

TECHNICAL ADVANCES

A robotic molecular method for *in situ* detection of marine invertebrate larvae

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Abstract

Knowledge of the temporal and spatial abundance of invertebrate larvae is critical to understanding the dispersal capabilities and recruitment potential of marine and aquatic organisms. Traditional microscopic analyses are time-consuming and difficult given the diversity of larval species and a frequent lack of discriminating morphological characteristics. Here, we describe a sensitive rRNA targeted sandwich hybridization assay (SHA) that uses oligonucleotide probes to detect and enumerate the larvae of invasive green crabs (*Carcinus maenas*), native blue mussels (*Mytilus*), native barnacles (*Balanus*) and polychaetes (*Osedax* and *Ophelia*) that occur in the Monterey Bay National Marine Sanctuary, California. Laboratory-based assays demonstrate specificity, high sensitivity, and a quantitative response to cultured samples from three of the target organisms. Oligonucleotide probes were then printed in arrays on nitrocellulose membranes and deployed in our robotic Environmental Sample Processor (ESP) to detect larvae *in situ* and autonomously. We demonstrate that the SHA-detection method and ESP robot can be used for near real-time, *in situ* detection of larval species in the marine environment.

Keywords: *Balanus*, *Carcinus*, Environmental Sample Processor, *Mytilus*, ribosomal RNA, sandwich hybridization

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Introduction

Understanding the dynamics of marine invertebrate populations requires knowledge of larval retention, dispersal and settlement. Invertebrate larvae were traditionally viewed as passive particles, capable of long-range dispersal (Scheltema 1986), but recent research has shown that larval behaviours of many species are not that simple. For example, some crabs show ontogenetic shifts in behaviour that selectively exploit tidal transport to increase retention (Queiroga & Blanton 2005; Marta-Almeida *et al.* 2006). Bivalve larvae also are capable of remaining near shore despite currents that should sweep them out to sea and away from areas suitable for settlement (reviewed in Shanks & Brink 2005).

Although significant progress has been made elucidating behaviours that interact with physical processes, our understanding of larval dispersal is limited by the time-

intensive methods of sampling, species identification and enumeration. Larval stages of many marine invertebrates are extremely difficult to identify morphologically (Brown & Roughgarden 1985; Hu *et al.* 1993; Garland & Zimmer 2002), which led researchers to seek genetic markers for species identifications (Hu *et al.* 1993; Baldwin *et al.* 1996; Bilodeau *et al.* 1999; MaKinster *et al.* 1999; Hare *et al.* 2000; Morgan & Rogers 2001; Deagle *et al.* 2003; Larsen *et al.* 2005; Patil *et al.* 2005; Vadopalas *et al.* 2006; Pradillon *et al.* 2007). The capacity to accurately and efficiently identify diverse marine larvae from complex mixtures is unparalleled by traditional methods. Nonetheless, molecular analyses are not readily conducted using samples from natural populations. Those studies that have attempted analysis of natural samples typically preserved them for subsequent analysis in land-based laboratories.

Here we describe a robotic, *in situ* instrument utilizing a molecular method that is capable of detecting and quantifying invertebrate larvae in real-time from marine and other aquatic environments. Molecular and environmental

data (temperature, salinity and chlorophyll-a) were obtained with the second-generation Environmental Sample Processor (ESP) (Scholin *et al.* 2006; Roman *et al.* 2007), a robot that is programmed to take water samples and autonomously conduct a variety of chemical tests including nucleic acid sandwich-hybridization assays (SHA) while at sea (www.mbari.org/microbial/esp). We previously demonstrated that the SHA method could detect barnacle larvae from marine samples (Goffredi *et al.* 2006). We now extend this methodology with printed arrays of various oligonucleotide probes that are capable of simultaneously detecting barnacle, mussel, polychaete, and crab larvae. Dosage–response curves were calibrated in the laboratory to demonstrate the quantitative nature and detection limits of these assays. During the spring of 2006, the 2G-ESP was deployed twice on a mooring in Monterey Bay, California. The environmental measurements and results of SHA tests were transmitted by radio telemetry to our on-shore laboratory. The ESP was also programmed to collect and preserve ‘archive’ samples of materials filtered from whole water for subsequent molecular probing (FISH) and morphological verification in our shore-based laboratories.

Materials and methods

Environmental Sample Processor

The second-generation ESP (Scholin *et al.* 2001) is capable of autonomously sampling, concentrating, detecting and preserving microorganisms and small invertebrate larvae (< 1500 µm) from marine or aquatic environments (Fig. 1). Control software and mechanical details of this methodology were previously described (Greenfield *et al.* 2006; Scholin *et al.* 2006; Roman *et al.* 2007). The current 2G-ESP consists of five subsystems. (i) A carousel stores up to 132 ‘pucks’ or reaction chambers (Fig. 1a inset) that house 25-mm diameter filters of varying mesh-size or printed membranes for SHA hybridization. (ii) A robotic manipulator delivers each puck from the carousel to the sample collection or processing positions. (iii) Pucks are then clamped into a temperature regulated (ambient to 100 °C) reaction manifold. (iv) Depending on the position of valves, syringe pumps then deliver seawater or dispense a variety of reagents to the manifold in a programmable sequence (see below). (v) Results of arrays (DNA and protein; Greenfield *et al.* 2006) are recorded by a CCD camera and transmitted via radio modem to the land-based laboratory.

