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Trophic analyses of opportunistic polychaetes (*Ophryotrocha cyclops*) at salmonid aquaculture sites

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A new species of dorvilleid polychaete, Ophryotrocha cyclops*, has been observed on the rocky seafloor underneath deep salmonid aquaculture sites on the south coast of Newfoundland, Canada. The distribution of these opportunistic worms is likely related to organic matter accumulation on the seafloor, and this species may have a role in remediation processes. To better understand the functional role of* O. cyclops *at aquaculture sites, it is important to know what they feed upon. Here, stable isotope analyses ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) and trace element analyses were performed on dorvilleids and their potential food sources at three aquaculture sites. Stable isotope analyses revealed spatial and temporal variation in the isotopic carbon signature of* O. cyclops*, highlighting possible differences in the food sources of individual dorvilleids within and between sites. The isotopic composition of dorvilleids was closest to that of fish pellets; the presence of abundant lipid droplets in gut epithelial cells of* O. cyclops *suggests the assimilation of fish pellet-derived lipids. Trace element analysis indicated that* O. cyclops *does not concentrate the aquaculture tracers Zn or Cu to a large extent. However, concentrations of sulphur were high in* O. cyclops *compared with other sources. Taken together, results show that* O. cyclops *most likely consume both fish pellets and flocculent matter-associated bacteria. As such, they are involved in sulphur cycling and fish pellet degradation at aquaculture sites.*

Keywords: Dorvilleidae, stable isotopes, sulphur, carbon, nitrogen, aquaculture, *Beggiatoa*, *Ophryotrocha*

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INTRODUCTION

At finfish aquaculture sites, organic waste (e.g. faecal matter and unconsumed feed), if not adequately dispersed, can alter seafloor biogeochemistry and benthic community structure (Ye *et al.*, 1991; Pereira *et al.*, 2004; Shahidul Islam & Tanaka, 2004; Carvalho *et al.*, 2006; Yokoyama *et al.*, 2006; Kutti *et al.*, 2007; Borja *et al.*, 2009, Husa *et al.*, 2013). Sedimentary infauna beneath aquaculture cages typically show a decreased biodiversity and a high abundance of opportunistic species compared with adjacent sites; consequently, ecosystem functioning in such sediments is affected (Ye *et al.*, 1991; Crawford *et al.*, 2001; Pereira *et al.*, 2004; Carvalho *et al.*, 2006; Kutti *et al.*, 2008; Borja *et al.*, 2009). In contrast, little is known on the impacts of aquaculture wastes on benthic community function at sites dominated by hard substrates.

Along the south coast of Newfoundland (NL), Canada, salmonid aquaculture takes place in bays and fjords with steep slopes and a patchy substrate, often dominated by coarse particles or bedrock (Hamoutene *et al.*, 2013). Using seafloor imaging, white microbial mats (likely *Beggiatoa* spp.) and opportunistic polychaete complexes (OPC) were found to be directly associated with aquaculture production in this region (Hamoutene *et al.*, 2013; Hamoutene, 2014). OPC are found at aquaculture sites worldwide and are composed of species tolerant of organic matter enrichment and associated reduced conditions (e.g. increased sulphide and methane concentrations), such as the capitellids *Heteromastus filiformis* Claparède, 1864 and *Capitella capitata* Fabricius, 1780 (Nickell *et al.*, 2003; Kutti *et al.*, 2008). In NL, OPC beneath salmon and steelhead trout cages consist of a single new dorvilleid species, *Ophryotrocha cyclops* Salvo *et al.*, 2014, with conspecifics also found on whalebones in Greenland (Salvo *et al.*, 2014). Other *Ophryotrocha* species colonize extreme (and often sporadic) habitats such as wood and whale-falls (Wiklund *et al.*, 2009a, b, 2012), aquaculture sites (Paxton, 2009; Paxton & Davey, 2010), cold methane seeps (Sahling *et al.*, 2002; Levin *et al.*, 2006, 2009, 2013; Thurber *et al.*, 2010) or sites with high forest litter accumulation (McLeod *et al.*, 2010).

Dorvilleids are highly tolerant of organic enrichment and even sulphidic conditions: in cold seeps, 80% of the total abundance of dorvilleids was restricted to sulphide patches (Levin *et al.*, 2006). In such habitats, they are taxonomically diverse and very abundant (>8000 ind. m^{-2}) (Thornhill *et al.*, 2012; Levin *et al.*, 2013) compared with other taxa, and likely play a role in the sulphur cycle. Dorvilleids are often associated with *Beggiatoa* spp. mats (Levin *et al.*, 2009, 2013), and some species within the family selectively feed on sulphide-oxidizing bacteria or methanotrophic archaea

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(Levin *et al.*, 2000, 2009, 2013; Levin & Michener, 2002; Decker & Olu, 2010; Thornhill *et al.*, 2012; Thurber *et al.*, 2012). At NL aquaculture sites, the accumulation of organic matter has likely triggered the development of OPC: using video-imaging, *Ophryotrocha cyclops* presence was mainly documented close to fish cages, where they were typically in high abundance and often co-occurring with *Beggiatoa* spp. mats (Hamoutene *et al.*, 2013; Hamoutene, 2014). The food source(s), habitat requirements, and physiology of this dorvilleid species are not well known. A better understanding of the biology of *O. cyclops* could indicate whether this species plays a role in the remediation of benthic habitats at aquaculture sites following organic waste accumulation.

