0014-4754/95/040414-05$1.50 + 0.20

Measuring toxicity in marine environments: critical appraisal of three commonly used methods

M. A. Becerro, M. J. Uriz and X. Turon[a]

Centre for Advanced Studies, Camí de Sta Bàrbara s/n, E-17300 Blanes, Girona and [a]Department of Animal Biology, University of Barcelona, Barcelona (Spain)
Received 31 May 1994; accepted 1 November 1994

Abstract. Toxicity quantification is important in environmental monitoring, in the field of natural products, and in chemical ecology. The sensitivity and precision of three commonly used methods detecting toxicity in marine environments were compared, using the toxic marine sponge *Crambe crambe* as a test organism. The paper disk diffusion method (run with marine bacteria) showed the least sensitivity and did not permit toxicity levels to be quantified. The sea urchin and the MICROTOX® tests showed greater sensitivity, and the latter had the higher precision. The relative performance of these methods is discussed. It is concluded that the MICROTOX® bioassay displays the best characteristics for toxicity quantification.
Key words. Environmental monitoring; natural products; chemical ecology; toxicity quantification; paper disk diffusion; MICROTOX (*Photobacterium phosphoreum*); *Paracentrotus lividus*.

Toxicity detection is crucial in several aspects of research, for example in assessing environmental contamination or searching for natural products with potentially useful activities. Moreover, as the release of pollutants in sewage is regulated by law, the establishment of standardized methods of quantification of toxicity is necessary both for setting the permitted pollutant levels and for their routine monitoring. Toxicological research has led to the application of some general bioassays to environmental monitoring. Tests on organisms such as sea urchins[1] or the fresh water cladoceran *Daphnia magna*[2] have been recommended to assess the levels of pollutants in water[3].

The search for active natural products from marine organisms has been vigorously pursued in recent decades[4] as a response to industrial demand. The various activities displayed by these compounds have led to their being used for a wide range of applications. Natural marine products have provided a never-ending source of valuable compounds for use in agriculture (herbicides, fungicides, pest control), as antifouling paints, and in pharmacology (antibiotic, antitumor, antiviral compounds), to cite only a few examples. The selection of potentially useful compounds relies heavily on accurate detection of their activities by means of different bioassays run with extracts of the organisms.

Selection of the bioassay is not a trivial step, but it has often been disregarded. Accuracy in detecting toxicity, in studies both of contamination and of natural products, depends on the test used. In our opinion, a suitable method to detect toxicity should display a response to a wide array of metabolites or toxicants (wide range). In addition, if toxicity is to be quantified, the method should discern slight changes in the amount of toxic compounds present (high sensitivity). Finally, any method used should have a great degree of repeatability between experiments (high precision), which would allow for world-wide use and comparisons of measurements made at different times or in different places. Of course, other characteristics such as rapidity or ease of use of the test should be considered, but they are secondary in importance to accurate quantification.

The encrusting sponge *Crambe crambe* (Schmidt) was selected for a comparative study on toxicity assessment methods. This species has been reported to have strong antimitotic[5], cytotoxic[5–7], antimicrobial[5,8,9], antifungal[5], and antiviral[5,7] properties. It also exhibited significant toxicity in assays with *Artemia salina*[6], *Lebistes reticulatus*[10], and *Gambusia affinis* (authors, unpubl. res.), and inhibited the reaggregation of cells of *Ephydatia fluviatilis*[10]. In addition, it also showed a strong activity as a Ca^{++} channel blocker (ionic channels are known to be important targets of the action of natural toxins[6]). These properties, together with the abundance of *C. crambe* in Mediterranean littoral waters, make this species a suitable object for research on toxicity evaluation.

This study has a twofold goal: firstly, to compare the sensitivity and precision of three commonly used bioassays in the quantification of the toxicity of *Crambe crambe* and secondly, to quantify variations of toxicity within the species by comparing sponges of different size ranges. Size has been claimed to have an important influence on the properties of encrusting, substrate-dependent benthic organisms[11,12]. The influence of biological or ecological variables on toxin production is a rarely-explored field[13–15]. Besides providing valuable bi-

ological information, a greater knowledge of variability in toxin production could increase the efficiency of exploratory surveys, for example in pharmacological research, by providing guidelines for selecting specimens of an organism that are likely to have high levels of toxin-production.

