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ORIGINAL PAPER

Identifying trophic variation in a marine suspension feeder: DNA- and stable isotope-based dietary analysis in *Mytilus* spp.

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Abstract Accurate field data on trophic interactions for suspension feeders are lacking, and new approaches to dietary analysis are necessary. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was integrated with stable isotope analysis to examine dietary patterns in suspension-feeding *Mytilus* spp. from seven spatially discrete locations within a semi-enclosed marine bay (Strangford Lough, Northern Ireland) during June 2009. Results of the two methods were highly correlated, reflecting dietary variation in a similar manner. Variation in PCR-DGGE data was more strongly correlated with the principal environmental gradient (distance from the opening to the Irish Sea), while values of $\delta^{13}C$ and $\delta^{15}N$ became progressively enriched, suggesting a greater dependence on animal tissue and benthic microalgae. Diatoms and crustaceans were the most frequently observed phylotypes identified by sequencing, but specific DNA results provided little support for the trophic trends observed in the stable isotope data. This combined approach offers an increased level of trophic insight for suspension feeders and could be applied to other organisms.

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Introduction

Suspension-feeding marine bivalves are dominant members of inshore macrobenthic communities. They commonly act as ecosystem engineers (Jones et al. 1994) and serve a critical role in coupling pelagic production with the benthos (Dame 1996). Bivalves occupy a broad trophic niche, which remains poorly defined, and ecologists attempting to characterize their overall diet face distinct challenges posed by the small size of the food they consume and its spatial and temporal variability. Historical investigations of marine bivalve diets indicated the importance of primary producers such as diatoms and dinoflagellates (Lotsy 1895; Galtsoff 1964), while more contemporary work has demonstrated the ingestion of secondary consumers such as copepods and invertebrate larvae (Davenport et al. 2000; Lehane and Davenport 2006), although their contribution as a food resource is not well understood. In addition, it is unknown whether individuals within bivalve populations display a level of

feeding specialization. Such intraspecific niche partitioning can affect a population's stability, intraspecific competition, fitness, ecological impact and its potential to diversify and speciate (Bolnick et al. 2003). These types of investigations have not been reported for suspension feeders, due in part to a lack of robust methodologies.

DNA-based approaches to dietary analysis have been effective at delineating trophic pathways and gaining insights into the feeding ecology of a variety of organisms (Jarman et al. 2002; Blankenship and Yayanos 2005; King et al. 2010). The combination of polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) has been recently applied to marine suspension-feeding bivalves (Maloy et al. 2009). In this approach, DNA extracted from gut contents is amplified by PCR with universal eukaryotic primers and separated on a denaturing gradient gel to create a profile. Each band within a profile represents a different sequence type (Fischer and Lerman 1983), and together, the bands represent the diversity of organisms within the gut, including both ingested organisms and resident parasites. Through the comparison of profile similarities, Maloy et al. (2009) demonstrated that different sympatric bivalve species had different gut contents and concluded that some level of food resource partitioning existed. Though this approach is sensitive and can detect a wide diversity of organisms, gut content profiles offer a short-term picture of ingestion and no information on long-term foraging behaviour or assimilation.

In contrast, biochemical methods such as fatty acid profile analysis (Allan et al. 2010; Ezgeta-Balić et al. 2012) and analysis of stable isotope ratios (Riera et al. 2002; Lefebvre et al. 2009) have been used to trace long-term (weeks–months) dietary patterns. Fatty acid analysis provides information on the lipids characterizing prey and consumers, and stable isotope analysis of assimilated elements, for example, carbon and nitrogen, is routinely used to analyse trophic relationships and energy flow within ecosystems (Peterson 1999; West et al. 2006). Isotopic turnover times for $\delta^{13}C$ and $\delta^{15}N$ in bivalve tissue are 2–6 months (Fukumori et al. 2008; Fertig et al. 2010), and the diet-consumer trophic fractionation of carbon ($\Delta^{13}C = 0.6–0.9$ ‰) and nitrogen ($\Delta^{15}N = 3.4–3.6$ ‰) isotope ratios (Yokoyama et al. 2005b) are within typically reported ranges (McCutchan et al. 2003). Stable isotope studies have also demonstrated the importance of phytoplankton (Yokoyama et al. 2005b; Leal et al. 2008) and microphyto-benthic material (Sauriau and Kang 2000; Kang et al. 2006) in the diets of suspension-feeding bivalves, while indicating that spatial, temporal and hydrodynamic features can influence the relative contributions of various organic matter sources (Decottignies et al. 2007; Dubois et al. 2007). In this respect, stable isotope data are sufficiently sensitive to detect shifts in assimilated carbon and nitrogen due to spatial or environmental variation, but do not identify specific trophic links. This is in contrast with DNA-based approaches, which have the potential to identify ingested organisms to the genus or species level, whereas stable isotope studies typically classify carbon sources into broad functional groups (e.g. phytoplankton v benthic microalgae). In fact, bivalves themselves are routinely used as a baseline proxy for primary production in stable isotope studies exploring higher trophic level relationships within freshwater (Cabana and Rasmussen 1996; Post 2002) and marine food webs (Fukumori et al. 2008; Mallela and Harrod 2008).

DNA dietary analyses based on universal PCR primers are inherently qualitative in nature. PCR bias and variation in gene copy numbers among different dietary species in the gut currently limit quantitative application (Kanagawa 2003; Zhu et al. 2005). Conversely, through the use of various mixing model approaches, stable isotope studies can provide a more quantitative perspective on dietary relationships. However, this is often complicated by the large number of potential dietary sources and a limited number of usable stable isotopes as trophic tracers (Phillips and Gregg 2003). To cope with this, stable isotope studies use multiple sites and times for comparative purposes, employ statistical models, use multiple tracer molecules or artificially add a tracer isotope to the study system (Phillips and Gregg 2003; Fry 2006). Despite the insights these approaches may provide, reducing the number of potential dietary sources required would simplify the mixing model calculations. To this end, it may be possible to use the taxonomic identifications provided by a PCR-DDGE approach to account for specific dietary sources or to set endpoints in stable isotope mixing models.

