

Real-time PCR assay for detection and relative quantification of *Liocarcinus depurator* larvae from plankton samples

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Received: 1 May 2007 / Accepted: 23 October 2007 / Published online: 7 November 2007
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Abstract Accurate species identification of decapod crustacean larvae is required to understand their population distributions, life cycle dynamics and interactions with their habitats. Analysis of plankton samples using morphological taxonomic methods and microscopy is time-consuming, requires highly skilled and trained operatives and may often be inaccurate. As complementary tools to classical identification methods, recent work has focused on the development of molecular approaches and shows their feasibility for species-specific identification. This study has developed real-time PCR assays utilising species-specific Taqman[®] probes designed in the cytochrome oxidase I (COI) gene of *Liocarcinus depurator*, *Necora puber*, *Carcinus maenas* and *Cancer pagurus*. Our study then employed the probe and primers designed for *L. depurator* to obtain accurate identification and relative abundance estimates of *L. depurator* larvae in plankton samples collected between March 2005 and October 2006. Ranges of larval abundances were derived from a standard curve created from plankton samples spiked with a known number of larvae reared in the laboratory. Inhibition of the PCR reaction was shown to be an important factor and our results suggested that 0.1 ng of DNA as template provided accurate identification and avoided inhibition. Real-time PCR was shown to provide accurate species identification on unsorted plankton samples

and could be suitable for the estimation of larval abundances in the plankton, although more work must be done to improve the accuracy of those estimations.

Introduction

Fisheries of decapod crustaceans are important economic resources, with many species of crabs, lobsters and shrimps being exploited around the world and of high importance in terms of landings and value. Besides the economic importance of decapod fisheries, adult decapods are important components of marine food webs, particular in coastal ecosystems, for their abundance and diversity. Their larvae can, at times, be abundant in the plankton and constitute an important part in the diets of many larval and juvenile fish (Bromley et al. 1997).

The taxonomy and identification of marine zooplankton has traditionally been based on morphological characteristics visualised using a light microscope. Developing the necessary skills for this identification requires a great deal of training, experience and dedication and usually requires a long time to process plankton samples. Independent of the degree of such expertise, the phenotypic variability caused by environmental factors increases the uncertainty in the identification of many species (Gimenez 2006). Furthermore, specimens preserved for long times or damaged during collection can be simply impossible to identify.

For decapods, whose larvae undergo great morphological changes between developmental phases, accurate identification of some species can be especially difficult or simply impossible even for expert taxonomists. Such is the case for the genus *Liocarcinus* (Stimpson, 1871) (subfamily Polybiinae, family Portunidae), represented in the north-eastern Atlantic by seven species: *L. arcuatus* (Leach,

Communicated by A. Atkinson.

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1814), *L. corrugatus* (Pennant, 1777), *L. depurator* (Linnaeus, 1758), *L. holsatus* (Fabricius, 1798), *L. marmoreus* (Leach, 1814), *L. pusillus* (Leach, 1815a) and *L. zariquieyi* (Gordon, 1968) (Ingle 1992). The complete larval development has been described from reared material for all except *L. zariquieyi*. Clark (1984) and Kim and Hong (1999) examined and compared *Liocarcinus* species (except *L. zariquieyi*), concluding that there is no single morphological character valid for species identification in the zoeal stages. In the study area, only *L. zariquieyi* and *L. arcuatus* are not expected to be present. *L. depurator* and *L. holsatus* have been recorded previously (d'Udekem d'Acoz 1999) and although we do not have previous records for the other species, their distribution indicates that their presence in the area can be expected.

Reflecting the problems associated with identification of many other taxa, there have been many reports of molecular techniques developed to provide accurate identification. Examples of molecular approaches applied to larval identification include species-specific oligonucleotide probes (Medeiros-Bergen et al. 1995); restriction length polymorphism (RFLP) analysis (Lindeque et al. 1999, 2004, 2006; Eimanifar et al. 2006; Wang et al. 2006); species-specific random amplified polymorphic DNA (RAPD) markers (Hughes and Beaumont 2004); DNA sequence comparison (Øines and Heuch 2005); multiplexed species-specific polymerase chain reaction (PCR) (Bucklin et al. 1999; Hill et al. 2001; Hare et al. 2000) and two-step nested PCR (Deagle et al. 2003). Recently, real-time PCR has been applied to the identification of marine invertebrate larvae and fish eggs and larvae (McBeath et al. 2006; Vadopalas et al. 2006; Fox et al. 2005; Watanabe et al. 2004; Taylor et al. 2002). The success obtained by those authors and the specificity, speed, sensitivity and possibility of develop quantification assays, indicated that this technique would be suitable for the work reported here.

The mitochondrial cytochrome c oxidase subunit I (COI) gene is a common target in phylogenetic and taxonomic analysis and it has been reported to enable the discrimination of closely allied species in all animal phyla except Cnidaria (Hebert et al. 2003). The COI gene has been successfully employed as a molecular marker for species identification in previous studies on copepods (Bucklin et al. 1999; Hill et al. 2001; Øines and Heuch 2005; McBeath et al. 2006), and importantly, this gene has enabled the design of robust primers (Folmer et al. 1994).

In this study the main objectives were: (1) to develop a reliable real-time PCR assay for accurate decapod larvae identification from unsorted plankton samples, including the design of specific probes and primers for *L. depurator*, *C. maenas*, *N. puber* and *C. pagurus*; and (2) by the use of the methodology developed, study temporal patterns of abundances for *L. depurator* larvae.

Materials and methods

Field sampling, collection and rearing

During spring-summer of 2005, adult specimens of *Liocarcinus depurator* (subfamily Polybiinae, family Portunidae), *Necora puber* (subfamily Polybiinae, family Portunidae), *Carcinus maenas* (subfamily Carcininae, family Portunidae) and *Cancer pagurus* (family Cancridae), were captured using traps placed offshore around the Fisheries Research Services monitoring station at Stonehaven (56°57.8'N 02°06.2'W) in the western North Sea, south of Aberdeen. The specimens captured were frozen to be used for DNA extraction.

Plankton samples were collected weekly from February 2005 until October 2006, from the monitoring station at Stonehaven, approximately where decapod adults were captured, in ~50 m water depth. One sample per month, from March 2005, was allocated for abundance analysis for *L. depurator* by real-time PCR, while the rest of the samples were used for preliminary tests. The sampling was carried out using a 40 cm diameter Bongo net (composed by two identical nets) of 200 µm mesh size, towed obliquely from the surface to ~5 m above the seabed. Since no flowmeter was fitted to the net, the filtered volume was estimated from the speed of the boat (2.5 knots), the haul duration (4 min on average), the depth reached by the net (45 m) and net mouth area (0.125 m²), and assuming 70% efficiency. The average volume of filtered water was 56 m³ per sample.

The Bongo net used provides two replicates per sample, allowing the conservation of one of them for molecular analysis and the other one for microscopy analysis if required. One Bongo net plankton sample was immediately preserved in 4% borax buffered formaldehyde in seawater and the other sample was preserved in 100% ethanol (for molecular analysis). The alcohol was changed after 24 h, allowing a ratio of at least 3:1 alcohol:plankton volume, and samples were stored at 4°C.

