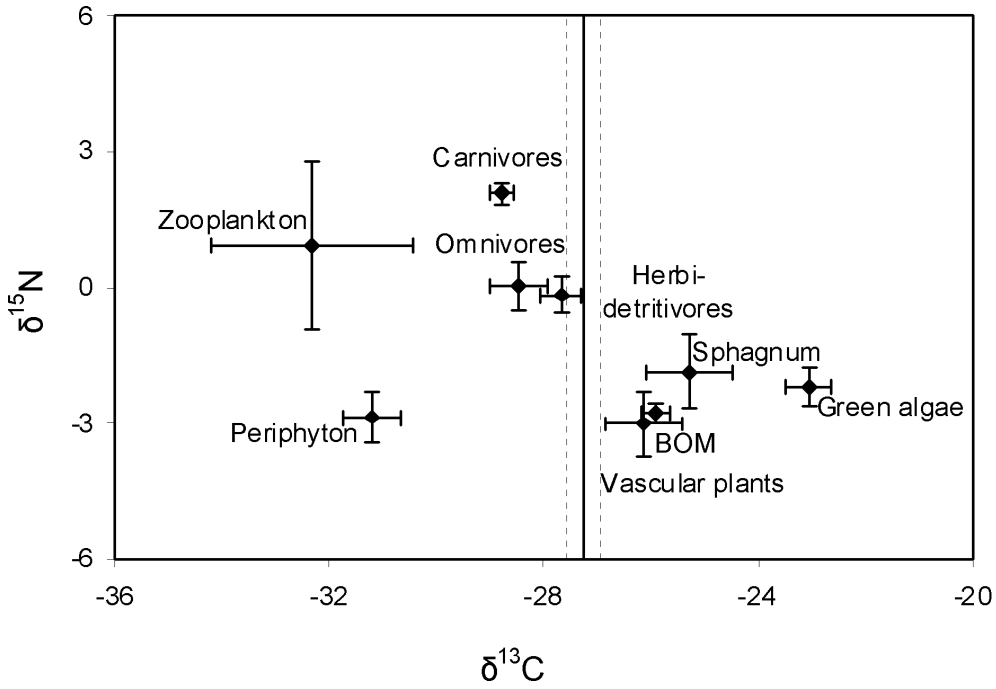

FIG. 2. Mean (±1 SE) of $\delta^{15}N$ and $\delta^{13}C$ of different groups of primary producers, benthic organic matter (BOM), and different trophic groups of invertebrate species in the bog pools N1, N2, and N3. Vertical lines indicate the mean (±1 SE) $\delta^{13}C$ of dissolved organic C (DOC) of the 3 bog pools. The classification of invertebrates in the different trophic groups is given in Table S2.

presented here. The PLFAs found in BOM and invertebrates were classified according to their likely origin (biomarkers for algae, MOB, etc.) based on previous studies. References are mentioned in Table S4 (available online from: http://dx.doi.org/10.1899/12-121.1.s1).

Results

Stable-isotopic signatures

Invertebrate species differed in their $\delta^{13}C$ values, indicating a differential use of basal C sources, and in their $\delta^{15}N$ values, indicating the presence of multiple trophic levels (Fig. 2, Table S2; available online from: http://dx.doi.org/10.1899/12-121.1.s1). The living and dead plant material had the lowest $\delta^{15}N$ values. Most invertebrate species collected were carnivorous according to literature. The highest $\delta^{15}N$ values, in the range of 1.2 to 10.3‰, were found for the water spider *Argyroneta aquatica* and several heteropteran and coleopteran species that are known top predators. The $\delta^{15}N$ values of corixid species and dipteran, isopteran, anisopteran, ephemeropteran, and trichopteran nymphs and larvae ranged between −2.0 and 2.8‰. The invertebrates with the lowest $\delta^{15}N$ values were the herbivores, herbidetritivores, or omnivores, e.g., zooplankton, ephemeropteran nymphs, and larvae of the chironomid genera *Psectrocladius* and *Chironomus*.