Each method has its respective strengths and weaknesses in trophic studies and only recently has an attempt been made to use them in conjunction. Hardy et al. (2010) used a combination of DNA microarray data and stable isotopes to determine the diets of a riverine fish assemblage. Here, we report on a combined PCR-DGGE and stable isotope approach to examine the trophic ecology of the suspension-feeding bivalves *Mytilus* spp., which are ubiquitous on North Atlantic rocky shores and intensively cultured within the region. It is presumed that the bivalve samples were *Mytilus edulis*, but surveys of mussel populations in the region suggest a complex history of introgressive hybridization with *Mytilus galloprovincialis* and *Mytilus trossulus* (Gosling et al. 2008; Dias et al. 2009). Using both short-term (PCR-DGGE) ingestion data and long-term (stable isotope) assimilation data, we examine whether these methods reflect an inferred environmental gradient within Strangford Lough, Northern Ireland, a site where mussels are currently not cultured. The two methods are compared and the stable isotope results interpreted with

respect to the sequence-based identification of organisms in the gut contents.

Materials and methods

Collection sites and field processing

Located on the NE coast of Ireland, Strangford Lough (Fig. 1) is a large (150 km^2) coastal embayment connected to the Irish Sea through a narrow (0.5 km wide) channel (8 km long) at its southern end, where currents may reach 3.5 m s^{-1} (Magorrian et al. 1995). The restricted inlet and long north–south orientation (30 × 8 km) creates an environmental gradient based on the distances to the open waters of the Irish Sea. The gradient includes various interacting factors including fetch, wave action, tidal currents (greater at the south of the lough), shifts in primary producer and benthic invertebrate communities (Erwin 1986; Portig et al. 1994), the influx of water and associated taxa from the Irish Sea and relative levels of intertidal exposure. The southern shores are rocky, high energy and heavily influenced by Irish Sea water, while the northern reaches are more sheltered and include expansive tidal flats of primarily sand/mud with longer periods of aerial exposure (Portig et al. 1994; Malvarez et al. 2001). The lough's two main sources of freshwater input are the River Comber near site HI and the River Quoile near site NP (see Fig. 1 for site codes and locations), but discharges are low and there is no overall salinity gradient with salinities of 32–34 (Erwin 1986).

Samples of *Mytilus* spp. were collected from seven intertidal sites over two days in June 2009. Fifteen individuals were collected from each site prior to their emergence on a falling tide and held on ice during processing. Approximately 1 mL of 95 % ethanol (EtOH) was injected into the digestive gland using a sterile needle and syringe prior to fixation of the whole mussel in 95 % EtOH. The samples were stored at 4 °C and transferred to fresh 70 % EtOH after 48 h. One-litre samples of seawater was collected from each site at the time of mussel collection.

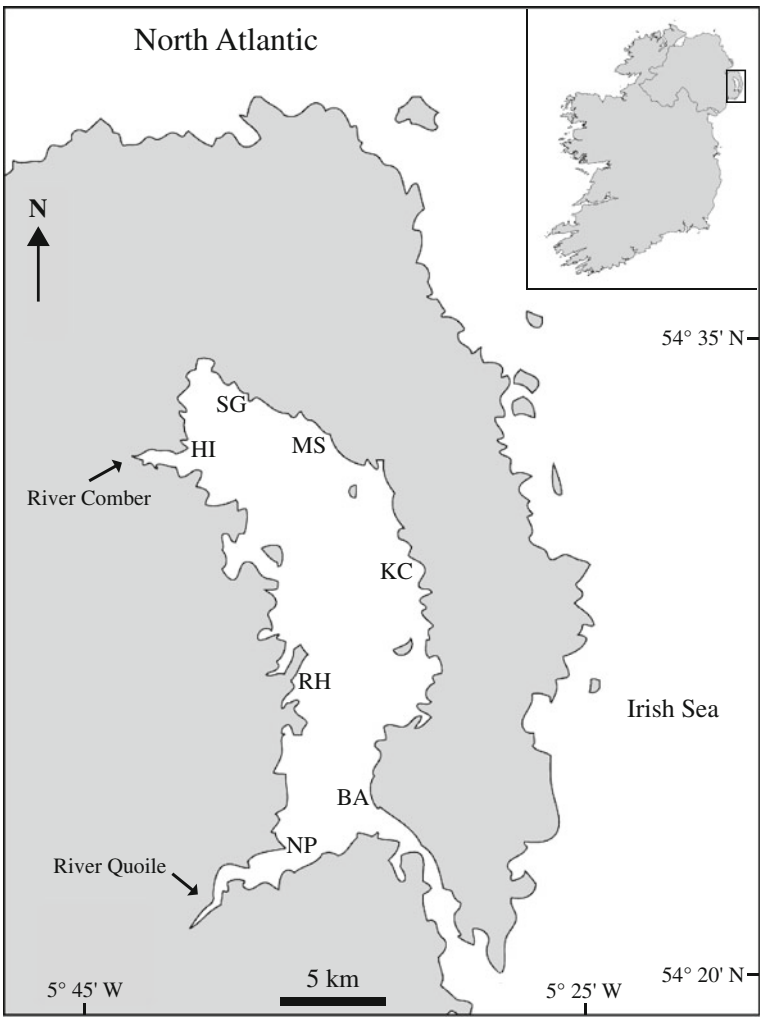

Fig. 1 Schematic map illustrating sampling sites in Strangford Lough, NI. Locations: Seagrass (*SG*), Horse Island (*HI*), Mount Stewart (*MS*), Kircubbin (*KC*), Ringhaddy (*RH*), Nickey's Point (*NP*), Ballywhite Bay (*BA*). Inlay depicts Strangford Lough within Ireland

