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Barnacle settlement: field experiments on the influence of larval supply, tidal level, biofilm quality and age on *Balanus amphitrite* cyprids

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ABSTRACT: A set of 3 field experiments lasting 24 h was conducted during April 1998 at the Duke University Marine Laboratory (Beaufort, North Carolina, USA) to: (1) assess the influence of larval supply, intertidal height, quantity and quality of biofilm and age of the larvae on the settlement of *Balanus amphitrite* Darwin and (2) examine the correspondence between small-scale planktonic distribution of larvae, the initial spatial pattern of newly settled larvae and the vertical distribution of adult barnacles. Precolonized methacrylate (Plexiglas) disks, arranged within 3 blocks and established so as to eliminate edge effects within 3 large experimental panels, were placed at 3 predetermined tidal heights (High, Medium, Low) corresponding to the upper limit, modal zone and the lower limit of adults of *B. amphitrite*. Split-split-plot ANOVAs were performed on densities of newly attached larvae (metamorphosis not completed) to test their habitat selection behavior to surfaces which had been precolonized by microbiota (bacteria and diatoms) at 3 heights (origin factor) for 0, 7, 14 or 21 d (age factor). The physical environment (salinity, temperature, current flow) was stable and comparable during the 3 experiments. *B. amphitrite* cyprids were uniformly distributed in the water column. Larval supply was poorly correlated with the intensity of settlement over the 1 wk experimental period. In fact, the same larval supply could induce either high (4×) or low (1×) settlement after 2 tidal cycles, and, inversely, similar settlement intensities were associated with planktonic larval abundance varying significantly at 2 d intervals (109 to 171 cyprids 923 l^{-1}). Settlement was homogeneous on each experimental unit (no significant block effect). Tidal height, however, was a significant factor in determining the vertical patterns of newly settled larvae during the first experiment where larvae were abundant but not during subsequent experiments for which fewer larvae were collected. The degree of microbial precolonization was the main parameter affecting the settlement of *B. amphitrite*. For the first 2 experiments, 'weighed cyprid settlement' significantly decreased as the age of the biofilm increased, revealing a strong preference of settlers for clean surfaces and avoidance of biofouled surfaces of all intertidal origins. Further analysis of biofilm samples showed that free-space availability in the microbial film and bacterial densities were significantly inversely correlated to settlement intensity. Moreover, settlement to 'favorable' substrata decreased by nearly ½ during our experimental period, suggesting changes in the selectivity of settling larvae. Our experiments confirm the role of larval supply in determining the vertical intertidal distribution of adults of *B. amphitrite*, but the short-term variability in the larval supply/settlement coupling observed over a 1 wk period must be integrated in models of recruitment dynamics of barnacles.

KEY WORDS: *Balanus amphitrite* · Barnacle · Larval settlement · Field experiments · Larval supply · Microbial biofilm · Microbial free-space availability · Energetic contents

INTRODUCTION

The structure of marine benthic communities varies at spatial and temporal scales with regards to the dominant invertebrate components; thus, determining how environmental factors influence benthic invertebrate populations is a prerequisite for understanding the dynamics of marine communities. Indeed, since the variability of marine benthic populations is strongly dependent on the recruitment process by settling invertebrate stages (larvae, post-larvae and juveniles), understanding the early phases of colonization is cru-

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cial to our understanding of community structure. Within rocky coastal systems, dynamics of adult populations are also closely related to processes acting before (pre-settlement phase) and after final attachment of larvae and growth to mature adults (post-settlement phase). The results pertaining to the importance of the cascade of processes are somewhat uncertain. Indeed, the references listed here report a positive relationship between patterns of settlement and recruitment or a relationship between planktonic distribution and settlement patterns, thus suggesting that pre-settlement processes determine the spatial distribution and the abundance of marine organisms, particularly for barnacle populations (Grosberg 1982, Gaines & Roughgarden 1985, Gaines et al. 1985, Underwood & Fairweather 1989, Sutherland 1990, Minchinton & Scheibling 1991, Gaines & Bertness 1992, 1993, Grosberg & Levitan 1992, Miron et al. 1995).

Pre-settlement influences can be divided into: (1) factors acting during the pelagic phase at large temporal and spatial scales, including water circulation (Young & Chia 1987, Farrell et al. 1991, Le Fèvre & Bourget 1991), predation (Young & Chia 1987) and larval behavior (Grosberg 1982, Minchinton & Scheibling 1991, Miron et al. 1995) and (2) factors acting within the boundary layer at small temporal and spatial scales, including larval habitat selection. Habitat selection is influenced by boundary layer structure (Crisp 1955, Rittschof et al. 1984, Wethey 1986, Eckman et al. 1990, Mullineaux & Butman 1991, Mullineaux & Garland 1993, Judge & Craig 1997), the nature of the surface (physico-chemistry: Raimondi 1988b, Holmes et al. 1997; surface energy: Rittschof & Costlow 1989a,b, Roberts et al. 1991, Maki et al. 1992, Becker 1993, O'Connor & Richardson 1994, Becker et al. 1997, Holmes et al. 1997; vibrations: Branscomb & Rittschof 1984, Rittschof et al. 1998; texture: Crisp & Barnes 1954, Letourneux & Bourget 1988, Raimondi 1990, Hills & Thomason 1996, 1998a,b; and small-scale heterogeneity: Bergeron & Bourget 1986, Chabot & Bourget 1988, Walters 1992, Grégoire & Bourget 1996, Lemire & Bourget 1996, Miron et al. 1996, Walters & Wethey 1996), substratum biological cues (associated with the presence of natural biofilms: Hudon & Bourget 1981, Letourneux & Bourget 1988, Wieczorek & Todd 1998; conspecific adults: Knight-Jones 1953, Rittschof et al. 1984, Rittschof 1985, Gabbott & Larman 1987, Letourneux & Bourget 1988, Chabot & Bourget 1988, Raimondi 1988a, Crisp 1990, Dineen & Hines 1994a,b, Miron et al. 1996, Noda et al. 1998; or conspecific larvae, e.g. cyprid footprints: Yule & Walker 1985, Clare et al. 1994; and presence of others species: Young & Chia 1981, Raimondi 1988a, Johnson & Strathmann 1989), free-space availability (Connell 1961, Chabot & Bourget 1988, Navarrete & Castilla 1990, Raimondi 1990, Minchinton & Scheibling 1993, Hills & Thomason 1998b) and larval energy contents (Kitamura & Nakashima 1996, Satuito et al. 1996, 1997, Jarrett & Pechenik 1997).

Among the extensive literature on larval settlement, few studies report the relative importance of each of the factors known to influence barnacle settlement. Therefore, the aim of our study was to use *in situ* experiments: (1) to assess the relative roles of larval supply, intertidal height and the nature of biofilm (intertidal location, degree of microbial colonization, microbial free-space availability and bacterial densities) on the settlement of *Balanus amphitrite* and (2) to examine the correspondence between small-scale planktonic distribution, initial spatial pattern of settlers and vertical adult barnacle distribution. Three successive experiments were used to study the temporal variability of the settlement intensity and habitat selection behavior.

MATERIALS AND METHODS

Study site. The study was carried out from 1 to 28 April 1998 under the pier of Pivers Island (Beaufort, North Carolina, USA) at the Duke University Marine Laboratory (DUML; 34° 43' 03" N, 76° 40' 18" W). The area is relatively well protected, waves essentially being generated by passing boats. Tidal oscillation is semidiurnal, with a mean range of 88 cm (Kirby-Smith & Costlow 1989). Water temperatures vary seasonally, reaching a mean minimum of 5°C in late January and a mean maximum of 30°C in late July and early August (Kirby-Smith & Costlow 1989). Salinities reach the ocean seawater values (35 ppt) and vary by 3 to 5 ppt tidally (Kirby-Smith & Costlow 1989). McDougall (1943), Sutherland & Karlson (1977), Sutherland (1981) and Walters & Wethey (1996) have extensively described the general features of the Beaufort fouling community, the dominant species of which are *Tubularia crocea* (hydroids), *Bugula neritina* (arborescent bryozoan), *Styela plicata* (ascidian), *Crassostrea virginica* (bivalve), *Hydroides dianthus* (annelid), *Balanus amphitrite*, *B. eburneus*, *B. improvisus*, *B. trigonus* and *Chthamalus fragilis* (barnacles). The target species in this study is *B. amphitrite* Darwin, the most common barnacle found in warm coastal and estuarine waters (Bishop 1950, Crisp & Molesworth 1951). Larval development includes 6 feeding nauplii stages and a terminal non-feeding cyprid stage, most larvae metamorphosing to the last stage within 4 d (Rittschoff et al. 1984, Raimondi 1992, Pechenik et al. 1993) in laboratory culture at 28°C.

Field experiments. Three experiments assessed the relative roles of larval supply, biofilm composition (intertidal origin and age) and tidal height on the

settlement and general fitness of larval cohorts of *Balanus amphitrite*.

General design: Three sets of 3 Plexiglas experimental blocks (1.22 × 1.22 m) were secured under the pier of the DUML 1 d before the beginning of the experiment at 3 distinct intertidal heights (Fig. 1): high (H; 0.96 m above Mean Low Water Level, MLWL), medium (M; 0.71 m above MLWL) and low (L; 0.53 m above MLWL). The height of each set (1 experimental unit) was based upon the vertical distribution of adult *Balanus amphitrite* on the pier piles: H and L corresponded respectively to the upper third and the lower third of the adult distribution, and M was the level of modal adult density. Within each block, 12 holes were cut from the Plexiglas sheet by laser beam (Mazak laser 2000 W), with a final cut width of <0.5 mm, providing 12 disks (20.32 cm diameter; area 324.3 cm^2). When placed in their final position, the disks caused no detectable interference with current flow. At the beginning of each experiment, such disks, which had been precolonized (see below for the conditions of precolonization) were randomly arranged within the blocks (Fig. 1) so that each block included 12 disks corresponding to the 3 intertidal origins (O_H, O_M, O_L) and 4 levels of microbial colonization (0, 7, 14, 21 d). This experimental design allowed us to assess the impact of block position (relative to the main direction of tidal currents) on settlement. We hypothesized that settlement would be related to differential larval supply between the flood and the ebb periods.