Here, we examine trophic relationships between *Ophryotrocha cyclops* at NL salmonid farm sites and fish pellets, flocculent matter (a complex mixture of sedimented material; Chou *et al.*, 2002; Yokoyama *et al.*, 2006) and other potential food sources such as macroalgae and suspended particulate organic matter (SPOM) using stable isotope analysis (SIA) ($\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$) and trace element analyses (TEA). The ratios of stable isotopes, especially those of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$), are often used in ecological studies to describe linkages between organisms and their food sources (e.g. Peterson & Fry, 1987; Cranford *et al.*, 2003; Carlier *et al.*, 2010). The $\delta^{13}C$ composition of primary producers is a function of available CO_2 and the degree of isotope fractionation during carbon fixation (which varies between types of primary producers), and animals show $\delta^{13}C$ signatures that are similar to those of their diet (DeNiro & Epstein, 1978; Peterson & Fry, 1987). The $\delta^{15}N$ values of primary producers reflect the available nitrogen source (Peterson & Fry, 1987), and animal $\delta^{15}N$ values increase by an average of 2.3‰ in successive trophic levels (McCutchan *et al.*, 2003). As fish pellets and fish faecal matter often bear characteristic $\delta^{13}C$ and $\delta^{15}N$ signatures depending on their composition (Chou *et al.*, 2002; Yokoyama *et al.*, 2006) and the relative importance of marine and terrestrial-derived products within them, the latter can be used as biomarkers of aquaculture-derived organic matter transfer along food webs. SIA of nitrogen and carbon have been used to examine the dispersion of aquaculture waste, its contribution to sedimented or suspended organic matter (Ye *et al.*, 1991; McGhie *et al.*, 2000; Franco-Nava *et al.*, 2004; Sarà *et al.*, 2004; Vizzini *et al.*, 2005; Yokoyama *et al.*, 2006) and its incorporation into organisms (Grey *et al.*, 2004). $\delta^{34}S$ can further clarify trophic relationships: sulphur isotopes have been used to discriminate between primary producers (Connolly *et al.*, 2004), benthic and pelagic organisms (Peterson, 1999), or terrestrial and marine sources (Moreno *et al.*, 2010). Little to no S fractionation occurs along the food web (Peterson, 1999; McCutchan *et al.*, 2003). At aquaculture sites, $\delta^{34}S$ is likely to be informative given the presence of mats of sulphur-oxidizing bacteria, a potential food source for *O. cyclops*. During bacterial sulphate reduction, isotopic fractionation results in sulphide depleted in ^{34}S (Canfield, 2001). The sulphur-oxidizing bacteria that assimilate this sulphide show ^{34}S depletion, as do organisms consuming these bacteria (Carlier *et al.*, 2010).

Zinc (from fish pellets) and copper (released during fish net cleaning) are two aquaculture-related trace elements that may accumulate in sediments (Chou *et al.*, 2002; Brooks & Mahnken, 2003; Mendiguchía *et al.*, 2006; Dean *et al.*, 2007; Sutherland *et al.*, 2007) and have been used as tracers of aquaculture waste (Chou *et al.*, 2002). Here, we compare the trace element composition of *Ophryotrocha cyclops* with that of their potential food sources (including fish pellets) to explore relationships between these dorvilleids and aquaculture waste.

MATERIALS AND METHODS

Sample collection

Newfoundland salmonid aquaculture occurs mainly along the south coast of the island in a complex of bays and fjords called Fortune Bay. Sampling was performed within 10 m from cages at aquaculture sites located in three bays: site S1 (depth: 54 m) in October 2012, site S2 (depth: 72 m) in November 2012, and site S3 (depth could not be precisely determined due to poor weather conditions but is estimated to be 35–40 m based on previous records) in August 2013. Sites S1 and S2 were in the second year of production while at site S3, salmon had been harvested the previous month. The coordinates of sampling sites are not disclosed herein at the aquaculture industry's request. There is a linear distance of 17.5 km between sites S1 and S3, of 44 km between sites S1 and S2, and of 25.6 km between sites S2 and S3.

We sampled *Ophryotrocha cyclops* during mandatory benthic monitoring surveys performed at aquaculture sites (as described in DFO, 2013). At site S1, *O. cyclops* were first located on the seafloor by video monitoring, at which point sampling was attempted. As grab sampling is inefficient at Newfoundland aquaculture sites (Hamoutene *et al.*, 2013; Hamoutene, 2014), we used a different strategy for worm collection. A net with a 0.2 μm mesh size, held open with a loop of iron, was affixed to one of the edges at the bottom of the cage frame used for video monitoring. We dragged the bottom while observing worm sampling using the video camera. At sites S2 and S3, *O. cyclops* presence was not confirmed prior to sampling (only flocculent matter was observed at site S2, and weather conditions precluded video sampling at site S3); however, we nonetheless attempted to collect worms from these two sites. *Ophryotrocha cyclops* identity was confirmed using genetic analyses as described in Salvo *et al.* (2014).

At each site, seawater was collected using a Niskin bottle at both 1 m from the surface and close to the seafloor, for the analysis of SPOM.