Capture probe development

The present capture probes target organisms that are of regional significance, as methods for the detection of alien invasive species is a priority for the Monterey Bay National

Marine Sanctuary. Thus, one of our probes targets the invasive European green crab, *Carcinus maenas*. Also included on arrays are probes to detect native invertebrates including barnacles of the genus *Balanus*. A long-term goal of MBARI’s research and development programs is to detect marine larvae and micro-organisms from deep-sea environments (e.g. hydrothermal vents, hydrocarbon seeps, seamounts and whale-falls), and we are currently testing a version of the ESP (deep-ESP) capable of sampling up to 1000-m depths (Scholin *et al.* 2006). Consequently, our molecular probe development targets several locally abundant deep-sea annelids (*Osedax* and *Lamellibrachia*).

The 18S rRNA sequences for target taxa (Table 1) and closely related nontarget taxa were obtained from GenBank (DQ079744, AY743951, AY527220, AF235964, AF235971, AY586485, AY586484, AF168742, AF168743, AF168744, AF315061, AF315062, AY577889, AY577886, AY577888, AF221638, AF221641, AF221645, DQ320503, AF120556, AY145381) or by direct sequencing (GenBank numbers J) (Table 1) as described in Goffredi *et al.* (2006). We designed five new 18S rRNA capture probes (Table 1) to specifically target specific species (*C. maenas*), higher taxa (polychaete, barnacle, mussel), or all eukaryotes. The 5′-biotinylated capture probes were synthesized commercially (Oligo’s Etc). Concentrated probes (100 µg/mL in TE) were kept at 4 °C until use. Working stocks of capture probes were made in a phosphate buffer (400 ng/mL in 0.1 M Na₂HPO₄), 0.5% Tween, pH 7.9–8.1 containing 1.25 µg/mL recombinant streptavidin (RSA, Pierce). Capture solutions were stable at 4 °C for several months. Universal consensus sequence probes (Euk519, Euk915, and Euk1194; Table 1) were used to prepare DIG-labelled signal probe cocktails (Goffredi *et al.* 2006) for use in the laboratory SHA tests.

The five new capture probes (Table 1) were initially tested against adult animal tissue (c. 25 mg) and lysis buffer only (negative) to determine specificity (Table 2). Larvae from three of the target taxa were available (*C. maenas*, *Ophelia* sp., and *Mytilus edulis*) and used to estimate the dynamic range of the assay (GCRAB, P1022, and M2B, respectively). All samples containing 100 or fewer larvae were counted by eye (W.J.J.) on an Olympus Research Stereo dissecting scope (SZH10) at ×10–20 power. Gravid female *C. maenas* were collected from Elkhorn Slough (Monterey Bay) and housed in aquaria until release of larvae. Polychaete larvae were obtained from whole water samples collected from Elkhorn Slough (Monterey Bay). Ten litres of native water was concentrated via a 20-µm Nitex mesh. Individuals of *Ophelia* (determined by sequencing of 18S rRNA) were individually picked out using a pipette and placed in a counting tray before being filtered (below). Cultured mussel larvae (*M. edulis*) of approximately 100 µm in size at a density of 130 individuals/mL were provided by Mr. Barry MacDonald (Bedford Institute of Oceanography). Five replicate counts of *M. edulis* larvae (1 mL each) were

