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Effect of nutrient inputs on growth, chlorophyll, and tissue nutrient concentration of *Ulva reticulata* from a tropical habitat

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ABSTRACT: "Green tides" caused by overgrowth of *Ulva* species are an increasing problem in tropical areas. The effect of dissolved nutrients on uptake rates, growth, chlorophyll, and tissue nutrient concentration of *Ulva reticulata* was examined in laboratory experiments lasting up to 7 d. Sterile seawater was enriched with nitrate, ammonium, phosphate, ammonium + phosphate and nitrate + phosphate. *U. reticulata* expressed luxury uptake of both nitrogen (N) and phosphorus (P). The maximum N-uptake rate was found when ammonium was added alone. The maximum relative growth rate was about 15.1% per day but this was in the nitrate-fed algae not the ammonia-fed algae. N-enrichment resulted in an increase in chlorophyll concentration on day 4 and a decrease on day 7, probably as a result of cell division. P-enrichment had no significant effect on chlorophyll concentration. Treatments with added N, P or N+P showed significant increase in tissue N and P content on day 4. On day 7, N content in macroalgal tissue decreased while P content continued to increase. *U. reticulata* responded most strongly to added N; responses to P were much lower than for added N and there was little or no evidence for an additive effect of N+P. The N:P ratio of *U. reticulata* of control material suggested that N was the most limiting nutrient at the collection site (Paklok, Phuket, Thailand).

KEYWORDS: *Ulva*, nutrient uptake, eutrophication, luxury accumulation, green tides

INTRODUCTION

Green tides are vast accumulations of green macroalgal biomass often found in estuaries and coastal water of areas undergoing eutrophication[1,2]. This phenomenon is becoming a major concern throughout the world (western Baltic Sea, Germany[3]; Finnish Baltic sea coast[4]; Washington State, USA[5]; Pacific coast of central and southern of Japan[6]; Pacific coast of USA[7]). In the summer of 2004, green tides, which were dominated by *Ulva reticulata* Forsskål, occurred in coastal waters of Patong beach, Phuket, a famous tourist beach in Thailand. This phenomenon had a strong economic impact. It occurred for almost two weeks; tourists were put off by the smelly, decomposing and free-floating macroalgae, which spoiled the white sand beach and made the water look unsafe to swim in. This event was assumed to be the result of waste-water discharge from human activities. *Ulva* spp. have been regularly forming green tides in several other places in Thailand *e.g.* Pattani Bay and Songkhla Lake, and Paklok, Phuket. However, little is known about the causes, extent or history of green algal blooms in Tropical SE Asia.

The excessive growth of these macroalgae not only creates an undesirable nuisance and lowers the recreation amenity of the beach but also causes adverse ecological effects including a decline of seagrass beds due to reduction of light penetration, gas, and nutrient exchange[8,9,10]. It also has a negative impact on fish and invertebrates because dissolved oxygen is consumed at night under thick algal mats and when the macroalgae decompose.

Excess nutrient load is supposed to be one of the major factors responsible for the occurrence of "green tides." Nitrogen and phosphorus are the two most common nutrients limiting macroalgal growth[2, 11,12]. Nitrogen has been described as the principal limiting nutrient[2,13,14,15] but in some places phosphorus supply may limit macroalgal production[16,17,18]. In order to find a proper method of controlling the blooms, the most limiting nutrient must be established.

Tissue nutrient composition can be a good index for evaluating macroalgal nutrient status but the choice

of the species to be used is important[13]. In the case of plants that are members of a resident or climax community (for example *Catenella nipae*[19,20]), the levels of nutrients in tissue result from the long-term integration and accumulation of nutrients from the surrounding water over periods of weeks or months. Analysis of an opportunistic species like various *Ulva* species might only give information on the nutrient status of the waters in which they are growing over only the previous few days[19, 20]. Water-column analysis would only detect the instantaneous nutrient concentrations at the time of sampling; intermittent surges of nutrients might go undetected. Alternatively, it has recently been shown that analyses of pigment content of long-lived resident species of macroalgae (*Gracilaria edulis*) can provide an accurate representation of the longer term nutrient status in a water body[21].