The vertical patterns of cyprid habitat selection were studied using precolonized disks at various intertidal heights for different durations to obtain a gradient in the abundance of microbiota. Prior to the start of the experiment, 3 sets of fouling community films were obtained from 1 to 27 April (7, 14, 21 d) by placing 9 sets of 27 disks at the 3 predetermined tidal levels (3 sets per tidal level). Disks were chosen instead of squares to avoid biases which could be related to the flow orientation: a tidal flow oriented obliquely to a square area would cover a greater distance than the same flow oriented perpendicularly. This might have induced spatial heterogeneity in the shear stress (Whitlatch & Osman 1998), since the latter varies with distance downstream from the leading edge of the faceplate (Schlichting 1979, Mullineaux & Butman 1991). These disks were previously sandblasted (40 µm grit) to create homogeneous rugosity suitable for barnacle settlement (Letourneux & Bourget 1988, Hills & Thomason 1998a,b) and were placed at random in 3 sets of experimental units (3 blocks per unit) made from the same material (Fig. 1). In an attempt to limit edge effects, each block was previously sanded to obtain a uniform rugosity (100 µm grit) higher than the disks' rugosity for technical reasons (use of an electric sander). Each holder was fastened horizontally to a wooden support which was fixed to the pillars of the pier at the H, M or L intertidal heights (Fig. 1). From 1 to 5 April, 3 sets of 27 disks were placed randomly in the holders at 2 d intervals, forming the 21 d biofilms of the 3 successive experiments (1 d intervals). New sets were added 1 and 2 wk later to obtain respectively 14 or 7 d biofilms. The sets of precolonized disks for the first, second and third experiments were collected at low tide respectively on 23, 25 and 27 April and transferred to a randomly assigned position within the experimental structure (see above).

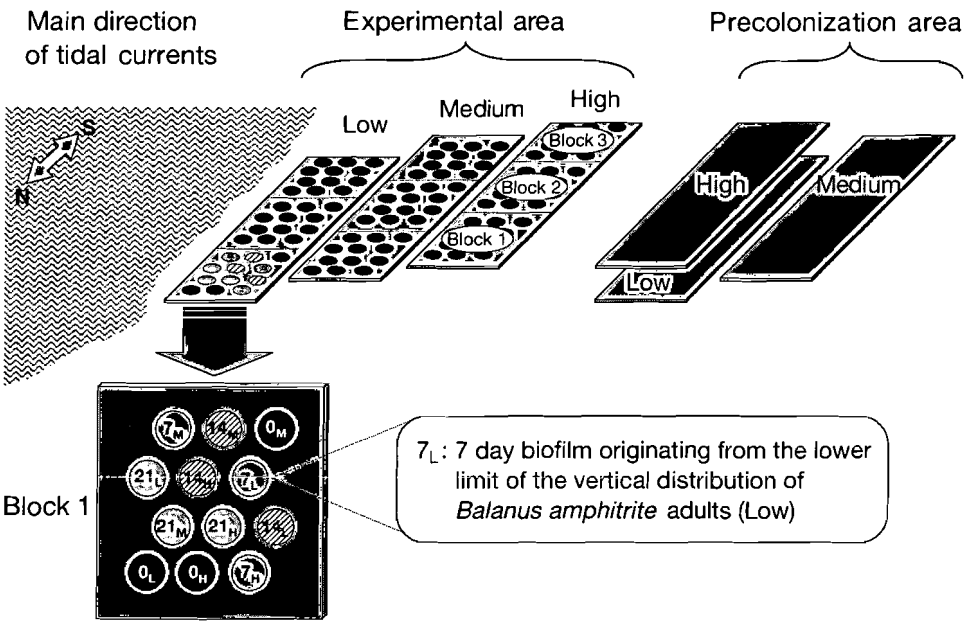

Fig. 1. Arrangement of the Plexiglas experimental units placed at 3 intertidal levels (Position factor: High, Medium, Low; tidal levels related to the vertical distribution of barnacle adults) for periphytic precolonization (from 1 to 27 April) and for the three 24 h settlement experiments (from 23 to 28 April). Each 'settlement' unit consists of 3 blocks (Block factor: Block 1, Block 2, Block 3), each of which included 12 biofilm treatments associated with the duration (Age factor: 0, 7, 14, 21 d) and the origin of precolonization (Origin factor: H, M, L)

Three successive experiments were conducted to assess the variability of the *Balanus amphitrite* settlement response to variations in larval supply (larval concentrations in the water column). In laboratory experiments, Rittschoff et al. (1984), Crisp (1988), Maki et al. (1988), Pechenik et al. (1993), O'Connor & Richardson (1994) and Satuito et al. (1996, 1997) have shown that extending the cyprid stage for 3 d has a major impact on the larval settlement. By conducting 3 successive experiments (every other day) over a 6 d period, we were thus expecting differing responses of cypris larvae to the biofouled surfaces over the duration of the experiment. The experiments were carried out during a period of spring tide. At the end of each barnacle settlement experiment, which lasted 2 tidal cycles (2 immersion periods) to limit the effect of post-settlement mortality, all the disks were quickly and carefully removed from the holders and transferred to running seawater aquaria in the DUML. In the laboratory, the settled larvae were sorted under binocular microscope, counted and then stored in a -80°C freezer for further energetic and genetic analyses. To assess a possible edge effect on the barnacle settlement on the disks, we distinguished the larvae which had settled on the periphery of the disks (2.54 cm width; area = 142 cm^2) from those collected in the center of the disks (area = 182 cm^2).

Vertical distribution of Balanus amphitrite adults: The vertical distribution of adults was determined by counting the individuals along 2 vertical transects on 2 northern pilings of the pier using contiguous 5 × 5 cm quadrats beginning 15 cm below the visible lower limit of *Balanus amphitrite*. Counts were stopped where adult densities were <1 ind. per quadrat.

Larval supply: During the 3 experiments, by high slack waters, plankton samples were collected through a 80 µm mesh Nitex net for 2 min using a self-priming centrifugal pump (Monarch BSGF-8, Type A). The outflow of the pump was equal to 461.5 l min^{-1}. Three 923 l replicate water samples were filtered from the northern side of the pier for each depth corresponding to the high, medium and low experimental disk levels. Each sample was immediately sorted to collect 20 living cyprids, which were immediately placed in a -80°C freezer for energy content measurements (R.T., F.O., E.B., D.R. unpubl. data). The remaining plankton were preserved in 95% ethanol for genetic analyzes. All the samples (n = 54) were sorted, and the remaining cypris larvae were counted.

Fouling community—microbial free-space availability and bacterial densities: The fouling community was examined on black flexible plastic cover sheets (21.6 × 27.9 cm), placed on each wooden supporting frame at the beginning of the colonization period of the fouling community and corresponding to one particular biofilm treatment (3 intertidal origins × 3 durations of precolonization). At the beginning of each settlement experiment, we punched out four 2 × 2 cm quadrats of 9 black flexible plastic cover sheets (21.6 × 27.9 cm); each fragment represented 0.7% of the available area.

Microbial free-space availability. Immediately after collection, all the 2 × 2 cm quadrats were placed in a freezer at -80°C. At the end of the experiments, samples were transferred to the laboratory using a cooler filled with dry ice. Due to the time needed to analyze the community, only the quadrats collected at the beginning of the first experiment were analyzed. One fragment of each biofilm treatment was randomly chosen among the 4 replicates, then fixed for scanning electron microscopy (SEM) using vapours of sodium tetraoxide (OsO_4 2% w/v) and dried in the air for a period of 24 h at ambient temperature. Before SEM observation, samples were sputter coated with gold (20 to 30 nm estimated thickness) for 4 min under an argon vacuum. Observations were carried out with a SEM JEOL JSM-35 CF at × 54 (gun potential = 15 k eV; 0° angle beam) determining a field of 4 mm^2. Ten polaroid pictures (Polapan 400 film) were randomly taken per sample to quantify the area free of detrital or biological material (fine sediment deposits). Black and white pictures were digitized at 600 pixels per inch using an Agfa© Studio Star scanner, and then analyzed using the image analysis software SigmaScan Pro©. The 21 d treatments were not analyzed in detail because the surface was 100% covered by deposits. A total of 60 frames were analyzed, corresponding to the 7 and 14 d treatments of the H, M and L intertidal levels.

Bacterial densities of the biofouling surfaces. To examine bacterial densities, 3 sub-samples (40 mm^2) were removed from each biofilm treatment, from a randomly chosen sample among the replicates of the plastic sheets of the first experiment. Each sub-sample was then placed in a 1 ml microtube and fixed with electron microscopy grade formaldehyde (1% final concentration). Filtered 4',6-diamidino-2-phenylindole (DAPI: Hobbie et al. 1977, Porter & Feig 1980, Lovejoy et al. 1996) solution (2.10^{-5} mg ml^{-1} final concentration) was added to the sample, vortexed for 30 s and then put in a cold and dark storage room (2 to 4°C) for 3 min. Biofilm samples, from which plastic samples were removed for surface measurements using a dissecting microscope, were then filtered through a 25 mm, 0.2 µm pore size Whatman® Anodisc inorganic membrane filter using a glass frit Millipore® base. Filters were mounted onto glass slides with Aquapolymount® immersion oil (Polysciences, Warrington, PA), which were frozen immediately and stored for 24 h before microscopic observations. Bacterial cells were counted in 60 fields per filter at a final magnification of 1000×, using a Zeiss Axiovert 100 epifluorescent microscope with a 50 W mercury lamp and UV filter blocks (exciter filter BP365/11,

beam splitter FT395 and barrier filter LP397). Although bacteria were found either as individual cells or trapped in detritus on the filter, we used only the densities of the free cells in the sub-sample (free bacterial cells cm^{-2}) to compare the biofilm treatments.

Physical data. Throughout the experimental period, water temperature and salinity as well as the direction and the intensity of the tidal currents were recorded at 2 min intervals using a S4 currentmeter (InterOcean Systems Inc., San Diego) located near the pier. Such measurements were made to characterize the study site as well as assessing the variability or the stability of environmental factors during the 3 experiments.