Sample processing

Upon collection, dorvilleids were immediately isolated using a transfer pipette. Some were fixed in 95% EtOH, 4% formaldehyde or 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer for identification, size determination or electron microscopy. Other specimens were kept at 4°C in separate 50 mL tubes with filtered seawater (0.7 μm) from the sampling site for a minimum of 24 h to allow gut content evacuation. Each replicate, consisting of 1 or 2 individuals, was frozen at −20°C in combusted vials. During the fasting period, filtered seawater was renewed and any mucus and faeces in the seawater were collected on pre-weighted and combusted (4 h, 450°C) GFF 47 mm filters (0.7 μm porosity), then dried for 48 h at 60°C. Dorvilleids were not rinsed with

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distilled water prior to freezing as it caused massive tissue rupturing. Separate frozen samples were used for either C&N SIA, for S SIA or for TEA.

All distinguishable types of organic matter found in the net were assumed to be potential food sources for the dorvilleids: pieces of macroalgae were cleaned of epibionts in filtered seawater, rinsed with distilled water and frozen at −20°C and flocculent matter was collected directly from the net and frozen at −20°C in 50 mL tubes. At site S2, partly degraded fish pellets were isolated from flocculent matter and frozen (−20°C) separately. Due to the sampling strategy, we were not able to isolate the bacterial mats that are known to coexist with OPC at NL aquaculture sites (Hamoutene *et al.*, 2013).

Fish pellets were graciously provided to us by the aquaculture companies managing sites S1 and S2, where production was occurring at the time of sampling. At site S2, two types of pellets were collected: with and without medication. At site S3, salmon had been removed from the site about a month prior to OPC sampling, and we did not obtain pellets.

Stable isotope analysis

All samples (except filters) were freeze-dried, manually ground, homogenized and weighed in tin capsules for SIA of carbon and nitrogen (run simultaneously), or sulphur. Filters (containing either SPOM or faeces/mucus) were scraped to collect contents for SIA of nitrogen or sulphur; for carbon analysis, dried filters (with SPOM) were fumigated under HCl vapours to remove carbonates, then dried and scraped. Prior to sulphur SIA, up to 0.200 μg of V_2O_5 was added to each sample. We used a Finnigan MAT252 interfaced with a Carlo Erba NA1500 Series II elemental analyser and an OI Analytical Aurora 1030 TOC Analyser at the CREAIT TERRA Facility Stable Isotope Lab (Memorial University, Canada) for all stable isotope determinations.

Stable isotope ratios are expressed as per convention: $R = {}^{13}C/{}^{12}C$ or ${}^{15}N/{}^{14}N$ or ${}^{34}S/{}^{32}S$, with the reference being Vienna Pee Dee Belemnite for $\delta^{13}C$, atmospheric air for $\delta^{15}N$ and Vienna-Canyon Diablo Triolite for $\delta^{34}S$ (see Coplen *et al.*, 2002). The maximal accuracy was < 0.3‰ for $\delta^{13}C$, <0.4‰ for $\delta^{15}N$ and 1.1‰ for $\delta^{34}S$.

Trace element analysis

A subset of freeze-dried samples was selected for TEA. The dorvilleids considered were exclusively from site S2 and each sample consisted of 1–2 (pooled) individuals (after 48 h of fasting); fish pellets analysed were also from site S2.

Samples were weighed in Teflon screw cap tubes, then bathed in 8N nitric acid for 1 or 2 days at 60°C until dissolution was complete. Then, 1 mL of 30% hydrogen peroxide was added to the samples, which were heated at 60°C for 12 h. Samples were then dissolved in ultrapure water and a 10% dilution of each sample in 0.2N nitric acid was made the following day. TEA were performed at the CREAIT trace element lab (Memorial University, Canada) using a Perkin Elmer Elan DCR II Inductively Coupled Plasma Mass Spectrometer. Each sample except dorvilleids was run twice, with mussels (NIST 2976 and NIST 2977) used as standards. Replicates from dorvilleid samples consisted of one or two individuals per sample and are not analytical replicates.

To localize any accumulations of Zn or Cu in dorvilleid tissues, three individuals from site S2 that had been fixed in glutaraldehyde and post-fixed in 1% osmium tetroxide were processed for elemental analysis using an X-ray detector attached to an environmental scanning electron microscope (ESEM). Dorvilleids were sectioned into anterior, median and posterior fragments, dehydrated and separately embedded in Epon resin. 1 μm thick sections were mounted on aluminium stubs and carbon coated prior to elemental analysis using a standard (solid state) backscattered electron detector (Bruker XFlash SSD 5030) in an FEI Quanta 650F ESEM. Additional 60 nm thick sections were mounted on Cu grids, post-stained with uranyl acetate and lead citrate, and observed using a Philips 300 transmission electron microscope.

Statistical analyses

Comparisons of the isotopic signatures of worms were made using Mann–Whitney and Kruskal–Wallis analyses in Statistica 7.1. We could not statistically compare stable isotope ratios of different food sources or replicates due to low sample size ($N < 5$). Given site-specific differences in isotopic signatures, we chose to analyse sites separately rather than pooling sites and sources within a single analysis. Our data did not meet the necessary conditions for determining either a single, or three site-specific Bayesian mixing models (Caut *et al.*, 2008, 2009; Moore & Semmens, 2008): available sources differed among sites and dates, and some important components (e.g. bacteria, fish faeces) could not be isolated. Moreover, mixing models need to consider isotopic fractionation, which varies between species or tissues considered (McCutchan *et al.*, 2003; Caut *et al.*, 2009). Although Thurber *et al.* (2010) assumed a null fractionation in some dorvilleid species from hydrothermal vents, fractionation has yet to be determined for the species investigated here. For these reasons, our interpretations are based on graphical representations of stable isotope ratios.

