

# A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga *Ulva pertusa* (Chlorophyta)

Taejun Han<sup>a,\*</sup>, Gye-Woon Choi<sup>b</sup>

<sup>a</sup> Division of Biology and Chemistry, University of Incheon, Incheon 402-749, South Korea

<sup>b</sup> Department of Civil and Environmental System Engineering, University of Incheon, Incheon 402-749, South Korea

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## Abstract

A 5-day aquatic toxicity test based on sporulation inhibition of the green macroalga *Ulva pertusa* Kjellman has been developed. Optimal test conditions determined for photon irradiance, salinity and temperature were 60–200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , 25–35‰ and 15–20 °C, respectively. Tests were conducted by exposing *U. pertusa* thallus disks to a reference toxicant (sodium dodecyl sulfate; SDS), metals ( $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Pb}^{2+}$ ) and elutriates of sludge collected from nine different locations. The  $\text{EC}_{50}$  values for SDS was 5.35  $\text{mg L}^{-1}$ . When four heavy metals were assayed, the NOECs were highest for lead (0.625  $\text{mg L}^{-1}$ ) and lowest for copper (0.031  $\text{mg L}^{-1}$ ). The  $\text{EC}_{50}$  values showed the following toxicity rankings:  $\text{Cu}^{2+}$  (0.061  $\text{mg L}^{-1}$ ) >  $\text{Cd}^{2+}$  (0.326  $\text{mg L}^{-1}$ ) >  $\text{Zn}^{2+}$  (0.738  $\text{mg L}^{-1}$ ) >  $\text{Pb}^{2+}$  (0.877  $\text{mg L}^{-1}$ ). The bioassay indicated also that the sporulation endpoint could be a sensitive indicator of toxicity effects of elutriates of sludge as reflected from the NOEC values equal to or lower than the lowest concentration employed (6.25%). Sporulation was significantly inhibited in all elutriates with the greatest and least effects observed in elutriates of sludge from industrial waste ( $\text{EC}_{50}$  6.78%) and filtration bed ( $\text{EC}_{50}$  15.0%), respectively. The results of the Spearman rank correlation analysis for  $\text{EC}_{50}$  data versus the concentrations of toxicants in the sludge presented a significant correlation between toxicity and four heavy metals ( $\text{Cd}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$ ). Introduction of the concept of toxicity unit (TU) showed that these metals were the main cause of toxicity in elutriates of at least four out of nine sludge samples. Members of the order Ulvales show a wide geographic distribution and have similar reproductive characteristics, thus making it possible to apply the present test method to other algae of this taxa, elsewhere. This novel method will be a useful tool for assessing the aquatic toxicity of a wide range of toxicants, once the respective sensitivities are demonstrated.

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**Keywords:** Aquatic toxicity; Sporulation; *Ulva pertusa*

## 1. Introduction

Marine and estuarine environments are often exposed to wastes from a range of industrial and municipal origins. Aquatic environments are generally thought to be resilient, providing a convenient and safe

\* Corresponding author. Tel.: +82 32 7708245;  
fax: +82 32 7708245.

E-mail address: hanalgae@incheon.ac.kr (T. Han).

location for disposal of this waste but, if the integrity of marine systems is to be maintained, environmental protection strategies, including effective monitoring and regulation, need to be developed (Bidwell et al., 1998). Traditionally, seawater quality has been estimated by chemical analyses employing sophisticated equipment but the approach is limited by the time required for analyses, particularly with mixtures of chemicals (Chu and Chow, 2002). A stronger limitation of performing solely chemical analysis is related with the fact that it is not possible to analyze all potential toxicants in the sample. In addition, chemical monitoring in most environmental studies does not necessarily reflect the toxicity to living organisms (Rai et al., 1981; Mallick and Rai, 2002). To circumvent these limitations, bioassay methods have been developed to complement chemical monitoring. Toxicity test methods using a range of microorganisms, invertebrates, fish and algae are being used increasingly (Williams et al., 1993; Klaine and Lewis, 1995).

Fewer tests employ algae, which is at least in part due to the misconception that algal species are less sensitive to toxicants than animals (Klaine and Lewis, 1995; Eklund and Kautsky, 2003). Tests based on algae are predominantly focused on microalgae with macroalgae being poorly represented, despite the importance of macroalgae as primary producers, providers of habitat and nursery grounds for fish and invertebrates in marine and freshwater systems. With the exception of reproductive tests on the red algae, *Ceramium strictum*, *Champia parvula* and the brown algae, *Laminaria saccharina* and *Fucus spiralis* and a growth test with the red alga *Gracilaria tenuistipitata* there are few standardized toxicological testing procedures using marine macroalgae (Eklund, 1993, 1998; Steele and Thursby, 1995; Haglund et al., 1996; USEPA, 1998). However, except for germination process of *Ulva fasciata* (Hooten and Carr, 1998), reproduction tests have not been developed with green macroalgae as it is considered extremely difficult to find a definite endpoint (Steele and Thursby, 1995).

