Chapter 7

Stress ethylene production in four marine macrophytes

ABSTRACT

To determine if stress ethylene production could be used to screen pollutants for sublethal toxicity on marine plants, four marine macrophytes (*Spartina alterniflora* Loisel, *Zostera marina* L., *Ulva lactucum* L., and *Ceramium* sp.) were exposed to phytotoxicants (Cu^{2+}, No. 2 fuel oil, 2,4-D, and naphthalene; $\approx 10^{-7}$ to 10^{-3} M or v/v). The response of each species to chemical stress varied greatly, but in all cases, Cu^{2+} induced the highest rates of stress ethylene production, and showed effects at lower concentrations than the other compounds tested. *Ulva* and *Zostera* significantly increased ethylene production when exposed to the highest concentrations of Fuel oil, but *Spartina* showed no response. Similarly, 2,4-D was a weak inducer of ethylene in *Ulva* and *Spartina*, and did not induce a response in *Zostera*, even at 10^{-4} M. None of the species produced ethylene in response to naphthalene exposure.

Ethane production was also produced by the plants in response to the phytotoxicants. Because significant increases in ethane production often co-occurred with increases in ethylene, the production of some of the ethylene observed may be from a peroxidation pathway, and indication of severe toxicity. These results suggest that this assay is not sensitive enough for assaying sublethal toxicity of pollutants in marine plants. Because Cu^{2+} induced an ethylene response in *Ulva* at 10^{-7} M, this assay may have limited use in assessing the relative toxicity of different algal species to Cu^{2+}. This is the first observation of

stress ethylene production in macroalgae, but other studies suggest ethylene production is widespread among phototrophs.

INTRODUCTION

Ethylene is a metabolite that controls fruit ripening, leaf senescence, and other physiological processes in higher plants (1,13). Photosynthetic aquatic organisms also produce ethylene, including submerged freshwater angiosperms during senescence and in response to IAA and kenetin (8), a green macroalga (*Ulva*) in response to IAA, a phytoplankter (*Scenedesmus*) in response to Cu^{2+} (12), and as a natural product in blue-green algae (7). It is unclear how ethylene production affects algal metabolism, or if production is ubiquitous among all algal groups.

In the 1970's it became apparent that ethylene can be induced in plants by a variety of mechanisms including physical injury, waterlogging and waterdeficit, freezing, or exposure to ozone, SO_2, NaCl, or soluble toxic compounds (4,5,6,9,11,15,17). The production of ethylene as a response to plant injury has become known as "wound ethylene" or "stress ethylene" and its production has been clearly identified as the degradation product of ACC which is derived from a methionine based precursor (6,9,17).

Tingey (15) and Rhodecap and Tingey (11) were the first to outline a rapid assay for testing the toxicity of phytotoxicants using stress ethylene production. With this assay, they were able to rank the relative toxicity of both organic and inorganic compounds applied to the rhizosphere of *Phaseolus*.

Ethane is often measured concurrently with ethylene and can also be used as a measure of stress. Its production, however, is dependant upon a different pathway--the peroxidation of fatty acids in membranes

(10)--thus it is considered an indicator of severe stress or cell death and less sensitive than ethylene production (9). Sometimes the production of ethylene is attributed to this pathway (10,11), but it is generally assumed that ACC metabolism is the primary pathway of ethylene production in higher plants. The induction of stress ethylene in marine macrophytes by toxic compounds has not been previously examined.

To assess the impact of pollutants in the marine environment, bioassays are needed which are fast, simple, and sensitive. The purpose of this study was to test if stress ethylene is produced in several taxonomically diverse macrophytes, and if so, to determine if it meets these criteria.

