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# Successful external fertilization in turbulent environments

**(Fucales/gamete release/reproductive ecology/spawning/water motion)**

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**ABSTRACT** **Mathematical and experimental simulations predict that external fertilization is unsuccessful in habitats characterized by high water motion. A key assumption of such predictions is that gametes are released in hydrodynamic regimes that quickly dilute gametes. We used fucoid seaweeds to examine whether marine organisms in intertidal and subtidal habitats might achieve high levels of fertilization by restricting their release of gametes to calm intervals. *Fucus vesiculosus* L. (Baltic Sea) released high numbers of gametes only when maximal water velocities were below *ca.* 0.2 m/s immediately prior to natural periods of release, which occur in early evening in association with lunar cues. Natural fertilization success measured at two sites was always close to 100%. Laboratory experiments confirmed that (*i*) high water motion inhibits gamete release by *F. vesiculosus* and by the intertidal fucoids *Fucus distichus* L. (Maine) and *Pelvetia fastigiata* (J. Ag.) DeToni (California), and (*ii*) showed that photosynthesis is required for high gamete release. These data suggest that chemical changes in the boundary layer surrounding adults during photosynthesis and/or mechanosensitive channels may modulate gamete release in response to changing hydrodynamic conditions. Therefore, sensitivity to environmental factors can lead to successful external fertilization, even for species living in turbulent habitats.**

Marine organisms with external fertilization must achieve high probabilities of gamete encounters in an environment that may rapidly dilute gametes in three dimensions. The most important ecological conditions for successful external fertilization are synchrony of gamete release, proximity of individuals, and low water motion (1–8). Synchronous release of gametes has evolved in many marine organisms (e.g., refs. 9 and 10); however, even if release occurs synchronously and from aggregated individuals, a large number of potential recruits could be lost due to the effects of gamete dilution if spawning coincides with high water motion. Experimental field studies (1, 5) show that commonly observed water velocities dilute gametes close to their source, leading to the fertilization of only a small proportion of eggs; in addition, theoretical models (11, 12) predict that fertilization success in turbulent flows may be lower than 1%. An important exception may be surge channels, where turbulent mixing occurs, but exchange with the adjacent water body is low, thereby reducing dilution of gametes (13). But under such turbulent conditions shear forces still limit fertilization success (14). However, the widespread occurrence of polyspermy blocks (15) and reproductive pheromones (16–18) in organisms living in intertidal and subtidal habitats suggests that fertilization may often occur under calmer conditions than those that usually prevail in these habitats because polyspermy blocks are required when eggs are likely to encounter high densities of sperm at fertilization and pheromones are effective at short range (micrometers to millimeters). Quantitative data on fertilization success during natural events of gamete release show that during periods when the majority of gametes are released, an average of 70–100% of the eggs are fertilized in organisms as diverse as fucoid algae (19), echinoderms (4, 7, 20, 21), and fish (refs. 22 and 23; but also see ref. 24). Whether these high levels of fertilization are widely representative is unknown, although a few observations of simultaneous spawning by many species during slack tides (25, 26) and selection of spawning locations and periods (23) suggest that organisms might regulate reproductive behavior to avoid suffering the reduced fertilization success due to high water motion that modeling and experimental field studies have predicted.

Fucoid algae dominate the biomass on many intertidal rocky shores in temperate regions, and in the atidal Baltic Sea the dioecious species *Fucus vesiculosus* L. is the only widely distributed, large macroalga (27). Gametes in fucoids are released from numerous spherical conceptacles distributed subepidermally in the reproductive tissue (receptacles); conceptacles are connected to the surface of the alga by means of multicellular pores (28). Both eggs and zygotes are negatively buoyant, and the absence of a planktonic larval phase means that settlement is directly related to gamete release. Three species of fucoid algae, *F. vesiculosus*, *Fucus distichus* L., and *Pelvetia fastigiata* (J. Ag.) DeToni, were used to investigate the effects of water motion on gamete release and reproductive success. Our data demonstrate that adults sense unfavorable hydrodynamic conditions and restrict their release of gametes to calm periods, thereby achieving successful fertilization.

