

## Molecular Detection of Marine Invertebrate Larvae

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### Abstract

The ecological patterns of many invertebrate larvae remain an ongoing mystery, in large part owing to the difficult task of detecting them in the water column. The development of nucleic-acid-based technology has the potential to resolve this issue by direct identification and monitoring of embryonic and larval forms in situ. We report herein on the successful development and application of nucleic-acid-based sandwich hybridization assays that detect barnacles using rRNA-targeted probes with both group-(order Thoracica) and species-(*Balanus glandula*) specificity. Primary results include the determination of target 18S rRNA sequences and the construction of "capture" probes for detection of larvae using hybridization techniques. In addition, we modified existing protocols for whole cell hybridization of invertebrate larvae as confirmation of the sandwich hybridization results. We used both hybridization techniques successfully in the laboratory on a plankton time series collected over 3 months, as well as a week-long in situ deployment of the technique in Monterey Bay, CA. The adaptability of this technology promises to be further applicable to various organisms and could be used to enhance our understanding of larval presence in the world's oceans.

**Keywords:** *Balanus* — barnacle — hybridization — larvae — probe — ribosomal RNA

### Introduction

Populations of marine invertebrates are controlled in large part by the supply of larvae in the water

column (Thorson, 1950; Strathman, 1993; Eckman, 1996). Yet little is known about the seasonal and spatial abundance of most invertebrate larvae, as abundance depends on the reproductive timing of adults, on the duration and behavior of larvae, and on ocean circulation patterns. Considerable difficulties exist in sampling, detecting, identifying, and quantifying marine invertebrate larvae from planktonic samples, owing to the small size (less than 1 mm) and scarcity of the target organisms, and the existence of a diverse community of microorganisms within which they exist. Knowledge of invertebrate larval stages has traditionally derived from studies involving laboratory cultures (Brown and Roughgarden, 1985; Strathmann, 1987) and microscopic examination of field samples collected with nets, pumps, or settlement traps (Greene, 1990). The complex life cycles of many marine invertebrates confound morphological identification, however, because larval and adult stages often differ greatly. Furthermore, discrimination among larvae of closely related species may be difficult, owing to species diagnostic differences that are typically restricted to adult stages (Branscomb and Vedder, 1982; Shanks, 1986). Consequently, progress in understanding the population biology and community structure of marine invertebrates could be improved by the development of morphology-independent methods for reliable detection and identification of larvae in the water column.

This article describes a molecular technique for laboratory-based detection of larval invertebrates in natural marine samples based on the sandwich hybridization method developed by Scholin and co-workers to detect and quantify toxic marine protists (Scholin et al., 1996, 1999, in press). This technique is highly amenable to automation and has been used to detect target species in near real-time, autonomously, and in situ. The sandwich hybridization technique as referred to here involves detection of ribosomal RNA (18S rRNA), a molecular marker

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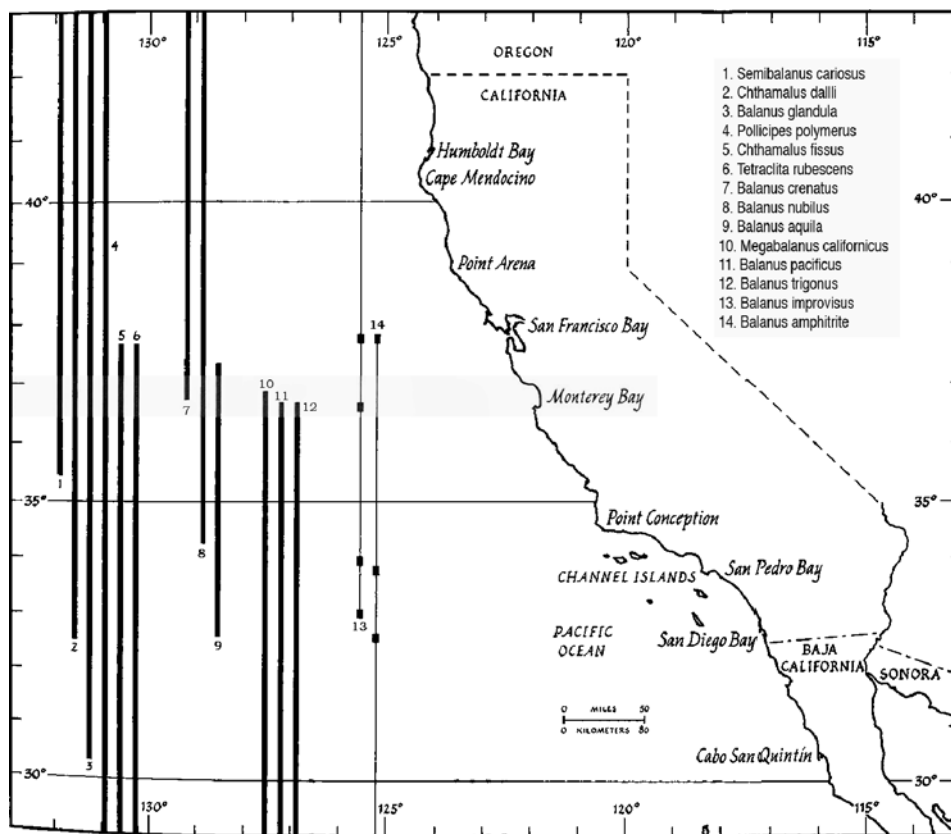
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that has been used to identify and assess species diversity for a broad range of organisms (Pace, 1997). To assess whether this method could be used to detect invertebrate larvae, we focused on near-shore barnacles (Cirrepedia), which are among the most abundant and intensively studied marine invertebrates inhabiting intertidal habitats along the west coast of North America, particularly in Monterey Bay (Figure 1). Adult barnacles brood fertilized eggs within the mantle cavity formed by their shells. A brood may contain thousands of nauplius larvae that hatch in about 30 days, feed and grow in the water column for about 11–21 days, undergo a series of molts, and eventually metamorphose into non-feeding cyprid larvae capable of settlement. Direct identification of barnacle larvae is difficult because the nauplii of many species (e.g., members of the genus *Balanus* in particular) tend to be similar in size, shape, and developmental pattern (Barnes and Barnes, 1959; Brown and Roughgarden, 1985). We have extended the scope of the nucleic-acid-based sandwich hybridization technology to include the detection of marine organisms larger than phytoplankton, specifically the larvae of shallow water

barnacle species. We also demonstrate deployment of these probes in situ as a possible means to assess the distribution of barnacle larvae in the water column remotely, in near real-time. With development of additional molecular probes the sandwich-hybridization method could be extended to other invertebrate species. With the incorporation of simultaneous, spatially separate, sampling events, this method also offers potential for studying the distribution, abundance, and dispersal of a variety of marine invertebrate larvae.

