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A TaqMan real-time PCR based assay targeting plaice (*Pleuronectes platessa* L.) DNA to detect predation by the brown shrimp (*Crangon crangon* L.) and the shore crab (*Carcinus maenas* L.) - Assay development and validation.

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Abstract

We describe a protocol for the preservation, extraction, and detection of plaice (*Pleuronectes platessa*) DNA from the stomach contents of the brown shrimp, *Crangon crangon* and the shore crab, *Carcinus maenas*. These two predatory species are thought to be important sources of mortality of small juvenile plaice on inshore nursery grounds. Previous studies of predation on juvenile plaice have used visual examination of stomach contents but this is time-consuming and may under-estimate true predation levels as remains may become un-identifiable due to maceration and digestion. Molecular based tools for detecting the presence of prey tissue in predator stomachs and scat are becoming increasingly used in marine ecology and provide an alternative or complementary approach to visual identification. We sequenced a part of the cytochrome-*b* region of plaice mitochondrial DNA and designed a species-specific, TaqMan real-time Polymerase Chain Reaction (PCR) based assay which was successfully tested for intra- and inter-species specificity. For application to predator stomach contents, two tissue preservation and two DNA extraction methods were tested followed by a set of aquarium experiments to determine the effect of digestion time on detectability. The quality of the extracted DNA was comparable for the two preservation and two extraction methods tested and the detectability remained similar for all of them. However, levels of PCR inhibition were significant for samples from both predators but could be overcome using serial dilution and 1.25 µg/µl Bovine Serum Albumin to reduce the incidence of false-negatives. Successful amplification and detection of plaice DNA from stomach contents was possible up to 24 h after ingestion for both predator species. For extracts of *C. crangon* stomachs the half-life detection rate (T50) was ~10 h at water temperatures of 14-16^{o}C. The effects of a wider range of temperatures were tested for stomach contents of *C. maenas* where the T50s were ~7 h at 6-10^{o}C and ~ 6 h at 14-16^{o}C but only 2 h at 19-20^{o}C. Our results indicate that the

TaqMan method is applicable in field studies providing species specific T50s, PCR inhibition and water temperatures are taken into account.

INTRODUCTION

For marine fish, year-class strength can vary considerably among years with important implications for conservation and fisheries management. Although it is generally accepted that much of this variation is generated during the early life stages, the controls on mortality are poorly understood. Predation is likely to be the main cause of mortality with other factors such as starvation, transport away from nursery grounds, disease and pollutants also being important in certain cases (e.g. Bailey and Houde, 1989; Bailey, 1994; Bax, 1998; Bunn et al., 2000). Studying predation in the field has proven challenging since accurate identification of partially digested fish remains in predator stomachs is difficult (Bailey and Houde, 1989; Heath, 1992). In this sense, Hunter and Kimbrell (1980) reported that although anchovy (*Engraulis mordax*) egg chorions remained identifiable for up to 8 h in anchovy stomachs, ingested larvae were unidentifiable after only 30 min; moreover, Folkvord (1993) reported that three-day old cod (*Gadus morhua*) larvae could only be identified in the stomachs of cannibalistic juvenile cod for 15-90 min post ingestion. Schooley et al. (2008) reported similarly rapid digestion times for larvae of native fish species ingested by non-native fish predators (only 50% of them being identifiable after 30 min post consumption, reducing to 3% after 60 min). Many other predators, particularly crustacea macerate their prey which can make it nearly impossible to identify the stomach contents (e.g. Garrod and Harding, 1981; Taylor, 2004), and previous laboratory and field studies have suggested that both *C. crangon* and *C. maenas* are important predators on recently settled juvenile plaice (*Pleuronectes platessa*) (e.g. van der Veer and Bergman, 1987; Pihl, 1990; Burrows et al., 2001). In some studies, identification of remaining hard-parts such as otoliths and eye lenses has been used to

assess predation (van der Veer and Bergman, 1987; Wennhage and Pihl, 2001; Nakaya et al., 2004); however, identifying these remains is time consuming and requires either skilled analysts or evidence that the target prey are the only species in the study area. For these reasons many studies of crustacean predation have restricted stomach content identification to broad taxonomic categories such as roundfishes and flatfishes (Pihl and Rosenberg, 1984; Pihl, 1985; del Norte-Campos and Temming, 1994; Ansell et al., 1999; Oh et al., 2001; Chen et al., 2004). A final problem with visually identifying prey is that prey may be abandoned after being only partially consumed - as noted for *C. crangon* predating on juvenile plaice (van der Veer and Bergman, 1987; Gibson et al., 1995). In these instances, the head and otoliths may not be ingested, again leading to an underestimate of the overall feeding incidence if using visual stomach analysis.

Molecular methods now offer an alternative of rapid and unambiguous identification of species present in gut contents (Symondson, 2002; King et al., 2008). Some studies have utilised polyclonal or monoclonal antibodies (Greenstone and Hunt, 1993; Symondson and Liddell, 1995; Hagler and Naranjo, 1997; van der Veer et al. 1998; Taylor, 2004), but DNA-based methods are now more widely applied being not only easier and cheaper to develop but allowing rapid screening against a multitude of different prey likely to be encountered in the field (Agustí et al., 2003; Harper et al., 2005; de León et al., 2006; Juen and Traugott, 2007). These methods have been most widely applied in terrestrial systems but some notable examples from marine systems include fishes (Rosel and Kocher, 2002; Smith et al., 2005), copepods (Nejstgaard et al., 2003) and appendicularians (Troedsson et al., 2007). Rapid progress has also been made in detection methods. Asahida et al. (1997) developed a conventional PCR based method with detection of stone flounder (*Kareius bicoloratus*) by agarose gel electrophoresis and Saitoh et al. (2003) applied a similar method to detect Japanese flounder (*Paralichthys olivaceus*). More recently real-time PCR has also been used in predation studies and is faster, more sensitive and offers improved specificity over

conventional PCR approaches (e.g. McBeath et al., 2006). The application of DNA probes takes advantage of the fact that conserved sequences of nucleotides unique to a species are present in mitochondrial or nuclear DNA enabling highly specific and sensitive assays to be developed. Real-time PCR (also known as quantitative real time PCR) has recently been successfully applied to identify visually indistinguishable early stage gadoid eggs to species (Taylor et al., 2002; Fox et al., 2005 and 2008), to identify and estimate the abundance of toxic algae (Galluzzi et al., 2004) and larvae of fish parasites (McBeath et al., 2006) in plankton samples and for the identification and quantification of algae in copepod stomachs (Durbin et al., 2008; Nejstgaard et al., 2008). This assay can also be extended to several target species using different fluorescent dyes (Taylor et al., 2002). Before use in a field study the specificity of molecular probe(s) should be checked against a range of potential targets. In addition, DNA based methods cannot distinguish between different life-stages of the target e.g. separating eggs from larvae. In some cases this can be achieved using antibody-based methods specific to yolk proteins (Taylor, 2004).

To date it is difficult to obtain quantitative estimates of prey consumption using molecular based methods and results usually indicate only a presence or absence of the prey. Although estimation of the amount of target DNA present may be possible using real-time (quantitative) PCR, signal strength will be affected by the length of post-capture digestion. DNA fragment length analysis may allow estimation of digestion times but requires careful laboratory calibration as digestion rates will be affected by temperature as well as the amount and type of prey ingested, mixed diets and predator size (Deagle et al. 2006; Troedsson et al., 2009). Such developments are beyond the scope of the present study where we were concerned with developing a presence/absence detection method suitable for further testing predictions from a size-selective predation model for juvenile plaice in the field (Burrows et al., 2001).

A real-time PCR based assay using a TaqMan probe (Holland et al., 1991; Lie and Petropoulos, 1998) targeting the cytochrome-*b* gene of plaice (*Pleuronectes platessa*) was developed and its specificity examined against a wide range of potential targets. We then tested the ability of the probe to detect plaice DNA in stomach contents of *Crangon crangon* and *Carcinus maenas* and investigated the time-course of detectability in the stomach contents of these potential predators.

MATERIALS AND METHODS

Plaice Detection Assay development

TaqMan assay is a hydrolysis probe (species-specific) based method that using the real-time PCR technique allows species-specific DNA identification and quantification by computing emission of fluorescence along the different cycles of the PCR. The TaqMan technique measures accumulation of a product via the emission of fluorescence during the exponential stage of the PCR (40 PCR cycles);briefly, the species-specific TaqMan probe attaches to the complementary target sequence during PCR, this TaqMan probe carries a fluorescent dye (reporter) along with an inhibitor of the emission of this fluorescence (quencher); during each PCR replication cycle the fluorescent dye and the inhibitor are separated due to the 5' → 3' exonuclease activity of the Taq polymerase (Holland et al., 1991; Lie and Petropoulos, 1998) allowing the emission of fluorescence that is quantified by a detector. Fluorescence intensities in the 40 PCR cycles are used to create amplification plots of fluorescence (Rn) versus cycle number. This allows us to calculate the Ct (threshold cycle), the number of PCR cycles at which a significant exponential increase in fluorescence (measured as ΔRn) is detected, which is directly correlated with the number of copies of target DNA present in the reaction,

giving us the quantification of target DNA present in predator's stomach. A lower Ct value means higher amount of target DNA in the stomach (Figure 1).

Tissue samples from adult plaice (*Pleuronectes platessa*), dab (*Limanda limanda*) and flounder (*Platichthys flesus*) were obtained from locations covering close to full the geographical range of these species (Table 1). DNA was extracted using a modified salt extraction protocol (Aljanabi and Martinez, 1997) from 77 individuals from 4 geographic regions for plaice, 36 individuals from 8 localities for dab and 24 individuals from 5 localities for flounder. 400 bp of the mitochondrial cytochrome-*b* (Cyt-*b*) gene was then PCR amplified using the universal mitochondrial primers GLU-(L)-TGACTTGAAGAACCAYCGTTG-3' (Palumbi, 1996) and CB2-(H)-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3' (Kocher et al., 1989), before PCR purification using exonuclease I (New England Biolabs) and shrimp-alkaline phosphotase (Invitrogen) and direct sequencing of the PCR product using BIGDYE version 3.1 chemistries on an ABI 3730xl genetic analyser (Applied Biosystems).

The sequences from plaice, dab and flounder along with other flatfish species retrieved from GenBank (Table 1) were then aligned using Bioedit (Hall, 1999). Primers, PLA-F and PLA-R (Table 2), were designed to amplify a 72 bp region of the Cyt-*b* gene using the Primer Express 3.0 software package (Applied Biosystems). A TaqMan MGB (minor groove binding) probe was designed for a target region that encompassed three variable sites between plaice, dab and flounder. The 5' end of probe was labelled with fluorescent dye VIC (Applied Biosystems), and the 3' end labelled with a non-fluorescent quencher (Table 2).