Stable isotope analysis

After 2–5 h in 95 % EtOH, the foot of each mussel was dissected, dried at 60 °C for 48 h and ground to a fine powder in an agate pestle and mortar, which was cleaned with 70 % ethanol and wiped dry between samples. Samples (0.55 mg) were loaded into tin cups and combusted in a Eurovector Isoprime continuous-flow isotope-ratio mass spectrometer. Isotopic composition (δ^{13}C and δ^{15}N) was determined following Mallela and Harrod (2008). Precisions of <0.1 ‰ (carbon) and <0.3 ‰ (nitrogen) were obtained through repeated analysis of internal standards after every six samples.

Gut samples

Following the protocol of Maloy et al. (2009), the stomach contents of each mussel were removed and transferred to 1.5-mL microfuge tubes. Recovered gut contents were mechanically disrupted and processed with a DNeasy® Blood and Tissue Kit (Qiagen) following the animal tissue protocol. Only mussels with sufficient gut contents could be analysed ($n = 7$ for MS, $n = 9$ for NP and BA, all other sites $n = 10$), were used for DNA and corresponding SI analysis.

Water samples

Water samples were immediately filtered onto a 0.2-μm filter membrane and stored at −20 °C until processing. Filters were cut into small pieces using sterile scissors and processed with an UltraClean™ Water DNA Isolation Kit (MoBio Laboratories, Inc.) according to the manufacturer's instructions.

PCR and DGGE preparation

A portion of the 18S ribosomal ribonucleic acid (rRNA) gene was amplified from each DNA sample using the eukaryotic specific GC-clamped primer 960FbGC and universal primer 1200R (Gast et al. 2004) following the protocol of Maloy et al. (2009). A total of 45 μL of PCR products was run on an 8 % acrylamide (37.5:1, acrylamide/bis-acrylamide) gel containing a 35–53 % denaturing gradient of formamide and urea. Samples were run in a Bio-Rad DCode™ system at 60 V for 16 h at a constant temperature of 60 °C.

DGGE image analysis

Gel images were captured and bands detected automatically with Phoretix 1D gel analysis software (Nonlinear Dynamics Ltd.) according to Maloy et al. (2009). Migration points within each gel were normalized using three standard lanes per gel, each containing six bands that served as migration reference points. Each reference point was assigned a retardation factor (Rf) between 0 and 1. The Rf position of each gut content phylotype was calculated based on its position relative to the migration standard, thus accounting for any variation between gel runs. Included in each standard lane was an amplification product corresponding to *Mytilus* spp. (Fig. 2). This served both as a migration reference point and as a means to identify and remove from analysis the phylotype within each gut content profile originating from the host tissue. Lane profiles and associated Rf migration values were exported to Phoretix 1D Pro (Nonlinear Dynamics Ltd.) for band matching. Bands were considered a match if Rf values were within a tolerance of 0.01. Once matched, a matrix of presence/absence data was available for each unique phylotype, and these values were used in subsequent statistical analysis.

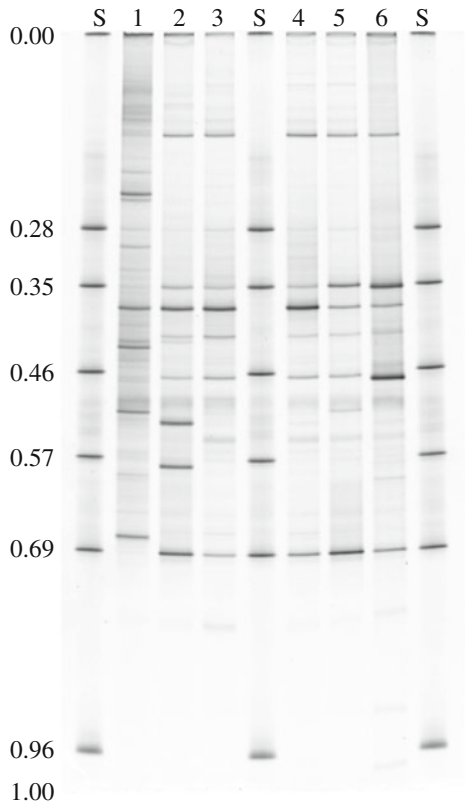

Fig. 2 DGGE gel image depicting experimental set-up. Each gel contained three standard *lanes* (S) containing six migration points, 1 L water profile (*lane 1*) and gut content profiles (*lanes 2–6*). Phylotype position was assigned a retardation factor (*Rf*) based on position relative to migration standard. Position 0.69 corresponds to *Mytilus* spp.

Statistical analysis

General linear ANOVA models (GLM) were used to test for site-associated variation in mussel size (shell height) and overall phylotypic diversity in mussel gut contents and

water column samples (Minitab 13.32). Independent two-sample *t* tests were used to compare within-site variation in phylotypic diversity from mussel diet and water samples. The matrix of phylotype presence/absence data (based on Rf migration points) was used to obtain a distance matrix based on Bray–Curtis similarities. From these two matrices, the relationship between gut content profiles across sites and their correspondence with untransformed $\delta^{13}C$ and $\delta^{15}N$ values based on Euclidean distances were assessed using principal coordinate analysis (PCO), analysis of similarity (ANOSIM), similarity percentage (SIMPER), RELATE, permutational multivariate analysis of variance (PERMANOVA) and canonical analysis of principle coordinate (CAP) routines in Primer 6.1.12 and PERMANOVA + (Clarke and Gorley 2006; Anderson et al. 2008). The relationship between $\delta^{13}C$ and $\delta^{15}N$ values was examined using least squares regression of untransformed data. An alpha level of 0.05 was used in all statistical comparisons. Frequency of occurrence data for phylotypes and higher taxons was calculated based on the number of mussels in which a particular phylotype or higher taxon was observed divided by total number of mussels sampled times 100.

