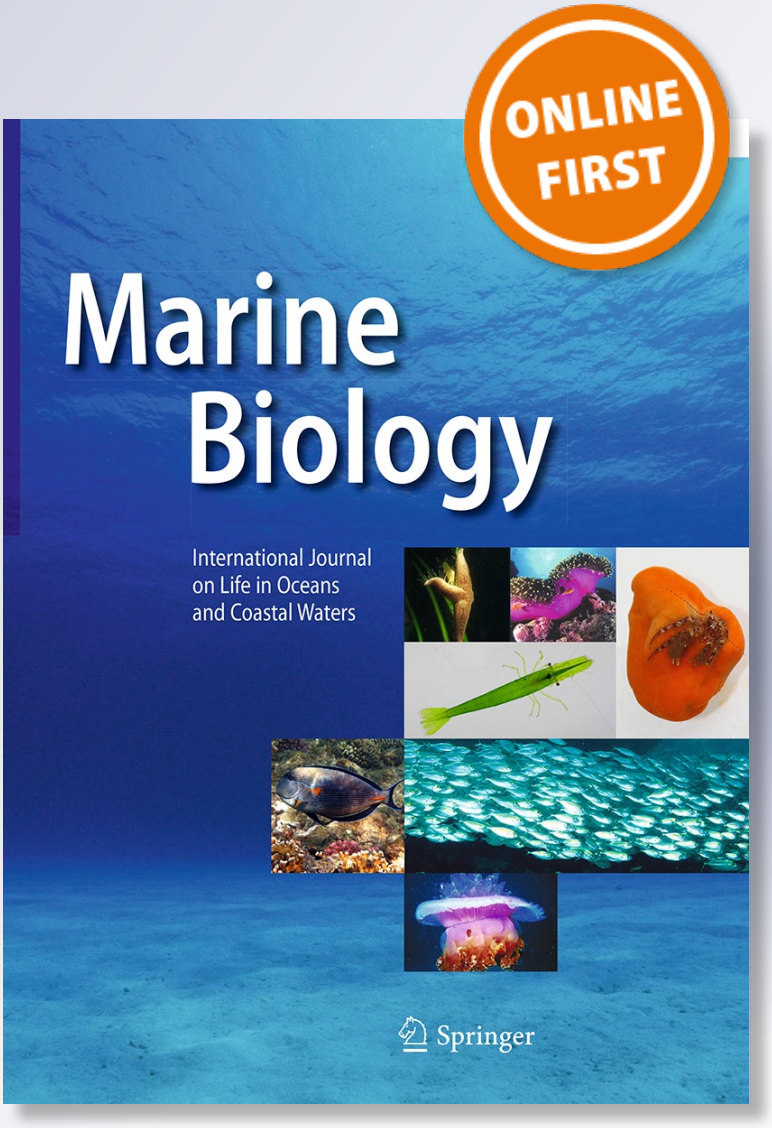
Feeding selectivity of bivalve larvae on natural plankton assemblages in the Western English Channel

Penelope K. Lindeque, Anna Dimond, Rachel A. Harmer, Helen E. Parry, Katharine L. Pemberton & Elaine S. Fileman

Marine Biology
International Journal on Life in Oceans and Coastal Waters

ISSN 0025-3162

Mar Biol
DOI 10.1007/s00227-014-2580-x



Springer

Mar Biol
DOI 10.1007/s00227-014-2580-x

ORIGINAL PAPER

Feeding selectivity of bivalve larvae on natural plankton assemblages in the Western English Channel

Penelope K. Lindeque · Anna Dimond · Rachel A. Harmer · Helen E. Parry · Katharine L. Pemberton · Elaine S. Fileman

Received: 22 July 2014 / Accepted: 10 November 2014

Abstract Meroplankton, including bivalve larvae, are an important and yet understudied component of coastal marine food webs. Understanding the baseline of meroplankton ecology is imperative to establish and predict their sensitivity to local and global marine stressors. Over an annual cycle (October 2009–September 2010), bivalve larvae were collected from the Western Channel Observatory time series station L4 (50°15.00′N, 4°13.02′W). The morphologically similar larvae were identified by analysis of the 18S nuclear small subunit ribosomal RNA gene, and a series of incubation experiments were conducted to determine larval ingestion rates on natural plankton assemblages. Complementary gut content analysis was performed using a PCR-based method for detecting prey DNA both from field-collected larvae and those from the feeding experiments. Molecular identification of bivalve larvae showed the community composition to change over the course of the sampling period with domination by *Phaxas* in winter and higher diversity in autumn. The larvae selected for nanoeukaryotes (2–20 µm) including coccolithophores (<20 µm) which together comprised >75 % of the bivalve larvae diet. Additionally, a small percentage of carbon ingested originated from heterotrophic ciliates (<30 µm). The molecular analysis of bivalve larvae gut content provided increased resolution of identification of prey consumed and demonstrated that the composition of prey consumed established through bottle incubations conferred with that established from in situ larvae. Despite changes in bivalve larvae community structure, clearance rates of each prey type did not change significantly over the course of the experiment, suggesting different bivalve larvae species may consume similar prey.

Communicated by U. Sommer.

P. K. Lindeque (✉) · A. Dimond · R. A. Harmer · H. E. Parry · K. L. Pemberton · E. S. Fileman
Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth, Devon PL1 3DH, UK
e-mail: pkw@pml.ac.uk

Introduction

Marine bivalves produce transient pelagic larvae which, like many marine invertebrates, differ considerably from their adult counterpart in terms of morphology, ecology, diet and habitat (Thorson 1950; Pechenik 1999). During their reproductive season, bivalves may produce large numbers of these planktotrophic larvae which can, at certain times of the year, contribute significantly to the meroplankton population and hence zooplankton community. These larvae grow and develop while in the plankton (Eckman 1996), allowing potential dispersal over hundreds or thousands of miles (Thorson 1950; Belgrano et al. 1995).

Bivalve larvae acquire initial energy reserves through maternal investment, but all other energy requirements must be met through feeding. While young, bivalve larvae are photopositive and crowd near the surface of the water column (Thorson 1950), where they compete with other plankton to graze on pico-, nano- and micro-plankton. Previous studies of bivalve larvae feeding consist mainly of those in laboratory environments and focus on commercially exploited bivalve species. From these studies, the larval diet of bivalves is commonly thought to be nanoplankton (<20 µm), including dinoflagellates, *Synechococcus* spp. (Baldwin 1995) and *Isochrysis* spp. (Rico-Villa et al. 2009). In addition, several studies have suggested bacteria and picoplankton as important sources of carbon for bivalve larvae, including detritus particles and smaller

Published online: 22 November 2014

plankton that escape predation by larger predators such as copepods (Sommer et al. 2000). The consumption of much larger cells (22–30 μm) has also been observed during a dinoflagellate bloom in Chesapeake Bay (Baldwin 1995).

Recruitment of new adult bivalves to the benthos, and maintenance of the population, relies on there being a sufficient food supply for the larvae to grow and survive to the point of metamorphosis of settled pediveligers (Raby et al. 1994; Laing 1995; Chicharo and Chicharo 2000; García-Esquivel et al. 2001; Bos et al. 2006). Bivalve larvae may be released into the plankton to take advantage of a phytoplankton bloom (Pulfrich 1997; Highfield et al. 2010); however, availability and quality of food may be patchy, both spatially and temporally (Haury et al. 1978), thereby possibly creating a mismatch in the distribution of the bivalve larvae and their preferred diet. Despite studies suggesting that bivalve larvae have a high tolerance to food deprivation (Moran and Manahan 2004; Ben Kheder et al. 2010; Matias et al. 2011), food quality and quantity have a notable influence on the duration of larval phases (Ben Kheder et al. 2010) with insufficient food resulting in delayed development. This meroplanktonic phase of bivalves is a critical period and if extended, the larvae may experience greater susceptibility to predation and environmental stresses.

Meroplanktonic larvae have been an object of study at the L4 monitoring site, an inshore site in the Western English Channel, where samples have been taken weekly since 1988 (Smyth et al. 2010; Fileman et al. 2014). This sampling program has shown that at brief times of the year, meroplankton can account for on average up to 32 % of the total zooplankton community (Fig. 1a) following spawning events linked to phytoplankton blooms (Highfield et al. 2010). Bivalve larvae can contribute up to 50 % of total meroplankton (Fig. 1b) with their abundance peaking between September and October (Fig. 1c) and contributing towards ~3.5 % of the total zooplankton community (Eloire et al. 2010).

Routine identification of the bivalve larvae to species level is not possible due to the extremely small size of the larvae and their lack of diagnostic morphological characters. In such cases, it is possible to use molecular techniques to improve the resolution of identification. Various molecular techniques have been developed for the identification of bivalve larvae based on PCR amplification of different target genes (Garland and Zimmer 2002; Livi et al. 2006; Wang et al. 2006; Zhan et al. 2008). During this study, we undertook to identify the bivalve larvae by barcoding a 550-bp region of the 18S nuclear small subunit ribosomal RNA gene (18S rRNA gene) which covers the hyper-variable V1–V3 regions.

While numerous studies have looked at bivalve larvae feeding on cultures in laboratory environments and for commercial exploitation, few studies have addressed their feeding patterns in the natural environment and none within the English Channel. Our main objectives were to identify the bivalve larvae occurring at L4 through molecular

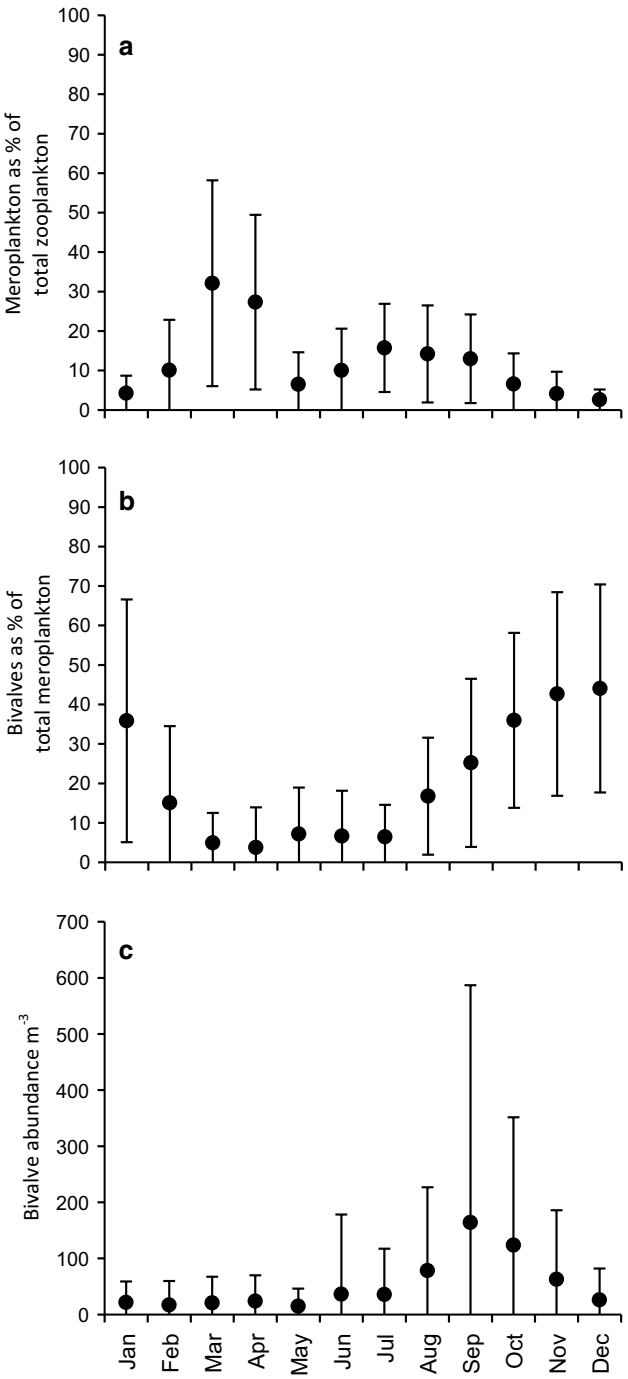


Fig. 1 Abundances (mean ± 1SD) from 1988 to 2010 at Station L4 in the Western English Channel of **a** meroplankton as a percentage of total zooplankton, **b** bivalves as a percentage of total meroplankton and **c** absolute bivalve abundance (m^{-3}). Each monthly data point is based on the mean of all samples taken in that month over the whole 22-year time series

barcoding and to experimentally determine their feeding selectivity in the natural plankton assemblage over a seasonal cycle. Due to the methodology problems with studying feeding in zooplankton (Bamstedt et al. 2000; Nejstgaard et al. 2001, 2003), we used molecular methods alongside traditional bottle incubations to increase the resolution of identification of ingested prey and compare feeding in the field with that under experimental conditions.