The TaqMan assay was then tested against a panel of adult tissue from plaice, dab and flounder (Table 1) as well as a panel of DNA extracts from a taxonomically wide range of species (Table 3). TaqMan assays were run on an Applied Biosystems 7900 real-time

sequence detection system. Twenty-five µl volume reactions were run in Optical 96-well reaction plates using Optical Adhesive Covers (Applied Biosystems). Each reaction contained 200 nM of plaice probe, 300 nM of the PLA-F and PLA-R primers, 12.5 µl of TaqMan Universal PCR Master Mix (NO UNG + ROX passive reference) (Applied Biosystems) and ~50 ng of DNA, and 4.9 µl tissue culture H_2O (Sigma). Plates were run under real-time conditions on a single dye layer with eight no template controls (NTCs) per 96- well plate. The assay was run using 40 PCR cycles using the default cycling conditions: after a first stage of 50ºC for 2 minutes followed by a 10 minutes one at 95ºC, the run comprised 40 cycles of 15 seconds at 95ºC followed by 60 seconds at 60ºC.

Post-PCR, the results were analysed using the Sequence Detection Software version 2.3 (Applied Biosystems). The ΔRn values for each cycle and dye layer were then exported to MS Excel and processed further manually. First, the mean and standard deviation of the endpoint (PCR cycle 40) ΔRn values of the NTCs were calculated for each dye layer. z*M-values [z*M = M + (3.89 x SD)] were then calculated where M = mean of the NTC ΔRn, SD is the standard deviation of the NTC ΔRn and 3.89 is the one tailed Z -value for the 99.999% confidence interval. Samples which had ΔRn values larger than the value of z*M from the above equation were deemed to have a fluorescence significantly greater than the NTCs, and were considered to be positive reactions (Taylor et al. 2002).

Assay specificity and reproducibility

Designed primers and probe were tested against plaice samples from their full geographic range, and also against dab and flounder from Table 1, and the real-time PCR based TaqMan assay tested negative against all dab and flounder DNA, whereas it was positive in all plaice tests. No positive reactions were found when using the plaice probe against a panel consisting

of a taxonomically wide range of samples (Table 3) confirming the specificity of the plaice probe and its utility for the analysis of stomachs.

Aquarium feeding experiments

C. crangon and *C. maenas* were collected from Red Wharf Bay (Anglesey, United Kingdom) in spring 2008 and acclimatised to aquarium conditions in separate 30 l, circulating seawater aquaria under a natural light cycle. *C. crangon* were fed on ground mussel (*Mytilus edulis*) and *C. maenas* on whiting (*Merlangius merlangus*) tissue for a minimum of 2 weeks after collection. They were then starved for 48h before feeding trials commenced (Asahida et al., 1997). Experimental animals were fed adult plaice tissue *ad libitum*. After 2 hours, approximately 10 individuals were preserved (end of the feeding period; t = 0 h) and the remaining animals were transferred into aquaria with clean water and no plaice tissue. Around 10 individuals were sampled at six-eight time points between 0 and 24h after the end of feeding; animals were preserved either frozen (-80ºC) or in 50 ml of 80% ethanol [preservation in 100% ethanol leads to tissue brittleness making subsequent stomach dissection difficult (Passmore et al., 2006)]. The 80% ethanol was changed twice within 24 h and samples were subsequently stored at -20^{o}C. Digestion trials were run at several temperatures for *C. maenas* but only one temperature for *C. crangon* (Table 4).

Detection of plaice DNA in stomachs

C. maenas stomach contents, including both cardiac and pyloric caecae, were dissected under a stereomicroscope. For *C. crangon*, the whole stomach was dissected due to the relatively small sizes of the animals. Sizes (cephalothorax length for *C. crangon* and cephalothorax width for *C. maenas*) were recorded for individual animals along with a stomach fullness

index (an index of 1 indicated stomachs with less than 1/10 of the volume filled; 2 indicated between 1/10 and 2/3 of the volume filled and 3 indicated more than 2/3 of the stomach volume filled). For *C. maenas*, animals without any visible solid remains in the stomach were indexed as 0. Dissecting tools were flame sterilized after each dissection.

Following dissection, whole stomachs (*C. crangon*) or stomach contents (*C. maenas*) were partially homogenized in 1.5 ml autoclaved Eppendorf tubes in 1 ml of extraction buffer (30mM Tris, 10mM EDTA and 1% SDS) and left overnight at 55°C to digest after addition of 10 µl Proteinase K (203% activity in extraction buffer; Qiagen). DNA was purified from 450 µl digest aliquots following modified salt (Aljanabi and Martinez 1997) or phenol-chloroform (Sambrook et al. 1989) protocols. In several experiments duplicate aliquots were purified using both salt and phenol-chloroform methods to compare extraction efficiency (Table 4). An extraction blank control (negative control) was added every 5 samples to detect cross-contamination. *Salt extraction method*: 150µl 5M NaCl was added to a 450 µl aliquot of the digested DNA aliquot and briefly vortexed. After centrifuging for 5 min at 13000rpm, 200 µl of the supernatant was collected in new 1.5ml eppendorf tube, 2 volumes of 100% ice cold ethanol added before leaving for 1h at -80ºC. After 30 min centrifugation at 13000rpm, the liquid phase was removed and 1ml of 70% ethanol was added to wash the remaining pellet. After 10 min centrifugation at 13000rpm the ethanol was poured off, the DNA pellet was then dried at 37^{o}C, then resuspend in 100µl ultrapure H_2O and stored at -20ºC. *Phenol-chloroform method*: 450µl equilibrated phenol was added to a 450µl aliquot of the digested DNA, and a standard phenol chloroform DNA extraction protocol protocol followed before resuspension of the DNA in 100µl ultrapure H_2O and storage at -20ºC.

TaqMan assay

Taqman assays were performed as for the probe specificity experiments with some minor modifications; 2.5 µl of the DNA extracted from the stomachs´ contents were added to each reaction (20 µl total volume). Plates were run under real-time conditions (40 PCR cycles) with seven non template controls (negative control) and two positive controls per 96-well plate, and a positive ΔRn threshold of 0.02 was set for the assay.

Experiment 1 – Effect of preservation and extraction method on DNA yield and purity

DNA yield (ng/µl) and purity indexes (determined from the absorbance (A) at different wave lengths (nm); A260/A280 and A260/A230 ratios) for the extractions were determined using a NanoDrop ND-1000 Spectrophotometer and, compared among both preservation (rapid freezing -80ºC or 80% ethanol) and extraction methods (*Salt* and *Phenol-chloroform* methods) where both salt and phenol-chloroform extractions were applied to the same stomach's contents (see Table 4). While the yield detection limit is set below 2 ng μl^{-1}, a ratio >1.8 for both sample absorbance indexes is associated with pure DNA (NanoDrop ND-1000 V3.5 User's Manual); because of this, when comparing NanoDrop data among salt and phenol-chloroform extractions, only samples with yields >2ng μl^{-1} were compared.

Experiment 2 – Investigation of PCR inhibition

Previous studies have identified PCR inhibitors in crab tissue extracts (Pan et al., 2008) which can lead to a high proportion of false-negatives. In order to determine the extent of inhibition we performed a series of 10-fold dilutions, from 1/10 to 1/100 for *C. crangon* and to 1/1000 for *C. maenas*, to the extracted DNA from those experiments (dilutions test; see Table 4). Only animals from 4 different digestion times, all of them preserved in 80% ethanol were used for the dilution test (Table 4).

Experiment 3 – Determination of minimum quantity of tissue (detection limit)

Four standard dilutions were undertaken (for each preservation and DNA extraction method) to test the efficiency of the TaqMan assay for detecting very low quantities of plaice DNA. Plaice DNA was extracted from raw adult tissue (where PCR inhibition is not a problem), the concentration was determined using a NanoDrop ND-1000 Spectrophotometer, and then serially diluted in 10-fold increments (until 1/10000) before applying the Taqman assay.

Experiment 4 – Determination of detectability over digestion time

Experimental data were analysed to determine the length of time that plaice DNA could be detected in predator stomachs. In order to determine the detectability of the DNA of plaice remains (the decline in DNA detection success) we calculated the half-life detection rate (T50) which is the time after which only half of the individuals test positive for prey DNA (Greenstone and Hunt, 1993). Based on PCR inhibition results, for all samples testing negative for plaice, we repeated the assay using a 1/10 dilution of the DNA extract for *C. crangon* while both 1/10 and 1/100 dilutions were assayed for *C. maenas*.

Experiment 5 – Validation of the assay with selected field samples

C. crangon were collected using a 1.5 m beam-trawl net in Tralee Beach (Ardmucknish Bay, West Scotland; 56" 29' N, 5" 25' W) in spring 2009, and preserved in 80% ethanol. Stomachs of predators in which juvenile flatfish remains could be identified visually were selected for a preliminary field validation of the assay. Briefly, an incision was made in field collected stomachs and conspicuous contents were identified to general categories when possible before

the DNA extraction process; DNA was extracted from five stomachs following the above commented salt protocol.

As in a first stage, using the same PCR protocol of the experimental animals, only one positive signal appeared (and with weak signal, Ct value close to 40) and, taking into account that plaice was the most abundant flatfish in the field (unpublished data), we tested the addition of 1.25 µg/µl Bovine Serum Albumin (BSA) (#B9001S New England Biolabs) to the PCR reaction of field samples as extra inhibition in the field could explain the results and the application of similar concentrations of BSA had proven to be effective reducing inhibition when applied to DNA extracts from stomachs of soil living insects (Juen and Traugott, 2006).

RESULTS

Assay specificity and reproducibility

The designed real-time PCR based TaqMan assay amplified 100% of plaice samples from different locations (Table 1) but did not react to any of the other species tested (Table 3) demonstrating its specificity to plaice DNA.

For all experiments and assays (Table 4), no negative controls (either extraction blanks, 1 every 5 samples, and PCR no template controls, 6 in each 96-well Optical reaction plate) tested positive, whilst all positive controls added to the PCR plate (2 in each 96-well Optical reaction plate; based on raw plaice tissue testing positive in previous runs) tested positive. Reproducibility among runs was high, with 24 repeated analyses of the same (positive control) sample yielding and average Ct of 21 and a standard deviation of 0.3.