### Materials and Methods

**Sample Collection.** Native seawater (approximately 10 L) was sampled weekly from each of two sites: the Coast Guard Pier in Monterey, CA, and the Capitola Wharf in Santa Cruz County, CA. Five liters of each sample were concentrated through a 20- $\mu\text{m}$  Nitex<sup>®</sup> mesh (Sefar America Inc., Monterey Park, CA) and filtered through a 5- $\mu\text{m}$  hydrophilic Durapore filter (Millipore, Bedford, MA). Specimens retained on the Durapore filters were treated immediately in 3.5 ml of lysis buffer (described below)



**Fig. 1.** Latitudinal ranges for barnacle species along the California coast (modified from Newman and Abbott, 1980). *Balanus improvisus* (no. 13) and *Balanus amphitrite* (no. 14) represent introduced species found mostly in bays or estuaries, thus the range is not thought to be continuous.

for sandwich hybridization, or stored in liquid nitrogen for later analysis. Samples stored in liquid nitrogen were empirically determined to yield a higher (2–4×) signal intensity in sandwich hybridizations, compared to duplicate samples stored in formalin, acid Lugol's iodine, RNA Later (Ambion, Inc., Austin, TX), ethanol, or methanol. Larvae of unknown identity, obtained directly from seawater samples using a dissection microscope, were extracted for DNA (see below) or preserved in "modified saline ethanol" (70% EtOH, 2.5 × SET; Miller and Scholin, 2000) for whole cell hybridizations.

Adults of the acorn barnacles *Balanus glandula*, *Chthamalus fissus*, and *Tetraclita rubescens* were collected from the intertidal zone adjacent to Hopkins Marine Station (Pacific Grove, CA) for molecular and morphological identification. Egg sacs were removed from brooding females and aerated in filtered seawater at 15°C until larvae hatched. Larvae were counted, filtered, and either frozen in liquid nitrogen or preserved in modified saline ethanol. Adult tissues were either frozen in liquid nitrogen or immediately processed for molecular analysis (DNA sequencing or sandwich hybridization).

**DNA Amplification and Sequencing.** The DNEASY kit (Qiagen, Valencia, CA) was used according to the manufacturer's protocol to extract total genomic DNA from larvae or adult tissue. An approximately 1800-bp fragment of the 18S rRNA gene was amplified with universal 18S rDNA primers, 18e (5'-CTggTTgATCCTgCCAgT-3') and 18P (5'-TAATgATCCTTCCgCAggTTCACCT-3'; Halanych et al., 1998). Polymerase chain reaction (PCR) products were sequenced directly with an ABI 3100 DNA sequencer (Applied Biosystems, Inc., Foster City, CA), using the internal primers 18M (5'-gAACC-CAAAGACTTTggTTTC-3') and 18Qf (5'-gTTATCg-gAATTAACCAgACA-3'; Halanych et al., 1998). Sequences were aligned and proofread using Sequencher v 4.1 (Gene Codes Corp., Ann Arbor, MI). Greater than 98% similarity in 18S sequence was determined to be positive identification of unidentified larvae when compared with 18S sequences from known species obtained either by us (Accession Nos. AY789456–AY789459), or available in GenBank (noted below).

**Sandwich Hybridization.** Samples for sandwich hybridization generally included hatched larvae (10–1000 individuals) or native water (5–10 L), both concentrated on Durapore filters, or adult tissue (approximately 50 mg). Filters or tissue were lysed in guanidine lysis buffer for 5 min at 85°C. Lysis buffer (modified from Scholin et al., 1999)

contained 3 M guanidinium thiocyanate (GuSCN), 50 mM Tris, 15 mM EDTA, 2% Sarkosyl, and 0.2% sodium dodecyl sulfate (SDS) (vol/vol), at pH 8.9. The lysate was allowed to cool to room temperature and then filtered through a 0.45- $\mu$ m hydrophilic Durapore syringe filter (Millipore, Bedford, MA). Signal intensity of heat-lysed samples was empirically determined to be twice as high as those produced at room temperature with sonication in sandwich hybridization assays. At this stage, lysates could also be extracted for DNA sequencing.

Sandwich hybridization employs the use of two oligonucleotide probes. A biotinylated "capture probe," specific for a target organism, is conjugated to streptavidin and immobilized on a biotinylated support medium, in this case a polystyrene prong (Saigene Corp.; available from ORCA Research Inc., Bothell, WA). The target sequence from a crude environmental sample is hybridized with the capture probe and then "sandwiched" between the capture probe and a "signal probe" labeled with digoxigenin. The "sandwich" hybrids are detected by an anti-digoxigenin antibody/horseradish peroxidase (HRP) conjugate, which reacts with a substrate to produce a product that can be detected colorimetrically (modified after Scholin et al., 1999).

All hybridization steps were carried out using a robotic workstation (Saigene, Redmond, WA, see Scholin et al., 1996, 1999) that transfers polystyrene biotin-coated prongs through each row of a standard 96-well plate (Evergreen, Los Angeles, CA), containing reagents (250  $\mu$ l/well) in the following order: row H, sample; row G, capture probe; row F, signal cocktail (see below); row E, wash buffer (50 mM Tris, 0.15 M NaCl, 0.05% Tween-20); row D, digoxin-antibody + HRP (ImmunoPure<sup>®</sup> Peroxidase Conjugated Mouse Anti-Digoxin IgG diluted 1:1000 in Guardian<sup>™</sup> Peroxidase Conjugate Stabilizer/Diluent/Blocker, Pierce, Rockford, IL); rows C and B, wash buffer; row A, HRP substrate (1-Step<sup>™</sup> Ultra TMB-ELISA, Pierce, Rockford, IL). The sandwich hybridization protocol is as follows: 8 min/application of capture probe to solid support—row G; 8 min/capture of template DNA—row H; 8 min/exposure of capture probe–template complex to signal probe cocktail—row F; 2 min/wash—row E; 5 min/antibody application—row D; 2 min/wash—row C; 2 min/wash—row B; 5 min/substrate application—row A. All reagents used in the sandwich hybridization assay are available from ORCA Research Inc. (Bothell, WA). The entire sandwich hybridization protocol was carried out at 25 to 30°C and totaled approximately 50 min in duration. Optical densities ( $A_{450}$ ) of the end products in row A were determined by a Spectromax plate reader

(Molecular Devices, Sunnyvale, CA), after addition of 50  $\mu$ l of 10% H<sub>2</sub>SO<sub>4</sub> (vol/vol) per well.