Material and methods

All the specimens used in this study were collected in Blanes (northwestern Mediterranean) from a community in which sponges were the dominant organisms at a depth of 10 m, by scuba-diving[12]. The specimens were individually collected and placed separately in plastic bags while still under water. All handling and treatment of the samples was done separately in order to ensure truly independent replicates. Once in the laboratory, the sponges were frozen and freeze-dried to obtain the dry weight (DW) of each specimen. They were then extracted three successive times with 5 ml dichloromethane (DCM) per 0.1 g (DW) of sponge for 5, 15, and then 30 min. The three extracts were pooled, the DCM evaporated, and the residue weighed and homogenized in different amounts of distilled water to obtain concentrations appropriate for each assay (see below). Sponges of three size categories were assayed separately: 1) smaller than 1000 mm^2 in area, 2) between 1000 and 10,000 mm^2 and 3) bigger than 10,000 mm^2. Sponges in these categories exhibited clear differences in shape and other biological parameters[12] and were, therefore, expected to show some differences in toxin production also. The quantification of these differences would provide an indication of the relative sensitivity of each method. A minimum of 70 specimens was used in each of the experiments involving these size classes. In order to assess repeatability, some randomly-chosen specimens were tested twice with the same method.

Description of bioassays. Three commonly used bioassays were analyzed in this study. The paper disk diffusion method[5,16–19], the sea urchin test[3,20,21], and the *Photobacterium phosphoreum* test using the commercially available MICROTOX® system[22–25]. Details of these methods can be found in the references cited. A brief description of the principles of the assays follows. The paper disk diffusion method has been widely used to detect antimicrobial activity in the screening of marine organisms[9,17]. In this study, the assay microorganism was a marine bacterium isolated from the vicinity of the sponges. This provided a more ecological basis for the interpretation of any toxicity detected than using a test organism from elsewhere would have done. Specimens were extracted as previously described. A solution was made in distilled water with 5 mg (referred to the sponge dry weight) per ml (5000 ppm). Five µl portions of this solution were deposited on sterile paper disks, 6 mm in diameter, with 4 replicate disks per specimen. The disks were placed on agar plates previously seeded with the bacteria. Controls had 5 µl of distilled water per disk. The toxic effect was recorded as the diameter of the inhibition zone (including the disk) measured to the nearest mm after incubation for 24 h at 30 °C[19].

The sea urchin test has been used in studies both of environmental contamination assessment[26,27] and the evaluation of natural toxicity of marine organisms[21]. The assay organism used was the sea urchin *Paracentrotus lividus* (Lamarck). Spawning was induced by peristomic injection of 1 ml of 0.5 M potassium chloride and both sperm and eggs were released into filtered (0.45 µm pore diameter) sea water. Cross fertilization of a pair of sea urchins was achieved by adding a small quantity of sperm to the container with the eggs. Experiments were run in 24-well plates. Solutions of the crude sponge extract were added in 0.1 ml portions to 0.9 ml of sea water containing the eggs, to obtain final concentrations of 18, 12, 6, 3, 1.5, and 1 µg of sponge extract per ml (ppm). Four replicates were used per concentration. Controls had 0.1 ml of distilled water. The percentage of embryos that did not reach the pluteus stage after 48 h was used as a measure of the toxicity. Finally, the concentration at which 50 percent of the embryos are expected to reach the pluteus stage (PT_{50}) was calculated by fitting the data to a bounded monotonic response function of the following form:

$$NE = \alpha + \frac{1-\alpha}{1+e^{(\beta-\gamma^* \text{ dose})}}$$

where NE = % of dead embryos, α = background response or rate of dying, β = a location parameter for the curve and γ = a slope parameter for the curve. The PT_{50} can be easily obtained from the former function:

$$PT_{50} = -\frac{\ln\left(\left(\frac{1-\alpha}{0.5-\alpha}-1\right)-\beta\right)}{\gamma}$$

