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Bacterial Bioluminescence in Marine Pollution Assessment

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ABSTRACT

Warm water marine luminous bacterial species, particularly *Vibrio harveyi*, *V. fischeri* and *Photobacterium leiognathi*, are easy to isolate, maintain and handle in the laboratories without strict temperature requirements, which is an important advantage in the tropics. With evaluation of their response to toxicants as the main objective, detailed studies on their physiology and optimisation of culture conditions were carried out. Quantitative output of luminescence from many strains of these species was monitored upon additions of micro and nanomolar concentrations of numerous toxic (heavy metals, pesticides, phenolic compounds, amines, aldehydes, organic solvents, commercial detergents, crude petroleum extracts) and nontoxic (essential salts, sugars, amino acids) substances. A very short time (300 sec) bioassay method was devised and on the basis of these and a few other studies, it was discernible that live cells of these luminous prokaryotes are helpful in reliable detection of even the picomolar concentrations of toxic materials. Results of this study highlight the possibility of employing these bacteria in a quick prescreening of the marine environment in the pollution monitoring programmes and other advantages with them.

INTRODUCTION

Bioluminescence, the phenomenon of light emission by living organisms, is widespread in the marine environment in all the trophic levels from bacteria to fish and is thought to play a crucial role in the general ecology of marine plants and animals [17]. Being an ecologically versatile group, luminous bacteria have continued to interest microbial ecologists. This is for the reason that they offer extensive possibilities to investigate problems in wide-ranging fields, such as medicine, ecology and marine biology [28,33]. Basically, the methods of measuring their luminescence — which is extremely sensitive — are simple, rapid, and provide instantaneous, continuous and precise information concerning the status of the system being studied [31]. A feature of particular importance here is that

the investigators actually observe the activity level of luminescent system without going into other details of cellular functions. This has possibly provided a stimulus for employing bioluminescence in an unabated research activity in wide ranging fields, such as medical sciences and molecular biology. The continuing quest for sensitive and nonradioactive methodologies has also contributed to the expanding repertoire of bioluminescent assays [7] for various situations. Live luminous bacteria offer themselves as a unique experimental system for studying various conditions [9]. More importantly, determinations *in vivo* can be made with ease and accuracy without disturbing other systems in these bacteria [30]. Ulitzur and associates [1,32] have shown that the genotoxic agents can be detected at ng.ml^{-1} level using intact and highly luminescent cells.

Stebbing [29] has reviewed various short-term biological assays that have been employed to screen for the presence of hazardous materials in the environmental samples. Problems encountered in these bioassays include the maintenance of animals or cell lines and cultures, technically complex procedures, longer assay times (usually in hours), expensive materials and, above all, subjective data analysis. These are certainly contrary to the known advantages of bioluminescence assays.

Knowing that bacterial bioluminescence is very sensitive to toxic materials, several investigations have tested its application for determining the toxicity of several chemicals [5,6,23,24,26]. In most of these studies, psychrotrophic luminous bacterium, *Photobacterium phosphoreum* has been employed for these assays [4,8,28]. This bacterium has a temperature optimum around 20°C and often is not suitable for handling in the tropics. Hence, it was felt necessary to make a detailed comparison of the effects of different chemicals on the luminescence of the commonly occurring tropical luminous species. Another aim of the study was to evaluate the effectiveness of a short-term bioassay for the detection of toxicity and adapt this information for marine pollution assessment in tropical seas; on the basis of these studies, we propose the use of bioluminescence in the programmes of pollution prescreening and monitoring. Since studies on the physiology of luminous bacteria isolated from warmer waters is fragmentary [35], we also aimed at comparing the growth and luminescence, particularly of *Vibrio harveyi*, *V. fischeri* and *Photobacterium leiognathi*.

MATERIALS AND METHODS

Ecology and biology of luminous bacterial species collected from various habitats in the Arabian Sea were studied. The results obtained are reported elsewhere [19,21]. The effects of different regimes of NaCl concentration, pH and temperature on the growth of many strains of these species were studied by monitoring the absorbance at 600 nm in a spectrophotometer. A complex liquid medium suggested by Baumann and Baumann [2] was used in these studies. Luminescence output (light units) and growth of a few strains under both static

and shake methods were monitored from the broth cultures grown in a complex medium at optimal pH and temperatures. Peak emission wavelengths of a few strains were also determined in a nano-second fluorescence spectrophotometer (Applied Physics, USA).

Bacterial bioluminescence assay for toxicity was done in a similar way as reported by Bulich *et al.* [6]. Fresh cultures of bacteria grown on SWC agar [22] were harvested and suspended in sterilized seawater and mixed well to attain homogeneity. Bioluminescence determinations were made with the luminometer (Turner Designs, USA) at 5, 10, 15, 30 and 40 min after the addition of chemicals to the bacterial suspensions held in a vial inside the luminometer chamber. All assays were performed at room temperature ($26 \pm 2^\circ\text{C}$). Bacterial suspensions were periodically monitored for their luminescence to ensure that these suspensions yielded substantial units of bioluminescence (>3000 units) during the prolonged periods of analyses.

