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SYMPOSIUM

Hydrodynamics of Larval Settlement from a Larva's Point of View

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Synopsis Many benthic marine invertebrate animals release larvae that are dispersed by ocean currents. These larvae swim and can respond to environmental factors such as chemical cues. However, larvae are so small (generally 0.01–1 mm) that they are often assumed to be passive particles whose trajectories are determined by the motion of the water in which they are riding. Therefore, marine larvae are useful model organisms to study the more general question of how the locomotion of very small animals in complex, variable natural habitats is affected by the motion of the fluid (water or air) around them. Studying larval locomotion under conditions of water flow encountered in nature is challenging because measuring the behavior of an individual microscopic organism requires high magnification imaging that is difficult to do in the field. The purpose of this article is to synthesize in one place the various approaches that we have been using to address the technical challenges of studying the locomotion of microscopic larvae in realistic ambient flow. The steps in our process include: (1) measuring water flow in the field; (2) mimicking realistic water movement in laboratory flumes to measure larval scale fluctuations in velocity of flow and concentration of chemical cues; (3) mimicking fine scale temporal patterns of larval encounters with a dissolved chemical cue to record larval responses; (4) using individual-based models to put larvae back into the larger scale environmental flow to determine trajectories; and (5) mimicking fine scale spatial and temporal patterns of larval encounters with water velocities and shear to determine the instantaneous forces on larvae. We illustrate these techniques using examples from our ongoing research on the settlement of larvae onto fouling communities and from our published work on settlement of larvae onto coral reefs. These examples show that water velocities and concentrations of chemical cues encountered by microscopic organisms can fluctuate in fractions of a second and vary over scales of less than a millimeter.

Introduction

Animals locomote in complex environments that vary in space and time. Studies of the mechanics of flight and swimming typically are done in still air or water, or in steady flow in a wind tunnel or flume (e.g., reviewed in Vogel 2003). However, when animals locomote in their natural habitats, they often are carried in ambient wind or water currents and can be buffeted by turbulence or waves. Therefore, to understand how animals locomote in the real world, we need to determine how their behavior and their trajectories are affected by ambient fluid motion.

The effects of ambient flow on the trajectories of very small organisms, (e.g., protozoans, marine larvae and other microscopic zooplankton, small insects) are more important than for larger organisms (e.g., adult fish or squid, whales, and birds) that are powerful swimmers or fliers. Thus, studying the effects of ambient fluid flow on the locomotion of tiny creatures is a good place to start unraveling how locomotion is altered by water flow or by wind, but measuring the behavior of very small organisms in realistic water currents or wind poses unique challenges. To determine how locomotory behavior affects the movement of very small organisms through the environment, their actions must be studied under conditions of water flow or wind like those they encounter in nature. However, measuring the behavior

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of an individual microscopic organism requires high magnification imaging of that organism, which is difficult to do in the field. We have faced these challenges in our past and ongoing studies of microscopic marine larvae.

The purpose of this article is to synthesize in one place a series of steps that we have been following to address the challenges of studying the locomotion of microscopic larvae in realistic ambient flow. We draw together here some approaches taken from different papers in our published body of work on the settlement of larvae onto coral reefs, as well as new techniques we are employing in ongoing studies of larval settlement into fouling communities. Our goal is not to review the topic of larval "settlement" (landing and attaching to a surface, the first step in recruitment of larvae into benthic communities), nor is it to present a new study of larval hydrodynamics. Rather, our intent is to outline technical approaches to studying small organisms in ambient flow, illustrated with examples from our past and ongoing studies of larvae. We hope that this compendium will also be useful to biologists who investigate the behavior and the settlement biology of marine invertebrate larvae.

Background: Approaches to studying larval hydrodynamics

The larvae of benthic marine invertebrates provide useful model systems for studying how the flow of ambient fluid affects animal locomotion. Many benthic marine invertebrates release microscopic larvae (generally 0.1–1 mm) that are dispersed by ocean currents. Although carried by ambient water movement, these larvae also swim and can respond to environmental factors such as light, shear, or chemical cues (reviewed by Koehl and Reidenbach 2007).

Richard Strathmann, together with his students and colleagues, led the way in studies of the fine scale hydrodynamics of larvae, including feeding mechanisms (Strathmann et al. 1972; Strathmann and Bonar 1976; Strathmann and Liese 1979; Strathmann 1982; Miner et al. 1999; Strathmann 2006; 2007), non-feeding ciliary currents (Emlet 1994; Hadfield et al. 1997), hydrodynamic consequences of drag and tethering (Emlet and Strathmann 1985; Emlet 1990) and of viscosity and temperature (Podolsky and Emlet 1993), as well as trade-offs between swimming and feeding performance (Byrne et al. 2001; Strathmann and Grünbaum 2006). All these studies were conducted in still water so that the details of the water currents produced by the larvae could be analyzed. Similarly, measurements of the swimming and sinking velocities of larvae typically have been made in still water (Butman et al. 1988a; Hadfield and Koehl 2004), as have studies of the kinematics of larval swimming (Williams 1994). Furthermore, most assays of larval responses to dissolved chemical cues have been done using uniform concentrations in still water (reviewed by Hadfield and Paul 2001). In contrast, larvae in the ocean swim, feed, and sink in moving water.

The importance of water flow to larval settlement has long been recognized (reviewed by Butman 1987; Abelson and Denny 1997; Koehl 2007). Some studies of larval behavior (Ableson 1997), trajectories (Jonsson et al. 1991; Tamburri et al. 1996; Finelli and Wethey 2003), or settlement onto the substratum (Butman et al. 1988b; Pawlik et al. 1991) were carried out in laboratory flumes in unidirectional currents, while other studies examined larval behavior in tanks in which vibrating grids created turbulence (Fuchs et al. 2004). None of these investigations incorporated the wave action typical of many shallow coastal areas, nor did they examine the instantaneous fine scale fluctuations in velocity or in the concentrations of chemical cues experienced by individual larvae carried in the moving water.

