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LUMINESCENT MARINE BACTERIA IN ACUTE TOXICITY TESTING

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ABSTRACT

Recently, a luminescent bacterial assay ("Microtox") to test the toxicity of pure substances and complex effluents, has been described. This test uses as biological reagent, a marine bacteria, Photobacterium phosphoreum, which has an optimal luminescence in saline medium.

The toxicity was evaluated by assessing an IC50 value, which is the concentration of the toxic substance or the dilution of the effluent, decreasing the bacterial luminescence by 50 % after a specified contact period (5, 10, or 30 min).

The bacterial luminescent test is interesting in marine toxicity screening at least for four reasons :

- simplicity ;
- rapidity (less than 60 min for preliminary and definitive tests) ;
- reproducibility (coefficient of variation < 20 %) ;
- sensitivity (of the same order of magnitude as for higher aquatic species).

IC50 values of various organic (benzenic and chlorinated hydrocarbons, phenolic compounds,carbamates, etc.) and inorganic chemicals (Zn^{2+}, Cu^{2+}, Cr^{6+}, Cd^{2+}, Hg^{2+}, etc.) have been determined in salt medium using this methodology. Results are presented and compared with the literature data on acute toxicity effects of these compounds on other marine species (fish, crustaceans, and algae).

The "Microtox" test appears to be a useful tool in toxicity screening of chemicals, complex effluents and chemically contaminated seawater. The rapidity and the simplicity of this method make it most convenient to detect toxicants in industrial sites.

KEYWORDS

Marine ecotoxicology, Methods, Luminescent bacteria, Photobacterium phosphoreum, Bioluminescence inhibition, Microtox test.

INTRODUCTION

A luminescent bacterial assay, designated as "Microtox", has been recently developed by Bulich (1979), to assess the acute toxicity of chemicals. This test has been shown to compare well with conventional bioassays using freshwater species such as algae, crustaceans, and fishes (Bulich et al., 1981 ; Dutka and Kwan, 1981 ; Qureshi et al., 1982). It is a rapid, simple and inexpensive method. For these reasons it has been adopted as a routine test to control the quality of rivers, domestic, and industrial wastewaters(Lebsack et al., 1981; Férard et al., 1983 ; Vasseur et al, 1983). This method has not yet been used to test seawaters. Nevertheless it would be a valuable tool to determine the toxicity of chemicals in the marine environment and to control the quality of the seawater in contaminated areas.

The Microtox test described in this paper uses the luminescent marine bacteria, Photobacterium phosphoreum, of which the luminescence decreases in the presence of toxicants. The principle of this bioassay is the evaluation of the percentage of inhibition of the luminescence after a few

minutes (5 to 30 min) following the addition of the toxic solution to the bacterial suspension, and the calculation of the concentration of toxic substance which inhibits the light normally produced by 50 % (IC 50).

Photobacterium phosporeum is an ubiquitous bacterium in the oceans, found on some marine fishes, non-pathogenic to humans and mammals. It is a gram-negative motile rod, sharing a number of morphological, physiological, and biochemical properties with the Enterobacteriaceae (Nealson and Hastings, 1979). The bioluminescence is produced metabolically by the luciferase enzyme (Fig. 1). This enzyme reacts with the flavin coenzyme, $FMNH_2$, and forms, with oxygen and an aliphatic aldehyde (C14), an activated complex which degrades and emits photons responsible of the luminescence (Nealson, 1979).

The first part this paper describes the effects of some organic and inorganic compounds on the bacterial luminescence in natural and synthetic seawater. In the second part these results are compared to the literature data on the acute toxicity of these compounds to other marine species.

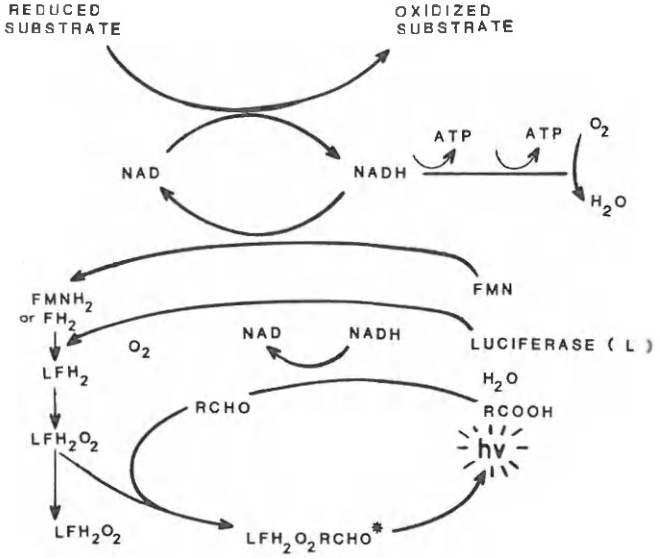

Fig. 1. Biochemical mechanism of the bacterial luminescence.