Ulva spp. are common fast-growing opportunistic macroalgae of the littoral zone and are generally known as one of the genera forming green tides. They are opportunistic in both ecology and physiology. *Ulva* of various species are usually the first colonizers on open substrata, and their cosmopolitan presence is attributed to their tolerance of a wide range of environments and opportunistic life strategy[1,22,23].

In the present study, we investigated nutrient uptake rates, growth, tissue nutrients and chlorophyll concentration of *Ulva reticulata* from Paklok, Phuket. We measured its responses to known amounts of added nutrients to determine which was the most limiting nutrient. This would help us to understand the blooming phenomenon and would be useful for promoting rational environmental wastewater management and planning in tourist areas.

MATERIALS AND METHODS

Ulva reticulata thalli was collected from the intertidal zone of a sheltered bay at Paklok, Phuket, Thailand (8°01′03″N, 98°24′38″E). Paklok is situated on the east of the island of Phuket and is surrounded by mangrove areas and seagrass beds. There are human residences, prawn farms, and other farmland nearby which may cause high nutrient loads to support significant growth of *U. reticulata*. Moreover, the soft-bottom substrate could also provide another source of nutrients for macroalgae and would act as a store for nutrients between surges of nutrients due to flooding and storms.

Individual plants were rinsed in seawater in order to remove small epiphytes and invertebrates. For consistent results, it is important to clean the plants properly[19,20]. They were kept for 3 days before starting the experiment. The background level of nutrients in the nutrient-depleted seawater was measured. Water was circulated by bubbling with compressed air under controlled temperature (25 °C) and light; daylight fluorescent lamps which provided approximately 300 μmol photons $m^{-2}s^{-1}$ of photosynthetically active radiation (PAR).

The added nutrients were KNO_3^-, NH_4Cl, and Na_2HPO_4. The experiments consisted of six treatments: ~200 $\mu M NO_3^-$, ~ 60 $\mu M NH_4^+$, ~14 μM HPO_4^{2-}, NO_3^- + HPO_4^{2-}, NH_4^+ + HPO_4^{2-} and the last treatment was maintained at the initial nutrient status to act as a low nutrient control (LNC). Seawater contains about 2 mM total inorganic carbon as CO_2, HCO_3^- and CO_3^{2-}. This carbonate system acts as an efficient buffering system at the normal pH of seawater (pH 8.1). The amounts of phosphate, nitrate and ammonia added in the present study would not have altered the pH of seawater in equilibrium with the air.

The concentrations used were 30 times higher than the background concentration measured *in situ*. Nutrient-rich seawater (with NO_3^-, NH_4^+ and HPO_4^{2-}) but without added algae was used as the control blank. During the experiments, approximately 10 g fresh weight (FW) of algae were placed in aerated 250 ml Erlenmeyer flasks with 200 ml of incubation medium under the same controlled environment. The tissue to media volume ratio was large and so metabolism of the added phosphate, nitrate and ammonia would have had little effect on the pH of the seawater. Water samples were taken at 0, 1, 2, 4, 8, 12, 24, 48 or 72 h, a total of 9 flasks making up one experimental nutrient treatment. All nutrient treatments were replicated 3 times. Multiple sampling from flasks at different times was avoided because it would alter the weight-to-volume ratio during incubation. There is also the statistical problem that multiple samples taken from a single flask are not truly statistically independent observations. Uptake rates of inorganic N and P were determined by measuring the disappearance of inorganic nutrients over time[24]. NO_3^-, NH_4^+ and HPO_4^{2-} were measured using cadmium reduction, the phenate method, and the ascorbic acid method, respectively[25]. Uptake, U, was determined using

$$U = \frac{(C_i - C_f)V}{tw}$$

where C_i and C_f are the initial and final nutrient concentrations, respectively, t is the incubation time interval, and w is the plant dry weight (DW), and V is the volume of seawater used for the incubation (200 ml).