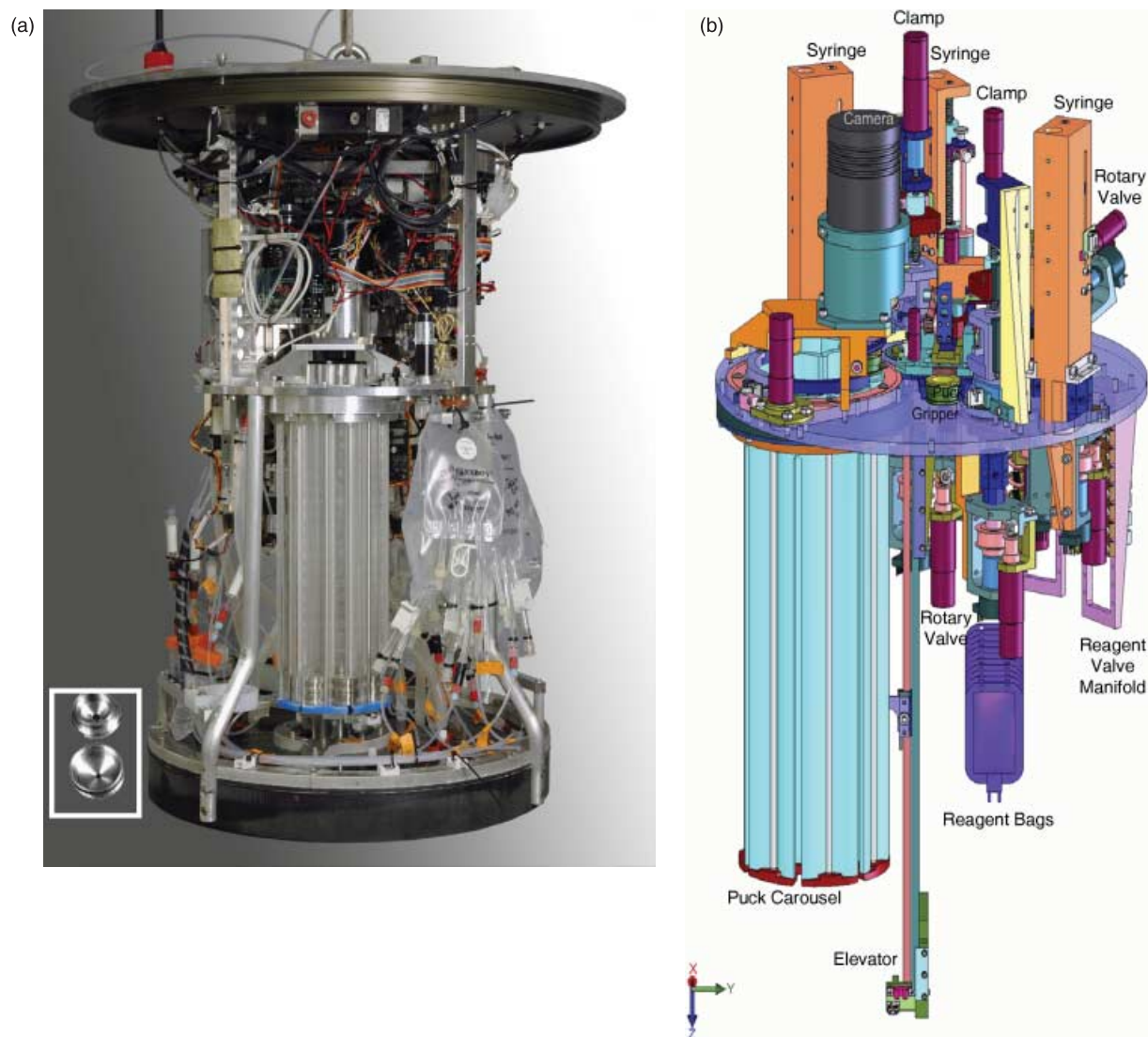


Fig. 1 (a) Second generation Environmental Sample Processor (ESP) with inset of a disassembled 'puck' chamber top and bottom. (b) SolidWorks™ schematic of the core 2G-ESP with main components labelled.

performed to estimate number of larvae per filter. Larvae were then collected, stored, and processed as described below.

In addition, individuals of *C. maenas*, *Ophelia*, and *M. edulis* were preserved for 2 h to 6 months in modified saline ethanol solution (Miller & Scholin 2000). Samples were processed using whole cell probing and fluorescent *in situ* hybridization for morphological identification as described below ('Whole cell probing').

Sample preparation for SHA and DNA extraction (laboratory)

Gentle vacuum filtration (10 mmHg) was used to concentrate larvae onto 25-mm diameter, 0.65- μ m pore size

hydrophilic Durapore membranes (Millipore). The filter was then transferred immediately to a 2-mL screw top cryovial (Nalge) with the sample side of the filter facing inward, then lysed immediately for the 96-well plate SHA, or archived in liquid nitrogen. To process frozen samples, vials were removed from liquid nitrogen and allowed to thaw for several minutes to near room temperature before addition of lysis solution. To lyse samples for the SHA assay, 2 mL of GuSCN lysis buffer [1.5 M GuSCN, 50 mM Tris, 15 mM EDTA, 2% Sarkosyl and 0.2% SDS (v/v), pH 8.9; modified from Scholin *et al.* 1999] was added to each filter. Each vial was tightly capped, vortexed, and placed in an 85 °C heating block for 5 min, with a brief vortexing at 2.5 min. Vials were removed from the heating block and allowed to cool for *c.* 5 min. The lysate was then

Table 1 18S ribosomal gene sequences for sandwich hybridization capture and signal probes, and whole cell hybridization signal probes

Target	Probe ID	Probe (5'→3') §	Source
Capture probes*			
<i>Carcinus maenas</i>	GCRAB	GGT TTC ACC TTA TAT GGG CTT G.. ... A.. C.. G.. ... A.. C.. G.. ... A.. C.. ...	<i>Carcinus maenas</i> (DQ079744) <i>Hemigrapsus sanguineus</i> (AY216710) <i>Pachygrapsus marmoratus</i> (DQ079763) <i>Cancer pagurus</i> (DQ079743)
Polychaete general	P1022	TCC AAC GAA TCG AGA AAG AGC TA A.. .C.AC A.. .C.AC C.. .C.A C	<i>Polychaete</i> general <i>Osedax frankpressi</i> (AY586485) <i>Lamellibrachia barhami</i> (AF168742) <i>Ridgeia piscesae</i> (AF168744) <i>Ophelia bicornis</i> (AF508122)
<i>Osedax</i> general	OS433	AGC ATC GTT AGA TAG CCC GCA TT C.. .AA ... G..T. C.. .AA ... G..T. .. G.. C.. .AA ... G.TT. .C	<i>Osedax</i> general <i>Osedax frankpressi</i> (AY586485) <i>Lamellibrachia barhami</i> (AF168742) <i>Ridgeia piscesae</i> (AF168744) <i>Ophelia bicornis</i> (AF508122)
Vestimentiferan/ Monliferan general	L54	GAA CGA TCC AAT AAA GAG TGA CCG AT. T.. T..T.T .G G.. CCA .A .T.	Vestimentiferan/Monliferan general <i>Osedax frankpressi</i> (AY586485) <i>Lamellibrachia barhami</i> (AF168742) <i>Ridgeia piscesae</i> (AF168744) <i>Ophelia bicornis</i> (AF508122)
Mytilidae general	M2B	AAG GG- CGA AAA ACC GG- GAG GTA GGTA ..G T.C G.. .C .T.C	Mytilidae general <i>Mytilus edulis</i> (L33448) <i>Bathymodiolus thermophilus</i> (AF221638) <i>Idas washingtonia</i> (AF221645) <i>Geukensia demissa</i> (GEKSRRNA) <i>Venerupis philippinarum</i> (EF426293)
Barnacle general	B1066	GTT GCG GAT TGC TGG TCG AC	Goffredi <i>et al.</i> (2006)
Universal eukaryotic	Euk338	CCT GCT GCC TTC CTT GGA TG	Goffredi <i>et al.</i> (2006)
Signal probes†			
Universal eukaryotic	Euk519	CTG GAA TTA CCG CGG CTG CTG	Goffredi <i>et al.</i> (2006)
Universal eukaryotic	Euk915	GTG CCC TTC CGT CAA TTC CTT	Goffredi <i>et al.</i> (2006)
Universal eukaryotic	Euk1194	CAT CTA AGG GCA TCA CAG ACC	Goffredi <i>et al.</i> (2006)
Whole cell probe‡			
Universal	UniC	GWA TTA CCG CGG CKG CTG	Goffredi <i>et al.</i> (2006)