MATERIALS AND METHODS

TESTED CHEMICAL COMPOUNDS

The tested chemical compounds were selected on the basis of their relative importance in the contamination of the sea and estuaries by industrial effluents. The following products were tested out :

inorganic salts	: $Cd(NO_3)_2.4H_2O$	(Merck No. 2 019)
	$K_2Cr_2O_7$	(Merck No. 4 864)
	$CuSO_4.5H_2O$	(Prolabo No. 23 174.23)
	$ZnSO_4.7H_2O$	(Prolabo No. 29 253.23)
organic compounds	: benzene	(Prolabo No. 21 803.29)
	toluene	(Merck No. 8 325)
	ethylbenzene	(Merck No. 801 372)
	phenol	(Merck No. 206)
	3,5-dichlorophenol	(Merck (No. 8 217)

These chemicals were diluted and tested in two kinds of saline medium, namely synthetic and natural seawater. The composition of the synthetic seawater is given in Table I. This medium was sterilized through filtration on 0.22 µm filters. The pH was 7.8 and salinity approximated 35 ‰. The natural seawater was supplied by the ISTPM (Institut Scientifique et Technique des Pêches Maritimes) of Nantes. It was collected in the Atlantic ocean, offshore Le Croisic, Brittany, and sterilized on 0.22 µm filters. The pH was 7.9 and the salinity 24 ‰.

Table I. Composition of the synthetic seawater commonly used in ISTPM (Le Baut, pers. commun.)

Compound	Quantity (g)
NaCl	22
$MgCl_2.6H_2O$	9.7
Na_2SO_4	3.7
$CaCl_2$	1.0
KCl	0.65
$NaHCO_3$	0.20
H_3BO_3	0.023
H_2O bi-distilled (quantum sufficit)	1 000 ml

MICROTOX ASSAY

The Microtox toxicity analyzer is equipped with a photomultiplier tube, adjacent to the reaction chamber provided with an adjustable temperature range of 10 °C to 25 °C and an air drying pump which prevents fogging of cuvettes and water condensation. An incubator chamber holds all solutions within ± 0.3 °C of the selected test temperature, which was 15 °C and 20 °C in the assays reported here. The photomultiplier is connected to a chart recorder and all values of the light output are derived from the length of the peaks in the chart recordings.

The Microtox bacterial reagent (lyophilized bacteria, supplied by Beckman Instruments Inc.) is reconstituted with 1 ml of distilled water. The suspension density of the viable luminescent bacteria is approximatively 10^8 per ml (Beckman, 1982).

The assay procedure, indicated in the Beckman Instruments Interim Manual (No. 015-555-879, 1982) may be summarized as follows :

- First 10 µl of the Microtox reagent is transferred to glass cuvettes and diluted with 490 µl of the saline medium. After 10 min of equilibration at the selected temperature, the luminescence intensity (I_o) of the bacterial suspension is measured.
- Then, 500 µl of the toxic solution is added, the final light level (It) is registered after the chosen time of exposure (5, 10, or 30 min).
- A preliminary test is conducted to assess the interval of concentrations of the toxic substance in the saline medium, decreasing 10 % to 90 % of the luminescence.
- Then, in a definitive test, at least six concentrations are chosen in this interval in such a manner that the percentages of the luminescence decrease fall within this scale. These concentrations are tested simultaneously to determine the IC50-value which is the concentration decreasing the light normally produced by 50 %. Every definitive test was run in duplicate.

CALCULATION OF THE IC50

Two different procedures may be used to assess the IC50 value :

- By calculating $\Delta\,\%$, which is the percentage of decrease in luminescence, taking the initial light level into account :

$$\Delta\,\% = \frac{\left(I_o \times \frac{B_t}{B_o}\right) - I_t}{I_o \times \frac{B_t}{B_o}} \times 100$$

Where I_o = initial luminescence of the bacterial suspension at t=o (before addition of the toxic solution);

I_t = final luminescence after t minutes following this addition;

B_o = initial luminescence of the control (t = o);

B_t = final luminescence of the control after t min.