Since the MICROTOX® bioassay was launched in the early 1980s, its use in environmental monitoring has steadily increased[22–25]. This method is based on measures of bioluminescence in living cell suspensions of the deep sea bacterium *Photobacterium phosphoreum*. A vial of freeze-dried bacteria (approx. 10^6 cells) was rehydrated in 1 ml of a reconstituent solution at 4 °C. This suspension was then added to 50 ml of 0.2 M saline solution. The toxic solution was prepared by adding 20 ml of distilled water to the extract of 10 mg of sponge (DW), resulting in a concentration of 500 ppm. Molarity was adjusted by means of a molarity adjustment solution (MOAS) added in the proportion of 250 µl of MOAS per 2500 µl of the toxic solution. In its basic procedure, MICROTOX® analyzes four decreasing concentrations (a dilution factor of two was used) and one control per sample. Experiments were run

during five minutes at a temperature of 15 °C by adding 500 µl of each concentration to the wells containing the 500 µl of bacteria suspension. The light produced by the bacteria was recorded before and after the addition in terms of Gamma Units (GU):

$$GU = \frac{R_t^* I_0}{I_t} - 1$$

where R_t = correction factor, I_0 = light at time O, I_t = light at time t.

Finally, the concentration at which GU is equal to 1 (50 percent of light reduction or estimated median effective concentration, EC_{50}) was calculated by fitting the data expressed in logarithmic form to a linear regression. The toxicity units (TU) used in this study were calculated as $100/EC_{50}$.

Parametric methods (ANOVA and Tukey HSD tests) for hypothesis testing were used whenever the data featured normality and homoscedasticity (according to Kolmogorov-Smirnov and Bartlett tests, respectively). Otherwise, analogous non-parametric procedures (Kruskal-Wallis and Dunn tests) were used[28].

Results and discussion

Sensitivity. Two out of the three methods used in this study (sea urchin and MICROTOX® test) detected differences in toxicity among the three size-categories of sponges (fig. 1) (MICROTOX®: Anova procedure $F = 6.649$, $p = 0.002$, $n = 100$ sponges; sea urchin: Kruskal-Wallis statistic = 12.514, $p = 0.002$, $n = 70$ sponges; paper disk: Kruskal-Wallis statistic = 1.658, $p = 0.436$, $n = 100$ sponges). It should be noted that toxicity is expressed in different units in each bioassay: inhibition diameter in the paper disk assay in mm, PT_{50} in the sea urchin assay in ppm, and TUs in the MICROTOX® assay. Accordingly, absolute toxicity values cannot be directly compared between assays. However, if the three tests were to be equally sensitive to toxic variations, they should display the same pattern of relative variation among size classes. This relative performance can indeed be compared across assays. Therefore, although the units in the ordinate axis have a different meaning in each test, they are presented together in figure 1 to highlight that the pattern of toxicity among different size classes varied according to the test employed.

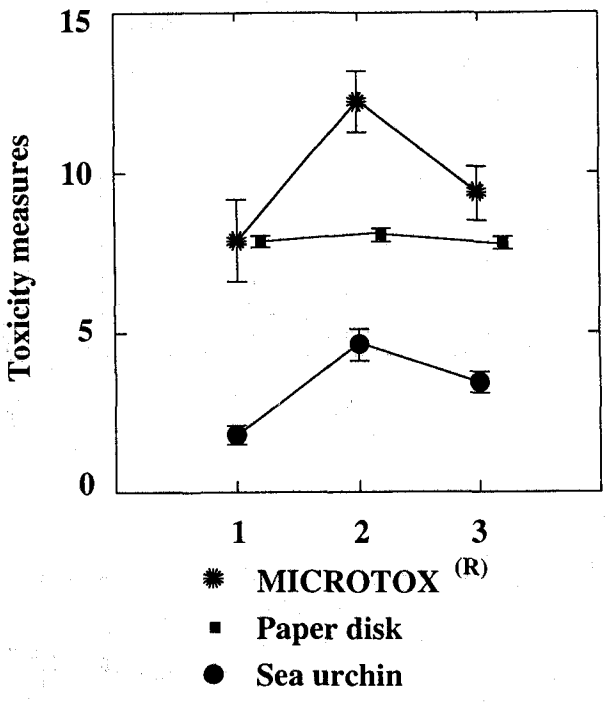

Figure 1. Toxicity measures obtained by the three methods for the size classes considered. Toxicity units in the ordinate differ in each test (TU, mm, and ppm for the MICROTOX®, paper disk, and sea urchin assays, respectively). Therefore, only the pattern of relative variation among size classes, not the absolute values, are comparable. Bars are standard errors.

