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Interference Tests with the Pheromone System of the Brown Alga *Cutleria multifida*

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ABSTRACT: Male gametes of the brown alga *Cutleria multifida* (Smith) Grev. are attracted to sources which release the sex pheromone multifidene into seawater. In interference tests the effect of additional pheromone or an analogue was studied. The threshold concentration significantly affecting gametes varies around 60 ng of multifidene l^{-1}. Crude oils interfered with the chemotactic response at concentrations between 1 mg and 23 μg l^{-1}; lower concentrations were not tested. But petroleum hydrocarbons did not initiate the characteristic search pattern which male gametes normally display in the presence of multifidene. In contrast, extracts containing liquid hydrocarbons and similar compounds of low polarity from different seawater samples caused this characteristic response. This indicates an ubiquitous presence of pheromone-like compounds in the sea.

INTRODUCTION

One difficulty in the quantification of a chemotactic response of male gametes of brown algae is lack of knowledge of the actual effective attractant concentration in the medium. In analyzing pheromone systems of marine brown algae (e. g. Müller, 1976; Müller and Seferiadis, 1977), concentrations were known only for the spiked hydrophobic droplets which acted as attractant sources. Only vague estimates exist on how much of the spike compound is lost from the droplets into the surrounding seawater. Quantification in these studies was thus restricted to relative concentrations in the medium, derived from the spike concentration in the hydrophobic solvent. In addition, the technique does not allow comparison between the efficiency of compounds of different chemical properties because differences in solubility would also change diffusion rates. Summarizing the situation, we are still ignorant of the absolute concentration of an algal pheromone necessary to induce a chemotactic response. In other words, we have no idea about naturally effective pheromone concentrations in the marine ecosystem. Consequently, it is even more difficult to estimate the possible interference of anthropogenic compounds with pheromone reactions (Derenbach and Gereck, 1980).

In this study we measured the effective concentrations of attractants in seawater by compensating the attraction of a pheromone source through mixing additional pheromone with the culture medium until a 'dead stop' was achieved. In case the pheromone concentration of the artificial egg was reduced just to attract gametes, a compensation of this attraction by adding a known amount of the pheromone into the medium anticipatedly revealed the chemotactic effective threshold concentration of the pheromone. With this gamete 'titration' – the interference test – the chemotactic effect of the proper pheromone, a pheromone analogue and other compounds could be measured.

MATERIAL AND METHODS

Culture Material

Male gametes of *Cutleria multifida* (Smith) Grev. were harvested from cultures as described by Müller (1975, 1976).

Attracting and Interfering Compounds

The following synthetic pheromones were used: The $C_{11}H_{16}$ hydrocarbon multifidene, cis-1-vinyl-2-(cis-1'-butenyl)-cyclopent-3-ene (Boland and Jaenicke, 1978) and the multifidene carboxylic acid methylester analogue E2, cis-2-(cis-1'-butenyl)-cyclopent-3-ene-1-car-

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boxylic acid methylester (Boland and Jaenicke, 1979). From both compounds dissolutions were prepared in FC78, a fluorinated hydrocarbon supplied by the 3M Company, Düsseldorf (FRG). 1.1×10^{-6} M multifidene was used in the attracting droplets (see below). Solutions of multifidene and E2 in seawater were prepared by thoroughly mixing 10 to 20 ml of culture medium with 1 µl of multifidene in FC78.

The following crude-oil samples were obtained from the US Bureau of Mines: Nigerian/Escravos, Tijuana/Lasalina, Orito/Colombia, Agha Sari/Kharg Island, Heavy Iranian/Point Tupper, Pensylvanian Atoka Congl./Delong Field, Boscan Venezuela/–, La Rosa crude/–, Languillas crude/–, South Lousiana crude/–, Wilmington crude/–. Light Diesel oil was obtained from a marine bunker station. Benzene and naphthalene were of p.a. grade purity. These last 3 compounds dissolved easily in FC78 and seawater at the desired concentrations. To dissolve at least some of the compounds contained in crude oils in FC78, 5 mg each were added to 1 ml solvent and thoroughly mixed for 5 min, using a vibrator. Similarly 1 to 0.5 mg of the Agha Sari, the Tijuana, the Wilmington, the Boscan Venezuela, and the Languillas crude oils were added to 15 ml culture medium and shaken vigorously for 10 min.