MATERIALS AND METHODS

An emergent marine angiosperm (*Spartina alterniflora* Loisel), a submerged angiosperm (*Zostera marina* L.), a green algae (*Ulva lactuca* L.), and a red alga (*Ceramium* sp.) were exposed to two or more of the following compounds: $CuSO_4$, naphthalene, 2,4-D, and the water soluble fraction (WSF) of No. 2 fuel oil (Bayton, Texas Exxon oil refinery)[2]. Test solutions were prepared using glass fiber (Whatman C) filtered seawater. Concentrations are given as molarity, except for No. 2 fuel oil solutions, which was reported as the concentration of the water soluble fraction (WSF) as v/v. The WSF test solutions were made from a 1 ppt WSF stock solution. The 1 ppt WSF was prepared by mixing 1 ml of No. 2 fuel oil and 1 l of GFC filtered seawater. This mixture was stirred vigorously in a flask with a stir bar for 2 hr, then allowed to

separate in a separatory funnel overnight. The WSF stock solution consisted of the aqueous phase.

Plants were collected in the field and acclimatized to laboratory conditions for at least 24 h. Plant segments of approximately equal size (0.1 to 0.4 g depending upon species) and were cut with a razor blade. These samples were inserted in 15 x 85 mm test tubes containing 4 ml of test solution, and sealed with a serum stopper. Conditions were altered for some experiments, but unless specified, the samples were incubated in a recirculating seawater aquarium at 18-20° C, with a 16:8 light:dark cycle under a light bank of incandescent and fluorescent light yielding ca. 180 µE m-2 sec-1 (PAR, measured with a Li-Cor Inc. calibrated light meter). Incubation time varied between experiments and typically ranged from 24 to 96 h. Within any one experiment, however, all samples were treated identically and generally consisted of 3 to 5 replicates at 4 or 5 concentrations plus controls and blanks. The *Ulva* time-course experiment consisted of 25 samples, 5 of which were sampled approximately every 24 h. The *Zostera* and *Spartina* samples consisted of healthy tissue with epiphytes removed, and unless specified otherwise, consisted of mid-leaf segments.

To measure ethylene and ethane concentrations, 1 ml gas samples (collected in gas tight syringes), were injected into a Varian 1400 gas chromatograph equipped with a flame ionization detector and supporting a 1800 x 6-mm Porapak N column (column temp. 65° C, N2 carrier 40 ml min-1). Standard curves were made from dilutions of 100 ppm ethylene and pure ethane (Suppleco Inc. Houston, TX). Background concentrations were 10 ppb, near the level of detectability. Blanks (test solutions