The digoxigenin labeled-signal probes (double labeled at both the 5' and 3' ends) were synthesized commercially (Oligo's Etc, Eugene, OR). Signal probes consisted of Euk519, Euk915, and Euk1194 (identified by start position within the 18S region, Table 1; J. Tyrell unpublished, modified from Medlin et al., 1988). Concentrated stocks of signal probe (100  $\mu$ g/ml of water) were kept at 4°C. A final signal cocktail solution (100 ng/ml for each probe) was made in 0.5 M GuSCN signal solution modified after Scholin et al. (1999) (produced via dilution of a 2 M GuSCN signal solution with a signal diluent consisting of 50 mM Tris, 10 mM EDTA, 0.5% Tween-20, pH 8.6, both available from ORCA Research Inc., Bothell, WA).

**Capture Probe Development.** Capture probes consisted of general and specific barnacle probes targeting the 18S region (see specific results below). To design probes, we sequenced a portion of the 18S ribosomal gene from four barnacle species (Balanomorpha) commonly found in the vicinity of Monterey Bay, CA: *B. glandula*, *Tetraclita rubescens*, *Chthamalus fissus*, and *Lepas* sp. Comparative sequences of these and other species were also obtained from GenBank (Accession Nos. AF201663, AF201665, AF201671, AY520624, AY520628, AY520629, AY520631, AY520633, L26510). Specific regions of the 18S gene were targeted for probe development based on the following criteria: (1) desired specificity for a particular target species or targeted group of species; (2) proximity to known signal probes

(usually within 100–250 bp); and (3) a G+C content of 40% to 60%. For our assays, capture probe design criteria consisted of a melting temperature ( $T_m$ ) between 69° and 74°C, secondary structure stability less than 34°C, and a homodimer stability less than 17°C (calculated using OligoTech primer design software, Oligo's Etc, Eugene, OR). In addition, capture probes could not have greater than 70% similarity with signal probes or possible background interference would result. We initially targeted variable regions that were unique to barnacles in general (order Thoracica) and to *B. glandula* specifically. These probes were identified as B42, B468, B941, and B1066 (Figure 2 and Table 1). Euk338, a universal probe for protists that also targets many eukaryotes, was used as a positive control for biomass during sandwich hybridization experiments (Table 1; J. Tyrell unpublished, modified from Medlin et al., 1988).

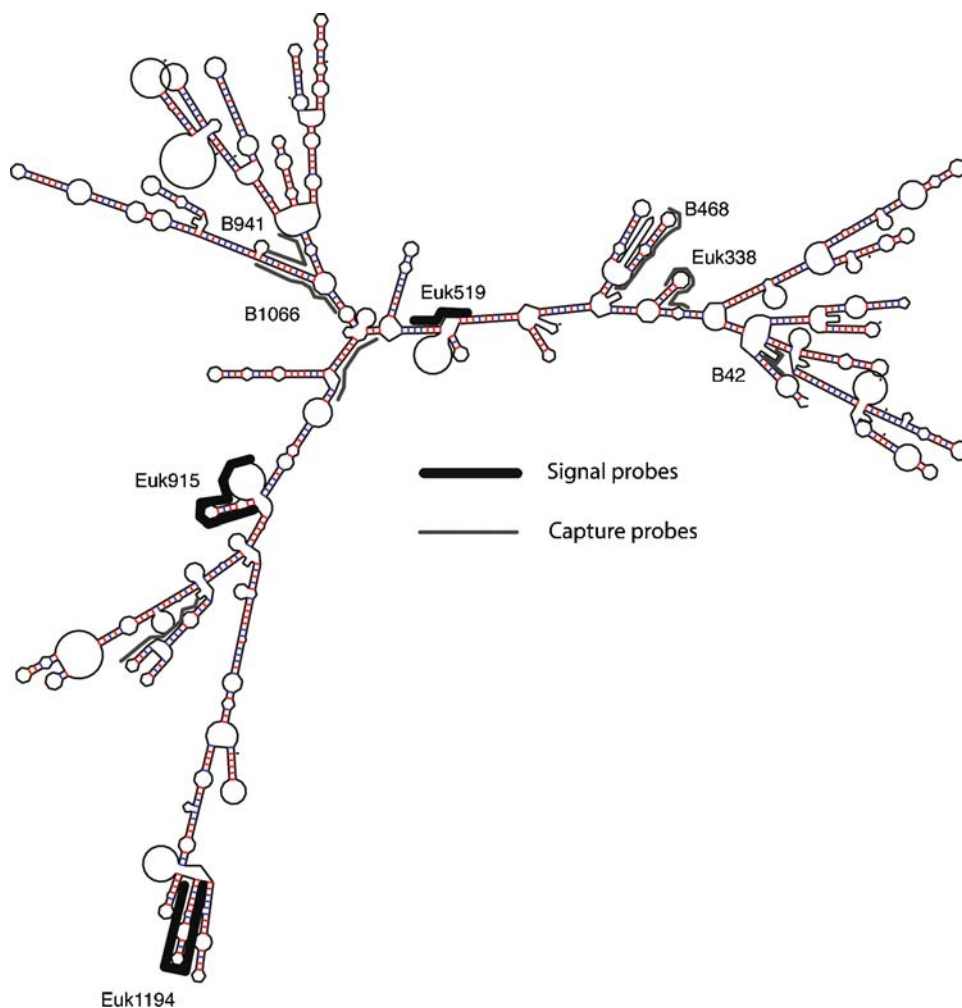
The 5'-biotinylated capture probes were synthesized commercially (Oligo's Etc, Eugene, OR). Concentrated probes (100  $\mu$ g/ml of water) were kept at 4°C until use. Working stocks of capture probes (400 ng/ml) were made in a phosphate buffer with the addition of streptavidin according to a proprietary formulation provided by Saigene Corporation (Seattle, WA). Capture solutions were stable at 4°C for several months.