*5' biotin TEG, three C9 spacers, probe; †5' Digoxigenin, one C9 spacer, probe, one C9 spacer, 3' Digoxigenin; ‡5' Fluorescein; §'- signifies deletion.

poured into a disposable polypropylene syringe fitted with a 0.45-µm Durapore syringe filter, and the filtered lysate was used in the 96-well SHA plate according to Goffredi *et al.* (2006). Upon completion of the SHA, the optical densities of reaction products in Row A were recorded immediately using an Emax plate reader (Molecular Devices) at 650 nm, and then again at 450 nm after acidification with 50 µL of 10% H₂SO₄ (v/v). The optical densities of the blue (650 nm, low sensitivity) and yellow (450 nm, high sensitivity) reaction products are proportional to the amount of rRNA captured from a sample which can be related to an estimate of cell density based on standard curves derived from cultured and/or natural samples, the volume the sample was lysed in, and the volume of water filtered (Scholin *et al.* 1999).

To obtain DNA from crude homogenate, 250 µL of filtered lysate was added to 40 µL 3 M NaOAc (pH 5.2) and mixed thoroughly followed by addition of 200 µL of 95% ethanol followed by vortexing. This solution was then added to a QIAGEN DNeasy Mini Spin column and processed according to manufacturer's instructions except for elution into 100 µL of sterile water instead of Buffer AE. DNA was amplified and sequenced as described above ('Capture probe development').

Preparation, processing and image analysis of ESP arrays

Detailed methods for the production of probe arrays were previously described (Greenfield *et al.* 2006). Briefly, a probe 'ink' consists of a biotinylated capture probes (as per Table 1)

Table 2 Specificity tests (via absorbance level) for capture probes upon exposure to various target species

Probe	Target species	No. of mismatches	A ₄₅₀	A ₆₅₀
GCRAB	<i>Carcinus maenas</i> (green crab)	0	3.496	3.770
	<i>Hemigrapsus nudus</i> (purple shore crab)	3	0.063	0.038
	<i>Pachygrapsus crassipes</i> (lined shore crab)	3	0.076	0.045
	1.5 M lysis negative	NA	0.064	0.039
OS433	<i>Osedax frankpressi</i> (bone-eating worm)	0	3.745	1.960
	<i>Lamellibrachia barhami</i> (cold-seep tubeworm)	5	0.060	0.042
	<i>Ridgeia piscesae</i> (hydrothermal vent tubeworm)	5	0.061	0.041
	1.5 M lysis negative	NA	0.069	0.045
P1022	<i>Osedax frankpressi</i>	0	3.768	3.554
	<i>Lamellibrachia barhami</i>	3	3.727	3.6553
	<i>Ridgeia piscesae</i>	3	3.746	3.876
	<i>Mytilus edulis</i> (blue mussel)	4	0.077	0.045
	<i>Carcinus maenas</i> (green crab)	4	0.073	0.053
	1.5 M lysis negative	NA	0.073	0.055
L54	<i>Osedax frankpressi</i>	4	0.117	0.065
	<i>Lamellibrachia barhami</i>	0	3.812	3.960
	<i>Riftia pachyptila</i>	0	4.084	4.136
	1.5 M lysis negative	NA	0.109	0.062
M2B	<i>Mytilus edulis</i> (blue mussel)	0	3.761	3.763
	<i>Bathymodiolus thermophilus</i> (hydrothermal vent mussel)	4 bp deletion	3.766	3.821
	<i>Idas washingtonia</i> (wood-/whale-fall mussel)	4 bp deletion	3.705	3.696
	<i>Geukensia demissa</i> (ribbed mussel)	3 bp deletion	3.357	1.709
	<i>Venerupis philippinarum</i> (Manila clam)	3-1 bp deletions, 6 mismatches	0.063	0.042

mixed with recombinant streptavidin in a buffered saline solution. A custom arrayer engineered at MBARI, was used to deposit capture probes in arrays on commercial membranes (Predator, Gelman). Internal chemistry standards and control target probes (see Greenfield *et al.* 2006) were included to validate positive signals, as illustrated in the array map (Fig. 2a).

The ESP was programmed to filter either 400 mL or 1000 mL of water through a 0.65- μ m Durapore filter and lyse the material retained in 1.5 M GuSCN. The resulting lysate was filtered through a 0.22- μ m Durapore filter and exposed to the array (Fig. 2), which was then processed using SHA chemistry (Fig. 3). The final step using the chemiluminescent substrate (Luminol/Enhancer Solution; Pierce Biotechnology) results in a light reaction that is detected with a CCD camera (Starlight Xpress model SXV-H9 England with a Fujinon model HF16HA-1B lens). The image was stored on a flash drive and transmitted to the shore-based laboratory via a radio modem. Array data presented in this paper are from a 10-s camera exposures with 2-by-2 binning.

Resulting mean intensity of each array probe spot was determined using IMAGEJ software (version 1.37, [\[rsb.info.nih.gov/ij/\]\(http://rsb.info.nih.gov/ij/\)\). The background level surrounding each spot was sampled four times, averaged and subtracted from the probe spot intensity. Between five and eight spots per capture probe were used to estimate signal. Corrected background values were averaged to estimate signal intensity associated with each capture probe. Background values on the arrays ranged from 2500 to 7000 counts with values typically around 3000 counts. For each array, the respective background level was subtracted from the raw positive spot values. A positive signal is defined as a spot having a value equal to or greater than 3 standard deviations above background following subtraction of background from the positive spots.](http://</p>
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ESP field operations

The ESP was deployed in Monterey Bay at 36.755°N, 122.025°W near the M0/CIMT mooring for two periods in 2006 (16–26 March and 10–23 April). Environmental data (temperature, salinity, chlorophyll-a and depth of ESP) were measured with a SBE-16 CTD (Seabird Electronics) mounted to the side of the ESP pressure housing. Configuration of the mooring is given by Scholin *et al.* (in press).