Data analysis. A 1-way ANOVA was used to examine the effects of intertidal height, period of sampling (end of the afternoon to sunset vs morning) and date of experiment on larval abundance over a 6 d period (n = 54). Data normalized using a log transformation and least square means tests (LS means, SAS Inc. 1991) were adopted for post hoc comparisons using either a 0.05 or a 0.05/3 α-threshold corrected for the total number of comparisons.

Similar statistical treatments were applied to examine the effect of tidal height and age of the biofouled surface on microbial free-space availability and bacterial densities (log-transformed). Data were normally distributed for both variables (n = 60 and n = 27, respectively), and LS means tests were used with a 0.05/9 α-threshold (9 post hoc comparisons).

Since no border effect was observed (*t*-paired parametric test, Zar 1984), we adopted split-split-plot factorial ANOVAs (Montgomery 1991) to analyze the effect of the origin of the biofilm treatment and its degree of microbial colonization on frequencies of newly settled cyprids at all 3 intertidal levels for each settlement experiment (Table 1). All the experimental data sets featured a factor Origin (O_H, O_M and O_L) and a factor Age of biofilm (0, 7, 14 and 21 d), thereby generating 12 different treatments (3 origins × 4 ages). These were randomly arranged within the 3 blocks chosen in relation to their position relative to the main direction of tidal currents (Fig. 1).

However, the 0 age of the biofilm could not be used to compare the qualitative origin factor since all the measurements at age 0 are indistinguishable control values (Addelman 1974). To solve this problem we used contrasts as adopted by D. Lauga, L. Lapointe, E.B., G. Daigle, L.-P. Rivest (unpubl. data).

Selectivity of *Balanus amphitrite* cyprids was analyzed by performing a 1-way ANOVA on ranked data (n = 324) with the 2 following factors: date (3 experiments) and age of the biofouled surface (4 treatments). LS means tests (SAS Inc. 1991) were adopted for post hoc comparisons using either a 0.05/3 or a 0.05/6 α-threshold dependent on the number of total comparisons.

Table 1. Split-split-plot design used for each settlement experiment [Block, position relative to the main direction of the tidal currents (North, Middle, South); Position, intertidal level of the experimental plates (H, M, L); Origin, intertidal level of biofilm precolonization (O_H, O_M, O_L); Age, degree of biofilm precolonization (0, 7, 14, 21 d)]

Source of variation	Degrees of freedom (df)	Total
Whole plot		
Block	2	
Position	2	
Block × Position	4	
		(8)
Sub-plot		
Origin	2	
Age	3	
Origin × Age	4	
Position × Origin	4	
Position × Age	6	
Position × Origin × Age	8	
		(27)
Sub-sub-plot		
Block × Origin	4	
Block × Age	6	
Block × Origin × Age	8	
Block × Position × Origin	8	
Block × Position × Age	12	
Block × Position × Origin × Age	16	
		(54)
Error	18	
Total	107	

Spearman's correlation analysis was used to study the relationship between the intensity of larval settlement during the first experiment and both microbial free-space availability and bacterial densities characterizing each biofilm treatment (for each intertidal level and for a fixed duration of immersion). No multiple regression analysis was performed since the positive correlation between the 2 variables was too strong (Spearman's correlation coefficient = 0.71; $p < 0.001$).

RESULTS

Physical data

Tidal currents: intensity and direction

The experimental period (23 to 28 April) fitted a period of spring tides (tidal range: 115 to 133 cm; Tides and Currents© 2.1 software data 1993 to 1996, Bluewater Books & Charts, Fort Lauderdale, FL). Current velocity patterns were comparable among experiments and were strongly related to tidal cycle (Fig. 2a). Tides are semidiurnal at the Beaufort inlet, each successive phase occurring at ~6 h 25 min intervals (Klavans 1983). The hydrography of our study site is typically estuarine

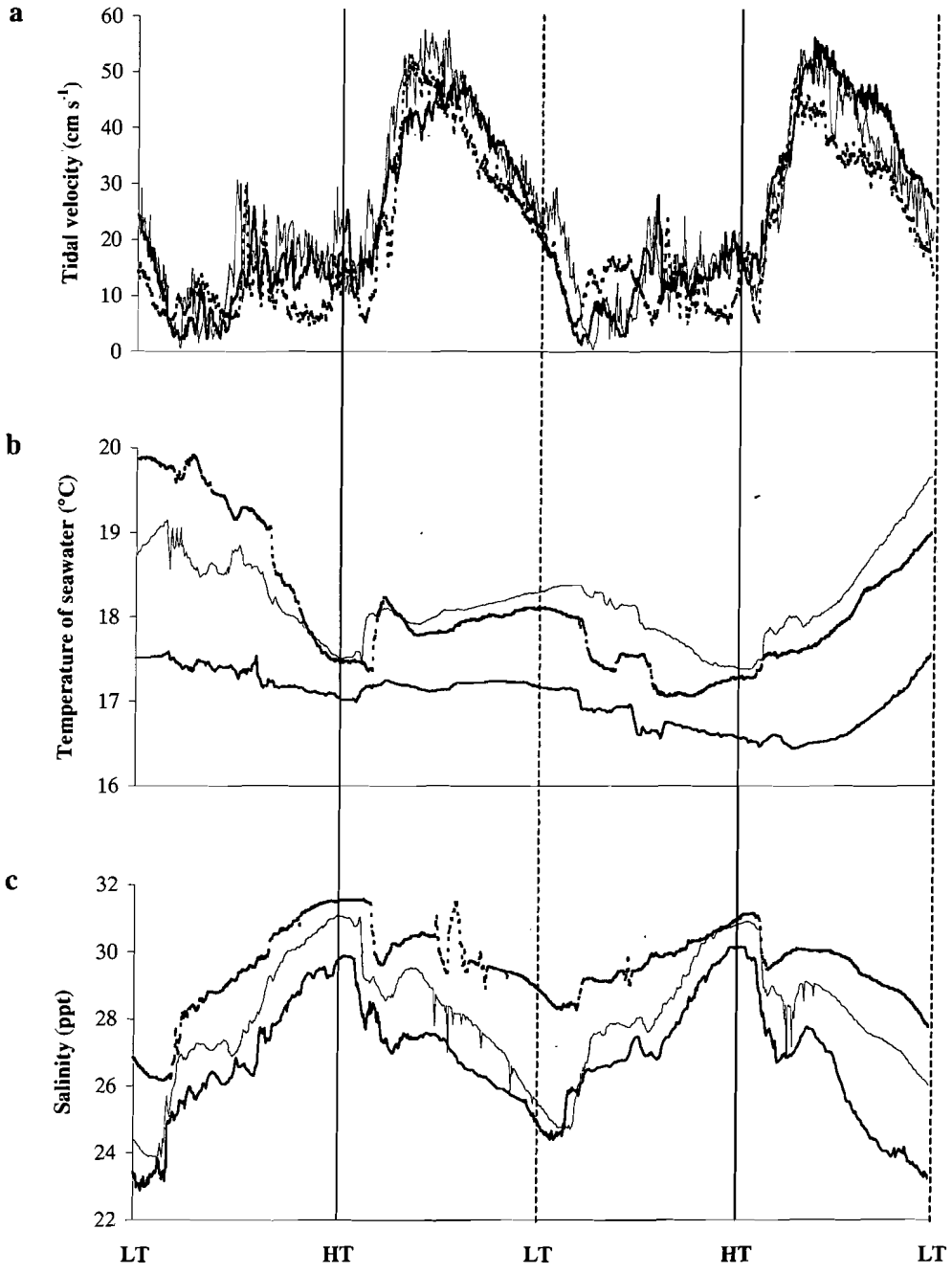

Fig. 2. Fluctuations of (a) tidal velocities (cm s^{-1}), (b) temperature (°C) and (c) salinity (ppt) of seawater recorded near the pier during each experiment (from 23 to 28 April), using a S4 currentmeter (InterOcean Systems Inc., San Diego) at a rate of 1 datum 2 min^{-1}. Note the close similarities of the fluctuations of these physical parameters among the 3 experiments. (——) Expt 1, 23-24 April; (----) Expt 2, 25-26 April; (——) Expt 3, 27-28 April. HT: high tide; LT: low tide

and characterized by asymmetrical tidal flows. Ebb tide currents were always oriented southward and stronger (mean = 34.1 cm s^{-1}; max. = 57.6 cm s^{-1}) than flood tide currents (mean = 12.1 cm s^{-1}; max. = 30.6 cm s^{-1}).

Temperature

Temperature was related mainly to the day/night cycle, highest values corresponding to the daytime (Fig. 2b). The influence of the tidal cycle could also be shown at night, the seawater becoming warmer during the ebb and colder during the flood period. During the 6 d experimental period, mean water temperature increased from 17.0°C during the first Experiment to 18.1°C for Expts 2 and 3.

Salinity

Salinity was directly related to the tidal cycle (Fig. 2c) with instant values increasing during flood

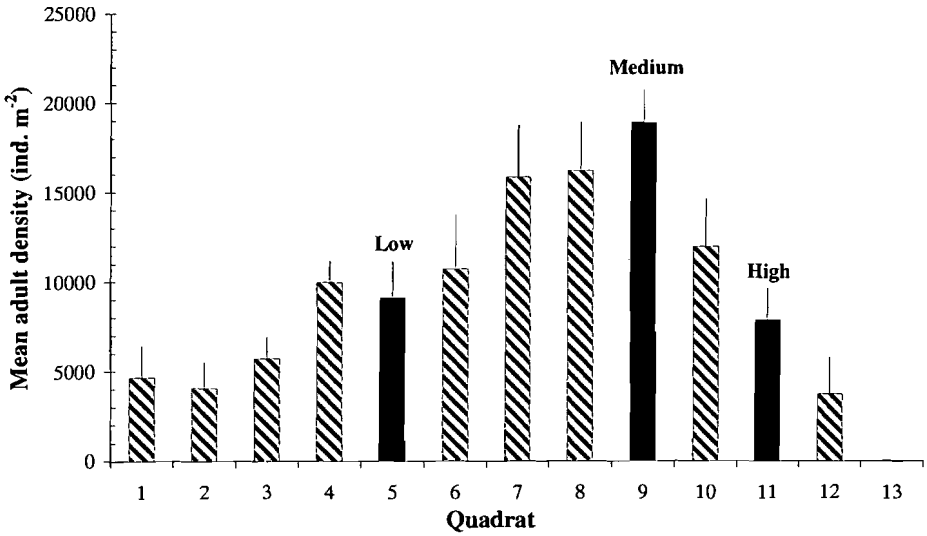

Fig. 3. *Balanus amphitrite*. Vertical distribution of adults observed on the pier piles; data are expressed as mean adult density (ind. m^{-2} ± SE, n = 6) counted within contiguous 5 × 5 cm quadrats. The starting point was located 15 cm below the *a priori* (determined by visual observations) lower limit of *B. amphitrite*. The measures were stopped where adult densities were equal to zero. Solid bars indicate the tidal levels chosen for our set of experiments (Position factor: High, Medium, Low)

tides (up to 31.6 ppt) and decreasing during the ebb tides (down to 22.9 ppt). Mean values increased during the experimental period from 26.8 ppt (Expt 1) to 29.6 ppt (Expt 3; Fig. 2c).