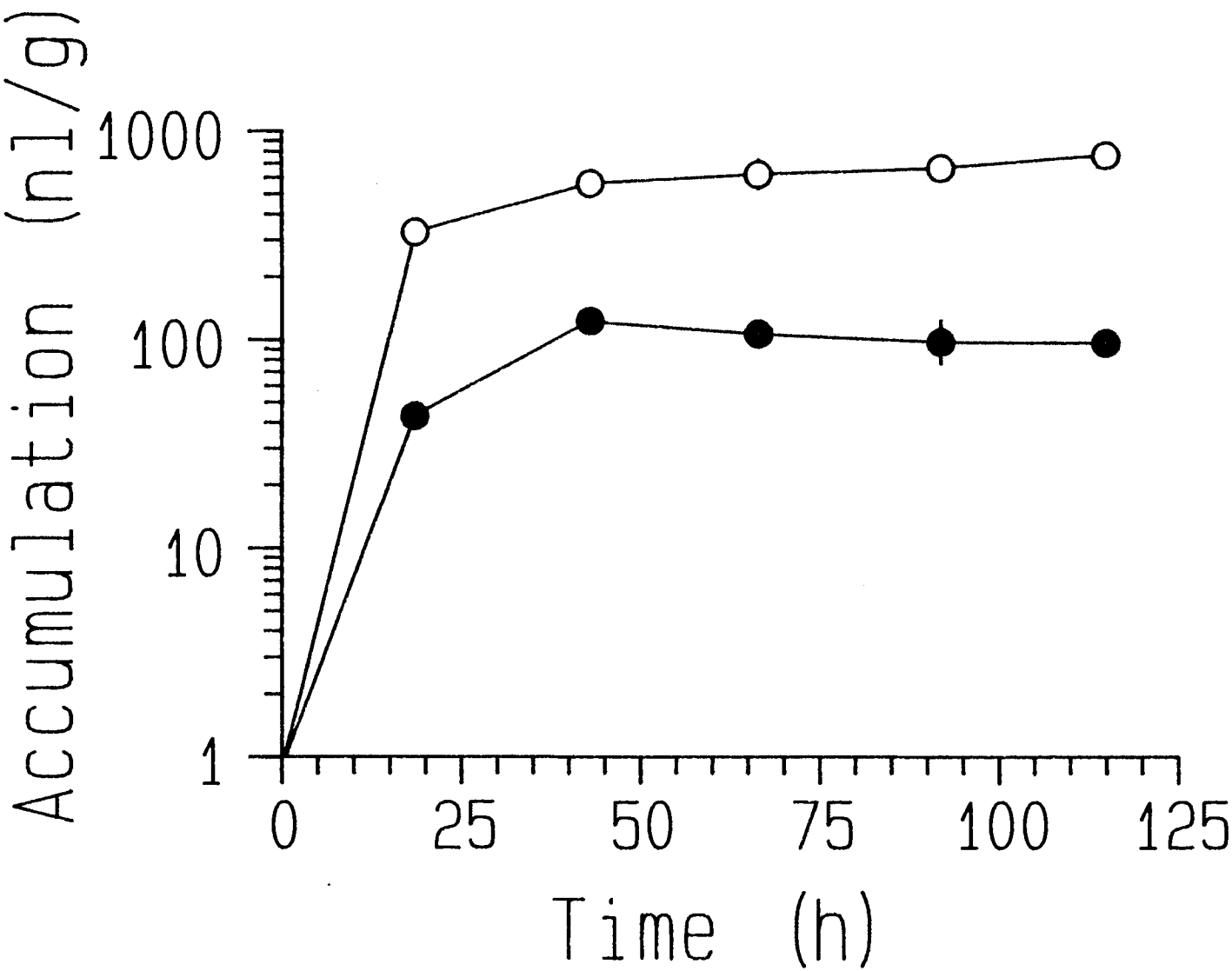
incubated without plant tissue) were indistinguishable from normal background levels except in the highest test concentration of No. 2 fuel oil, but even then it was not high enough to alter the interpretation of the results. Ethylene production was corrected for sample volume, pressure reduction, gas solubility, and plant weight which was measured at the end of each experiment and is presented as total nl accumulated per g wet wt. Concentrations were not adjusted for length of the incubation because most ethylene accumulates during the first 24 h and long incubation times do not result in proportionally large accumulations (see RESULTS). Ethylene production was log-transformed to normalize variance (11) and all treatments were compared using one-way anovas. If the phytoxicant demonstrated a significant in the anova test, the first treatment concentration illiciting a ethylene response higher than the control was identified using a GT2 paired mean test (14). Both tests were assessed at $\alpha = 0.05\%$.

RESULTS

All the macrophytes showed stress ethylene production, but response varied among species and test compounds. A time course experiment for *Ulva* exposed to 10^{-4} M Cu^{2+} is shown in Fig. 1. Ethylene production was most rapid in the first 24 h (80% of total accumulation) and stopped after 48 h. The decrease in ethylene after 48 h was probably due to diffusion out of the tubes, whereas the increases in ethane probably resulted from continual peroxidation of cell membranes.

These results are similar to observations of ethylene production in higher plants, which terminate ethylene production between 6 and 60

Fig. 1. Ethylene (●) and ethane (○) production in *Ulva* exposed to 10^{-4} M Cu. Production is shown as total accumulation over time. The mean ± SE of 5 samples at each time are shown for 25 different samples.