Recently, Eklund and Kautsky (2003) reported that the number of macroalgal species used for toxicity testing was 65 of which only 11 species are green algae. Conversely, the species for which most information relating to chemical toxicants has been gathered is the green macroalga *Ulva intestinalis* (*Enteromorpha intestinalis*). The taxon *Ulva* appears to have great

potential as a bioindicator due to the large size of plants, their wide distribution, ease of collection, and bioaccumulation of metals (Haritonidis and Malea, 1999; Lee and Wang, 2001). Furthermore, many *Ulva* species have been used as biofilters of waste inorganic nutrients (Vandermeulen and Gordin, 1990; Cohen and Neori, 1991; Chung et al., 2002; Mata and Santos, 2003). For these reasons, *Ulva* spp. may have been seen as tolerant species and, thus unsuitable for toxicological tests (Steele and Thursby, 1983). However, a recent study has revealed that the reproductive cells of *U. fasciata* are more sensitive to some toxicants than the sensitive stages of sea urchins which are commonly used marine test organisms, indicating that *Ulva* species are sensitive organisms that may be appropriate for toxicity tests (Hooten and Carr, 1998).

Most toxicity test methods employing macroalgae have been established using single toxicants. Only few studies have been conducted to investigate the impact of toxicants in combination, despite the fact that toxic compounds in natural environments usually occur in mixtures (Eklund and Kautsky, 2003). For example, sewage or waste sludge is contaminated with a mixture of heavy metals, PAHs, PCBs and organic compounds that can affect organisms at different trophic levels (Teal et al., 1982; Otte et al., 1993; Vikelsøe et al., 2002).

The present study was undertaken to develop a bioassay using sporulation of the green macroalga *U. pertusa* as endpoint. The proposed novel assay was evaluated by determining the phytotoxic potential of a reference toxicant (sodium dodecyl sulphate), four heavy metals (cadmium, copper, lead and zinc) as pure compounds and sewage or waste sludge elutriates as mixtures of contaminants.

*U. pertusa* is a fast growing opportunistic alga forming massive mats in the intertidal zone and occurs on most coasts in Korea all year round. The alga is easy to maintain in culture and its sporulation is simple to induce and observe (Han et al., 2003a,b).

## 2. Materials and methods

### 2.1. Culture of plants

*U. pertusa* was collected at sites near Ahnin on the eastern coast of Korea (37.4°N, 129.1°E). Immediately

after transport to the laboratory, the plants were maintained in plastic tanks with aerated artificial seawater medium prepared by dissolving commercial sea salts (Coralife, Energy Savers, CA, USA) in deionized water to a concentration of 35‰. To the medium was added 1 mM KNO<sub>3</sub> and 0.1 mM K<sub>2</sub>HPO<sub>4</sub> as nutrients. The unialgal stock cultures were maintained at 15 °C under 5–10 μmol photons m<sup>-2</sup> s<sup>-1</sup> of continuous white fluorescent light (FL400, Kum-Ho, Seoul, South Korea) with a 12:12 h light–dark cycle.

## 2.2. Experimental procedures

Reproduction of *U. pertusa* is restricted to the marginal area of thalli (Han et al., 2003a). Disks (ø 6 mm) were cut from the marginal region of the thallus and distributed to plastic, six-well cell plates (15 ml volume per well; Iwaki, Chiba, Japan) filled with 10 ml of medium. The cell plates were exposed during 5 days to various levels of environmental factors: photon irradiance (5–200 μmol photons m<sup>-2</sup> s<sup>-1</sup>), salinity (5–55‰) and temperature (5–25 °C). While testing each single factor, the other environmental conditions were kept optimal: photon irradiance at 80–100 μmol photons m<sup>-2</sup> s<sup>-1</sup>, photoperiod at 12:12 h LD, salinity at 35‰ and temperature at 15 °C. The medium was not replaced during the period of the test. After a 5-day culture period, disks were harvested for determination of the extent of sporulation using a computer-assisted image analyzer (MV200, Samsung, Seoul, Korea). Formation of reproductive cells in *Ulva* is accompanied by a change in thallus color from forest or yellow green (vegetative state) to dark olive or olive drab (reproductive state) and even to white or white cream (after release of reproductive cells). Percentage sporulation was therefore assessed from the proportion of the surface area with the latter two colorations.

## 2.3. Test procedure

Algal disks cut from the margins of the thallus were placed in the test cell plates with different concentrations of toxicants. Static cultures were established at 15 °C under 80–100 μmol photons m<sup>-2</sup> s<sup>-1</sup> of white fluorescent light with a 12:12 h light–dark cycle. Controls consisted of the artificial seawater medium without toxicants.

A reference toxicant test was conducted with sodium dodecyl sulphate (SDS, Sigma, St. Louis, USA). A dilution series of SDS ranging from 0.75 to 12 mg L<sup>-1</sup> was prepared using the artificial seawater medium.

Toxicity of heavy metals with *U. pertusa* was tested using stock solutions of cadmium, copper, lead and zinc as concentrated standards in deionized water acidified with 1N hydrochloric acid (HCl) or 1 mol L<sup>-1</sup> nitric acid (HNO<sub>3</sub>) (Junsei, Tokyo, Japan). Appropriate volumes of the stock solution were added so that the final concentrations were in the ranges, 0.313–5 mg L<sup>-1</sup> for lead and zinc nitrate, and 0.0313–0.5 mg L<sup>-1</sup> for cadmium and copper chloride.