Larvae of *L. depurator* were obtained from an ovigerous female collected in the same area by traps. The berried crab was maintained in sea water at a temperature of 15°C and gently aerated until the eggs hatched. Larvae at the zoea I stage were collected in 100% ethanol and stored at 4°C to be used as a positive material for plankton spiking.

DNA extraction

DNA was extracted from muscle obtained from the pereopods of three adult specimens of *Liocarcinus depurator*, *Carcinus maenas*, *Necora puber* and *Cancer pagurus* using the DNeasy[®] Tissue Kit (Qiagen) following the manufacturer's animal tissue protocol.

Total DNA from plankton samples was extracted using the same kit, with some modifications, as follows. Prior to lysis, samples were filtered and collected on an autoclaved 200- μm mesh of known weight and placed in a previously weighed tube. Tests previously applied to the mesh showed that it would not interfere in the PCR reaction (results not shown). The ethanol was evaporated to allow measurement of the dry weight. Each sample was resuspended in 360 μl Buffer ATL (Qiagen) and 40 μl Proteinase-K (Qiagen) per 25 mg of sample and lysed at 55°C overnight. Following lysis, 200 μl aliquots were used for DNA extraction. DNA was eluted in 200 μl of Elution Buffer (Qiagen) and stored at 4°C. The concentration of DNA was estimated by fluorometry, using PicoGreen® dsDNA Quantitation Kit (Invitrogen).

Whole larvae preserved in ethanol were rehydrated in distilled water for 30 min at room temperature prior to lysis. DNA from single and multiple whole *L. depurator* larvae (1, 10 and 100 larvae) was extracted by incubation overnight at 55°C in 100 μl of lysis buffer containing 1 \times TE Buffer, 0.45% Tween 20 (Sigma), 0.45% IGEPAL-CA630 (Sigma) and 20 mg ml⁻¹ proteinase-K (Sigma) followed by heating at 95°C for 5 min to inactivate the enzyme.

DNA sequencing

A fragment of approximately 700 bp of the mitochondrial COI gene of three specimens of *L. depurator*, *C. maenas*, *N. puber* and *C. pagurus* was amplified using the universal primers LCO-1490 (5'-GGTCAACAAATCATAAAGATAT TGG-3') and HCO-2198 (5'-TAAACTTCAGGGTGA CCAAAAATCA-3') (Folmer et al. 1994). PCR amplifications were set up in 50 μl reactions containing 1 \times NH₄ Buffer, 1 mM MgCl₂, 2 mM dNTPs (Invitrogen), 0.56 μM each primer, 2.5 U *Taq* polymerase (Bioline) or 1 \times Accuzyme Buffer, 1.5 mM MgCl₂, 1 mM dNTPs (Invitrogen), 0.28 μM each primer and 2.5 U Accuzyme DNA polymerase (Bioline). The cycling parameters included an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 37°C for 1.5 min, and 72°C for 2 min when using *Taq* polymerase, and 40 cycles of 94°C for 1 min, 37°C for 1 min and 72°C for 2 min when using Accuzyme. In both cases a final extension of 72°C for 5 min was carried out.

PCR products were purified using QIAquick Gel Extraction Kit Protocol (Qiagen) and the concentrations were estimated on 1% ethidium-bromide stained agarose gel using a Low DNA Mass Ladder (Invitrogen). PCR products were ligated into pGEM®-T Easy Vector (Promega) and subsequently used to transform Select96™ Competent Cells (Promega) following the manufacturer's instructions. Recombinant clones were screened for inserts of correct

size and positives were cultured and later purified using QIA prep Spin Miniprep kit (Qiagen). The positive clones were sequenced using the primers LCO-1490, HCO-2198, T7(5'-TAATACGACTCACTATAGGG-3') and Sp6(5'-ATTAGGTGACACTATAAGAATACTCAAGC-3'), and Big Dye™ Ready Reaction Mix Version 3.1 (Applied Biosystems) according to the manufacturer's protocol. Sequencing was performed on an ABI 377 automated DNA-sequencer (Applied Biosystems) and resulting sequences were analysed using Sequencher software (Gene Codes). A consensus sequence for each species was obtained from the alignment of sequences obtained: eight replicates for specimens 1 and 2 and three replicates for specimen 3 in the case of *L. depurator*; for *N. puber*, six replicates from specimen 1, eight for specimen 2 and two for specimen 3; for *C. maenas*, nine, four and four replicates respectively, and for *C. pagurus* a total of eighteen replicates were employed.

The consensus sequences were aligned to related species available from GenBank using ClustalW. For *L. depurator*, the alignment was performed with the species shown in Table 1. The sequences of the other species of interest were aligned to those species and others that were shown to be related to them. Nucleotide sequences of *L. depurator* and *N. puber* have been deposited in the GenBank database under accession numbers DQ480363 and DQ480362 respectively.

Probe and primers design and real-time PCR

Suitable species-specific primers and Taqman®-MGB probes for the four decapod species sequenced, *L. depurator*, *N. puber*, *C. pagurus* and *C. maenas* (Table 2), were identified using Primer Express Version 2.0 software (Applied Biosystems), although only those for *L. depurator* were applied to plankton samples. To ensure their specificity and avoid potential cross-reaction, the sequences were compared to the GenBank database using BLAST and the best set of probe and primers for each species was chosen. The probes were labelled on the 5'-end with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and on the 3'-end with a non fluorescent quencher and a minor groove binder (MGB). The FAM dye is separated from the quencher during the reaction, causing a fluorescent emission captured by a detector while the reaction is proceeding. This real time data allows setting a threshold at the moment when the exponential phase of the polymerase chain reaction (PCR) is happening and the amount of product is proportional to the amount of starting template. The cycle number of the reaction when the fluorescence signal passes that threshold is known as the Ct-value, thus higher Ct-values indicate there is less starting template and vice versa.