Accumulation (nl/g)
1000
100
10
1
0
25
50
75
100
125
Time (h)

hr depending upon conditions (6,15). Consequently, even though the incubations were of somewhat different length in each of the experiments, comparisons of maximum rates of ethylene production among species are valid because most experiments continued for more than 20 h, and also because the ethylene production response among species often differed by an order of magnitude or more.

Stress ethylene was induced by Cu^{2+} in each species (Fig 2). At 10^{-3} M Cu^{2+} ethylene production was highest in *Ulva* (250 nl g^{-1}) and lowest in *Spartina* (11 nl g^{-1}). *Ulva* showed a significant increase in ethylene production at 10^{-6}. *Ceramium* showed a slight increase 10^{-6}, and a significant increase in ethylene production at 10^{-5}. The angiosperms were less sensitive to Cu^{2+}, both of which showed significant increases in ethylene production only at 10^{-3} M. For all species, the samples became chlorotic or necrotic at 10^{-3} M, and except for *Spartina*, which showed some discoloration at 10^{-4} M Cu^{2+}.

Neither *Ulva* nor *Zostera* responded to naphthalene, even at 1.6 x 10^{-3} M (data not shown). These plants also did not become chlorotic. The responses of *Ceramium* and *Spartina* to naphthalene were not tested.

The WSF of No. 2 fuel oil induced a slight but statistically significant increase in ethylene production in *Zostera* leaves at 1 ppt , but no measurable effects on *Zostera* root and rhizome samples at that concentration (Fig. 3). *Ulva* showed an ethylene response at 100 ppm WSF (Fig. 3), but *Spartina* did not show increased ethylene production (not shown). The maximal rate of ethylene production in *Ulva* in response to fuel oil (32.6 nl/g, Fig. 3) was far less than observed with exposure to Cu^{2+}. *Ceramium* was not tested with fuel oil.

Fig. 2. Ethylene and ethane production after Cu^{2+} exposure. *Ulva*: duration 65.5 h, mean and standard deviation of 5 replicates at each concentration; *Ceramium*: 61.5 h, 5 replicates each; *Zostera*: 123 h, 3 replicates each; *Spartina*: leaf base, 48 h., 2 replicates each except for the control (4 replicates) and 10^{-7} M (3 replicates). Asterisk indicates statistically significant difference from the control (C) (see text).