## MATERIALS AND METHODS

**Field Sites.** Natural gamete release and fertilization were studied in two dense beds of *F. vesiculosus* from Askö, Sweden: (*i*) Granlösa, at 0.3–1 m depth, exposed to westerly winds; and (*ii*) Björkholmen, at 0.5–2 m depth, exposed to south-southwesterly winds, which produce high water velocities at this site. The surface temperature of the water at Granlösa is often 1–2°C above that at Björkholmen, and the seasonal cover of epiphytic algae on *Fucus* is high at Granlösa and low at Björkholmen.

**Egg Settlement and Gamete Release.** Egg settlement (in 1994) was studied by collecting eggs and zygotes on disks (28.3 $cm^2$) of Sea-goin' poxy putty (Permalite Plastics, Newport Beach, CA) cast using no. 6 (Swedish) sandpaper (to provide uneven surfaces that retained most egg-sized particles) and detoxified (as in ref. 29). At each site, two disks were placed on inverted plastic lids of collection boxes attached to bricks under each of 10 randomly chosen females. Samples were retrieved between 2030 and 2300 (after the natural period of maximal release) by capping the box over the lid underwater

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*Abbreviation*: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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to prevent loss of sample. Eggs per disk were counted with a dissecting microscope.

Release of eggs and sperm in the field (in 1995) was studied directly by placing receptacles (10 female or 20 male receptacles per replicate) inside 50 ml polypropylene centrifuge tubes (10 replicates per sex) with the side walls replaced by nylon mesh (Nybolt, Seidengaze, Zurich, Switzerland; 40-$\mu$m mesh for females or 1-$\mu$m mesh for males). The nylon mesh allowed for diffusion of seawater and dissolved gases, which is accelerated during agitation, despite reducing water flow. The receptacles inside the tubes were still exposed to levels of agitation as close to natural as possible because the tubes were anchored at the depth of the receptacles in the canopy at each site (2 replicates per sex in each of 5 locations per site, totaling 20 tubes per site). The gametes were collected daily at 1000–1100, and counted with a hemocytometer (sperm) or with a dissecting microscope (eggs).

In 1994, water motion was described qualitatively as calm or rough by visual observation of whether receptacles in the field were motionless or being displaced by the current near to the anticipated period of gamete release (early evening). In 1995, two quantitative measurements of water velocity supplemented these observations: (*i*) release of fluorescein dye and (*ii*) measurements with a current meter (Marsh-McBirney 511, Frederick, MD) using a time constant of 0.2 s. Maximal current velocities were recorded every 5 min during 1 h with the probe positioned at the level of the receptacles (approximately 0.4 m above the bottom). These measurements could not be performed every day; instead, measurements were taken at each site during periods of both calm ($n = 5$) and rough ($n = 4$) water motion to establish a quantitative threshold lying between our qualitative levels.

**Fertilization Success.** The proportion of eggs fertilized during natural shedding events (natural fertilization success) was studied in the two populations of *F. vesiculosus* at Granlösa (1993 and 1994) and Björkholmen (1994). Eggs and zygotes were collected on plates (100 $cm^2$) of Sea-goin' poxy putty (29) placed on inverted plastic lids of food storage boxes under randomly chosen females (for each site and date, 10–20 plates were placed in the field; of those, settlement occurred on 6–12 plates, and these were analyzed). At 2000–2300 (i.e., after the natural period of gamete release), each sample was retrieved, by capping the plastic box underwater, and immediately fixed in 1:3 acetic acid:ethanol, before collecting the next plate. A subsample (*ca*. 50 eggs per plate) was stained with aceto-iron hematoxylin (19). Under their natural conditions, Baltic zygotes do not start adhering to the substrate for at least the first 8 h after fertilization; thus, there is no reason to expect differences in collecting efficiency between eggs and zygotes.

At each site, the positions of every male and female were mapped throughout most of the sampled area (31 $m^2$ in Granlösa, 35 $m^2$ in Björkholmen); these data were used to determine the densities of males and females, and the average distance between each female and the closest male.

**Agitation Experiments.** Consequences of the time and duration of high water motion on gamete release were studied with *F. vesiculosus* from Drivan, Sweden. Receptacles (20 per replicate, stored in the dark at 4°C for 4 days) were cultured (10°C, 325 $\mu$mol photons $m^{-2}{\cdot}s^{-1}$) from 0200 to 0000 in 100-ml flasks (3 replicates per treatment) containing 20 ml of Baltic water (5 ‰S). Agitation intervals were from 0200 to 0800, 0200 to 1200, 0200 to 1600, 0200 to 1800, 0200 to 2000, 1800 to 2000, 1600 to 2000, 1200 to 2000, 0800 to 2000, and unagitated (control treatment). Agitation was provided by an orbital shaker (KS250, IKA, Staufen, Germany) at 150 rpm.