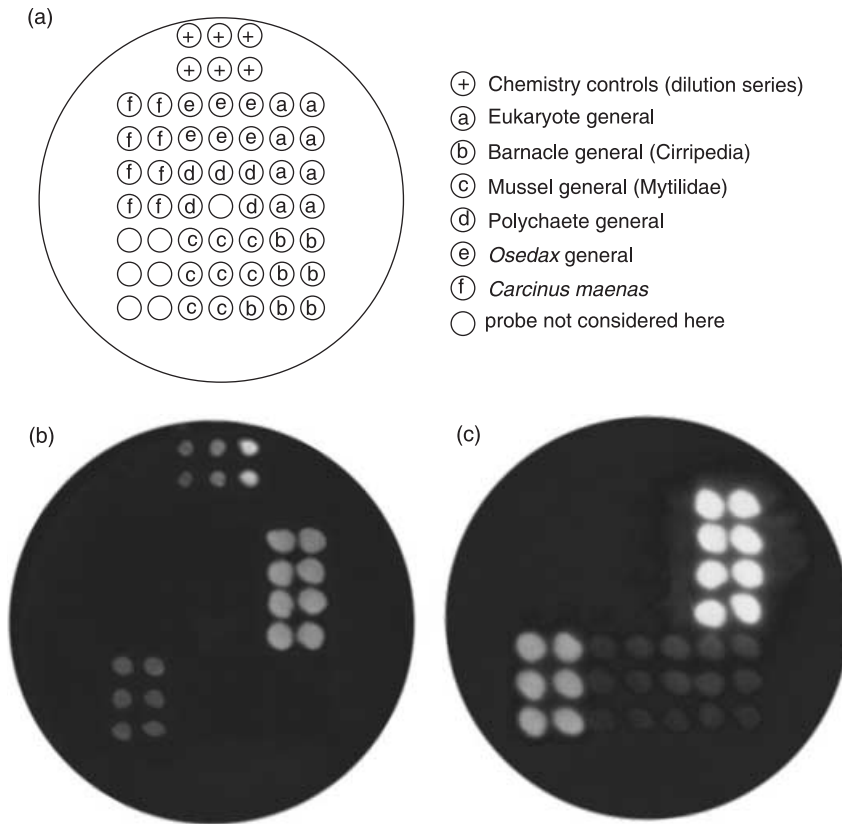


Fig. 2 Example of a 25-mm-diameter Environmental Sample Processor array printed with larval invertebrate probes. (a) Map showing location of capture probes. (b) Imaged array (#1) from deployment #1 showing only detection of eukaryotes. Dilution series of internal chemistry control spots are also shown. (c) Imaged array (#4) from deployment #1 showing simultaneous detection of eukaryotes, mussels, and barnacles. No internal chemistry controls are present on this particular array due to a printing error.

Whole cell probing (ESP)

The SHA approach detects RNA from the target species, but it is incapable of determining whether the nucleic acids were from intact larvae or in some deteriorated form in the water (e.g. in copepod faeces). To remedy this deficiency, we programmed the ESP to collect 'archive' samples for subsequent laboratory-based verification. Fifty-millilitre 'archive' samples were taken approximately 2 h after each SHA water sample. 'Archive' samples were filtered through a 1.2- μm Isopore polycarbonate membrane (Millipore) with a 0.45- μm Metricell (Millipore) backing and fixed in modified saline ethanol solution (described in Goffredi *et al.* 2006). This filter was then used to morphologically verify the presence of larvae by microscopy and molecular probing using the whole cell hybridization method (FISH).

Whole cell probing (laboratory)

Preserved samples (manually pulled onto filters) were washed with 1 mL of 5 \times SET hybridization buffer (5 min; Miller & Scholin 1998), then suspended in 0.5 mL 5 \times SET hybridization buffer containing a 5' fluorescein-labelled (UniC, Table 1) at a final concentration of 5 ng/mL. Samples were incubated for 45–60 min at 45 °C. After incubation,

excess probe was removed via washing with 1 mL of 5 \times SET at room temperature for 5 min. Filters were removed from the manifold and mounted, sample side up, on glass slides with Slowfade Lite (Molecular Probes). Fluorescently labelled intact cells were visualized using epifluorescence microscopy (Zeiss Axiophot 2 microscope; Chroma Technology filter, excitation 465–496 nm, dichroic 505 nm, emission 515–555 nm). Digital images were captured using a Spot SP100 cooled, charge coupled device camera (Diagnostic Instruments). The presence of target organisms was also verified by morphology.

Results

Capture probe development

A suite of capture probes was developed to detect the presence of larval invertebrates in water samples (Table 1). A universal probe (Euk338) was used to detect the presence of eukaryotic rRNA. Then, general probes were used to detect polychaetes, with nested probes for both vestimentiferans and *Osedax*. Specific probes targeted *C. maenas* and mytilid mussels.