Thus, in general, the physical environment was relatively stable for an estuary and comparable during the 3 experiments.

Vertical distribution of *Balanus amphitrite* adults

Densities (*d*) of *Balanus amphitrite* adults varied along the vertical axis from 0 ind. m^{-2} at the highest sampled quadrat to 18 627 ind. m^{-2} at the Medium intertidal height (Fig. 3). The 3 heights selected for our experiments were accordingly the upper (H; d = 7867 ind. m^{-2}), lower (L; d = 9133 ind. m^{-2}) and maximum-density regions (M; d = 18 933 ind. m^{-2}) of the *B. amphitrite* vertical distribution.

Larval supply

The concentration of cypris larvae in the water column varied during the experimental period (F = 10.65, $p < 0.001$), reaching a maximum of 171 ind. 923 l^{-1} during Expt 3 (Fig. 4), whereas cyprid densities were not statistically different between Expts 1 and 2 (109 ind. 923 l^{-1}). Larval abundance in the plankton did not vary according to a day/night cycle (F = 0.00, p = 0.9991). There was a significant interaction between the daytime period and the date of the experiment (F = 53.61, $p < 0.001$). In fact, during the first 2 experiments, cyprids were more abundant during the early morning than during the end of the afternoon to night period, in contrast with the pattern observed for Expt 3 (Fig. 4). Vertical distribution of *Balanus amphitrite* cyprids in the plankton was not related to the intertidal height (Fig. 5; F = 1.29, p = 0.2873).

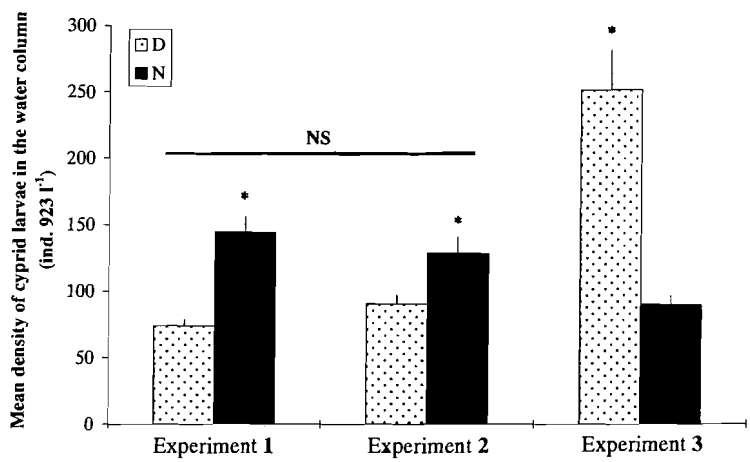

Fig. 4. *Balanus amphitrite*. Mean densities of cyprid larvae in the water column (ind. 923 l^{-1} ± SE, n = 9) sampled at the high tide slack water during day (D) or night (N) for each of the 3 experiments. Data were pooled because no significant differences between tidal levels and to show the interaction between 2 factors of the ANOVA, period and experiment (NS: cyprid densities not statistically different between Expts 1 and 2). Mean cyprid densities were significantly (*) higher during the night period for Expts 1 and 2, and higher during the day period for Expt 3

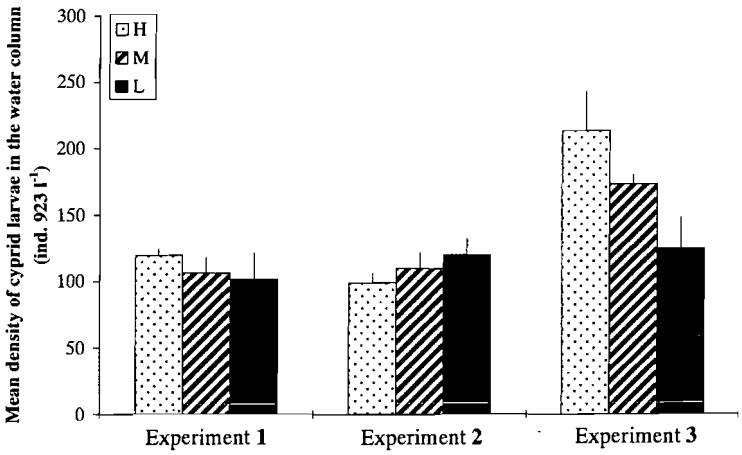

Fig. 5. *Balanus amphitrite*. Mean densities of cyprid larvae in the water column (ind. 923 l^{-1} ± SE, n = 3) sampled at the high tide slack water at the 3 tidal levels (H, High; M, Medium; L, Low) for each of the 3 experiments. No significant differences were emphasized between tidal levels, although the statistical analysis (ANOVA) performed on the data of the last experiment alone showed higher cyprid densities at the High level than at the Low level (LS means, p = 0.0112)

Settlement experiments

Abundance of newly settled cyprids

Expt 1. Because there was no significant edge effect (*t*-paired = 0.43 < $t_{0.05(2),107}$ = 1.98), we used abundances of the cyprids on the whole disks. The number of competent larvae settling during 2 tidal cycles was maximum on the clean surfaces, decreased gradually with the degree of microbial precolonization, and was related to the tidal height. At the end of this experiment 400 settlers were collected. Age of the biofilm and the intertidal level significantly affected the number of newly settled cypris larvae (Table 2a). For the 0 and 7 d biofilms, larval abundance was significantly lower at the H than at M and L levels, while no differences were observed for the 14 and the 21 d old biofilms (Table 2b). Settled larvae were most abundant on the 0 d disks, at all levels (Table 2b). At the H level, settlement was not significantly different among the 7, 14 and 21 d treatments, whereas the number of cyprids was higher on the 7 d disks of the M level than on the 14 and 21 d disks. At the L level, the abundance of newly settled larvae significantly decreased with increasing age of the biofilm (Table 2b). Settlement of *Balanus amphitrite* larvae was not related to tidal flow (block factor), nor to the intertidal origin of the biofilm (Table 2a). There was an interaction between block and position factors (Table 2a). At the L level, the number of settlers was significantly higher in Block 1, while no differences were found between the upper levels (Table 2c). Newly settled larvae were significantly greater at the L level of the first block in Block 1 and lowest at the H level of the third block of Block 3; on Block 2, larval abundance was significantly different between H and M levels (Table 2c).

Expt 2. Overall larval settlement was reduced by a factor of 4, compared to the first experiment, only 100 cyprids were collected during the 25 to 26 April experiment. Age of the biofilm was the only factor significantly affecting the abundance of cyprids (Table 3).

Table 2. (a) Results of the split-split-plot ANOVA examining the effects of Block (position relative to the main direction of the tidal currents: North, Middle, South), Position (intertidal level of the experimental plates: H, M, L), Origin (intertidal level of biofilm precolonization: O_H, O_M, O_L) and Age (degree of biofilm precolonization: 0, 7, 14, 21 d) on the density of newly settled cyprids in the first settlement experiment. (b) Results of multiple comparison tests examining the interaction between Position and Age factors. (c) Results of multiple comparison tests examining the interaction between Block and Position factors. Effects connected by lines do not differ significantly from one another at p = 0.05. Significant differences: *p ≤ 0.05; **p ≤ 0.01

(a)

Source of variation	SS	df	MS	*F*	p
Block	25.57	2	12.787	2.2112	0.11940
Position (intertidal level)	96.07	2	48.037	8.3067	0.00072***
Block × Position	85.04	4	21.259	3.6762	0.01017*
Origin	14.89	2	7.444	1.2873	0.28434
Age	1402.74	3	467.580	80.8554	0.00000***
Origin × Age	26.81	4	6.704	1.1592	0.33905
Position × Origin	10.15	4	2.537	0.4387	0.78006
Position × Age	92.81	6	15.469	2.6750	0.02402*
Position × Origin × Age	22.15	8	2.769	0.4787	0.86589

(b)

Age	Position			Position	Age			
0	H	M	L	H	0	7	14	21
7	H	M	L	M	0	7	14	21
14	H	M	L	L	0	7	14	21
21	H	M	L					

(c)

Block	Position			Position	Block		
1	H	M	L	H	3	2	1
2	H	L	M	M	1	3	2
3	H	L	M	L	2	3	1

Although no significant differences in settlement were observed among the 4 treatments, there was a general decrease in the mean number of newly settled larvae with the duration of the precolonization period (0 d: 4.89 ind. disk^{-1}; 7 d: 3.33 ind. disk^{-1}; 14 d: 1.67 ind. disk^{-1}; 21 d: 1.2 ind. disk^{-1}).

Expt 3. The intensity of larval settlement during the 27 to 28 April period was comparable with that of the second experiment (122 cyprids settled). The number of new settlers was related neither to the exposure to tidal flow (block factor), nor to intertidal origin, age of the biofilm or intertidal level (Table 4).