The elutriate test was developed to evaluate the potential effects of the release of water-soluble constituents from sewage or waste sludge to the water column during the disposal processes of sludge. Sewage or waste sludge was sampled from various sources, such as industrial, agricultural, domestic, rural and livestock farm sewage treatment plants. Sludge collected in plastic bags was brought to the laboratory and extracted in seawater. The ratio of sludge to seawater was 1:10 (w/w) and extraction was made in 1 L separating funnels at speed of 300 rpm for 8 h and finally filtered through a 0.45 μm membrane filter (Advantec, Tokyo, Japan). All extracts were stored in a refrigerator (<0.5 °C) before use. The chemical compositions of sludge samples, from each source, were analyzed by the Korean Ocean Research and Development Institute (KORDI), Ansan, Korea using standard analytical methods (APHA et al., 1995).

Toxicity tests were conducted with an elutriate concentration series made by dilution with artificial seawater medium to produce the desired concentration range, i.e. 100, 50, 25, 12.5 and 6.25% of the original elutriates. The pH and salinity of the test solutions were checked at the start and at the end of the culture, and found to be 7.5–8.4 and 26–34‰, respectively. After 5–8 days of culture, the algal disks were withdrawn for determination of the extent of sporulation, as an endpoint. Percentage sporulation of the control should be higher than 75% for the validity of the test.

## 2.4. Data analysis

One-way analyses of variance (ANOVA) followed by the least significance difference (LSD) post-hoc

test at  $P < 0.05$  were used to determine the difference between the treatments. We have taken the value for the error mean square from the ANOVA table, divided it by the number of replicates in each sample, multiplied by two and then calculated the square root,  $\text{SQRT}(2 \times \text{ErrMS}/n)$ . This value was multiplied by  $t$  for  $P = 0.05$  and the degree of freedom for error from the ANOVA table to give the LSD. The sporulation percentage data were arcsine-transformed before the ANOVA. The results were reported as NOECs and  $\text{EC}_{50}$ s with 95% confidence intervals estimated by the linear interpolation method (Toxical 5.0, Tidepool Science, CA, USA). The NOEC ranges represent the highest concentration that did not result in a significant difference in sporulation percentage from the controls. The  $\text{EC}_{50}$  value is the effect concentration at which 50% inhibition of sporulation occurs. The Spearman rank correlation coefficient was calculated to find out the possible links between the concentrations of each toxicant contained in the sludge and the  $\text{EC}_{50}$ s calculated from the corresponding sludge elutriates. The correlation was considered to be significant at  $P < 0.05$  when the calculated value of  $r_s$  was greater than the critical value. In order to estimate the metal concentrations in the elutriates, we applied the reported average mobilization factors of Cu (48), Zn (51) and Pb (15) to the metal concentrations of the sludge samples in this study, and transformed these concentrations ([M]) into toxicity units (TU) according to the expression,  $\text{TU} = [\text{M}]/\text{EC}_{50}$  (Beiras et al., 2003). The summation of TU was made for each sludge sample to estimate if the toxicity of the corresponding elutriate was due to these three metals.

### 3. Results

The percentage sporulation of *U. pertusa* grown for 5 days under different photon irradiances, salinities and temperatures is shown in Fig. 1. Sporulation was completed at irradiances higher than  $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  while the percentage decreased with decreasing irradiances with no sign of sporulation in the dark. Sporulation percentage reached a maximum in the salinity range 25–35‰, followed by a decrease thereafter with increasing salinity. The alga exhibited significantly lower or no sporulation at all at reduced salinities. In response to different temperature

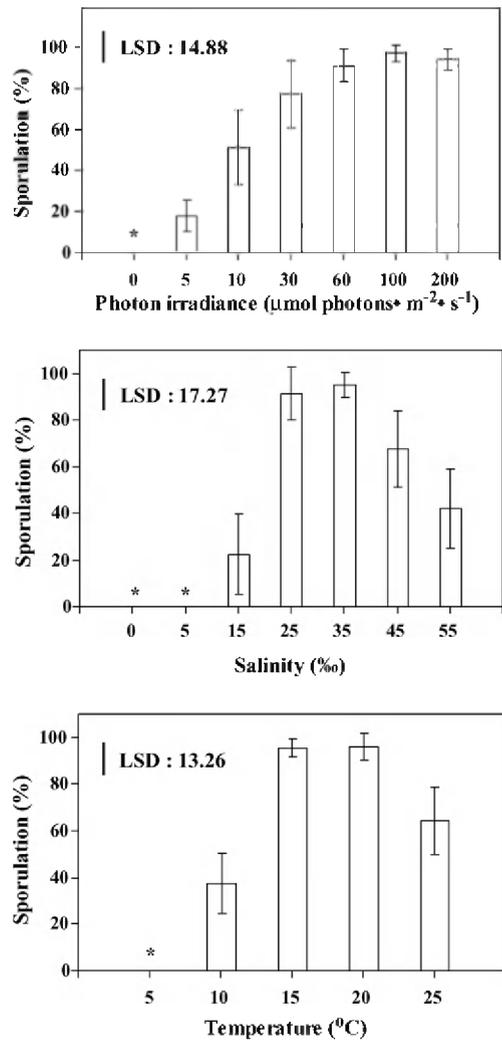


Fig. 1. Effects of photon irradiance, salinity and temperature on sporulation of *U. pertusa*. The bar denotes least significance difference (LSD) at 5% level and each error bar indicates 95% confidence interval ( $n = 8$ ). Asterisks denote no sporulation.