To examine the effect of water motion on males and females, receptacles of *F. vesiculosus* (four per replicate, no storage) from Askö were cultured in 150-ml flasks (five replicates per treatment) containing 25 ml of Baltic water (6.5 ‰S, 13°C, 16 h light/8 h dark, 340 $\mu$mol photons $m^{-2}{\cdot}s^{-1}$) during 4-day periods coincident with the third quarter moon (experiment with females) or new moon (experiment with males). Water motion was provided by an orbital shaker (OS 71, Chiltern Scientific, Wendover, U.K.) at 150 rpm; controls were not agitated. Gametes released during each 8-h interval were counted.

*F. distichus* was collected at night from Chamberlain, Maine, and kept in culture overnight. Fragments with six to eight receptacles (0.5–1.0 g fresh weight were placed in 250-ml flasks (three replicates per treatment) containing 100 ml of seawater ($1 \pm 1$°C). Agitation was provided by an orbital shaker (Lab Line, Melrose Park, IL) at 200 rpm. In the simulated tidal treatment, the period of agitation was coincident with the period when the tidal pools were flushed during high tide (from 2 h prior until 2 h after high tide). The seawater in the flasks was changed 2 h prior to natural high tide periods (i.e., independently of the photoperiod, 11 h light/13 h dark, 550–600 $\mu$mol photons $m^{-2}{\cdot}s^{-1}$), and eggs were counted under a dissecting microscope.

Receptacles of *P. fastigiata* (six per replicate) from Pacific Grove, California, stored at 5°C in darkness for 4 days, were placed in 250-ml flasks (four replicates per treatment) containing 50 ml of seawater (15°C) and agitated with an orbital shaker (Lab Line, 140 rpm) or kept calm (controls) in the light (210–220 $\mu$mol photons $m^{-2}{\cdot}s^{-1}$). After 5 h of illumination, flasks with receptacles were transferred to darkness for 30 min to stimulate gamete release (30). Eggs released during the 5 h in light plus 30 min in darkness were counted under a dissecting microscope.

**Experiments with 3-(3,4-Dichlorophenyl)-1,1-Dimethylurea (DCMU).** Receptacles of *P. fastigiata* (six per replicate) stored for 5 days at 5°C in darkness were placed in 250-ml flasks (six replicates per treatment) containing 50 ml of seawater with 0 (control), 1, or 10 $\mu$M of DCMU. After 5 h of illumination (210–220 $\mu$mol photons $m^{-2}{\cdot}s^{-1}$, 15°C) flasks with receptacles were transferred to darkness for 30 min to stimulate gamete release (30). Receptacles of *F. vesiculosus* from Askö, Sweden (four per replicate, no storage) were placed in flasks (150 ml, five replicates per treatment) containing 25 ml of Baltic water (6.5 ‰S) with 0 (control), 1, or 10 $\mu$M of DCMU. Cultures (13°C, 340 $\mu$mol photons $m^{-2}{\cdot}s^{-1}$) were started at 1600 before the natural period of release; at 0000 the water was collected and eggs counted. DCMU was dissolved in ethanol; 0.1% ethanol was shown in previous experiments to have no effect on gamete release from either species.

**Statistical Analysis.** Data were log-transformed to homogenize variances (as verified with Cochran's test). Comparisons of means were performed with analysis of variance (experiments on duration of agitation period, agitation on *P. fastigiata*, and DCMU), or repeated measures analysis of variance (with Huynh–Feldt adjustment) for the experiments in which gamete release was measured more than once from the same receptacles (effects of agitation on *F. vesiculosus* and *F. distichus*). Tukey's test was used for multiple comparisons between means, except for the experiment on duration of agitation, where every mean was compared to the control using Dunnet's test. All statistical tests were performed with SYSTAT 5.2.1 or by performing the calculations on a spreadsheet. Where mentioned in the text and figures, "significant" is reported at the 0.05 level.