All real-time PCR reactions were conducted in Micro-Amp Optical 96-well reaction plates (Applied Biosystems)

Table 1 COI sequences aligned using ClustalW to design species-specific probes and primers for *L. depurator*

Taxa	Accession number
<i>Aegla obstipa</i>	AY595646
<i>Callinectes sapidus</i>	AY682072
<i>Cancer antennarius</i>	AF060773
<i>Cancer borealis</i>	AF060767
<i>Cancer branneri</i>	AF060774
<i>Cancer gracilis</i>	AF060769
<i>Cancer magister</i>	AF060766
<i>Cancer novaezealandiae</i>	AF060768
<i>Cancer oregonensis</i>	AF060772
<i>Cancer pagurus</i>	AF060771
<i>Cancer productus</i>	AF060770
<i>Carcinus maenas</i> (haplotype 1)	AY616437
<i>Carcinus maenas</i> (haplotype 2)	DQ523682
<i>Carcinus maenas</i> (haplotype 3)	DQ523683
<i>Carcinus maenas</i> (haplotype 4)	DQ523684
<i>Carcinus maenas</i> (haplotype 5)	AY616438
<i>Carcinus maenas</i> (haplotype 6)	AY616439
<i>Carcinus maenas</i> (haplotype 7)	DQ523685
<i>Carcinus maenas</i> (haplotype 8)	DQ523686
<i>Carcinus maenas</i> (haplotype 10)	AY616440
<i>Carcinus maenas</i> (haplotype 13)	AY616441
<i>Carcinus maenas</i> (haplotype 23)	AY616442
<i>Carcinus maenas</i> (haplotype 24)	AY616443
<i>Carcinus maenas</i> (haplotype 29)	AY616444
<i>Carcinus aestuarii</i> (haplotype 60)	AY616445
<i>Chionoectes opilio</i>	AB211151
<i>Eriocheir formosa</i>	AF105250
<i>Eriocheir japonica</i>	AF105246
<i>Hemigrapsus nudus</i>	AF060775
<i>Hyas coarctatus alutaceus</i>	AB244632
<i>Munida armilla</i>	AY350937
<i>Necora puber</i>	DQ480362
<i>Petrolisthes cinctipes</i>	AF060776
<i>Portunus pelagicus</i>	AF082732
<i>Portunus trituberculatus</i>	AB093006
<i>Pseudocarcinus gigas</i>	AY562127
<i>Rhithropanopeus harrisi</i>	DQ094789

The COI sequences and others were used to design probes and primers for *N. puber*, *C. maenas* and *C. pagurus*

in a volume of 20 μ l containing: 1 μ l DNA template, 1 \times Taqman[®] Universal PCR Mastermix, 900 nM each primer, 200 nM Taqman[®] probe, 1 \times exogenous internal positive control (IPC) primer and probe mix and 1 \times exogenous IPC target. Each plate also contained four no template controls (NTC). Definitions for IPCs and NTCs can be found in Table 3. The reactions were run on the ABI Prism 7000

Table 2 Probe and primers designed for species-specific identification by real-time PCR

Taxon	Forward primer (5'–3')	Probe (5'–3')	Reverse primer (5'–3')
<i>L. depurator</i>	TGATTACTCCACCTTCGCTAAC	TCTTCTCCTCATAAGAGGCAT	GTTGAAAGAGGTTTGGTACTGGA
<i>N. puber</i>	GGTGTGGTACTGGTTGAACTGTT	TATCTGCAGCTATTGCCAC	TTCAGTTGATTTAGGTAATTTTCGG
<i>C. maenas</i>	CAGTTGATTTAGGGATTTCTCTTACA	CAATAAGCGTTCTTTCGGCAT	TAGACCAGATACCTTTATTTGTGTGAGC
<i>C. pagurus</i>	GCTGTCTTATTACTGCTATCCTTTACTTC	TATCACCTCCCTGCTTAGCT	CATCACTATACTTTTAAACAGACCCGAAACC

Only those for *L. depurator* were applied to plankton samples to obtain relative quantification

Table 3 Important terms and controls used in the application of Real-time PCR

	Definition	Purpose
NTC	“No Template Control” PCR reaction with no test template but including IPCs	To verify amplification quality and to provide a reference Ct-value to assess inhibition in the samples
IPC	“Internal Positive Control” Composed of template DNA, primers and probe, always added to every PCR reaction	To assess inhibition of amplification in the samples
Artificial plankton samples	Filtered sea water containing representative specimens of planktonic taxa, excluding brachyurans, extracted from plankton samples	“Clean” samples with no brachyurans
Brachyuran negative samples	Plankton samples analysed microscopically from which brachyurans were removed	Samples without whole brachyurans
Spiked samples	Samples with known numbers of <i>L. depurator</i> larvae added or where the lysate of a known number of larvae was added.	To check sensitivity and efficiency of PCR and relative quantification

sequence detection system (Applied Biosystems) with the following conditions: 50°C for 2 min; 95°C for 10 min; 45 cycles of 95°C for 15 s and 60°C for 1 min.

Specificity and efficiency

Probes and primers designed for each of the four species were tested on DNA extracted from adult tissue of conspecifics, DNA from each other, DNA extracted from *Inachus phalangium* (family Majidae)—which was commonly found in the traps, and DNA extracted from one *L. depurator* larva.

The efficiency of the PCR chemistry for each set of probe and primers (except for *N. puber*, which was not evaluated due to limitation in time and resources) was assessed using triplicate tenfold serial dilutions from DNA extracted from adult tissue of every species. The slopes from the calibration curves created with these serial dilutions (where the Ct-values obtained are plotted versus the logarithm of the dilution) were used to calculate the efficiency of the PCR according to the equation $E = 10^{(-1/\text{slope})}$ (Pfaffl 2001).

Plankton trials

All subsequent tests on plankton samples were focused on the detection of *L. depurator*. Their larvae can be found in the water column throughout the year (Clark 1984; Martin 2000) so it is not possible to know a priori if they are absent from a plankton sample. Initial tests were performed on winter plankton samples ($n = 4$), when there is less likelihood of finding *L. depurator* larvae in the water column. In addition, artificial plankton samples were created ($n = 2$). Real plankton samples were examined by microscope and several specimens of all the taxa present, excluding any decapod larvae, were removed to filtered sea water. In this way we ensured the absence of *L. depurator*. All these samples (the winter plankton samples and artificial plankton samples) were divided into two subsamples (HML beaker technique, van Guelpen et al. 1982), and one whole larva of *L. depurator* was added to one of the subsamples in order to test the capability of the technique to detect a single larva in a mixed plankton sample.

The complexity of a real plankton sample, where many inhibitors can be present and influence the results, was not properly represented by the winter plankton samples, which are too small compared with plankton samples from the rest of the year, or by artificial plankton samples. For that reason, new plankton samples ($n = 4$) were examined by microscope and all brachyurans present were removed (these samples were called “brachyuran negative samples”, Table 3). The aim of these tests was to examine specificity of the probe and primers, the PCR efficiency, the degree of inhibition if any, and the appropriate dilution of extracted

DNA that should be used when analysing plankton samples. These plankton samples were lysed as described above. After lysis, 100 μl aliquots were taken from two of the brachyuran negative samples (tests 1 and 2) and the lysate of 0, 1, 10 or 100 larvae was added to each aliquot. The DNA was extracted and used undiluted, or in 10^{-1} and 10^{-2} dilutions, as templates in the real-time PCR.

Because adding larvae that had previously been lysed could artificially increase the concentration of target DNA and increase the degree of inhibition, in the other two brachyuran negative samples (tests 3 and 4), the larvae were added to the plankton samples without prior lysis. After plankton lysis, four 200- μl aliquots were spiked with 0, 1, 10 or 100 *L. depurator* larvae. Another 180 μl of buffer ATL and 20 μl of proteinase K were added to each aliquot and lysis continued for another 8 h. DNA was extracted from 200 μl of the lysate. Undiluted, 10^{-1} , 10^{-2} and 10^{-3} dilutions of the DNA extracted from each subsample were used as template in the real-time PCR. Besides, DNA concentration from the four aliquots spiked with larvae was obtained by fluorometry and 1, 0.1, 0.01 and 0.001 ng was used for real-time PCR analysis.