treatments, the alga showed maximal sporulation percentage at 15–20  $^{\circ}\text{C}$  with a significant decline at 10 and 25  $^{\circ}\text{C}$ . No sporulation was recorded at 5  $^{\circ}\text{C}$ .

The NOEC and  $\text{EC}_{50}$  (95% CI) of the SDS test with *U. pertusa* were 1.5 and 5.35 (2.66–7.17)  $\text{mg L}^{-1}$ , respectively (Fig. 2 and Table 1).

Sporulation of *U. pertusa* in response to different concentrations of cadmium, copper, lead and zinc is shown in Fig. 3. Table 1 lists the NOECs and  $\text{EC}_{50}$ s for the toxicants. The NOEC was lowest for  $\text{Cu}^{2+}$

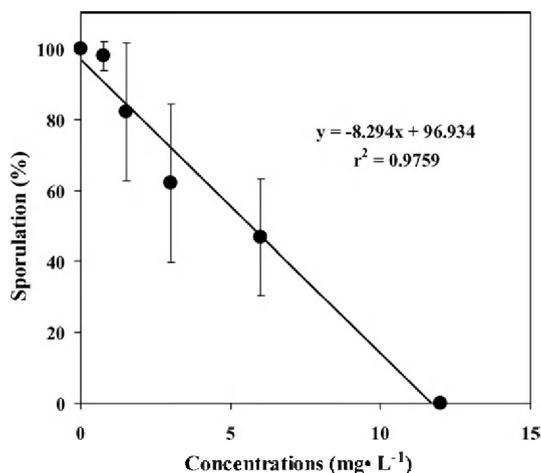


Fig. 2. Sporulation inhibition of *U. pertusa* exposed to different concentrations of SDS for 5 days. Mean and 95% confidence interval are shown ( $n = 8$ ).

(0.0313 mg L<sup>-1</sup>) and highest for Pb<sup>2+</sup> (0.625 mg L<sup>-1</sup>). The toxicity of the heavy metals as estimated by EC<sub>50</sub> values was found to be in the decreasing order of Cu<sup>2+</sup> > Cd<sup>2+</sup> > Zn<sup>2+</sup> > Pb<sup>2+</sup>.

Chemical constituents of sludge samples from all sources are presented in Table 2. The sludge from S7 contained the highest concentration of total heavy metals, followed by S3, S1, S4, S2, S9, S6, S5 and S8. The differences in concentrations of PAHs, total organic P and PCBs among sludge samples were variable. The dose response of *U. pertusa* and calculated NOEC and EC<sub>50</sub> for the nine sludge elutriates are reported in Fig. 4 and Table 1. In general, all tested elutriates had adverse effects on *U. pertusa* with NOECs of 6.25%

Table 1

NOEC and EC<sub>50</sub> values for sporulation inhibition of *U. pertusa* exposed to different toxicants for 5 days

| Sludge source                          | NOEC  | EC <sub>50</sub> (95% CI range) |
|--|-------|---------------------------------|
| SDS (mg L <sup>-1</sup> )              | 1.5   | 5.35 (2.66–7.17)                |
| Cd <sup>2+</sup> (mg L <sup>-1</sup> ) | 0.063 | 0.326 (0.254–0.360)             |
| Cu <sup>2+</sup> (mg L <sup>-1</sup> ) | 0.031 | 0.061 (0.049–0.081)             |
| Pb <sup>2+</sup> (mg L <sup>-1</sup> ) | 0.625 | 0.877 (0.745–0.947)             |
| Zn <sup>2+</sup> (mg L <sup>-1</sup> ) | 0.313 | 0.738 (0.554–0.880)             |
| Industrial waste (S1) (%)              | 6.25  | 9.15 (7.96–9.85)                |
| Livestock waste (S2) (%)               | 6.25  | 10.60 (9.43–12.70)              |
| Leather waste (S3) (%)                 | 6.25  | 13.45 (10.30–16.50)             |
| Urban sewage (S4) (%)                  | <6.25 | 8.25 (5.42–9.40)                |
| Food waste (S5) (%)                    | 6.25  | 9.47 (7.80–10.50)               |
| Mixed waste (S6) (%)                   | 6.25  | 9.53 (9.04–10.33)               |
| Industrial sewage (S7) (%)             | <6.25 | 6.78 (4.74–8.46)                |
| Filtration bed (S8) (%)                | 6.25  | 15.00 (11.53–19.08)             |
| Rural sewage (S9) (%)                  | 6.25  | 13.96 (11.90–16.00)             |

or lower, which was the lowest concentration tested. Elutriates with the highest and lowest toxic effect, as estimated by an EC, were those from industrial sewage (S7; 6.78%) and from filtration bed (S8; 15.0%), respectively.