One of the water extracts has been sampled by adsorption onto Amberlite XAD-2 from the open Baltic in the Gotland Sea during May 1979 (Ehrhardt et al., 1980). The other extracts originate from the tropical Atlantic Ocean (Equator near 22 ° West, March 1979), from the Gotland Sea (May 1979), and from coastal waters near Bergen (Norway, June 1979). They were prepared by purging either boiling seawater or a suspension of collected particulate material with nitrogen and collecting the purged compounds in a cool trap at – 25 °C. Compounds of low polarity and of a volatility similar to that of n-alkanes with 8 to 17 carbon atoms were thus collected at an average efficiency of 73 %. All extracts were dissolved in pentane. This was evaporated at – 30 °C under a nitrogen stream and the residue redissolved in an approprivate amount of FC78, before being added in a 1 µl portion to 10 ml culture medium.

Attractivity and Interference Tests

In attractivity tests the response of *Cutleria multifida* gametes to droplets of spiked FC78 was compared with the response to a nonspiked droplet (Müller, 1976). In interference tests the response of gametes was measured in a culture medium, spiked or 'contaminated' with the compound to be tested for its interfering effect.

Measurement of Interference Compounds

As pointed out below, we could not rely on the amount of the interference compound dissolved in the test medium to equal its concentration during the actual test. Loss due to adsorption was measured in 7 series of simulated experiments: multifidene spiked culture medium (300 ng l^{-1}) was exposed in test chambers without adding fluorocarbon droplets and gametes. After 8 min (equal to the residence time of the medium in test chambers under test conditions, including preparatory steps) the contents of several test chambers per series were collected for extraction with methylene chloride. The extracts were sealed in glass vials and later concentrated under a nitrogen stream at –25 °C to about 10 µl for GLC analysis. GLC conditions were: OV101 coated 20 m × 0.3 mm glass capillary column in a Carlo Erba 2150 Fractovap; H_2-carrier gas at 2.5 ml min^{-1}; splitless injection 1 min; isothermal programme at 50 °C. A procedural loss of ca 25 % was measured for multifidene and different alkanes near the molecular weight of multifidene. All data were therefore corrected for a 25 % loss.

The crude-oil concentrations in seawater were measured fluorimetrically (UNESCO, 1976). Extraction was with hexane and calibration by known concentrations of the individual crude oils in hexane. The loss of spike material due to adsorption was measured in one simulated experiment for each type of oil tested.

RESULTS AND DISCUSSION

For an evaluation of the results from the interference tests it is necessary to know the concentration of the pheromone in the spiked medium, as well as in the spiked fluorocarbon droplets. Though the latter lose some of the spike material into the surrounding medium during the experiment, this effect is neglected and the concentration of the spike compound assumed to be equal to what had been added to the solvent. The test medium, on the other hand, will not retain all of the dissolved hydrophobic spike compound. A large amount will be adsorbed onto the walls of the experimental chambers. The dissolved portion that may be regained with a suitable solvent from the spiked medium after exposure during the experiment, is thought to be equal to what had been available to the organisms. It is assumed that the concentration of this dissolved fraction of the pheromone remains constant during the experiment. Its concentration was measured in simulated experiments and an average of 22 % (standard deviation ± 7) of the multifidene added to the medium was found in a dissolved state. All

pheromone concentrations given in Figure 1 are corrected for this average loss of 78 %.

Knowing the actual pheromone level in the experiment, we tried to find the threshold concentration for multifidene which compensates for attraction. In our interference tests the fluorocarbon droplets were spiked to contain 1.1×10^{-6} M multifidene. Without multifidene in the medium they attracted about 3 times more gametes as did the blank droplets: the exact value for the quotient (Q) formed from numbers of spermatozoids counted at spiked droplets divided by numbers counted at blank droplets was 2.7 (standard deviation ± 0.9).