Our recent research on the process of larval settlement has focused on studying the responses of larvae on the fine spatial scales pioneered by Strathmann, but doing so when the larvae are exposed to instantaneous patterns of water flow and concentrations of chemical cues similar to those they encounter in the field. The steps we follow in this research are: (1) measuring water flow in the field; (2) mimicking realistic water movement in laboratory flumes to measure larval scale fluctuations in velocity of flow and concentration of chemical cues; (3) mimicking fine scale temporal patterns of larval encounters with a dissolved chemical cue to record larval responses; (4) using individual-based models to put larvae back into the larger scale environmental flow to determine trajectories; and (5) mimicking fine scale spatial and temporal patterns of larval encounters with water velocities and shear to determine the instantaneous forces on larvae.

In this article we describe these steps for studying the fine scale hydrodynamic environment of microscopic organisms by using examples from our ongoing research on the settlement of larvae onto fouling communities (assemblages of organisms growing on hard surfaces in harbors and estuaries) and from our published work on settlement of larvae onto coral reefs. Fouling communities have been used for

many years as model systems for studying ecological succession (Sutherland and Karlson, 1977; Bram et al. 2005), the process whereby communities are established and develop over time. Therefore, fouling communities are ideal for investigating how the rugosity of the substratum affects the flow experienced by larvae (reviewed by Koehl 2007; Rittschoff et al. 2007), because the topography of the community changes with time as surfaces are first colonized by a biofilm of bacteria and other micro-organisms, and then by larger multicellular organisms (Holm et al. 2000). In the past our investigation of the effects of surface rugosity focused on taller, more complex topography when we studied coral reefs. In addition, we also used coral reefs to study how water-borne chemical cues affect larval settlement in ambient water flow, focusing on larvae of the sea slug *Phestilla sibogae*. We selected larvae of *P. sibogae* because they had been shown in the laboratory (in still water) to settle and undergo metamorphosis in response to a dissolved species-specific metabolite of their prey, *Porites compressa*, an abundant coral that forms reefs in shallow, wave-dominated habitats in Hawaii (Hadfield 1977).

Step 1: Measuring water flow in the field

Our first step in determining how ambient water flow affects settling larvae is to measure water velocities in the field in habitats in which the larvae recruit into benthic communities. Such data on field flow enable us to design realistic flow in laboratory flumes where we can conduct further studies on even finer scales.

When water flows past a stationary substratum, a velocity gradient (the "boundary layer") develops in the water between the surface of the substratum and the free stream current (reviewed for biologists by Nowell and Jumars 1984; Vogel 1994; Koehl 2007). Therefore, the velocities encountered by larvae near benthic communities are slower near the substratum than they are higher in the water column. Boundary layers in marine habitats over macroscopic surfaces are turbulent; the velocity gradient in a turbulent boundary layer is steepest close to the solid surface. Therefore, to assess the water motion experienced by larvae as they approach benthic communities, we measure water velocity profiles above those surfaces on the scale of centimeters, rather than just determining free stream current speeds at field sites. As eddies swirl around in a turbulent boundary layer, water and the larvae and dissolved chemical cues it carries are transported to and from the benthos. Therefore, we also characterize the rapid fluctuations in flow velocity due to turbulence and waves, rather than simply determining mean velocities.

Flow across fouling communities in a harbor

In our present study of fouling communities, we are using an acoustic Doppler velocimeter, "ADV" (Sontek 16 MHz MicroADV) to measure the water velocity profiles near panels on which fouling communities are growing. The panels (25 cm × 30.5 cm sheets of PVC) are mounted vertically on submerged racks rigidly attached to a dock in Pearl Harbor on the Island of Oahu, HI, where fouling communities develop on them over time (details described by Holm et al. 2000). An ADV is a very useful instrument for characterizing the fine scale velocity fluctuations due to turbulence because it can be deployed close to surfaces ($\geq$1 cm) (Finelli et al. 1999) and has a small sampling volume (0.25 cm^3), a rapid response time and a high sampling rate (25 Hz). To determine water velocity profiles in the boundary layers that develop in the water flowing past the fouling communities, we measure velocity as a function of time at positions 2, 4, 8, 16, and 22 cm from panels on which fouling communities have developed. We have been measuring water velocities past fouled panels at two sites in Pearl Harbor at different stages of the tidal cycle. An example of our ADV data shown in Fig. 1A for a position 4 cm from a fouled panel illustrates the rapidly fluctuating nature of the water flow. In order to mimic such flow conditions in the laboratory, we characterize turbulence and identify wave frequencies by calculating the power spectral density of each velocity record by Welch's method (Welch 1967; Rabiner and Gold 1975) using Matlab Signal Processing Toolbox 4.3 software. Such spectra indicate how much of the variation in velocity is due to fluctuations at different frequencies (an example of such a spectrum is shown in Fig. 1B). Flow in our laboratory wave flumes is designed so that the wave frequencies and fine scale turbulence spectra match the spectra of our field data (techniques described by Reidenbach et al. 2009).

Although our study of flow over fouling communities is still in progress, our measurements of velocity as a function of time near fouled surfaces on docks are already revealing the rapid variations in velocity of the water in which larvae swim. Although mean velocities are quite low (a few cm/s) in a harbor, instantaneous velocities fluctuate a great deal due to turbulence and to the oscillations in velocity due to wind chop, which has periods of 1–1.5 s (Fig. 1A). We are also finding that fouling