Experiment 1 – Effect of preservation and extraction method on DNA yield and purity

A comparison of salt and phenol-chloroform extraction techniques for *C. crangon* and *C. maenas* revealed that DNA yields were significantly higher using phenol-chloroform extraction compared with salt (Figure 2). Furthermore DNA quality, as determined using the A260/A280 and A260/A230 absorbance indexes, was high for both preservation and extraction methods for *C. crangon* (>1.8 in all cases) but lower in *C. maenas* extracts. No apparent differences in these patterns were noted if considering all the different digestion times together or separately

Experiment 2 – Investigation of PCR inhibition

Results dealing with the inhibition effects on the TaqMan assay were limited to 80% ethanol preserved animals using the digestion times where both extraction methods were applied (dilutions test; see Table 4). In total, 38 *C. crangon* (times 0, 1, 6 and 18h; 14-16 °C) and 41 *C. maenas* (times 0, 1, 6 and 12h; 19-20 ^{o}C) stomach extracts were subjected to 10-fold dilutions. The extent of the inhibition on plaice detectability is expressed in terms of percentage of false-negatives; a false-negative corresponds to a stomach where plaice DNA was positively detected in some or all of the dilutions but not in the undiluted (1/1) extract. Overall 7% and 25% of the *C. crangon* stomachs presented a false-negative result for, respectively, salt and phenol-chloroform extractions, while these values increased up to 86% and 88% for *C.* maenas (Table 5); no apparent differences in the inhibition extent were found if considering all the different digestion times together or separately. Running the assay for an additional 1/10 dilution (apart from the undiluted DNA one), reduces the incidence of false-negatives to zero for *C. crangon* extracts while running the assay for an additional 1/10 and 1/100 dilutions achieves the same result for *C. maenas* extracts except for 1 case (out of 13)

where a 1/1000 one was needed (Table 5). For both species extracts it was also noted that higher DNA yields needed a higher dilution to overcome inhibition indicating that inhibition is caused by some factor extracted from the stomachs along with the DNA. Consequently in the digestion experiments, all *C. crangon* testing negative with the TaqMan probe were diluted to 1/10 and re-tested while *C. maenas* testing negative were diluted to both 1/10 and 1/100 and re-tested with the TaqMan probe.

Experiment 3 – Determination of minimum quantity of tissue (detection limit)

Standard graphs (Figure 1) showed the magnitude of the detection signal (expressed as Ct value: see Methods for further explanation) along the decreasing values of template (raw Plaice) DNA; the Taqman assay was able to detect 0.001ng of plaice DNA (corresponding to a 1/10000 dilution of salt extracted DNA from frozen preserved raw plaice tissue).

Experiment 4 – Determination of detectability over digestion time

Visual assessment of stomach contents during the course of the digestion experiments showed that stomach fullness reduced progressively with increasing digestion times in both species from total fullness in the first hours to nearly or totally empty at the end of the experiment (Table 6). Out of thirty nine *C. maenas* stomachs visually identified as empty (index 0, see Methods), four tested positive for plaice DNA. Quantification of plaice DNA in the stomachs (expressed as Ct value; by plotting the obtained Ct value in the standard graph shown in Figure 1 we obtain the ng of plaice DNA in the stomach contents) showed comparable values for both extraction methods along with the decrease of detectable plaice DNA with increasing digestion times (Table 6).

Half-life detection rates of the target DNA (T50 values; see Methods for further explanation) and maximum detection times were estimated for both predator species in the different experimental setups using the dilutions described above to overcome inhibition. Plaice DNA could be amplified from *C. crangon* and *C. maenas* stomachs up to a maximum of 24 h after feeding (Figures 3 and 4). For *C. crangon* extracts the T50 was ~10 h at water temperatures of 14-16^{o}C. The effects of a wider range of temperatures were tested for *C. maenas* where the half-life detection rates (T50) were ~7 h at 6-10^{o}C, ~ 6 h at 14-16^{o}C but only 2 h at 19-20^{o}C. The extraction method had little effect on T50s for either species.

Experiment 5 – Validation of the assay with selected field samples

When applying the TaqMan assay with ten-fold dilutions to the DNA extracted from the five field collected stomachs of *C. crangon* (sample codes O27, Q38, S7, S38 and U58; Table 7), none tested positive for plaice DNA in the undiluted and 1/10 diluted extract, and one tested positive for the 1/100 one (sample S7), and all but one (Q38) tested positive at a 1/1000 dilution. However, by adding 1.25 µg/µl Bovine Serum Albumin (BSA) to the PCR reaction, all the samples tested positive from 1/10 dilution through to 1/1000 with the exception of one sample that tested negative for all dilutions (Q38).

DISCUSSION

Knowledge of trophic interactions is critical in addressing many ecological and conservation issues in marine ecosystems. Trophic interactions have usually been studied by identifying stomach contents (also referred as gut contents) of predators. Although visual identification of prey items has yielded considerable insights, a large component of marine food webs are not amenable to this approach. Prey items may be cryptic e.g. early stage eggs of cod, haddock

411 and whiting which are indistinguishable visually (Taylor et al., 2002); predators may damage
412 the prey items during ingestion e.g. maceration of winter flounder eggs and larvae by shrimp
413 (Taylor, 2004) or prey items may lack hard-parts and be rapidly digested beyond recognition.
414 The last factor will apply especially to fish larvae (Folkvord, 1993).

415 Previously published studies on predation of recently settled plaice juveniles all used
416 visual gut content identification. Although plaice begin spawning early in the year compared
417 with other species, their spawning and nursery grounds often include several species with
418 similar larvae, especially later in the season. Unambiguous identification of the early
419 developmental stages of plaice from closely related species as dab and flounder requires
420 counts of anal and fin rays (Nichols, 1971) but this is often only possible in un-damaged
421 specimens. The identification of plaice larvae and juveniles in the stomachs of crustacean
422 predators is further complicated by maceration making it even more difficult to identify the
423 stomach contents visually although some studies have used the presence of plaice otoliths in
424 predator stomachs as an index (van der Veer and Bergman, 1987; Wennhage and Pihl, 2001;
425 Nakaya et al., 2004). However, this may under-estimate the incidence of predation where
426 whole fish have not been consumed (Gibson et al., 1995). Recent progress in the development
427 and application of molecular methods for predation studies (Symondson, 2002; King et al.,
428 2008) suggests that species specific DNA probes may overcome some of these problems and
429 be a useful tool for examining predation of flatfish larvae and juveniles in the field.
430
431 The application of molecular tools to ecology relies both on the specificity and sensitivity of
432 the PCR assay (Harwood et al., 2007). Our results demonstrated that the real-time PCR based
433 TaqMan assay for plaice was highly specific as it reacts positively with plaice samples
434 collected from a variety of locations (North Sea, Irish Sea, Scottish west coast and English
435 Channel) but fails to react to DNA from a wide range of other species (Table 1 and 3). The
436 risk that the TaqMan assay cross hybridizes with DNA from other species and the risk of a

sequence polymorphism between populations from different localities that would create a mismatch when hybridizing is therefore minimal. Regarding sensitivity, the standard graphs (Figure 1) show the TaqMan probe is capable of detecting as little as 0.001 ng of raw (undigested) plaice DNA with high repeatability. This compares well with detection limits of 0.002 ng and 0.006 ng for, respectively, a real time PCR assay for the identification of *Anguilla japonica* eggs and larvae (Minegishi et al., 2009) and for a PCR based assay for stomach contents analysis in the insect genus *Homalodisca* (de Leon et al., 2006). Because of the high sensitivity of PCR, field sampling and subsequent laboratory analysis should include rigorous blank procedures designed to detect any cross-contamination (King et al., 2008).

A key factor for successful application of PCR is the use of appropriate tissue preservation and DNA extraction methods (e.g. Passmore et al., 2006; Lopera-Barrero et al., 2008; Simonelli et al., 2009). Although the effect of different preservation and extraction methods on the subsequent success of PCR is fairly well understood for high quality tissue, there is a paucity of reported research on protocols for degraded material, including stomach contents. Passmore et al. (2006) working with krill stomach contents concluded that 80% ethanol was superior to freezing for maintaining the integrity of prey DNA as nucleases may degrade DNA in stomachs if not rapidly deactivated. Our results showed little difference in detectability using the TaqMan plaice assay on DNA extracted from predator stomachs which had either been rapidly frozen (-80°C) or preserved in 80% ethanol (Figure 3). For field sampling either preservation method appears suitable. Preservation of crustacea in 80% ethanol has the advantage of partially dehydrating the specimens making subsequent dissection easier but ethanol is flammable and this may make it a poor choice if samples have to be shipped following collection. In addition there are ethical concerns about sampling of decapod crustaceans and the recommended procedures for working with these animals in Europe at least are currently being reviewed. Rapid freezing may be a more humane sampling method and for field-work dry-shippers which are charged with liquid nitrogen are both safe

463 and effective. However, care must be taken that frozen samples do not become accidentally
464 thawed as enzyme activity, especially in stomach contents, may lead to further DNA
465 degradation.

466 We also compared phenol-chloroform and salt extraction of the samples. Although
467 both extraction methods have shown similar performance when applied to human blood
468 (Miller et al., 1988) and fish tissue (Lopera-Barrero et al., 2008), phenol-chloroform is usually
469 considered to yield greater quantities of higher quality DNA compared with salt extraction and
470 this was generally confirmed in our comparisons (Figure 2) although the comparatively
471 smaller portion of the supernatant that was taken in the salt extraction method, as to prioritize
472 quality of the DNA, would explain most of that difference. However, salt extraction is
473 cheaper, safer (from a health risk view) and faster. To our knowledge, this is the first time salt
474 extraction methods have been applied to invertebrate stomach contents and compared with
475 phenol-chloroform extraction. For all the samples where salt and phenol-chloroform
476 extractions were compared we obtained comparable results for DNA purity, T50 detection
477 values and maximum TaqMan probe detection times. For either method the purity of DNA
478 extracted from *C. maenas* stomach contents was lower compared with the *C. crangon*
479 stomachs but this may be partly explained as the amount of predator DNA, which would be
480 non-digested, co-extracted along with the stomach contents was probably proportionately
481 lower for the crab samples where only the contents, and not the whole stomach, were
482 dissected. Because of its low toxicity, cost and speed and the comparable TaqMan results we
483 suggest that salt extraction could be more widely used for DNA extraction from crustacean
484 stomachs.