The IC 50 is determined graphically, by writing out the results on semilog paper with the log scale corresponding to the concentration values.

- By calculating the ratio of the lost light to the final luminescence:

$$\Gamma = \frac{\left(I_o \times \frac{B_t}{B_o}\right) - I_t}{I_t}$$

The Γ-values corresponding to the different test concentrations are plotted on log-log paper. The IC50 equals the concentration corresponding to $\Gamma = 1$. In that case the quantity of light lost is equivalent to the remaining luminescence.

ASSAY OF COLOURED SOLUTIONS

Coloured toxic solutions interfere with the bacterial luminescence and modify the light intensity registered by the analyzer, which results in an erroneous evaluation of the luminescence. In this case, the data have been corrected by taking the absorbance of the coloured test sample into account. These corrections have been made for $K_2Cr_2O_7$ solutions, according to the method recommended in the Beckman Instruments Inc. manual (1982).

RESULTS

Fig. 2 shows a characteristic evolution of the bioluminescence of Photobacteriumphosphoreum exposed to copper. The evolution of the IC50-values upon exposure to other metals is presented in Table II. From these data it can be concluded that the bacterial response to metals is slow. When the bacteria are exposed to benzenic and phenolic hydrocarbons the response is, however, faster (Table III).

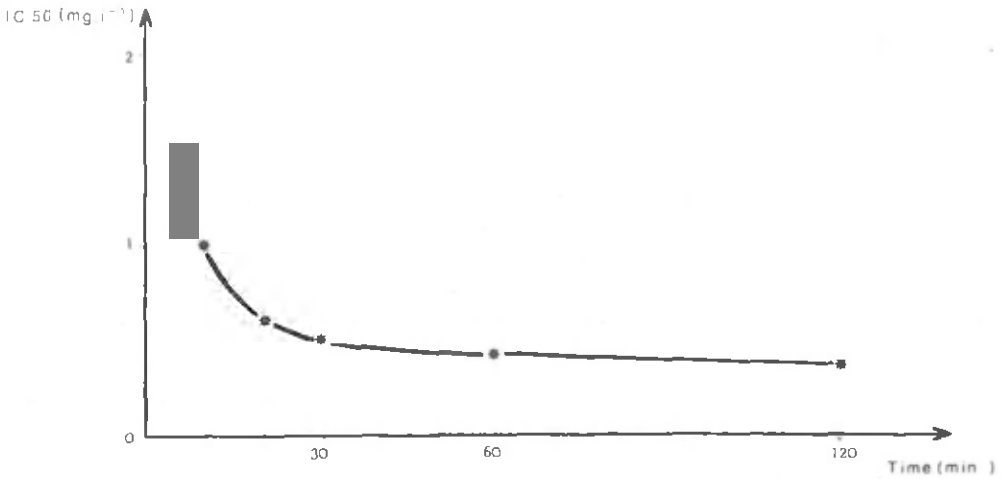

Fig. 2. Evolution of the bacterial luminescence (IC50-value) of Photobacterium phosphoreum exposed to Cu^{2+}. Test conditions : natural seawater, 15 °C.

Two test media have been compared, a natural and a synthetic seawater of which the composition is given above. The exposure time was 5 min for organics and 30 min for metals, taking the results obtained earlier in natural seawater into account (Tables II and III). The IC50 values of organics, tested at 15 °C are given in Table IV. This table shows that the toxicity of phenol and 3,5-dichlorophenol is higher in natural seawater than in synthetic water whereas it is lower in natural seawater for toluene, ethylbenzene, and benzene.

Table II. Evolution of the IC50-values ($mg.l^{-1}$) upon exposure of the bacteria to metals. Test conditions : natural seawater, 15 °C ± 0.3 °C

Metal	Exposure time (min)			
	5	10	15	20
Cu^{2+}	1.50	1.05	0.62	0.50
Zn^{2+}	40	25	7.5	5.4
Cd^{2+}	95	57	25	15.5

Table III : Evolution of the IC50-values ($mg.l^{-1}$) upon exposure of the bacteria to organic compounds. Test conditions : natural seawater, 15 °C ± 0.3 °C

Compounds	Exposure time (min)	
	5	10
Benzene	440	460
Ethylbenzene	110	110
Toluene	135	135
Phenol	24	23.5
3,5-dichlorophenol	4.7	4.2

Table IV. Acute toxicity (IC50-value in $mg.l^{-1}$, after 5 min exposure) of organic compounds to luminescent bacteria, according to the composition of the seawater medium