Table 3. Results of the split-split-plot ANOVA examining the effects of Block (position relative to the main direction of the tidal currents: North, Middle, South), Position (intertidal level of the experimental plates: H, M, L), Origin (intertidal level of biofilm precolonization: O_H, O_M, O_L) and Age (degree of biofilm precolonization: 0, 7, 14, 21 d) on the density of newly settled cyprids in the second settlement experiment. Significant differences: ***$p \leq 0.001$

Source of variation	SS	df	MS	*F*	p
Block	3.5741	2	1.787 04	0.959 67	0.389 45
Position (intertidal level)	7.7963	2	3.898 15	2.093 37	0.133 16
Block × Position	5.2037	4	1.300 93	0.698 62	0.596 27
Origin	1.9506	2	0.975 31	0.523 76	0.595 27
Age	25.2593	3	8.419 75	4.521 55	0.006 70***
Origin × Age	7.6790	4	1.919 75	1.030 94	0.399 77
Position × Origin	8.1975	4	2.049 38	1.100 55	0.365 79
Position × Age	19.9074	6	3.317 90	1.781 77	0.120 27
Position × Origin × Age	9.9506	8	1.243 83	0.667 96	0.71715

Table 4. Results of the split-split-plot ANOVA examining the effects of Block (position relative to the main direction of the tidal currents: North, Middle, South), Position (intertidal level of the experimental plates: H, M, L), Origin (intertidal level of biofilm precolonization: O_H, O_M, O_L) and Age (degree of biofilm precolonization: 0, 7, 14, 21 d) on the density of newly settled cyprids in the third settlement experiment

Source of variation	SS	df	MS	*F*	p
Block	10.9630	2	5.481 48	2.149 25	0.126 44
Position (intertidal level)	12.5741	2	6.287 04	2.465 11	0.094 52
Block × Position	7.9815	4	1.995 37	0.782 37	0.541 61
Origin	3.2840	2	1.641 98	0.643 81	0.529 27
Age	9.3704	3	3.123 46	1.224 69	0.309 66
Origin × Age	4.3457	4	1.086 42	0.425 98	0.789 21
Position × Origin	5.6790	4	1.419 75	0.556 68	0.695 02
Position × Age	17.1296	6	2.854 94	1.119 40	0.363 48
Position × Origin × Age	14.4691	8	1.808 64	0.709 16	0.682 20

Cyprid availability

Since cyprids were uniformly distributed in the water column (see 'Larval supply' section), differences in settler densities among the 3 intertidal heights (Expt 1) may have been due to differences in the immersion period of each holder. To assess the role of immersion time in determining the settlement pattern, we adjusted the initial cyprid abundances for the percentage duration of the immersion period (see Minchinton & Scheibling 1991, Noda et al. 1998). Data expressed as weighted cyprid settlement were then analyzed as before.

Expt 1. The 'settlement/availability' of newly settled cypris larvae was not significantly related to the position factor but always to the age of the biofilm. The interaction between position and age factors was not significant (Table 5a). Weighted cyprid settlement significantly decreased as the duration of precolonization increased (Fig. 6a), passing from 23.1 to 1.6 ind. disk^{-1} on the 0 and 21 d biofilms, respectively. This response variable was not related to the block position, to the intertidal height or to the origin of the biofilm, but a significant interaction was shown between the intertidal height and block (Fig. 6b, Table 5a). At the L level, weighted cyprid settlement was significantly higher for the first block than for the other blocks, while no differences were found at the upper levels (Table 5b). Moreover, weighted larval settlement was significantly higher at the M level than at the level of Block 2, whereas it did not vary within either of the last 2 blocks.

Expt 2. The block position, the intertidal height and the origin of disks did not significantly influence the weighted cyprid settlement (Table 6). Age of the biofilm was again critical and responsible for a significant decreasing gradient of the weighted cyprid settlement as the duration of precolonization increased (Fig. 7, Table 6), from 3.7 to 1.0 ind. disk^{-1} on the 0 and 21 d biofilms, respectively.

Expt 3. None of the factors studied significantly influenced the cyprid 'settlement/availability' (Table 7).

Selectivity of newly settled cyprids

Selectivity of settlers was studied over the experimental period in relation to the age of the biofilm. Selectivity of the settling cyprids is expressed by larvae settling upon the surfaces with shorter biofilming pretreatments. Selectivity decreased over the

Table 5. (a) Results of the split-split-plot ANOVA examining the effects of Block (position relative to the main direction of the tidal currents: North, Middle, South), Position (intertidal level of the experimental plates: H, M, L), Origin (intertidal level of biofilm precolonization: O_H, O_M, O_L) and Age (degree of biofilm precolonization: 0, 7, 14, 21 d) on the weighted cyprid settlement in the first settlement experiment. (b) Results of multiple comparison tests examining the interaction between Block and Position factors. Effects connected by lines do not differ significantly from one another at p = 0.05. Significant differences: $^{*}p \leq 0.05$; $^{***}p \leq 0.001$

(a)

Source of variation	SS	df	MS	*F*	p
Block	102.28	2	51.14	1.7450	0.184 35
Position (intertidal level)	23.25	2	11.62	0.3966	0.674 55
Block × Position	305.38	4	76.34	2.6049	0.045 80*
Origin	58.21	2	29.11	0.9931	0.377 07
Age	7822.18	3	2607.39	88.9663	0.000 00***
Origin × Age	115.79	4	28.95	0.9877	0.422 12
Position × Origin	32.90	4	8.23	0.2807	0.889 24
Position × Age	318.19	6	53.03	1.8095	0.114 54
Position × Origin × Age	85.94	8	10.74	0.3666	0.933 65

(b)

Block	Position			Position	Block		
1	M	L	H	H	3	2	1
2	L	H	M	M	1	3	2
3	H	L	M	L	2	3	1

Table 6. Results of the split-split-plot ANOVA examining the effects of Block (position relative to the main direction of the tidal currents: North, Middle, South), Position (intertidal level of the experimental plates: H, M, L), Origin (intertidal level of biofilm precolonization: O_H, O_M, O_L) and Age (degree of biofilm precolonization: 0, 7, 14, 21 d) on the weighted cyprid settlement in the second settlement experiment. Significant differences: $^{***}p \leq 0.001$

Source of variation	SS	df	MS	*F*	p
Block	23.212	2	11.6059	1.163 70	0.320 04
Position (intertidal level)	19.705	2	9.8526	0.987 90	0.378 98
Block × Position	28.475	4	7.1188	0.713 79	0.586 14
Origin	13.667	2	6.8334	0.685 17	0.508 33
Age	130.652	3	43.5507	4.366 74	0.007 97***
Origin × Age	51.651	4	12.9127	1.294 74	0.283 68
Position × Origin	45.545	4	11.3862	1.141 67	0.346 87
Position × Age	72.943	6	12.1572	1.218 98	0.311 02
Position × Origin × Age	69.137	8	8.6421	0.866 52	0.55014

experimental period (Table 8a,b, Fig. 8). During the first experiment, barnacle larval settlement decreased with the increasing age of the biofouled surface: 66% total collected larvae settled on the 0 d disks (Table 8b). During the second experiment 44% were observed on 0 d disks, which again collected more cyprids than with the other treatments (Table 8b). During the last experiment, differences could no longer be observed between the age treatments (Table 8b), and the percentage of cyprids settling on control disks dropped from 44 to 35%. Moreover, more larvae were collected during the first experiment for the 0, 7 and 14 d treatments than in the second and third experiments. *Balanus amphitrite* settlement was equal for the 3 experiments on the 21 d biofilm (Table 8b).

Microbial free-space availability and bacterial density

Preliminary SEM observations on the substrata from different treatments indicated greater colonization by sediments and bacteria than by diatoms. We therefore assessed 2 features of the various 'biofilm' treatments, microbial free-space availability (MFSA) and bacterial density, both known to influence barnacle larval settlement (Maki et al. 1988, Avelin Mary et al. 1993, Wieczorek & Todd 1998).

MFSA on the biofouled surfaces

We excluded the 21 d samples from the analysis and statistical treatments because these disks were always totally covered by inorganic deposits (0% MFSA). MFSA was positively related to the duration of biofilm precolonization ($F = 24.82$, $p < 0.001$) and to their intertidal position ($F = 15.95$, $p < 0.001$), with a significant interaction between these 2 factors ($F = 21.26$, $p < 0.001$). Mean MFSA of the 7 d samples was significantly larger at the M level (83.8%) than at the other 2 levels (69.7%), and there was a significant decreasing gradient from the upper (85.1%) to the lower (75.9%) shore level for the 14 d treatments (Fig. 9a). At the H level, the mean 7 d MFSA (66.9%) was significantly lower than the 14 d one (85.1%). There were however no significant variations between the 7 and 14 d mean MFSA at the M (LS means, p = 0.3740) or the L level (LS means, p = 0.1372).

Bacterial densities on the biofouled surfaces

Bacterial densities were positively related to the age ($F = 11.41$, $p < 0.001$), and intertidal origin of the

biofilm ($F = 118.45$, $p < 0.001$), with a significant interaction between these 2 factors ($F = 10.12$, $p < 0.001$). Bacterial densities were highest at the lowest level independent of the biofilm's age (LS means, $p < 0.001$) and, for the other 2 levels, values were significantly higher at the M level of the 21 d treatment (Fig. 9b; LS means, $p < 0.001$). No significant differences were found between the age treatments at the highest level (LS means, $p > 0.05$). At the M level, bacteria were significantly more abundant on the 21 d surfaces than on the 7 and 14 d biofilms (LS means, $p < 0.001$), which did not differ significantly (LS means, $p = 0.448$). At the L level, there was a significant increasing gradient in bacterial abundance from the youngest to the oldest surface (Fig. 9b).

Biofilm features and settlement intensity

The intensity of settlement increased with MFSA and decreased with the density of bacteria attached to the substratum. Indeed, significant correlations (Spearman's correlation test, $p < 0.001$) between the number of settlers and either MFSA ($p_{High\ level} = +0.61$, $p_{Medium\ level} = +0.54$, $p_{Low\ level} = +0.64$) or bacterial densities ($p_{High\ level} = -0.45$, $p_{Medium\ level} = -0.57$, $p_{Low\ level} = -0.48$) were observed.