485 PCR inhibition has been reported with DNA extracted from a wide range of sources including
486 human blood and soil (Rådström et al., 2004; Kermekchiev et al., 2009). Blood components
487 including hemolymph, several enzymes, proteins, polysaccharides and humic acid have all
488 been reported as powerful inhibitors but no studies have analyzed in detail the reasons for

489 inhibition in DNA extracted from invertebrates. PCR inhibition has been reported in extracts
490 from insect stomach contents (Juen and Traugott, 2006) whilst Pan et al. (2008) stated that
491 inhibition was a significant factor in application of molecular methods for species
492 identification of crab larvae. Dilution of template DNA to 0.1 ng overcame this inhibition in
493 the study of Pan et al. (2008) and this is in line with our results. Alternative methods for
494 overcoming inhibition have been proposed including commercial reagents. Some of these
495 have been tested e.g. DNeasy which failed to reduce inhibition in crab DNA extracts (Pan et
496 al., 2008). Juen and Traugott (2006) reported that addition of ≥1.28 µg μl^{-1} bovine serum
497 albumin (BSA) to the PCR reaction mix was effective. In our experimental study (aquarium
498 animals) PCR inhibition was apparent in extracts from both *C. crangon* and *C. maenas* but
499 was much stronger in extracts from the latter species (approx 87 % of false-negatives for
500 undiluted template). The relative size of the two types of predators (sizes in Table 6) used in
501 our experiments may have had an effect. Because *C. crangon* expected to predate on plaice in
502 the wild are typically larger than the ones used in our experiments (≥7mm cephalotorax length
503 [CL] following van der Veer and Bergman, 1987), we performed another dilution test in
504 spring 2009 involving 23 *C. crangon* between 7 mm and 14 mm (CL) killed at t = 0 h after
505 fed with plaice, to more precisely define the dilutions required for field studies. For these
506 individuals, yielding an average of 591 ng/µl of DNA per individual (in contrast with the
507 average 53 ng/µl DNA yielded for the experimental samples) the incidence of inhibition was
508 also around 85%, similar to that found in *C. maenas*, but was overcome by dilutions up to
509 1/1000. While higher dilutions were generally needed to overcome the inhibition in higher
510 yield samples, as reported for the digestion experiments, a combination of 1/10 + 1/100
511 dilutions overcame the inhibition in these larger *C. crangon*, except for 1 case (out of 23)
512 where a 1/1000 dilution was needed. An extra 1/100 dilution appears to be required to
513 overcome inhibition when testing large *C. crangon*'s stomachs. Taking into account that an
514 extra ten-fold dilution increases the Ct value of the assay by 3.75 units (Figure 1), the

515 detectability curves (and T50 values) for the large *C. crangon* are expected to be more similar
516 to the *C. maenas* curves (at the same water temperature). Thus, the detectability in the large *C.*
517 *crangon* will be slightly reduced as samples that would have yielded Ct values close to the
518 detection limit of 40 (see methods) if a lower dilution (1/10) had been enough to overcome
519 inhibition (as in the experimental, relatively small size ones, *C. crangon* case), will probably
520 test negative when applying the extra dilution of 1/100.
521 For a preliminary field validation of the assay, five field collected stomachs of *C. crangon*
522 containing juvenile flatfish remains were tested (see Methods); extracted DNA yields fell
523 within the range of the ones obtained for the big *C. crangon* reported above (Table 7). While
524 the sample Q38 tested negative for every treatment and most likely corresponded to a different
525 flatfish species (flounder and dab also occur in the same area), the rest of samples tested
526 positive for plaice; however, and taking into account that the relatively low digested remains,
527 as identified visually, pointed to low digestion times, the relatively high Ct values recorded
528 along with the practical absence of positives with dilutions lower than 1/1000 suggest a higher
529 inhibition in the field than in the aquarium experiments (Table 7). However, when applying
530 1.25 µg/µl Bovine Serum Albumin (BSA) to the PCR reaction, positive reactions were found
531 from 1/10 to 1/1000 dilutions; moreover, Ct values with BSA in the field samples showed
532 values within the range of those obtained in the big *C. crangon* reported above (sampled at
533 digestion time = 0 h and kept in the aquarium), when the same dilution was applied (data not
534 shown), as expected due to the relatively low digested state of the flatfish remains in the field
535 collected stomachs. However, applying BSA does not remove the need to apply dilutions as
536 shown by the undiluted (1/1) samples results. This suggests that the addition of BSA
537 compensates for extra inhibition in field samples when comparing with the aquarium samples,
538 and suggests BSA should be added to the PCR reaction along with the above recommended
539 dilutions when applying the assay to field samples. Similar concentrations of BSA
540 considerably reduced inhibition in insect stomach contents analysis (Juen and Traugott, 2006),

where the authors suggested the presence of PCR inhibitory substances within the soil ingested while feeding as the source of PCR inhibition. In this study, mud-like remains, including sand grains in many of the cases, were found in most of the dissected stomachs, as has been reported elsewhere for both *C. crangon* and *C. maenas* (e.g. Pihl and Rosenberg, 1984; Ansell et al., 1999; Oh et al., 2001), suggesting a potential reason of the higher inhibition reported in the field when comparing to the aquarium experiments, where clean tanks, containing only sea water, were used.

For field applications we recommend testing stomach DNA extracts in a non diluted sample and 1/10 dilution for *C. crangon* presenting yields < 100 ng μl^{-1} (extracting the whole stomach) and for *C. maenas* <10 ng μl^{-1} (extracting only the stomach's contents), while a combination of 1/10 and 1/100 dilution are needed for *C. crangon* yielding > 100 ng μl^{-1} and for *C. maenas* yielding > 10 ng μl^{-1}; in every case, 1.25 µg/µl BSA should be added to the PCR reaction. Moreover, to save time and cost we recommend initially performing the TaqMan assay using the first of the suggested dilutions and repeating samples which present a negative result with the second dilution. Recently, Kermekchiev et al. (2009) showed that a mutational alteration of the polymerase was able to overcome inhibition in blood and soil samples when using Taq polymerase. If results are confirmed for marine crustacea, changing the Taq could overcome inhibition and reduce the cost of re-analysing negative samples with dilutions avoiding the above commented negative effect of these on the detectability.

A key consideration in applying either visual or molecular stomach content analysis is the impact of digestion on the prey detectability. Molecular probes for predation studies tend to target short-sequences of DNA in order to improve their effectiveness with degraded material (e.g. Hoogendoorn and Heimpel, 2001; Symondson, 2002; King et al., 2008; Troedsson et al., 2009). In addition, probes to multicopy regions of the DNA, most typically

567 mitochondrial DNA or nuclear ribosomal gene cluster (rDNAs), will likely be more effective
568 than probes for single copy DNA in predation studies (King et al., 2008). The design of our
569 assay, with a 15 bp probe hybridizing over an amplified (PCR mediated) 72 bp region of the
570 mitochondrial Cyt-*b* gene (Table 2), assists in this manner and allowed us to detect plaice
571 DNA in predator stomachs up to 24 h after ingestion. In several incidences a positive result
572 was even obtained with stomachs which appeared visually empty. This shows that it is
573 possible to detect traces of target DNA after almost complete digestion or gut clearance.
574 Water temperature is another factor which must be taken into account as it often has a strong
575 effect on digestion rates in marine ectotherms including fish (Dos-Santos and Jobling, 1991;
576 Singh-Renton and Bromley, 1996), medusae (Martinussen and Båmstedt, 2001) and
577 cephalopods (Grigoriou and Richardson, 2008) although the effect is not always found
578 (Bernreuther et al., 2008). Temperature must therefore be taken into account in relating
579 detectability by visual or molecular means to digestion time. Previous studies have reported
580 prey detection up to 5 h after ingestion for stone flounder, *Kareius bicoloratus* fed to shrimp
581 *Crangon affinis* at ~9^{o}C, (Asahida et al., 1997) and up to 12 h for cod (*Gadus morhua*) fed to
582 mackerel (*Scomber scombrus*) at 10^{o}C (Rosel and Kocher, 2002). In both studies detection
583 was by classic (not real-time one) PCR. Ideally we need to be able to detect a high proportion
584 of the predators which have fed, say >90%. As expected, the amount of time the TaqMan
585 method could detect plaice after feeding decreased with increasing water temperature (*C.*
586 *maenas* experiments; Figure 4) as previously demonstrated in insect predator guts assayed
587 with immunoassays (Hagler and Naranjo, 1997) and PCR (Hoogendoorn and Heimpel, 2001).
588 In our study TaqMan T90s (the time after which 90% of the individuals test positive for prey
589 DNA) decreased to ~2 h at 19-20^{o}C but were around 5 h at temperatures <16^{o}C. Water
590 temperatures in European plaice nursery grounds can certainly exceed 19^{o}C in inter-tidal
591 pools in late spring/early summer but would more typically be <16^{o}C at water depths where
592 most juvenile plaice are found (Gibson et al., 2002). We can use the detectability curves to

593 estimate the sampling frequency required but this will only work for predators which show a
594 clear diel cycle in feeding. However, as both crabs and shrimp tend to feed most heavily
595 around dawn and dusk (e.g. Pihl and Rosenberg, 1984; del Norte-Campos and Temming,
596 1994; Ansell et al., 1999), field sampling carried out within 5 h of these periods followed by
597 TaqMan analysis should detect >90% of the predators which have fed on plaice.

598 Digestion rates may be further affected by meal size (Dos-Santos and Jobling, 1991),
599 mixed diets (Andersen and Beyer, 2005) and starvation. Pre-experimental starvation is often
600 used in laboratory studies to ensure that the predators feed when exposed to the prey (Asahida
601 et al., 1997) but reports on the effects of pre-starvation on digestion rates and the subsequent
602 detectability by molecular methods are conflicting (Symondson and Liddell, 1995). In this
603 sense, van der Veer and Bergman (1987) found that higher periods of starvation increased the
604 rate of digestion in shrimp fed juvenile plaice, while Wallace (1973) reported that feeding
605 crabs that have been starved previously showed higher metabolic rates. This would suggest
606 that our T50/T90 values could be considered as a minimum when applying to the field where
607 starvation is less common. Furthermore, predators in the wild usually ingest a range of prey
608 and this may reduce the amount of target tissue ingested. However, the high sensitivity of the
609 TaqMan assay should enable detection even where only small quantities of plaice tissue have
610 been ingested.

611

612 **CONCLUSIONS**

613

614 We developed a rapid, highly sensitive, TaqMan real-time PCR based assay for a sequence of
615 the cytochrome-*b* gene on the mitochondrial DNA of plaice (*Pleuronectes platessa*), a
616 common European flatfish and tested the probe specificity against a wide range of co-
617 occurring organisms. We successfully amplified plaice DNA from the stomach contents of
618 two species of crustacea known to be common predators of recently settled, juvenile plaice,

namely the brown shrimp, *Crangon crangon* and the shore crab, *Carcinus maenas*. Two preservation (rapid freezing to -80^{o}C frozen and 80% ethanol) and two DNA extraction methods were tested (salt and phenol-chloroform). All combinations led to comparable results with similar target detectabilities. Although not widely used, salt extraction may be beneficial as it reduces time and costs and eliminates the health concerns associated with phenol-chloroform. However, significant levels of PCR inhibition occurred, particularly for *C. maenas* and large *C. crangon* stomach extracts. The occurrence of false-negatives could be overcome using serial dilution of the DNA template and 1.25 µg/µl Bovine Serum Albumin whilst the degree of inhibition was related to the extracted DNA yield this being a useful proxy to set suitable dilutions.

Experiments on the effect of digestion time on the detectability of plaice DNA in predator stomachs showed that successful amplification was possible up to 24 h after ingestion. For extracts of *C. crangon* stomachs the T50 was ~10 h at water temperatures of 14-16^{o}C. The effects of a wider range of temperatures were tested for stomach contents of *C. maenas* where the detection rate half-lives (T50) were ~7 h at 6-10^{o}C and ~ 6 h at 14-16^{o}C but only 2 h at 19-20^{o}C. For field application in European plaice nursery grounds, sampling of crabs and shrimp within 5 h of dawn and dusk should ensure detection rates of >90% of predators which have fed on plaice.