Individuals of the large predatory invertebrate species *Acilius canaliculatus*, *Acilius sulcatus*, *Dytiscus dimidiatus*, *Dytiscus lapponicus*, and *Notonecta glauca* were analyzed separately. Various individuals of the same species captured in the same water body on the same day (Table S1) differed strongly in C and N isotopic signature. Differences also were found among different size classes of the same taxa.

Many of the invertebrates were more depleted in ^{13}C (<−28‰) than the dominant primary producers (vascular plants, mosses, filamentous and branched green algae), their dead organic matter, and DOC (Fig. 2). Periphyton (mainly consisting of green algae) was the most depleted potential basal C source. Periphyton varied considerably in $\delta^{13}C$ values among the 3 bog pools (Table S2), but it accounted for the $\delta^{13}C$ value of at least the more-depleted ½ of the invertebrate food web, assuming an enrichment of 0 to 1‰ for the $\delta^{13}C$ values between trophic levels.

The mixing models indicated that the mean contribution of periphyton to the trophic group of the carnivores was 55% (minimum = 44%; 1st percentile). Its estimated mean dietary contribution was somewhat lower for omnivores (49%) and herbidetritivores (34%). The contribution estimated

TABLE 2. Mean (1^{st}–99^{th} percentiles) feasible contribution of potential basal C sources to the different trophic groups of invertebrates. The classification of invertebrate species in the trophic groups is given in Table S2. BOM = benthic organic matter, DOC = dissolved organic C.

Basal C source	Omnivores	Carnivores	Herbidetritivores
BOM	0.10 (0–0.37)	0.09 (0–0.33)	0.14 (0–0.47)
DOC	0.14 (0–0.49)	0.13 (0–0.44)	0.18 (0–0.63)
Sphagnum	0.09 (0–0.33)	0.08 (0–0.29)	0.12 (0–0.42)
Vascular plants	0.11 (0–0.38)	0.10 (0–0.34)	0.14 (0–0.49)
Periphyton	0.49 (0.37–0.60)	0.55 (0.44–0.65)	0.34 (0.18–0.49)
Green algae	0.07 (0–0.24)	0.06 (0–0.21)	0.09 (0–0.30)

for the other potential basal C sources was considerably lower, with means between 6 and 18% (Table 2). The dietary contribution to zooplankton, could not be assessed because their mean $\delta^{13}C$ was more depleted than those of the potential basal C sources.

PLFA composition

PLFAs diagnostic for MOB were found in BOM and in all invertebrates. Relative concentrations of PLFAs diagnostic for MOB type I (16:1ω8c and 16:1ω5t) were $\leq$1.1% (Fig. 3, Table S5; available online from: http://dx.doi.org/10.1899/12-121.1.s1). PLFAs diagnostic for MOB type II (18:1ω8c), were found in BOM (2.6%), but not in invertebrates. The peak of PLFA 18:1ω9c in the GC-MS analysis, which was present in high concentrations in all invertebrates but in a lower concentration in BOM, could have obscured the peak for 18:1ω8c. In addition, PLFAs 18:2ω6c,12c and 18:2ω7c,12c, which are diagnostic biomarkers of MOB II, were detected in BOM and 2 insect species.

The PLFA composition of insects was dominated by PUFAs (36–53%). These PUFAs were diagnostic for algae, or were associated with algae, other plants, fungi, or protozoans grazing on MOB (Fig. 3, Table S5). In the insect species, only 2 to 11% of the PLFA content was diagnostic for bacteria. The PLFA composition of BOM was dominated (58%) by PLFAs diagnostic for bacteria (Fig. 3, Table S5). Zooplankton had a higher saturated-PLFA content than did BOM and insects, and these PLFAs were not diagnostic for algae, bacteria, or other food sources. In zooplankton, 5% of the PLFAs were diagnostic for algae, or were associated with algae, other plants, fungi, or protozoans. Eleven percent was diagnostic for bacteria, and another 11% could be associated with either bacteria or algae (Fig. 3). These results show that the food web in bog pools is sustained by both algae and bacteria, including MOB. Insects in bog pools probably are mainly sustained by algae, whereas pelagic zooplankton are sustained by bacteria.