Materials and methods

Sampling

Samples were collected weekly at the Western Channel Observatory (www.westernchannelobservatory.org.uk/) long-term time series station L4 (50°15.00′N, 4°13.02′W) between September 2009 and September 2010 (Fig. 2). On each sampling date, two replicate vertical WP2 net hauls (mesh size = 200 µm, mouth aperture = 57 cm diameter) were taken from 50 m to the surface. Water depth at the site averages 54 m. The samples were washed immediately from the cod end into a cool box with fresh surface seawater, stored on board at ambient surface water temperature and returned to the laboratory within 2 h of collection. Concurrently, 10 L of fresh surface seawater was collected, immediately screened using a 200-µm mesh to remove potential mesozooplankton predators, returned to the laboratory, where it was screened a second time through an 80-µm mesh to remove potential micrometazoan competitive predators, and left overnight in the dark at ambient sea temperature. Approximately 250 active bivalve larvae were isolated from the live net samples on the day of collection using a stereomicroscope (Wild M5). These were transferred in aliquots of 50 into 10-mL wells of 0.2-µm-filtered seawater (FSW) and left overnight at ambient sea surface temperature to allow gut clearance. To determine bivalve larvae size for each sampling date, approximately 30 bivalve larvae were photographed with a camera affixed to an inverted microscope (Olympus IX71). Images were analysed with cellSens (Olympus) software to determine shell length (µm).

Experimental procedure

Bivalve larval feeding experiments consisted of bottle incubations of the screened naturally occurring microplankton community with and without the addition of bivalve larvae. The screened surface water was gently mixed and divided between 8 × 500-mL acid-washed clear glass Duran^{R} bottles. Fifty bivalve larvae were added to each of the four experimental bottles, and four control bottles were left with screened surface water only. All eight bottles were topped up with the screened surface water to remove any air bubbles and sealed. The bottles were attached to a revolving plankton wheel rotating at 1 rpm and left in the dark for 24 h at ambient sea surface temperature. The remaining bivalves (~50) were left for 24 h at ambient sea surface temperature in 0.2-µm-filtered seawater (FSW) for gut evacuation as a negative control for the molecular gut content analysis.

At T_{zero}, 100 mL of screened surface water was filtered onto a glass fibre filter (GF/F) and frozen at −20 °C prior to chlorophyll-*a* (chl-*a*) analysis. Triplicate 250 mL sub-samples were also taken from the screened surface water and fixed in acid Lugol's iodine solution (2 % final

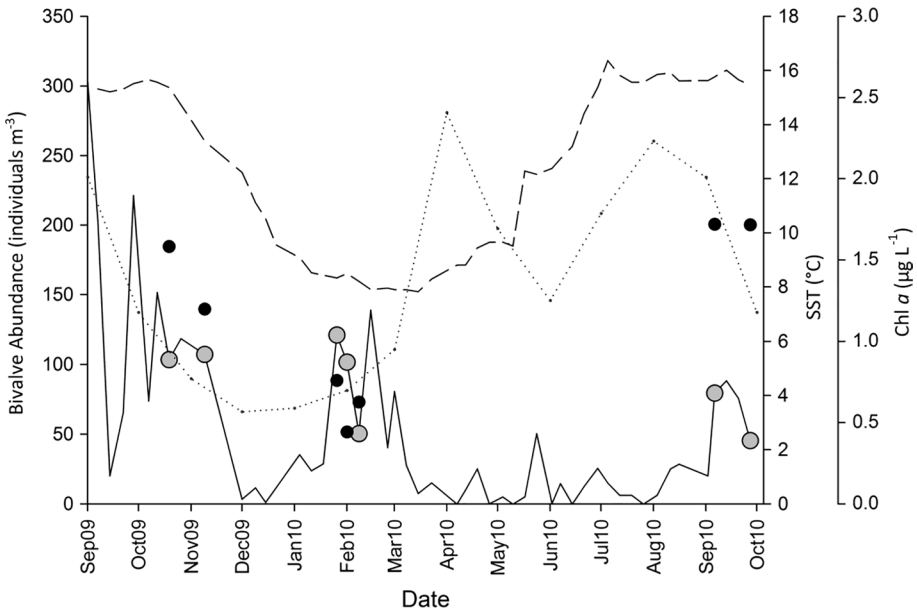


Fig. 2 Abundance of bivalve larvae (*solid line*) and sea surface temperature (*dashed line*) at station L4, Western English Channel, over 1 year from September 2009 to September 2010. Dates of feeding selectivity experiments (*grey circles*) and chlorophyll concentration (*black circles*) are indicated. *Dotted line* represents the monthly average chlorophyll concentration (µg L^{-1})

concentration), and triplicate 2 mL sub-samples were fixed in paraformaldehyde (1 % final concentration) for approximately 1 h before being flash-frozen with liquid nitrogen and then stored at −20 °C prior to flow cytometric analysis.

After 24 h, the experiments were stopped and the bivalve larvae were prepared for molecular identification and characterisation of gut content. For experiment 1, bivalves ($n = 200$) were pooled following the feeding incubations, repeatedly washed in 0.2-µm-filtered seawater (FSW), collected in a 1.5 mL centrifuge tube, spun to remove excess water and stored at −20 °C for DNA analysis. Thereafter, for experiments 2–7, the larvae were pooled for each experiment ($n = 200$), repeatedly washed in 0.2-µm-filtered seawater and sedated by dipping the mesh (100 µm)-bottomed plexiglass chamber in 200 mL 0.37 mg mL^{-1} tricane methane sulphonate (Sigma) (Simonelli et al. 2009). The sedated larvae were picked into a 1.5-mL centrifuge tube, flash-frozen in liquid nitrogen and then stored at −80 °C until DNA analysis.

From each experimental bottle, 100 mL of sample was filtered onto a GF/F and frozen at −20 °C, triplicate sub-samples of 250 mL were fixed in Lugol's solution and 2 mL sub-samples were fixed in paraformaldehyde as described for T_{zero}.

Taxonomic composition of bivalve larvae

Bivalve larvae are morphologically near-identical, and therefore, it is not possible to identify them to species or even genus level by microscopy. During this study, we undertook to identify the bivalve larvae by barcoding a region of the 18S rRNA gene. The 200 pooled bivalve larvae from each feeding experiment were prepared as described above and the DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen) following the standard protocol for animal tissue. An RNase step was included; 4 µL RNase A (100 mg mL^{-1}) was added to the lysis and incubated at room temperature for 2 min. Total lysis was left for 2.5 h and the DNA was eluted in 2 × 100 µL MilliQ water. Efficiency of the DNA extraction was checked by running on a 0.8 % electrophoresis gel. This genomic DNA was also used for the molecular analysis of gut content (see below).

Extracted DNA (2 µL) from each of the feeding experiments 1–7 were used as a DNA template for PCR amplification of a partial region of the 18S rRNA gene with universal eukaryote primers 18S for 5′-GCCAGTAGGA TATGCTTGTCTC-3′ and 18S rev 5′-AGACTTGCCTC CAATGGATCC-3′ (Holland et al. 1991). PCR ingredients include 10 µL 5× GoTAQ DNA polymerase buffer (Promega UK Ltd), 4 µL 2 mM dNTPS (Promega UK Ltd), 4 µL 25 mM $MgCl_2$, 10 µM of each forward and reverse primer and 1.25 U GoTAQ DNA polymerase (Promega UK Ltd). Cycling was carried out in a G Storm or VWR thermocycler with the following parameters: 94 °C (2.5 min) followed by 35× cycles of 94 °C (45 s), 50 °C (1 min), 72 °C (2 min), a final extension phase at 72 °C (10 min) and storage at 10 °C. Ten microlitre aliquots of the amplification reaction were analysed by gel electrophoresis (1 %) to check amplification efficiency. PCR products (25 µL) were cleaned using a QIAquick purification kit (Qiagen UK Ltd), and 2 µL of each PCR product was ligated and transformed using the pGEM®-T Easy Vector System and JM109 competent cells (Promega UK Ltd) to construct clone libraries, following the standard protocol. White colonies ($n \geq 40$ for each PCR product) were picked, inoculated into 5 µL MilliQ water and following denaturation at 95 °C for 5 min used for colony PCR with the addition of 10 µL 5× GoTAQ DNA polymerase buffer (Promega UK Ltd), 4 µL 2 mM dNTPS (Promega UK Ltd), 5 µL 25 mM $MgCl_2$, 10 µM of primers M13f and M13r (Heidecker et al. 1980) and 1.25 U GoTAQ DNA polymerase (Promega UK Ltd). Cycling conditions included an initial denaturation step at 96 °C (5 min) followed by 30× cycles of 94 °C (1 min), 53 °C (1 min), 72 °C (1.5 min), a final extension phase at 72 °C (5 min) and storage at 10 °C. Following colony PCR, over 40 colonies were sequenced (LGC Genomics GmbH, Germany) for each clone library. Sequences were trimmed to the forward and reverse primers using Mega 5 (Heidecker et al. 1980) and assigned to OTUs at the 97 % similarity level using the Qiime pipeline (Caporaso et al. 2010). A representative sequence for each OTU was generated in the Qiime pipeline. By default, the representative sequence for an OTU is chosen as the most abundant sequence present in that OTU. This is computed by collapsing identical sequences and choosing the one that was read the most times as the representative sequence (Caporaso et al. 2010). In addition, 10 (or if <10 sequences in any OTU, as many as are available) random sequences (www.randomizer.org/form.htm) were selected. For each OTU, the representative sequence selected by the Qiime pipeline and the additional randomly picked sequences were manually assigned taxonomy by a BLASTN (Basic Local Alignment Tool) search of the GenBank DNA database for sequence similarities. The top hits for each BLASTN output for the representative and random sequences within an OTU were compared and assigned identity based on the lowest shared taxonomic level.

Analysis of prey

To determine overall feeding on the <80 µm phytoplankton assemblage, chl-*a* was used as a proxy. For analysis of chl-*a* concentration, frozen filters were placed into 10 mL 90 % acetone and left overnight at 4 °C in the dark. Samples were analysed using a Turner fluorometer, and chl-*a* concentrations were measured in µg mL^{-1}.