Application and semiquantification

Three plankton samples were tested in order to find an appropriate sample to be used as a standard curve. This standard would allow relative quantification of *L. depurator* in plankton samples. The samples were subsampled into four equal parts (HML beaker technique, van Guelpen et al. 1982) spiked with 0, 1, 10 and 100 whole larvae respectively. Subsamples were filtered by a 200 μm mesh and the ethanol was evaporated before addition of Buffer ATL and proteinase-K. Following extraction, the concentration of DNA was calculated by fluorometry and 0.1 ng μl^{-1} dilutions were prepared. Tenfold dilutions from the subsample spiked with one single larva were prepared, obtaining 0.01 and 0.001 ng μl^{-1} of total DNA. Triplicate real-time PCR reactions were performed for each dilution.

One of these samples tested was used to create a standard and five ranges of Ct-values were used, corresponding with a range of numbers of larvae present in the samples: lower than 1 larva, between 1 and 10 larvae, between 10 and 50 larvae, between 50 and 100 larvae and more than 100 larvae. The value for 50 larvae was calculated theoretically during the exponential phase of the PCR curve (Table 4). These values were later transformed into abundances (number of larvae m^{-3}).

One plankton sample per month, from March 2005 until October 2006 (Q1–Q20), was analysed by real-time PCR, following the methodology explained above. To each reaction, 0.1 ng of extracted DNA from a sample was added and two replicates were prepared from each sample.

Table 4 Ranges of values used for relative quantification of *L. depurator* on plankton samples analysed by real-time PCR. Ct-values for 50 larvae were calculated theoretically during the exponential phase of the amplification ($y = 2^x$)

No. of <i>L. depurator</i> larvae in the sample	Abundances (no. of larvae/ m^3)	Range of ct-values
>100	>2	<26.41
100–50	2–1	(26.41–27.41)
50–10	1–0.2	(27.41–29.67)
10–1	0.2–0.02	(29.67–32.20)
<1	<0.02	>32.20

Results

Sensitivity and specificity

Real-time PCR primers and probes were specific for each of the four species and did not cross-react with any of the other species tested. The calibration curves performed for three of the four species showed a high efficiency of 1.97 for *L. depurator*, 1.94 for *C. maenas* and 1.91 for *C. pagurus* (Fig. 1).

In two of the winter samples tested, only the subsamples spiked with one *L. depurator* larva were positive, while another subsample, which had not been spiked showed a positive signal, indicating the “unwanted” presence of *L. depurator*. The last winter sample tested showed high inhibition levels and no reliable conclusions could be drawn.

Two artificial plankton samples were tested for *L. depurator* and in both cases only the subsamples spiked with one *L. depurator* larva were positive, showing the capability of the technique to detect a single larva in a mixed plankton sample. However, the inhibition was high in these samples: the IPCs were not detected in one of the samples and in the second, the Ct-values for the IPCs varied between 22.93 and 39.16, when the average values for the NTCs were 28.70.

In subsequent tests with new plankton samples, even after microscopic analysis confirmed that apparently all brachyuran specimens had been eliminated (“brachyuran negative samples”), three of the samples yielded positive results in real-time PCR tests for *L. depurator*.

Inhibition of PCR was found when high concentrations of template were used. For the first two tests (tests 1 and 2), where the lysate of 0, 1, 10 and 100 *L. depurator* larvae was added, the results showed high inhibition, which decreased with increasing dilutions. Even when 100-fold dilutions were used, the samples containing the lysate of 100 larvae showed a delay in the Ct-values of IPCs compared to the NTCs. When whole larvae were added instead of their

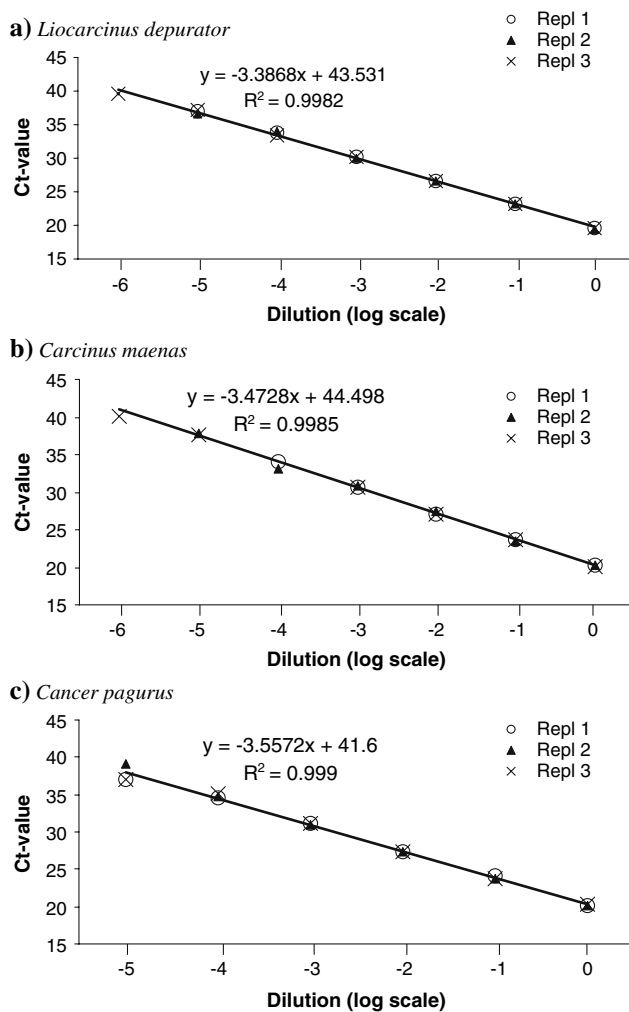


Fig. 1 Real-time PCR efficiencies of probes and primers, tested on tenfold dilution series of DNA template extracted from adult tissue. Three replicates were used. Standard curves showing cycle threshold values (Ct-value) plotted against logarithm of the initial DNA template. The efficiency, was calculated from the equation $E = 10^{(-1/\text{slope})}$. A PCR efficiency of 100% is achieved when the slopes of the curves approach to the theoretical value of -3.32 i.e. $E = 2$. **a** *Liocarcinus depurator*, $E = 1.97$ (slope = -3.3868). **b** *Carcinus maenas*, $E = 1.94$ (slope = -3.47). **c** *Cancer pagurus*, $E = 1.91$ (slope = -3.55)

lysate (tests 3 and 4), the inhibition was again high, especially when 100 larvae were added (Fig. 2). After these results, the exact amount of DNA was calculated and it was deduced that use of 0.1 ng of DNA avoided inhibition due to high DNA concentrations or from unknown inhibitors. Consequently it was decided to use this DNA concentration as template in subsequent analysis of plankton samples.