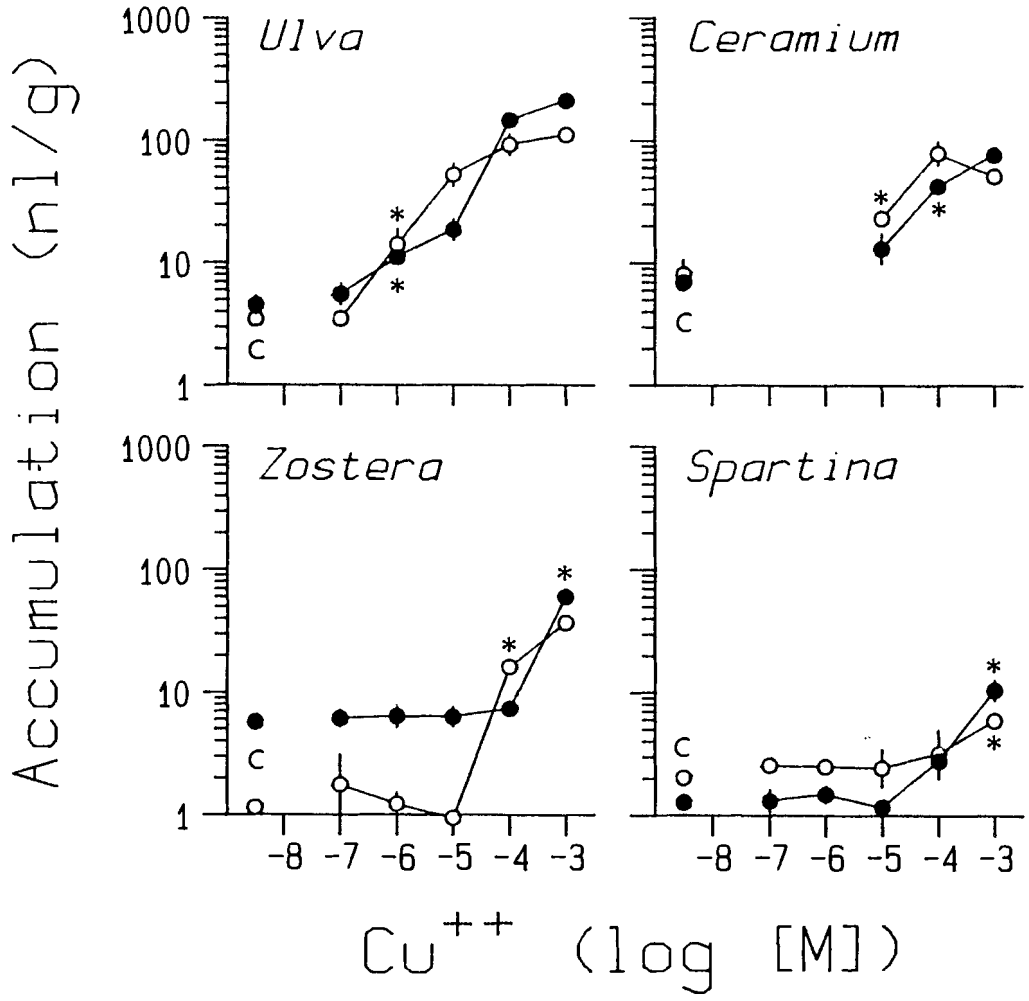

Ulva
Ceramium
Zostera
Spartina
1000
100
10
1
c
*
-8 -7 -6 -5 -4 -3
Accumulation (nl/g)
Cu++ (log [M])

Fig. 3. Ethylene and ethane production induced by the water-soluble fraction of No. 2 fuel oil. *Ulva*: 13 h, in sunlight at 31°, 5 replicates at each concentration; *Zostera* 13 h in sunlight at 31°, 3 replicates at each concentration.