Compound	Synthetic seawater	Natural seawater
Benzene	405	440
Ethylbenzene	19	110
Toluene	25	135
Phenol	68	24
3,5-dichlorophenol	25	4.7

Table V. Acute toxicity (IC50-values in $mg.l^{-1}$ after 30 min exposure) of metals to luminescent bacteria, according to the composition of the seawater medium and the temperature

Metal	Synthetic seawater		Natural seawater	
	15 °C	20 °C	15 °C	20 °C
Cd^{2+}	60	7	15.5	7
Cr^{6+}	58	57	41.5	43
Cu^{2+}	1.8	2	0.5	0.5
Zn^{2+}	8	1.6	5.4	1.4

Metals were tested at two different temperatures (15 °C and 20 °C) for the two different media. From the data in Table V it appears that :

- for the two seawaters, the toxicity of cadmium and zinc increases substantially when the temperature is higher.
- all tested metals, especially cadmium and copper, exhibit a greater toxicity in the natural seawater than in the synthetic one ; at 15 °C the toxicity of cadmium and copper is increased by a factor four, whereas it is only slightly increased for zinc and chromium.

DISCUSSION

The kinetic study of the luminescence inhibition allows to distinguish two kinds of bacterial response to toxic substances :

- a rapid response, which is characteristic of the organic chemicals tested ; the decrease of luminescence occurs as soon as the toxic medium is added to the bacterial suspension and does not last beyond 5 min ;
- a slow and progressive response, which is characteristic of the heavy metals ; the luminescence intensity decreases when the time of exposure is extended.

An increase in the toxicity of the metals was noticed when the tests were done with natural seawater at 20 °C. This fact might be explained by the lower salinity of natural seawater as compared to that of the synthetic water. The influence of temperature and salinity on the toxicity of particular substances on various marine species is well-known and has been pointed out by several authors (Eisler, 1971 ; Cairns et al., 1975 ; Fales, 1978). Negilski (1976) underlined the greater toxicity of chromium when the test temperature was risen and the salinity reduced. Similar results were obtained by Frank and Robertson (1979) studying the toxic effects of chromium and cadmium in crustaceans. In the case of cadmium, this increased toxicity has been correlated with an enhanced uptake rate of the toxic substance at a higher temperature (O'Hara, 1973 ; Hutcheson, 1974 ; Jackim et al., 1977).

The comparison of the acute effects of the tested chemicals to luminescent bacteria and other organisms, frequently selected for marine toxicity testing, has been made with the literature data. From Table VI it appears that the sensitivity of luminescent bacteria is of the same order of magnitude than the sensitivity of algae (benzene), and of crustaceans (toluene). Even though the 96 h IC50-value of toluene for the marine fish Oncorhynchus gorbuscha is lower than the 10 min IC50 for Photobacterium phosphoreum, it is difficult to conclude that fishes display a higher sensitivity to organics than bacteria , since only very few references relating to this topic are presently available. As for heavy metals, the data referred to in Table VII show that the Microtox test yields results similar to other tests. Luminescent bacteria may be considered as sensitive as the other marine species tested.

Table VI. Comparison of the acute toxicity of benzene and toluene to luminescent bacteria and other marine species

Species	Reference	Test conditions				IC50 or LC50 ($mg.l^{-1}$)	
		Salinity ‰	Temperature (°C)	pH	Exposure time	Benzene	Toluene
Luminescent bacteria							
Photobacterium phosphoreum		24	15	7.9	10 min	460	135
		35	15	7.8	-	445	30
Algae							
Skeletonema costatum	Brooks et al. (1977)	*	*	*	8 h	300	20
Crustacea							
Eualus spp.	Korn et al. (1979)	26 - 28	12	*	96 h		14.7
Corolana borealis	Bakke and Skjoldal (1979)				3 h		125
		34	10	*	69 h		12.5
					400 h		5.7
Fish							
Oncorhynchus gorbuscha	Korn et al. (1979)	26 - 28	12	*	96 h		8

* not indicated

Table VII. Comparison of the acute toxicity of benzene and toluene to luminescent bacteria and other marine species