Tissue samples were taken after 4 day and 7 day incubations. Dry weight, tissue nitrogen, and phosphorus and total chlorophyll concentration of the algae with 3 replicates from each treatment were analysed. Tissue phosphate was extracted using hot nitric/perchloric acid digestion[25] and assayed using atomic absorption spectrophotometric methods. Tissue nitrogen was extracted using standard preparatory methods for Kjeldahl analysis[26]. Chlorophyll calculations were based on the Jeffrey and Humphrey equations[27] and calculated as mg total chlorophyll. The relative growth rate (μ) of *U. reticulata* was calculated from changes in FW biomass for each experimental period using

$$\mu = \frac{1}{t} \ln \frac{B_t}{B_0}$$

where B_0 and B_t are the initial and final FW biomasses

Statistical analysis

One-way ANOVA with a significance level of 95% was used to test the effect of the enrichment of distinct forms of nutrients on growth rate, nutrients uptake, tissue nutrients, and chlorophyll concentration of *U. reticulata* after 4 and 7 days[28]. Two-way ANOVA was used to test differences among uptake rates of N as NO_3^- or NH_4^+ alone or in combination with P. Significant differences between pairs of means were identified using the Tukey test[28].

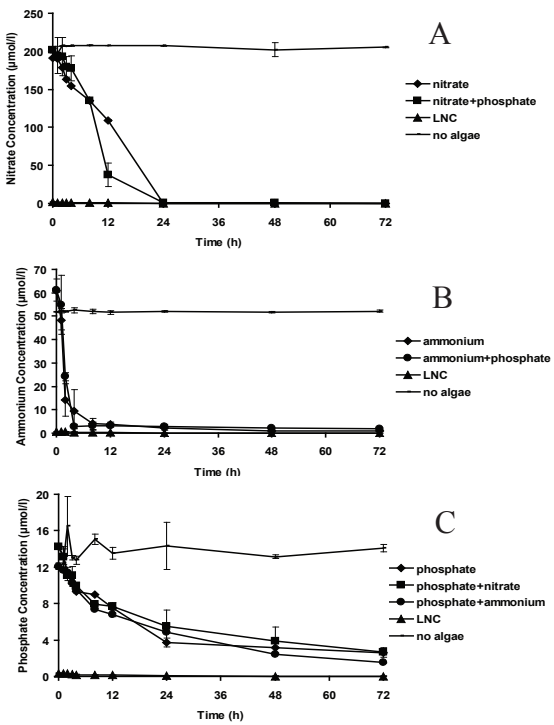

Fig. 1 Nutrients concentration during 72 h of treatment: A: nitrate; B: ammonium; C: phosphate.

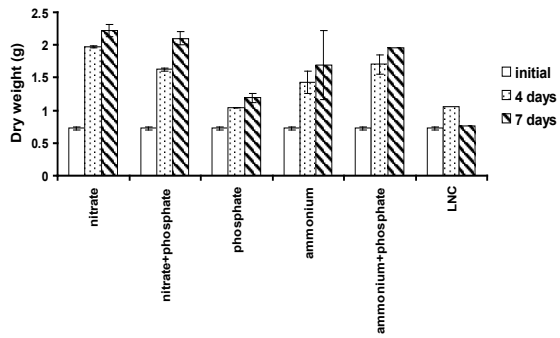

Fig. 2 Average biomass of *Ulva reticulata* in each treatment at 0, 4, 7 days of incubation.

RESULTS

Nutrient uptake rates

The uptake of N and P by *U. reticulata* treated with added NO_3^-, NH_4^+, and HPO_4^{2-} were significantly higher than that with the LNC treatment (Fig. 1). There was little evidence for additive effects of N+P. On the contrary, uptake of each nutrient (NO_3^-, NH_4^+ and HPO_4^{2-}) was slightly higher when it was added alone than in combination with others ($p < 0.001$).

Water column N decreased significantly over time. Rapid uptake was observed during the experiment in the first 4 to 24 h when NH_4^+ and NO_3^- were added as the nitrogen source. The maximum uptake rate was found in the case of the NH_4^+ treatment (V_{max} = 9.39 $\pm$ 1.26 μmol g^{-1} DW h^{-1} 1–2 h of incubation). After 24 h, approximately 96% of the added NO_3^- and approximately 90% of the added NH_4^+ was taken up. At the end of the experiment N was depleted in both NO_3^- and NH_4^+ treatments (Figs. 1A, 1B).

Trends of phosphate concentration were different from the changes in nitrate and ammonium concentrations. There was no obvious rapid uptake of P. Over a 3-day period, dissolved phosphate concentration decreased continuously but had still not completely disappeared at the end of the experiment (approximately 2.3 μM was left in the water column) (Fig. 1C).