Phylotype identification

Based on a SIMPER analysis, bands contributing to the first 75 % of the intraspecific similarity from each site were targeted for sequence-based identification (Online Resource 1). Gut content profiles containing the selected bands were reamplified and run on a DGGE gel. Bands at corresponding Rf positions were excised and placed in 150 μL of nuclease-free H_2O overnight at 4 °C. A 1.0-μL aliquot was used as template for reamplification and the migration point of each excised band confirmed with an additional DGGE run prior to sequencing (Cogenics, Essex, UK). Sequence reads were assembled and manually edited using Geneious Pro 5.3.3 (Drummond et al. 2009). Contiguous sequences (GenBank accession numbers JF799987–JF800015) were compared to the 'nr/nt' database provided by NCBI using BLAST (Altschul et al. 1997). BLAST results were used to determine a putative taxonomic affiliation for each sequenced phylotype, and the top five matches were used to assign each to a higher taxonomic group.

Results

Shell heights

Mean mussel shell heights differed among sites ($F_{6,\ 60} = 4.61$, $p \leq 0.001$), but were representative of typical adult *Mytilus* spp. shell heights (mean range = 43.0–49.1 mm). Based on the increased areal exposure and potential associated feeding stress at northern sites, mussels there were likely slower growing and older relative to mussels of similar size at less stressful locations. No geographical trends in mean shell height were apparent (Online Resource 2), and possible variation in the age structure or growth rate of mussels at the seven sites is unknown.

Gut content profiles

Phylotypic richness and selectivity

A total of 89 unique phylotypes (based on Rf positions) were identified. There was a significant difference in phylotypic richness (total number of bands) among sites (GLM: $F_{6,\ 73} = 6.78$, $p \leq 0.001$) and sample type (water column and gut content profile) ($F_{1,\ 73} = 139.08$, $p \leq 0.001$) (Online Resource 3), with more phylotypes present in the water column relative to the gut content profiles (two-sample *t* test: $t = -10.58$, $p \leq 0.001$, $df = 20$). The first two axes of an unconstrained PCO based on Bray–Curtis similarities captured 50.1 % of the total variation (Fig. 3). In all cases, gut content profiles were different from those of the water column.

SIMPER and phylotype identification

The subset of phylotypes identified within the first 75 % similarity (SIMPER) for each site was strongly correlated with the full complement of gut content phylotypes and

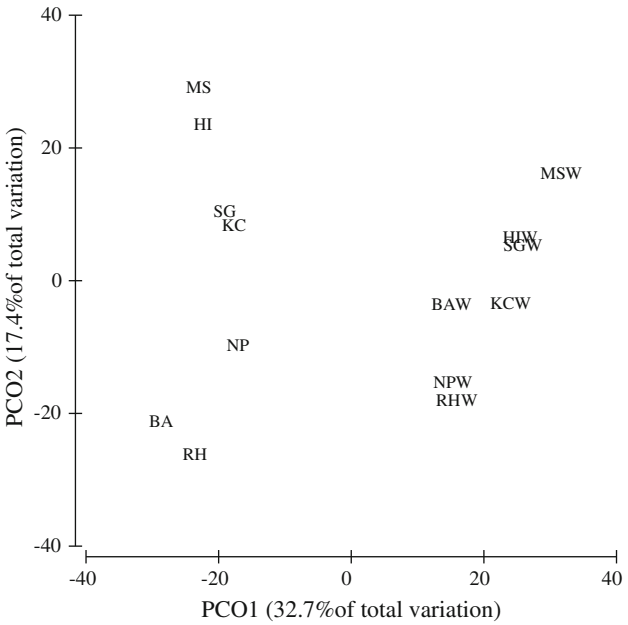

Fig. 3 Unconstrained PCO displaying distance between centroids for gut content (site code) and water column profiles (site code W) based on Bray–Curtis similarities

captured 93.2 % of the total variability (RELATE, Rho = 0.932, $p \leq 0.0001$). From this subset of 32 phylotypes, 29 were successfully sequenced and identified (Table 1); sequences for the remaining three phylotypes were not recovered. Nine higher taxonomic groups were identified, including autotrophic, heterotrophic, mixotrophic and parasitic taxa (Online Resource 4). Bacillariophyta and Crustacea were observed at all sites, while Dinophyceae and Apicomplexa occurred at six and five of the seven sites, respectively. Cnidaria and Chlorophyta were observed at four sites, with cnidarians dominant at the RH and BA sites. The remaining groups of Phaeophyceae, Nematoda and Cryptophyta were observed at three or fewer sites (Table 1; Fig. 4).