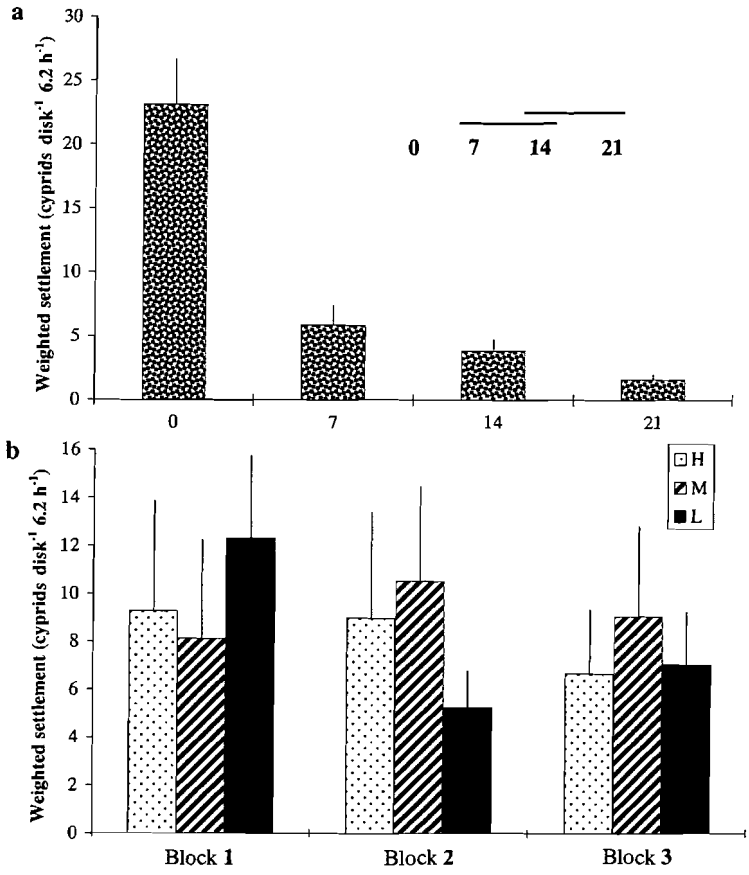

Fig. 6. *Balanus amphitrite*. (a) Mean 'weighted densities' (mean settlement intensity divided by the duration of immersion associated with each tidal level: High = 28.8%, Medium = 45.1%, Low = 55.4% of 1 tidal cycle; data expressed as ind. disk^{-1} 6.2 h^{-1} ± SE, n = 27) of newly settled larvae sampled at the end of the first experiment on biofilms of different ages (0, 7, 14, 21 d of precolonization). Results of LS means tests are shown directly on the graph (effects connected by lines do not differ significantly from one another at p = 0.05). (b) Mean 'weighted densities' of newly settled larvae (ind. disk^{-1} 6.2 h^{-1} ± SE, n = 9) sampled at the end of the first experiment on disks from the 3 tidal levels (High, Medium, Low) for each block (Block 1, North; Block 2, Middle; Block 3, South)

DISCUSSION

The aim of this set of *in situ* experiments was: (1) to assess simultaneously the relative roles of larval supply, intertidal height and nature of biofilm (intertidal location, degree of microbial colonization, MFSA and bacterial densities) on the settlement of *Balanus amphitrite* cypris larvae and (2) to examine the correspondence between the initial spatial pattern of settlers and the adult barnacle distribution. Three successive experiments were used to study the temporal variability of the settlement intensity and habitat selection.

Table 7. Results of the split-split-plot ANOVA examining the effects of Block (Position relative to the main direction of the tidal currents: North, Middle, South), Position (intertidal level of the experimental plates: H, M, L), Origin (intertidal level of biofilm precolonization: O_H, O_M, O_L) and Age (degree of biofilm precolonization: 0, 7, 14, 21 d) on the weighted cyprid settlement in the third settlement experiment

Source of variation	SS	df	MS	F	p
Block	92.499	2	46.2493	2.694 90	0.076 63
Position (intertidal level)	32.422	2	16.2111	0.944 61	0.395 16
Block × Position	91.133	4	22.7833	1.327 56	0.271 54
Origin	28.096	2	14.0482	0.818 57	0.446 46
Age	25.771	3	8.5904	0.500 55	0.683 48
Origin × Age	38.812	4	9.7030	0.565 38	0.688 80
Position × Origin	50.329	4	12.5821	0.733 15	0.573 35
Position × Age	92.185	6	15.3641	0.895 25	0.505 11
Position × Origin × Age	106.814	8	13.3518	0.777 99	0.623 71

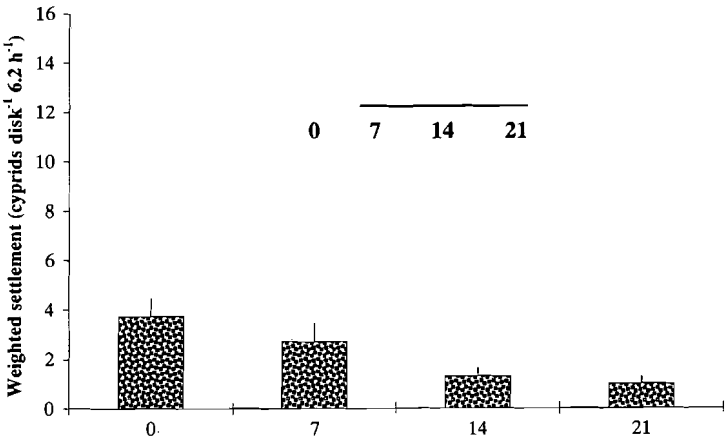

Fig. 7. *Balanus amphitrite*. Mean 'weighted densities' (mean settlement intensity divided by the duration of immersion associated with each tidal level: High = 28.8%, Medium = 45.1%, Low = 55.4% of 1 tidal cycle; data expressed as ind. disk^{-1} 6.2 h^{-1} ± SE, n = 27) of newly settled larvae sampled at the end of the second experiment on biofilms of different ages (0, 7, 14, 21 d of precolonization). Results of LS means tests are shown directly on the graph (effects connected by lines do not differ significantly from one another at p = 0.05)

Larval supply

We were unable to find a strong correlation between larval supply and the intensity of settlement at the short-term scale (1 wk). The abundance of larvae in the water column is not a true measure of larval supply, and the actual flux of larvae to the substratum, which is related to the flow velocities (Gaines & Bertness 1993, Todd 1998), provides a much better index (Harvey et al. 1995). In the present study, care was taken to conduct repeated experiments lasting 2 tidal cycles (same fluctuations in tidal currents) during a short spring tide period (6 d) to ensure the best relationship possible between larval concentrations in the plankton and larval supply to the experimental panels. We found that larval concentrations in the water column did not vary during the first 2 experiments, whereas densities of newly settled cyprids decreased significantly and drastically on a 2 d temporal scale. Moreover, larval supply was maximum during the last experiment but settlement was identical to the previous one. This result contrasts with recent supply-side studies focusing on barnacle settlement (Grosberg 1982, Gaines & Roughgarden 1985, Gaines et al. 1985, Underwood & Fairweather 1989, Sutherland 1990, Minchinton & Scheibling 1991, Gaines & Bertness 1992, 1993, Grosberg & Levitan 1992, Miron et al. 1995, 1999). Miron et al. (1995) recommended that 'special attention be paid to the precise distribution of larvae in the water column to explain the abundance of newly settled spat and adults in the subtidal or intertidal zones before selecting indices of larval abundance and subsequently making predictions'. In this study, we measured the vertical distribution of cyprids of *Balanus amphitrite* in the plankton, and no variations were observed in relation to intertidal height. Given the constancy of physical parameters during the experimental period (S4 meter data), the weak correlation between larval supply and larval settlement could be related to a shift in larval behavior of *B. amphitrite* between the first and the 2 subsequent experiments.

The cyprid is a non-feeding larval stage of barnacles. Individuals use lipids (Lucas et al. 1979, Crisp 1988, West & Costlow 1988) or proteins ('Cyprid Major Protein': Satuito et al. 1996, Shimizu et al. 1996) as energy sources during the free-swimming phase until subsequent metamorphosis. Thus, energy content decreases with the age of the larvae until a level when metamorphosis is no longer possible (Lucas et al. 1979, Crisp 1988, Pechenik et al. 1993). A few authors have studied the role of larval age on the intensity of settlement or on the substratum specificity of the *Balanus amphitrite* cyprids in the laboratory (Branscomb & Rittschof 1984, Rittschof et al. 1984, Crisp 1988, Maki et al. 1988, 1992, O'Connor & Richardson 1994, Kitamura & Nakashima 1996, Satuito et al. 1996, 1997). But the rare and recent field

Table 8. (a) Results of the ANOVA analysis performed on rank transformed data, examining the effects of Age (degree of biofilm precolonization: 0, 7, 14, 21 d) and the Experimental period (settlement experiment A, B, C) on the weighted cyprid settlement. (b) Results of multiple comparison tests examining the interaction between Age and Experimental period (Expt) factors. Effects connected by lines do not differ significantly from one another at p = 0.05. Significant differences: ***p ≤ 0.001

(a)

Source of variation	SS	df	MS	*F*	p
Age	272 514.89	2	136 257.44	22.69	0.0001***
Expt	291 345.52	3	97 115.17	16.17	0.0001***
Expt × Age	114 515.90	6	19 085.98	3.18	0.0048***

(b)

Age	Expt			Expt	Age			
0	B	C	A	A	21	14	7	0
7	B	C	A	B	21	14	7	0
14	B	C	A	C	21	7	14	0
21	B	C	A					

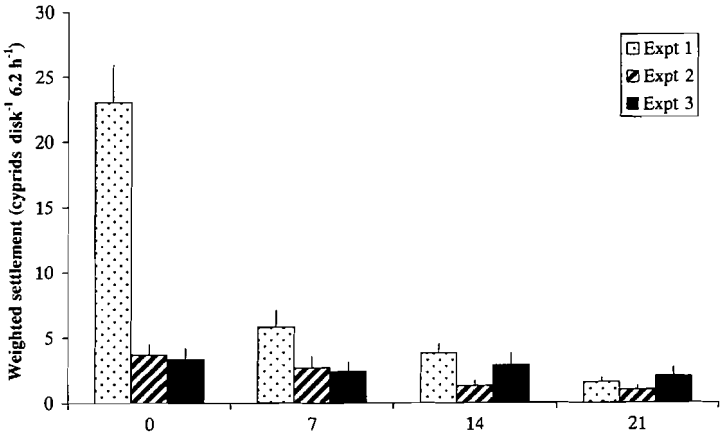

Fig. 8. *Balanus amphitrite*. Mean 'weighted densities' (mean settlement intensity divided by the duration of immersion associated with each tidal level: High = 28.8%, Medium = 45.1%, Low = 55.4% of 1 tidal cycle; data expressed as ind. disk^{-1} 6.2 h^{-1} ± SE, n = 27) of newly settled larvae sampled at the end of the 3 experiments on biofilms of different ages (0, 7, 14, 21 d of precolonization). Note that settler selectivity decreased from the first to the last experiment, with 66, 44 and 35% of the newly settled larvae sampled on the clear surfaces (0 d) during the first, second and third experiment, respectively