An unlabeled custom oligonucleotide template ("linker") was used as an internal standard for the performance of the sandwich hybridization assay. This linker consisted of the reverse complement of the B1066 capture probe coupled to the reverse complement of the Euk915 signal target sequence via 3 T residues (Oligo's Etc, Eugene, OR). The

**Table 1. 18S Ribosomal Gene Sequences for Sandwich Hybridization Capture and Signal Probes, Whole Cell Hybridization Probes, and a Synthetic Positive Control "Linker" Oligonucleotide**

Target	Probe ID	Probe	$T_m$
Capture probes			
Barnacle general	B42	5'-Biotin-(C9)3-ATTCggCggTTCACCTTAgtgT-3'	71.3
Barnacle general	B468	5'-Biotin-(C9)3-gTTAACgAgACCTCTgTAAgg-3'	68.9
<i>Balanus glandula</i>	B941	5'-Biotin-(C9)3-TCgTCCAACggTCACAggATTTC-3'	73.5
Barnacle general	B1066	5'-Biotin-(C9)3-gTTgCggATTgCTggTCgAC-3'	72.3
Universal eukaryotic	Euk338	5'-Biotin-(C9)3-CCTgCTgCCTTCCTTggATg-3'	72.3
Signal probes			
Universal eukaryotic	Euk519	5'-Dig-CTggAATTACCgCggCTgCTg-dig-3'	74.7
Universal eukaryotic	Euk915	5'-Dig-gTgCCCTTCCgTCAATTCCTT-dig-3'	70.8
Universal eukaryotic	Euk1194	5'-Dig-CATCTAAgggCATCACAgACC-dig-3'	70.8
Whole cell probes			
Universal	UniC	5'-Fluor-gWATTACCgCggCKgCTg-3'	69.1
Negative	UniR	5'-Fluor-CAgCMgCCgCggTAATWC-3'	69.1
<i>Balanus glandula</i>	B941	5'-Fluor-TCgTCCAACggTCACAggATTTC-3'	73.5
Barnacle general	B1066	5'-Fluor-gTTgCggATTgCTggTCgAC-3'	72.3
Linker			
Barnacle general	B1066L	gTCgACCAgCAATCCgCAAC-(T)3-CAgCAgCCgCggTAATTCAG	Na

Dig, digoxigenin; Fluor, fluorescein; Na, not applicable.



**Fig. 2.** Predicted secondary structure of *B. glandula* 18S rRNA using GeneBee (<http://www.genebee.msu.su/genebee.html>). Approximate location of capture and signal probes (listed in Table 1) are indicated.

linker was resuspended in water, then diluted in lysis buffer at 0.01 and 0.04 ng/ml, giving expected absorbance values ( $A_{450}$ ) of 0.4 and 1.2, respectively. By comparing actual versus expected absorbance values for linker (control) samples, we were able to standardize the absorbance readings for the environmental and larval culture samples, to account for slight changes in final reading based on reagent batch and/or prong variability. Actual values did not vary by more than  $\pm 20\%$  from the expected value, and always occurred with a consistent over- or underestimate with regard to both concentrations.

**Whole Cell Hybridizations.** Native seawater or larval samples were filtered onto a 1.2- $\mu\text{m}$  pore size Isopore polycarbonate membrane (Millipore, Bedford, MA) and preserved for 2–24 h in a variety of fixatives. To achieve adequate fixation of environmental samples we evaluated the stability, and

suitability for in situ preservation for whole cell hybridization, of samples stored in modified saline ethanol solution (Miller and Scholin, 2000) versus other available preservatives, including methanol (90%), acid Lugol's iodine (5% wt/vol), RNA Later (Ambion, Inc., Austin, TX), Streck Tissue Fixative (S.T.F.; Streck Laboratories, Inc., La Vista, NE), and InSituFix and TissueFix (bio-World, Dublin, OH), for a 3-week period of time.

Preserved samples (pulled onto filters) were washed with 1 ml of  $5 \times$  SET hybridization buffer (5 min; Miller and Scholin, 1996), then suspended in 0.5 ml  $5 \times$  SET hybridization buffer containing a capture probe labeled with fluorescein (Table 1) at a final concentration of 5 ng/ $\mu\text{l}$ . Samples were incubated for 45 to 60 min at 45°C. After incubation, excess probe was removed via washing with 1 ml of prewarmed  $5 \times$  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, Eugene, OR). Fluorescently labeled oligonucleotide probes hybridized to the rRNA contained within intact cells, and were visualized using epifluorescence microscopy (Zeiss Axiophot 2 microscope; Chroma Technology filter, excitation 465–496 nm, dichroic 505 nm, emission 515–555 nm). The relative intensity of the capture probes was compared against positive (UniC probe) and negative (UniR, complementary to UniC) control probes to determine specificity and sensitivity (after Miller and Scholin, 1996; Table 1). Digital images were captured using a Spot SP100 cooled, charge coupled device camera (Diagnostic Instruments, Sterling Heights, MI).