In the sea urchin test, specimens of the smaller size class showed significantly less toxicity than medium-sized and larger specimens ($p < 0.01$ for both pairwise comparisons in a Dunn test), while the toxicity of the medium and large specimens shared a small and not significant difference ($p > 0.5$). The MICROTOX® bioassay exhibited results with a similar pattern, but the higher toxicity of the medium-sized sponges was more marked. A multiple comparison test (Tukey HSD), showed significant differences between medium-sized sponges and both the smaller ($p = 0.002$) and larger ($p = 0.035$) size classes, while the smaller and larger sponges were not significantly different ($p = 0.35$). The paper disk diffusion test showed no significant difference between the toxicity of extracts from the three size classes.

The three methods showed high correlation values when their readings were compared for the three size classes (fig. 2). However, the probabilities of Type I error associated with these values were above the 0.05 level, due to the low number of points (3) in the analysis (fig. 2; sea urchin and MICROTOX® $r = 0.976$, $p = 0.138$; sea urchin and paper disk diffusion $r = 0.932$, $p = 0.236$; paper disk diffusion and MICROTOX® $r = 0.988$, $p = 0.098$).

The MICROTOX® and the sea urchin methods exhibited bigger toxicity differences among the size classes compared with the paper disk diffusion test. Therefore, the study of the degree of repeatability of the experiments was performed on these two methods only.

Precision. Precision in evaluating toxicity has been measured as the percentage of variation between experiments run with the same samples. Results of this analysis on the sea urchin test (17 repeated samples) and the MICROTOX® test (13 repeated samples) showed significant differences in the percentage variation between these two methods (T-test on data arcsine transformed,

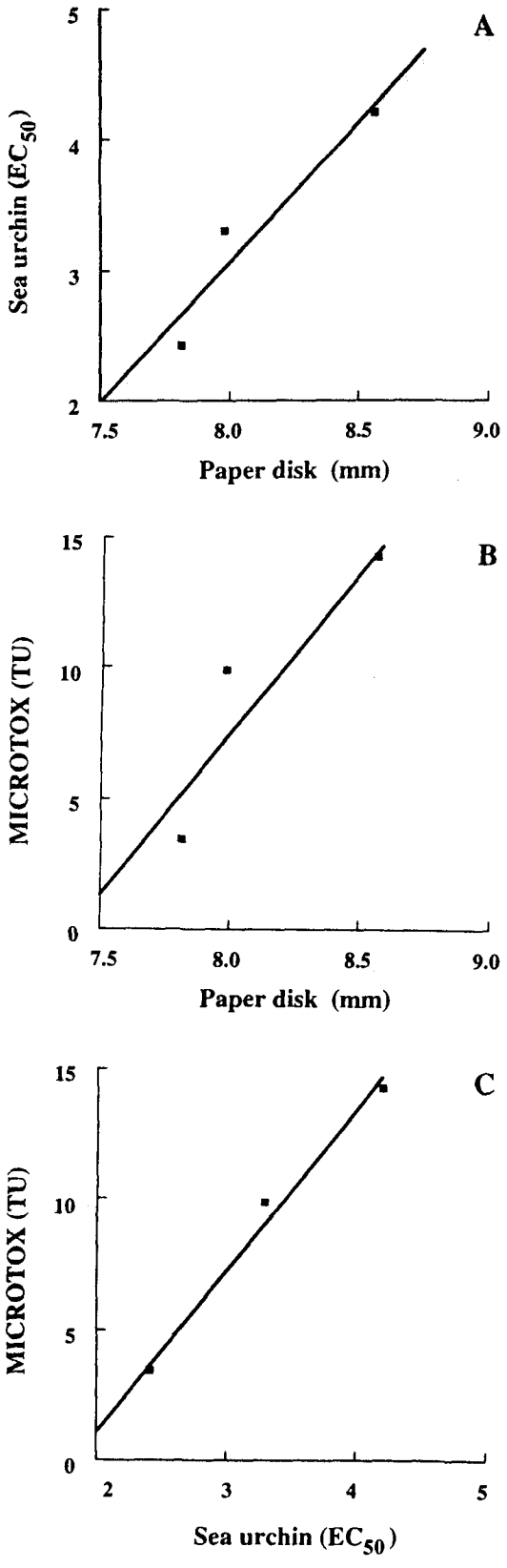

Figure 2. Regressions between the mean toxicity values obtained for each size class: *A* sea urchin (measured in EC_{50}) and paper disk diffusion (measured in mm), *B* MICROTOX® (measured in TU) and paper disk diffusion (mm), and *C* MICROTOX® (TU) and sea urchin (EC_{50}).