Spatial variation

The first two coordinates of an unconstrained PCO captured 47.2 % of the total variation within gut content profiles (Fig. 5). Groups clustered within the ordination on a largely site-specific basis with the northern sites (SG, HI and MS) significantly different from central and southern sites (PERMANOVA: Pseudo-$F_{6,\ 58} = 12.3$; $p \leq 0.0001$). Subsequent pairwise comparisons revealed significant

Table 1 Taxonomic relatedness of DGGE phylotypes. Crustaceans (*C*) are followed by their respective order. Trophic mode: unknown (*U*); heterotrophic (*H*); mixotrophic (*M*); parasitic (*P*); autotrophic (*A*)

Rf position	Sequence length (bp)	Accession number	Sequence identity (%)	Closest relative	Higher taxon	Trophic mode
0.075	–	–	–	Sequence not recovered	–	U
0.108	230	JF79987	100	*Harpacticus* sp. France_RJH_2007	(C) Harpacticoida	H
0.138	232	JF79988	99	*Heterocapsa triquetra*	Dinophyceae	M
0.144	221	JF79989	94	*Selenidium serpulae*	Apicomplexa	P
0.165	225	JF79990	98	Uncultured eukaryote clone DSGM-1	Apicomplexa	P
0.207	226	JF79991	93	*Lankesteria chelyosomae*	Apicomplexa	P
0.216	–	–	–	Sequence not recovered	–	U
0.277	234	JF79992	100	*Stephanodiscus hantzschii* isolate UTCC 267	Bacillariophyta	A
0.296	225	JF79993	93	Uncultured eukaryote clone TAGIRI-29	Apicomplexa	P
0.313	231	JF79994	93	Uncultured eukaryote clone DSGM-12	Apicomplexa	P
0.330	228	JF79995	99	Uncultured cryptophyte clone CS050L18	Cryptophyta	A
0.339	232	JF79996	100	Uncultured eukaryote clone NA1_1G6	Dinophyceae	M
0.349	230	JF79997	97	Uncultured eukaryote clone OTU_D	Cnidaria	H
0.378	229	JF79998	100	Uncultured marine eukaryote clone mj223	Cnidaria	H
0.409	231	JF79999	94	Uncultured Syndiniales clone CS050L01	Dinophyceae	M
0.413	233	JF80000	93	*Cytheromorpha acupunctata*	(C) Podocopida	H
0.441	230	JF80001	100	Uncultured eukaryote isolate DGGE CHL5	Chlorophyta	A
0.459	232	JF80002	100	*Heterocapsa triquetra*	Dinophyceae	M
0.466	234	JF80003	100	*Cyclotella meneghiniana* isolate HYK0210	Bacillariophyta	A
0.485	232	JF80004	98	*Symbiodinium* sp. Clade C clone 8	Dinophyceae	M
0.525	234	JF80005	97	*Paradoxostoma setoense*	(C) Podocopida	H
0.540	235	JF80006	100	*Navicula tripunctata*, strain AT-202.01	Bacillariophyta	A
0.557	232	JF80007	96	*Trohicola entericus*	(C) Poecilostomatoida	H
0.597	227	JF80008	99	*Spirinia parasitifera*	Nematoda	H
0.634	232	JF80009	100	*Sacculina carcini*	(C) Kentrogonida	H
0.638	203	JF80010	99	*Fucus distichus*	Phaeophyceae	A
0.653	230	JF80011	97	*Nemesis* sp. SMD-2008 isolate 9R3n	(C) Siphonostomatoida	H
0.697	231	JF80012	99	*Itunella muelleri*	(C) Harpacticoida	H
0.740	234	JF80013	100	*Semibalanus balanoides*	(C) Sessilia	H
0.782	234	JF80014	100	*Semibalanus balanoides*	(C) Sessilia	H
0.801	235	JF80015	96	*Paramenophia* New Caledonia-RJH-2007	(C) Harpacticoida	H
0.827	–	–	–	Sequence not recovered	–	U

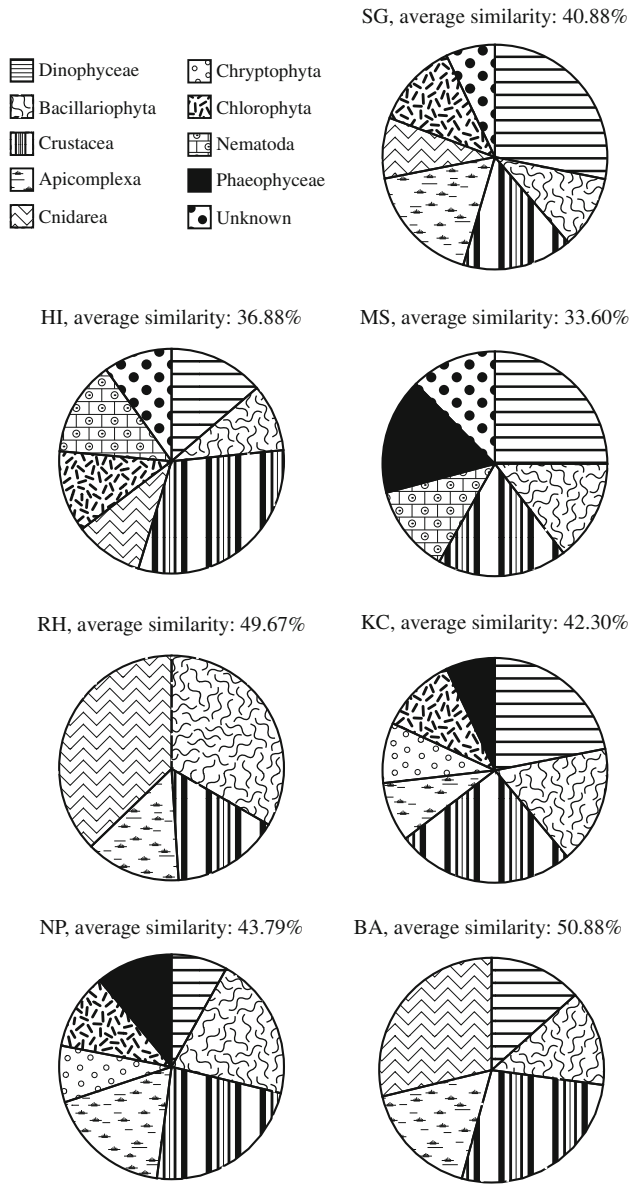

Fig. 4 Sequence-based identification of gut content phylotypes. Values represent frequency at which phylotypes from each higher taxonomic group were observed in gut content profiles of sampled mussels at each site