## RESULTS AND DISCUSSION

*F. vesiculosus* (dioecious) lives permanently submerged in the Baltic Sea, and natural gamete release and settlement of zygotes usually occur close to all four lunar phases (Fig. 1 and ref. 27). However, high release and settlement occurred exclusively under calm conditions (Fig. 1), and they were absent or low when high water motion was present at the site in late

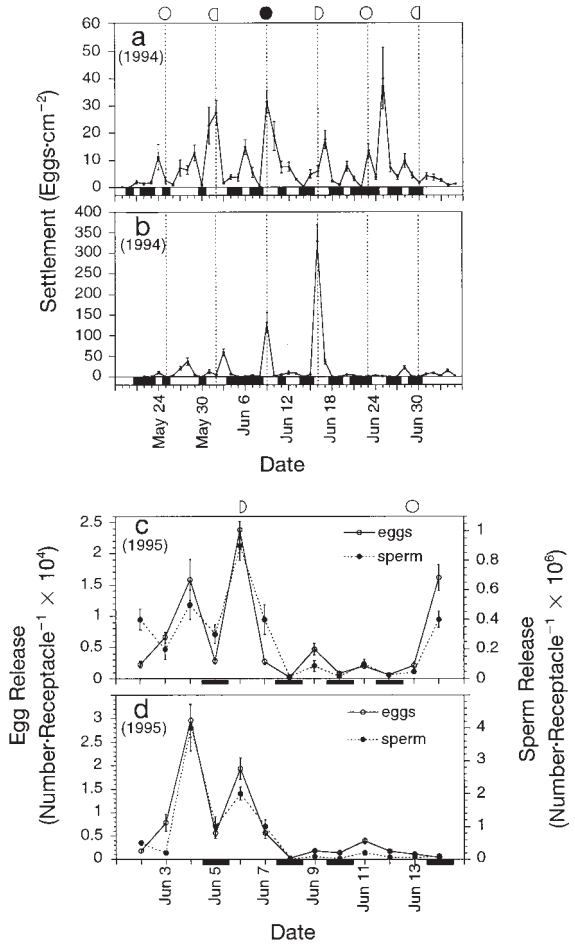


FIG. 1. Daily egg settlement (*a* and *b*) and gamete release (*c* and *d*) (mean ± SE) for *F. vesiculosus* from the Baltic Sea during the reproductive season of 1994 (egg settlement) and 1995 (gamete release) for two sites at Askö, Sweden: (*a* and *c*) Granlösa, (*b* and *d*) Björkholmen, showing that high release and settlement of eggs occurred only on calm days. Phases of the moon are shown above the graphs. Solid bars on the *x* axis represent rough days when currents caused movement of receptacles shortly prior to and during the natural time of high release in early evening.

afternoon. Maximal water velocity recorded with a current meter at the two field sites varied from 0.2 to 0.4 m/s during rough periods and from 0.04 to 0.17 m/s during calm periods; average currents estimated by dispersal of fluorescein dye varied from 0.09 to 0.13 m/s during rough periods and from 0.01 to 0.06 m/s during calm periods. Although maximal velocities (peak currents) reflect the hydrodynamic forces that the receptacles are experiencing, the measurements with fluorescein are a better estimate of the average effects of water motion in diluting sperm.

Male and female receptacles responded in the same way to simulated turbulence in laboratory experiments. Simulated turbulence in laboratory experiments (Fig. 2) showed that agitation inhibited release of eggs if it occurred near to the natural period of release of Baltic gametes (*ca.* 1700–2000). The duration of agitation (a total period of between 2 and 18 h) was unimportant, whereas the time during the day when receptacles were agitated was critical (Fig. 2). These data show that the algae respond quickly and very sensitively to hydrodynamic conditions. Gamete release was inhibited by turbulence during a period when release was high both in calm cultures (Fig. 3) and in the field (data not shown). Following the cessation of agitation, large numbers of gametes were released from both male and female receptacles that had been