Application and semiquantification

One of the three samples tested to find a suitable standard curve to allow relative quantification of *L. depurator* in plankton samples was naturally negative for the target

species. The efficiency of the PCR was 2.1 (Fig. 3) and the coefficient of variation between replicates was very low, ranging from 0.1 to 0.7%.

The results obtained from plankton sample analysis by real-time PCR appear in Table 5. The relative quantification of *L. depurator* larvae in the plankton samples calculated from the standard curve is represented in Fig. 4. Four ranges of abundances have been indicated to facilitate comprehension of these data: less than 0.02 larvae m^{-3} , between 0.02 and 0.2 larvae m^{-3} , between 0.2 and 2 larvae m^{-3} and more than 2 larvae m^{-3} . In 40% of the samples analysed larval density was less than 0.02 larvae m^{-3} , the abundances for 20% of the samples were between 0.02 and 0.2 larvae m^{-3} and in further 20% of samples the abundances registered were between 0.2 and 2 larvae m^{-3} . In 20% of the samples analysed *L. depurator* was absent.

L. depurator larvae were not present from December 2005 to March 2006 and the highest abundances (0.2–2 larvae m^{-3}) appeared during the Summer-Autumn periods (from July to September in 2005 and from August to September in 2006). In both years, a similar abundance pattern can be observed, with two abundance peaks; in spring and in summer-autumn. During 2005, the first peak was smaller than in 2006 with a maximum value reached in April, while in 2006 this first peak is reached in May. The second peak during 2005 is more protracted and the abundances were kept high through a 3-month period (from July to September). During the next year, this second peak in *L. depurator* abundance happened a month later and its duration was shorter.

Discussion

Accurate identification of decapod larvae, as well many other marine invertebrates or their larval stages, can be difficult and on occasions represents an obstacle not only for the study of their population dynamics but also for the study of zooplankton communities.

In this study, a fragment of the COI gene from each of the decapod species *Liocarcinus depurator*, *Cancer pagurus*, *Carcinus maenas* and *Necora puber* was analysed to identify regions containing species-specific sequences, and was used to design specific probes and primers for each of these species. Although specific probes and primers were identified for the four species, particular focus was placed on *L. depurator* due to known identification problems. The other three brachyuran species were selected based on their high abundance in the study area, the economical importance in the case of *C. pagurus* and *N. puber* and their taxonomic proximity to *L. depurator*. Unfortunately, no other species of the genus *Liocarcinus* were captured.

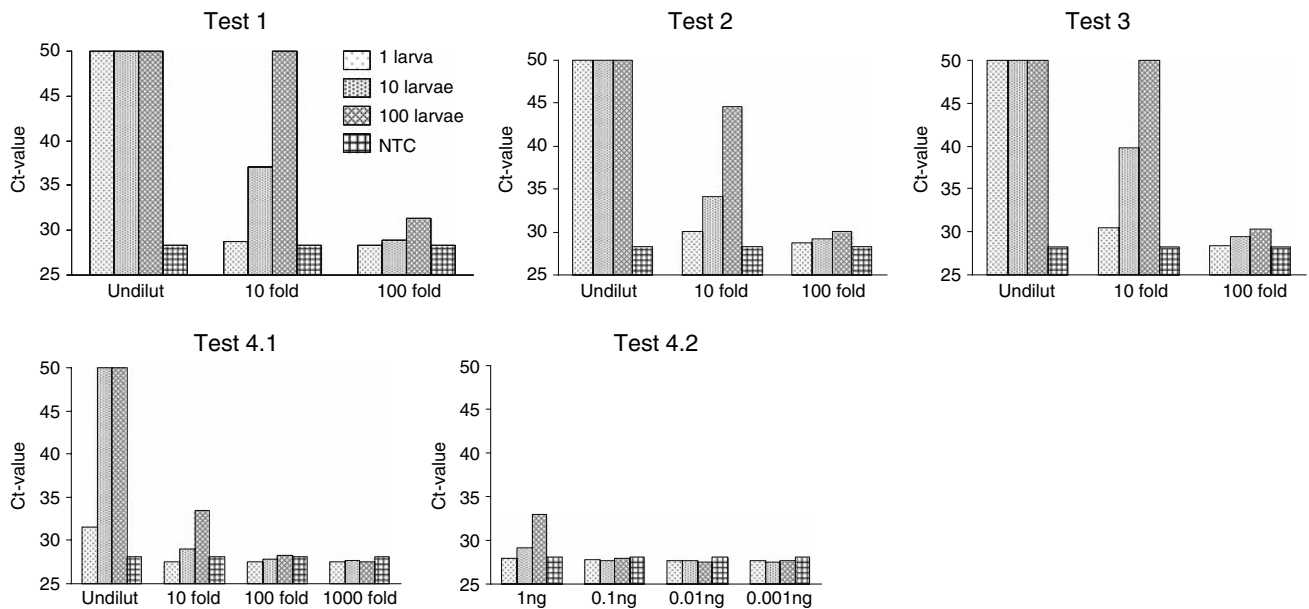


Fig. 2 Ct-values obtained for internal positive controls (IPCs) added to every sample. Tests performed on brachyuran negative samples; in tests 1 and 2 the lysate from 0, 1, 10 and 100 larvae was added to the

sample. In tests 3 and 4, whole larvae were added. In test 4.2 the exact amount of DNA added was previously measured

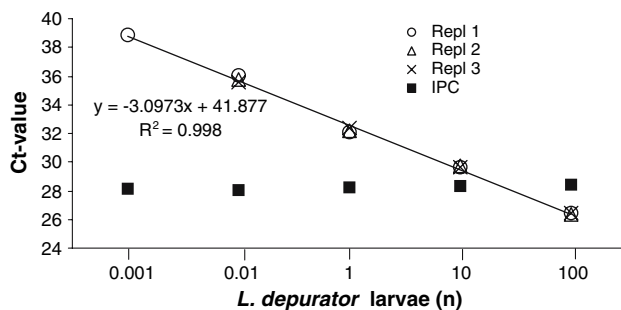


Fig. 3 Real-time PCR efficiency of the standard for *L. depurator* semi quantification, $E = 2.1$ (slope = -3.0973); IPC values are represented in the graph. Ct-values are plotted versus *L. depurator* larvae added

Although real-time PCR has been used recently for both identification and quantification of invertebrate larvae (larval abalone by Vadopalas et al. 2006 and sea lice by McBeath et al. 2006) to our knowledge, this is the first time that this technique has been applied to decapod crustacean larvae and the first time that it has been used to obtain data on relative abundance. The real-time PCR technique and the probe and primers developed were shown to be sensitive and specific for detection of *L. depurator*, and able to detect even one zoea I single larva in a mixed plankton sample. Although the probes and primers designed for *C. maenas*, *N. puber* and *C. pagurus* were not applied to plankton samples due to limitations in time and resources, we are confident that this methodology can be extrapolated to them and to other taxa.