The main analysis of prey was undertaken with flow cytometry to make a more detailed characterisation of the <20 μm phytoplankton assemblage. *Synechococcus* (0.8–1.5 μm) (SYN), picoeukaryotes (0.2–2.0 μm) (PEUK) and nanoeukaryotes (2–20 μm) (NEUK) were distinguished based on their light scattering and fluorescence properties using a Becton Dikinson FACSort™ flow cytometer equipped with an air-cooled laser providing blue light at 488 nm following the method of Tarran et al. (2006). Cryptophytes and coccolithophores (<20 μm) were counted in addition to the general nanoeukaryote group as cryptophytes can be distinguished from other nanoeukaryotes by their orange-fluorescing phycoerythrin and coccolithophores by enhanced side light scatter relative to their size. PEUK, NEUK and cryptophyte abundances were converted to carbon using a conversion factor of 0.22 pg C μm^3 (Booth et al. 1988) and for coccolithophores a conversion factor of 0.285 pg C μm^{-3} (Tarran et al. 2006). These conversion factors were applied to cell volumes calculated from median cell diameter measurements (Tarran et al. 2006).

To determine the community concentrations (cells mL^{-1}) of both high nucleic acid and low nucleic acid (LNA) bacteria, 500 μL of sample was stained with 5 μL SYBR Green (10^{-4}) plus 45 μL potassium nitrate (300 mM) and left for 1 h in the dark at room temperature. The samples were run through the flow cytometer for 1 min. Bacteria abundance was converted to carbon using a conversion factor of 19 fg C cell $^{-1}$ (Zubkov et al. 1998).

To enumerate ciliates (<30 μm), which cannot be enumerated by flow cytometry, sub-samples of between 25 and 100 mL were taken from Lugol's fixed samples and concentrated by sedimentation onto a counting chamber (Utermöhl 1958); the whole chamber was examined at 200× magnification using an Olympus IMT-2 inverted microscope, and all ciliates were enumerated. The carbon content of each ciliate was determined using the carbon to volume conversion equations of Putt and Stoecker (1989). Diatoms were not enumerated in this study because they were very low in abundance and the few that were present were assumed too large to be ingested by the bivalve larvae (Hansen et al. 1994). Dinoflagellates were not counted as a separate group, but those <20 μm were included in the nanoeukaryote fraction of the flow cytometry analysis.

Clearance rate, ingestion rate and selectivity

Bivalve larval clearance and ingestion rates were calculated for total phytoplankton using chl-*a* concentrations as a proxy. Clearance and ingestion rates of bacteria, *Synechococcus* spp. picoeukaryotes, cryptophytes, coccolithophores, nanoeukaryotes (excluding coccolithophores and cryptophytes) and ciliates were estimated from the differences in the rates of change of prey abundance in experimental bottles with and without the addition of bivalve larvae (Frost 1972).

Selective feeding by the bivalve larvae was evaluated from positive feeding rates using the electivity index (E_i) of (Vanderploeg and Scavia 1979). E_i ranges from −1 to +1 where 0 corresponds to no selectivity; negative values correspond to avoidance, and positive values represent selection. We used a one-sample Student's *t* test to determine whether electivity significantly deviated from 0.

Statistical analysis

All data analyses were carried out using Sigmaplot v.12 unless otherwise specified. A Kruskal–Wallis nonparametric ANOVA was used to determine whether there was a significant difference in bivalve size between experiments. Due to unequal sample sizes, further analysis was carried out with Dunn's method pairwise multiple analyses.

Student's *t* tests (Excel 2010) were used to determine whether there was a significant difference in prey concentration between controls and experimental bottles (df_3). The tests were performed to the significance level $\alpha = 0.05$. Only when the Student's *t* test demonstrated a significant difference were clearance rates calculated.

To test for statistical differences in clearance rates over the seven experiments, individual tests were performed for each prey type (one-way ANOVA or Kruskal–Wallis).

To ascertain whether bivalve larvae demonstrated preferences for any prey types, a Kruskal–Wallis test was used. As clearance rates did not change significantly between experiments for any prey type, clearance rates from all experiments were pooled together for each prey type. Further analysis was then undertaken by applying Tukey's multiple pairwise comparisons.

Molecular analysis of gut content

There were two sources of bivalve larvae used for molecular analysis of gut content; those collected straight from the bottle incubation experiments and, for 3 of the sampling dates (Table 1), those picked straight from the water samples, immediately preserved and used as "in situ" comparative samples. The small size of the bivalve larvae, ~360 μm, makes dissection of their stomachs impractical; therefore, DNA was extracted from pooled whole individuals (see above). For experiments 3, 6 and 7, further bivalve larvae ($n = 40$–50) were sorted directly from the field sample and, with no incubation in FSW for gut evacuation, immediately washed and sedated following the method above, flash-frozen in liquid nitrogen and then stored at −80 °C until DNA analysis. For both experimental larvae and in situ larvae picked straight from the water sample, process time

Table 1 Dates of bivalve larvae feeding selectivity experiments including dates in situ samples were collected straight from the field for molecular gut content analysis, sea surface temperature (°C), mean length of larvae ± SD (µm) and initial community analysis of <80 µm plankton prey given as prey abundance (cells mL^{-1})

Expt. no.	Date	In situ field samples taken for molecular analysis	Temperature (°C)	Mean length of larvae ± SD (µm)	Prey abundance (cells mL^{-1})					
					Ciliates	NEUK	Cocco	Crypto	PEUK	Syn
1	19.10.09	No	15.4	422 ± 60	2.6	1,427	146	436	11,997	13,466
2	10.11.09	No	13.4	371 ± 41	3	1,716	95	521	13,800	27,421
3	26.01.10	Yes	8.3	330 ± 22	3.05	821	47	136	5,177	9,370
4	02.02.10	No	8.5	322 ± 27	0.9	513	36.9	52	3,188	3,512
5	08.02.10	No	8.2	328 ± 34	1.58	1,002	66.59	89	5,058	5,023
6	06.09.10	Yes	15.8	443 ± 73	7.6	1,353	50.8		7,953	33,618
7	27.09.10	Yes	15.5	385 ± 60	12.66	2,256	153		16,305	61,918

from removal from natural feeding environment to sedation and snap freezing was kept to a minimum (<5 min) to limit digestion of gut content or gut evacuation. Starved bivalves, which had been incubated overnight in FSW for gut evacuation, were washed and sedated as described above and stored at −80 °C for a negative control for molecular analysis of gut contents.

Feeding incubation experiments determined that the majority of the bivalve larvae diet was composed of nanoeukaryotes and coccolithophores. We therefore used primers to target the haptophyta, a group of nanoeukaryotes including the orders coccolithales, isochrysidales, phaeocystales and prymnesiales. Partial 18S rDNA solely found in haptophytes was selectively amplified using the forward primer Prym-429f: 5′-GCG CGT AAA TTG CCC GAA-3′ (Coolen et al. 2004) and the reverse primer PRYM02: 5′-GGA ATA CGA GTG CCC CTG AC-3′ (Simon et al. 2000). Two microlitres of extracted genomic DNA from experiments 1–7, in situ field animals and starved bivalves were used for PCR amplification. PCR ingredients include 10 µL 5× GoTAQ DNA polymerase buffer (Promega UK Ltd), 4 µL 2 mM dNTPS (Promega UK Ltd), 4 µL 25 mM $MgCl_2$, 10 µM of each forward and reverse primer and 1.25 U GoTAQ DNA polymerase (Promega UK Ltd). Amplifications were carried out in a G Storm or VWR thermocycler. The cycling parameters included an initial denaturation step at 96 °C (4 min) followed by 35× cycles of 94 °C (30 s), 55 °C (40 s), 72 °C (40 s). A final extension phase at 72 °C (10 min) was followed by storage at 10 °C. Ten microlitre aliquots of the amplification reaction were analysed by gel electrophoresis (1 %) to check amplification efficiency. To increase amplification of any haptophyte DNA from the bivalve larvae guts, internal nested PCR primers were designed specifically from an alignment of 12 haptophyta sequences. These consisted of sequences from within the orders coccolithales, isochrysidales, phaeocystales and prymnesiales. One microlitre of each PCR product was used as a template for a nested PCR with 10 µM of the custom-designed forward and reverse primers Nest-Hapto-F 5′-TGA CAC AGG GAG GTA GTG ACA AG-3′ and Nest-Hapto-R 5′-GGT CGA AAC CAA CAA AAT AGC ACC-3′. Remaining PCR components included 10 µL 5× GoTAQ DNA polymerase buffer (Promega UK Ltd), 4 µL 2 mM dNTPS (Promega UK Ltd), 4 µL 25 mM $MgCl_2$ and 1.25 U GoTAQ DNA polymerase (Promega UK Ltd). Amplifications were carried out in a G Storm or VWR thermocycler with the following cycling parameters: initial denaturation step at 96 °C (4 min) followed by 30× cycles of 94 °C (1 min), 60 °C (1 min), 72 °C (1 min). A final extension phase at 72 °C (5 min) was followed by storage at 10 °C. Five microlitres of PCR product was run on a 1 % agarose gel to check for successful amplification. For each successful amplification, 25 µL of PCR product

Table 2 Multiple comparisons between bivalve larval size for each sampling date using Dunn's procedure

Expt. date	*q* statistic					
	19.10.09	10.11.09	26.01.10	02.02.10	08.02.10	06.09.10
10.11.09	2.743					
26.01.10	6.032*	3.064*				
02.02.10	7.384*	4.583*	1.837			
08.02.10	6.607*	3.788*	0.955	0.842		
06.09.10	0.473	2.834	5.581*	6.800*	6.128*	
27.09.10	2.045	0.611	3.609*	5.055*	4.288*	2.236

* $p < 0.05$

was cleaned using a QIAquick purification kit (Qiagen UK Ltd). To separate individual amplicons from the PCR products for sequencing, 2 µL of each PCR product was ligated and transformed using the pGEM®-T Easy Vector System and JM109 competent cells (Promega UK Ltd) following the standard protocol. White colonies ($n = 32$ for each PCR product) were picked, inoculated into 5 µL MilliQ water and following denaturation at 95 °C for 5 min used for colony PCR as described above. Twenty microlitres of each successful amplification was sent away for sequencing by LGC Genomics GmbH, Germany. Sequences were opened in Mega 5 (Heidecker et al. 1980) and trimmed to the forward and reverse primers. The Qiime pipeline (Caporaso et al. 2010) was used to assign the sequences to operational taxonomic units (OTUs) at the 97 % similarity level and to generate representative sequences. The representative sequences were then manually assigned taxonomy by searching the GenBank DNA database for sequence similarities using a basic local alignment search tool (BLAST).

Trophic impact

The potential trophic impact of bivalve larval feeding on each of the different prey groups was calculated by multiplying the bivalve carbon ingestion rate for each prey group by the abundance of bivalves. This was then compared to the average standing stocks of each prey group present at L4 at 10 m depth. Using field abundance data, the potential trophic impact in the field of bivalve larvae on each prey type was calculated as the percentage of the total standing stocks grazed daily (assuming that all prey were evenly distributed throughout the water column).

Results

During the sampling period September 2009 to September 2010, bivalve abundance was highest during autumn 2009, winter 2010 and autumn 2010 (Fig. 2). The peaks of bivalve abundance in the autumns of 2009 and 2010 occurred in a period of high sea surface temperature and relatively high chl-*a* concentrations. The peak in abundance in winter (January/February) 2010 occurred when sea surface temperature and chl-*a* concentrations were both lower. During the sample period, sufficient numbers of larvae were available to conduct seven feeding experiments, 2 in October/November 2009, 3 in January/February 2010 and 2 in September 2010 (Fig. 2).