RESULTS

Using video monitoring, *Ophryotrocha cyclops* colonies were observed either directly underneath, or within 10 m from finfish cages. In the first bay (site S1), high abundances of dorvilleids were visible at the surface of the rocky substrate (Figure 1) whereas in the second bay (site S2), worms were

Fig. 1. Image of the substrate beneath aquaculture site B (June 2012) in Fortune Bay, NL, extracted from benthic monitoring surveys, showing abundant masses of *Ophryotrocha cyclops*. The inner frame measures 25 × 25 cm.

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not visible using video sampling but were present within flocculent matter. No direct seafloor observation was possible at site S3; however, dorvilleids were collected from this site in association with flocculent matter.

Stable isotope analysis

CARBON

Dorvilleids from sites S1 and S2 differed from those at site S3 in $\delta^{13}C$ values (Table 1), the latter being about 2.5‰ lighter; Mann–Whitney U-tests revealed a significant difference between specimens from sites S2 and S3 ($P < 0.001$; S1 was not included in the analysis because N = 2). Compared with dorvilleid tissues, the faeces and mucus of dorvilleids were depleted in $\delta^{13}C$ (Table 1).

Fish pellets from different sites had a similar carbon isotopic composition (average throughout sites and samples, with or without medication: -19.52 ± 0.35‰). A degraded fish pellet (site S2, −20.05‰) and flocculent matter had slightly lighter carbon isotope values (averages: site S1, -21.31 ± 0.10‰ and site S3, -21.22 ± 1.28‰) than fresh pellets (Table 1).

SPOM differed less in $\delta^{13}C$ between sites than between surface and bottom water samples: the carbon signature at the bottom was lighter (-27.96 ± 1.20‰) than at the surface (-25.47 ± 1.31 ‰) at sites S1 and S2. At site S3, SPOM at the bottom had heavier $\delta^{13}C$ values closer to surface SPOM samples from sites S1 and S2. The various macroalgae sampled had $\delta^{13}C$ values characteristic of estuaries, ranging from −20.48 to −16.88‰, except for red algae, which had lighter $\delta^{13}C$ values (approximately −34‰).

NITROGEN

The nitrogen isotopic composition of dorvilleids from sites S1 and S2 was similar (5.92 ± 0.63‰) and differed from that of worms at site S3 (3.77 ± 0.26‰); a Mann–Whitney U-test revealed a significant difference between samples from sites S2 and S3 ($P < 0.05$; S1 was not included in the analysis because N = 2, Table 1). The $\delta^{15}N$ composition of dorvilleid faeces was close to that of dorvilleid tissues.

The $\delta^{15}N$ composition of fish pellets differed between sites, with values in site S1 being heavier than at site S2. The $\delta^{15}N$ of the degraded fish pellet was slightly heavier than that of fresh pellets (4.88‰) and flocculent matter was lighter at site S3 than at site S1.

The $\delta^{15}N$ values of SPOM ranged from 3.84 to 7.20‰ according to site and depth in the water column. Macroalgae had light $\delta^{15}N$ values ranging from 2.94 to 5.23‰, as expected for marine primary producers.

SULPHUR

Sulphur stable isotope data were not obtained for all material types due to the restricted number of samples available and the limited quantity of sulphur within samples.

The sulphur stable isotopic composition of dorvilleid worms varied greatly between individuals (coefficient of variation = 51%) and among sites: no site-specific significant differences were found using Kruskal–Wallis comparisons ($P > 0.05$). Dorvilleid faeces are more ^{34}S enriched than are individuals (Table 1). The $\delta^{34}S$ signature of algae ranges from 16.86 to 22‰.

Fish pellets are distinctive in $\delta^{34}S$ (5.21 ± 1.85‰) and are the isotopically lightest of samples examined. The degraded fish pellet from site S2 was more ^{34}S enriched (18.03‰) than fresh pellets, as was flocculent matter from S1 site (14.52‰). SPOM (site S2 only) had a $\delta^{34}S$ signature typical of marine phytoplankton, near 20‰.

Table 1. Carbon, nitrogen and sulphur stable isotope ratios of samples from the study sites. Values in $\delta^{15}N+2.3$ represent those expected in a particular source's consumer, assuming a fractionation of 2.3. Values are averages ± SD, with number of replicates in parentheses when greater than 1.