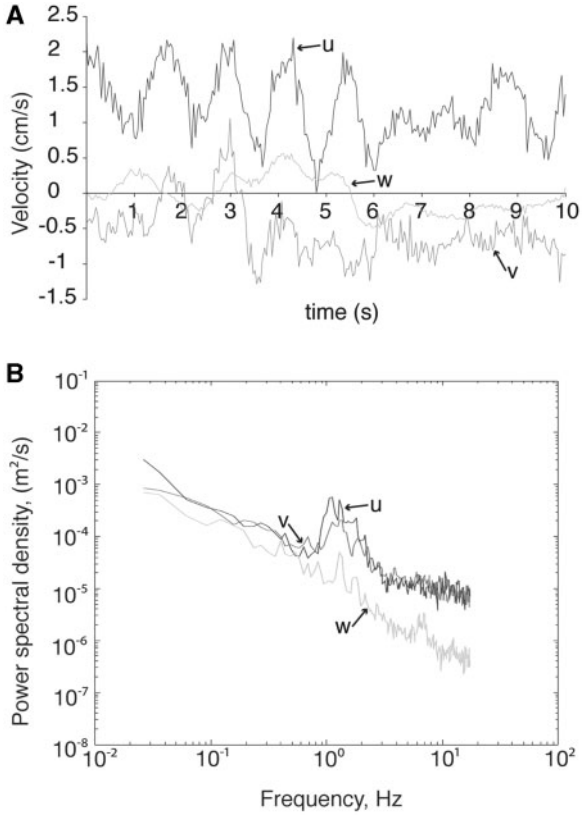

Fig. 1 An example of water velocities measured with an acoustic Doppler velocimeter (ADV) at a position 4 cm from the surface of a late stage fouling community growing on a panel mounted vertically on a dock in Pearl Harbor, HI, USA. Velocities were recorded for a 3 min period during which no ship wakes hit the dock. (A) Water velocity plotted as a function of time for a 10 s segment of the flow record. The black tracing shows the horizontal component of the water velocity parallel to the surface of the panel (u), the dark grey tracing indicates the vertical component of the velocity parallel to the surface of the vertically mounted panel (v), and the light gray tracing shows the component of velocity normal to the surface of the panel (w). The large oscillations in velocity at 0.7 Hz are due to small waves caused by the wind ("wind chop"), and the other fluctuations in velocity are due to turbulence. (B) Power spectral density (PSD) plotted as a function of frequency for the 3 min record of velocities shown in (A). The peak in PSD at 0.7 Hz shows the fluctuations in velocity due to wind chop, and the PSD at higher frequencies indicates the fluctuations in velocity due to the small scale turbulent eddies that must be mimicked in our laboratory flumes.

communities in a harbor can be exposed frequently to transient larger waves (with peak velocities generally 10–20 cm/s just 2 cm above fouling communities) due to the wakes of boats and ships passing nearby. Measurements of water velocities as a function of time near docks at other sites have also shown that instantaneous velocities vary due to turbulence and to the oscillations of velocity caused by wind chop and the wakes of boats (Okamura 1984; Hunter 1988; Schabes 1992; Koehl 2007; Rittschoff et al. 2007). Although our harbor field site is not exposed to rapid currents, other sites where fouling communities occur can be exposed to swift tidal currents (Rittschoff et al. 2007).

Flow above and through coral reefs

For our study of the hydrodynamics of settling larvae of *Phestilla sibogae*, we measured water velocity profiles above and within shallow coral reefs dominated by *Porites compressa* in Kaneohe Bay on the island of Oahu, HI (Koehl and Hadfield 2004; Koehl and Reidenbach 2007; Reidenbach et al. 2008). We selected two reef sites dominated by living *Porites compressa* from which *P. sibogae* had been collected. We measured water velocities at the sites at different times during the tidal cycle, and on days characterized by different wind and wave conditions. Those reefs were in a shallow central region of Kaneohe Bay where wave-driven flow moves onshore (Bathen 1968; Lowe et al. 2009). In addition to measuring water velocity profiles above the reefs using ADV, we simultaneously employed electromagnetic flow meters to measure flow velocities through the interstices within reefs at different distances below the reef surface where ADV's could not be deployed. These field measurements revealed that the shallow reef habitats in which the larvae of *P. sibogae* settle onto corals are subjected to turbulent, wave-driven flow. At our reef sites, the oscillatory flow due to waves had periods of 5–10 s and peak instantaneous free stream velocities of ~10 cm/s (similar to those measured at other Kaneohe reef sites by Lowe et al. 2005), but on very wavy days peak instantaneous velocities reached 30–40 cm/s. We measured peak velocities 5 cm above the reef surface that were about half the magnitude of free stream. Water in waves moves up and down as well as back and forth, and we measured oscillatory vertical flow into and out of the porous surface of the reef, finding peak velocities of ~12 cm/s. We measured much slower vertical and horizontal oscillations through the spaces within reefs, with peak velocities of only 2–4 cm/s. As the waves sloshed back and forth above the reefs, there was a slow net flow shoreward at ~3 cm/s (within the range of values reported by Lowe at al. 2009 for other sites in Kaneohe Bay). In contrast, we found that net shoreward flow within the reefs was only ~0.5 cm/s. We also measured slow (~1.5 cm/s) net upward flow coming out of the reefs in areas where the reef surface was flat or convex, and net

downward flow into the reefs in areas where there were depressions in the surface.

The physical forcing mechanisms driving large scale water flow in Kaneohe Bay have been studied using long term, spatially coarse measurements of water velocities and wave heights (Lowe et al. 2005, 2009). Unfortunately, those data are not useful to biologists who need to recreate the fine scale flow encounterd by settling larvae. While a single hydraulic roughness length can be used to describe the effect of reefs on large scale flow in the bay (Lowe et al. 2005), the local effects on water flow of bumps, depressions, and interstices of coral heads are critical to the fates of settling larvae.

In summary, our field measurements reveal that water flow in the boundary layers near fouling communities and coral reefs are characterized by velocity oscillations due to waves and by rapid velocity fluctuations due to turbulence. These non-steady-state features of the flow should be mimicked in laboratory studies of larval behavior.

Step 2: Mimicking realistic water movement in the laboratory to measure larval scale fluctuations in velocity of flow and concentration of chemical cues

We have been using wave-current flumes in the laboratory to produce boundary layer velocity profiles, waves, and fine scale turbulence spectra to mimic the water flow we measured near fouling communities or coral reefs in the field. Details of how such water flow is produced in a flume and ground-truthed to field flow are given by Koehl and Reidenbach (2007) and Reidenbach et al. (2007). By working in laboratory flumes, we are able to measure on the fine scales experienced by larvae (milliseconds and fractions of millimeters) the instantaneous water velocities and the concentrations of chemical cues released by the benthos. Such fine scale measurements are made by using laser-based techniques: (1) particle–image velocimetry (PIV) and laser Doppler velocimetry (LDV) for determining water velocities; and (2) planar laser-induced fluorescence (PLIF) for measuring instantaneous spatial distributions of concentrations of dissolved substances.