The results of the interference tests, that is the compensating effect of different amounts of multifidene in the culture medium, are given in Figure 1. The response of gametes is plotted versus the concentration of the interfering compounds multifidene and the multifidene analogue E2. In our tests both compounds were found to be similarly effective though in earlier attractivity measurements (Boland, Jaenicke, Müller, in preparation) the multifidene analogue E2 seemed to be more effective by some orders of magnitude. The differences in polarity between both compounds, resulting in an improved solubility of E2 in seawater, would explain these former findings. Much less pronounced, this also might affect our data for E2, since they are corrected for the same adsorption that had been measured for multifidene. Accordingly, if E2 should be slightly less adsorbed, it would be less efficient as a pheromone than given in our graph.

The main result of our interference tests is the measurement of the effect of a known concentration of a pheromone in the medium. The amount of about 60 ng of multifidene l^{-1} seawater compensates for about 50 % of a normally observed chemotactic response of *Cutleria multifida* gametes. As a first approximation this concentration of about 60 ng multifidene l^{-1} is thought to be equal to a naturally effective threshold concentration of multifidene. However, a slight shift to values below 60 ng could be obtained by using less intensly spiked fluorocarbon droplets; a similar effect was observed in earlier experiments with *Fucus vesiculosus* gametes (Derenbach and Gereck, 1980). Consequently, the result depends to some degree on whether a threshold concentration is defined as the lowest possible concentration to attract a few out of a large number of gametes or whether it is expected to affect a large portion of the gametes present.

In earlier experiments fossile oils were shown to mimic sexual chemotaxis of *Fucus vesiculosus* gametes. Similar attractivity tests with *Cutleria multifida* gametes gave different results. These gametes seemed to flee from fluorocarbon droplets spiked with different crude oils from Iran, Nigeria, Venezuela, and USA, rather than to mistake the oils as a pheromone. Diesel oil (1 µl in 1 ml of fluorocarbon), benzene (10^{-3} M), and naphthalene (10^{-4} M) also did not affect

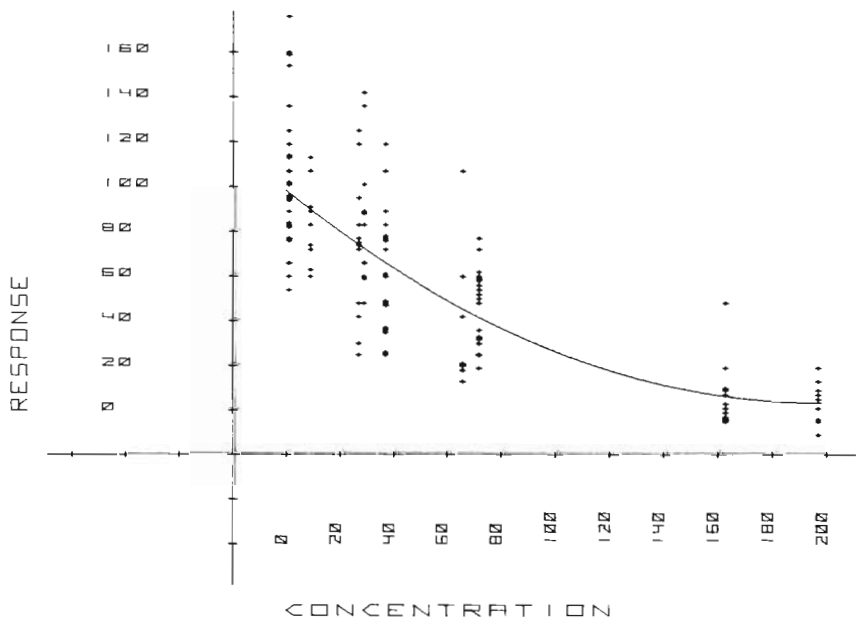

Fig. 1. *Cutleria multifida*. Responses of gametes plotted vs concentration of interfering compounds multifidene and E2. Response Q: number of gametes counted at a multifidene-spiked fluorocarbon droplet divided by the number counted at a blank droplet. Average Q-values measured in clean medium (no interfering compound present) taken as 100 % A Q-value of 1 = 0 %. Concentration expressed as ng l^{-1}. Most measurements made with interfering compound multifidene; at 8, 26, and 162 ng l^{-1} the multifidene analogue E2 was taken instead. As an approximation a least-squares quadratic curve was fitted to the data set

the gametes, except for 10^{-3} M naphthalene in fluorocarbon droplets, which tended to immobilize gametes in the vicinity of the droplets.