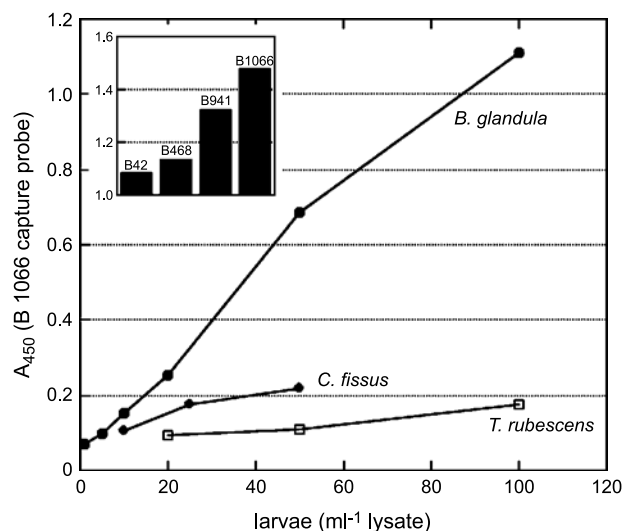
**In situ Larval Detection.** We employed the sandwich hybridization technique in situ during a deployment of an oceanographic instrument known as the Environmental Sample Processor (ESP; Scholin et al., 2001) off of Monterey, CA in October 2002. The ESP is an electromechanical/fluidic instrument system that remotely collects discrete subsurface water samples, concentrates microorganisms, and automates application of molecular probes in an array formatted sandwich hybridization assay (Scholin et al., 1998, in press). In addition, the ESP archives discrete samples for nucleic acid analysis, microscopy, and other types of analytical procedures after the instrument is recovered (e.g., by applying the modified saline ethanol fixative to discrete samples collected on filters stored in individual reaction chambers).

The ESP consists of five major subsystems: carousel, shuttle, clamp, syringe pump, and charge coupled device (CCD) camera. The carousel stores up to 100 reaction chambers, or “pucks,” which accommodate a wide variety of user-defined 25-mm diameter filters or chemically adsorptive media. An elevator and linear shuttle are used to move a chamber from the carousel to the processing position, where it is sealed in a clamp, thus providing connections to the sample port and reagent valve manifolds. The seals used in the clamp have embedded heater pads for temperature control from ambient to approximately 100°C at any time during a protocol. A syringe pump draws in seawater samples and dispenses the required reagents in a specified timed sequence. Modular valves support the use of up to 16 different reagents. Users control the ESP through a simple ASCII text-based language that defines the sequence of steps to be performed by the instrument. The sandwich hybridization chemistry used in the ESP was the same as that employed in the 96-well plate version of the assay

except that capture probes were arrayed on a filter medium (Scholin et al., unpublished) and solutions were passed sequentially over that array, a positive reaction resulted in the emission of light (SuperSignal ELISA Femto Maximum Sensitivity Substrate, Pierce, Rockford, IL), results of the assay were recorded by CCD camera, and an image of the resulting array was transmitted via radio modem to a remote location for interpretation. Reaction intensity of individual capture probes was scored as CCD counts [average spot intensity [raw signal from a given capture probe]—average intensity of background [signal from membrane outside of the spotted capture probe]]. The ESP was deployed at a fixed depth on a subsurface mooring.

## Results

**Capture Probe Evaluation.** Candidate capture probes were evaluated for activity, specificity, response, and sensitivity. The overall reactivity of each capture probe was evaluated against live *B. glandula* larvae (300 larvae/ml of lysate). Probes B42 and B468 had low levels of activity compared to the negative control. In contrast, probes B941 and B1066 had much higher activities (Figure 3, inset) and were therefore advanced to specificity trials. High guanidine concentrations were used to lyse cells and



**Fig. 3.** Absorbance values ( $A_{450}$ ) for capture probe B1066 after exposure to a larvae dilution series of three different barnacle species (closed circle, *B. glandula*; closed diamonds, *C. fissus*; open squares, *T. rubescens*). Regression lines for activity were:  $y = 0.0581 + 0.0109x$ ,  $R = 0.995$  for *B. glandula*,  $y = 0.0892 + 0.0027x$ ,  $R = 0.961$  for *C. fissus*, and  $y = 0.0659 + 0.0011x$ ,  $R = 0.985$  for *T. rubescens*. Inset, absorbance values of four separate capture probes after exposure to 300 *B. glandula* larvae/ml of lysate.

**Table 2. Sandwich Hybridization Specificity Test (via Absorbance Level) for Capture Probe B1066 on Exposure to Various Species**

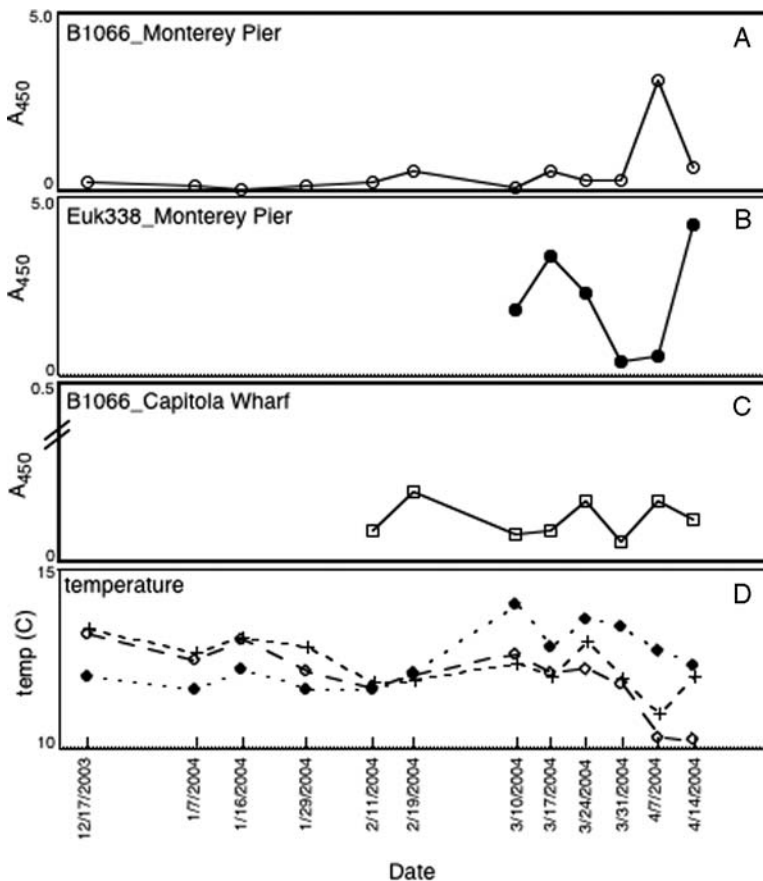
Species	Target	A <sub>450</sub>
<i>Balanus glandula</i> (barnacle)	Yes	2.886
<i>Tetraclita rubescens</i> (barnacle)	Yes	3.598
<i>Chthamalus fissus</i> (barnacle)	Yes	2.443
<i>Policipes polymerus</i> (barnacle)	Yes	3.459
<i>Asterina miniata</i> (starfish)	No	0.051
<i>Perampithoe humeralis</i> (amphipod)	No	0.063
<i>Pagurus samuelis</i> (crab)	No	0.056
Negative	None	0.051

ensure high fidelity in near-ambient temperature hybridizations (Van Ness and Chen, 1991). Consequently, it was difficult to predict how candidate probes would behave in high guanidine concentrations using conventional estimates of base mismatches, melting/dissociation temperatures, and secondary structure. For instance, in high GuSCN concentrations, the base pair mismatch G:U can be stable (G. Congelosi, personal communication; Van Ness and Chen, 1991).