Fine scale flow over fouling communities

As fouling communities develop on surfaces, their topographic relief increases as settlers accumulate and grow. Newly submerged surfaces in Pearl Harbor are rapidly overgrown by a biofilm of bacteria and other micro-organisms, and then by a succession of larger multicellular organisms (Holm et al. 2000; Shikuma and Hadfield 2006). In this bay, biofilmed surfaces are first colonized by the tubeworm *Hydroides elegans*, which builds to great densities within a month. After a year, surfaces are densely coated by a complex, lumpy assemblage of other invertebrates, including sponges, bryozoans, solitary, and compound ascidians, oysters, sabellid polychaetes, and barnacles. For our ongoing flume studies we prepare surfaces with topographies representing different successional stages of developing fouling communities by freeze-drying PVC panels that have been accumulating organisms over different periods of time in Pearl Harbor. We cannot use living fouling communities because reflections from their surfaces interfere with the laser techniques we employ. The communities that we freeze-dry (in a Vertis Freezemobile 12ES Lyophilizer) retain the topography of living communities and can be spray painted matte black for PIV, LDV, and PLIF studies. An example of an early stage fouling community dominated by *H. elegans* that was prepared in this way is shown in Fig. 2A, and of a late-stage community in Fig. 2B.

In our present research we expose the lyophilized fouling communities to various realistic regimes of water flow and use simultaneous PIV and PLIF to map the time-varying fine scale distributions in the water near each community of concentrations of dissolved substances released from the surface and of water velocities (Fig. 2). Fine scale water flow can affect larval trajectories in several ways. The local instantaneous velocity of the water around a larva moves the larva relative to the substratum, and the water shear a larva experiences may reorient it (Grunbaum and Strathmann 2003) or elicit a behavioral response (Fuchs et al. 2004). Dissolved chemicals released by benthic organisms also can induce behavioral responses by the larvae of some species of benthic animals (e.g., sea slugs, Hadfield and Pennington 1990, Hadfield and Koehl 2004; oysters, Tamburri et al. 1996). Although water-borne chemicals from the benthos do not affect the settlement behavior of *H. elegans* (Carpizo-Ituarte and Hadfield 1998; Huang and Hadfield 2003), odors from the substratum may influence the behavior of larvae of other species in the fouling community.

Maps of instantaneous water velocities are constructed using PIV. Neutrally buoyant particles (silver-coated, air-filled glass spheres, diameter = 11 μm, Potter Industries) carried in the water are illuminated by a thin plane (2 mm thick) of laser light (Melles Griot DPSS 546 nm green 3 W laser) and recorded at 63 frames/s using a digital camera (AOS High Speed Digital Imaging System and AOS