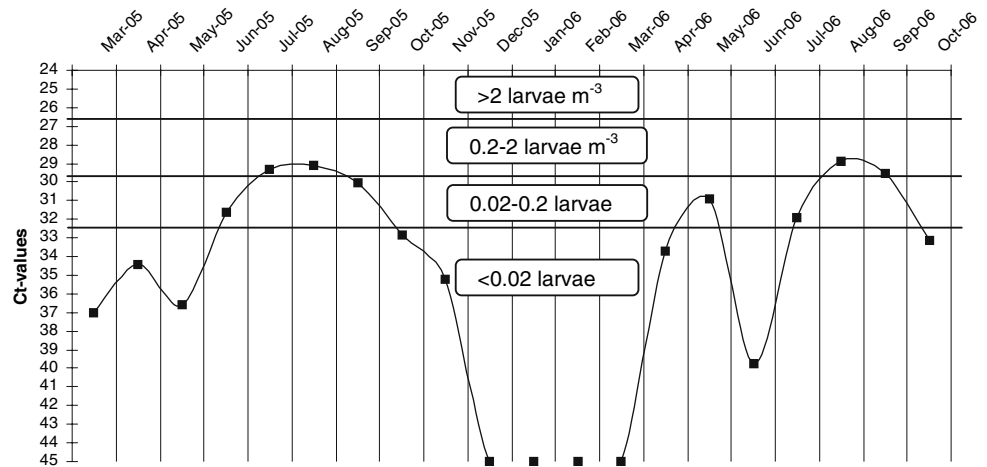
Other molecular methodologies previously developed for species identification have been shown to be accurate

Table 5 Ct-values obtained for the detection of *L. depurator* larvae in plankton samples and for internal positive controls (IPCs)

Month	<i>L. depurator</i>		IPC	
	Mean \pm SD	%CV	Mean \pm SD	%CV
March 2005	37.035 \pm 0.9	2.42	27.87 \pm 0.05	0.20
April 2005	34.46 \pm 0.1	0.29	27.91 \pm 0.12	0.43
May 2005	36.563 \pm 1.01	2.78	28.03 \pm 0.17	0.61
June 2005	31.65 \pm 0.065	0.21	27.88 \pm 0.16	0.56
July 2005	29.33 \pm 0.12	0.40	28.03 \pm 0.07	0.27
August 2005	29.08 \pm 0.09	0.33	27.92 \pm 0.07	0.24
September 2005	30 \pm 0.05	0.19	27.96 \pm 0.09	0.32
October 2005	32.85 \pm 0.34	1.04	27.91 \pm 0.03	0.10
November 2005	35.18 \pm 0.5	1.41	27.72 \pm 0.14	0.50
December 2005	Undetected	Undetected	27.75 \pm 0.04	0.15
January 2006	Undetected	Undetected	27.7 \pm 0.14	0.51
February 2006	Undetected	Undetected	27.79 \pm 0.21	0.76
March 2006	Undetected	Undetected	27.88 \pm 0.07	0.27
April 2006	33.7 \pm 0.36	1.07	27.81 \pm 0.03	0.12
May 2006	30.92 \pm 0.12	0.39	28.03 \pm 0.21	0.76
June 2006	39.72 \pm 2.17	5.46	27.87 \pm 0.29	1.05
July 2006	31.93 \pm 0.2	0.62	28.05 \pm 0.08	0.29
August 2006	28.88 \pm 0.06	0.22	28.02 \pm 0.09	0.32
September 2006	29.51 \pm 0.08	0.28	28.10 \pm 0.04	0.16
October 2006	33.15 \pm 0.38	1.15	28.31 \pm 0.07	2.49

and specific (e.g. Medeiros-Bergen et al. 1995; Bucklin et al. 1999; Lindeque et al. 1999; Ellison and Burton 2005). Medeiros-Bergen et al. (1995), by the use of oligonucleotide

Fig. 4 Relative quantification of *L. depurator* larvae present in the plankton samples analysed by real-time PCR, representing mean Ct-values of 2 replicate reactions per sample; when larvae were not detected, these are represented by a Ct-value of 45



probes, amplification by PCR and a subsequent detection by isotopic and non-isotopic assays, studied the spatio-temporal distribution of three otherwise indistinguishable species of holothurian larvae; Lindeque et al. (2004) analysed the distribution of four species of *Calanus* in the North Atlantic by Restriction Fragment Length Polymorphism (RFLP) analysis. In both cases, the larvae needed to be removed from plankton samples before application of the technique. Ellison and Burton (2005) studied the dynamics of eight phytoplankton species simultaneously by bead array technology, while in our case three species and one IPC could be multiplexed. However, as Vadopalas et al. (2006) noted, the specificity of bead array technique could be lower than that of real-time PCR. In our opinion, the complexity of some of these technologies compared with the assay described here makes them less suitable for use by non-molecular ecologists. Real-time PCR is easy to apply, quick (<2 h once the DNA has been extracted), does not require any post-PCR processing, does not require previous sorting of the samples, provides high levels of accuracy and specificity and has potential for application in ecological studies, as this work demonstrates with the application of the technique to a 1.5-year series of samples.

The high specificity of real-time PCR is provided by the use of a specific primer pair and a specific oligonucleotide probe situated between the primers. Theoretically, a single base difference between probe and target can prevent the probe annealing, thus good probe design is essential for specificity. This high specificity is also a potential weakness since the presence of the target species could go undetected if there is any difference between probe and target sequence. Unknown intraspecific genetic variation could cause false negatives. On the other hand, 100% specificity cannot be assured without comparisons with all related taxa and coexisting groups; otherwise the detection of false positives is possible. These possibilities were reduced in the present study by the sequencing of several specimens,

comparisons with related taxa and the selection of an appropriate area of the genome to design the specific probe. As part of the protocol and good practice to follow when applying these techniques, the sequencing of the COI gene from species closely related to the species of interest and specimens from different geographic regions, must continue to ensure the specificity of the probe designed. We did not capture any other species of *Liocarcinus* during this study and unfortunately, there are no sequences available in the genome databases, so this should be a priority for future work.

Real-time PCR is a relatively new technique and its use has mostly been concentrated in molecular biology. However, there are several studies, which have started to extend this technique to identification of marine invertebrate larvae (Vadopalas et al. 2006; McBeath et al. 2006) and fish eggs and larvae (Taylor et al. 2002; Watanabe et al. 2004; Fox et al. 2005). The methods developed by Watanabe et al. (2004) and Fox et al. (2005) included sorting of the samples prior to PCR, increasing the time employed in sample preparation, which is not necessary in the method described here. Vadopalas et al. (2006) for abalone larvae (*Haliotis kamtschatkana*) and McBeath et al. (2006) for two species of sea lice (*Lepeophtheirus salmonis* and *Caligus elongatus*), did not use previous sorting of the plankton samples. These studies explored the possibility of obtaining relative quantification of the species of interest and verified the high correlation between the number of larvae predicted to be present by the technique and the number of larvae actually present in environmental samples, previously spiked by a known number of larvae or previously analysed by microscopy.