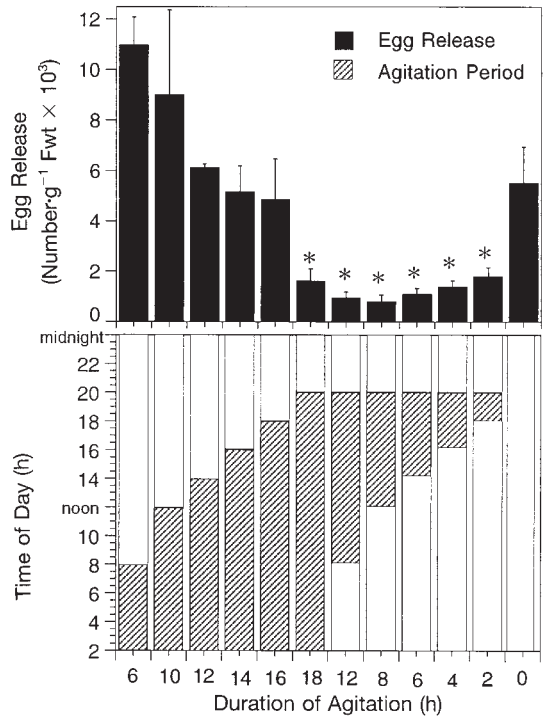


FIG. 2. Effect of time and duration of the period of agitation on the release of eggs (mean ± SE) from Baltic *F. vesiculosus*. Asterisks indicate results that differ significantly from the control (calm).

agitated (Fig. 3), but not immediately after agitation was stopped (at midnight); instead, high release occurred in the

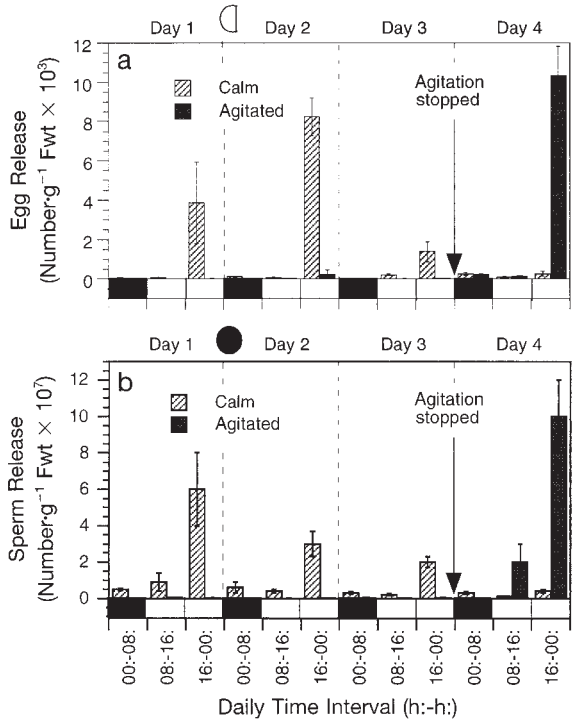


FIG. 3. Inhibitory effect of water motion on the release of gametes (mean ± SE: *a*, eggs; *b*, sperm) from Baltic *F. vesiculosus.* Calm treatments (controls) were not agitated. Solid bars on the *x* axis represent dark portions of the photoperiod. During the normal time (1600–0000) of gamete release, agitated cultures released significantly fewer eggs and sperm; after the cessation of agitation, previously agitated cultures released significantly more eggs and sperm than calm cultures.

early evening of the following day showing the same daily pattern observed in calm cultures and in the field (the interval 1600–0000 in Fig. 3 includes the natural period of release, *ca.* 1700–2000). Thus, *F. vesiculosus* in the Baltic Sea finely times gamete release within the 2-month-long reproductive season with a set of lunar, circadian, and environmental cues.

The synchronized release of gametes under calm conditions resulted in levels of fertilization success between 95 and 100% (Fig. 4) in two sites with different population densities: 2.6 females/$m^2$ and 2.2 males/$m^2$ (Granlösa), and 5.8 females/$m^2$ and 5.5 males/$m^2$ (Björkholmen). Despite the densities of reproductive individuals at Granlösa being approximately half those in Björkholmen, the degree of aggregation of males and females was high at both sites; the average distance between each female and the nearest male was 0.38 m ± 0.03 (mean ± SE, $n$ = 81 females) at Granlösa and 0.21 m ± 0.01 (mean ± SE, $n$ = 203 females) at Björkholmen. Thus, external fertilization in these populations occurs under the most favorable ecological conditions to maximize sperm:egg encounters: (*i*) gamete release is synchronous, (*ii*) it occurs during low water motion, and (*iii*) males and females are in close proximity.