Spearman rank correlation coefficient between the concentrations of one particular toxicant in the tested sludge samples and the EC<sub>50</sub> values for the corresponding elutriates revealed a significant relationship for cadmium ( $r = 0.783$ ), copper ( $r = 0.800$ ), lead ( $r = 0.717$ ) and zinc ( $r = 0.733$ ) at  $P < 0.05$  ( $r_s = 0.600$ ) (Table 3).

In comparison with one TU, that predicts a 50% inhibition of sporulation, the summation of TU was between 0.87 and 3.3 for four sludge samples (S1, S2, S4, S7) and 0.11 or lower for the remaining samples (S3, S5, S6, S8, S9) (Table 4).

Table 2

Physico-chemical analyses of sludge samples collected from different locations

| Sludge source | pH  | Salinity (%) | Chemicals (mg L <sup>-1</sup> DW) |                  |                  |                  |                  |                  |                  |                  |        |                 |         |
|---------------|-----|--------------|-----------------------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|--------|-----------------|---------|
|               |     |              | As <sup>2+</sup>                  | Cd <sup>2+</sup> | Co <sup>2+</sup> | Cr <sup>2+</sup> | Cu <sup>2+</sup> | Hg <sup>2+</sup> | Pb <sup>2+</sup> | Zn <sup>2+</sup> | PAHs   | Total organic P | PCBs    |
| S1            | 8.3 | 32           | 68.9                              | 100.5            | 16.5             | 168.8            | 3728.8           | 1.2              | 892.1            | 4778.0           | 5768.9 | 4765.7          | 20.7    |
| S2            | 8.2 | 26           | 2.6                               | 0.9              | 5.1              | 14.7             | 928.4            | 0.0              | 7.6              | 2009.9           | 665.0  | 1240.4          | –       |
| S3            | 8.2 | 26           | 1.7                               | 0.0              | 7.2              | 24053.7          | 21.6             | 0.0              | 7.8              | 171.1            | 4080.7 | 3163.4          | –       |
| S4            | 7.9 | 30           | 10.3                              | 30.9             | 7.8              | 82.9             | 1044.0           | 107.1            | 356.7            | 1945.9           | 3709.0 | 2013.1          | 59.2    |
| S5            | 7.5 | 26           | 1.2                               | 0.5              | 7.8              | 95.0             | 100.0            | 0.0              | 23.1             | 227.0            | 4640.3 | 1672.7          | –       |
| S6            | 8.4 | 34           | 10.7                              | 2.2              | 14.2             | 68.4             | 365.7            | 1.0              | 88.7             | 807.3            | 3176.9 | 1797.2          | 12.7    |
| S7            | 8.2 | 30           | 21.6                              | 27.0             | 90.9             | 4220.5           | 1839.1           | 1.4              | 1007.6           | 20784.0          | 2718.0 | 6210.5          | 21874.4 |
| S8            | 7.7 | 30           | 27.4                              | 0.2              | 13.8             | 56.6             | 33.2             | 0.0              | 43.3             | 166.6            | 1487.5 | –               | –       |
| S9            | 8.3 | 30           | 8.8                               | 2.1              | 12.6             | 52.8             | 259.5            | 3.1              | 80.5             | 970.0            | 2059.8 | 2338.5          | 4.6     |

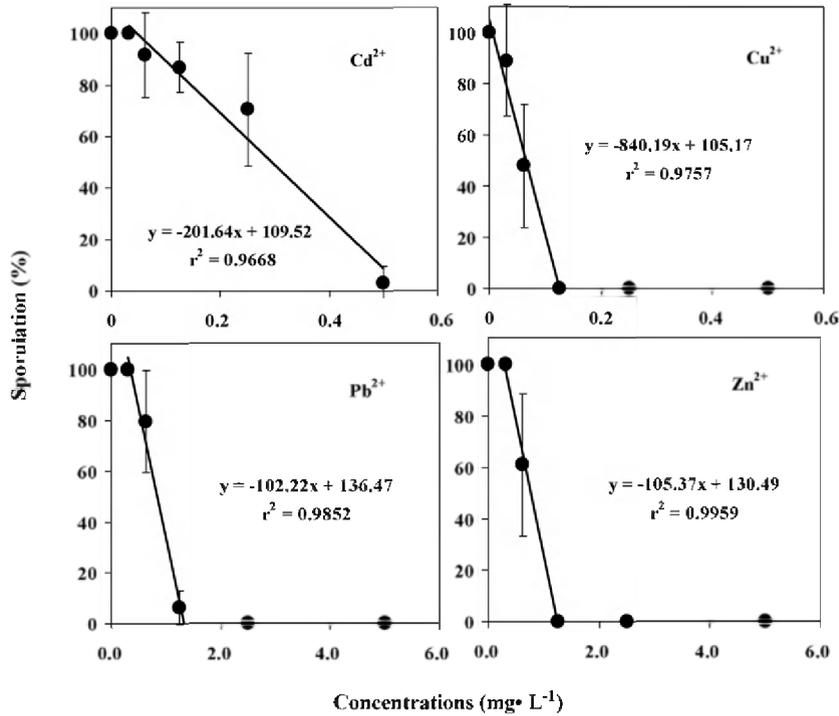


Fig. 3. Sporulation inhibition of *U. pertusa* exposed to different concentrations of heavy metals for 5 days. Mean and 95% confidence interval are shown ( $n = 8$ ).