Initial tests applied in order to develop the technique and investigate the appropriate manner of dealing with the samples required the use of numerous plankton samples and *L. depurator* larvae. During these initial tests, winter samples, selected because the possibility of finding

L. depurator is lower at this time of the year, were used to check the specificity of the technique. However, *L. depurator* larvae can be found in the water column throughout the year (Clark 1984; Martin 2000) making it difficult to obtain a plankton sample without its presence. Consequently, due to the impossibility of predicting whether samples were naturally negative and the limited number of *L. depurator* larvae available, we could not compare spiked samples with the value obtained by real-time PCR although this possibility will be explored in the future. Nevertheless the results suggested by the studies of Vadopalas et al. (2006) and McBeath et al. (2006), our initial tests with spiked samples, and the high efficiency obtained in the standard curve used for relative quantification ($E = 2.1$) in this study, provide a good indication of its reliability. In addition, the Ct-values obtained from brachyuran negative plankton samples, subsampled into four after lysis and then spiked with 0, 1, 10 and 100 larvae, were very similar to those obtained for the standard curve used for relative quantification. This provided more evidence of the reliability of the standard curve created, but because the methodology employed was different, detailed data has not been shown. The alternative possibility of prior microscope analysis of the samples could not be applied in this case since the unequivocal identification of *L. depurator* larvae by morphological characters is not possible (Clark 1984; Kim and Hong 1999), and although we were not able to get any other *Liocarcinus* species from the study area, their presence is expected.

The preparation of “artificial” plankton samples was intended to solve the lack of naturally negative plankton samples for *L. depurator*, although their use was restricted to the initial stages of the study in order to prove the capability of the technique to detect one single larva in a mixed plankton sample. We avoided the use of artificial plankton samples in subsequent analysis since they do not adequately represent the complexity of a real plankton sample, which could have led to an overestimation of the applicability of the technique in field studies.

Subsequent tests were performed on plankton samples that had been previously checked via light microscope and from which all brachyurans had been removed to ensure negative samples. Nevertheless, in some cases, positive results for *L. depurator* were obtained. The Ct-values were relatively high indicating a low amount of target DNA in the samples. This could be caused by the presence of a single larva or remnants of larvae such as appendages or moulted exuvia that were not removed, or possibly contamination events when dealing with the samples. The high specificity of this technique makes it vulnerable to contamination errors that can lead to inaccurate results, so great care must be taken during the whole process and appropriate control reactions are required.

Another possible source of bias is the presence of inhibitory components, originally present in the samples or created through the DNA extraction and purification processes. These inhibitors, such as heparin, humic acid, urea, EDTA, hemoglobin or high DNA concentrations, can cause false negative results or changes in the Ct-values, generating inaccurate results. This problem is extremely important when relative quantification is being assessed. The degree of inhibition was assessed by the use of an internal positive control (IPC) added to all reactions, and no-template controls (NTC). The background signal obtained from the NTCs is considered as the negative control for the IPCs. Differences between the Ct-value obtained for the IPC added to the sample and the average Ct-value from the NTCs indicate inhibition. During the tests carried out on plankton samples we observed significant inhibition, which decreased when dilutions were increased. High DNA concentrations, either from total genomic DNA or from target template are the likely reason for that inhibition. The concentration of the starting template is extremely important for the efficiency of the PCR reaction and our findings suggest that 0.1 ng of DNA is effective in order to avoid inhibition while maintaining the high efficiency of this technique.

Vadopalas et al. (2006) and McBeath et al. (2006) demonstrated the possibility of obtaining relative quantification of the species of interest using real-time PCR. In our study, 20 monthly plankton samples from March 2005 to October 2006 were analysed by real-time PCR, obtaining a pattern of relative abundances for *L. depurator* larvae in a coastal location in the North Sea. The comparison of Ct-values obtained from plankton samples with those obtained from a standard spiked with known numbers of larvae (Table 4) enabled the estimation of larval abundances in the plankton samples. The seasonal cycle of larval abundance inferred was consistent with the annual cycle of zooplankton, characterised by an abundance peak in the late spring, extending into the summer and followed by a secondary peak in the autumn (Valdes and Moral 1998; Siokou-Frangou 1996). Phytoplankton concentration and water temperature are key factors in the occurrence of zooplankton (Raymont 1963). The relatively high phytoplankton biomass in spring, resulting from increasing water temperature and light, is reduced during the summer due to nutrient depletion and grazing by herbivores. At the end of the summer, when nutrients become available again, there is generally a second peak in primary and secondary production (Bot et al. 1996). Temperature also has a major influence on larval development and low temperatures may affect larval survival or condition, adult reproduction and embryonic development (Anger 2001). Therefore, changes in temperature and phytoplankton have a strong influence on larval productivity and the timing and occurrence of decapod

larvae. Those variables, among other biotic and abiotic factors could be the reason for the differences observed between 2005 and 2006.

Ideally, the data obtained by this assay should be compared with microscopy analysis of the samples. However, *L. depurator* larvae cannot be identified by classical methods. The congruent data obtained on seasonal changes and abundances and the positive results found by Vadopalas et al. (2006) and McBeath et al. (2006) when comparing results from morphology and molecular methods, support the consistency of these results.

Consideration should however, be given to a possible bias introduced in the standard, since only larvae of zoea I, obtained by rearing, were used in the spiking experiments in this study. *L. depurator* larvae pass through five zoeal stages and a megalopa stage before metamorphosis into juveniles. A useful and commonly taken measurement in zoeal stages is the distance between the tip of the dorsal and rostral spines (T.T.) which in *L. depurator* larvae increases from 1.52 mm in zoea I to 2.80 mm in zoea V (Ingle 1992). This clearly reflects the increase in biomass through larval development, accompanied by the consequent increase in cell number. It could even be supposed that, since COI is involved in the mitochondrial respiratory chain, the number of copies of COI might vary even within an instar, e.g. increasing closer to moulting. The influence of the larval stage on the Ct-value obtained has not been investigated during this study since only zoea I were available, but this issue should be subject to further study to improve the accuracy of the assay. However, even if the estimations of abundances are considered to be potentially inaccurate, real-time PCR can provide useful information about peaks of abundances and relative differences among samples. When settlement and recruitment to adult populations is being studied, identification and quantification of the different larval stages is highly important. In those cases, pre sorting of the samples could be carried out similar to that reported by Fox et al. 2005 when working with fish eggs, selecting all brachyurans from the samples, separating them by their different larval stages and then applying the technique. Obviously, the time employed in sample preparation will considerably increase and it will only be practical for those cases when the identification of a species cannot be done by taxonomic methods.

Recent publications and the work presented here show a new trend in the use of molecular techniques, especially real-time PCR, and the possibilities for its application to field studies. These methods can complement the classical methods of plankton taxonomy. Nevertheless, the cost of the technique and its requirements will limit its use to problematic species. In the case of its applicability for abundance estimation, the methodology must be improved

but the potential for this type of work has been demonstrated.

Acknowledgments The authors would like to thank J. Dunn and *Temora* crew (R. Cargill, M. Leys, A. MacDonald, P. MacDonald, T. Morrice, J. Smith) for their efforts and invaluable help in collecting plankton samples and supplying ovigerous females.