Stable C isotope ratio of PLFAs

Several PUFAs diagnostic for algae, or associated with algae, other plants, fungi, or protozoans had lower $\delta^{13}C$ values than the PLFA 18:1ω8c diagnostic for MOB or the PLFAs diagnostic for bacteria. The PLFA 18:1ω8c, diagnostic for MOB II and found in BOM, had a $\delta^{13}C$ value of −38.0‰ (Table S6; available online from: http://dx.doi.org/10.1899/12-121.1.s1). The $\delta^{13}C$ values of the PLFAs diagnostic for MOB I could not be measured because of low concentrations ($\leq\sim$1% of the total amount of PLFA). The PLFA 18:1ω7c, typical for bacteria and a major PLFA in MOB, had $\delta^{13}C$ values between −35.9 and −29.8‰. The $\delta^{13}C$ values of other PLFAs typical for bacteria varied between −38.5 and −29.9‰.

The PUFA 20:4ω? was the most depleted PLFA in BOM and zooplankton with $\delta^{13}C$ values of −45.5‰ and −47.1‰, respectively, but was less depleted in the insects, with $\delta^{13}C$ values between −40.3 and −33.2‰. In contrast, the PUFA 20:5ω3 was more depleted in ^{13}C in the invertebrates than in BOM. In most insects, the PUFA 18:3ω6, derived from algae, other plants, fungi, or protozoans, was the most depleted PLFA with $\delta^{13}C$ values between −42.3 and −37.2‰. This PUFA was not detected in zooplankton and had a low concentration in BOM. Therefore, no reliable $\delta^{13}C$ value could be obtained for 18:3ω6 in BOM.

Discussion

Combining the outcomes of stable isotopes and PLFA analyses

The PLFA composition data showed that algae, MOB, and other bacteria were ingested by invertebrate species of different trophic levels, directly or via their prey (Fig. 4). Pelagic zooplankton seemed to rely more on bacteria, whereas algae were more important for insects. The bulk $\delta^{13}C$ values indicated that periphyton sustained at least the depleted ½ of the invertebrate food web ($\delta^{13}C < -28$‰). Living or