Biomass

Algal biomass significantly increased after 4 days of incubation in all treatments with added nutrients ($p < 0.001$) and continued to increase until the

end of the experiment (Fig. 2). LNC treatment showed little or no growth. This indicated that the alga had little or no luxury accumulations of either N or P that the plant could draw upon when brought into the laboratory. Final biomass compared to initial biomass ranged from 100.3% to 288.2% in different experiments and treatments. In the example shown (Fig. 2), the lowest value was observed in the LNC treatment and the highest value was observed when NO_3^- was added alone ($\mu = 0.151 \pm 0.006$ day^{-1}). This relative growth rate of 15.1% per day is equivalent to a doubling time of only 4.6 ± 0.2 days.

Total chlorophyll concentration

Chlorophyll concentration of *U. reticulate*, in all treatments where N was added, significantly increased after 4 days of incubation ($p < 0.001$) and decreased after 7 days of incubation ($p < 0.001$) (Fig. 3). Maximum chlorophyll concentration was found on the fourth day of incubation in the NO_3^- treatment (0.62 ± 0.01 mg g^{-1} FW), while the initial chlorophyll was 0.13 ± 0.01 mg g^{-1} FW. This significant greening of the plants is good evidence that they were N-limited. Chlorophyll content responded more strongly to added NO_3^- than NH_4^+. Phosphate had little effect either by itself or in combination with either NO_3^- or NH_4^+.

Tissue nutrient content

In N added treatments (Fig. 4), tissue N (Kjeldahl N) of *U. reticulata* significantly increased at 4 days ($p < 0.001$) of incubation and was followed by a decrease after 7 days of incubation ($p < 0.005$). Maximum %N was found in the NO_3^- treatment at 4 days of treatment (%N = 0.47 ± 0.01). These results are consistent with excess NO_3^- being easily stored but there is a more limited capacity to store NH_4^+.

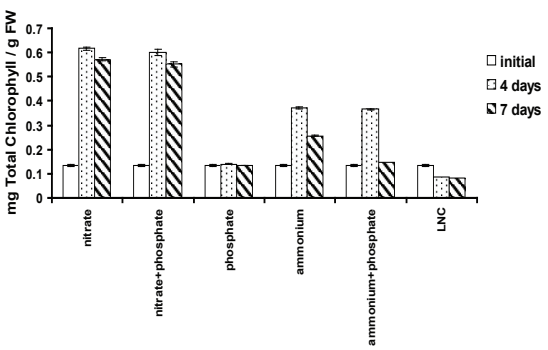

Fig. 3 Variation in total chlorophyll in *Ulva reticulata* in each treatment at 0, 4, 7 days of incubation.

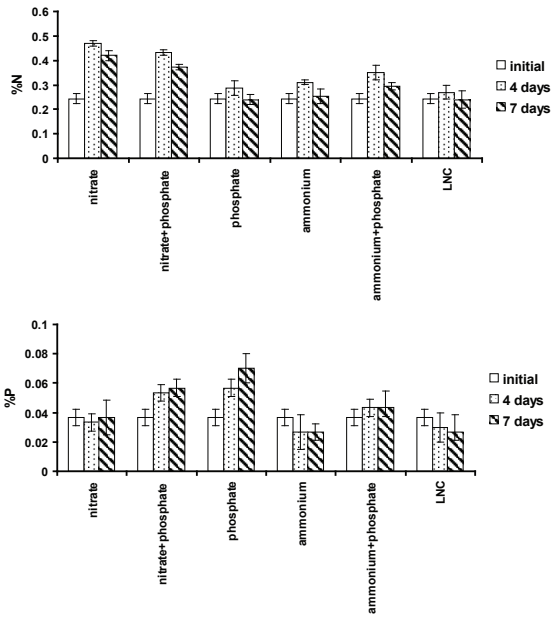

Fig. 4 Kjeldahl nitrogen and phosphorus content as percentage of fresh weight of *Ulva reticulata* at 0, 4, 7 days of incubation.

Tissue P of *U. reticulata* showed significant increase after 4 days of incubation only in the treatments where HPO_4^{2-} was added alone and where NO_3^- was added in combination with HPO_4^{2-} ($p < 0.005$). There was no difference between tissue P at 4 days and 7 days. Maximum %P was found in the HPO_4^{2-} treatment after 7 days (%P = 0.07 ± 0.01) (Fig. 5).