Three probes (GCRA, P1022, and M2B) for which larvae were available were tested in laboratory-based, 96-well

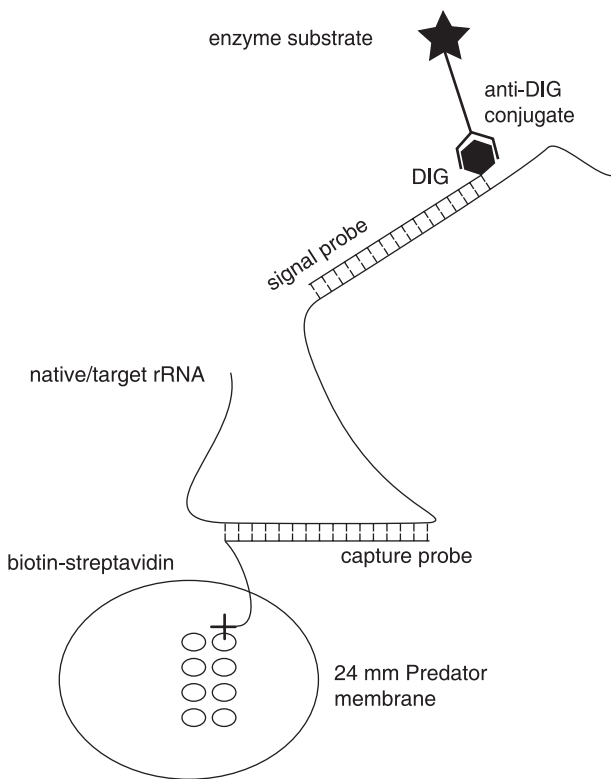


Fig. 3 Sandwich hybridization assay (SHA). A 'sandwich' consisting of a capture probe (attached to a solid-phase substrate via biotin-streptavidin), target rRNA and a DIG-labelled signal probe is detected via an enzymatic substrate reaction (after Scholin *et al.* 1996).

plate SHA to determine the relationship between reaction intensity (at A_{450} and A_{650}) as a function of larvae abundance (Fig. 4). The optical densities of the blue (650 nm, low sensitivity) and yellow (450 nm, high sensitivity) reaction products provide a wider range of detection given the different levels of sensitivity. Two larvae per millilitre of lysate was the lower limit of detection (LLD, Fig. 4a) for *C. maenas* zooea I (GCRAB probe, at A_{450}). The estimated upper limit of detection of *C. maenas* larvae with the GCRAB SHA probe was 90 larvae per millilitre of lysate (predicted using the lower sensitivity wavelength, A_{650}). The response was linear within this range. The polychaete probe (P1022) also provided a linear response to larval numbers (Fig. 4b). Its LLD was three *Ophelia* larvae per millilitre of lysate. The mytilid general probe (M2B) was linear up to 750 *M. edulis* larvae per millilitre of lysate, where it saturated (Fig. 4c). The LLD of the M2B probe was less sensitive than the other two capture probes (35 larvae per millilitre of lysate at A_{450}).

ESP field deployments

Ocean temperatures increased almost 2 °C during the two deployments (Fig. 5a), corresponding to the spring-

summer transition. Salinity was relatively stable, except for sudden decreases on Julian days 85 and 105 (Fig. 5b). Depth of the instrument varied with tidal cycles: generally 2–5 m for the first deployment and 3–6 m for the second deployment (Fig. 5c). A distinct peak in chlorophyll-a occurred at the start of the first deployment and then declined (Fig. 5d). Backgrounds on arrays varied but were generally between 2000 and 8000. Signal intensities above background were consistently observed for the general eukaryote (EUK338) probe. Of the four larval arrays that were developed during our first deployment, three showed relatively constant values for the EUK338 probe (*c.* 30 000 CCD counts; Fig. 5e), but the fourth (Julian day 85) was higher (*c.* 50 000 counts). During our second deployment, the first two arrays showed relatively low activity for the EUK338 probe (*c.* 20 000 counts) and increased on the last two arrays (*c.* 30 000 counts).

Although unidentified eukaryotic rRNAs appeared in all of our samples, the targeted larval species were only detected on Julian day 85, during the first deployment (Fig. 5e). A strong positive signal for the eukaryotic probe (EUK338) corresponded with weak signals for the general barnacle (B1066; 95% confidence interval 2283–2778 counts) and general mytilid (M2B; 95% confidence interval 1769–2930 counts) (Fig. 5e, also Fig. 2c). The positive signal for B1066 and M2B corresponded to an increase in temperature and a decrease in salinity (Fig. 5a–d, black dashed lines at Julian day 85) relative to the previous array sampled. Whole cell probing (as described above) using the UniC probe (Table 1) on the archived filter collected just after the positive array for B1066 and M2B could not confirm the presence of barnacle or mussel larvae, although a bivalve larva was observed (data not shown).

Discussion

Sandwich hybridization assay chemistry has been adopted into the fields of harmful algal species detection (Anderson *et al.* 2005), food industry monitoring (Umek *et al.* 2001), and bioweapon detection (Service 2002). It is also among the first molecular assays successfully used for autonomous detection of nucleic acids in the ocean (Scholin *et al.* 2001, 2006; Paul *et al.* 2007). Instruments such as the autonomous microbial genosensor (Paul *et al.* 2007) and the ESP (Scholin *et al.* 2001) hold promise in providing a way for continuous monitoring of aquatic environments.