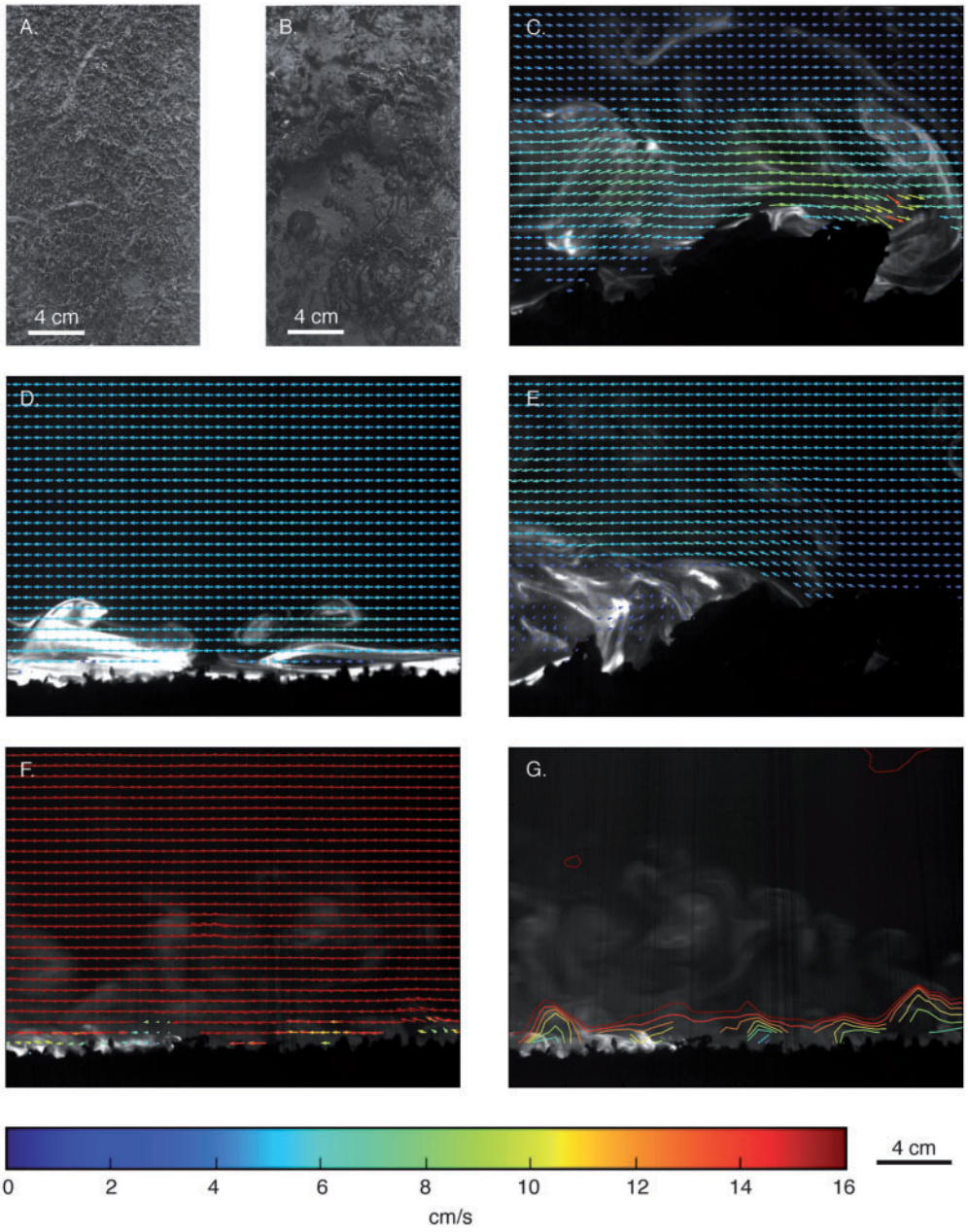

Fig. 2 Flat panels bearing an early stage fouling community (A, D, F, G) and a more rugose late stage fouling community (B, C, E) studied using simultaneous PLIF and PIV in a wave flume (M. Koehl, M. Hadfield, and J. Jaffe, unpublished data). (A) Surface view of an area within an early stage fouling community dominated by *H. elegans* on a panel that had been lyophilized and painted black. (B) Surface view of an area within a late stage fouling community composed mainly of ascidians, sponges, oysters, and barnacles on a panel that had been lyophilized and painted black. (C–G) are single frames from high speed videos of fluorescent dye (an analog of dissolved substances, "cues", from the benthos) swept from the substratum by moving water and illuminated by a sheet of laser light (PLIF). The brighter (i.e., lighter) a pixel, the higher the concentration of "cues" at that position in the water. In (C–F), colored velocity vectors measured using simultaneous PIV are superimposed on these video frames. (C) Late stage fouling community subjected to flow typical of wind chop. (D) Early stage community exposed to wind chop, shown at the same stage in the wave cycle as illustrated in (E) (E) Late stage fouling community subjected to wind chop, but shown at a different stage of the wave cycle than that shown in (C). (F) Early stage community subjected to water flow like that encountered in the field when a ship wake hits a dock. (G) Velocity contours calculated from simultaneous PIV data superimposed on a PLIF image of an early stage community exposed to flow like that from a ship's wake, as shown in (F). The closer together the contours, the steeper the velocity gradient and the higher the shear at that position in the water. (Scales for velocity and size scales for C–G at bottom of figure).

Imaging Studio V2 software; 50 mm AF Nikkor lens). We use PIV software (MatPIV 1.6.1) to calculate water velocities. Each video frame is divided into a large number of windows; the spatial pattern of pixel brightness in each window indicates the positions of particles in that area of the water. The location in the next frame of the video of that group of particles (i.e., pattern of pixel brightness) is calculated using a cross-correlation technique. The displacement vectors for the groups of particles in each of the windows are divided by the time between the video frames to produce of map of velocity vectors. Examples from our ongoing study of such velocity vector maps are shown in Fig. 2C–F. The relative velocities of adjacent vectors are then used to calculate local instantaneous velocity gradients (Fig. 2G).

While the PIV measurements of velocity are being made, we simultaneously use PLIF to measure fine scale instantaneous distributions of concentrations of dissolved chemicals released from the substratum. To mimic the release of dissolved substances from fouling organisms, we fill a reservoir below a panel with fluorescent dye dissolved in seawater (0.2 g/L Rhodamine WT, light excitation peak 558 nm, emission peak 582 nm) that oozes out of the substratum via a row of small holes (2 mm diameter) along the midline of the panel. Using dye to measure the dispersal of dissolved substances by moving water is justified because the Schmidt number, Sc, for water is high (>1000). Sc is a dimensionless number that represents the ratio of the rate at which momentum is spread through a fluid by viscosity to the rate at which chemical structures (like odor filaments) are dispersed through the fluid by molecular diffusion ($Sc = \nu/D$, where ν is the kinematic viscosity of the fluid, and D is the molecular diffusivity of the dissolved substance in the fluid). At high Sc's, the millimeter-scale patterns of the concentration of dye and dissolved chemical cues swept from the substratum will be very similar to each other because molecular diffusivity is so low relative to the water's kinematic viscosity (Koehl and Reidenbach 2007). The plane of green laser light used for PIV also illuminates a slice of the dye plume, causing the dye to fluoresce. We record the motions of the dye using a second high speed camera (the "PLIF camera") synchronized to the camera recording the particle trajectories for PIV. The PLIF camera is fitted with a 552 nm high pass filter (Oriel Corporation) so that only emitted light from the fluorescent dye is imaged. Pixel brightness recorded by the PLIF camera is proportional to the dye's concentration (calibration described in Crimaldi and Koseff 2001).

Although not completed, our PIV/PLIF studies are already revealing how water velocities and concentrations of dissolved chemicals released from the substratum vary with space and time on the fine scales relevant to larvae. For example, laser illumination of just a slice of the cloud of dye (i.e., of dissolved odors released by the community) in the water moving above fouling communities shows that fine filaments of odor swirl around in odor-free water (Figs 2C–G). Therefore, as microscopic larvae swim or sink through the water, they move into and out of strips of odor rather than encountering a continuous diffuse concentration gradient, as had been assumed in earlier models of larval settlement (Eckman et al. 1994). Our pilot data show that odor hugs the surface of a fouling community in the rapid flow caused by a ship wake (Fig. 2C) more so than during slower wind chop (Fig. 2E). Furthermore, odor is mixed farther away from diverse, rugose late stage fouling communities (Fig. 2D) than from less rugose early stage communities dominated by *H. elegans* exposed to the same ambient water flow (Fig. 2E). In the realistic turbulent wave-driven flow used in our studies, flow-velocity fields and distributions of odor change rapidly with time (compare Fig. 2D and E, which show the velocity and dye distributions measured less than a second apart near a late stage fouling community exposed to wind chop flow conditions). Furthermore, velocity gradients are steeper, and thus shear is greater, near the substratum than in the water column farther away from the fouling community (Fig. 2G).