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REFERENCES

Agustí, N., Shayler, S., Harwood, J.D., Vaughan, I.P., Sunderland, K.D., Symondson, W.O.C., 2003. Collembola as alternative prey sustaining spiders in arable ecosystems: prey detection within predators using molecular markers. Mol. Ecol. 12, 3467-3475.

Aljanabi, S.M., Martinez, I., 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR based techniques. Nucleic Acids Res. 25, 4692-4693.

Andersen, N.G., Beyer, J.E., 2005. Gastric evacuation of mixed stomach contents in predatory gadoids: an expanded application of the square root model to estimate food rations. J. Fish Biol. 67, 1413-1433.

Ansell, A.D., Comely, C.A., Robb, L., 1999. Distribution, movements and diet of macrocrustaceans on a Scottish sandy beach with particular reference to predation on juvenile fishes. Mar. Ecol. Prog. Ser. 176, 115–130.

Asahida, T., Yamashita, Y., Kobayashi, T., 1997. Identification of consumed stone flounder, *Kareius bicoloratus* (Basilewsky), from the stomach contents of sand shrimp, *Crangon affinis* (De Haan) using mitochondrial DNA analysis. J. Exp. Mar. Biol. Ecol. 217, 153-163.

Bailey, K.M., Houde, E.D., 1989. Predation on eggs and larvae of marine fishes and the recruitment problem. Adv. Mar. Biol. 25, l-83.

Bailey, K.M., 1994. Predation on juvenile flatfish and recruitment variability. Neth. J. Sea Res. 32, 175-189.

673

674 Bax, N.J., 1998. The significance and prediction of predation in marine fisheries. ICES J.
675 Mar. Sci. 55, 997–1030.

676

677 Bernreuther, M., Herrmann, J.-P., Temming, A., 2008. Laboratory experiments on the gastric
678 evacuation of juvenile herring (*Clupea harengus* L.). J. Exp. Mar. Biol. Ecol. 363, 1-11.

679

680 Bunn, N.A., Fox, C.J., Webb, T., 2000. A literature review of studies on fish egg mortality:
681 implications for the estimation of spawning stock biomass by the annual egg production
682 method. The Centre for Environment, Fisheries and Aquaculture Science, Science Series
683 Technical Report, 111, 37 pp.

684

685 Burrows, M.T., Gontarek, S.J., Nash, R.D.M., Gibson, R.N., 2001. Shrimp predation on 0-
686 group plaice: contrasts between field data and predictions of an individual-based model. J. Sea
687 Res. 45, 243-254.

688

689 Chen, R.B., Watanabe, S., Yokota, M., 2004. Feeding habits of an exotic species, the
690 Mediterranean green crab *Carcinus aestuarii*, in Tokyo Bay. Fish. Sci. 70, 430–435.

691

692 Deagle, B.E., Eveson, J.P., Jarman, S.N., 2006. Quantification of damage in DNA recovered
693 from highly degraded samples – a case study on DNA in faeces. Front. Zool. 3, 11.

694

695 Dos-Santos, J., Jobling, M., 1991. Factors affecting gastric evacuation in cod, *Gadus morhua*
696 L., fed single meals of natural prey. J. Fish Biol. 38, 697-713.

697

698 Durbin, E.G., Casas, M.C., Rynearson, T.A., Smith, D.C., 2008. Measurement of copepod
699 predation on nauplii using qPCR of the cytochrome oxidase I gene. Mar. Biol. 153, 699–707.
700
701 Folkvord, A., 1993. Prey recognition in stomachs of cannibalistic juvenile cod (*Gadus*
702 *morhua* L.). Sarsia 78, 97-100.
703
704 Fox, C.J., Taylor, M.I., Pereyra, R., Villasana-Ortiz, M.I., Rico, C., 2005. TaqMan DNA
705 technology confirms likely over-estimation of cod (*Gadus morhua* L.) egg abundance in the
706 Irish Sea: implications for the assessment of the cod stock and mapping of spawning areas
707 using egg based methods. Mol. Ecol.14, 879-884.
708
709 Fox, C.J., Taylor, M.I., Dickey-Collas, M., Fossum, P., Kraus, G., Rohlf, N., Munk, P., van
710 Damme, C.J.G., Bolle, L.J., Maxwell, D.L., Wright, P.J., 2008. Mapping the spawning
711 grounds of North Sea cod (*Gadus morhua*) by direct and indirect means. Proc. R. Soc. Lond.
712 B 275, 1543-1548.
713
714 Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garces, E., Magnani, M., 2004.
715 Development of a Real-Time PCR Assay for Rapid Detection and Quantification of
716 *Alexandrium minutum* (a Dinoflagellate). Appl. Environ. Microbiol. 70(2), 1199–1206.
717
718 Garrod, C., Harding, D., 1981. Predation by fish on the pelagic eggs and larvae of fishes
719 spawning in the west central North Sea in 1976. ICES CM 1981/I: 11, 6pp.
720
721 Gibson, R.N., Yin, M.C., Robb, L., 1995. The behavioural basis of predator-prey size
722 relationships between shrimp (*Crangon crangon*) and juvenile plaice (*Pleuronectes platessa*).
723 J. Mar. Biol. Ass. U. K. 75, 337-349.

Gibson, R.N., Robb, L., Wennhage, H., Burrows, M.T., 2002. Ontogenetic changes in depth distribution of juvenile flatfishes in relation to predation risk and temperature on a shallow-water nursery ground. Mar. Ecol. Prog. Ser. 229, 233-244.

Greenstone, M.H., Hunt, J.H., 1993. Determination of prey antigen half-life in *Polistes metricus* using a monoclonal antibody-based immunodot assay. Entomol. Exp. Appl. 68, 1–7.

Grigoriou, P., Richardson, C.A., 2008. The effect of ration size, temperature and body weight on specific dynamic action of the common cuttlefish *Sepia officinalis*. Mar. Biol. 154, 1085-1095.

Hagler, J.R., Naranjo, S.E., 1997. Measuring the sensitivity of an indirect predator gut content ELISA: detectability of prey remains in relation to predator species, temperature, time and meal size. Biol. Control 9, 112–119.

Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. 41, 95-98.

Harper, G.L., King, R.A., Dodd, C.S., Harwood, J.D, Glen, D.M., Bruford, M.W., Symondson, W.O.C., 2005. Rapid screening of invertebrate predators for multiple prey DNA targets. Mol. Ecol. 14, 819–827.

Harwood, J.D., Desneux, N., Yoo, H.J.S., Rowley, D.L., Greenstone, M. H., Obrycki, J. J., O'neil R. J., 2007. Tracking the role of alternative prey in soybean aphid predation by *Orius insidiosus*: a molecular approach. Mol. Ecol. 16, 4390–4400.

Heath, M.R., 1992. Field investigations of the early life stages of marine fish. Adv. Mar. Biol. 28, 1-174.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5'→3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc. Natl. Acad. Sci. 88: 7276-7280.

Hoogendoorn, M., Heimpel, G.E., 2001. PCR-based gut content analysis of insect predators: using ribosomal ITS-1 fragments from prey to estimate predation frequency. Mol. Ecol. 10, 2059–2067.

Hunter, J.R., Kimbrell, C.A., 1980. Egg cannibalism in the northern anchovy, *Engraulis mordax*. Fish. Bull. 78, 811-816.

Juen, A., Traugott, M., 2006. Amplification facilitators and multiplex PCR: tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. Soil Biol. Biochem. 38, 1872–1879.

Juen, A., Traugott, M., 2007. Revealing species-specific trophic links in soil food webs: molecular identification of scarab predators. Mol. Ecol. 16, 1545–1557.

Kermekchiev, M.B., Kirilova, L.I., Vail E.E., Barnes, W.M., 2009. Mutants of Taq DNA polymerase resistant to PCR inhibitors allow DNA amplification from whole blood and crude soil samples. Nucleic Acids Res. 37(5), e40, doi:10.1093/nar/gkn1055.

King, R.A., Read, D.S., Traugott, M., Symondson, W.O.C., 2008. Molecular analysis of predation: a review of best practice for DNA-based approaches. Mol. Ecol. 17, 947–963.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S.F., Villablanca, F.X., Wilson, A.C., 1989. Dynamics of mtDNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86, 6196–6200.

de León, J.H., Fournier, V., Hagler, J.R., Daane, K.M., 2006. Development of molecular diagnostic markers for sharpshooters *Homalodisca coagulata* and *Homalodisca liturata* for use in predator gut content examinations. Entomol. Exp. Appl. 119, 109–119.

Lie, Y.S., Petropoulos, C.J, 1998. Advances in quantitative PCR technology: 5′ nuclease assays. Curr. Opin. Biotechnol. 9 (1), 43-48.

Lopera-Barrero, N.M., Povh, J.A., Ribeiro, R.P., Gomes, P.C., Jacometo, C.B., da Silva-Lopes, T., 2008. Comparison of DNA extraction protocols of fish fin and larvae samples: modified salt (NaCl) extraction. Cien. Inv. Agr. 35(1), 65-74.

Martinussen, M.B., Båmstedt, U., 2001. Digestion rate in relation to temperature of two gelatinous predators. Sarsia, 86, 21-35.

McBeath, A.J.A., Penston, M.J., Snow, M., Cook, P.F., Bricknell, I.R., Cunningham, C.O., 2006. Development and application of real-time PCR for specific detection of *Lepeophtheirus salmonis* and *Caligus elongatus* larvae in Scottish plankton samples. Dis. Aquat. Org. 73, 141–150.

Miller, S.A., Dykes, D.D., Polesky, H.F., 1988. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16(3), 1215.

Minegishi, Y., Yoshinaga, T., Aoyama, J., Tsukamoto, K., 2009. Species identification of *Anguilla japonica* by real-time PCR based on a sequence detection system: a practical application to eggs and larvae. ICES J. Mar. Sci. 66, 1915–1918.

Nakaya, M., Takatsu, T., Nakagami, M., Joh, M., Takahashi, T., 2004. Spatial distribution and feeding habits of the shrimp *Crangon uritai* as a predator on larval and juvenile marbled sole *Pleuronectes yokohamae*. Fish. Res. 70, 445-455.

Nejstgaard, J.C., Frischer, M.E., Raule, C.L., Gruebel, R.C., Kohlberg, K.E., Verity, P.G., 2003. Molecular detection of algal prey in copepod guts and faecal pellets. Limnol. Oceanogr. Methods 1, 29-38.

Nejstgaard, J.C., Frischer, M.E., Simonelli, P., Troedsson, C., Brakel, M., Adiyaman, F., Sazhin, A.F., Artigas, L.F., 2008. Quantitative PCR to estimate copepod feeding. Mar. Biol. 153, 565–577.