Because of the nontraditional behaviour of oligonucleotide probes in a guanadidium solution (van Ness & Chen 1991), standard methods used to design probes such as for PCR do not readily apply. Rather, SHA probes are designed based on available sequence data and then rigorously tested in the laboratory using the benchtop SHA to validate specificity and range of utility. While single base discrimination is possible with the SHA (Haywood *et al.* in press),

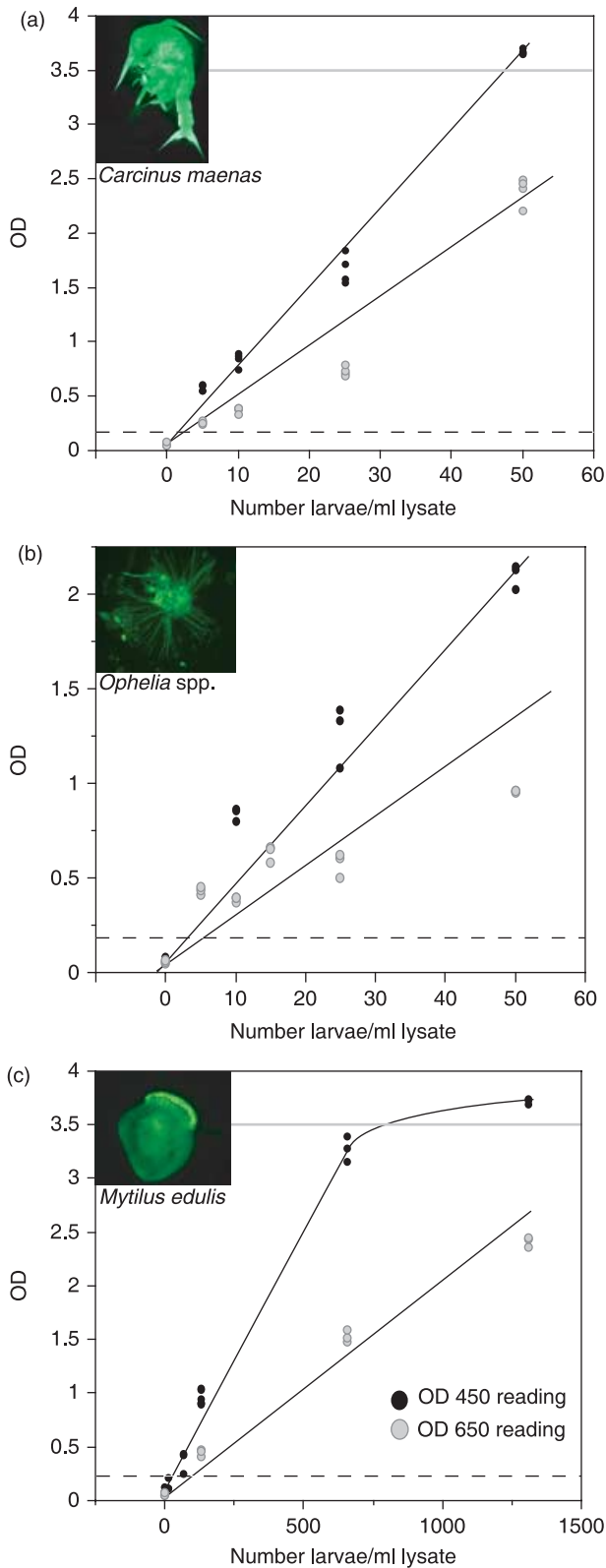


Fig. 4 Standard curve relationships for SHA 96-well plate activity (A_{450} and A_{650} optical densities) as a function of increasing number of larvae: (a) GCRAB probe with *Carcinus maenas* zoea I (b) P1022 probe with *Ophelia* sp. larvae, and (c) M2B probe with *Mytilus*

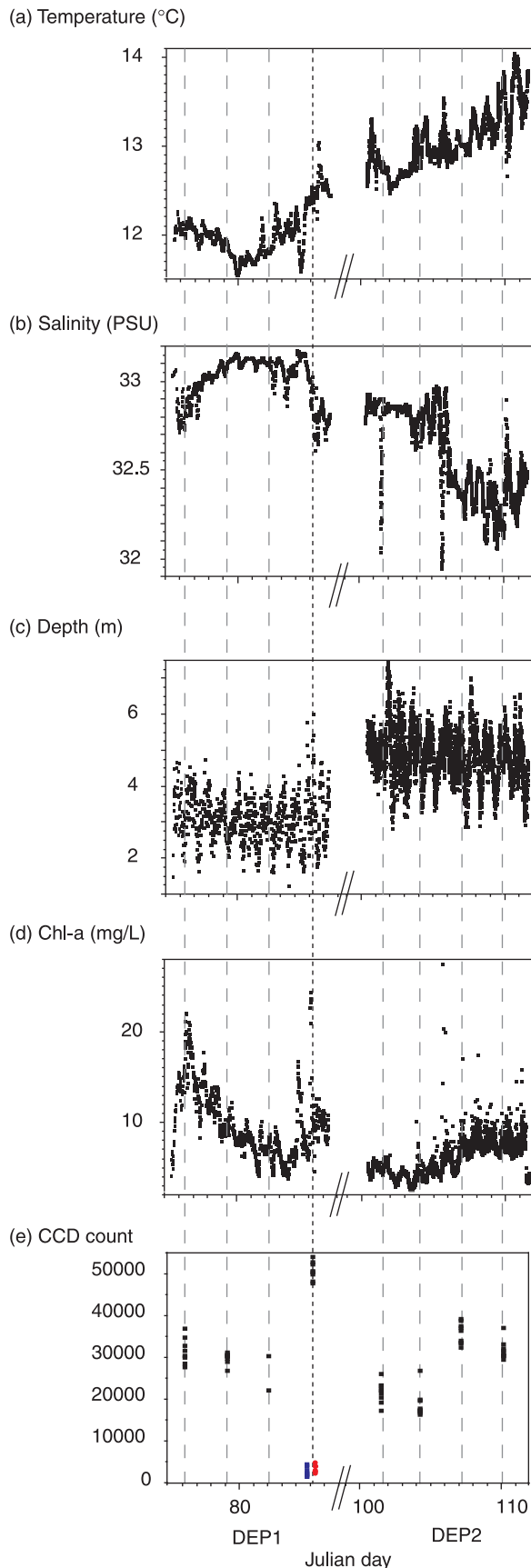
the probes presented in this paper do show reactivity with taxa which may not be targets (i.e. P1022, Table 2). It is suspected that stem-loop structure of the areas around the probe may allow a level of specificity that is not known a priori to testing the probe on the benchtop SHA (Tyrrell *et al.* 2001; Haywood *et al.* in press). Secondary structure of the 18S rRNA likely plays a role in allowing probes access to hybridize (Haywood *et al.* in press).