Species	References	Test conditions				IC50 or LC50 ($mg.l^{-1}$)			
		Salinity ‰	Temperature (°C)	pH	Exposure time	Cd^{2+}	Cr^{6+}	Cu^{2+}	Zn^{2+}
Luminescent bacteria									
Photobacterium phosphoreum		24	15	7.9	30 min	35.5	41.5	0.5	5.4
			20		30 min	7	43	0.5	1.4
		35	15	7.8	30 min	60	58	1.8	8
			20		30 min	7	57	2	1.5
Algae									
Skeletonema costatum	Berland et al. (1977)	35	20	7.8	96 h	0.05	–	0.05	–
	Overnell (1976)	*	*	*	15 min	–	–	3.2	–
Molluscs									
Mytilus edulis	Ahsanullah (1976)	34	20	8	96 h	1.62	–	–	2.5
	Davenport (1977)	33	15	*	108 h	*	–	0.25	*
Polychaetes									
Neanthes arenaceodentata	Reish (1978)	*	*	*	96 h	12.1	3.2	0.3	1.8
Crustaceans									
Crangon crangon	Prince and Uglow (1975)	34	12	*	148 h	0.35			
					130 h			1.6	14.4
Palaemonetes pugio	Pales (1978)	20	15				107		
			20				78		
Fish									
Aldrichetta forsteri	Negilski (1976)	34	19	7.9	120 h	14			
					96 h		31		12
Fundulus heteroclitus	Fisler (1971)	20	20	8	96 h	55		78	66

The Microtox test offers a number of advantages as a marine aquatic toxicity test. It is a simple test, very easy to perform and rapid. The average time necessary to run a sample is approximatively 1 h , including the preparation of the solutions and dilutions. It is also an inexpensive test. Indeed, after the initial acquisition of the analyzer, the only cost is the purchase price of the lyophilic reagent and the time to run a wages of a technician. This last factor is minor, when the Microtox test is compared to the time required to culture marine species such as crustaceans or fishes to be added to the test time itself.

An additional advantage of the luminescent bacterial test is the small quantity or sample volume necessary to carry out the test, in comparison to most other aquatic bioassays, especially those with fishes.

CONCLUSION

This study underlines the interest of the bacterial luminescence-inhibition test in the toxicity assessment for the marine environment. Because the method is simple, rapid, and inexpensive, this test is especially convenient in broad toxicity screening.

This study shows that the sensitivity of the Microtox test is comparable to that of the bioassays with algae, crustaceans, and fishes to test the acute toxicity of chemicals.

Further investigations are needed to improve the reliability of the test to detect the toxicity of contaminated seawater and to determine its possible limits as a toxicity indicator. In these conditions, the Microtox toxicity-testing system will be well-suited to survey estuaries and to control the quality of seawater. As such it constitutes a useful contribution to the battery of marine ecotoxicological screening tests.

LITERATURE CITED

Ahsanullah M. 1976.
Acute toxicity of cadmium and zinc to seven invertebrate species from Western Port, Victoria. Aust. J. Mar. Freshwater Res. 27(2):187-196.

Bakke T. and H.R. Skjoldal. 1979.
Effects of toluene on the survival, respiration and ademplate system of a marine isopod (Cirolana borealis). Mar. Pollut. Bull. 10(4):111-115.

Beckman Instruments, Inc. 1982.
Microtox system operating manual No. 015-555 879.

Berland B.R., D.J. Bonin, O.J. Guérin-Anety, V.I. Kapkov, and D.P. Arlhac, 1977.
Action des métaux lourds à des doses sublétales sur les caractéristiques de la croissance chez la Diatomée, Skeletonema costatum. Mar. Biol. 42(1):17-30.

Brooks J.M., G.A. Fryxell, D.F. Reid, and W.M. Sackett. 1977.
Effects of hydrocarbons on phytoplankton. p. 45. In : Pollutant effects on marine organisms. Giam C.S. (Ed.). Lexington Books, Farnborough. 213 p.

Bulich A.A. 1979.
Use of luminescent bacteria for determining toxicity. p. 171-180. In : Aquatic toxicology. Eaton J.G., P.R. Parish, and A.C. Hendricks (Eds). American Society for Testing and Materials, Philadelphia. STP 667. 403 p.

Bulich A.A., M.W. Greene, and D.L. Isenberg. 1981.
Reliability of the bacterial luminescence assay for the determination of the toxicity of pure compounds and complex effluents. p. 338-347. In: Aquatic toxicology and hazard assessment. Branson D.R. and K.L. Dickson (Eds). American Society for Testing and Materials, Philadelphia. STP 737. 466 p.

Cairns J., J.R. Alan, G. Heath, and B.C. Parker. 1975.
Temperature influence on chemical toxicity to aquatic organisms. J. Water Pollut. Control Fed. 17(2):267-280.