Table 3  
Significance of correlation between the toxicants retained in the sludge and EC<sub>50</sub> values for the corresponding sludge elutriates

| Chemicals               | <i>r</i> value | <i>P</i> value |
|-------------------------|----------------|----------------|
| Cu <sup>2+</sup>        | 0.77           | <0.05          |
| Cd <sup>2+</sup>        | 0.73           | <0.05          |
| Zn <sup>2+</sup>        | 0.72           | <0.05          |
| Pb <sup>2+</sup>        | 0.67           | <0.05          |
| Total organic P         | 0.53           | >0.05          |
| Hg <sup>2+</sup>        | 0.51           | >0.05          |
| Cr <sup>2+</sup>        | 0.50           | >0.05          |
| PAHs                    | 0.47           | >0.05          |
| Co <sup>2+</sup>        | 0.37           | >0.05          |
| As <sup>2+</sup>        | 0.18           | >0.05          |
| Standard <i>r</i> value | 0.60           |                |

Table 4  
The summation of toxicity unit for the nine tested sludge elutriates

| Sludge source | Heavy metals     |                  |                  | ∑ TU(= [M]/EC <sub>50</sub> ) |
|---------------|------------------|------------------|------------------|-------------------------------|
|               | Cu <sup>2+</sup> | Pb <sup>2+</sup> | Zn <sup>2+</sup> |                               |
| S1            | 2.934            | 0.015            | 0.330            | 3.279                         |
| S2            | 0.730            | 0.000            | 0.139            | 0.869                         |
| S3            | 0.017            | 0.000            | 0.012            | 0.029                         |
| S4            | 0.822            | 0.006            | 0.134            | 0.963                         |
| S5            | 0.079            | 0.000            | 0.016            | 0.095                         |
| S6            | 0.070            | 0.014            | 0.025            | 0.109                         |
| S7            | 0.660            | 0.017            | 1.436            | 2.113                         |
| S8            | 0.026            | 0.001            | 0.012            | 0.039                         |
| S9            | 0.204            | 0.001            | 0.067            | 0.272                         |

#### 4. Discussion

A variety of endpoints have been employed in many macroalgae, including egg production, gametogenesis, germination, growth, photosynthesis, respiration, rhizoid regeneration (see review by Eklund and Kautsky,

2003). The endpoint examined in the present study was spore production; the endpoint relating to reproduction is particularly useful because, in general, the reproductive process is impaired prior to cessation of growth, suggesting that the former is more sensitive to damaging agents (Steele and Thursby, 1983). In this respect, the response of sporulation can be considered as an adequate endpoint for toxicity test with *U. pertusa*.

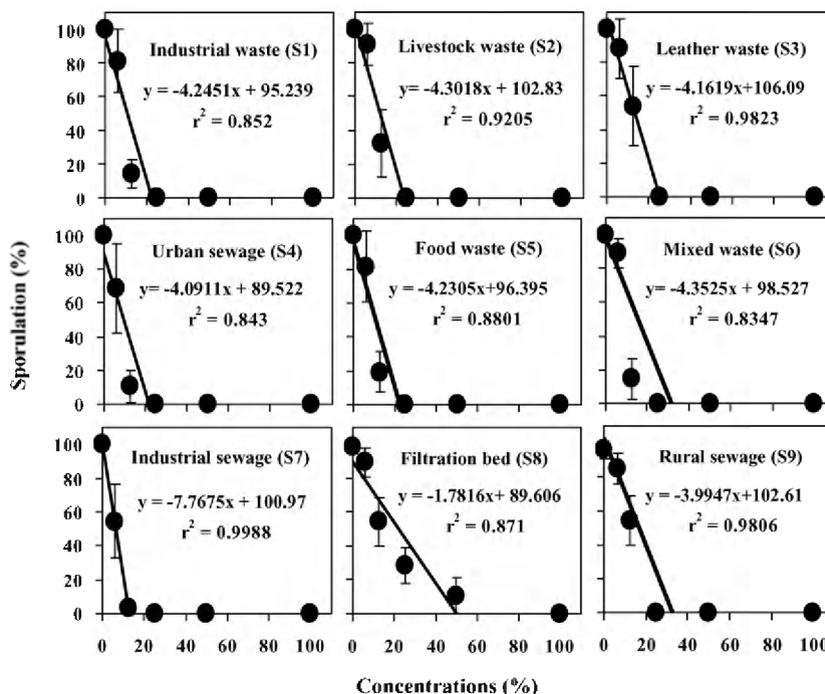


Fig. 4. Sporulation inhibition of *U. pertusa* exposed to different concentrations of sludge elutriates for 5 days. Mean and 95% confidence interval are shown ( $n = 8$ ).