Sample	Site	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{15}N + 2.3$ (‰)	$\delta^{34}S$ (‰)
Ophryotrocha cyclops	S1	-18.64 ± 0.03 (2)	6.12 ± 0.79 (2)		9.95 ± 2.12 (2)
	S2	-18.11 ± 0.18 (9)	5.99 ± 0.42 (9)		6.75 ± 4.64 (9)
	S3	-20.59 ± 0.35 (3)	3.77 ± 0.26 (3)		11.83 ± 1.80 (3)
O. cyclops faeces	S1	-21.89 ± 1.32 (2)	6.15 ± 2.42 (2)		14.2
	S2	-20.17 ± 0.08 (2)	3.92 ± 0.31 (2)		19.35 ± 0.65 (2)
FP, fresh	S1	-19.56 ± 0.27 (4)	4.50 ± 0.43 (4)	6.80	3.54
FP, fresh	S2	-19.42 ± 0.14 (5)	3.21 ± 0.20 (5)	5.51	NA
FP, medicated	S2	-19.94 ± 0.50 (3)	3.25 ± 0.53 (3)	5.55	6.87 ± 2.28 (3)
Average (FP)		-19.59 ± 0.35	3.65 ± 0.71	5.95	5.21 ± 1.85
FP, degraded	S2	−20.05	4.88	7.18	18.03
Filamentous algae	S1	−18.54	2.94	5.24	21.78
Green algae	S1	−17.91	4.70	7.00	20.16
Fucus sp.	S1	−20.48	5.41	7.71	20.04
Ulva sp.	S2	−19.15	2.39	4.69	16.86
Rhodophyceae	S2	−34.26	5.23	7.53	18.05
Green algae	S2	−16.88	3.09	5.39	19.14
SPOM – surface	S1	−26.39	6.29	8.59	NA
SPOM – bottom	S1	−28.81	4.85	7.15	NA
SPOM – surface	S2	−24.54	7.07	9.37	19.66
SPOM – bottom	S2	−27.11	3.84	6.14	20.20 ± 0.23 (2)
SPOM – bottom	S3	−25.14	7.20	9.5	NA
Flocculent matter	S1	-21.31 ± 0.10 (2)	4.35 ± 0.06 (2)	6.65	14.52 ± 0.42 (2)
Flocculent matter	S3	-21.22 ± 1.28 (5)	2.99 ± 0.39 (5)	5.29	12.91 ± 3.14 (9)

FP, fish pellets; NA, not available; SPOM, suspended particulate organic matter.

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Relationships between worms and potential food sources at each site

At site S1, the two dorvilleid $\delta^{13}C$ signatures were close to those of fresh fish pellets and green algae (Figure 2). Dorvilleids also had the heaviest $\delta^{15}N$ values (with the exception of surface SPOM and one of the dorvilleid faeces samples), roughly one trophic level above many of the potential sources, considering a fractionation of 2.3 (Table 1). In plots of carbon *vs.* sulphur isotopic signatures, all green algae were grouped together, with sulphur values corresponding to a pelagic marine source whereas: (1) the dorvilleids were placed between fresh fish pellets (light in $\delta^{34}S$) and marine algae, and (2) dorvilleids were closer to, but slightly lower in $\delta^{34}S$ than flocculent matter and their own faeces (Figure 2).

At site S2, dorvilleids showed the same pattern as in site S1 in $\delta^{13}C$, their signature being close to fresh and degraded fish pellets, their own faeces and algae, and dissimilar to red algae and SPOM (Figure 2). The $\delta^{15}N$ signature of dorvilleids was close to SPOM from the surface. Dorvilleids showed the lightest sulphur isotope values, which did not correspond to typical marine signatures and were closest to fish pellets.

No fish pellets were collected from site S3 because no salmon were present at the time of sampling, but dorvilleids showed isotopic carbon values similar to those of flocculent matter (Figure 2). As in sites S1 and S2, dorvilleids from site S3 are highly variable in $\delta^{34}S$.