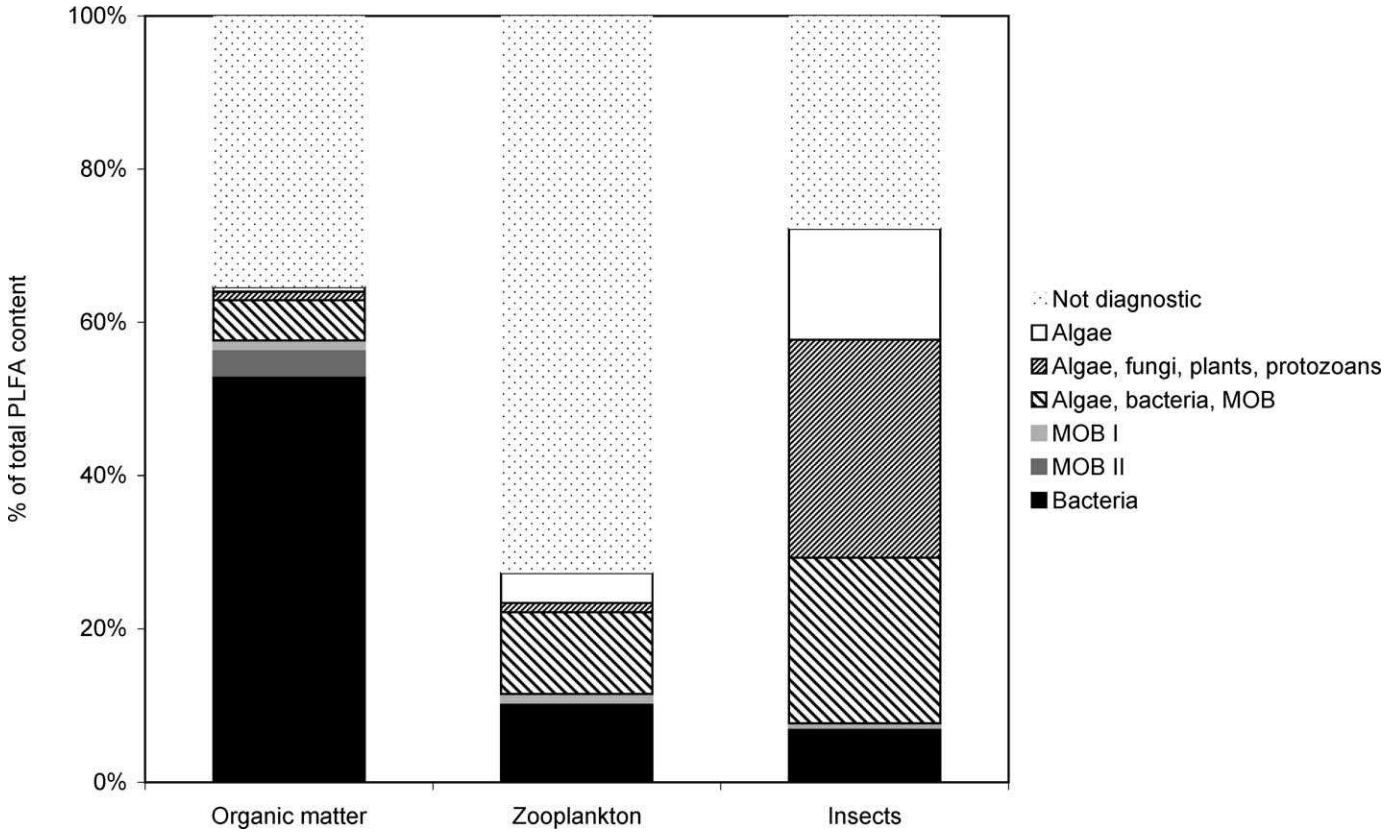

FIG. 3. Phospholipid-derived fatty acid (PLFA) composition, as percentage of total PLFAs, in benthic organic matter, pelagic zooplankton, and aquatic insects in the bog pools N1, N2, and N3. The last bar gives an average of 11 insect species. PLFAs are classified according to their potential origin. See Table S4 for details. MOB = methane-oxidizing bacteria.

dead organic material of the dominant primary producers in the bog pools (*Sphagnum* mosses and vascular plants) could be involved in sustaining the other, less-depleted ½. However, the range of $\delta^{13}C$ values of the different algae sampled (periphyton and larger algae, −35 to −19‰) implies that different species of algae could sustain the whole invertebrate community.

Invertebrates grazing on periphyton may ingest MOB associated with periphyton. The result that PUFAs diagnostic for algae were more depleted in ^{13}C than the PLFA diagnostic for MOB and PLFAs typical for bacteria (Table S6) could indicate use by algae of depleted C, possibly derived from CH_4 via MOB (indicated with the grey curved arrows in Fig. 4). Thus, depleted $\delta^{13}C$ values of whole organisms or PLFAs do not necessarily implicate a direct pathway between MOB and these organisms. Instead, algae could use CH_4-derived C, converted to CO_2 by MOB, like submerged *Sphagnum* mosses do (Raghoebarsing et al. 2005, Kip et al. 2010). Algae and MOB are then intermediates linking CH_4 and aquatic invertebrates.

Stable isotopic signatures and the role of periphyton

The periphyton collected in the bog pools consisted predominantly of green algae, but probably also contained different kinds of bacteria, protozoans, and other microbes. The microbial assemblage living in the periphyton may contribute to its depleted signature. Possibly MOB, which are known to be depleted in ^{13}C (Boschker and Middelburg 2002) and present in *Sphagnum* mosses (Kip et al. 2010), constitute a significant portion of periphyton biomass. Furthermore, the relatively depleted $\delta^{13}C$ values of the bulk of periphyton and of algae-derived PUFAs in insects may be caused by algal assimilation of depleted CO_2 released by MOB.