The specificity of probes B941 and B1066 was determined via exposure to a defined series of tar-

get and nontarget species, including several barnacle species and crustacean outgroups. For example, the activity of the Thoracica group-specific probe (B1066), when assayed against tissue lysates from the target species versus two closely related and one distant nontarget species (Table 2), shows a higher absorbance with the specific target and a level comparable to the negative for nontargets. It is important to note that this particular specificity experiment using B1066 versus adult tissue was qualitative for reactivity only to the targets; thus, the absolute A<sub>450</sub> values measured are not quantitative and can vary depending on rRNA level, tissue type, and so forth.

The response of each capture probe was evaluated for the estimation of abundance of specific larvae via the sandwich hybridization assay. Dilution series of the target larvae were created, allowing for the establishment of standard curves and determination of lower limits of detection (sensitivity). Probes B941 and B1066 were evaluated against *B. glandula* larvae hatched in the laboratory. Both probes showed significant linearity over the range of larvae used (0–100 larvae/ml of lysate; see Figure 3 for B1066 results). For example, the linear



**Fig. 4.** Sandwich hybridization results (absorbance at 450 nm wavelength) for environmental samples collected during a time-course experiment. (A) Results from the barnacle specific B1066 capture probe for native water samples from the Coast Guard Pier–Monterey. (B) Results using the universal Euk338 capture probe for native water samples from the Coast Guard Pier–Monterey. (C) Results from the barnacle specific B1066 capture probe for native water samples from the Capitola Wharf, Santa Cruz, CA. (D) Temperature measurements at Hopkins Marine Station, Pacific Grove, CA, (closed diamonds), at the Monterey Bay Aquarium Research Institute, Moss Landing, CA M1 buoy (10 miles offshore, crosses), and the MBARI M2 buoy (28 miles offshore, open diamonds).

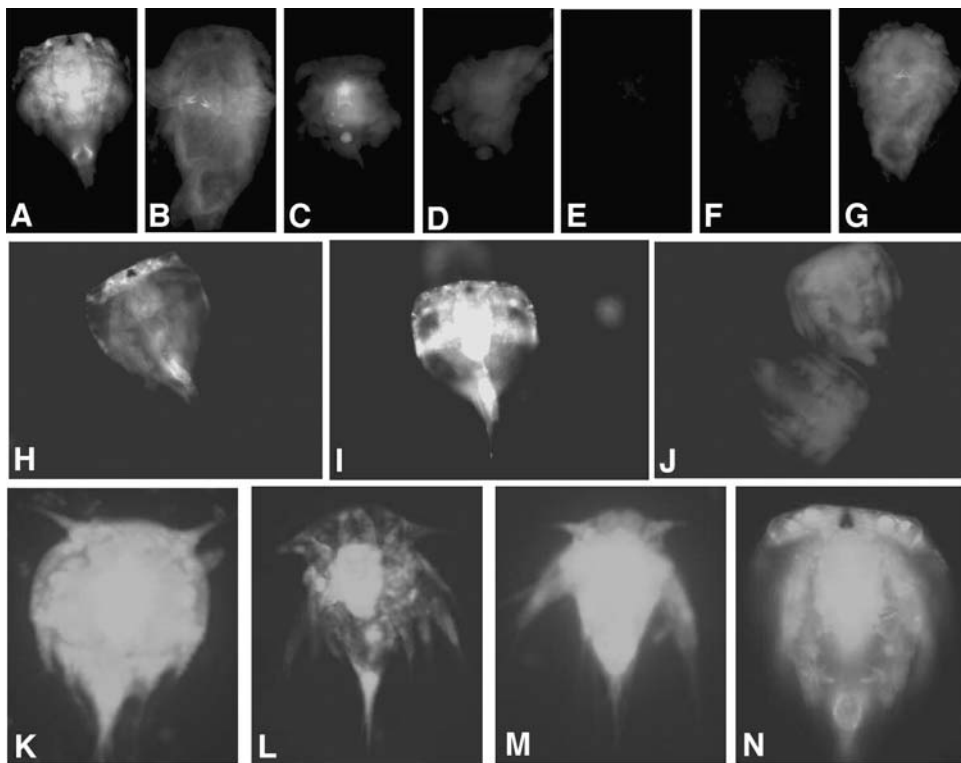
regression for B1066 tested against *B. glandula* larvae was  $y = 0.011x + 0.058$  ( $R = 0.99$ ) with a lower limit of detection at approximately 5 larvae/ml of lysate.

Although designed to be universal for all barnacles, B1066 showed significant variability to the three different barnacle larval species tested (Figure 3). The variable response of the B1066 capture probe may have resulted from differences in the amount of rRNA present in different organisms, possibility owing to developmental stage, and/or accessibility of the target sequences, among other factors. To account for this variability, larval abundance estimates for native water samples were calculated as averages based on linear regressions for all three species (Figure 3).

**Environmental Sampling—Benchtop Sandwich Hybridization.** Between December 17, 2003 and April 14, 2004, we conducted a time-course experiment in which native water samples were collected

one to four times per month from the Coast Guard Pier in Monterey, CA, and the Capitola Wharf in Santa Cruz County, CA. Temperature data was compiled from the near shore Hopkins Marine Station (Pacific Grove, CA) as well as offshore stations including the M1 and M2 buoys (10 and 28 miles offshore, respectively; Figure 4) managed by the Monterey Bay Aquarium Research Institute in Moss Landing, CA.