Nichols, J.H., 1971. *Pleuronectidae*. International Council for the Exploration of the seas, Fiches d'identification des oeufs et larves de poissons, 4-6, 18 pp.

del Norte-Campos, A.G.C., Temming, A., 1994. Daily activity and rations in gobies and brown shrimp in the northern Wadden Sea. Mar. Ecol. Prog. Ser. 115, 141-153.

827 Oh, C.-W., Hartnoll, R.G., Nash, R.D.M., 2001. Feeding ecology of the common shrimp
828 *Crangon crangon* in Port Erin Bay, Isle of Man, Irish Sea. Mar. Ecol. Prog. Ser. 214, 211–
829 223.
830
831 Palumbi, S.R., 1996. Nucleic acids II: The polymerase chain reaction. In: Hillis, D.M., Moritz,
832 C., Mable, B.K. (Eds.) Molecular Systematics. Sinauer, Sunderland, Massachusetts, pp. 205–
833 247.
834
835 Pan, M., McBeath, A.J.A., Hay, S.J., Pierce, G.J., Cunningham, C.O., 2008. Real-time PCR
836 assay for detection and relative quantification of *Liocarcinus depurator* larvae from plankton
837 samples. Mar. Biol. 153, 859–870.
838
839 Passmore, A.J., Jarman, S.N., Swadling, K.M., Kawaguchi, S., McMinn, A., Nicol, S., 2006.
840 DNA as a dietary biomarker in Antarctic krill, *Euphausia superba*. Mar. Biotechnol. 8, 686–
841 696.
842
843 Pihl, L., Rosenberg, R., 1984. Food selection and consumption of the shrimp *Crangon*
844 *crangon* in some shallow marine areas in western Sweden. Mar. Ecol. Prog. Ser. 15, 159–168.
845
846 Pihl, L., 1985. Food selection and consumption of mobile epibenthic fauna in shallow marine
847 areas. Mar. Ecol. Prog. Ser. 22, 169–179.
848
849 Pihl, L., 1990. Year-class strength regulation in plaice (*Pleuronectes platessa* L.) on the
850 Swedish west coast. Hydrobiologia 195, 79–88.
851

852 Rådström, P., Knutsson, R., Wolffs, P., Lövenklev, M., Löfström, C., 2004. Pre-PCR
853 processing: strategies to generate PCR-compatible samples. Mol. Biotechnol. 26, 133–146.
854
855 Rosel, P.E., Kocher, T.D., 2002. DNA-based identification of larval cod in stomach contents
856 of predatory fishes. J. Exp. Mar. Biol. Ecol. 267, 75-88.
857
858 Saitoh, K., Takagaki, M., Yamashita, Y., 2003. Detection of Japanese flounder-specific DNA
859 from gut contents of potential predators in the field. Fish. Sci. 69, 473–477.
860
861 Sambrook, J., Fritsch, F.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual. Cold
862 Spring Harbor Laboratory, Cold Spring Harbor, NY.
863
864 Schooley, J.D., Karam, A.P., Kesner, B.R., Marsh, P.C., Pacey, C.A., Thornbrugh, D.J., 2008.
865 Detection of larval remains after consumption by fishes. Trans. Am. Fish. Soc. 137, 1044-
866 1049.
867
868 Simonelli, P., Troedsson, C., Nejstgaard, J.C., Zech, K., Larsen, J.B., Frischer, M.E., 2009.
869 Evaluation of DNA extraction and handling procedures for PCR-based copepod feeding
870 studies. J. Plank. Res. 31(12), 1465–1474.
871
872 Singh-Renton, S., Bromley, P.J., 1996. Effects of temperature, prey type and prey size on
873 gastric evacuation in small cod and whiting. J. Fish Biol. 49, 702-713.
874
875 Smith, P.J., McVeagh, S.M., Allain, V., Sanchez, C., 2005. DNA identification of gut
876 contents of large pelagic fishes. J. Fish Biol. 67, 1178-1183.
877

878 Symondson, W.O.C., Liddell, J.E., 1995. Decay rates for slug antigens within the carabid
879 predator *Pterostichus melanarius* monitored with a monoclonal antibody. Entomol. Exp.
880 Appl. 75, 245–250.
881
882 Symondson, W.O.C., 2002. Molecular identification of prey in predator diets. Mol. Ecol. 11,
883 627-641.
884
885 Taylor, M.I., Fox, C.J., Rico, I., Rico, C., 2002. Species-specific TaqMan probes for
886 simultaneous identification of cod (*Gadus morhua* L.), haddock (*Melanogrammus aeglefinus*
887 L.) and whiting (*Merlangius merlangus* L.). Mol. Ecol. Notes 2, 599-601.
888
889 Taylor, D.L., 2004. Immunological detection of winter flounder (*Pseudopleuronectes*
890 *americanus*) eggs and juveniles in the stomach contents of crustacean predators. J. Exp. Mar.
891 Biol. Ecol. 301, 55-73.
892
893 Troedsson, C., Frischer, M.E., Nejstgaard, J.C., Thompson, E.M., 2007. Molecular
894 quantification of differential ingestion and particle trapping rates by the appendicularian
895 *Oikopleura dioica* as a function of prey size and shape. Limnol. Oceanogr. 52, 416–427.
896
897 Troedsson, C., Simonelli, P., Nägele, V., Nejstgaard, J.C., Frischer, M.E., 2009.
898 Quantification of copepod gut content by differential length amplification quantitative PCR
899 (dla-qPCR). Mar. Biol. 156, 253–259.
900
901 van der Veer, H.W., Bergman, M.J.N., 1987. Predation by crustaceans on a newly settled O-
902 group plaice *Pleuronectes platessa* population in the western Wadden Sea. Mar. Ecol. Prog.
903 Ser. 35, 203-215.

van der Veer, H.W., Feller, R.J., Weber, A., Witte, J.IJ., 1998. Importance of predation by crustaceans upon bivalve spat in the intertidal zone of the Dutch Wadden Sea as revealed by immunological assays of gut contents. J. Exp. Mar. Biol. Ecol. 231, 139-157.

Wallace, J.C., 1973. Feeding, starvation and metabolic rate in the shore crab *Carcinus maenas*. Mar. Biol. 20, 277-281.

Wennhage, H., Pihl, L., 2001. Settlement patterns of newly settled plaice (*Pleuronectes platessa* L.) in a non-tidal Swedish fjord in relation to larval supply and benthic predators. Mar. Biol. 139, 877–889.

916 **TABLE LEGENDS**

917 Table 1. Species used for the design of the assay. Number of individuals used from each site
918 for the screening of cytochrome-*b* gene sequence variation along with gene accession numbers
919 (GenBank).

920

921 Table 2. Primers and probe sequences.

922

923 Table 3. Species used to validate the assay specificity.

924

925 Table 4. Summary of the aquarium experiments. Last column shows samples where serial ten-
926 fold dilutions were performed for the inhibition test. DNA extraction of the whole stomach
927 (*C. crangon*) or stomach contents alone (*C. maenas*) was performed using both salt and
928 phenol-chloroform (two 450 µL aliquots of the same stomach) or one method depending of
929 the experiment.

930

931 Table 5. Results of the dilutions test for *C. crangon* and *C. maenas*. Serial ten-fold dilutions
932 were applied to the extracted DNA to estimate the inhibition effect in detectability; only
933 individuals testing positive for at least one of the treatments were included. Results are shown
934 pooling all the studied animals together (last row) and also splitting these into different
935 categories based in the extracted yield (ng/µl) measurement; a) corresponds to salt extraction
936 method while b) to phenol-chloroform. The percentage of false negatives is shown for either
937 undiluted (1/1), 1/10 and 1/100 diluted extractions in *C. crangon* (top table), and for the
938 former and 1/1000 dilution in the *C. maenas* case (bottom table); results for the combination
939 of more than one dilution are also shown. A false negative is assigned to each
940 dilution/combination of dilutions case if a negative test is reported there but it has been

reported positive in any of the other dilutions; highlighted are the best results within each combination of dilutions (see text for further explanation).

Table 6. Detectability of plaice DNA during the digestion by *C. crangon* and *C. maenas*. Results show the number of TaqMan testing positive by treatment following application of the dilution protocol to overcome PCR inhibition as described in the Methods section. Average Ct values along with standard deviation values are reported, in brackets, after the number of positives for both extraction methods, only for samples where all dilutions were performed (dilutions test animals; Table 4); these Ct values correspond to the minimum Ct value estimated from the whole set of dilutions applied, after correcting for the effect of ten-fold dilutions in their value (3.75 units increase in the Ct value for each ten-fold dilution; Figure 1), and represent the closest approximation to the amount of plaice DNA that we would get if no inhibition were present. Sizes (cephalotorax length for *C. crangon* and cephalothorax width for *C. maenas*) along with a stomach fullness index were recorded (see Methods). N/A (Not Available); note that the fullness index for the crabs kept at 6-10°C was not recorded.

Table 7. Validation of the assay with selected field samples. Serial ten-fold dilutions were applied to the extracted DNA from five field collected *C. crangon* stomachs containing juvenile flatfish remains (see Methods). Yield (ng/µl) measurements are reported along with the results for the TaqMan assay, both without and with the addition of 1.25 µg/µl Bovine Serum Albumin to the PCR reaction, for four replicates of each sample; the number of positive signals (highlighted) along with the average Ct value (in brackets) are shown.

963 **FIGURE LEGENDS**

964 Figure 1. Standard graphs for the TaqMan real-time PCR based assay. Ct (threshold cycle; see
965 Methods) values for the assay against serial dilutions of plaice template DNA extracted from
966 rapid freezing to -80^{o}C raw plaice tissue (top graph) and Ct values for the assay against serial
967 dilutions of plaice template DNA extracted from 80% ethanol preserved raw plaice tissue
968 (bottom graph); both salt and phenol-chloroform extractions' results are shown.

969

970 Figure 2. Quality of the extracted DNA. Yield and purity data for DNA extracts from *C.*
971 *crangon* stomachs and *C. maenas* stomach contents (see Methods for further explanation).
972 Boxplots indicate the minimum, lower quartile, median, upper quartile and maximum.
973 Significant differences between extraction methods by Mann-Whitney U test are shown. ***,
974 $p<0.001$; **, $p<0.01$; *, $p<0.05$, NS, Not Significant.

975

976 Figure 3. Detectability curves for *C. crangon* aquarium experiments. a) Animals kept at water
977 temperature 14-16^{o}C, samples rapid frozen to -80^{o}C, b) animals kept at 14-16 ^{o}C, samples
978 preserved in 80% ethanol. Note that the phenol-chloroform extraction method in *C.crangon*
979 was performed in only four of the digestion times (Table 4).

980

981 Figure 4. Detectability curves for *C. maenas* aquarium experiments. a) Animals kept at water
982 temperature 6-10^{o}C, samples rapid frozen to -80^{o}C, b) animals kept at 14-16 ^{o}C, samples rapid
983 frozen to -80^{o}C, c) animals kept at 19-20 ^{o}C, samples preserved in 80% ethanol. Note that salt
984 extraction method was not performed in the 6-10 ^{o}C experiment (Table 4).