Reproduction as an endpoint is also of ecological significance since failure to reproduce would affect later recruitment of the adult population, and subsequently marine productivity cycles with macroalgae as the base.

In order to establish a standardized toxicity test method, incubation conditions of the alga for reproduction had to be determined so that any inhibitive effects observed could be ascribed to toxicants alone. It is well known that environmental factors, such as light and temperature exert significant influences on the initiation of algal sporulation (Lüning, 1990; Lobban and Harrison, 1994) and that the variability of physico-chemical factors can modify the effects of chemicals on the alga, thus complicating the evaluation of the results (Eklund and Kautsky, 2003).

The light requirements for the maximal sporulation of *U. pertusa* in static cultures were found at photon irradiances higher than  $60 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . These values are higher than the previously reported  $30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  for the same species although in this latter case sporulation was carried out under aerated conditions (Han et al., 2003b). It

has often been observed that *U. pertusa* sporulates faster in aerated water than in still water (unpublished data), and one possible explanation for the differential effects could be a steady supply of available nutrients in moving water (Norton et al., 1981).

This study indicated that the salinity could also be an influential factor for sporulation of the intertidal alga, allowing maximal sporulation in the range of 25–35‰. Occurrence of reproduction in *Ulva fenestrata*, growing from the mid to low intertidal zone, was reported to be limited to between 19 and 55‰ although the salinity range for maximal reproduction was not indicated (Van Alstyne et al., 2003).

Optimal temperature for sporulation was in the range 15–20 °C, coincident with the fact that *U. pertusa* is a temperate species (Tseng, 1983). In *Ulva* plants collected from the Netherlands, spore formation was observed over a similar temperature range (Malta et al., 1999).

When the reliability of the test procedure was tested using SDS as a reference toxicant, the  $\text{EC}_{50}$  range ( $2.66\text{--}7.17 \text{ mg L}^{-1}$ ) was comparable to that for ger-

mination inhibition in *U. fasciata* ( $1.75\text{--}4.29\text{ mg L}^{-1}$ ) and to that for inhibition of sea urchin fertilization ( $1.76\text{--}8.56\text{ mg L}^{-1}$ ) and embryo development ( $1.93\text{--}7.07\text{ mg L}^{-1}$ ) (Hooten and Carr, 1998). The levels of sensitivity shown by the studied alga indicate that the sporulation inhibition test may be successfully used for toxicity testing.

The metals tested were found to have adverse effects on sporulation of *U. pertusa* and the calculated  $EC_{50}$  values revealed the toxicity of the metals in the decreasing order of  $Cu^{2+} > Cd^{2+} > Zn^{2+} > Pb^{2+}$ . According to the toxicity classification system GESAMP (1989), the present results imply that all four metals are very toxic ( $EC_{50} < 1\text{ mg L}^{-1}$ ), indicating high sensitivity of *U. pertusa* sporulation in response to these metals. Comparison of the test results obtained from the present method with those of other algal assay protocols would provide information on the sensitivity of sporulation inhibition as a suitable endpoint. Bearing in mind that the different sensitivities of taxa are not well understood (Lewis, 1995), we compared  $Cu^{2+}$  toxicity of *U. pertusa* with that of *U. compressa* and found that *U. pertusa* was 10–100 times more sensitive to copper than *U. compressa* (Reed and Moffat, 1983). The endpoints used for the latter species were growth, rhizoid regeneration and cell viability, and so a comparison of the results appears to confirm that reproduction is a sensitive indicator of copper toxicity, as has been previously shown in the brown alga *Macrocystis pyrifera* (Anderson et al., 1990). In fact, the sensitivity to  $Cd^{2+}$ ,  $Pb^{2+}$  and  $Zn^{2+}$  was also greater in the present test using *U. pertusa* than in some well developed and standardized tests using freshwater green alga and aquatic vascular plants (Kay et al., 1984; Bengtsson et al., 1999). The threshold toxicity in duckweed and water hyacinths was in the range,  $0.087\text{--}0.5\text{ mg L}^{-1}$  for  $Cd^{2+}$ ,  $1.8\text{--}7.0\text{ mg L}^{-1}$  for  $Zn^{2+}$  and higher than  $2.0\text{ mg L}^{-1}$  for  $Pb^{2+}$ .

Effects of toxic metals on algal metabolism have been extensively documented by Rai et al. (1981). In general, exposure to heavy metals, such as those tested in the present study, is known to inhibit growth and photosynthesis, to suppress cell division, to cause loss of photosynthetic pigments and to damage plasma membranes (Rai et al., 1981). The differential cause of cellular toxicity by different metals might partly be related with the stability of the metal ions binding to cellular targets and/or with mobility of the met-