The $\delta^{13}C$ values of periphyton varied considerably among the 3 pools. In pool N1, periphyton had a $\delta^{13}C$ value of −27.4‰. Periphyton is a mixture of different components (algae, bacteria, protozoa), so components with a $\delta^{13}C$ value <-30‰ were possibly present in N1, like in N2 and N3. High variation in $\delta^{13}C$ values was found among different samples of periphyton and filamentous algae (Table S2). Other

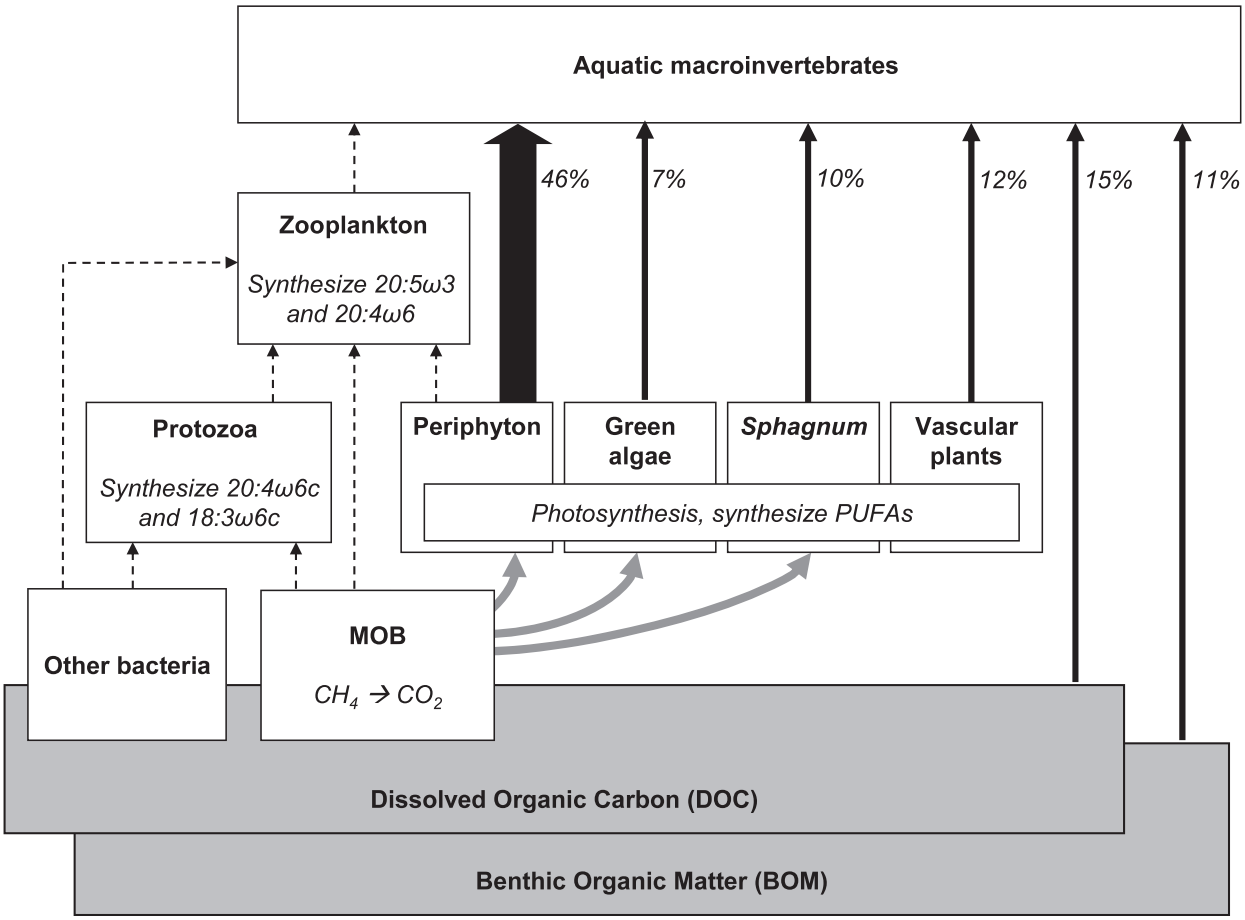

FIG. 4. Schematic representation of the food web in raised bog pools. The percentages next to the black arrows indicate the mean estimated contribution of the basal food sources to the aquatic macroinvertebrates, as derived from the bulk stable-isotope ratios (Table S2). The dashed arrows indicate possible additional trophic relations discussed in our paper based on phospholipid-derived fatty acid (PLFA) data. The grey curved arrows indicate the possible role of methane-oxidizing bacteria (MOB) in the C supply to the primary producers. PUFAs = polyunsaturated fatty acids.

investigators also found considerable variation in $\delta^{13}C$ values of algae during the year and among algae species (e.g., Bontes et al. 2006). In all 3 bog pools, the $\delta^{13}C$ values probably vary seasonally, and periphyton with associated microbes probably is important in sustaining at least the more-depleted ½ of the invertebrate food web. In our study, all sampling was done in the same season (late summer), but sampling of periphyton and invertebrates at various times during the year would give more detailed information about the seasonal fluctuation in the $\delta^{13}C$ values of potential food sources and their consumers. The abundance of green algae and the presence of some pieces of *Sphagnum* mosses and other macrophytes in the guts of several insects in the pools (Odonata nymphs and larvae of Chironomidae and Trichoptera; GAvD, personal observations) support the conclusion that algae, bacteria, and possibly macrophytes are important basal food sources in the bog-pool food web.