The mean length of bivalve larvae ranged from 322 µm (SD ± 27) in experiment 4 (February 2010) to 443 µm (SD ± 73) in experiment 6 (September 2010) (Table 1). There was a significant difference in bivalve size between experiments (Kruskal–Wallis nonparametric ANOVA; $H = 108.382$; df_6; $p < 0.001$). Bivalves in winter experiments (January and February 2010) were significantly smaller than those in autumn experiments (Table 2).

Taxonomic composition of bivalve larvae

The bivalve larvae were successfully identified by barcoding a region of the 18S rRNA gene. From the 18S amplicon clone libraries constructed for each bivalve larval feeding experiment, a total of 341 sequences were returned, with at least 40 sequences originating from each library. These sequences clustered to 10 OTUs at the 97 % similarity level (Accession numbers KJ542098-KJ542107; Table 3). A representative sequence generated in the Qiime pipeline, and up to 10 randomly picked sequences from each OTU were used in a BLASTN search against the NCBI non-redundant nucleotide data set, using the criteria that the *E* value was 0, the BLASTN coverage over 550 bp and the BLASTN homology ≥98 % (Table 3). The top hits for each BLASTN output for the representative and randomly selected sequences within an OTU were compared and assigned identity based on the lowest shared taxonomic level. The results were checked to ensure the assigned taxonomy contained local species. This list of local species was derived from adult bivalves collected as part of the PML benthic survey time series which includes five sample sites around Plymouth (stations L4, Eddystone, Jennycliff Bay, Cawsand Bay and Rame Head) and covers an area of approximately 150 km^2. When both the representative and random sequences for an OTU showed the highest homology to a single species, the GenBank database was checked

Table 3 Taxonomic description of bivalve larvae identified by barcoding a region of the 18S rDNA gene

OTU	Accession number	No. of seqs.	Assigned ID ≥98 %	Score (bits)	*E* value	Homology (%)
1	KJ542098	126	*Phaxas pellucidus*	1,014–1,026	0.0	99
2	KJ542099	48	Genus *Barnea*	1,026–1,031	0.0	99
3	KJ542100	38	Genus *Musculus*	950–972	0.0	98
4	KJ542101	6	Family Montacutidae	959–972	0.0	99
5	KJ542102	57	Family Hiatellidae	966–1,009	0.0	99
6	KJ542103	6	Family Mytilidae	924–970	0.0	98
7	KJ542104	1	None			
8	KJ542105	1	Family Pectinidae	953–998	0.0	99
9	KJ542106	55	Order Veneroida	924–1,000	0.0	99
10	KJ542107	3	Family Mactridae	989–1,038	0.0	99

Sequences clustered at the 97 % similarity level to 10 OTUs (operational taxonomic units). Annotation of each OTU was achieved by BLASTN searching a representative sequence generated in the Qiime pipeline and 10 (or as many as are available) randomly selected sequences from each OTU, against the NCBI non-redundant nucleotide data set. The top hits for each BLASTN output for the representative and randomly selected sequences within an OTU were compared and assigned identity based on the lowest shared taxonomic level

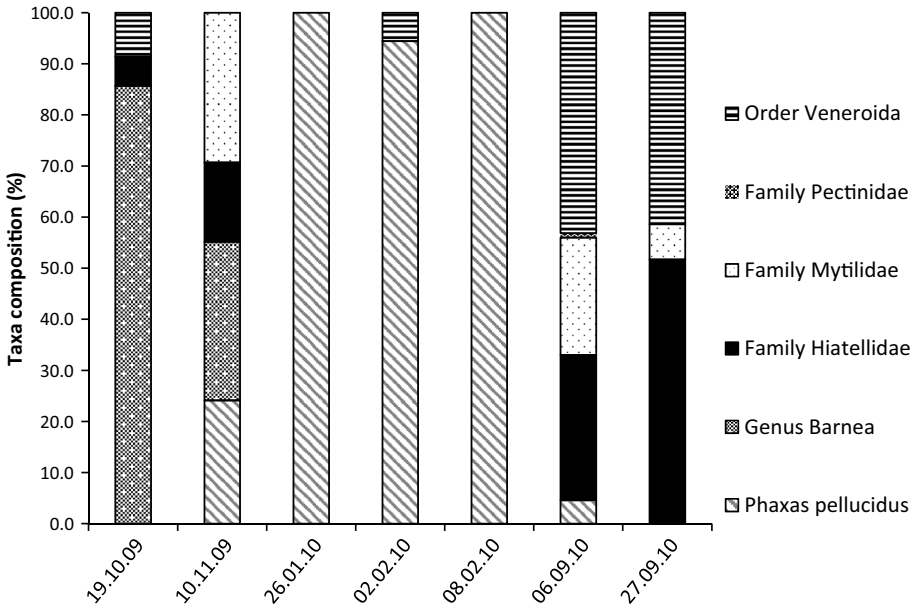


Fig. 3 Composition of bivalve larvae in each experiment derived from the number of sequences for each operational taxonomic unit as a percentage of the total number of sequences for each experiment

to ascertain whether 18S sequences for other local species within the same genus were represented. If such sequences were not found on the database, the OTU was annotated to the genus level. In addition, when an OTU showed highest homology to a single genus, the GenBank database was checked to ascertain whether 18S sequences from other local genera within the same family were represented. If it was found that other local genera from within the same family were not on GenBank, then the OTU was assigned to the family level. Where an OTU contains a genus or family member which falls within the same family or order of a different OTU, these OTUs have been combined and assigned identity at the lowest common taxonomic level. For example, OTU 3 was assigned to the genus *Musculus* which is a member of the Mytilidae family (OTU 6); therefore, OTU 3 and 6 have been combined. Similarly, OTU 4 originally assigned to the family Montacutidae falls within the order Veneroida (OTU 9) as does OTU 10 assigned as family Mactridae; therefore, OTUs 4, 9 and 10 have been combined and assigned to the order Veneroida. Combining OTUs that are annotated as members of the same taxonomic family or order, as above, and following the criteria that any OTU not showing homology greater or equal to 98 % (such as OTU 7), the original 10 OTUs (Table 3) were condensed to 6 OTUs. Of these 6 combined OTUs, 1 was identified to species level, 1 to genus, 3 to family and 1 to order (Fig. 3).

The composition of bivalve larvae changed over the course of the feeding experiments (Fig. 3). Experiments 1

Table 4 Student's *t* test results to determine whether there is a significant difference (** $p \leq 0.01$; * $p \leq 0.05$) in the concentration of each prey group between the controls and experimental bottles

Experiments	*t* statistic								
	Chl-*a*	LNA	HNA	Syn	Crypto	PEUK	NEUK	Cocco	Ciliates
1	11.20**	0.00	1.51	0.68	0.76	0.69	10.58**	3.38*	2.33
2	5.74**	2.36*	0.98	2.97*	3.37*	2.84*	4.56**	3.15*	1.81
3	7.86**	1.60	0.65	2.36*	0.79	1.62	4.16*	24.30**	25.95**
4	4.44*	0.60	1.10	3.33*	0.79	8.84**	3.93*	8.12**	1.29
5	14.91**	2.79*	0.74	0.00	1.99	4.53*	3.78*	3.69*	4.64**
6	5.42**			4.42*		2.58*	9.00**	8.39**	1.13
7	45.54**			0.00		3.21*	5.30**	13.21**	1.44

Clearance rates were calculated when *t* was significant ($p \leq 0.05$). Chlorophyll-*a* (Chl-*a*), low nucleic acid bacteria (LNA), high nucleic acid bacteria (HNA), *Synechococcus* (Syn), cryptophytes (Crypto), picoeukaryotes (PEUK), nanoeukaryotes (NEUK), coccolithophores (Coccos) and ciliates

and 2 (October/November 2009) showed a mix of *Phaxas pellucidus*, members of the genus *Barnea*, the families Mytilidae and Hiatellidae, and order Veneroida. In winter 2010 (experiments 3, 4 and 5), the composition of bivalve larvae was dominated by larvae of the razor shell, *P. pellucidus*. By the following autumn, when the larvae again increased in abundance, the composition of bivalve larvae was mixed, with experiments 6 and 7 showing the greatest diversity with *P. pellucidus*, the families Mytilidae, Hiatellidae and Pectinidae and order Veneroida being represented.

Bivalve larvae clearance and grazing rates

A significant difference in chl-*a* concentration between controls and experimental bottles was seen in all feeding experiments, indicating grazing of the <80 μM plankton community (Student's *t* test; df_3; $\alpha = 0.05$) (Table 4). To determine the diets and preferred prey of the bivalve larvae, the potential planktonic prey groups were investigated in further detail by flow cytometry and microscopy (ciliates). Significant grazing of nanoeukaryotes (2–20 μM) and coccolithophores was determined for all experiments and for picoeukaryotes (<2 μM) in all experiments except experiment 1 and 3. However, significant grazing was only detected for LNA bacteria in experiments 2 and 5; *Synechococcus* in experiments 2, 3, 4 and 6; and cryptophytes (~7–10 μM) in experiment 2. No significant grazing was determined on high nucleic acid bacteria. Significant feeding was only detected on ciliates (<30 μm in length) in experiments 3 and 5 (Table 4).

Analysis of potential prey at the beginning of each bivalve larval feeding experiment is given as both prey abundance (cells mL^{-1}) (Table 1) and carbon biomass available (Fig. 4a, b). Over the annual cycle, the composition of prey available changed considerably, but consistencies were apparent within a particular season. In general, as we would expect, there was less carbon available in the winter months (experiments 3, 4 and 5) than in late and early autumn. In early autumn 2010 (experiments 6 and 7), it appears that there were fewer cryptophytes available.

The amount of carbon (ng) ingested per day per individual for each prey group (Fig. 4c) shows that the bivalve larvae ate a wide size range of foods from *Synechococcus* to small ciliates. However, nanoeukaryotes and coccolithophores provided the vast majority of carbon available and made up at least 75 % of the bivalve larvae diet (Fig. 4d), with *Synechococcus* spp. and picoeukaryotes contributing little. During each season, a small percentage (0.6–2.5 %) of the carbon ingested came from small (<30 μm) heterotrophic ciliates.

Although temperature, prey availability and bivalve larval size varied over the course of the experiments, clearance rates did not change significantly between experiments for any prey type (one-way ANOVA or Kruskal–Wallis non-parametric tests performed per prey type; Table 5).

Selectivity

The highest clearance rates measured were of coccolithophores and on some occasions nanoeukaryotes (Fig. 5). Between food types, there were significant differences in clearance rates ($H = 40.8$; df_5; $p < 0.001$), with clearance rates of coccolithophores higher than all other prey types except nanoeukaryotes, and clearance rates of nanoeukaryotes significantly higher than those of cryptophytes and *Synechococcus* (Tukey's multiple pairwise comparison; Table 6).

This was further confirmed by the electivity index E_i (Fig. 6), which showed selection of coccolithophores in all experiments except experiment 2 and selection of nanoeukaryotes in experiment 2. There was negative selection of *Synechococcus*, picoeukaryotes, cryptophytes and ciliates in various experiments according to the electivity index (Fig. 6).