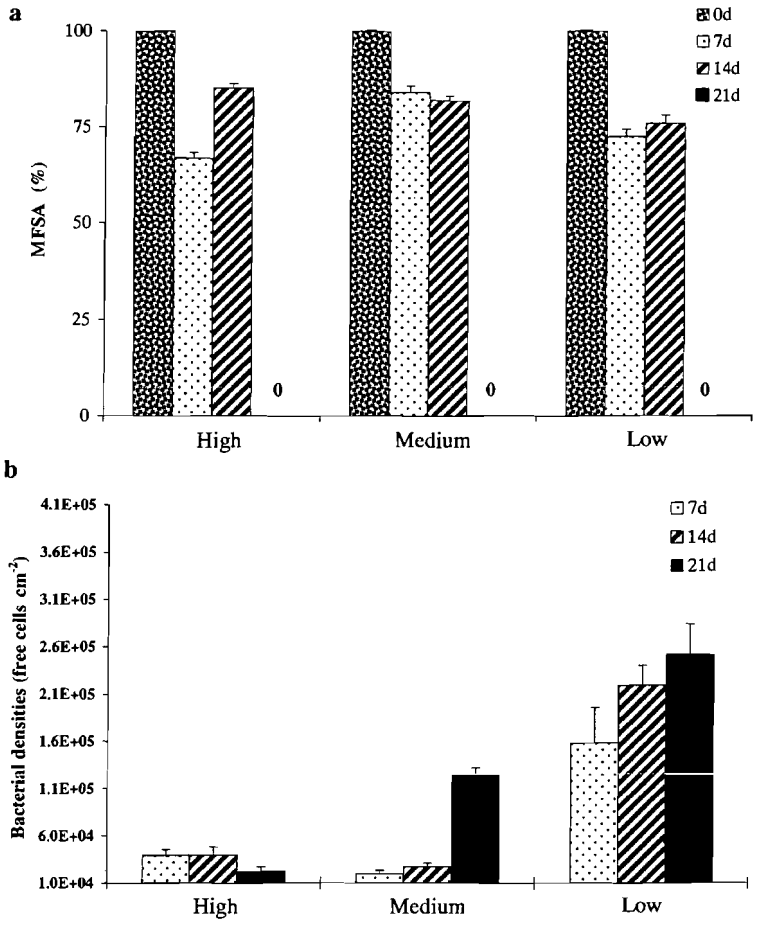

Fig. 9. (a) Mean microbial free space availability (% of MFSA ± SE, n = 10) of biofilm treatments of increasing age (0, 7, 14, 21 d of precolonization) and originating from 3 tidal levels (High, Medium, Low) used in the first experiment. Note the absence of free space for biofilms of 21 d. (b) Mean bacterial densities (free cells cm^{-2} ± SE, n = 3) on biofilm treatments of increasing age (7, 14, 21 d of precolonization) and originating from 3 tidal levels (High, Medium, Low) used in the first experiment

studies which have dealt with such topics involved the boreo-arctic barnacle *Semibalanus balanoides* (Jarrett 1997). The latter study showed that specificity of daily cohorts of settling cyprids for a conspecific cue varied considerably during the recruitment season, while Jarrett & Pechenik (1997) went further in suggesting, from laboratory experiments on field-sampled individuals, that variations in cyprid organic content during the pre-settlement period may explain the observed 'dramatic' temporal variation in the fate of newly settled cyprids (metamorphosis success, post-metamorphosis survival and juvenile growth capacity). Larval supply-side ecologists should therefore take this short-term variability into account when modeling recruitment dynamics of barnacles and, to a greater extent, of marine sessile invertebrates.

Tidal height

Once competent, i.e. able to settle and metamorphose, larvae must first contact the substratum before exploring it (see Rittschof et al. 1984, Rittschof 1985, Roberts et al. 1991, Walters et al. 1999). Within the intertidal zone, contact with a surface is correlated to a particular height on the shore, a parameter directly related to the duration of immersion (see Miron et al. 1995). In our study, tidal position was a significant factor in determining the vertical patterns of newly settled larvae during the first experiment. The 'position' factor encompasses several influences, such as duration of larval presence above the substratum and the quality of biofilms (see discussion below; 'Substratum features: the role of biofilm composition' section). The analysis performed on the weighted cyprid settlement data (or 'cyprid availability' in Minchinton & Scheibling 1991,

Noda et al. 1998) eliminates the observed effect of shore level. The settlement response of *Balanus amphitrite* observed vertically represents a good illustration of one prediction of the model proposed by Miron et al. (1995). There was good concordance between the observed distribution of newly settled spat in the intertidal zone with that expected from a theoretical species whose larvae are uniformly distributed in the water column within an oscillating system. *B. amphitrite* planktonic distribution contrasts with that of other species of barnacles which display neustonic (*B. glandula*, Grosberg 1982) or suprabenthic (*B. crenatus*, Grosberg 1982; *Semibalanus balanoides*, Minchinton & Scheibling 1991, Miron et al. 1995) distributions in the water column. The good correlation between the short-term vertical patterns of larvae in the water column and the short-term vertical patterns of settlement are in agreement with supply-side studies (see the 'Larval supply' section of the 'Discussion'), though the longer term patterns (the 3 experiments) are not. Indeed, the study shows that while short-term spatial patterns may be explained by larval supply, longer temporal patterns are at odds with the supply-side theory.

Substratum features: the role of biofilm composition

Once in contact with the substratum, competent cyprids test the suitability of the habitat through a chain of behavioral events ('broad exploration', 'close exploration' and 'inspection'; Crisp 1961) in response to facilitating or inhibiting environmental cues (Crisp 1974, Lewis 1978, Hui & Moyse 1987, Bourget 1988, Letourneux & Bourget 1988).

Physical cues known to influence cypris larvae involve salinity (Dineen & Hines 1994a,b), temperature (O'Connor & Richardson 1994, Kitamura & Nakashima 1996, Satuito et al. 1996), light (Crisp & Barnes 1954, Raimondi 1990), wave strength (Raimondi 1990), benthic boundary layer flows (Crisp 1955, Wethey 1986, Rittschof et al. 1984, Eckman et al. 1990, Mullineaux & Butman 1991, Mullineaux & Garland 1993, Judge & Craig 1997) and the characteristics of the surface: physico-chemistry (Raimondi 1988b, Holmes et al. 1997), surface energy (Rittschof & Costlow 1989a,b, Roberts et al. 1991, Maki et al. 1992, Becker 1993, O'Connor & Richardson 1994, Becker et al. 1997, Holmes et al. 1997), vibration (Branscomb & Rittschof 1984, Rittschof et al. 1998), texture (Crisp & Barnes 1954, Letourneux & Bourget 1988, Raimondi 1990, Hills & Thomason 1996, 1998a,b) and small-scale heterogeneity (Bergeron & Bourget 1986, Chabot & Bourget 1988, Walters 1992, Lemire & Bourget 1996, Miron et al. 1996, Walters & Wethey 1996).

To assess the role of biological cues (see below), we designed a specific field experiment to minimize the influence of physical cues: 24 h experiments (2 tidal cycles) conducted during a short 6 d period to obtain comparable fluctuations of temperature, salinity, velocity and light within an area protected from waves; initial surface features were controlled by adopting flat black Plexiglas plates of fine surface roughness suitable for barnacle settlement (Letourneux & Bourget 1988, Hills & Thomason 1998a,b), which were precisely inserted into a frame (to minimize edge effects) at 3 different tidal heights. Cyprids of barnacles respond to a large spectrum of biological cues, including the presence of conspecific adults (Knight-Jones 1953 but see the review of Gabbott & Larman 1987 and more recently Chabot & Bourget 1988, Letourneux & Bourget 1988, Raimondi 1988a, Crisp 1990, Dineen & Hines 1994a,b, Miron et al. 1996, Noda et al. 1998), cyprid footprints (Yule & Walker 1985, Clare et al. 1994) and the presence of other species (Young & Chia 1981, Raimondi 1988a, Johnsson & Strathmann 1989).

Biological cues related to the presence of natural biofilms may play an important role in determining suitability of a particular settlement site for subsequent juvenile and adult survival and growth. In 2 experiments, we found that the presence of a microbial biofilm was the main factor affecting the settlement of *Balanus amphitrite*. The number of competent larvae settling during 2 tidal cycles was maximum on the clean surfaces and then decreased gradually with the degree of microbial precolonization. Moreover, the origin of the biofouled surfaces did not affect the densities of newly settled cyprids of *B. amphitrite*, revealing their lack of discrimination for a specific tidal height. To our knowledge, this study constitutes the first attempt to test the habitat selection abilities of settling larvae of *B. amphitrite* in the field (but see the review of Wieczorek & Todd 1998). There has been emerging evidence in the last 2 decades that 3 major features of the rocky substratum have a considerable role in controlling the settlement of sessile marine invertebrates: microbiota, microheterogeneity and free-space availability. Of these, to our knowledge, the biofilm is probably the least studied factor, particularly in the field.

Bacterial densities

Bacterial densities increased with the duration of immersion, with maximum values found at the lowest tidal level, and with the age of the biofilm. This is in accordance with previous works focusing on experimental (Maki et al. 1988, Wieczorek et al. 1995) or natural microbial biofilms (Hudon & Bourget 1981, Becker 1993, Becker et al. 1997, Tsurumi & Fusetani 1998).