Considerable interest exists among marine and aquatic biologists in the development of convenient field methods for detecting and monitoring invertebrate larvae. The present methodology is based on a robust chemistry that has been adopted for ISO-certified identification of *Pseudonitzschia australis*, a toxic diatom, in New Zealand (Ayers *et al.* 2005). The SHA 96-well plate chemistry has also been demonstrated to be adaptable to shipboard measurements (Anderson *et al.* 2005) that allow a rapid response and processing time. The SHA method has been recently adopted for biosecurity and detection of invasive seastars in New Zealand (L. Rhodes, Cawthron, personal communication). With a low limit of detection, the SHA is an appropriate method for monitoring ballast water samples for invasive larval species such as the green crab (*C. maenas*).

In this paper, we used a laboratory-based assay in the 96-well plate format to demonstrate the quantitative nature of SHA capture probes for the detection of marine invertebrate larvae. Dosage–response curves for target marine invertebrate larvae for the ESP mooring arrayed format remain to be tested. Comparative tests have been performed between the 96-well plate and the ESP mooring on harmful algal species (Greenfield *et al.* 2006) with a significant positive correlation between the two assay measures. As with any molecular assay, assay intensity and the relevant variables (e.g. abundance, developmental stage, size) particular to a study will need to be assessed.

During the two spring deployments of the 2G-ESP in 2006, we autonomously detected, in near real-time, the presence of barnacle and mussel larvae in a higher temperature and lower salinity water mass. This result confirms our first detection of barnacle larvae in Monterey Bay using the 1G ESP mooring (Goffredi *et al.* 2006). In both instances, detection of targeted marine larvae occurred following the intrusion of a warmer, less saline water mass into the study site. For our research goals, we have focused on a suite of taxa from four groups (crustacean, bivalve, decapods, and polychaete) whose larvae are commonly found in Monterey Bay. An important advance facilitating this increase in target

edulis larvae (c. 100 μ m). The lower limit of detection is indicated by the dashed line. The upper limit of the dynamic range is indicated by the solid grey line. Inset images of target organisms were taken from whole cell probing (FISH) as described in the Materials and methods.



taxa is the use of a macroarray spotter (detailed in Greenfield *et al.* 2006), which allows 49 capture probe spots and six chemistry control spots to be printed on a 25-mm DNA array membrane (Fig. 2). The 2G-ESP mooring also provides a number of important advances that results in a more robust autonomous mooring: more efficient electronics and lower power consumption, more array storage, more flexible water sampling protocols (i.e. ESP detects filter clogging by monitoring pressure changes over the filter membrane in the puck), and a more user-friendly software interface (Ruby, detailed in Roman *et al.* 2007). Future modifications to the ESP arrays involve higher density spotting for increased probe spot density as well as an integrated microfluidics block for PCR applications. Future goals for the ESP mooring include event response sampling, for example based on temperature, salinity, and optical measurements from the CTD.

Molecular biology provides a number of tools for identifying and quantifying marine larvae (Baldwin *et al.* 1996; Bilodeau *et al.* 1999; MaKinster *et al.* 1999; Hare *et al.* 2000; Morgan & Rogers 2001; Deagle *et al.* 2003; Larsen *et al.* 2005; Vadopalas *et al.* 2006). Consequently, a suite of morphology-independent methods allows rapid and accurate identification of larval invertebrate species by nonspecialists. Choosing among the alternative molecular approaches will depend on a study's goals and the need for real-time monitoring. SHA methods employed in 96-well laboratory-based and ESP mooring-based technologies provide a highly flexible chemistry that is subject to modification for specialized efforts, e.g. additional capture probes, postrecovery whole cell FISH, and ELISA assays (Greenfield *et al.* 2006). The SHA chemistry is a robust molecular assay that has been successfully deployed and operated in the field for detection of marine larvae (see also Goffredi *et al.* 2006) and harmful algal species (Greenfield *et al.* 2006).

If oceanographic conditions alone (e.g. temperature, nutrients, currents, etc.) determine the temporal and spatial abundance of invertebrate larvae, then correlated patterns should exist across invertebrate taxa. Our methodology allows researchers to autonomously observe such correlations *in situ*. On the other hand, if invertebrate taxa show species-specific behaviours (e.g. diel migrations, Marta-

Fig. 5 Array and environmental data for deployments #1 and #2 of the second generation ESP in Monterey Bay, California. (a) temperature (°C) (b) salinity (PSU) (c) instrument depth (meters), and (d) chlorophyll-a (mg/L); (e) CCD counts for all probes that showed at least one positive signal during the two deployments (black EUK338 probe, red M2B probe, blue B1066 probe). Dashed lines indicate days when marine invertebrate larval arrays were processed in the ESP. Black dashed line corresponds to Julian day 85 when positive signals for the barnacle (B1066) and mussel (M2B) probes were detected.

Almeida *et al.* 2006), discrepancies would exist among taxa (Dibacco *et al.* 2001) despite the existence of common oceanographic conditions. By coupling a suite of current and traditional methodologies with the ESP instrument, previously unanswerable questions regarding larval identification and distribution can be addressed. If recruitment pulses are indeed short and episodic (Levin 2006), a larval detection mooring would provide an appropriate means for detection. To that end, we are currently constructing four additional copies of the 2G-ESP to provide an arrayed network of sensors. These copies will also serve the purpose of initiating technology transfer to interested parties.

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