differences between all pairs of sites (t statistic range = 2.04–4.70, p values all $\leq$0.004). A discriminate CAP also detected significant differences between sites ($\delta_1^2 = 0.879$, $p \leq 0.0001$) and captured 92.7 % of the variation present in the original matrix of gut content similarities. Cross-validation of the model separated gut content profiles into the correct site allocations with 80 % success. Of the 13 misclassified gut content profiles, 10 belonged to mussels from the three northern sites: SG (2), HI (3) and MS (5) (Table 2). To ensure that phylotypes representing potentially resident gut parasites were not biasing site-based measures of similarity, the five phylotypes of suspected

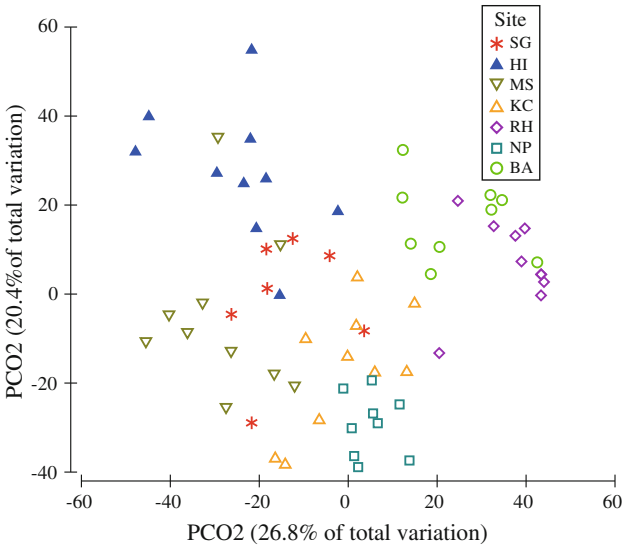

Fig. 5 Unconstrained PCO ordination of gut content profiles clustering largely on a site-specific basis with clear separation of northern (SG, HI and MS) from central and southern sites

parasite origin were removed from the analyses (see Table 1). Results (PERMANOVA: Pseudo-$F_{6,\ 58} = 12.3$; $p \leq 0.0001$) (pairwise comparisons: t statistic range = 1.90–4.86, p values all $\leq$0.008) suggest that parasite sequences have little to no effect on the overall results.

Correlation with environmental gradient: PCR-DGGE

Initial ordinations (Fig. 5) showed a distinct separation between sites in the northern portion of the lough (SG, HI and MS) from those in central (KC and RH) and southern locations (NP and BA). To test whether the gut content profiles were accurately reflecting the spatial variation and underlying environmental gradient, an additional CAP analysis was used. In this instance, the matrix of gut content similarities was constrained against the Euclidean (i.e. straightline) distance of each site to the mouth of the lough (Fig. 6a), with a significant canonical correlation ($\delta_1^2 = 0.805$, $p \leq 0.0001$) between the gut content profiles and distance to the inlet.

Stable isotope ratios

Spatial variation

Mussels showed considerable isotopic variation (Fig. 7): δ^{13}C values varied between −19.0 and −16.2 ‰, while δ^{15}N values ranged between 8.9 and 11.5 ‰. Variation in the δ^{13}C and δ^{15}N values of individual mussels was closely and positively related ($F_{1,\ 66} = 124.0$, $p \leq 0.0001$; $R^2 = 0.65$). Isotopic variation followed an apparent spatial

Table 2 Cross-validation results from discriminate CAP analysis of gut content profiles and stable isotope data

Original site	Classified by model							Total	% Correct
	SG	HI	MS	KC	RH	NP	BA		
Gut content profiles									
SG	5	0	1	1	0	0	0	7	71
HI	3	7	0	0	0	0	0	10	70
MS	3	1	5	1	0	0	0	10	50
KC	0	0	0	10	0	0	0	10	100
RH	0	0	0	0	10	0	0	10	100
NP	0	0	0	1	0	8	0	9	89
BA	0	0	0	1	1	0	7	9	78
	Total correct: 52/65 (80 %)				Misclassification error: 20 %				
Stable isotopes									
SG	1	1	2	3	0	0	0	7	14
HI	1	7	0	0	0	2	0	10	70
MS	2	1	6	1	0	0	0	10	60
KC	0	0	1	7	0	1	1	10	70
RH	0	0	0	0	8	1	1	10	80
NP	2	2	0	0	1	4	0	9	44
BA	0	0	0	2	1	0	6	9	67
	Total correct: 39/65 (60 %)				Misclassification error: 40 %				