SEM observations (see next section) show that the biofouled surfaces related to the 9 experimental treatments (tidal height: High, Medium, Low × age: 7, 14, 21 d) corresponded to young microbial biofilm (Wahl 1989, Lovejoy pers. comm.), classically dominated by bacteria and detrital material, by a few protozoa and diatoms and without any filamentous algae. Such results were unexpected considering the maximum duration of precolonization (21 d), but they may be explained by the strong environmental variations which characterize the intertidal zone (see Hudon & Bourget 1981).

Settlement of *Balanus amphitrite* larvae was strongly negatively correlated to the number of bacteria attached to the substratum. Indeed, increasing bacterial densities induced a decrease of the number of newly settled larvae at all levels ($p_{High\ level} = -0.45$, $p_{Medium\ level} = -0.57$, $p_{Low\ level} = -0.48$) examined. The role of bacteria on the attachment of *B. amphitrite* has been well documented from bioassay studies with monospecific (Maki et al. 1988, 1990, 1992, Mitchell & Maki 1988, Rittschof & Costlow 1989a, Avelin Mary et al. 1993) or natural multi-species biofilms (Maki et al. 1990, Wieczorek et al. 1995). Our results contrast with those of Wieczorek et al. (1995), who showed, from laboratory experiments, that 'older' (12 and 18 d) natural multi-species biofilms were more attractive for *B. amphitrite* settlers than 'younger' ones. Several factors may explain such differences in the biofilm attractiveness.

First, to our knowledge, our study is the earliest attempt to assess the role of microbial biofilms in controlling *Balanus amphitrite* settlement in the field. As pointed out by Wieczorek & Todd (1998), 'major difficulties are perceived in extrapolating from laboratory to the field', especially given that bioassay experiments ignore the role of benthic boundary layer flows which have been shown to be of primary importance in the settlement of marine sessile invertebrates (Eckman 1990, Eckman et al. 1990, Havenhand & Svane 1991, Mullineaux & Butman 1991, Mullineaux & Garland 1993, Abelson et al. 1994, Abelson & Denny 1997, Judge & Craig 1997) including *B. amphitrite* (Walters et al. 1999). In this context, the use of nets (100 or 260 µm mesh size) adopted in some studies to create 'natural biofilms' (Todd & Keough 1994, Keough & Raimondi 1995, Wieczorek et al. 1996) may affect the dynamics of the microbial community by altering the benthic boundary layer flows (Laws & Livesey 1978, Pouliot et al. 1995, Snelgrove et al. 1995) and so may not reflect the field situation. This artifact could be particularly important given the results of Neal & Yule (1994a) and Neal et al. (1996), showing that attachment and exploration behavior of cyprids of *Elminius modestus* were more pronounced on high-shear versus low-shear precolonized multi-species biofilms.

Second, comparing results of studies conducted at different sites, with contrasting environmental conditions, could be very misleading particularly when focusing on the effect of the age of microbial biofilms. For example, 14 d biofilms from the St. Lawrence estuary are expected to be much less developed than similar films developing in the Beaufort area, because of seasonal variations in physical conditions (temperature, salinity, ...; see Hudon & Bourget 1981). Therefore, systematic characterization of the microbial biofilms used to test the selectivity of invertebrate larvae should be a prerequisite to allow a robust interpretation of the bacterial settlement relationships, between sites or in species comparisons, in an attempt to understand and model the related processes. For example, the *a priori* contradictory results of our study with those of Wieczorek et al. (1995), although both groups worked on the same species and with similar age treatments of biofouled surfaces, could be explained by differences in the dynamics of microbial colonization in the different regions. Maximum bacterial densities determined in Beaufort did not exceed 5×10^5 cells cm^{-2} (21 d of the low tidal height) and were lower than those facilitating settlement effects in Wieczorek et al. (1995).

Third, bacterial composition may vary in relation to the dynamics of microbial colonization (Hudon & Bourget 1981, Avelin Mary et al. 1993, Wieczorek et al. 1995), the influence of which could therefore switch from inhibitory to facilitatory within several days, as demonstrated by Unabia & Hadfield (1999) for the polychaete *Hydroides elegans*. In our case, such a switch may not be applicable in light of the study by Avelin Mary et al. (1993), working in Tuticorin, India, on *Balanus amphitrite*, who identified 16 isolates of bacteria from surface fouling, belonging to 5 major groups (*Aeromonas*, *Alcaligenes*, *Flavobacterium*, *Pseudomonas* and *Vibrio*), all of which were inhibitory on cyprid settlement. Moreover, spatial scale may be important as shown by Bourget (1988) and Letourneux & Bourget (1988).

Finally, the inhibitory mechanism of bacterial films on the settlement of barnacles is not clear but some explanations have been proposed, e.g. larval rejection behavior of the surface in response to cells, exopolymer, extracellular materials or leachate cues (Maki et al. 1990, Holmström et al. 1992, O'Connor & Richardson 1996) or to a reduced tenacity or adhesion of the antennulary disk with the substratum (Yule & Crisp 1983, Yule & Walker 1984, Crisp et al. 1985, Maki et al. 1988, 1989, 1994, Neal & Yule 1994a,b, Tsurumi & Fusetani 1998). We think that progress in studies focusing on the role of microbial films on the settlement of marine sessile invertebrates is closely dependent on the standardization of the key parameters

characterizing such biofilms (densities and biodiversity of the microbiota). A good, recent illustration of such an attempt has been provided by Tsurumi & Fusetani (1998), who used a confocal laser microscope and 3-D image analysis to assess the role of the biofilm volume on the settlement of *Balanus amphitrite* cyprids (Tsurumi & Fusetani 1998). We think that such a method, though interesting (Norton et al. 1998), requires much more time and money than DAPI analysis. DAPI also has the advantage of marking DNA as it stains organic material (Mostajir et al. 1995), which allows more precise determinations of bacterial densities than the acridine orange method when applied to samples placed in high-sedimentation coastal areas (C. Lovejoy pers. comm.).

MFSA

We have shown from SEM observations of biofilm samples that MFSA was one other major factor influencing the number of newly settled larvae in the field. MFSA and the intensity of settlement were found positively correlated at the limits and within the vertical adult distribution ($p_{High\ level} = +0.61$, $p_{Medium\ level} = +0.54$, $p_{Low\ level} = +0.64$). The majority of *Balanus amphitrite* cyprids settled intensively in response to high MFSA surfaces and avoided, in bulk, substrata lacking clean microsites. Such a relationship linking the intensity of settlement and the free-space availability has been highlighted in some field studies focusing on *Semibalanus balanoides* (Connell 1961, Chabot & Bourget 1988, Minchinton & Scheibling 1993, Hills & Thomason 1998b) or other barnacles (Navarette & Castilla 1990, Raimondi 1990). For example, Chabot & Bourget (1988) showed that larval settlement of *S. balanoides* increased with increasing conspecific adult cover up to 30% and decreased afterwards. They suggested that the observed reduction was probably caused by the reduction of free space. However, in assessing the effect of MFSA on barnacle settlement through percentages of adult cover, many authors do not isolate MFSA from other variables which may act simultaneously such as: (1) the release of conspecific chemical cues (summarized in Rittschof et al. 1998) and (2) modifications of the benthic boundary layer flows due to the presence of barnacles whose shells can be considered 'roughness elements' affecting the passive and/or active patterns of settlement (Eckman 1990, Havenhand & Svane 1991, Pawlik et al. 1991, Mullineaux & Garland 1993, Pawlik & Butman 1993, Harvey et al. 1995, Grégoire et al. 1996, Miron et al. 1996, Olivier et al. 1996, Walters & Wethey 1996, Abelson 1997, Abelson & Denny 1997, Harvey & Bourget 1997). Few studies have focused on the hydrodynamic influence of barnacle colonization, but in recent flume work barnacle density has been shown to have complex effects on the benthic boundary layer flows, with low, medium and high adult densities of *B. amphitrite* generating independent, interactive and skimming flows, respectively (Eckman et al. 1981, Nowell & Jumars 1984, Vogel 1994, Thomason et al. 1998). Furthermore, Thomason et al. (1998) pointed out that the hydrodynamic impact of the filter-feeding activity of barnacle adults may, in addition, have an impact on settlement through predation of settling larvae (Young & Gotelli 1988, André et al. 1993). In our study, we assessed the role of MFSA (surface free of detrititus) on settlement. The observed positive correlation between barnacle settlement and MFSA supports the results of others (Chabot & Bourget 1988, Raimondi 1990, Minchinton & Scheibling 1993, Hills & Thomason 1998b), while contradicting those of Bertness et al. (1992), Pineda (1994) and Pineda & Caswell (1997). We think that attention should be paid to experimental field studies to effectively control the variables which are likely to be important at differing spatial and temporal scales before concluding on the impact of a single factor on the settlement of barnacles. Our results may be useful to the models of Roughgarden et al. (1985) and Iwasa & Roughgarden (1986), who assume that settlement rate is proportional to the amount of suitable substrata. Unfortunately, we cannot determine how much variance in settlement intensity can be attributed to MFSA or bacterial densities, since both factors were statistically correlated. Further experiments are thus required for assessing their relative importance in the field. Moreover, our experiments were conducted at the beginning of the *B. amphitrite* recruitment period to limit the influence of conspecific interactions during attachment. It would be interesting to conduct further studies during the high recruitment period to confirm or refute the correlations between MFSA-bacterial densities and settlement that we determined during this low recruitment period.

Conclusions

This set of field experiments confirms the role of larval supply in determining the short-term vertical intertidal distribution of adults of *Balanus amphitrite*, but the longer-term variability in the larval supply/settlement coupling observed over a week may be related to daily variations in the physiology of settling larvae; this concept could be integrated in theoretical models of recruitment dynamics of barnacles. Our results suggest that post-settlement processes (predation, competition) may be predominant at the low intertidal level.

Once in contact with the substratum, cyprids of *B. amphitrite* settle upon clean surfaces where microbial free-space is maximum and avoid biofouled surfaces where microbial free-space is limited. Further analysis of the energetic contents of settling and settled larvae are currently being performed to assess, for the first time in the field, the role of larval physiology in controlling the habitat selection behavior leading to permanent attachment.

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