Biomarker PLFAs and pathways in the food web

The importance of algae in the bog-pool food web is further confirmed by the relatively high content of PUFAs diagnostic for algae that were found in the insects. In addition, the PLFA data show that MOB and other bacteria are assimilated by invertebrates of different trophic levels. However, the elucidation of the relative importance of these basal food sources to the invertebrate food web of bog pools is somewhat constrained because many of the recorded PLFAs cannot be attributed unambiguously to either macrophytes, algae, MOB, or other bacteria. Furthermore, protozoans and zooplankton might synthesize the PUFAs 18:3ω6, 20:4ω6, and 20:5ω3 that are commonly

used as biomarkers for algae (Caramujo et al. 2008, Murase et al. 2010). Whether this pathway is important in bog pools is not known, but it is unlikely to be the only pathway conveying these PUFAs to insects. The amounts of these PUFAs and their possible ω6 and ω3 precursors in BOM and zooplankton are small compared to the high amounts of these PUFAs in the insects (Table S5), although preferential assimilation of PUFAs by insects might contribute to the higher relative amount of these PUFAs in the insects than in their food.

The amount of 20:5ω3, diagnostic for diatoms and also present in some other groups of algae but absent in bacteria, might provide a rough estimate of the relative amount of PLFAs originating from bacteria or algae, when a more-or-less-constant ratio between 20:5ω3 and the other PLFAs in the algal community is assumed. The relative amount of the PLFA 20:5ω3 was 6× higher in zooplankton and 14 to 37× higher in insects than in BOM (Table S5). Thus, much larger proportions of the PLFA content of the invertebrates originated from algae, and the insects seem to rely on algae more than pelagic zooplankton do (Fig. 3).