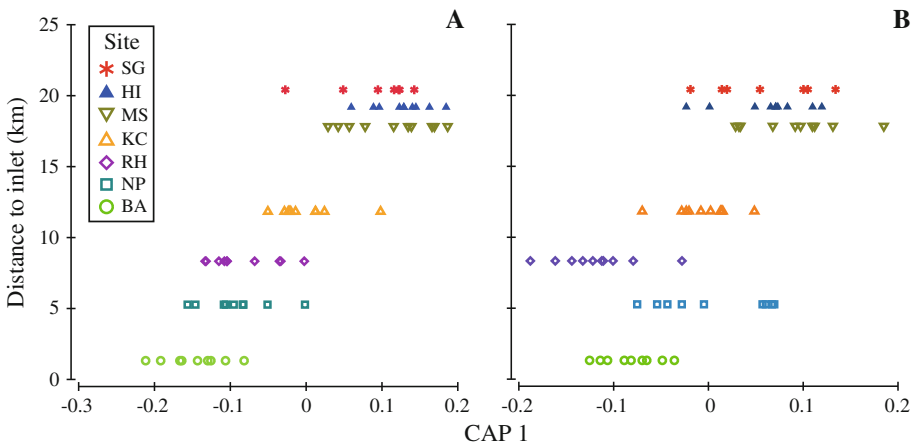

Fig. 6 CAP analysis of gut content similarities (Bray–Curtis) (**a**) and stable isotope similarities (**b**) analysed against Euclidean distance between site and inlet

pattern reflecting the location of sites within Strangford Lough (See Figs. 1 and 7). The northern sites of SG, HI and MS were ^{15}N enriched relative to sites of RH and BA. NP and KC fell at intermediate levels of δ^{15}N. Mussels from RH were ^{13}C depleted, while individuals from SG and MS were ^{13}C enriched relative to other locations. These apparent site differences were supported by PERMANOVA of combined δ^{13}C and δ^{15}N data (Pseudo-$F_{6,\ 58} = 27.16$; $p \leq 0.0001$). Subsequent pairwise comparisons revealed significant differences between all but two site combinations (t statistic range 2.22–8.91, p values all $\leq$0.03). The δ^{13}C–δ^{15}N centroids of northern sites SG and MS ($t = 1.63$, $p = 0.096$) and sites NP (south) and HI (north) ($t = 1.9$, $p = 0.059$) overlapped. A discriminate CAP analysis also detected significant differences between sites ($\delta_1^2 = 0.757$, $p \leq 0.0001$) and captured 89 % of the variation in the original matrix of stable isotope similarities. Cross-validation indicated that individual mussels could be classified isotopically to capture location with a 60 % success rate. The highest misclassification rates were in the northern sites SG and MS and the southernmost site NP (Table 2).

Correlation with environmental gradient: stable isotopes

Visual comparison of stable isotope data (Fig. 7) indicated separation between sites in the northern portion of the lough (MS, HI and SG) from those in central (KC and RA)

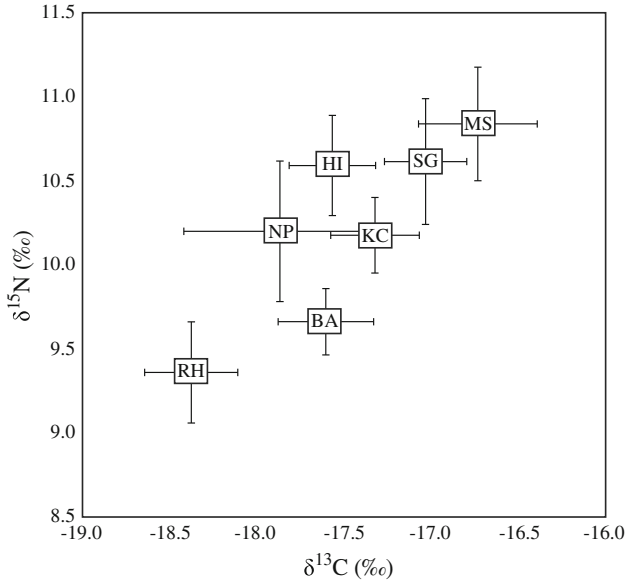

Fig. 7 Spatial variation in mean (±1 SD) δ13C and δ15N

and southern locations (NP and BA). As previously performed with the gut content profiles, a CAP analysis was used to test whether the stable isotope data reflected the inferred environmental gradient (Fig. 6b). Results demonstrated a significant canonical correlation ($\delta_1^2 = 0.462$, $p \leq 0.0001$) between the stable isotopes and the larger spatial variation between sites, though the overall correlation was weaker than for the gut content profile data.

Comparison of gut content profiles and stable isotope ratios

As noted above, both gut content profiles and stable isotope data reflected the spatial differences between sites; there was also a significant correlation between the two data matrices (RELATE, Rho = 0.24, $p \leq 0.0001$). A comparison of stable isotope and gut content phylotype data using CAP revealed a significant canonical correlation ($\delta_1^2 = 0.800$, $p = 0.0004$) between the two data sets, indicating that spatial variation in the mussel gut was captured in a similar way by both approaches.

Discussion

The analysis of stable isotope ratios (δ^{13}C and δ^{15}N) and gut content profiles obtained with PCR-DGGE demonstrated considerable variation in potential food sources of *Mytilus* spp. within and between sample locations. As a relatively new technique, it is essential to evaluate the PCR-DGGE approach against established techniques to determine whether ecologically relevant inferences can be drawn from the patterns observed within the gut content profiles. Both PCR-DGGE and stable isotope approaches for estimating consumer diet have their respective strengths and weaknesses, but no previous comparison of the techniques had been made. PCR-DGGE gut content profiles of mussels differed between all seven sites, and individuals could be classified to a site with an 80 % success rate. Though differences were detected in most pairwise comparisons of sites, cross-validation of the CAP model based on stable isotope ratios resulted in a 60 % classification success rate; however, the overall misclassification pattern was not similar to the PCR-DGGE spatial pattern. In addition, when the gut contents were analysed against the environmental gradient, the PCR-DGGE method found a stronger correlation between the distances of each site to the lough's inlet. In this respect, the PCR-DGGE assay closely reflected the inferred spatial gradient in Strangford Lough. This is likely a consequence of the number of variables in each analysis. For isotopic analysis the relationship among sites was determined with just two variables, that is, δ^{13}C and δ^{15}N values. With PCR-DGGE, 32 variables were used, each representing a different gut content phylotype.