Our data show that there is a general mechanism for coordinating gamete release with low water motion in fucoid seaweeds, as we have found that species from two other habitats (intertidal pools, intertidal zone) respond in a way similar to Baltic *F. vesiculosus. F. distichus* (monoecious) is restricted in Maine to rock pools in the high intertidal zone. Peaks of gamete release occur exclusively during daytime low tides (G.P. and S.H.B., unpublished data) when water motion is very low in the isolated pools. Cultured reproductive material continues to release in synchrony with daytime low tides when cultured under both constantly calm conditions and simulated tides (Fig. 5: tidal phase not shown). However, daytime release of gametes was almost abolished in cultures that were continuously agitated (Fig. 5). Thus, there appears to be an endogenous cycle of release coincident with daytime low tides that requires calm conditions. This is consistent with the observation that daytime lows occur during neap tides when tidal pools are often not washed for 3–4 days. Under these conditions, natural fertilization success in *F. distichus* is high (78–100%; G.P. and S.H.B., unpublished data). Interestingly, water motion did not affect the low release of gametes at night. High water motion also inhibits release of gametes from the intertidal alga *P. fastigiata* in cultures (significantly fewer eggs were released by agitated cultures (338 ± 131, mean ± SE) than by calm cultures (34,228 ± 5,147, mean ± SE), $n$ = 4 cultures per treatment).

Water motion affects fertilization success in several ways; some motion is advantageous for mixing eggs and sperm (1, 11, 14), but higher levels will cause rapid sperm dilution (1, 5, 11, 12) in addition to causing damage to zygotes (14). In field experiments where echinoderms were artificially induced to spawn, currents above 0.2 m/s reduced fertilization success from *ca.* 60% to *ca.* 20% at 10 cm away from the sperm source (1), and in even slower currents, average fertilization success was reduced from *ca.* 30% at 0.002 m/s to *ca.* 10% at 0.047 m/s (5). However, at low velocities of 0.00–0.12 m/s (2) or 0.003–0.009 m/s (3), current speed and/or direction did not affect fertilization success in experiments with hydroids (2) and echinoderms (3), and it has been suggested that spawning during calm intervals (e.g., during slack tide) might help organisms to avoid the negative effects of turbulence on external fertilization (25, 26), as demonstrated by our results. In addition to the restriction of gamete release to the calm conditions described here, organisms with external fertilization have evolved a variety of strategies [e.g., synchronous spawning (9, 10), high quantity and longevity of gametes (4, 7, 31), spawning in pairs or groups (23, 24)] that reduce the negative effects of water motion on fertilization success. Indeed, recent suggestions that sperm are limiting in the sea (32) rely heavily on theory and on experimental field studies in which gametes were released artificially, thus not reflecting what the fertilization success would be under the hydrodynamic conditions and the concentrations of gametes that are likely to occur during natural periods of gamete release. Recent reports on natural fertilization success in a variety of "broadcast spawners" (4, 7, 19–23) show that the majority of eggs released is fertilized; thus, high fertilization success occurs in nature and may even be the rule rather than the exception if organisms are able to release their gametes under ideal ecological conditions. Some individuals in any population may have limited reproductive success; for example, natural fertilization success in some echinoderms has been observed to fall from 83–99% (at the peak of a major spawning event) to 9–28% for eggs released after the majority of individuals had spawned (4, 7, 20), or to be as low as 0–2% for eggs from an isolated female with delayed spawning [other conspecific females had 81–90% of eggs fertilized (20)]. These values

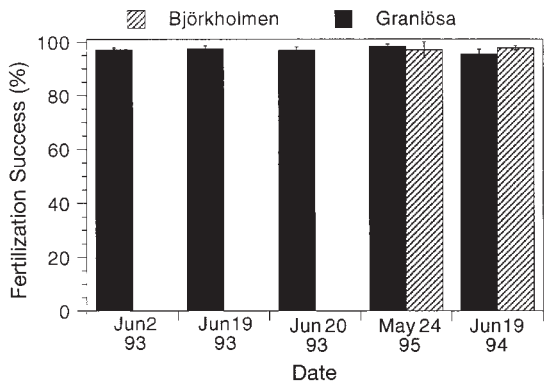


FIG. 4. Success of fertilization (mean ± SE) during natural gamete release in *F. vesiculosus* from the Baltic Sea at Askö: Granlösa (1993 and 1994) and Björkholmen (1994).