Compared to insects, zooplankton assimilated more fatty acids originating from MOB (ingested directly or via protozoans). The relative amount of the PLFA 16:1ω5t, diagnostic for MOB I, was 2 to 3× higher in the zooplankton than in BOM and insects (Table S5). Moreover, zooplankton PLFA composition was different, with a high amount of the PLFAs 14:0, 16:0, and 16:1ω7c in the zooplankton relative to both BOM and insects. These PLFAs were the main ones in which labeled CH_4 was incorporated in forest soil samples (Knief et al. 2003). Differences between BOM and zooplankton in their PLFA composition and in the $\delta^{13}C$ values of PLFAs could result from preferential assimilation of PLFAs by zooplankton. Preferential ingestion of bacteria by the protozoa upon which the zooplankton preys is another possibility. For example, Murase et al. (2010) found that protozoans preferred MOB I to MOB II. Last, the composition of the bacterial community may differ between the seston ingested by zooplankton (collected in open water) and the BOM (collected at the bottom of the pool), with a higher relative abundance of MOB in the seston. Elucidation of the various pathways by which MOB-derived C is assimilated by zooplankton and the relative importance of MOB in bog pools requires further investigation, but the available data indicate that MOB are a significant direct food source for pelagic zooplankton in bog pools (Fig. 3), a finding also reported in lake pelagic food webs (Bastviken et al. 2003, Taipale et al. 2007, Sanseverino et al. 2012).

The PLFA composition of the BOM shows that the living biomass in BOM is dominated by bacteria. In a variety of sediments dominated by bacteria, the sum of the relative amounts of 5 PLFAs diagnostic for bacteria (i14:0, a15:0, i15:0, i16:0, and 18:1ω7c) is 28 ± 4% (Middelburg et al. 2000). Much higher amounts were found in the BOM of the bog pools studied here (41%) because of the high abundance of the PLFA 18:1ω7c (31%). This PLFA is probably the prevailing lipid in methanotrophs in *Sphagnum* moss (Bodelier et al. 2009, van Winden et al. 2010). Using the relative amount of the MOB-specific PLFAs 16:1ω8c and 18:1ω8c in BOM and the fairly constant ratio between these specific PLFAs and nonspecific PLFAs found in MOB strains (Bodelier et al. 2009), we may infer that MOB make up ~10% of the bacterial population in the BOM.

Pathways of CH_4 to higher trophic levels

CH_4 and MOB are depleted in ^{13}C, so the $\delta^{13}C$ values of invertebrates assimilating CH_4-derived C are similarly depleted (Taipale et al. 2007, 2009). The low $\delta^{13}C$ values of the zooplankton samples compared to most insects, including all insects of low trophic level (Table S2), correspond to the larger reliance of zooplankton on MOB inferred from the PLFA data. However, the $\delta^{13}C$ values of the PLFAs 16:1ω7c, possibly derived from MOB (as suggested above), and 18:1ω7c, derived from MOB and other bacteria, were generally less depleted in zooplankton than in the insects (Table S6). In addition, overall, the PUFAs diagnostic for algae or other plants (18:3ω6, 20:4ω?, 20:5ω3) were more depleted than the PLFAs diagnostic for bacteria, including the PLFAs 18:1ω7c and 16:1ω7c, which presumably are partly derived from MOB. For some of these PUFAs, this result might be explained by the possibility that they also can be synthesized by protozoans (cf. Murase et al. 2010) or zooplankton (cf. Caramujo et al. 2008). However, the PLFA 20:4ω? was much more depleted in ^{13}C in the zooplankton than in the insects, indicating a difference in C pathways. CH_4 is depleted in ^{13}C, so this result suggests that the zooplankton synthesized this PLFA from precursor fatty acids (cf. Caramujo et al. 2008) ingested via MOB, or that they ingested protozoa that synthesized this PLFA (cf. Murase et al. 2010), whereas the insects might get the PLFA 20:4ω? via algae and herbivorous prey. Protozoa and zooplankton synthesizing PUFAs commonly used as biomarkers for algae may play a role in the pathway from CH_4 and MOB to invertebrates of the higher trophic levels (Fig. 4). As suggested above, MOB living as a constituent of the

periphyton may play a role in a pathway via algae, oxidizing CH_4 to CO_2, which is consequently assimilated by the algae. This is the most parsimonious explanation for the relatively depleted $\delta^{13}C$ values of algae-derived PUFAs in the insects. Labeling studies with ^{13}C-bicarbonate or $^{13}CH_4$ (Raghoebarsing et al. 2005, Deines et al. 2007, Pace et al. 2007) are required to verify the existence and importance of such intriguing pathways in the food web of bog pools.

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