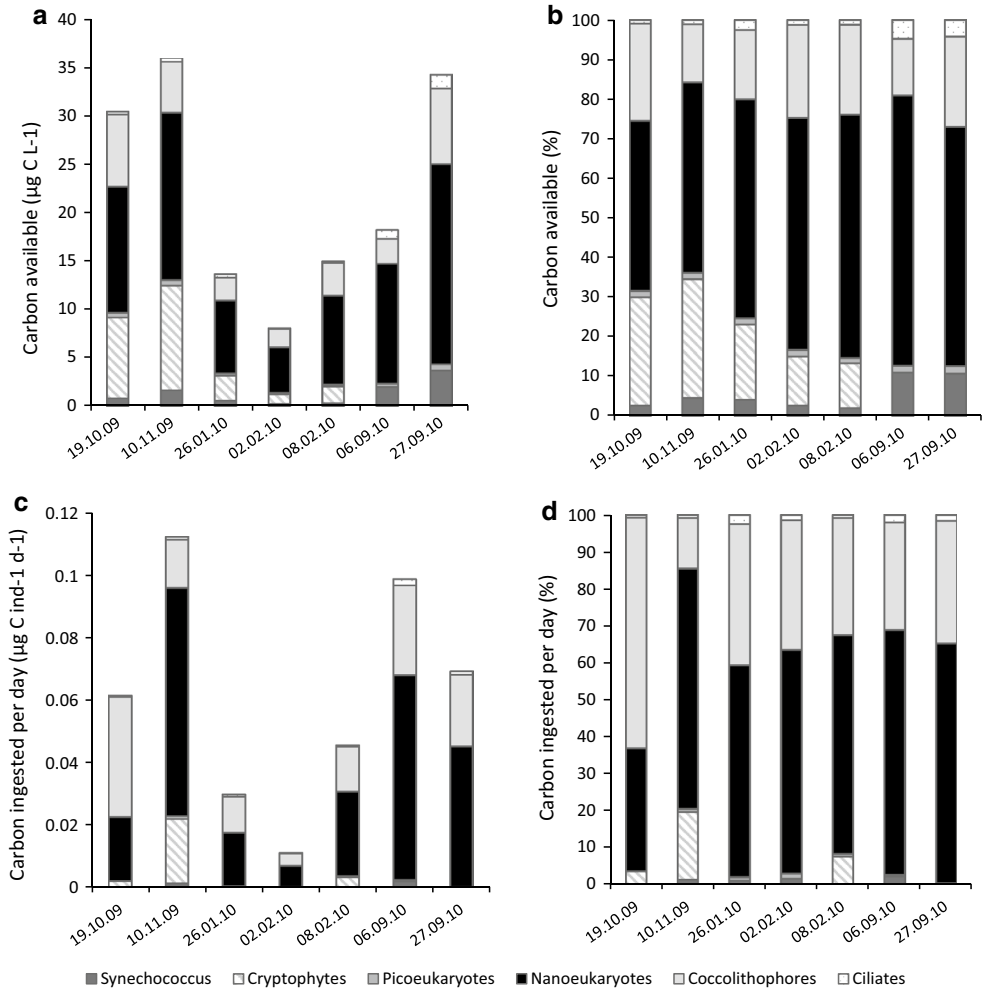


Fig. 4 Contribution of the different prey types to the measured carbon in the incubation water for each experiment **a** as the absolute carbon available (µg C L^{-1}) **b** as a percentage of the total carbon available. Bivalve larvae ingestion rates of ciliates, nanoeukaryotes, coccolithophores, cryptophytes, picoeukaryotes and Synechococcus for each experiment 1–7 shown as **c** carbon (µg) ingested individual^{-1} day^{-1} **d** carbon ingested individual^{-1} day^{-1} as a percentage of total

Molecular analysis of gut content

A total of 282 sequences were returned from 7 feeding experiments and the in situ field samples supplementing experiments 3, 6 and 7. Analysis using the Qiime pipeline assigned these sequences to ten different OTUs, numbered 0–9 (Accession numbers KF878247-KF878256; Table 7). OTU 3 contained only one sequence and was therefore not included in any further analysis.

The percentage of the total haptophyte diet made up by each of the OTUs for each experiment, as a percentage of the total number of sequences assigned to each OTU, is shown in Table 7. The detection of sequences from different prey species is subject to primer efficiency and copy number biases, and therefore, these proportions may only be deemed semi-quantitative. The table also shows the species or genus likely to be represented by that OTU, as determined by the highest similarity from comparison with sequences in the GenBank database using BLASTN. In some cases, sequences from two or more species showed the same similarity to the OTU representative sequence. In these cases, the genus or family common to both or all species listed is shown in brackets.

The haptophyte species represented in the diet were all from class Prymnesiophyceae. Within this class, four orders were represented: Coccosphaerales (*Braarudosphaera bigelowii*); Isochrysidales (*Isochrysis galbana*; *Gephyrocapsa oceanica*; *Emiliania huxleyi*); Phaeocystales (*Phaeocystis* spp.) and Prymnesiales (*Chrysochromulina* spp. and *Prymnesium* spp.). For three of the experiments, all of the sequences were assigned to just one OTU. For two of these, 100 % of the diet was from OTU 9, likely to be *I. galbana*, and for the other one, 100 % of the diet was from OTU 7, whose representative sequence was equally similar to several species of *Prymnesium*. Three species from two

Table 5 Summary of one-way ANOVA and Kruskal–Wallis nonparametric tests to compare clearance rates of prey types over the seven experiments: ciliates, nanoeukaryotes (NEUK), coccolithophores (Coccos), cryptophytes (Crypto), picoeukaryotes (PEUK) and *Synechococcus* (Syn)

Prey type	Statistical test	*F*/*H* statistic	*p* value
Syn	ANOVA	$F = 1.971$	0.12
PEUK	ANOVA	$F = 0.834$	0.56
Crypto	ANOVA	$F = 2.48$	0.09
Coccos	ANOVA	$F = 0.54$	0.77
NEUK	Kruskal–Wallis	$H = 11.64$	0.07
Ciliates	Kruskal–Wallis	$H = 2.89$	0.82

Df6, $\alpha = 0.05$

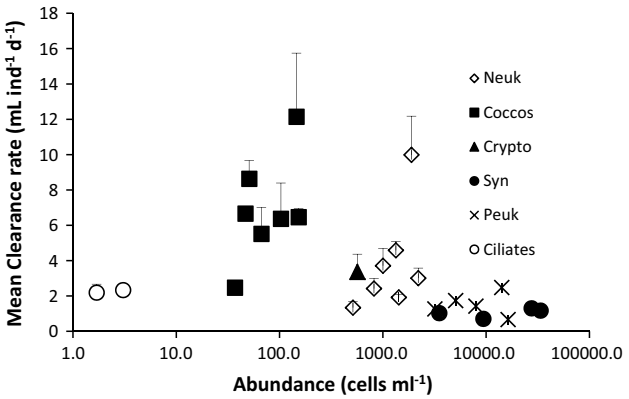


Fig. 5 Clearance rates (mLs individual^{-1} day^{-1}) of bivalve larvae (mean + 1SD) as a function of prey availability (cells mL^{-1}) for each of the prey groups: ciliates, nanoeukaryotes (NEUK), coccolithophores (Coccos), cryptophytes (Crypto), picoeukaryotes (PEUK) and Synechococcus (Syn)

Table 6 Multiple pairwise comparisons between bivalve larvae clearance rates of each prey type: ciliates, nanoeukaryotes (NEUK), coccolithophores (Coccos), cryptophytes (Crypto), picoeukaryotes (PEUK) and Synechococcus (Syn) using Tukey's method

Prey type	*q* statistic				
	Ciliates	NEUK	Coccos	Crypto	PEUK
NEUK	3.35				
Coccos	4.18*	0.83			
Crypto	1.77	5.12*	5.94*		
PEUK	0.64	3.99*	4.81*	1.13	
Syn	2.92	6.27*	7.10*	1.15	2.28

* $\alpha = 0.05$

different orders showed 100 % similarity with the representative sequence from OTU 4 (*Chrysochromulina brevifilum*, *Chrysochromulina parkeae* and *B. bigelowii*). Samples from experiment 7 showed the greatest variety, with

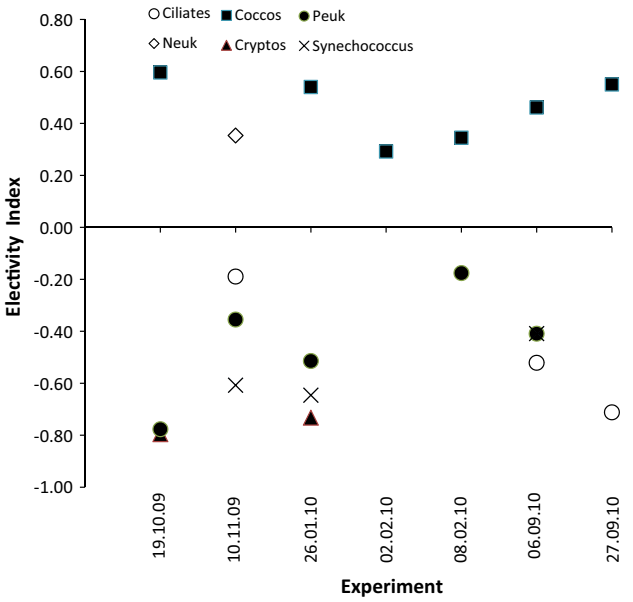


Fig. 6 Electivity index (E_i) of the different prey groups (ciliates, nanoeukaryotes, coccolithophores, cryptophytes, picoeukaryotes and *Synechococcus*) for all experiments where clearance rates were significant. Only the data points where the electivity index was shown to deviate significantly from 0 (one-sample Student's *t* test) are shown

the sequences assigned to five different OTUs, although four of these OTUs were likely to be *Phaeocystis*.

The representative sequences from four different OTUs (0, 1, 2 and 8) were all shown to be most similar to species of *Phaeocystis*. The representative sequence from OTU 0 was 100 % homologous to a sample of *Phaeocystis globosa* from the English Channel. For the other three OTUs, it was more difficult to assign a likely species confidently. This is due to the fact that the representative sequence showed the highest similarity to an unspecified *Phaeocystis* spp. or to a non-local *Phaeocystis* spp. sequence whereby no 18S sequences for that species from sources local to the study existed. To simplify our understanding of the diet, sequences assigned to OTUs 0, 1, 2 and 8 were compiled in one group containing all *Phaeocystis* spp. (Fig. 7).

There appears to be a good correlation between the haptophyte portion of the bivalve larval diet determined using molecular techniques from larvae analysed after the feeding experiments and those larvae whose gut contents were assessed directly after collection from the field (Fig. 7). For experiment 3, both field and experimental samples showed the haptophyte portion of the diet of larvae to be approximately 50 % *E. huxleyi*/*G. oceanica* and *I. galbana* with the remainder being made up of *Phaeocystis* spp. for experimental larvae and *Chrysochromulina* spp. for in situ larvae. Comparison of larval diet in experiment 6 with field samples shows the haptophyte portion of their diet to be approximately 70 % *E. huxleyi*/*G. oceanica*

Table 7 Percentage of the total haptophyte portion of the bivalve larvae diet made up by each of the OTUs for each experiment, measured as the percentage of the total number of sequences assigned to each OTU, where E = experiment, F = field and *N* is the number of sequences in each category

OTU (*N*)	Accession number of representative sequence	Highest similarity from blast (common denominator)	Experiment or field number (*N*)									
			E 1 (21)	E 2 (20)	E 3 (21)	F 3 (20)	E 4 (30)	E 5 (27)	E 6 (19)	F 6 (31)	E 7 (31)	F 7 (31)
0 (19)	KF878247	*Phaeocystis globosa* (Genus *Phaeocystis*)	0	0	43	0	0	0	0	0	3	29
1 (7)	KF878248	*Phaeocystis* spp. (Genus *Phaeocystis*)	0	0	14	0	0	0	0	0	10	3
2 (8)	KF878249	*Phaeocystis* spp. *(Genus Phaeocystis)*	0	0	0	0	0	0	0	0	26	0
4 (6)	KF878251	*Chrysochromulina brevifilum*, *C. parkeae* or *Braarudosphaera bigelowii* (Class Prymnesiophyceae)	0	0	0	0	0	0	32	0	0	0
5 (108)	KF878252	*Emiliania huxleyi* or *Gephyrocapsa oceanica* (Family Noelaerhabdaceae)	0	0	24	25	93	0	68	87	29	68
6 (12)	KF878253	*Chrysochromulina* spp. Family Prymnesiaceae	0	0	0	35	3	0	0	13	0	0
7 (59)	KF878254	*Prymnesium* spp. Genus *Prymnesium*	0	0	0	0	3	100	0	0	0	0
8 (10)	KF878255	*Phaeocystis* spp. (Genus *Phaeocystis*)	0	0	0	0	0	0	0	0	32	0
9 (52)	KF878256	*Isochrysis galbana*	100	100	19	40	0	0	0	0	0	0

with the remaining portion consisting of either OTU 4 (*C. brevifilum*, *C. parkeae* or *B. bigelowii)* or OTU 6 (*Chrysochromulina* spp.) for the experimental and in situ samples, respectively. The haptophyte portion of the diet for larvae from experiment 7 and field 7 both comprises of *E. huxleyi/G. oceanica and Phaeocystis* spp. but in different proportions.