Fine scale flow over coral reefs

Our field measurements of water velocities above coral reefs were used to design the water flow in a large wave-current flume (12.5 m long by 1.2 m wide) in which a section of coral "reef" was constructed from skeletons of *Porites compressa* (technical details given by Reidenbach et al. 2007, 2008; Koehl and Reidenbach 2008). Use of coral skeletons to study the flow of water over reefs was justified by the data of Baird and Atkinson (1997), who found little difference between the overall drag, Reynolds stress, roughness length scale, and mass-transfer coefficient of living *P. compressa* and of their skeletons. The spatially and temporally varying wave-driven water flow in the flume was characterized using ADV. To mimic the release of a chemical cue from the corals, the coral skeletons were painted with a thin coating of gelatin and fluorescent dye (Rhodamine 6G); as the gelatin slowly dissolved,

dye was released into the water to simulate dissolved cue leaching continuously from *P. compressa*.

PLIF was employed to determine the fine scale, rapidly changing spatial distribution of dye (i.e., settlement cue) released from coral surfaces into the water above the reef. These measurements were done using techniques described in detail by Reidenbach et al. (2007) and Koehl and Reidenbach (2007). Athough the wave-driven water movement over coral reefs was faster and more turbulent than the flow we are now measuring over fouling communities in a harbor, PLIF revealed that fine filaments of cue swirled around in cue-free water above a reef (Fig. 3) (Reidenbach et al. 2007; Koehl et al. 2007; Koehl and Reidenbach 2007).

Our flume studies of fouling communities (ongoing) and coral reefs (published) show that, across a range of flow habitats near shallow benthic communities, microscopic larvae swim in wavy turbulent environments characterized by thin patches of chemical signals and by velocities that vary over spatial scales of millimeters and temporal scales of fractions

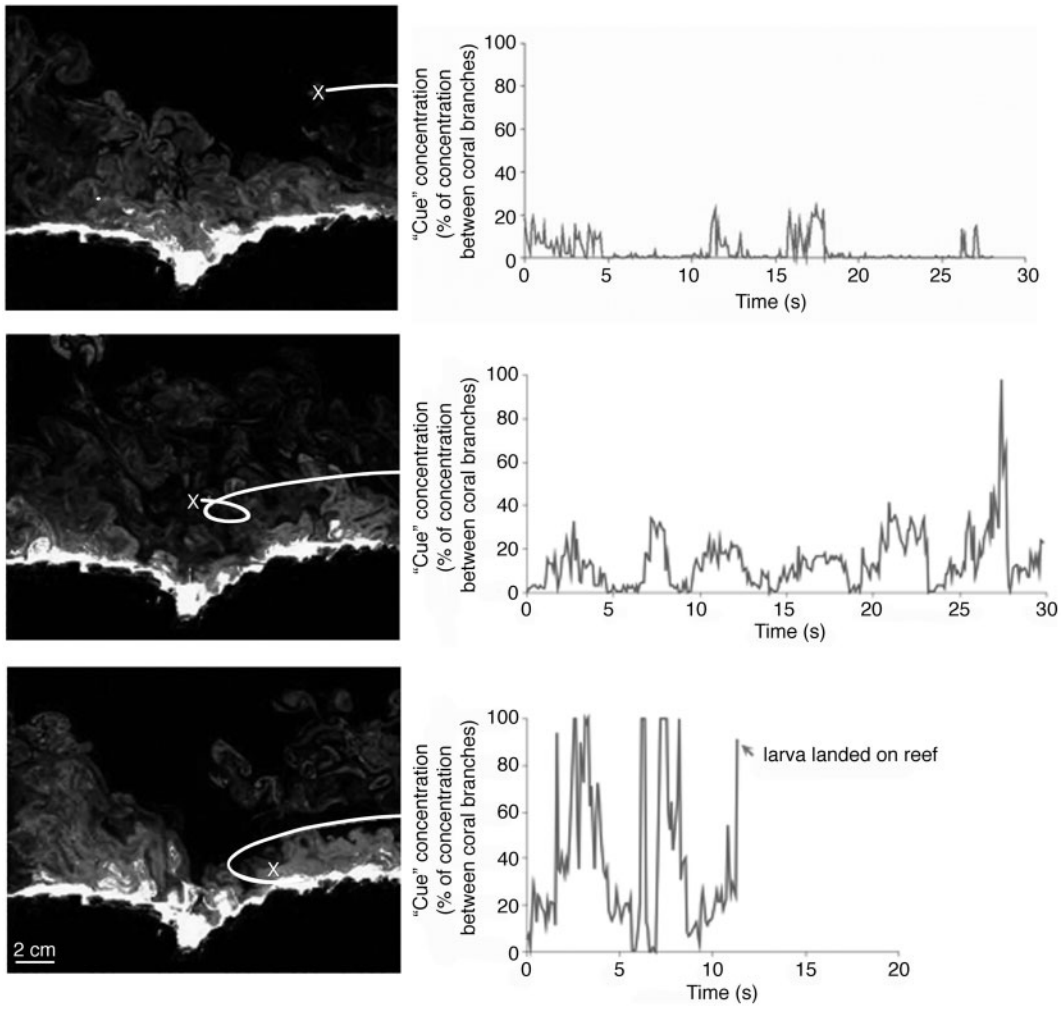

Fig. 3 Calculated patterns of cue concentration encountered by larvae of *P. sibogae* carried in the water above a coral reef. Images on the left are frames from a PLIF video (made by M. Reidenbach) of dye dissolving from the surface of a "reef" of *P. compressa* (black shapes at the bottom of each image) exposed in a flume to wave-driven turbulent water flow like that measured in the field. Dye dissolving from the reef surface was mixed into the water flowing above the reef and was illuminated by a sheet of laser light (PLIF). The brighter (i.e., lighter) a pixel in the water above the coral, the higher the concentration of "cue" at that position in the water. The "x" in each image shows the calculated position of a larva in that frame of the video, and the white line shows the calculated trajectory that the larva followed during previous frames (calculations described in Koehl et al. 2007). The graph to the right of each image plots the concentration encountered by the larva as a function of time as it moved along the trajectory shown in the image. The top image and graph show "cue" encounters for a larva higher above the reef than for the larva shown in the middle image and graph. The bottom image and graph show the "cue" encounters for a larva just before it landed on the reef.

of a second. The next challenge is to reproduce these fluctuating conditions in fine scale laboratory studies of the behavior of microscopic larvae.