Initially, known concentrations of various chemicals were checked for their effects on the light output by different strains of *Vibrio harveyi*, *V. fischeri* and *P. leiognathi* to 50% level or below. Of a total of 53 chemicals tested, as many as 34 reduced the luminescence in all these species within 300 sec. A series of toxicant solutions of lower concentrations were prepared and tested for reduction in luminescence. EC_{50} values (i.e. concentrations of a toxic chemical which reduce the luminescence to 50%) of all these toxic chemicals were determined by plotting the bioluminescence readings at the end of the assay period (usually 5 min, though there were assays for longer durations).

RESULTS

The growth of different strains of all the four species of luminous bacteria under different culture conditions is depicted in Fig.1. Growth of all strains was maximum around 30°C (except for *P. phosphoreum*), 3% NaCl concentration and at pH 7. However, in terms of cell numbers (1 unit of $\text{OD}_{600} = 9.98 \times 10^9 \cdot \text{ml}^{-1}$ medium) the strains of *P. phosphoreum* showed the least growth and their maximum growth was about less than half of that of all other species. None of the *P. phosphoreum* strains grew at 30°C or above indicating their true psychrophilic nature. On the other hand, growth of most strains of the other three species at 15°C or below was poor or none. A wide tolerance for changes in pH and NaCl concentrations was discernible. None of the strains grew in the absence of NaCl, pH below 3 and at temperatures above 40°C . Slight growth of a few *V. harveyi* strains was noticeable at 37°C .

Shake culture method was found to enhance the growth substantially. This method yielded 1.5-2 times the cell numbers recorded in the static cultures (Fig.2). Production of light was greatly enhanced by shake culture, which was seen to yield light units of over one order of magnitude and maintain higher levels at the end of 3 days (Fig.3). Peak emission wavelengths (nm) and relative intensities of

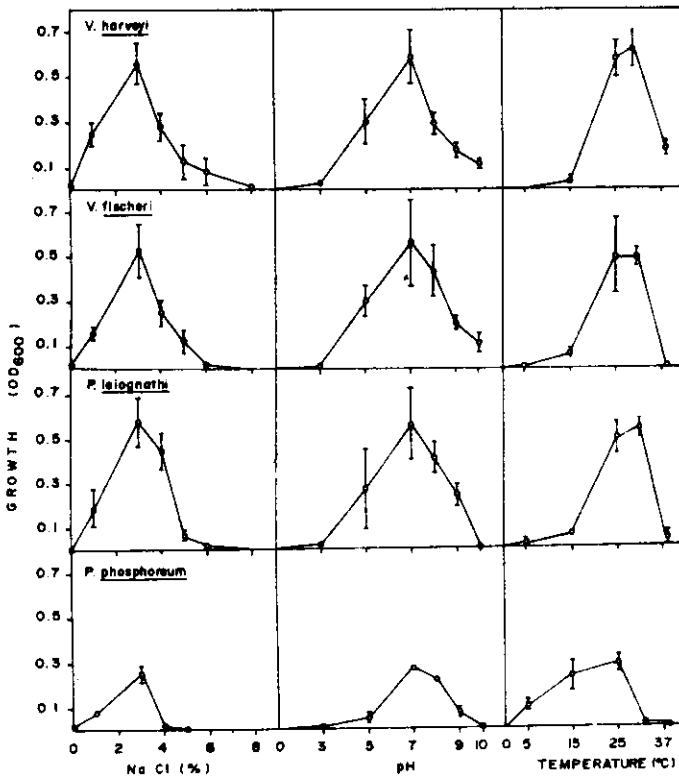


Fig. 1 — Growth of different species of luminous bacteria under various cultural conditions

bioluminescence of 20 different strains are shown in Fig.4. These wavelengths varied from 466 to 474 nm for the strains of *Vibrio harveyi*, which generally had higher intensities of luminescence. For *V. fischeri*, it was in the ranges 464-470 and 465-470 nm. It is seen from Fig.4 that the relative intensity varied widely (a mean of 82% for *V. harveyi*, 70% for *V. fischeri* and 72% for *P. leiognathi*).

The EC₅₀ value of different heavy metals to *V. harveyi* appeared to be in the range 0.16 - 6.33 ppm. These limits were similar if not identical (Table 1) in the case of *V. fischeri* (0.17 - 5.15 ppm) and *P. leiognathi* (0.19 - 9.29 ppm). Mercuric salts appeared to be most toxic. Generally, similar concentrations of pesticides and phenolic compounds (Table 2), organic solvents (Table 3) and various other chemicals (Table 4) were observed to cause 50% reduction in bioluminescence in all three species.