Trophic impact

The potential trophic impact of bivalve larval feeding on each of the different prey groups in the field was minimal. The bivalves grazed up to 0.4 % of the standing stocks much of which appears to be made up of nanoeukaryotes including coccolithophores. The potential trophic impact in

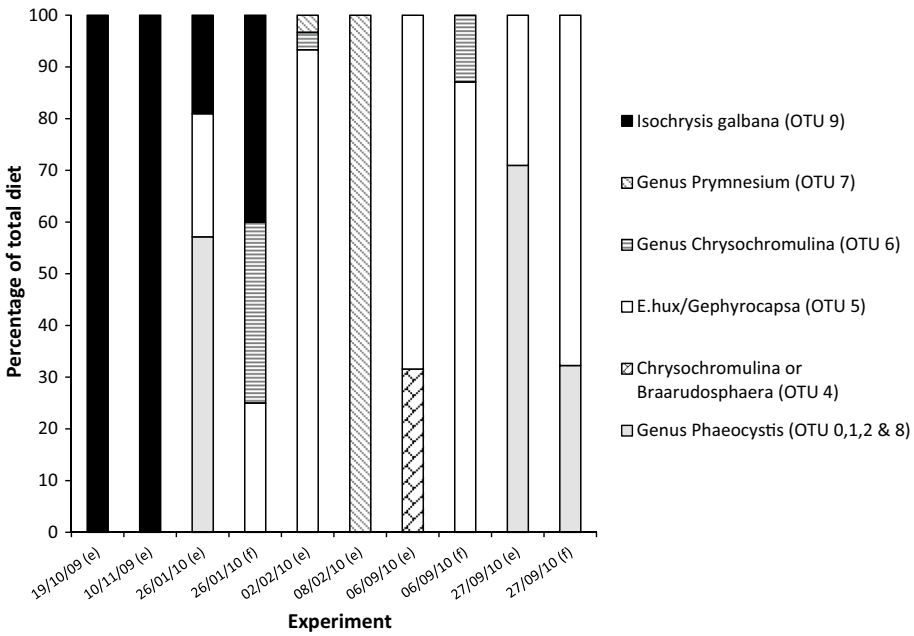


Fig. 7 Percentage of the total haptophyte diet made up by each of the OTUs for each sampling date, as a percentage of the total number of sequences assigned to each OTU; e = bottle incubation experiment, f = field. The species or genus likely to be represented by that OTU, as determined by the highest similarity from comparison with sequences in the GenBank database using BLAST (Basic Local Alignment Tool) is shown

the field was low and did not vary greatly over the whole season (Fig. 8).

Discussion

During the period of this study, we saw three peaks in bivalve abundance over the year from September 2009 to September 2010, allowing seven feeding experiments to be undertaken over the seasonal cycle. In both autumn 2009 and autumn 2010, the high abundance of bivalve larvae correspond to relatively high sea surface temperature (SST) and chl-*a* concentration; these peaks correlate well with the long-term time series characterisation of bivalve larval abundance. During the time series analysis of meroplankton at Station L4, bivalves have been routinely enumerated but, due to their morphological similarities, they have never been identified beyond the classification of 'bivalve larvae'. During this study, barcoding a region of the 18S rRNA gene has enabled us to determine how the composition of bivalve larvae changes throughout the year. Molecular analysis was performed on whole genomic DNA pooled from all bivalve larvae from a particular experiment/time. Therefore, despite the size of the bivalve larvae being relatively similar, it should be taken into consideration that the proportion of species detected at any one time may be influenced by biases in the amount of genetic material, copy number or primer homology between species.

Molecular characterisation of the bivalve larvae from these feeding experiments showed the composition of the larvae to change quite considerably between the peaks of abundance. The bivalve larvae in October/November 2009 (experiments 1 and 2) consisted of a total of five OTUs: comprising of *P. pellucidus*, the genus *Barnea*, the families Mytilidae and Hiatellidae, and order Veneroida. In winter 2010 (experiments 3, 4 and 5), the composition of bivalve larvae was dominated by larvae of the razor shell, *P. pellucidus*. In September 2010 (experiments 6 and 7), the peak of bivalve abundance showed the greatest diversity with 5 OTUs being represented in experiment 6 alone; these were annotated to be *P. pellucidus*, the families Mytilidae, Hiatellidae and Pectinidae, and the order Veneroida. Considering that bivalve larvae are often spawned in response to elevated SST (Highfield et al. 2010) or to take advantage of increased prey during a phytoplankton bloom (Pulfrich 1997; Highfield et al. 2010), it was unusual to see a peak of bivalve larvae in winter 2010 (experiments 3, 4 and 5). Indeed, peaks in bivalve larvae abundance in winter are only seen on occasional years (our unpublished L4 data). This winter peak of abundance occurred when SST and chl-*a* concentration were half of that seen in the autumn. These bivalve larvae (experiments 3, 4 and 5) were established by molecular identification to be predominantly (≥94 %) *P. pellucidus*. All 11 sequences (1 representative sequence and 10 randomly selected sequences) which were used to classify OTU 1 showed 99 % homology to *P. pellucidus*. Considering that this is the sole local species of the *Phaxas* genus and that homology with any other members of the Pharidae family was below our homology threshold for assigning taxonomy, we are confident to classify this OTU to species level. *P. pellucidus* is a razor shell, <4 cm in length, which is found buried in fine mixed sands in coastal waters of north-western Europe including the coasts of the British Isles (Neish 2008) and is known to spawn in

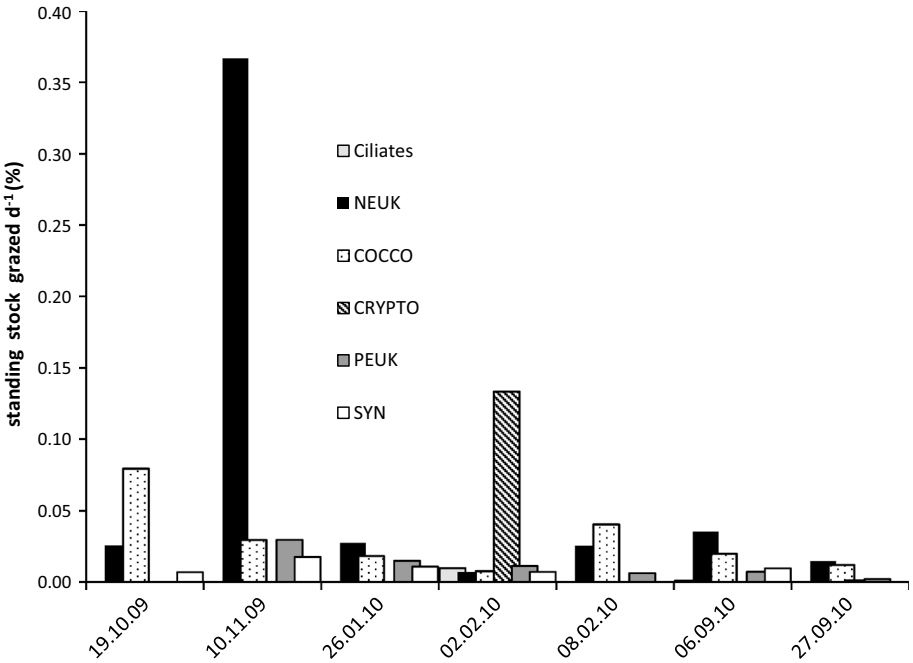


Fig. 8 Potential trophic impact in the field of bivalve larvae on each prey group: ciliates, nanoeukaryotes (NEUK), coccolithophores (Coccos), cryptophytes (Crypto), picoeukaryotes (PEUK) and Synechococcus (Syn), calculated as the percentage of the total standing stocks grazed daily (assuming that all prey were evenly distributed throughout the water column)

both late-summer and winter months (MBA 1957). The taxonomy of bivalve larvae, assigned by molecular characterisation, was compared to adult bivalves collected and identified from the same station (L4) as part of the benthic survey of the Western Channel Observatory (www.westernchannelobservatory.org.uk/). Nineteen species of bivalves were collected and identified over a 2-year period. These species belonged to 8 orders of which 5 orders were also represented in the bivalve larvae results presented here. Of the three orders that were not represented by the bivalve larvae, the 18S sequences of the local species within 2 of the orders (Lucinoida and Anomalodesmata) are present in the GenBank database; for the third order Nuculida, not all local species are represented in GenBank. This comparison with adult bivalves from Station L4 has served to confirm that our molecular characterisation of the bivalve larvae is likely to be a good representation of the bivalves present at station L4 on our sampling dates; however, it cannot be overlooked that bivalve larvae can be advected to the sampling site and therefore may have originated from adult bivalves some distance away.

This study is novel in that it looks at naturally occurring bivalve larvae in the field and assesses the feeding rate and selectivity of these larvae on a naturally occurring plankton assemblage. The results of this study show that bivalve larvae feed on a wide range of prey from picoeukaryotes to ciliates within the <30 µm plankton assemblage. In particular, the larvae appear to select for nanoeukaryotes, especially coccolithophores, with these prey groups contributing >75 % of the carbon ingested. The focus of many previous studies on larval bivalve feeding has been based around commercially important species in laboratory environments (Baldwin and Newell 1995; Rico-Villa et al. 2009; Ben Kheder et al. 2010). From these studies, it is thought that the diet of bivalve larvae consists mostly of nanoplankton (<20 µm), including dinoflagellates, *Isochrysis* spp. (Rico-Villa et al. 2009) and *Synechococcus* spp. (Baldwin 1995). Despite these studies being based solely on oyster larvae, our study largely concurs with the results, demonstrating that the diet of naturally occurring bivalve larvae is also based heavily on cells <20 µm.