Details of locomotory behaviour in male gametes of *Cutleria multifida* seem to be an important aspect connected with their response to the sexual pheromone. Without multifidene in the culture medium or up to a level of about 10 ng l^{-1} they move freely through the water in rather straight lines. In the presence of the pheromone at a concentration of around 100 ng l^{-1} they are stimulated to move in loops and circles, similar to the reaction of *Ectocarpus siliculosus* gametes (Müller, 1978). Further increase of the pheromone concentration gradually seems to immobilize a large portion of the gametes.

In the following experiments the interference of the test compounds in the medium prohibited a significant attraction of gametes. Therefore, we concluded from the visually observed type of motion of the gametes onto the efficiency of a compound as a pheromone substitute. The results should be regarded as semiquantitative at best. They were recorded by mere visual observation. In addition, we are unable to give more than an estimate on the concentrations for the compounds dissolved in seawater.

Saturated solutions of Diesel oil and a series of different crude oils in seawater almost suppressed the natural response of gametes in interference tests. The type of movement of gametes did not differ from what is usually observed in a medium without any pheromone. They moved freely in straight lines. Although petroleum hydrocarbons did not mimic sexual chemotaxis of *Cutleria multifida* gametes, the attraction of a pheromone source, which normally would attract 3 times more gametes than the blank droplets, was suppressed when the medium contained a concentration of petroleum hydrocarbons in the medium of about 1 mg l^{-1}. Only two oils were tested at lower concentrations. The Boscan Venezuela crude at 23 μg l^{-1} and the Languillas crude at 870 μg l^{-1} had the same interference effect. The values given are corrected for an average loss of 66 % (standard deviation $\pm$ 18) due to adsorption.

We then examined the interfering potential of water extracts, and extracts of particulate material from the tropical Atlantic Ocean, from coastal waters near Bergen (Norway), and from the open Baltic Sea. A portion of the extracts representing 5 to 10 l of seawater was added to 10 ml of test medium. Adsorption onto the walls of the test chambers was estimated to reduce the actual concentration to an extract from between 1 to 2 l 10 ml^{-1} medium. Interference tests with these preparations resulted in almost total suppression of the natural response of gametes towards the pheromone source, as found for crude oils in the medium. However, in contrast to oil interference, the gametes now moved in circles and loops observed before only by adding the pheromone or a pheromone analogue to the medium. Since algal pheromones known so far are olefines and since they had been extracted among other compounds at pg- concentrations l^{-1} from seawater, this group of compounds most likely contains the substances that have caused the observed so called 'pheromone effect'. In addition, olefinic hydrocarbons are not to be found in crude oils, though they contain a wide range of hydrocarbons.

The same culture media that had been spiked with crude oils or with seawater extracts were also tested for possible interference with a still unknown pheromone of *Laminaria digitata* (Müller et al., 1979). The spermatozoid release reaction was taken as an indication for 'pheromone effects'. Preliminary results (Lüthe and Müller, personal communication) were similar to our findings with *Cutleria multifida*. Crude oils did not initiate the pheromone reaction. In their presence, however, normally effective pheromone concentrations could no longer trigger spermatozoid release. The different water extracts, on the other hand, caused some release of male gametes, but again no further release of gametes could be stimulated with the natural pheromone.

In addition to the interference of crude oils with natural pheromone systems, we found that all the different seawater extracts tested contained compounds which influence sensitive natural systems. The effects observed are similar to those initiated with the native sexual pheromone or a pheromone analogue. The same reaction could not be stimulated with a wide range of petroleum hydrocarbons. The response is thus regarded as being rather specific. Hence our findings may perhaps be taken as an indication for the ubiquitous presence of low concentrations of pheromone-like compounds in seawater which might have originated from plankton algae.

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