DISCUSSION

Nutrient uptake

Ulva took up large amounts of nutrients from the water column when offered nutrients in the laboratory. Although *Ulva* depleted NO_3^- and NH_4^+ in less than 24 hours, positive growth responses to these nutrients persisted over the 7-day duration of our experiment and may have even lasted longer than this. This result supports conclusions drawn by Pedersen and Borum[29] and Runcie *et al*[19,20] that *Ulva* species are capable of luxury uptake of nutrients, particularly N. The maximum N-uptake rate was found in the NH_4^+ treatment. Similar result was found in other macroalgae (*Pilayella littoralis* and *Enteromorpha intestinalis*[30]; *Ulva lactuca* and *Catenella nipae*[19,20]; *Ulva fenestrata* and *Gracilaria pacifica*[31]; *Chaetomorpha linum*[32]). NH_4^+ is usually considered to be the preferred form of nitrogen because no energetically costly enzymatic reduction reactions are required for NH_4^+ to be available for assimilation[33]. Although NO_3^- is usually the more

common form of nitrogen in nearshore waters, it is more metabolically costly for macroalgae to use NO_3^-. However, NO_3^- can be stored in large amounts in the vacuoles of *Ulva lactuca*[19] whereas the capacity of algae to store large amounts of ammonia in the vacuoles is more limited. To confirm this notion it would be necessary to set up experiments where algae were fed repeated doses of ammonia, nitrate and combinations of N-sources. It would be very interesting and environmentally relevant to determine how long such growth surges continue after a surge in nutrient supply and whether the type of N-source provided and whether or not N and P provided simultaneously has an effect on the time course of growth surges.

In our experiments on *U. reticulata*, we observed indications of surge uptake of NH_4^+ during the first four hours. During the later time intervals (12–72 h), we found that the tissue uptake rate decreased. The uptake rate of NH_4^+ may have been controlled by diminishing nutrient supplies in the medium. Pedersen separated uptake of NH_4^+ by *Ulva lactuca* into three phases[34]. The first phase, surge uptake, is the transiently enhanced nutrient uptake and lasts for a few hours. The second phase, internally controlled uptake, is characterized by a relatively constant uptake rate occurring at high substrate concentration which may be controlled by the nutritional status of the cell. The final phase, externally controlled uptake, occurs at decreasing substrate concentrations and is regulated by the rate of transport of nutrients across the alga surface. Unfortunately, not enough data was available to estimate the K_m for uptake of NH_4^+ from the depletion experiments shown in Fig. 1, but Runcie *et al*[19] give estimates of the K_m for NH_4^+ of about 100 μM and 30μM for NO_3^-.

Large amounts of NO_3^- were taken up by *U. reticulata*. We found that *Ulva* grew fastest on nitrate even though ammonia was taken up by *U. reticulata* faster than nitrate. Nitrate was almost completely depleted in 24 h indicating the large NO_3^- storage capacity of *U. reticulata*. NO_3^- can represent a significant storage pool in algae[31,35]. In *Chaetomorpha linum*, N is stored as simple organic compounds if ammonia is the N-source but as NO_3^- if N is provided as NO_3^-. Both storage forms of N provide a temporary mechanism to buffer against the asynchrony of N supply and demand[25]. However, the N uptake rate of *U. reticulata* observed in our research was much lower than the N uptake of other *Ulva* species[19,36,37,38,39]. Some of these differences can be attributed to possible differences in the N-status of the material used in different studies. Other differences can be attributed to different experimental methods, particularly the times over which uptake measurements were made. For example, Runcie *et al*[19] measured rates of uptake of up to 400 μmol g^{-1} DW h^{-1} but such high rates were sustained for only the first few minutes of an incubation. Thus our relatively slow rates might indicate a relatively high N-status of our experimental material and the nutrient concentrations we provided for the algae, which were 30 times higher than background nutrient measured *in situ* (6.5 μM NO_3^-, 2.03 μM NH_4^+, 0.46 μM HPO_4^{2-}), might have quickly satisfied the demands of the algae for extra N. Several researchers have shown that nutrient uptake by macroalgae was regulated by substrate nutrient concentration[19,20,29,37–39].