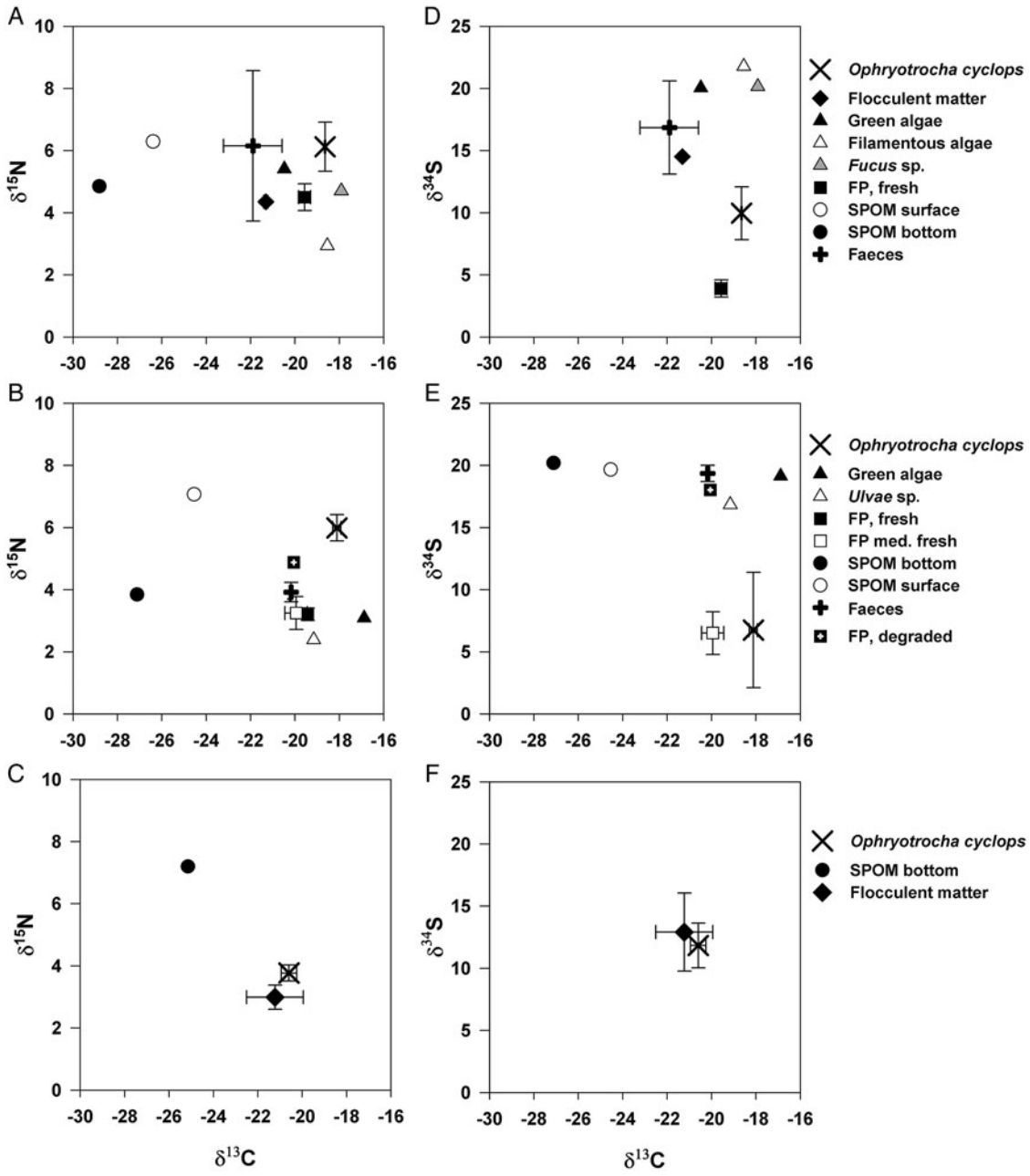

Fig. 2. Stable isotope composition of samples from sites S1 (A, D), S2 (B, E) and S3 (C, F), with data presented as averages ± SD. (A–C) Nitrogen and carbon stable isotope ratios; (D–F) Sulphur and carbon isotope ratios.

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Table 2. Average and SD of Cu, Zn, Fe, Mn and S concentrations (ppm) using trace element analysis. N, number of analytical replicates, except for *Ophryotrocha cyclops*, where each replicate consists of either 1 or 2 whole individuals.

	N	Cu (ppm)	Zn (ppm)	Fe (ppm)	Mn (ppm)	S (ppm)	Site
Ophryotrocha cyclops	4	<4	53 ± 37	330 ± 148	354 ± 2	21 894 ± 81 464	S2
Fish pellet	2	9 ± 1	149 ± 15	462 ± 8	136 ± 18	7587 ± 330	S2
Flocculent matter	2	135 ± 17	433 ± 51	7362 ± 79	59 ± 1	14 539 ± 1203	S3

Trace metal element analysis

Dorvilleid tissues had zinc concentrations ranging from 23 to 96 ppm (Table 2), whereas Zn concentrations were higher in flocculent matter (433 ± 51 ppm). Copper concentrations were relatively low (<10 ppm) in most samples, with flocculent matter again showing the highest values (135 ppm). Fe is most concentrated in flocculent matter (7×10^3 ppm), while in other samples Fe concentrations ranged from 2 to 5×10^2 ppm.

The lowest concentrations of sulphur were found in fish pellets (7×10^4 ppm). Dorvilleids showed a great variability in sulphur concentration ($2.19 \pm 8.15 \times 10^4$ ppm) and range, as did flocculent matter (1×10^4 to 2×10^4 ppm).

The X-ray analysis of dorvilleid tissues using SEM revealed no localized accumulations of Zn or Cu. However, we noted abundant lipid droplets in gut epithelial cells using transmission electron microscopy (Figure 3).

DISCUSSION

We studied several potential *Ophryotrocha cyclops* food sources (SPOM, macroalgae, fish pellets both fresh and degraded, and flocculent matter) collected from aquaculture sites. Despite site-specific variations in isotopic composition, we were able to identify likely and unlikely contributors to the diet of this annelid at aquaculture sites, as detailed below.

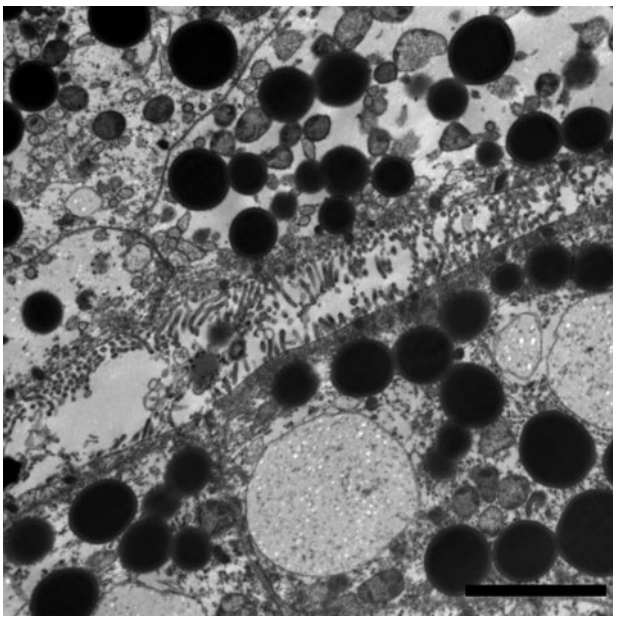

Fig. 3. Transmission electron micrograph of gut epithelial cells of *Ophryotrocha cyclops*. Lipid droplets appear as dark structures within epithelial cells. Scale bar: 5 µm.