Table 1. Species used for the design of the assay.

Species	Sample site	n sequenced	Accession numbers (GenBank)
Plaice (*Pleuronectes platessa*)	East Irish Sea	20	**GU168804-GU168823**
	Iceland	19	**GU168824-GU168842**
	North Sea	20	**GU168843-GU168862**
	Norway	18	**GU168863-GU168880**
Dab (*Limanda limanda*)	English Channel (Boulogne)	5	**GU168881-GU168885**
	Irish Sea (Burbo Bank)	2	**GU168886-GU168887**
	Iceland	4	**GU168888-GU168891**
	Irish Sea (Inner Cardigan Bay)	5	**GU168892-GU168896**
	English Channel (Lyme Bay)	5	**GU168897-GU168901**
	North Sea (Dogger Bank)	5	**GU168902-GU168906**
	Sweden	5	**GU168907-GU168911**
	Liverpool Bay	5	**GU168912-GU168916**
Flounder (*Platichthys flesus*)	North Sea (Smiths Knoll)	5	**GU168917-GU168921**
	North Sea (Alde)	5	**GU168922-GU168926**
	Irish Sea (Mersey)	4	**GU168927-GU168930**
	Irish Sea (Morecombe Bay)	5	**GU168931-GU168935**
	Sweden	5	**GU168936-GU168940**
GenBank sequences			
Microstomus kitt			**EU492290**
Platichthys bicoloratus			**NC_003176**
Pleuronectes mochigarai			**AB126392**
Hippoglossoides dubius			**AB126391**
Clidoderma asperrimum			**AB126393**
Reinhardtius hippoglossoides			**L77944**
Glyptocephalus stelleri			**AB114910**

Table 2. Primers and probe sequences.

Primer/probe	Sequence 5’ to 3’	5’ label	3’ label
Forward (PLA-F)	CTTAAAATCGCAAACRATGCTTTA		
Reverse (PLA-R)	CCCAAAGTTTCATCAGACAGAGATG		
Plaice probe	TCGATCTCCCAGCCC	VIC	Dark quencher

Table 3. Species used to validate the assay specificity.

Group	Species
Crustacea	*Scalpellum scalpellum, Munida rugosa, Crangon crangon, Crangon allmanni, Pagurus prideaux, Inachus dorsettensis, Carcinus maenas, Liocarcinus holsatus, Pycnogonum littorale, Pandalidae sp., Processa sp., Pasiphaea sp., Cirolana sp.*
Teleostei	*Lophius piscatorus, Scomber scombrus, Buglossidium luteum, Myoxocephalus scorpius, Balistes carolinensis, Platichthys flesus, Limanda limanda, Crenilabrus melops, Blennius ocellaris, Microchirus variegatus, Callionymus maculatus, Labrus bergylta, Merlangius merlangus, Zeus faber, Trisopterus luscus, Capros aper, Myxine glutinosa, Gadus morhua, Hyperoplus immaculata, Arnyrhombus regius, Mullus surmuletus, Spondylissoma cantharus, Arnoglossus laterna, Microstomus kitt, Trisopterus minutus, Merluccius merluccius, Merlangius aeglefinus.*
Elasmobranchii	*Scyliorhinus canicula*
Mollusca	*Mytilus edulis, Buccinum undatum, Tritonia hombergi, Aequipecten opercularis, Loligo forbesi*
Echinodermata	*Cellaria sp., Antedon bifida, Luidia sarsi, Ophiura ophiura, Psammechinus miliaris, Thyone sp.*
Cnidaria	*Sponge (indet.), Nemertesia antennina, Alcyonium digitatum, Anemone (indet.), Hylanoecia tubicola*
Tunicata	*Ascidiella scabra*

Table 4. Summary of the aquarium experiments.

Species	Temp. (°C)	Preservation	Time (h)	n	Salt	Phenol-	Dilutions test
C. crangon	14-16	-80°C	0	10	✓	✓	
			1	10	✓	✓	
			2	10	✓		
			4	10	✓		
			6	10	✓	✓	
			9	10	✓		
			18	10	✓	✓	
			24	10	✓		
C. crangon	14-16	80%EtOH	0	10	✓	✓	✓
			1	10	✓	✓	✓
			2	10	✓		
			4	8	✓		
			6	10	✓	✓	✓
			9	10	✓		
			18	8	✓	✓	✓
			24	10	✓		
C. maenas	6-10	-80°C	2	7		✓	
			4	6		✓	
			6	7		✓	
			8	7		✓	
			12	6		✓	
			18	6		✓	
			24	6		✓	
C. maenas	14-16	-80°C	0	8	✓	✓	
			3	8	✓	✓	
			6	8	✓	✓	
			9	8	✓	✓	
			24	8	✓	✓	
C. maenas	19-20	80%EtOH	0	10	✓	✓	✓
			1	8	✓	✓	✓
			2	10	✓	✓	
			3	10	✓	✓	
			6	10	✓	✓	✓
			12	10	✓	✓	✓
			18	10	✓	✓	
			24	10	✓	✓	

Table 5

Table 5. Results of the dilutions test for *C. crangon* and *C. maenas*.

Crangon crangon

a) Salt

Yield (ng/µl)	Total positives	1/1	1/10	1/100	1/1+1/10	1/10+1/100
		% false negatives	% false negatives	% false negatives	% false negatives	% false negatives
<10	2	0	0	50	0	0
10-100	28	7.14	7.14	14.29	0	7.14
>100	1	0	0	100	0	0
All	31	6.45	6.45	19.35	0	6.45

b) Phenol-Chloroform

Yield (ng/µl)	Total positives	1/1	1/10	1/100	1/1+1/10	1/10+1/100
		% false negatives	% false negatives	% false negatives	% false negatives	% false negatives
<10	3	0	0	0	0	0
10-100	21	19.05	9.52	19.05	0	9.52
>100	8	50	12.50	12.50	0	12.5
All	32	25	9.38	15.63	0	9.38

Carcinus maenas

c) Salt

Yield (ng/µl)	Total positives	1/1	1/10	1/100	1/1000	1/1+1/10	1/10+1/100	1/100+1/1000	1/1+1/10+1/100	1/10+1/100+1/1000
		% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives
<10	5	80	20	60	100	0	20	60	0	20
10-100	13	92.31	61.54	7.69	53.85	61.54	0	7.69	0	0
>100	4	75	50	0	75	50	0	0	0	0
All	22	86.36	50	18.18	68.18	45.45	4.55	18.18	0	4.55

d) Phenol-Chloroform

Yield (ng/µl)	Total positives	1/1	1/10	1/100	1/1000	1/1+1/10	1/10+1/100	1/100+1/1000	1/1+1/10+1/100	1/10+1/100+1/1000
		% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives	% false negatives
<10	7	85.71	28.57	85.71	71.43	14.29	14.29	71.43	0	14.29
10-100	4	75	0	50	75	0	0	50	0	0
>100	13	92.31	61.54	15.38	38.46	61.54	7.69	7.69	7.69	0
All	24	87.50	41.67	41.67	54.17	37.50	8.33	33.33	4.17	4.17

Table 6

Table 6. Detectability of plaice DNA during the digestion by *C. crangon* and *C. maenas*.

Species	Temp. (°C)	Preservation	Time (h)	n	Mean size (std. dev.)	Mean fullness (std. dev.)	Positives Salt	Positives Phenol-
C. crangon	14-16	-80°C	0	10	4.95 (0.27)	2.8 (0.32)	10	10
			1	10	5.1 (0.6)	N/A	10	10
			2	10	4.93 (0.59)	N/A	10	N/A
			4	10	5.1 (0.77)	1.8 (0.64)	9	N/A
			6	10	4.85 (0.57)	N/A	8	8
			9	10	5.33 (0.69)	1.2 (0.32)	6	N/A
			18	10	4.78 (0.63)	N/A	0	0
			24	10	5.08 (0.28)	1 (0)	1	N/A
C. crangon	14-16	80% EtOH	0	10	5.25 (0.55)	2.9 (0.18)	10 (25.6; 2.5)	10 (24; 2)
			1	10	5.25 (0.7)	N/A	10 (26.3; 1.7)	10 (26.8; 2.3)
			2	10	5.75 (0.5)	N/A	10	N/A
			4	8	5.31 (0.69)	1.88 (0.22)	8	N/A
			6	10	5.35 (0.62)	N/A	8 (29.2; 3.9)	9 (29.8; 4.7)
			9	10	5.58 (0.84)	1.3 (0.42)	5	N/A
			18	8	5.31 (0.48)	N/A	3 (34; 3.7)	3 (31.7; 4.7)
			24	10	5.63 (0.55)	1 (0)	1	N/A
C. maenas	6-10	-80°C	2	7	49.57 (4.94)	N/A	N/A	6
			4	6	54.83 (7.83)	N/A	N/A	6
			6	7	54.14 (7.27)	N/A	N/A	5
			8	7	58.86 (4.41)	N/A	N/A	3
			12	6	58.67 (3.44)	N/A	N/A	1
			18	6	54.33 (6.44)	N/A	N/A	1
			24	6	57.17 (7.5)	N/A	N/A	0
C. maenas	14-16	-80°C	0	8	55.69 (6.31)	3 (0)	8	8
			3	8	55.75 (5.56)	2.5 (0.5)	7	8
			6	8	53.25 (8.69)	0.75 (0.56)	6	2
			9	8	53.25 (9.5)	0.5 (0.63)	2	3
			24	8	49 (6.38)	0.13 (0.22)	0	1
C. maenas	19-20	80% EtOH	0	10	54.25 (9.3)	2.6 (0.48)	9 (26.7; 2.7)	9 (26.6; 2.9)
			1	8	56.88 (7.25)	2.88 (0.22)	7 (26.9; 1.5)	8 (27; 2.8)
			2	10	58.95 (9.36)	2.5 (0.7)	5	5
			3	10	55.85 (8.78)	1.6 (0.72)	4	3
			6	10	55.4 (8.12)	0.4 (0.48)	4 (34.6; 3.1)	4 (33.9; 3)
			12	10	55.25 (10.55)	0.5 (0.5)	1 (29.7)	2 (34.6; 0.8)
			18	10	54.4 (9.1)	0.4 (0.48)	0	0
			24	10	53.75 (8.6)	0.7 (0.42)	1	0

Table 7

Table 7. Validation of the assay with selected field samples.