$p < 0.01$), the *P. phosphoreum* test being twice as precise as the sea-urchin bioassay: only 14.5% (average) variation between the experiments compared to the 32.6% in the sea-urchin bioassay.

Of the three methods analyzed, the paper disk diffusion test appears to be the least sensitive. It failed to detect the differences in toxicity among size classes that were demonstrated by the other two assays. This assay may not be a satisfactory means of obtaining information on the toxic effects as a function of the toxicant concentration, since the area of inhibition of bacterial growth might well depend on the physical, rather than toxicological, properties of the molecules (e.g. diffusion rate in the agar). Consequently, this method is not recommended for environmental management or any other study where pollutants or toxicity levels are to be quantified. However, it could be a useful method for assessing the biological or ecological roles of a particular bactericidal activity in marine organisms. Bacteria from the surface, the inside or the near vicinity of the organism which produces the bactericidally active molecules can easily be cultured, and their vulnerability to the bactericide then assayed[19].

The sea urchin bioassay detected the higher toxicity of the medium-sized specimens well, but it showed less sensitivity and precision than the MICROTOX® test, despite the fact that eggs show a higher sensitivity to toxic compounds than adult organisms (i.e., fishes and crustacea) or even larvae[21]. Some advantages of this method have been pointed out by various authors: the ease of obtaining adult sea urchins and their gametes, easy fertilization and maintenance of constant experimental conditions[1], and the possibility of investigating effects on sperm[20], embryos[14,21,29] and larvae (ref. 29 and authors unpubl. res.) with a sole species. Moreover, it allows for detection of toxicity as a function of time. However, the low repeatability exhibited must be considered as an important disadvantage, since it makes it difficult to compare bioassays separated in time or space.

The toxicity assay using the bacterium. *P. phosphoreum*, performed with the commercially available MICROTOX® system, showed the greatest sensitivity and highest procision of the three methods analyzed. It is conducted on lyophilized marine bacteria and guarantees constant experimental conditions, which permits standardization. Possible sources of variation other than toxicity (genetic variations in the test organism or variation in experimental conditions) are reduced to a minimum. *P. phosphoreum*, is sensitive to an array of different toxins[30,31] and permits both a quantitative (EC_{50}) and qualitative interpretation of the results (the slope of regression between toxic concentration and toxicity units indicate different mechanisms of action). It is rapid and easy to use.

Consequently, we fully recommend its use for toxicity quantification in marine environments, especially when the toxicant is water-soluble (the assay can also be used for particles in suspension).

Quantifying toxicity is a common aim in contamination studies, and may even become a routine procedure for monitoring water quality in marine environments in order to protect the environment from human activities. Quantifiation has rarely been carried out in studies of natural products[13-15,21]. According to the results obtained in this study with the MICROTOX® test, specimens of *C. crambe* between 1000 and 10,000 mm^2 in surface area showed higher toxicity than either smaller or larger specimens. To our knowledge, this is the first time that such a study has been conducted on modular, substrate-dependent organisms. This kind of information is not only of ecological interest, but may also aid in organizing pharmacological surveys, since it may help in the selection of specimens with a high concentration of active substances.

Acknowledgments. This study is a part of a Ph.D. thesis supported by a Basque Government Predoctoral Fellowship to M. A. Becerro. The Spanish Marine Resources and Aquaculture Programme (project CICYT MAR91-0528) and Commission of the European Communities (project MAST-CT91-004) provided funds for this research. The authors are indebted to G. Benito and the staff of the 'Junta de Sanejament' of the Environment Department, Generalitat of Catalunya, for providing MICROTOX(R) and laboratory facilities.