Macroalgae and SPOM probably contribute very little, if at all, to the diet of *Ophryotrocha cyclops*. The macroalgae retrieved using the net were rare, mostly fragmented, and occasionally degraded according to their shape and colour but with $\delta^{13}C$ signatures similar to those reported in a study of salmonid farms in Tasmania (*Ulva*: – 19.82‰, Rhodophyceae: −30‰; Ye *et al.*, 1991). While most macroalgae are close in their $\delta^{13}C$ and $\delta^{15}N$ composition to *O. cyclops*, both $\delta^{34}S$ differences (≈10‰) and rarity indicate that macroalgae are unlikely to constitute a perennial food source for the worms, considering a null fractionation for sulphur along the food web (McCutchan *et al.*, 2003). It is also unlikely that *O. cyclops* feeds extensively on suspended or freshly deposited POM, as the $\delta^{13}C$ signature of SPOM at sites S1 and S2 was lighter (by at least 6‰) than that of *O. cyclops*, while the SPOM $\delta^{34}S$ was heavier (≈10‰) and typically marine. Moreover, the shape of the jaw in dorvilleids species is not suggestive of a deposit or suspension-feeding behaviour (Salvo *et al.*, 2014).

The $\delta^{13}C$ and $\delta^{15}N$ signatures of fresh fish pellets were respectively about 1 and 2‰ lower than those of *Ophryotrocha cyclops* from sites S1 and S2, indicating that fish pellets are a likely food source for those worms once average fractionation is considered. Also, the $\delta^{34}S$ of fresh fish pellets was notably lighter (>10‰) than other marine sources, suggesting a terrestrial sulphur component (i.e. plant matter, FAO, 2014) and providing a means of tracing fresh fish pellet-derived organic matter at our study sites. Sulphur isotopic signatures indicate that fresh fish pellets likely form a major part of the diet of *O. cyclops*. The changes in isotopic signature that take place as fish pellets degrade on the seafloor are noteworthy: their $\delta^{13}C$ becomes slightly lighter, while $\delta^{15}N$ and $\delta^{34}S$ become heavier (the latter remarkably so, see Figure 2, S2). These changes may be due to differential decay of various organic matter components (Lehmann *et al.*, 2002), and/or to colonization by microbes; the widely ranging *O. cyclops* $\delta^{34}S$ signatures may reflect their consumption of fish pellets (and associated microbes) at various stages of decay. Moreover, the fish pellets used by aquaculture companies at the time of sampling (or, for site S3, prior to harvesting), were oil-rich (the pellets most commonly used by aquaculture companies in NL, Skretting Optiline, have a lipid content >30%; http://www.skretting.ca). The large, electron-dense droplets observed in gut epithelial cells of *O. cyclops* suggest that they may be assimilating large quantities of fish pellet-derived lipids. A high proportion of lipids could modify the fractionation between food and consumer (Post *et al.*, 2007).

Ophryotrocha cyclops may also consume a fraction of the materials present in flocculent matter. Although the exact composition of flocculent matter at our study site is

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unknown, its isotopic and trace element composition indicate relationships with fish pellets. Flocculent matter is composed of roughly 60% organic matter (unpublished data) and shows high concentrations of Zn and Cu (Table 2), likely due to Zn enrichment in fish pellets, selective Cu excretion in fishes, and Cu use in antifouling paints, as observed at other aquaculture sites (Chou *et al.*, 2002; Brooks & Mahnken, 2003; Mendiguchía *et al.*, 2006; Dean *et al.*, 2007; Sutherland *et al.*, 2007). *Ophryotrocha cyclops*, however, does not accumulate Zn and Cu to a large extent.

Flocculent matter contains microbes, fish faeces, dorvilleid mucus and other sedimented organic matter, which collectively contribute to the viscosity and thickness of this material and explains the intermediate position of flocculent matter in Figure 2. The isotopic signature of flocculent matter is variable at different scales (within S3 and between sites), distinct from SPOM, and likely influenced by resident microbes: a mixture of heterotrophs and chemoautotrophs including sulphur oxidizers such as *Beggiatoa* spp. Chemoautotrophic bacteria show different degrees of specificity for ^{12}C and ^{13}C during carbon fixation, depending on which form of the Rubisco enzyme they contain (Robinson & Cavanaugh, 1995). The relative importance of heterotrophic to chemoautotrophic processes in flocculent matter should vary according to redox conditions and influence the carbon isotope signature. The $\delta^{13}C$ of microbes is also influenced by available inorganic carbon: for instance, isotopically light methane at some seeps leads to light $\delta^{13}C$ in microbes and in organisms that consume them, including dorvilleids (see Levin & Michener, 2002; Levin *et al.*, 2013).