U. reticulata did not demonstrate the same high and sustained affinity for P that it did for N. Fig. 5 shows that the baseline phosphate content of *U. reticulata* was already high. However, the LNC material showed little or no growth (Fig. 2). Added P alone resulted in some growth but large amounts of growth required extra N whether or not P was also added. Considerable amounts of HPO_4^{2-} remained in the water column at the end of the experiment relative to background HPO_4^{2-} concentrations. The highest rate of uptake of P was found when P was added alone. Phosphate supplied with nitrate or ammonium was taken up much more slowly in the first four hours and about 20% remained unassimilated even after 3 days (Fig. 5). Thus, this study suggested that N is the most limiting nutrient to *U. reticulata*. Considering the difference between treatments fertilized with two forms of N, with or without added P, we found that N uptake rate was slightly higher when it was added alone than in combination with P. Uptake of P was highest when offered alone rather than with an added source of N. Phosphate (> 1 μM) has been shown to inhibit N uptake in a marine diatom as reported by Terry[40]. On the other hand, high concentrations of N (>100 μM) were thought to inhibit HPO_4^{2-} uptake[41].

Effect of nutrient supply on growth, chlorophyll, and tissue nutrient concentration of *Ulva reticlata*

Both P and N enrichment resulted in an increase of biomass after 4 days of incubation and the biomass continued increasing over the 7 days of the experiments. The maximum growth rate was found when NO_3^- was added alone, demonstrating the large NO_3^- uptake capacity in *U. reticulata*. This could give it an advantage by being able to grow well on stored N for several days without additional nutrient supplies. *U. reticulata* can take advantage of temporary surges of nutrients. Some studies have shown that excess

N is stored primarily in pools of inorganic N (NO_3^- and to a lesser extent NH_4^+) and simple organic compounds[19]. We have shown that N supplied as NO_3^- can be stored by *U. reticulata* as NO_3^- but it is less capable of storing N supplied as NH_4^+. The algal growth rate was significantly higher when N was added, indicating that N is the most limiting nutrient to *U. reticulata*. Chlorophyll-bound N typically changes in response to macroalgal N status[42]. It is largely considered as a metabolically active pool, but because of its small size is not considered to be significant for N storage[35]. Nevertheless, chlorophyll content can be used as a useful index of conversion of N sources into organic N because chlorophyll is so easily assayed.

As noted earlier, photosynthetic pigment content is sensitive to the N status of the alga. N-enrichment was followed by an increase in chlorophyll content 4 days after treatment and chlorophyll concentration decreased at day 7 when the water column N was depleted probably due to growth and a lack of sufficient ambient N for continued synthesis of new chlorophyll. This effect is probably due to cell division because the doubling time for *U. reticulata* under favorable conditions is about 4.6 days. At day 4 the cells had large amounts of chlorophyll but by day 7 the cells had largely divided and so the amount of chlorophyll per cell had dropped. The maximum chlorophyll content was found on day 4 when NO_3^- was added alone (0.62 mg g^{-1} FW). This could be a result of the larger molar amount of N added as NO_3^- than as NH_4^+ in the experiment in this study. A tissue N:P ratio greater than 12–24 is indicative of P limitation, whereas a ratio less than 8–16 is indicative of N limitation for *Ulva fenestrata*[13]. Our results showed that *U. reticulata* was limited by N in all treatments and also limited by P in NH_4^+ and NO_3^- treatments. The initial N:P ratio of *U. reticulata* in this experiment was 6.69 $\pm$ 0.57; this should have been a N-limited experimental condition.

In conclusion, *Ulva reticulata* can take up large amount of nutrients, particularly N. Added N results in rapid increase of chlorophyll concentration, tissue nutrient concentration, and biomass. These give *U.reticulata* a selective advantage to form dense blooms in only a few days. For example, a relative growth rate of about 15.1% per day is equivalent to a doubling time of only about 4.6 days. Since our results indicate that the most limiting nutrient to *U. reticulata* is N, top priority should be given to limiting N loads to avoid blooms of *U. reticulata*. We suggest that N concentration in the sea and point sources or in the receiving water should be measured to provide evidence for the most important contributors of N in order to recommend some appropriate action. However, *Ulva reticulata* is able to take advantage of surges of nutrients from storms, sewage overflows, draining of prawn ponds, etc., which could easily be missed in routine sampling schedules. *Ulva reticulata* would be useful for biomonitoring provided that only short incubation periods consistent with its rapid growth cycle are used[7,19,43].

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