The selection of nanoplankton including coccolithophores by bivalve larvae may be passive selection or active selection; choice exercised by the predator in accepting or rejecting a prey type (Almeda et al. 2011). Passive selection is partly based on prey size, and as such, our results would suggest nanoeukaryotes are of optimum size to be captured and ingested by the bivalve larvae. However, observations on meroplankton larvae with double cilia bands, such as bivalve larvae, suggest that these meroplankton have a predator/prey size ratio ranging between 30:1 and 125:1 (Hansen et al. 1994). For the bivalve larvae, in our study, this would equate to a preferred prey size of 2.8–12 µm. The positive selection seen for nanoeukaryotes and coccolithophores in this study could therefore be active selection and potentially based on food quality. Coccolithophores, such as *E. huxleyi*, have in fact been shown to be of exceptionally high nutritional quality (Pond and Harris 1996). The fact that cryptophytes are not selected for, and yet are of a similar size to the species of coccolithophores eaten during this study, as determined by the molecular assessment of gut content, suggests that the bivalve larvae

are indeed actively selecting for coccolithophores. This is also evident from the clearance rates and electivity index which clearly demonstrate a significant and positive selection for coccolithophores and an avoidance of picoeukaryotes, cryptophytes, *Synechococcus* and ciliates.

Previous studies have suggested the importance of picoplankton in the diet of bivalve larvae as well as bacteria and detritus (Baldwin and Newell 1991; Raby et al. 1997; Sommer et al. 2000). However, for this study, the contribution of picoplankton to the carbon ingested by the larvae is extremely small. In addition, during this study, the clearance rate of picoplankton was significantly lower than that for coccolithophores, and no positive selection was seen. It is worth considering, however, that our low grazing rates on picoplankton could be attributable to masking by a more significant cascade of trophic effects caused by the removal of other predatory groups. For example, as suggested by (Almeda et al. 2011), if the bivalve larvae remove other grazers of picoplankton such as ciliates and heterotrophic nanoflagellates, this may result in a relaxation of grazing pressure on small-sized prey which in turn may overshadow the effects of bivalve larvae grazing on these food items and lead to underestimation of grazing. However, in our study, grazing on ciliates was minimal; therefore, it is unlikely that the low grazing on picoplankton is attributable to masking by a cascade of trophic effects.

Similarly, the grazing rates on bacteria may also be masked by growth which could occur as a result of food chain effects or as a result of excretion by the bivalve larvae in the experimental bottles. In only 3 experiments was the ingestion of LNA bacteria significant and no significant ingestion of HNA bacteria was seen in any experiments. LNA bacteria may be representative of senescent bacteria and therefore are not in growth phase. HNA bacteria are indicative that the population of bacteria is growing and therefore significant ingestion on these bacteria could potentially be masked by growth of the HNA bacteria in the control bottles (Gasol et al. 1999). We may also speculate that there is a proportion of the bivalve larvae diet that we were not able to analyse, such as detritus or dissolved organic matter (Manahan and Crisp 1982).

The diet of the bivalve larvae does not appear to change dramatically over the seasonal cycle. This is despite the composition of prey available changing, the size of the bivalves being significantly different and indeed the diversity of bivalves changing between seasons. In all experiments, over 80 % of the diet consists of nanoeukaryotes including coccolithophores. For experiments 3, 4 and 5, where the bivalves are of a smaller size and comprised predominantly of *P. pellucidus*, it is interesting to note that the clearance rate of smaller prey such as picoeukaryotes and *Synechococcus* did not significantly increase. During winter when chl-*a* concentrations and prey abundance are lower than that at other times of the year, it might be predicted that bivalve larvae eat a greater range of prey; however, from these studies, this does not appear to be the case with the larvae in winter still feeding predominantly (>90 %) on nanoeukaryotes and coccolithophores. To conclude, despite the changes in bivalve larvae community structure, clearance rates of each prey type did not change significantly over the course of the experiments, therefore suggesting that different bivalve species may in fact consume similar prey.

The potential trophic impact in the field was low and did not vary greatly over the whole season. It is possible that the trophic impact is underestimated as our bivalve numbers are relatively low. We know from our time series data that bivalve larvae can reach double the abundance we saw during our study period and that the relatively low numbers of bivalve larvae seen in our study may in part be attributable to the size of mesh used for sampling (Riccardi 2010). However, even considering abundance of bivalve larvae may reach twice that used in our trophic impact calculations; the impact is unlikely to exceed 1 % of standing stock grazed per day. These findings are in concurrence with those described by (Almeda et al. 2011) who also found that the trophic impact of bivalve larvae was <1 % of the biomass of the standing stock grazed daily. From the observations of this study and that of Almeda, it may be implied that bivalve larvae are unlikely to be food-limited at any time and in turn therefore, one may advocate that mortality is potentially controlled by predation as opposed to limitation of prey.

Performing bottle incubation experiments can provide a good insight into what naturally occurring bivalve larvae may prey upon in a natural plankton assemblage. However, given that availability and quality of food may be patchy, both spatially and temporally, running a laboratory-based experiment on natural assemblages of bivalve larvae and prey may still not give a true representation of feeding in the field. Many studies suggest that bivalve larvae may have a high tolerance to food deprivation (Moran and Manahan 2004; Ben Kheder et al. 2010; Matias et al. 2011). However, food quality and quantity have a notable influence on the duration of larval phases (Ben Kheder et al. 2010), and delayed development leaves larvae open to predation and therefore reduced recruitment. The only alternative way to gain information on the diet of pelagic larvae is by identifying the food actually found in the stomachs of the larvae in situ. However, dissection and identification of gut content is unrealistic due to the small size of the bivalve larvae. In such circumstances, molecular analysis of gut content of individuals taken directly from the field may provide valuable information on predator–prey relationships. As part of this study, we performed molecular analysis of gut content of bivalve larvae following the bottle incubations,

and where there were sufficient larvae, on the gut content of larvae taken directly from the field. Recent studies have used DNA-based techniques for dietary analysis including PCR amplification of 18S genes from the gut content of zooplankton (Nejstgaard et al. 2003; Troedsson et al. 2007; Nejstgaard et al. 2008; Troedsson et al. 2009; Durbin et al. 2012), including meroplanktonic larvae (Maloy et al. 2009; Riemann et al. 2010; Fileman et al. 2014). If the gut content of the predator can first be removed to reduce co-amplification of host DNA, then general or universal primers designed to conserved regions (Holland et al. 1991) targeting the 18S gene can be used. However, for small organisms such as bivalve larvae where gut content cannot be extracted, universal primers would result in a high concentration of predator DNA which would likely mask amplification of the small amount of prey DNA. As such, this study utilised 18S primers designed to target the haptophyte group of nanoeukaryotes, known to be positively selected for, which by nature of their specificity would not amplify the predator DNA. This was possible during this study as the bottle incubations gave a good idea of the main portion of the larval diet and allowed a comparison between the results seen for the feeding experiments and the larvae taken directly from the field. It must, however, always be taken into consideration that this method of DNA amplification is not quantitative. Biases in primer efficiency and DNA copy number between species, as well as DNA degradation of prey during gut passage, may all lead to a skew in the proportion of the diet detected. This study has, however, clearly shown that using a DNA-based technique for analysing the gut content of bivalve larvae provides a better resolution of identification of the prey consumed, compared with flow cytometric or routine microscope analysis of prey, and also that the composition of prey established through traditional bottle experiments confers quite well with that established from in situ larvae.

The application of DNA-based techniques to establish the predator–prey interactions of meroplanktonic larvae and microplankton is likely to gain more interest with the development of new techniques. Next generation sequencing (NGS) allows sequencing of several thousands of sequences simultaneously, increasing the efficiency and decreasing the cost and time associated with more traditional amplification, cloning and sequencing techniques. DNA metabarcoding using NGS has the potential to reveal many consumed species simultaneously (O'Rorke et al. 2012; Pompanon et al. 2012; Lindeque et al. 2013). In addition, the most recent studies have developed a method whereby the dietary analysis of consumers can be made with universal primers using predator-specific endonuclease restriction enzymes or blocking primers, including the analysis of small planktonic consumers such as bivalve larvae (Maloy et al. 2013).

Conclusion

Despite the ecological and commercial importance of adult populations of many invertebrate species, little is known regarding specific trophic interactions of their larval stages, including feeding selectivity, ontogenetic shifts in prey preference or levels of feeding competition between various species (Maloy et al. 2013). While physical transport processes play major roles in delivering larvae to the shore, first, larvae must survive the perils of life in the plankton, where they usually suffer great mortality (Vargas et al. 2006). This study has demonstrated that bivalve larval size and species composition can change over a seasonal cycle. Despite these changes, larval food preferences and clearance rates remain constant, even though their prey's composition and availability also vary. A more informed understanding of the role of meroplanktonic larvae in pelagic marine food webs, such as we have undertaken to provide in this study, will allow a better prediction of how shifting plankton community structure may affect larval survival and recruitment to the benthos.

Acknowledgments We are grateful to the crew of the RV Plymouth Quest for collection of samples and to Denise Cummings for collection of water. We would also like to thank Dr Angus Atkinson for his insightful comments on the paper, Dr Glen Tarran for his flow cytometric help and Dr John Bruun for his invaluable help with statistics. This work was in part funded by the EU INTERREG IV CHARM 3 project (www.charm-project.org) (Management number 4037/1938) and the Natural Environment Research Council National Capability funding for the Western Channel Observatory (Agreement number R8-H12-84).

References

Almeda R, Messmer AM, Sampedro N, Gosselin LA (2011) Feeding rates and abundance of marine invertebrate planktonic larvae under harmful algal bloom conditions off Vancouver Island. Harmful Algae 10:194–206. doi:10.1016/j.hal.2010.09.007

Baldwin BS (1995) Selective particle ingestion by oyster larvae (*Crassostrea virginica*) feeding on natural seston and cultured algae. Mar Biol 123:95–107. doi:10.1007/bf00350328

Baldwin BS, Newell RIE (1991) Omnivorous feeding by planktotrophic larvae of the eastern oyster *Crassostrea virginica*. Mar Ecol Prog Ser 78:285–301. doi:10.3354/meps078285

Baldwin BS, Newell RIE (1995) Feeding rate responses of oyster larvae (*Crassostrea virginica*) to seston quantity and composition. J Exp Mar Biol Ecol 189:77–91

Bamstedt DJ, Gifford X Irigoien, Atkinson A, Roman M (2000) Feeding. Academic press, London

Belgrano A, Legendre P, Dewarumez JM, Frontier S (1995) Spatial structure and ecological variation of meroplankton on the French-Belgian coast of the North Sea. Mar Ecol Prog Ser 128:43–50

Ben Kheder R, Quere C, Moal J, Robert R (2010) Effect of nutrition on *Crassostrea gigas* larval development and the evolution of physiological indices Part B: effects of temporary food deprivation. Aquaculture 308:174–182. doi:10.1016/j.aquaculture.2010.08.030

Booth BC, Lewin J, Lorenzen CJ (1988) Spring and summer growth-rates of subarctic pacific phytoplankton assemblages determined from carbon uptake and cell columes estimated using epifluorescence microscopy. Mar Biol 98:287–298. doi:10.1007/bf00391207

Bos OG, Hendriks IE, Strasser M, Dolmer P, Kamermans P (2006) Estimation of food limitation of bivalve larvae in coastal waters of north-western Europe. J Sea Res 55:191–206. doi:10.1016/j.seares.2005.10.006

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Pena AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Tumbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336. doi:10.1038/Nmeth.F.303

Chicharo LMZ, Chicharo MA (2000) Short-term fluctuations in bivalve larvae compared with some environmental factors in a coastal lagoon (South Portugal). Scientia Marina 64:413–420

Coolen MJL, Muyzer G, Rijpstra WIC, Schouten S, Volkman JK, Damste JSS (2004) Combined DNA and lipid analyses of sediments reveal changes in Holocene haptophyte and diatom populations in an Antarctic lake. Earth Planet Sci Lett 223:225–239. doi:10.1016/j.epsl.2004.04.014