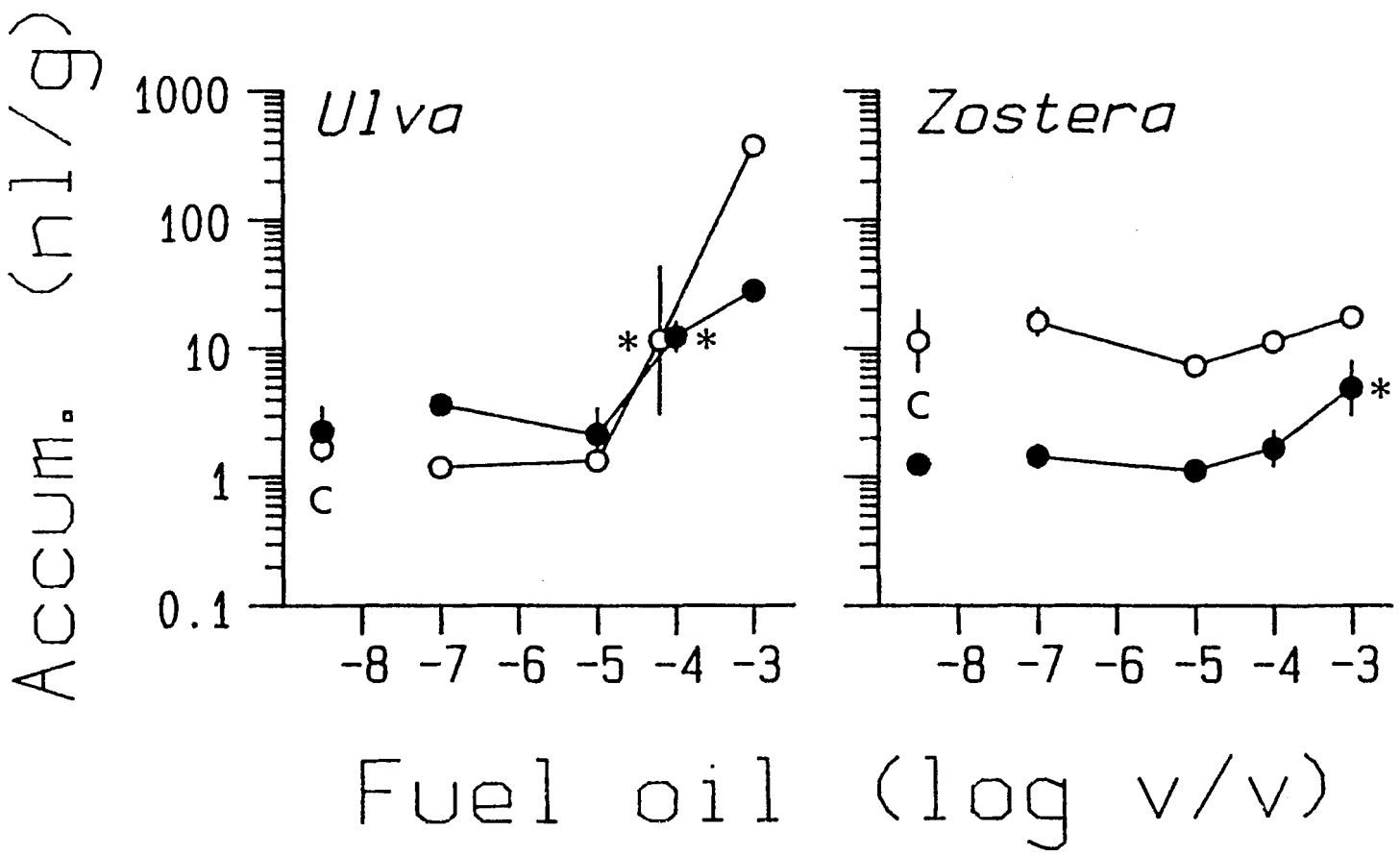
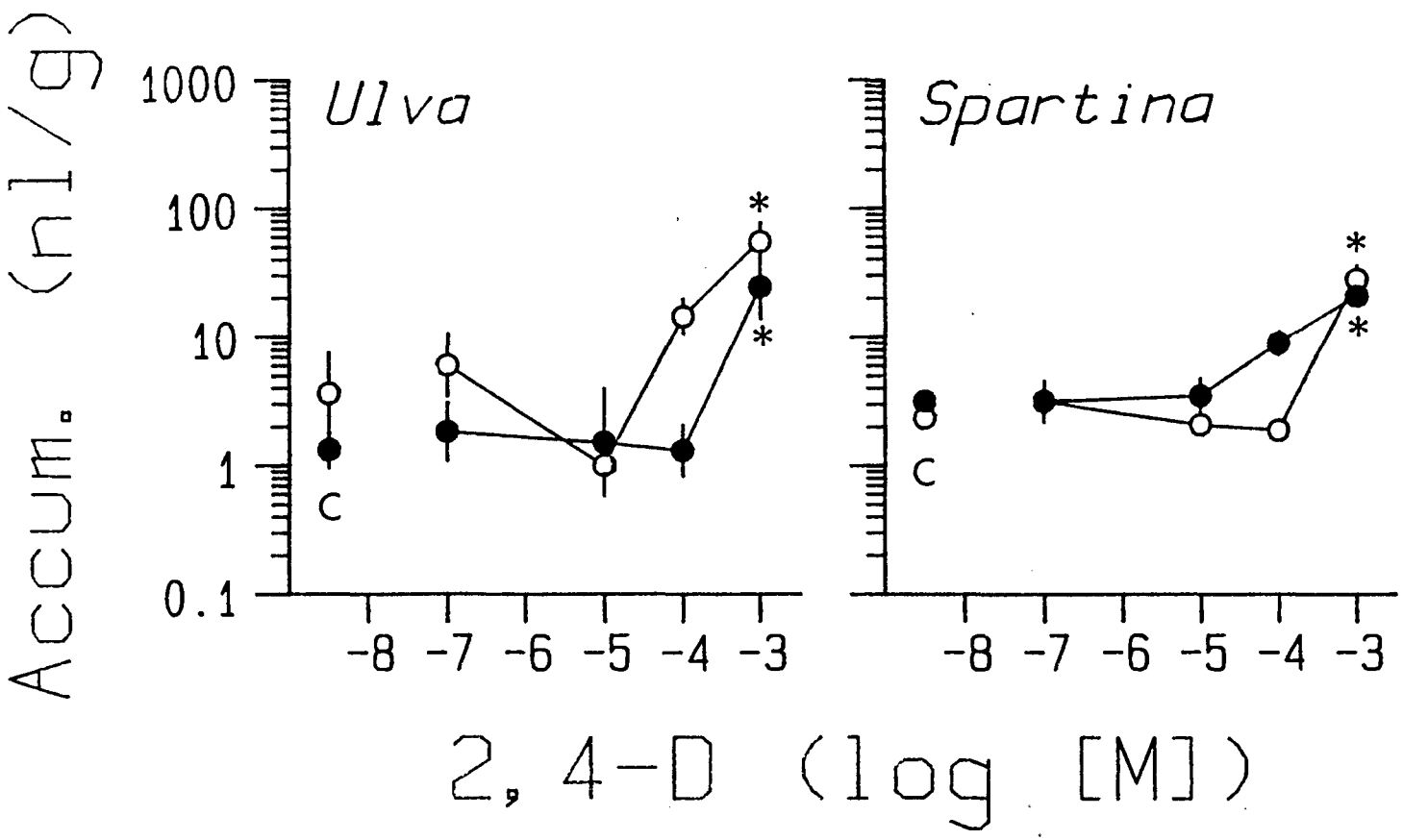