Davenport J. 1977.
A study of the effects of copper applied continuously and discontinuously to species of Mytilus edulis (L.). J. mar. biol. Ass. UK 57:63-74.

Dutka B.J. and K.K. Kwan. 1981.
Comparison of three microbial toxicity screening tests with the Microtox test. Bull. Environ. Contam. Toxicol. 27:753-757.

Eisler R. 1971.
Cadmium poisoning in Fundulus heteroclitus (Pisces : Cyprinodontidae) and other marine organisms. J. Fish. Res. Bd Can. 28:1225-1234.

Fales R.R. 1978.
The influence of temperature and salinity on the toxicity of hexavalent chromium to the grass shrimp, Palaemonetes pugio (Holthuis). Bull. Environ. Contam. Toxicol. 20:447-450.

Férard J.F., P. Vasseur, L. Danoux, and G. Larbaigt. 1983.
Application d un test d'inhibition de luminescence bactérienne à l'étude toxicologique d'effluents complexes et de substances chimiques. Sciences de l'Eau, 2:221-237.

Frank P.M. and P.B. Robertson. 1979.
The influence of salinity on toxicity of cadmium and chromium to the blue crab, Callinectes sapidus. Bull. Environ. Contam. Toxicol. 21 (1/2):74-78.

Hutcheson M.S. 1974.
The effect of temperature and salinity on cadmium uptake by the blue crab, Callinectes sapidus. Chesapeake Sci. 15(4):237-241.

Jackim E., G. Morrison, and R. Steele. 1977.
Effects of environmental factors on radiocadmium uptake by four species of marine bivalves. Mar. Biol. 40:303-308.

Korn S., D.A. Moles, and S.D. Rice. 1979.
Effects of temperature on the median tolerance limit of pink salmon and shrimp exposed to toluene, naphtalene and cook inlet crude oil. Bull. Environ. Contam. Toxicol. 21(4-5):521-525.

Lebsack M.E., A.D. Anderson, G.M. Degraeve, and H.L. Bergman. 1981.
Comparison of bacterial luminescence and fish bioassay results for fossil-fuel process waters and phenolic constituents. p. 348-356. In : Aquatic toxicology and hazard assessment. Branson D.R. and K.L. Dickson (Eds). American Society for Testing and Materials, Philadelphia. STP 737. 466 p.

Nealson K.H. 1979.
Alternative strategies of symbiosis of marine luminous fishes harboring light-emitting bacteria. Trends Biochem. Sci. 4:105-109.

Nealson K.H.and J.W. Hastings. 1979.
Bacterial bioluminescence its control and ecolcgical significance. Microbiol. Rev. 43(4):496-518.

Negilski D.S. 1976.
Acute toxicity of zinc, cadmium and chromium to the marine fishes, yellow-eye mullet, Aldrichetta fosteri (C & V) and small-mouthed hardyhead, Atherinasoma microstoma whitley. Aust. J. Mar. Freshwater Res. 27:137-149.

O'Hara J. 1973.
Cadmium uptake by fiddler crabs exposed to temperature and salinity stress. J. Fish. Res. Bd Can. 30(6):846-848.

Overnell J.1976.
Inhibition of marine algal photosynthesis by heavy metals. Mar. Biol. 38:335-342.

Price R.K.J. and R.F. Uglow. 1975.
Some effects of certain metals on development and mortality within the moult cycle of Crangon crangon (L.). Mar. Environ. Res. 2(4):287-299.

Qureshi A.A., K.W. Flood, S.R. Thompson, S.M. Janhurst, C.S. Inniss, and D.A. Rokosh. 1982.
Comparison of a luminescent bacterial test with other bioassays for determining toxicity of pure compounds and complex effluents. p. 179-195. In : Aquatic toxicology and hazard assessment. Pearson J.G., R.B. Foster, and W.E. Bishop (Eds). American Society for Testing and Materials, Philadelphia. STP 766. 396 p.

Reish D.J. 1978.
The effects of heavy metals on polychaetous annelids. Rev. int. Océanogr. méd. 49(3):99-104.

Vasseur P., J.F. Férard, C. Rast, and G. Larbaigt. 1983.
Luminescent marine bacteria in ecotoxicity screening tests of complex effluents. In : Proc. 1st symp. toxicity testing using bacteria. May 17-19 1983, Burlington, Ontario, Canada. (in press).