Sample code	O27	Q38	S7	S38	U58
Yield (ng/µl)	613.88	360.96	535.38	912.25	511.45
			40 cycles rtPCR, no BSA		
Dilution					
1/1	0/4 (-)	0/4 (-)	0/4 (-)	0/4 (-)	0/4 (-)
1/10	0/4 (-)	0/4 (-)	0/4 (-)	0/4 (-)	0/4 (-)
1/100	0/4 (-)	0/4 (-)	4/4 (37.14)	0/4 (-)	0/4 (-)
1/1000	4/4 (35.62)	0/4 (-)	4/4 (34.15)	2/4 (39.23)	4/4 (35.21)
			40 cycles rtPCR, 1.25 µg/µl BSA		
Dilution					
1/1	0/4 (-)	0/4 (-)	0/4 (-)	0/4 (-)	0/4 (-)
1/10	3/4 (34.39)	0/4 (-)	4/4 (28.89)	4/4 (31.42)	4/4 (28.48)
1/100	4/4 (32.68)	0/4 (-)	4/4 (32.45)	4/4 (34.41)	4/4 (31.81)
1/1000	4/4 (35.56)	0/4 (-)	4/4 (35.94)	3/4 (37.24)	4/4 (34.94)

Figure 1

Figure 1.

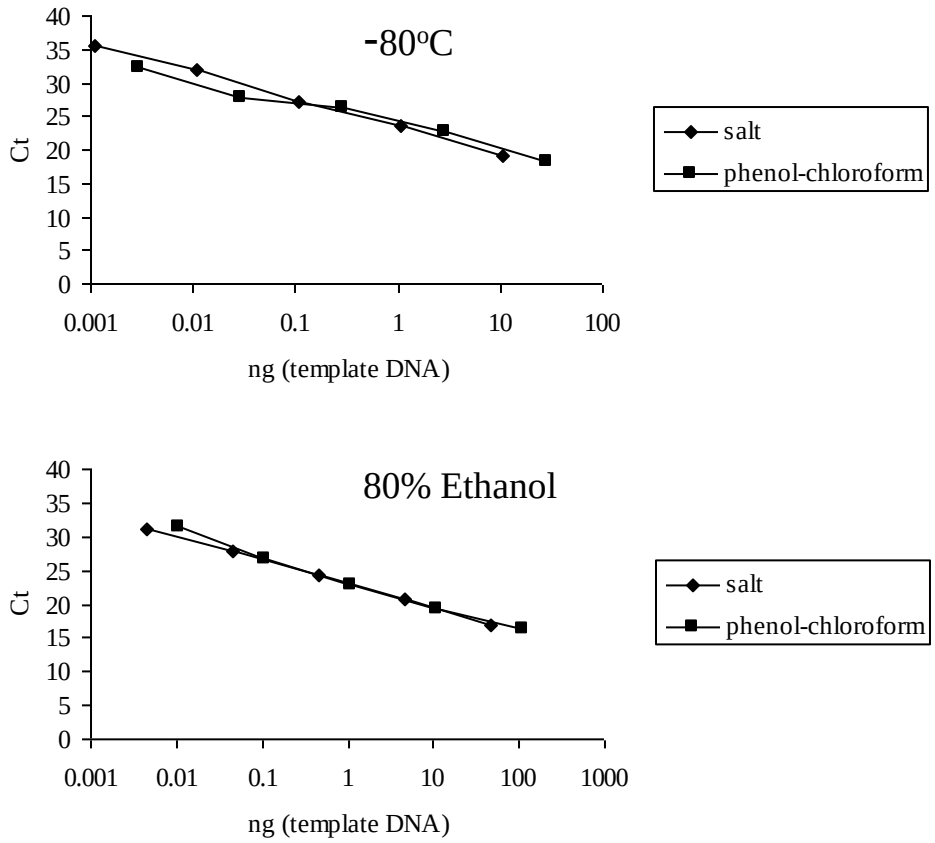


Figure 2

Figure 2.

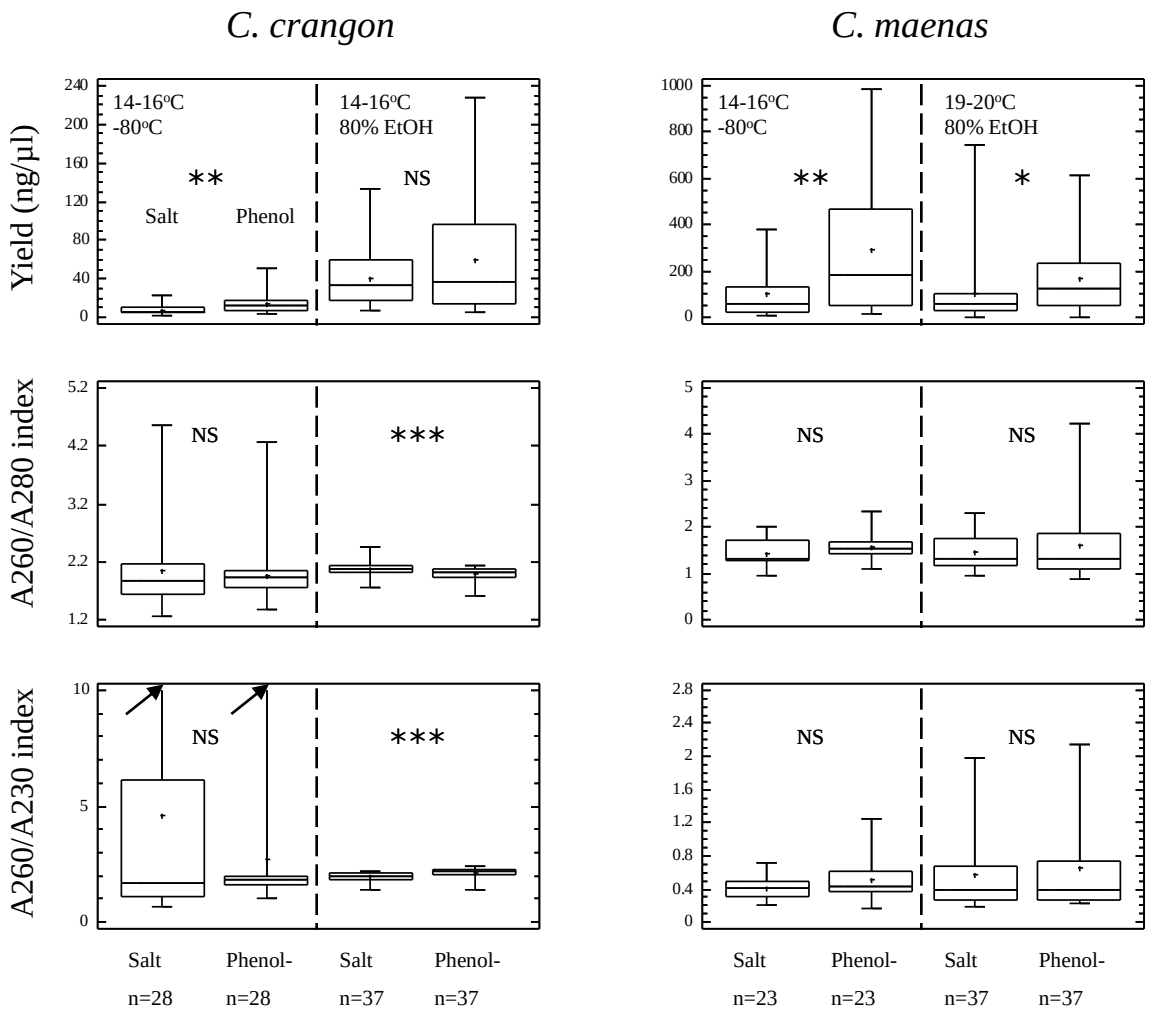
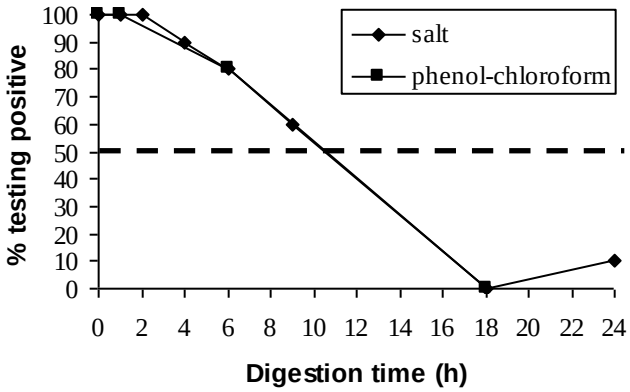


Figure 3.

a) *Crangon crangon* 14-16ºC (-80ºC)



b) *Crangon crangon* 14-16ºC (80% Ethanol)

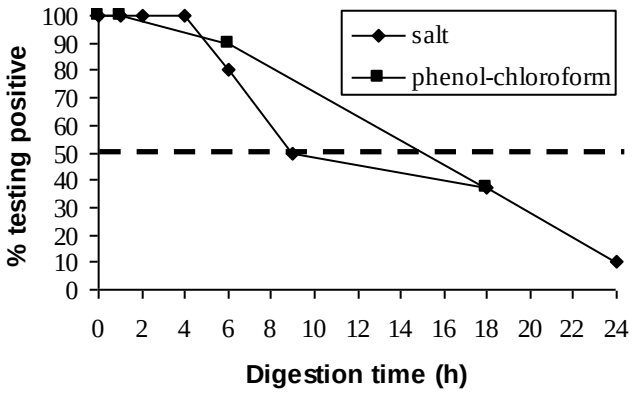
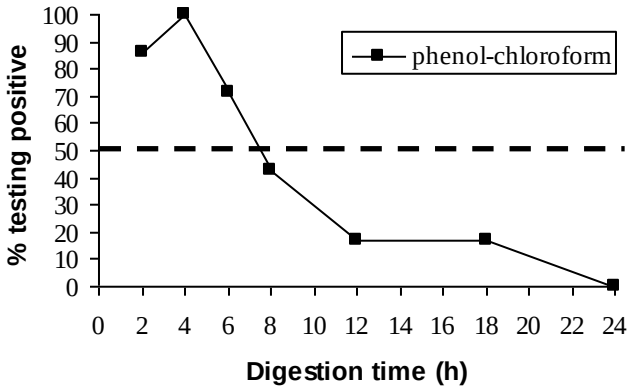
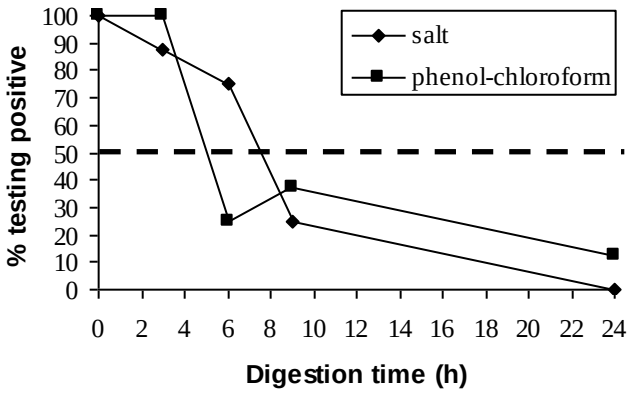


Figure 4.

a) *Carcinus maenas* 6-10ºC (-80ºC)



b) *Carcinus maenas* 14-16ºC (-80ºC)



c) *Carcinus maenas* 19-20ºC (80% Ethanol)