Accum. (nl/g)
1000
100
10
1
0.1
Ulva
Zostera
C
C
*
*
*
-8 -7 -6 -5 -4 -3
-8 -7 -6 -5 -4 -3
Fuel oil (log v/v)

Fig. 4. Ethylene and ethane production after 2,4-D exposure. *Ulva*: 13 h incubation with sunlight, 31° C, 3 replicates at each concentration except control (4 repl.) and 10^{-5} and 10^{-3} M (2 each); *Spartina*: leaves 48 h sunlight/dark incubation, 31° C, 3 replicates at each concentration.


Accum. (nl/g)
1000
100
10
1
0.1
Ulva
Spartina
C
C
*
*
*
*
-8 -7 -6 -5 -4 -3
-8 -7 -6 -5 -4 -3
2, 4-D (log [M])

Ulva and *Spartina* showed a significant increase in ethylene production when exposed to 10^{-3} M 2,4-D (Fig 4). Both species became necrotic at the two highest concentrations, thus ethylene production coincide with cell death. Only the areas near cut leaf margins became chlorotic in *Spartina*, illustrating that 2,4-D did not pass through the epidermis, but instead diffused through the vascular tissue. *Spartina* meristems were also tested (not shown), and they showed a slight increase throughout the range of treatments, down to 10^{-8} M (not shown). This observation may be due to higher sensitivity of dividing cells to 2,4-D. 2,4-D toxic effects are known to be most acute in dicots, and monocots do not generally show lethal effects at low concentrations. . *Zostera* showed no ethylene production at the highest concentration that it was exposed to (1.6×10^{-4} M, not shown), and it did not become necrotic or chlorotic. These results were surprising because 2,4-D had been described as effective in destroying eelgrass beds with water-bourne applications (3). *Ceramium* did not show increased ethylene production at the highest concentration to which it was exposed (10^{-5} M 2,4-D).