The $\delta^{13}C$ signature of *Ophryotrocha cyclops* was about 2‰ heavier than that of flocculent matter collected from sites S1 and S3, suggesting that the worms may have consumed part of this organic matter (considering fractionation). Also, the slightly more positive $\delta^{15}N$ values of *O. cyclops* compared with flocculent matter are concordant with assimilation of this resource and a trophic level increase. However, the difference in $\delta^{34}S$ (4.5 and 1‰ for S1 and S3, respectively) between flocculent matter and *O. cyclops* may indicate selective feeding (possibly on bacteria with lighter $\delta^{34}S$; Canfield, 2001) within the flocculent matter pool. Interestingly, the $\delta^{34}S$ of flocculent matter from site S1 is similar to that of the sample of dorvilleid mucus and faeces from the same site, supporting the idea that *O. cyclops* feeds on flocculent matter (i.e. material that has gone through the gut shares the signature of flocculent matter). However, the difference in $\delta^{34}S$ between dorvilleids and their faeces may indicate that they assimilate a particular fraction of flocculent matter (possibly the microbial component) and reject the rest (including most of the Cu and Zn-enriched fraction). Other observations support the likely consumption of microbes by *O. cyclops*: specimens collected from whalebones in Greenland were observed to consume white microbial filaments (K. Worsaae, pers. comm.) and dorvilleids from cold seeps contained filamentous microbes in their gut (Levin & Michener, 2002).

The relatively high abundance of sulphur in *Ophryotrocha cyclops* provides further evidence that this dorvilleid likely consumes filamentous bacteria, and accumulates sulphur. The storage of elemental sulphur in *Beggiatoa* spp. is a defining feature of the genus (Teske & Nelson, 2006). While different species of *Beggiatoa* differ metabolically, elemental sulphur likely acts as a reservoir of electron donors within cells (Teske & Nelson, 2006); in some strains, elemental sulphur accumulation was linked to the availability of sulphide or thiosulphate (Nelson & Castenholz, 1981). The high variability in sulphur concentrations among *O. cyclops* samples may reflect small-scale differences in elemental sulphur content between sulphur-metabolizing bacteria at the sampling site.

Factors leading to observed spatial and temporal differences in *Ophryotrocha cyclops* isotopic signatures are not yet known. The $\delta^{13}C$ of individuals from sites S1 and S2 was different from that of individuals from site S3, possibly reflecting differences in: (1) fish pellet composition and abundance (those sites were managed by different companies); (2) state of fish pellet degradation (there were no freshly deposited pellets at site S3); (3) sulphate reduction rates, which vary spatially and temporally around cages according to fine-scale conditions (Holmer & Kristensen, 1994, 1996); and (4) microbial species composition. The lower $\delta^{15}N$ values of both *O. cyclops* and flocculent matter at site S3 also suggest that chemoautotrophic bacteria may comprise a relatively larger fraction of this organic matter pool at this site, and that dorvilleids are consuming these bacteria. Dorvilleids may also be opportunistic and able to prey on different food sources, as suggested by the presence of *O. cyclops* conspecifics at both aquaculture sites in NL and whalebones in Greenland (Salvo *et al.*, 2014). In cold seep dorvilleids, wide isotopic ranges were observed among conspecifics within a site, suggesting some degree of dietary flexibility; however, community analyses revealed trophic partitioning such that dorvilleid species specialized on different types of microbes (Levin *et al.*, 2013). Compared with these cold seep dorvilleids (and considering differences in the inorganic carbon pool at cold seeps and at aquaculture sites), *O. cyclops* from this study most resemble, in their isotopic signature, cold seep species such as *Ophryotrocha maciolekae* and *O. platykephale* which are thought to consume mainly sulphur-oxidizing filamentous bacteria (Levin *et al.*, 2013).

Our investigations at Newfoundland aquaculture sites lead us to conclude that: (1) there are trophic linkages between *Ophryotrocha cyclops* and fish pellets (reflected in Zn signatures, isotopic composition and lipid accumulation in gut epithelial cells); (2) *O. cyclops* select specific components from the flocculent matter and likely consume *Beggiatoa*-like bacteria (isotopic composition and sulphur content); and (3) both fish pellets and bacteria contribute to the complex nature of flocculent matter. Fatty acid or lipid composition/stable isotope analyses may be useful in determining the relative importance of microbes and fish pellets to the diet of *O. cyclops*.

At aquaculture sites, *Ophryotrocha cyclops* presence often coincides with that of *Beggiatoa* spp., based on visual observations of benthic images (Bungay, 2013; Hamoutene *et al.*, 2013). Considering that *O. cyclops* most likely consume aquaculture-derived organic matter (including fish pellets undergoing degradation) and microbes such as *Beggiatoa* spp., we can put forward some hypotheses regarding the functional role of *O. cyclops* at these sites. First, feeding on organic waste can help accelerate the remineralization of this excess organic matter and aid in the recovery of benthic habitats at aquaculture sites. Through their feeding activities, *O. cyclops* may facilitate the transfer of energy and organic matter to higher trophic levels in these environments. Second, selective feeding on microbes can stimulate microbial productivity and accelerate nutrient cycling at the seafloor; in particular,

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O. cyclops may play important roles in the sulphur cycle. Third, the ability of *O. cyclops* to live and move within the flocculent matter layer may further enhance remineralization by increasing oxygenation rates. Further research is needed to clarify the relative importance of *O. cyclops* to nutrient cycling during periods of aquaculture production and fallowing, considering fluxes in population size for this species.

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