References

- Anger K (2001) The biology of decapod crustacean larvae. crustacean issues, vol 14. A.A. Balkema, The Netherlands
- Bot PVM, van Raaphorst W, Batten S, Laane RWPM, Philippart K, Radach G, Frohse A, Schultz H, van den Eynde D, Colijn F (1996) Comparison of changes in the annual variability of the seasonal cycles of chlorophyll, nutrients and zooplankton at eight locations on the north-west european continental shelf. *German J Hydrogr* 48:349–364
- Bromley PJ, Watson T, Hislop JRG (1997) Diel feeding patterns and the development of food webs in pelagic 0-group cod (*Gadus morhua* L.), haddock (*Melanogrammus aeglefinus*, L.), whiting (*Merlangius merlangus* L.), saithe (*Pollachius virens* L.), and Norway pout (*Trisopterus esmaki* Nilson) in the northern North Sea. *ICES J Mar Sci* 54:846–853
- Bucklin A, Guarnieri M, Hill R, Bentley A, Kaartvedt S (1999) Taxonomic and systematic assessment of planktonic copepods using mitochondrial COI sequence variation and competitive species-specific PCR. *Hydrobiologia* 401:239–254
- Clark PF (1984) A comparative study of zoeal morphology in the genus *Liocarcinus* (Crustacea: Brachyura: Portunidae). *Zool J Linn Soc-Lond* 82:273–290
- Deagle BE, Bax N, Hewitt CL, Patil JG (2003) Development and evaluation of a PCR-based test for detection of *Asterias* (Echinodermata: Asteroidea) larvae in Australian plankton samples from ballast water. *Mar Freshw Res* 54:709–719
- d'Udekem d'Acoz C (1999) Inventaire et distribution des crustacés décapodes de l'Atlantique nord-oriental, de la Méditerranée et des eaux continentales adjacentes au nord de 25°N. *Patrimoines naturels (M.N.H.N./S.P.N.)*, 40:383 p
- Eimanifar A, Rezvani S, Carapetian J (2006) Application of RFLP analysis to identify cyst populations of *Artemia urmiana* Günther, 1899 (Branchiopoda, Anostraca) from Urmia Lake, Iran. *Crustaceana* 78:1311–1323
- Ellison CK, Burton RS (2005) Application of bead array technology to community dynamics of marine phytoplankton. *Mar Ecol Prog Ser* 288:75–85
- Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* 3:294–299
- Fox CJ, Taylor MI, Pereyra R, Villasana MI, Rico C (2005) TaqMan DNA technology confirms likely overestimation of cod (*Gadus morhua* L.) egg abundance in the Irish Sea: implications for the assessment of the cod stock and mapping of spawning areas using egg-based methods. *Mol Ecol* 14:879–884
- Gimenez L (2006) Phenotypic links in complex life cycles: conclusions from studies with decapod crustaceans. *Integr Comp Biol* 46:615–622
- Hare MP, Palumbi SR, Butman CA (2000) Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. *Mar Biol* 137:953–961
- Hill RS, Allen LD, Bucklin A (2001) Multiplexed species-specific PCR protocol to discriminate four N. Atlantic *Calanus* species,

- with an mtCOI gene tree for ten *Calanus* species. Mar Biol 139:279–287
- Hebert PDN, Ratnasingham S, deWaard JR (2003) Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc R Soc Lond B (Suppl) 270:S96–S99
- Hughes G, Beaumont AR (2004) A potential method for discriminating between tissue from the European Lobster (*Homarus gammarus*) and the American Lobster (*H. americanus*). Crustaceana 77:371–376
- Ingle R (1992) Larval stages of northeastern Atlantic crabs. Chapman & Hall, Natural History Museum publications, London
- Kim KB, Hong SY (1999) Larval development of the wrinkled swimming crab *Liocarcinus corrugatus* (Decapoda: Brachyura: Portunidae) reared in the laboratory. J Crust Biol 19:792–808
- Lindeque PK, Harris RP, Jones MB, Smerdon GR (1999) Simple molecular method to distinguish the identity of *Calanus* species (Copepoda: Calanoida) at any developmental stage. Mar Biol 133:91–96
- Lindeque PK, Harris RP, Jones MB, Smerdon GR (2004) Distribution of *Calanus* spp. as determined using a genetic identification system. Sci Mar 68:121–128
- Lindeque PK, Hay SJ, Heath MR, Ingvarsdottir A, Rasmussen J, Smerdon GR, Waniek JJ (2006) Integrating conventional microscopy and molecular analysis to analyse the abundance and distribution of four *Calanus* congeners in the North Atlantic. J Plank Res 28:221–238
- Martin J (2000) Les larves de crustacés décapodes des côtes françaises de la Manche. Identification, période, abondance. Ifremer, Plouzané, France
- McBeath AJA, Penston MJ, Snow M, Cook PF, Bricknell IR, Cunningham CO (2006) Development and application of real-time PCR for specific detection of *Lepeophtheirus salmonis* and *Caligus elongatus* larvae in Scottish plankton samples. Dis Aquat Organ 73:141–150
- Medeiros-Bergen DE, Olson RR, Conroy JA, Kocher TD (1995) Distribution of holothurian larvae determined with species-specific genetic probes. Limnol Oceanogr 40:1225–1235
- Øines O, Heuch PA (2005) Identification of sea louse species of the genus *Caligus* using mtDNA. J Mar Biol Ass UK 85:73–79
- Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:2002–2007
- Raymont JEG (1963) Plankton and productivity in the oceans. 2nd edn., vol 2. Pergamon, Zooplankton
- Siokou-Frangou I (1996) Zooplankton annual cycle in a Mediterranean coastal area. J Plank Res 18:203–223
- Taylor MI, Fox C, Rico I, Rico C (2002) Species-specific TaqMan probes for simultaneous identification of cod (*Gadus morhua* L.), haddock (*Melanogrammus aeglefinus* L.) and whiting (*Merlangius merlangus* L.). Mol Ecol Notes 2:599–601
- Vadopalas B, Bouma JV, Jackels CR, Friedman CS (2006) Application of real-time PCR for simultaneous identification and quantification of larval abalone. J Exp Mar Biol Ecol 334:219–228
- Valdes L, Moral M (1998) Time-series analysis of copepod diversity and species richness in the southern Bay of Biscay off Santander, Spain, in relation to environmental conditions. ICES J Mar Sci 55:783–792
- van Guelpen L, Markle DF, Duggan DJ (1982) An evaluation of accuracy, precision, and speed of several zooplankton subsampling techniques. ICES J Mar Sci 40:226–236
- Wang S, Bao Z, Zhang L, Li N, Zhan A, Guo W, Wang X, Hu J (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 DH-PLC. J Plank Res 28:375–384
- Watanabe S, Minegishi Y, Yoshinaga T, Aoyama J, Tsukamoto K (2004) A quick method for species identification of Japanese Eel (*Anguilla japonica*) using Real-Time PCR: an onboard application for use during sampling surveys. Mar Biotechnol 6:566–574