1 Hagström, B., and Lönning, S., Pharmac. Toxic. *32* (1973) 1.
2 Janssen, C. R., and Persoone, G., Environ. Toxic. Chem. *12* (1993) 711.
3 Reish, D. L., and Oshida, P. S., Manual of methods in aquatic environment research. Part 10. Short-term statics bioassays. FAO Fish. Tech. Pap. *247* (1987) 269.
4 Faulkner, D. J., Nat. Prod. Rep. *7* (1990) 269.
5 Uriz, M. J., Martín, D., and Rosell, D., Mar. Biol. *113* (1992) 287.
6 Berlinck, R., Bifulco, G., Braeckman, J. C., Bruno, I., Daloze, D., Pem. S., Riccio, R., Spampinato, S., and Speroni, E., in: Proceedings 7th Int. Symp. Marine Natural Products, p. 56. Eds M. Piatelli and L. Minale. Consiglio Nazionale delle Ricerche, Capri, Italy 1992.
7 Jares-Erijman, E. A., Sakai, R., and Rinehart, K. L., J. org. Chem. *56* (1991) 5712.
8 Burlholder, P. R., and Rüetzler, K., Nature *222* (1969) 983.
9 Amade, P., Charroin, C. Baby, C., and Vacelet, J., Mar. Biol. *94* (1987) 271.
10 Van der Vyver, G., Huysecom, J., Braekman, J. C., and Daloze, D., Vie Milieu *40* (1990) 285.
11 Turon, X., and Becerro, M. A., Mar. Ecol. Prog. Ser. *82* (1992) 235.
12 Becerro, M. A., Uriz, M. J., and Turon, X., Mar. Biol. *121* (1994) 301.
13 Rinehart, K. L. Jr., Gloer, J. B., Wilson, G. R., Hughes, R. G. Jr., Li, L. H., Renis, H. E., and McGovern, J. P., Fedn. Proc. *42* (1981) 87.
14 Maida, M., and Coll, J. C., J. chem. Ecol. *19* (1993) 2285.
15 Paul, J. V., and van Alstyne, K. L., Coral Reefs *6* (1988) 263.
16 Bergquist, P. R., and Bedford, J. J., Mar. Biol. *46* (1978) 215.
17 Thompson, J. E., Walker, R. P., and Faulkner, D. J., Mar. Biol. *88* (1985) 11.
18 Uriz, M. J., Martín, D., Turon, X., Ballesteros, E., Hughes, R., and Acebal, C., Mar. Ecol. Prog. Ser. *70* (1991) 175.
19 Becerro, M. A., Lopez, N. I., Turon, X., and Uriz, M. J., J. expl. mar. Biol. Ecol. *179* (1994) 195.
20 Dinnel, P. A., Link, J. M., and Stober, Q. J., Archs environ. Contam. Toxic. *16* (1987) 23.
21 Martín, D., and Uriz, M. J., J. expl mar. Biol. Ecol. *173* (1993) 11.
22 Vasseur, P., Ferard, J. F., Vial, J., and Larbaigt, G., Envir. Pollution *34* (1984) 225.
23 Reteuna, C., Vasseur, P., and Cabridene, R., Hydrobiologia *188/189* (1989) 149.
24 Ribo, J. M., and Rogers, F., Toxic. Assess *5* (1990) 135.
25 Ribo, J. M., and Kaiser, K. L. E., Toxic. Assess. *2* (1987) 305.
26 Kobayashi, N., Publ. Seto mar. biol. Lab. *28* (1971) 376.
27 Oshia, P. S., Goochey, T. K., and Mearns, A. J., Biological Monitoring of Marine Pollutants, p. 389. Eds F. S. Vernberg, A. Calabrese, F. P. Thurberg and W. B. Vernberg. Academic Press, New York 1986.
28 Zar, J. H., Biostatistical Analysis, Prentice-Hall, Inc., Englewood Cliffs, New Jersey 1984.
29 Cariello, L., Giudici, M. N., and Zanetti, L., Comp. Biochem. Physiol. *65* (1980) 37.
30 Kaiser, K. L. E., and Ribo, J. M., Toxic. Assess. *3* (1988) 195.
31 Blum, D. J. W., and Speece, R. E., Journal J. Wat. Pollut. Control Fed. *63* (1991) 198.