For native water samples collected during this time, the sandwich hybridization assay, using B1066 as a capture probe, detected three periods of larval presence near the Monterey Coast Guard Pier (Figure 4). The two lesser peaks occurred on February 19 and March 17–31, 2004 and consisted mainly of *Balanus crenatus* and *Balanus nubilus* larvae, respectively, based on sequencing of individual larvae. The largest peak observed on April 7, 2004 consisted primarily of *B. glandula* larvae, determined by sequencing of individual larvae. The signal detected at the Capitola Wharf



**Fig. 5.** Whole cell hybridizations using the universal probe UniC (labeled with a single fluorescein on the 5' end) versus *B. glandula* larvae after 3-week fixation in modified saline EtOH (A), RNALater (B), 10% formalin (C), InSitu Fix (D), TissueFix (E), S.T.F. (F), as well as a "no-probe" negative with modified saline ethanol (G). Comparison of whole cell hybridizations using the universal probe UniC labeled with one (H) versus two fluorescein molecules (I), labeled on both 5' and 3' ends), and the corresponding negative UniR control (J), labeled on both 5' and 3' ends). Whole cell hybridizations using B1066 on larvae of known identity including *Chthamalus fissus* (K), *B. nubilus* (L), *B. glandula* (M), and *B. crenatus* (N). All images were taken as color channel corrected and printed in grayscale using Adobe Photoshop 7.0.

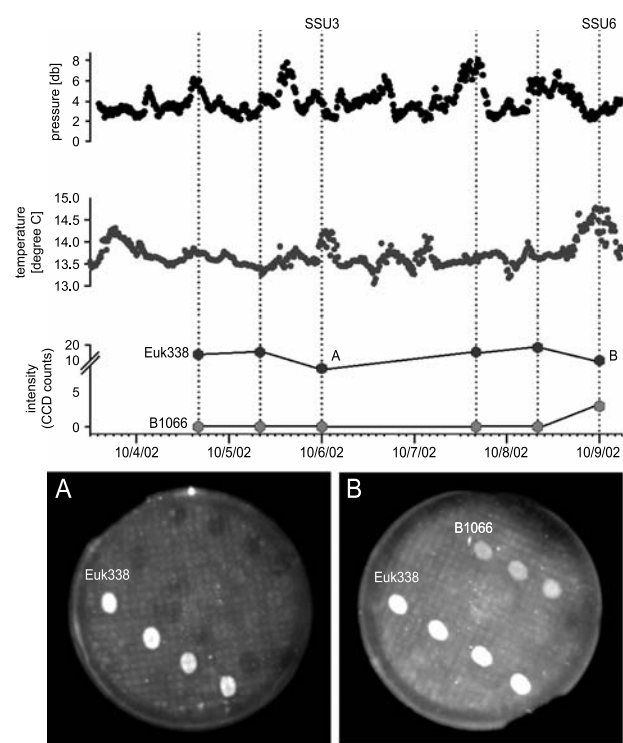
(approximately 20 nautical miles north of the Monterey Coast Guard Pier) followed the same pattern; however, signal intensity (inferred as total abundance of barnacle larvae) was lower by a factor of 10 (Figure 4).

The presence of barnacle larvae was not correlated with the signal resulting from total overall biomass (measured using the universal capture probe Euk338; Figure 4), suggesting the presence of organisms other than barnacles. This was confirmed via microscopy and/or direct sequencing, in which a variety of organisms were observed at each time point, including tintinnid ciliates, dinoflagellates (*Ceratium* and *Protoperidnium*), diatoms (*Pseudonitzschia*, *Chaetoceros*, *Asterionella*), cyclopoid and calanoid copepod larvae, and scolecid polychaete larvae.

**Whole Cell Assays.** Using net tow samples, native water, and newly hatched larvae, we evaluated a variety of common fixative types for invertebrate larval preservation and subsequent whole cell probing. Samples were treated with acid Lugols, modified saline EtOH, RNA Later, methanol, S.T.F., InSituFix, and TissueFix, for 2–6 weeks (Figure 5A–G). Post-fixation, samples were subjected to the whole cell hybridization protocol using the UniC universal probe, as well as the B941 and B1066 probes. “No probe” and UniR negative control treatments were used to gauge background autofluorescence and nonspecific probe binding, respectively. Modified saline EtOH proved to be the best fixative, based on preservation of cell integrity and optimal signal from fluorescent probes. This result was similar to previous studies on phytoplankton, in which samples were successfully stored for up to one month depending on the level of desiccation (Miller and Scholin, 2000). Labeling of whole cell hybridization probes with two fluorescein molecules greatly increased the signal, and only slightly increased the background (Figure 5H–J). Whole cell hybridizations with the universal UniC and barnacle B1066 probes were performed on barnacle larvae of known identity (Figure 5K–N) as well as environmental samples taken on February 19, March 17–31, and April 7, 2004, corresponding to peaks in sandwich hybridization intensity. Results of the whole cell assays confirmed the presence of barnacle larvae within the native water samples collected and analyzed with the sandwich hybridization assay.

**Environmental Sampling—In Situ Sandwich Hybridization.** Between October 4, 2002 and October 9, 2002, we employed the sandwich hybridi-

zation technique in situ during a deployment of an oceanographic instrument known as the Environmental Sample Processor (ESP), off of Monterey, CA (moored at approximately 5 miles offshore and 3 m below the surface). A conductivity–temperature–density (CTD) sensor was mounted to the ESP and provided contextual data. At all six time points, the Euk338 probe yielded a strong signal (example on array SSU3; Figure 6A), suggesting reliable, remote, automated sampling of organisms and development of sandwich hybridization arrays in situ. For most of the time points, the general barnacle probe B1066 produced very low signal, indistinguishable from background. On October 9, 2002, however, we re-



**Fig. 6.** In situ application of the sandwich hybridization technology. Pressure (db) and temperature ( $^{\circ}\text{C}$ ) as recorded by a conductivity–temperature–density (CTD) recorder, and signal intensity (in CCD counts) of the Euk338 and B1066 capture probes, as measured by a CCD camera, during a 6-day deployment of the Environmental Sample Processor (ESP) in Monterey Bay, CA in October 2002, at approximately 5 miles offshore and 3 m below the surface. Dotted lines represent time points during which the sandwich hybridization assay, using membranes carrying a suite of capture probes, was processed automatically and remotely. On all six days, the Euk338 probe yielded a strong signal (example on membrane array SSU3; (A), suggesting reliable sampling of the water column. A positive signal with the general barnacle probe (on membrane array SSU6; (B), was observed on the final day (October 9, 2002), which coincided with a low tide and warm water mass (arrowheads).

ceived a positive signal with B1066 (Figure 6B). The indication of barnacle larvae coincided with a low tide and warm water mass, as recorded by the CTD (Figure 6).