DISCUSSION

These experiments illustrate that stress ethylene production in aquatic producers is not a sensitive enough assay to assess sublethal effects of phytotoxicants on aquatic plants because the species tested only responded to acutely toxic concentrations, if at all. This results show, however, that the mechanism of stress ethylene production can be

studied in aquatic plants and algae in ways that may be difficult in terrestrial studies.

Two trends are apparent from the responses of the four plants to Cu^{2+}. First, these algae were more sensitive to Cu^{2+} than the angiosperms. For example, *Zostera* became necrotic at both 10^{-3} M and 10^{-4} M--the same concentrations at which both ethane and ethylene were induced--therefore ethylene was produced only at acute concentrations. The second trend is that the absolute rate of ethylene production per plant weight positively correlates with surface:volume ratios of the four plants. *Ulva* is a sheetlike bilayered algae and all cells come in contact with the test solution, and its ethylene production peaked at 250 nl g^{-1}. The next highest rates occurred in *Ceramium*, a pseudoparenchemous filamentous algae that has fewer cells in contact with the test solution. *Zostera* has parenchymous, strap-like parenchymous leaves with a thin, multiperforate cuticle had lower rates than the algae, *and Spartina*, which has thicker leaves covered with a waxy cuticle, and had the lowest rates of ethylene production. Thus, the per unit weight ethylene production rate is probably a function of the degree of contact between the test solution and the plant cells.

In higher plants, Cu^{2+} and Cd^{2+} are strong inducers of stress ethylene production in other plants (4,10), and here, Cu^{2+} induced stress ethylene production in algae nearest to sublethal concentrations. Therefore this assay may have limited use in assessing the relative sensitivity of different algal species to certain metals.

In some trial experiments, the samples were exposed to elevated temperatures (27-30° C) and direct sunlight. This elevated the response

of the plants, but too few trials were made to determine if this also increased the sensitivity of the assay. Light plays an important role in ethylene production (7), and this explains why eelgrass leaves and not roots showed increased ethylene production when exposed to fuel oil.

This is the first account of stress ethylene production in macroalgae. In a pilot experiment I observed ethylene production in *Fucus vesiculosus* L. (not shown), and elsewhere non-stress ethylene production has been observed in green microalgae (12), a blue green algae (7) and in the green macro-alga *Codium* (16). Thus it appears that ethylene production may be ubiquitous in diverse groups of algae. The significance of this is not clear, however, since ethylene is not recognized as a hormone in algae. It is not known if the production of stress ethylene in algae is involved with in tissue senescence or some another similar role that ethylene performs in higher plants, and this area needs further study.

It is also unclear by which pathway algae produce ethylene. Sandmann and Boeger (12) assumed that ethylene production in *Scenedesmus* is derived from the peroxidation of lipids as is ethane. In this paper, increased ethane production often occurred at the same concentration of phytotoxicant that induced increased ethylene production. That is, ethylene was produced only at acutely toxic concentrations. One possibility that could explain this result is that a large fraction of the ethylene produced by the algae is, in fact, derived (like ethane) from peroxidation of membranes. Alternatively, both pathways may be triggered by similar concentrations of phytotoxicants. There is evidence that the ACC pathway exists in algae because blue-green algae

have been shown to metabolize ACC to ethylene (7), and I have also observed high rates of ethylene production in *Ulva* when exposed to ACC (not shown).

One additional ramification of these results, is that the production of stress ethylene by algae may affect laboratory measurement of N-fixation in sediments. This is because N-fixation is usually measured by the reduction of acetylene to ethylene. Thus the presence of microalgae could lead to artificially high estimates of N-fixation if plant cells have been chemically or physically stressed during sample processing.

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