Mussel δ^{13}C and δ^{15}N values recorded across the seven sites were within the range typically reported for marine bivalves (Riera et al. 2002; Yokoyama et al. 2005a; Kang et al. 2006). On average, mussels from the central site of RH were the most ^{13}C depleted (−18.4 ‰), while congeners from the northern sites of SG and MS were the most ^{13}C enriched (−17.0 ‰ and −16.7 ‰, respectively). The two main carbon sources for bivalve growth are phytoplankton, measured as particulate organic carbon (POM), and benthic microalgae (BMA) (France 1995; Riera et al. 2002; Yokoyama et al. 2005b; Lefebvre et al. 2009). Within these studies, average POM (−20.8 ‰) values are ^{13}C depleted relative to average BMA (−16.5 ‰), suggesting that mussels in Strangford Lough were feeding on a combination of these two sources. Mussels with ^{13}C depleted values from sites in the southern and central portion of the bay likely relied more heavily on phytoplankton-derived carbon, while northern reaches of the bay were more dependent on carbon derived from benthic microalgae. Such a scenario is consistent with the hydrography of the bay, with sites closer to the southern inlet being more heavily influenced by the Irish Sea. Variation in mussel δ^{15}N was considerable, and mussels were ^{15}N enriched at the northern sites relative to the rest of the lough, suggesting a greater proportion of the diet contained animal tissue. Assuming a trophic fractionation value (Δ^{15}N) of 3.5 ‰ for bivalves (Post 2002; Yokoyama et al. 2005b), the trophic level of individual mussels varied by ca. 0.8 of a trophic level, with an average between-site difference of ca. 0.5 of a trophic level. This may either

reflect variation in baseline δ^{15}N (not measured here) or omnivory. Some support for these mussels having assimilated energy and nutrients at different trophic levels was provided by the positive linear relationship between mussel δ^{13}C and δ^{15}N values ($R^2 = 0.65$).

However, the DNA-based analyses found no evidence that, at the time of sampling, there was a greater consumption of ^{13}C-enriched benthic algae or animal prey in mussels from northern sites. DNA-based identification of ingested organisms did not show a shift towards benthic microalgae in the northern sites. In fact, only one diatom phylotype (0.540) identified as *Navicula tripunctata* and observed only at the central site RH is a benthic microalga. As a group, the phytoplankton (diatoms, chlorophytes, dinoflagellates and cryptophytes) were detected at similar frequencies at all sites, suggesting a common spatial dependence.

Differences between stable isotope data and anticipated results of the PCR-DGGE were even more evident for mussel δ^{15}N values, where individuals from the three northern sites were ^{15}N-enriched relative to congeners from most other sites. Animal prey (crustaceans, cnidarians and nematodes) was most frequently observed in mussels from sites HI, BA and RH, which, with the exception of HI, were the two most ^{15}N-depleted sites. Furthermore, stable isotope values were similar at HI and NP. Though spatially separated within Strangford Lough, these two sites were near freshwater inputs, potentially explaining their similar isotopic composition. This connection was not observed in the PCR-DGGE data. Gut content profiles were distinct for each site, and no identified phylotypes could be attributed to organisms from a freshwater source.

The overall abilities of the two techniques to detect spatial variation in the gut contents of mussels were significantly correlated, but in pairwise comparisons, they diverged. Based on δ^{13}C and δ^{15}N values, the expected groups of organisms were not identified in the PCR-DGGE data. Three factors potentially contribute to this discrepancy. First, the PCR-DGGE technique is inherently qualitative. PCR-DGGE data represent only the presence/absence of an organism in the gut contents of an individual mussel and, as such, do not provide quantitative data. To partially account for this, the perceived importance of PCR-DGGE-detected gut contents was assessed by the frequency with which each phylotype occurred among sampled mussels ($n = 65$). Secondly, it is known that bivalves can pass ingested organisms through their digestive system intact (Galtsoff 1964). Thus, the organisms identified by PCR-DGGE do not necessarily contribute to the observed assimilation values. Finally, PCR-DGGE data represent short-term (hours) ingestion data, while stable isotopes reflect longer-term (months) assimilation patterns. We assume that this temporal offset in the respective measurements is the largest factor contributing to the overall discrepancy.

The PCR-DGGE method was effective in determining the scope of gut content diversity and identifying unanticipated trophic connections. PCR-DGGE gut content data clearly show that mussels ingest and presumably assimilate a wide diversity of secondary consumers from the plankton. Furthermore, many of these taxa are not routinely considered in isotopic studies, for example, for inclusion in mixing models. Given the wide trophic plasticity exhibited by *Mytilus* spp., it may not be an appropriate species to use as a proxy for primary production as has been proposed for other suspension feeders (Post 2002; Fukumori et al. 2008). Moreover, a similar level of trophic plasticity has been observed in PCR-DGGE studies in other bivalve species (Maloy et al. unpublished). Such trophic plasticity raises concerns regarding the use of suspension-feeding bivalves as environmental proxies for primary production in stable isotope studies (Post 2002; Fukumori et al. 2008). Future work using bivalves as a primary production proxy should pay careful attention to the natural history and feeding patterns of the species being considered.

A combined stable isotope and PCR-DGGE approach provides a more complete picture of the trophic relationships within mussels from Strangford Lough than either approach alone provides. Both methods detected variations on the lough wide scale, though the PCR-DGGE method provided greater taxonomic specificity. However, organisms identified in the gut contents of mussels did not necessarily reflect the overall isotopic values. This is presumably explained chiefly by the temporal lag between the approaches. Future work to develop food web analysis tools should also consider alternative DNA technologies such as next-generation sequencing approaches and how they may complement techniques such as stable isotope analysis and/or fatty acid analysis.

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