### Discussion

Understanding the fate of larvae in the plankton and the influence of oceanographic biophysical processes, such as circulation patterns and temperature/salinity gradients on larval dispersal remains a daunting task (Scheltema, 1986; Roughgarden et al., 1988; Mullineaux and Butman, 1991; Pineda, 2000; Palumbi, 2003). Certainly, no single method is likely to be robust enough to understand the complexity of larval movement within the marine realm. Therefore, a combination of methods concerning biophysical processes, larval life history, and actual confirmation of larval presence is necessary. The present molecular technology is a potentially useful tool for detecting, identifying, and even quantifying marine invertebrate larvae in situ. Although the concept of probe-based detection of species and biochemical substances is well established, routine application with environmental samples is only very recent (Levin et al., 1993; Scholin et al., 1997; MaKinster et al., 1999; Deagle et al., 2003; Caron et al., 2004).

Using morphology-independent nucleic acid based sandwich hybridization technology, we successfully detected larvae from water samples collected off the coast of central California. Between December 17, 2003 and April 14, 2004, we observed three peaks in larval abundance, corresponding to the balaniid barnacles, *B. crenatus* and *B. nubilus* (peaks on February 19 and March 17–31, 2004) and *B. glandula* (April 7, 2004). Previously published results suggest that *B. glandula* is typically present in February–May and again in December–June, however, *B. glandula* in our study did not appear in measurable numbers (the largest peak measured) until April. *B. crenatus*, the subtidal equivalent of *B. glandula*, was present for much of the spring. Typically, the nauplii larvae of this species enter shallower waters to develop, before descending to deeper recruitment depths (Branscomb and Vedder, 1982).

In addition to the benchtop assay, we also successfully adapted the sandwich hybridization for in situ field measurement of invertebrate larvae. This was accomplished using a new technology known as the Environmental Sample Processor, a compact, low-power, adaptable platform that is capable of performing a wide variety of sample processing, distribution, and analysis functions (Scholin et al.,

1998, in press). During our deployment, we successfully, and remotely, sampled the water column approximately 3–5 m below the surface at a distance of approximately 5 miles from shore. These samples were processed automatically using the sandwich hybridization protocol and the resulting image captured by CCD camera was relayed to shore for evaluation. During 6 days of monitoring the water column, the ESP reliably collected and processed organisms less than 500  $\mu\text{m}$  in diameter based on samples preserved by the ESP and examined by microscopy after the instrument was recovered, and by positive reactions with the “biomass control probe,” Euk338. On the last day of the deployment, we also received a positive signal from the barnacle group-specific probe, B1066, suggesting the presence of barnacle larvae in the water column. These larvae were not *B. glandula* based on a negative reaction with the *B. glandula* specific probe, B941. The positive B1066 signal correlated with notable changes in the physicochemical characteristics of the water column, including a low tide and increased temperature, signaling the movement of a water mass apparently carrying the barnacle larvae past the ESP at the time it acquired its sample.

Successful archival of discrete samples, for nucleic acid analysis or whole cell microscopy to name a few, is an important complementary sampling strategy to the sandwich hybridization assay. Invertebrate larvae, collected both by hand in native water samples and during automated archival sampling/preservation by the ESP during experiments presented here, were successfully hybridized and imaged using sandwich hybridization capture probes labeled with fluorescein rather than biotin. In this way, fluorescent whole cell examination not only allowed for confirmation of the sandwich hybridization results but also provided a better understanding of larval morphology.

Additional possibilities for validating sandwich hybridization results include independent molecular measures of barnacle larvae presence/abundance. We are currently optimizing both southern blotting and direct PCR/cloning of environmental ribotypes for larval identification and independent confirmation of the sandwich hybridization results. There are a number of methodological challenges to overcome, including establishing specificity and sensitivity limits, especially for blotting. For example, even pooled larvae ( $n = 20$ ), positively identified as *B. crenatus* and *B. nubilus* based on direct sequencing are currently below the detection limit of our blotting assay. A quantitative PCR technique (qPCR) could also be involved if truly quantitative results were desired. Although the probes used in

this study appear to detect relative abundance of organisms, as we observed linear responses with dilution curves, we currently consider the assay to be qualitative.

The sandwich hybridization technique employed here targeted regions of the nuclear ribosomal subunits, including 18S and 28S. These sequences, however, do not always distinguish between species of interest (e.g., shallow water mytilid mussels). Consequently we are assessing the utility of probes targeting other regions of DNA that offer finer taxonomic resolution. To that end, we are exploring the application of probes that target the mitochondrial cytochrome *c* oxidase (*mtCOI*) gene.

Sandwich hybridization technology can be used to detect a wide range of microorganisms, even to the species level, within complex native samples collected from the marine environment. In particular, it has proven to be a reliable and rapid benchtop screening method for the presence of larvae. It overcomes many of the difficulties that exist in detecting and identifying marine invertebrate larvae from planktonic samples. For example, small size is not a factor because the larvae are detected via unique nucleic acid signatures. The scarcity of the target organisms within a diverse community of microorganisms can also be overcome to some extent by optimizing the amount of sample collected and the sensitivity of the assay so that relatively few individuals of the target species are required to elicit a positive reaction. Larval dispersal, however, is more complicated than can be estimated from a limited sampling of one site over a very small time scale. The small size of invertebrate larvae (less than 500  $\mu\text{m}$ ) in relation to the potential scale for larval dispersal (on the order of kilometers) would benefit greatly from in situ detection and automated sampling technology. To that end, we also demonstrated the feasibility of applying molecular probes in an autonomous sensor system, the ESP, to the great task of linking environmental variability to the presence and movement of invertebrate larvae.

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