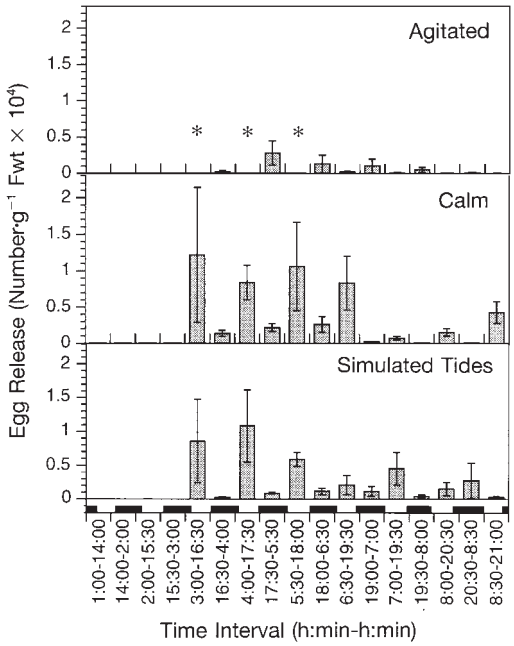


FIG. 5. Effects of water motion on the release of eggs (mean ± SE) from *F. distichus* under constant agitation, calm conditions, or agitation during natural high tides (simulated tides). Black bars (*x* axis) represent dark portions of the photoperiod. Asterisks indicate significant differences between the agitated treatment and the other two treatments at the time intervals shown. There were no differences between constantly calm and simulated tidal cultures at any time interval.

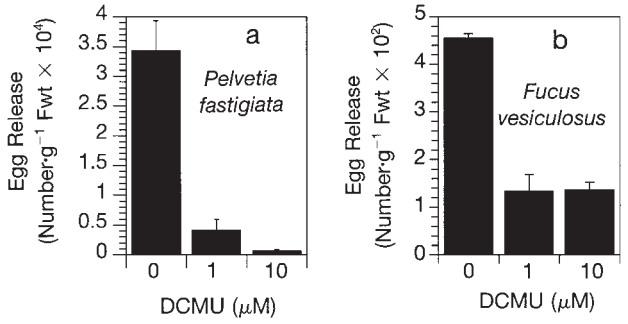


FIG. 6. Inhibitory effect of DCMU on egg release (mean ± SE) under calm conditions in (*a*) *P. fastigiata* and (*b*) *F. vesiculosus*. Significantly higher numbers of eggs were released in the controls than in the treatments.

establish that even small changes from the optimal conditions for fertilization during natural gamete release may have drastic effects. Sperm dilution may thus become a major limitation for the reproductive success of some females; for example, those that spawn slightly later or in isolation from the majority of individuals. However, even in these cases, the proportion of eggs that are fertilized is still high for the population as a whole, because the majority of eggs is released within a favorable temporal and spatial window for successful fertilization (4, 7, 20).

How do fucoid seaweeds sense changing hydrodynamic conditions? Gamete release in each of the three species we have studied occurred predominantly in calm water and with a light requirement that included photosynthetic competence: release in *P. fastigiata* and in *F. vesiculosus* is inhibited by DCMU (Fig. 6), which specifically inhibits photosystem II electron transport. Release may thus be stimulated by chemical changes occurring in the boundary layer surrounding the receptacles during photosynthesis under calm conditions, such as carbon limitation, increasingly alkaline pH, or oxygen supersaturation. Alternatively, the response to water motion may be mediated by specific mechanical sensitivity to agitation, perhaps via stretch-activated channels (33, 34). The signaling mechanism could result in turgor changes that regulate gamete release, analogous to stomatal responses in plants (35). More information on the mechanisms to detect water motion is needed to specify which aspects of water motion inhibit gamete release (i.e., both average water flow and maximal currents are likely to reduce boundary layer effects, but the latter are perhaps more likely to be effective in activating putative stretch-activated channels). The particular mechanism for responding to water motion in fucoids may not be general, because it is tied in some way to photosynthesis; however, all organisms with external fertilization have sensory systems that could mediate similar responses. In conclusion, our results provide direct evidence for a functional link between synchronous gamete release and an environmental variable (water motion) that plays a key role in the reproductive success of organisms with external fertilization.

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