Durbin EG, Casas MC, Rynearson TA (2012) Copepod feeding and digestion rates using prey DNA and qPCR. J Plankton Res 34:72–82

Eckman JE (1996) Closing the larval loop: linking larval ecology to the population dynamics of marine benthic invertebrates. J Exp Mar Biol Ecol 200:207–237

Eloire D, Somerfield PJ, Conway DVP, Halsband-Lenk C, Harris R, Bonnet D (2010) Temporal variability and community composition of zooplankton at station L4 in the Western Channel: 20 years of sampling. J Plankton Res 32:657–679. doi:10.1093/plankt/fbq009

Fileman E, Lindeque PK, Harmer R, Halsband C, Atkinson A (2014) Feeding rates and selectivity of decapod larvae in the Western English Channel. Mar Biol 161:2479–2494. doi:10.1007/s00227-014-2520-9

Frost BW (1972) Effects of size and concentration of food particles on the feeding behavior of the marine planktonic copepod *Calanus pacificus*. Limnol Oceanogr 17:805–815

García-Esquivel Z, Bricelj VM, Gonzalez-Gomez MA (2001) Physiological basis for energy demands and early postlarval mortality in the Pacific oyster, *Crassostrea gigas*. J Exp Mar Biol Ecol 263:77–103

Garland ED, Zimmer CA (2002) Techniques for the identification of bivalve larvae. Mar Ecol Prog Ser 225:299–310. doi:10.3354/meps225299

Gasol JM, Zweifel UL, Peters F, Fuhrman JA, Hagström A (1999) Significance of size and nucleic acid content heterogeneity as measured by flow cytometry in natural planktonic bacteria. Appl Environ Microbiol 65:4475–4483

Hansen B, Bjornsen PK, Hansen PJ (1994) The size ratio between planktonic predators and their prey. Limnol Oceanogr 39:395–403

Haury LR, McGowan JA, Wiebe PH (1978) Patterns and processes in the time-space scales of plankton distributions. In: Steele JH (ed) Spatial pattern in plankton communities: NATO conference on marine biology. Plenum Press, New York

Heidecker G, Messing J, Gronenborn B (1980) A versatile primer for DNA sequencing in the M13mp2 cloning system. Gene 10:69–73. doi:10.1016/0378-1119(80)90145-6

Highfield JM, Eloire D, Conway DVP, Lindeque PK, Attrill MJ, Somerfield PJ (2010) Seasonal dynamics of meroplankton assemblages at station L4. J Plankton Res 32:681–691. doi:10.1093/plankt/fbp139

Holland PWH, Hacker AM, Williams NA (1991) A molecular analysis of the phylogenetic affinities of Saccoglossus-cambrensis Brambell and Cole (Hemichordata). Philos Trans R Soc Lond Ser B Biol Sci 332:185–189. doi:10.1098/rstb.1991.0048

Laing I (1995) Effect of food supply on oyster spatfall. Aquaculture 131:315–324

Lindeque PK, Parry HE, Harmer RA, Somerfield PJ, Atkinson A (2013) Next generation sequencing reveals the hidden diversity of zooplankton assemblages. PLoS ONE 8:e81327. doi:10.1371/journal.pone.0081327

Livi S, Cordisco C, Damiani C, Romanelli M, Crosetti D (2006) Identification of bivalve species at an early developmental stage through PCR-SSCP and sequence analysis of partial 18S rDNA. Mar Biol 149:1149–1161. doi:10.1007/s00227-006-0296-2

Maloy AP, Culloty SC, Slater JW (2009) Use of PCR-DGGE to investigate the trophic ecology of marine suspension feeding bivalves. Mar Ecol Prog Ser 381:109–118. doi:10.3354/meps07959

Maloy AP, Culloty SC, Slater JW (2013) Dietary analysis of small planktonic consumers: a case study with marine bivalve larvae. J Plankton Res 35:866–876. doi:10.1093/plankt/fbt027

Manahan DT, Crisp DJ (1982) The role of dissolved organic material in the nutrition of pelagic larvae: amino acid uptake by bivalve veligers. Integr Comp Biol 22:635–646

Matias D, Joaquim S, Ramos M, Sobral P, Leitao A (2011) Biochemical compounds' dynamics during larval development of the carpet-shell clam Ruditapes decussatus (Linnaeus, 1758): effects of mono-specific diets and starvation. Helgol Mar Res 65:369–379. doi:10.1007/s10152-010-0230-3

MBA (1957) Plymouth marine fauna. Marine Biological Association of the United Kingdom, p. 547

Moran AL, Manahan DT (2004) Physiological recovery from prolonged "starvation" in larvae of the Pacific oyster *Crassostrea gigas*. J Exp Mar Biol Ecol 306:17–36

Neish A (2008) Phaxas pellucidus. A razor shell. Marine life information network. Marine Biological Association of the United Kingdom, Plymouth

Nejstgaard JC, Naustvoll LJ, Sazhin A (2001) Correcting for underestimation of microzooplankton grazing in bottle incubation experiments with mesozooplankton. Mar Ecol Prog Ser 221:59–75. doi:10.3354/meps221059

Nejstgaard JC, Frischer ME, Raule CL, Gruebel R, Kohlberg KE, Verity PG (2003) Molecular detection of algal prey in copepod guts and fecal pellets. Limnol Oceanogr Methods 1:29–38

Nejstgaard JC, Frischer ME, Simonelli P, Troedsson C, Brakel M, Adiyaman F, Sazhin AF, Artigas LF (2008) Quantitative PCR to estimate copepod feeding. Mar Biol 153:565–577. doi:10.1007/s00227-007-0830-x

O'Rorke R, Lavery S, Chow S, Takeyama H, Tsai P, Beckley LE, Thompson PA, Waite AM, Jeffs AG (2012) Determining the diet of larvae of western rock lobster (*Panulirus cygnus*) using high-throughput dna sequencing techniques. PLoS ONE 7. doi:10.1371/journal.pone.0042757

Pechenik JA (1999) On the advantages and disadvantages of larval stages in benthic marine invertebrate life cycles. Mar Ecol Prog Ser 177:269–297

Pompanon F, Deagle BE, Symondson WOC, Brown DS, Jarman SN, Taberlet P (2012) Who is eating what: diet assessment using next generation sequencing. Mol Ecol 21:1931–1950. doi:10.1111/j.1365-294X.2011.05403.x

Pond DW, Harris RP (1996) The lipid composition of the coccolithophore *Emiliania huxleyi* and its possible ecophysiological significance. J Mar Biol Assoc U K 76:579–594

Pulfrich A (1997) Seasonal variation in the occurrence of planktic bivalve larvae in the Schleswig-Holstein Wadden Sea.

Helgolander Meeresuntersuchungen 51:23–39. doi:10.1007/bf02908751

Putt M, Stoecker DK (1989) An experimentally determined carbon–volume ratio for marine oligotrichous ciliates from estuarine and coastal waters. Limnol Oceanogr 34:1097–1103

Raby D, Lagadeuc Y, Dodson JJ, Mingelbier M (1994) Relationship between feeding and vertical-distribution of bivalve larvae in stratified and mixed waters. Mar Ecol Prog Ser 103:275–284. doi:10.3354/meps103275

Raby D, Mingelbier M, Dodson JJ, Klein B, Lagadeuc Y, Legendre L (1997) Food-particle size and selection by bivalve larvae in a temperate embayment. Mar Biol 127:665–672. doi:10.1007/s002270050057

Riccardi N (2010) Selectivity of plankton nets over mesozooplankton taxa: implications for abundance, biomass and diversity estimation. J Limnol 69:287–296. doi:10.3274/JL10-69-2-10

Rico-Villa B, Pouvreau S, Robert R (2009) Influence of food density and temperature on ingestion, growth and settlement of Pacific oyster larvae, *Crassostrea gigas*. Aquaculture 287:395–401. doi:10.1016/j.aquaculture.2008.10.054

Riemann L, Alfredsson H, Hansen MM, Als TD, Nielsen TG, Munk P, Aarestrup K, Maes GE, Sparholt H, Petersen MI, Bachler M, Castonguay M (2010) Qualitative assessment of the diet of European eel larvae in the Sargasso Sea resolved by DNA barcoding. Biol Lett 6:819–822. doi:10.1098/rsbl.2010.0411

Simon N, Campbell L, Ornolfsdottir E, Groben R, Guillou L, Lange M, Medlin LK (2000) Oligonucleotide probes for the identification of three algal groups by dot blot and fluorescent whole-cell hybridization. J Eukaryot Microbiol 47:76–84. doi:10.1111/j.1550-7408.2000.tb00014.x

Simonelli P, Troedsson C, Nejstgaard JC, Zech K, Larsen JB, Frischer ME (2009) Evaluation of DNA extraction and handling procedures for PCR-based copepod feeding studies. J Plank Res 31(12):1465–1474

Smyth TJ, Fishwick JR, Al-Moosawi L, Cummings DG, Harris C, Kitidis V, Rees A, Martinez-Vicente V, Woodward EMS (2010) A broad spatio-temporal view of the Western English Channel observatory. J Plankton Res 32:585–601. doi:10.1093/plankt/fbp128

Sommer F, Stibor H, Sommer U, Velimirov B (2000) Grazing by mesozooplankton from Kiel Bight, Baltic Sea, on different sized algae and natural seston size fractions. Mar Ecol Prog Ser 199:43–53. doi:10.3354/meps199043

Tarran GA, Heywood JL, Zubkov MV (2006) Latitudinal changes in the standing stocks of nano- and picoeukaryotic phytoplankton in the Atlantic Ocean. Deep Sea Res Part 2 Top Stud Oceanogr 53:1516–1529. doi:10.1016/j.dsr2.2006.05.004

Thorson G (1950) Reproductive and larval ecology of marine bottom invertebrates. Biol Rev 25:1–45

Troedsson C, Frischer ME, Nejstgaard JC, Thompson EM (2007) Molecular quantification of differential ingestion and particle trapping rates by the appendicularian *Oikopleura dioica* as a function of prey size and shape. Limnol Oceanogr 52:416–427

Troedsson C, Simonelli P, Nagele V, Nejstgaard JC, Frischer ME (2009) Quatification of copepod gut content by differential length amplification quantitative PCR (dla-qPCR). Mar Biol 156:253–259. doi:10.1007/s00227-008-1079-8

Utermöhl H (1958) Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. Mitt int Ver Theor Angew Limnol 9:1–38

Vanderploeg HA, Scavia D (1979) Calculation and use of selectivity coefficients of feeding: zooplankton grazing. Ecol Model 7:135–149. doi:10.1016/0304-3800(79)90004-8

Vargas CA, Manriquez PH, Navarrete SA (2006) Feeding by larvae of intertidal invertebrates: assessing their position in pelagic food webs. Ecology 87:444–457. doi:10.1890/05-0265

Wang S, Bao ZM, Zhang LL, Li N, Zhan AB, Guo WB, Wang XL, Hu JJ (2006) A new strategy for species identification of planktonic larvae: PCR-RFLP analysis of the internal transcribed spacer region of ribosomal DNA detected by agarose gel electrophoresis or DHPLC. J Plankton Res 28:375–384. doi:10.1093/plankt/fbi122

Zhan A, Hu X, Bao L, Lu W, Peng W, Wang M, Hu J (2008) Molecular identification of scallop planktonic larvae using species-specific microsatellites. Acta Oceanol Sin 27:134–146

Zubkov MV, Sleigh MA, Burkill PH (1998) Measurement of bacterivory by protists in open ocean waters. FEMS Microbiol Ecol 27:85–102. doi:10.1111/j.1574-6941.1998.tb00527.x