Step 3: Mimicking fine scale temporal patterns of larval encounters with a dissolved chemical cue to record larval responses

Our study of how larvae of *Phestilla sibogae* behaved during realistic encounters with a dissolved settlement cue released into turbulent, wave-driven water flow by benthic *Porites compressa* provides an example of how flume data can be used to design experiments on the scale of a larva (Hadfield and Koehl 2004; Koehl et al. 2007).

As larvae swim or sink through water in which filaments of a chemical cue from the benthos swirl around in cue-free water, the larvae move into and out of cue filaments. We used larvae of *Phestilla sibogae* to study how brief encounters with filaments of the dissolved cue from *Porites compressa* affect swimming behavior (Hadfield and Koehl 2004). Because a larva of *P. sibogae* is only ~230 μm long and swims by beating cilia along the edges of its velum, a two-lobed swimming organ, we had to observe swimming behavior through a microscope. However, the field of view and depth of focus of a microscope were too small to permit us to follow the velar actions of freely swimming larvae. Therefore, we made video records through a microscope of the actions of the cilia and velar lobes of individual larvae tethered in a small flume (''mini-flume'') that moved water past each larva at the velocity of water motion 'relative to' an untethered swimming larva (0.17 cm/s, determined by measuring the speeds in low magnification videos of untethered larvae swimming in aquaria). Tethered larvae in the mini-flume were exposed to filaments of test solutions (filtered sea water, or various concentrations of dissolved cue from *P. compressa*) labeled with fluorescein that were carried past them in the flowing water, as though the larvae were swimming through the filaments. The mini-flume, which permitted us to expose larvae to different concentrations of the dissolved cue and to a range of widths and spacings of cue filaments, was a flow-through system so that background levels of dye and chemical cue did not accumulate during an experiment.

Our measurements of the instantaneous responses (to the nearest 0.017 s) of larvae of *P. sibogae* to cue filaments were reported by Hadfield and Koehl (2004). We found that swimming competent larvae (larvae developmentally advanced enough to undergo metamorphosis) did not respond to fluorescein dye alone, but that they stopped beating their cilia and retracted their velum into the shell when they encountered cue above threshold concentration. If they had not been tethered, the larvae would have sunk through the water at 0.12 cm/s (sinking speed measured from low magnification videos of competent larvae in aquaria filled with solutions of the cue in sea water). The larvae re-expanded the velar lobes and resumed ciliary beating (i.e., resumed swimming) upon exiting an inducer filament. We found that there was a threshold cue concentration below which the larvae did not respond, and that the threshold concentration was only ~17% of the concentration of cue found in the ''in reef'' water collected between branches of *P. compressa* in the field (Hadfield and Koehl 2004). The ''responsivity'' (percentage of encountered cue filaments that induced velar retraction) of competent larvae of *P. sibogae* was 27% at the threshold cue concentration, but was 80% for ''in reef'' cue concentrations (Hadfield and Koehl 2004; Koehl et al. 2007).

By recording larval behavior at high magnification, we discovered that larvae in cue filaments kept the foot protruded from the shell when the velum was retracted. The drag on the protruded foot causes the asymmetical larvae to descend in spirals rather than straight lines and explains why living larvae moving downwards in cue in still water fell more slowly than sinking dead, fully-retracted larvae that had more compact body profiles and experienced lower drag (Hadfield and Koehl 2004). Past analyses of larval settlement onto surfaces have used sinking velocities measured for dead or anesthetized larvae (reviewed by Hadfield and Koehl 2004), but our results illustrate the importance of using high magnification measurements of postures and behaviors of larvae before assuming that living larvae move like passive (i.e., anesthetized or dead) larvae.

Step 4: Using individual-based models to put larvae back into the larger scale environmental flow to determine trajectories

How do the instantaneous behavioral responses of larvae of *P. sibogae* to brief encounters with filaments of cue from *P. compressa* affect their motion relative to a coral reef in nature? To address this question, we had to put the microscopic larvae (whose responses to cue were measured in the mini-flume, and whose swimming and sinking velocities were measured in still water in aquaria) back into the turbulent wave-driven flow over a coral reef.

We did this using a computer simulation of larvae in ambient flow.

We developed an individual-based model of the transport of larvae of *Phestilla sibogae* through the fine scale, changing cue-concentration distributions and water velocity fields we measured over a reef of *Porites compressa* in the wave-current flume (details explained by Koehl et al. 2007). Competent larvae in the water column were simulated by behavioral algorithms based on our measurements of the swimming and sinking velocities of larvae, and of the responses of larvae to brief encounters with cue of different concentrations (Hadfield and Koehl 2004). We placed these simulated larvae at random starting positions in a frame of a PLIF video (i.e., in an instantaneous spatial distribution of cue concentrations). The larva's instantaneous speed and direction through the water depended on its response to the local cue concentration (i.e., pixel brightness). At that instant, the larva was also carried by the moving water around it. Therefore, the vector sum of the motion of a larva through the water and the local instantaneous velocity of the parcel of water carrying the larva (as measured in the flume) were calculated to determine where the larva would be in the next frame of the PLIF video. By repeating such calculations for successive frames of the PLIF videos, the trajectory of a larva swimming and sinking in the turbulent, wave-driven, cue-laden water above the reef was determined. The cue concentrations encountered by a larva following this trajectory through the changing flow and concentration fields could thus be plotted as a function of time. The example of such a trajectory in Fig. 3 illustrates that a larva in the water above a reef passed into and out of filaments of cue, and that when a larva was close to the reef's surface, it encountered patches of cue of above-threshold concentrations more often than it did when higher above the reef.