als (Kay et al., 1984; Nieboer and Fletcher, 1996). The stability of metal ion–cellular target complexes increases with the magnitude of the covalent and ionic index. The metals tested in the present study have a similar ionic index with a descending order of covalent index as  $Pb^{2+}$ ,  $Cu^{2+}$ ,  $Cd^{2+}$  and  $Zn^{2+}$ . This order is similar to that of toxicity estimated by  $EC_{50}$  values except for lead which was found to be far less toxic than  $Cu^{2+}$  and  $Cd^{2+}$ . In water hyacinths, it was noted that solutions containing  $0\text{--}5\text{ mg L}^{-1}$   $Cd^{2+}$ ,  $Cu^{2+}$  and  $Pb^{2+}$  had differential effects on the plant growth. The result was explained by differential mobility of the metals (Kay et al., 1984). The order of increasing mobility of the metals  $Pb^{2+} < Cu^{2+} < Cd^{2+}$  corresponded with the degree of toxicity, although  $Cu^{2+}$  becomes more mobile than  $Cd^{2+}$  at lower levels of exposure (Kay et al., 1984). The same principle might seem to be applicable to the present results where metal toxicity as measured by  $EC_{50}$  ranging from 0.061 to  $0.877\text{ mg L}^{-1}$  was shown to be in the descending order of  $Cu^{2+} > Cd^{2+} > Zn^{2+} > Pb^{2+}$ . The same order of toxicity has also been reported for natural phytoplankton (Gächter, 1976).

The toxicity of the sludge from different sources was assessed by means of elutriate bioassay with *U. pertusa*. Salinity and pH were checked prior to the bioassay since these were important physico-chemical factors known to affect the toxicity of pollutants (Rai et al., 1981). Heavy metals become more toxic in acidic conditions than in alkaline pH due to higher bioavailability in the former (Rai et al., 1981). There is no consensus on the role that salinity plays in toxicity, but enhanced toxicity has been often reported in response to effluents of reduced salinity (Doblin and Clayton, 1995; Burrige et al., 1999). In the present conditions, similar salinity and pH values were recorded among elutriates. The  $EC_{50}$  concentrations obtained here were different depending on sludge samples, indicating that the toxicity test based on sporulation inhibition is an effective means to differentiate mixtures from different origins. At higher elutriate concentrations, the toxic effects appear to cause injuries characterized by thallus discoloration. The mechanism of toxic expression by the elutriates and contribution to toxicity by individual constituents are uncertain since it is difficult to identify the differing degree of extractability and the potential of synergistic, antagonistic and additive effects (Pardos et al., 1998). However, sludge contains

a complex mixture of constituents including heavy metals, organic particulates, PAHs and PCBs, etc., and many of these have already been shown to cause toxicity to various algal taxa (Rai et al., 1981; Klaine and Lewis, 1995). When the non-parametric Spearman rank correlation analysis was performed for the association between the endpoint and the concentrations of pollutants retained in the sludge, a significant correlation was found ( $P < 0.05$ ,  $r = 0.717$  or higher,  $n = 9$ ) between the biological response and the levels of  $\text{Cu}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Pb}^{2+}$ . This does not necessarily mean that these elements are responsible for the observed toxicity of the elutriates. Information on mobilization of metals from the sludge into the liquid phase would be required but it was not possible to perform extensive toxicant analysis of the elutriates. Instead, we adopted the reported average mobilization factors of three heavy metals ( $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Pb}^{2+}$ ) and estimated the metal concentrations in the elutriates in terms of toxicity units (TU) (Beiras et al., 2003). The TU concept is commonly used with complex mixtures of contaminants with similar modes of action. For elutriate samples in which the summed TU was greater than one, adverse effects were expected since one TU predicts a 50% inhibition of sporulation. Therefore, the toxicity found for the four sludge samples with TU values greater than 0.8 may be explained on the basis of the predicted  $\text{Cu}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Pb}^{2+}$  contents of their elutriates while the toxicity of the other sludge samples might involve other pollutants. Chemicals present in complex mixtures, such as sludge elutriates can adversely affect the physiological functions of the test organism in more complex manner than single toxicants. Elutriate testing using the sporulation inhibition of *U. pertusa* seems to give information about the bioavailability and toxicity of extractable pollutants, but not on the potential effects of contaminants retained in the sludge. As the elutriate test may provide useful ancillary information on sludge-associated contaminants, it might be advisable to use elutriate toxicity data as estimates of the mixing and dilution of sludge before disposal and the determination of the size of mixing zone.

Development of marine algal test methods noting effects of a particular chemical class would be useful for hazard assessment of any liquid samples previously unstudied, thus reducing the time and cost associated with blind chemical scanning for a wide array of contaminants and providing information for subsequent

chemical characterization efforts (Bisson et al., 1989). In this sense, the *Ulva* toxicity test may prove a novel approach being useful for an integrated assessment of toxicity of both well-defined and unknown wastes.

The algal toxicity test using the sporulation inhibition of *U. pertusa* exhibits practical advantages in many ways. This method does not require much expertise or equipment because it uses microscopic observation of the thallus color change. Unialgal cultures of *U. pertusa* can be set up with ease and maintained as stock cultures in the laboratory. Samples are also available all the year round in the field. With further scaling-up of quantification of the toxicity versus concentration of single or multiple toxicants, we have no doubt that this bioassay could be applicable to the routine monitoring of coastal water environments as a complement to the chemical analysis.

Members of *Ulva*es show a wide range of geographic distribution and have similar reproductive processes. Therefore, the test method developed in the present study has a potential for application to the species belonging to the same taxa elsewhere.

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