We ran our model for thousands of "larvae" randomly placed in the water column above a reef, and compared transport rates into the reef of larvae that sank in cue versus those that ignored cue (Koehl et al. 2007). Our calculations revealed that the simple sinking response of competent larvae of *P. sibogae* during brief encounters with cue filaments enhanced the rate of larval settlement onto a reef by ~20%. Our model also predicted that most larvae of *P. sibogae* should land on the seaward portion of a reef. We tested that prediction in the field by conducting a three-year study of recruitment of *P. sibogae* onto reefs, and by making measurements of the touch-down locations on reefs of larval mimic particles (Hadfield et al. 2006). Both studies yielded results consistent with the prediction of the model.

Our fine scale measurements and the results of our model revealed that, from a larva's point of view, a plume of dissolved chemicals from a benthic source is not the diffuse concentration gradient so often used in models of odor tracking, but rather is a series of on-off encounters with patches of odor above threshold concentration, the frequency and concentration of which increase as the larva nears the substratum. Furthermore, our model revealed that the behavioral responses of slowly moving microscopic larvae to chemical cues can affect their trajectories in the environment, even in turbulent, wave-driven ambient water flow, and can thus bring about settlement in appropriate sites.

Step 5: Mimicking fine scale spatial and temporal patterns of larval encounters with water velocities and shear to determine the instantaneous forces on larvae

Temporal patterns of larval encounters with shear

Water is sheared when neighboring layers move at different velocities. Some larvae sink in response to shear in the water around them (Fuchs et al. 2004), while the swimming direction of others can be changed by ambient shear, depending on larval morphology (Grunbaum and Strathmann 2003). Various types of larvae have been reported to orient their locomotion relative to the direction of ambient water flow or shear (barnacles, Crisp 1955; bivalves, Jonsson et al. 1991; bryozoans, Abelson 1997). Our ongoing PIV studies of flow over fouling communities are showing that shear in the wave-driven turbulent flow over a benthic community varies across fine spatial scales (Fig. 2G) and changes rapidly as the water sloshes back and forth. In the future the passive reorientation and behavioral responses of larvae to such fluctuating shear should be measured and incorporated into individual-based models like the one we used to evaluate the effects of sinking in chemical cue to larval transport. Since PIV and PLIF can be done simultaneously, future models can incorporate responses both to odors and to shear simultaneously.

Temporal and spatial patterns of velocities and forces on settled larvae

For a larva to settle into a benthic habitat, it must stick to the substratum after landing. Not only does water motion transport larvae to benthic habitats, but it may also sweep away larvae that have landed

on surfaces. In turbulent boundary layers, high instantaneous velocities and shear stresses occur along surfaces when eddies "sweep" through the thin viscous sublayer along the surface while water near the surface "bursts" up into the overlying flow (Abelson and Denny 1997; Crimaldi et al. 2002). How do such instantaneous peaks in hydrodynamic force on settling larvae affect their probability of successful settlement at a spot on the substratum?

We used larvae of the nudibranch *Phestilla sibogae* settling onto coral reefs as a system to study how turbulent wave-driven flow interacts with a complex substratum to affect the hydrodynamic forces encountered by microscopic larvae that have landed on surfaces at different positions on the terrain (Reidenbach et al. 2008). Since larvae of *P. sibogae* are only ~230 μm long, we had to measure the water velocities very close to coral surfaces to quantify the flow microhabitats experienced by larvae on surfaces of the reef. To do this we used laser Doppler velocimatry (LDV) to measure water velocities encountered 200 μm from coral surfaces at different locations within a reef in a laboratory flume in which field-flow conditions were mimicked. Comparing realistic wave-driven flow to unidirectional flow of the same mean velocity, we found that peak hydrodynamic forces on settled larvae were about an order of magnitude higher under wave conditions. Peak forces on larvae on surfaces at the top of the reef were three to ten times greater than on larvae on surfaces 5–10 cm below the top of the reef. Furthermore, intermittent bursts of high velocity occurred more often at the top of the reef than within it. We calculated the probability of successful attachment for larvae in different reef microhabitats, using the approach described by Crimaldi et al. (2002) and values for the adhesive strength of *P. sibogae* measured by Koehl and Hadfield (2004). We predicted that larvae of *P. sibogae* can only successfully stick to sheltered surfaces within reefs. This analysis illustrates the importance of the interactions of substratal topography with flowing water in determining where larvae can settle (i.e., land on and stick to the substratum) in benthic communities.

Conclusion

Although studies of swimming are often conducted in still water or in unidirectional currents in flumes, the motion of microscopic organisms such as larvae swimming in their natural habitats cannot be understood without considering how ambient water flow affects their behavior and trajectories. Our goal in this paper has been to lay out a series of steps that we have found useful in recreating field-relevant flow conditions in the laboratory to study the locomotion of microscopic organisms. We have illustrated these steps using examples from our published and ongoing studies of larval settlement.

Moving water in the environment transports larvae relative to the substratum and can reorient them, and ambient flow determines the spatial and temporal distributions of dissolved chemicals and of shear, both of which may stimulate behavioral responses by larvae. While most studies of the effects of flow on larval settlement have been carried out in unidirectional currents, we found that even in protected harbors, the flow across fouling communities is turbulent and characterized by the velocity oscillations due to waves (wind chop). Although on our human scale, turbulent water flow across a rough substratum is well described by boundary shear velocity, and dispersal of dissolved substances is modeled as a diffusing cloud, examination of these processes on the scale encountered by an individual microscopic larva reveals a more complex and variable world. For example, larvae have rapid on-off encounters with chemical cues while swimming through fine filaments of odor swirling in unscented water, and after they land they experience rapidly fluctuating hydrodynamic forces with peaks that depend on their location within the fine scale topography of the habitat. Therefore, to be ecologically relevant, laboratory studies of larval behavior or mechanical properties should be conducted under conditions that mimic the fine scale fluctuations in velocity and chemical stimuli encountered by larvae in nature. Such studies are revealing that, although microscopic organisms (such as the larvae of *Phestilla sibogae*) are small and swim slowly relative to ambient water flow, their locomotory behavior in response to the environmental conditions they encounter can affect where they are transported by ambient water movement.

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