Essential salts, amino acids and sugars increased the bioluminescence output substantially (Fig.5). Subsequent to such additions, higher levels of light persisted

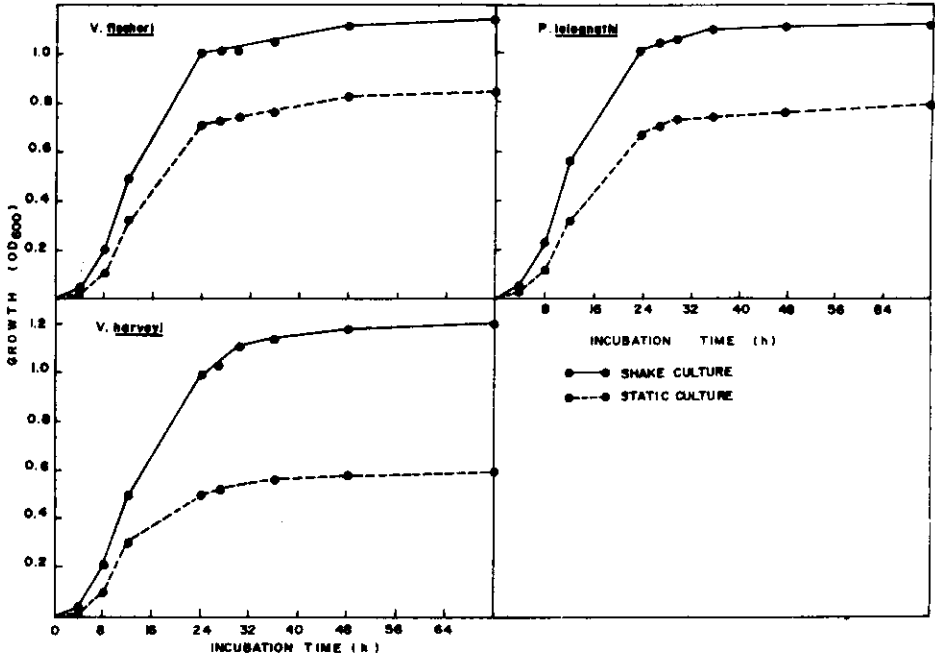


Fig.2 — Growth of *Vibrio harveyi*, *Vibrio fischeri* and *Photobacterium leiognathi* under static and shake cultures

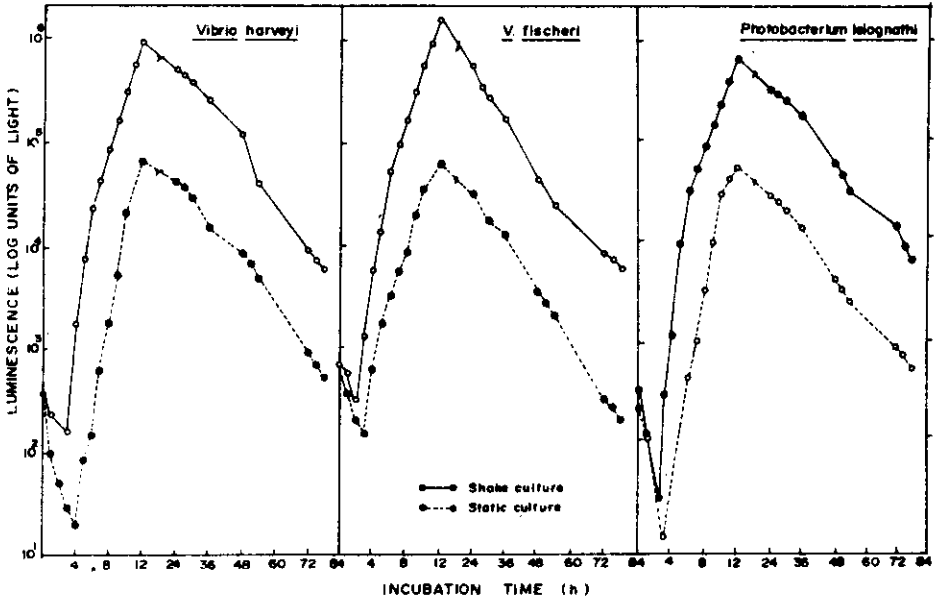


Fig.3 — Relative luminescence (log units of light) of different species of luminous bacteria

Table 1 — EC₅₀ (ppm) for Heavy Metal Salts at 5 min for Different Species of Luminous Bacteria

Salt	<i>Vibrio harveyi</i>	<i>Vibrio fischeri</i>	<i>Photobacterium leiognathi</i>
Mercuric chloride	0.16	0.17	0.19
Mercurous nitrate	0.27	0.27	0.27
Copper sulphate	0.37	0.38	0.31
Silver nitrate	0.57	0.69	0.66
Zinc chloride	1.80	1.62	1.60
Lead acetate	1.50	2.01	3.63
Cadmium chloride	4.26	3.91	3.56
Cobaltus chloride	6.33	5.15	9.29

Table 2 — EC₅₀ (ppm) for Pesticides and Phenolic Compounds at 5 min for Different Species of Luminous Bacteria

Chemical	<i>Vibrio harveyi</i>	<i>Vibrio fischeri</i>	<i>Photobacterium leiognathi</i>
DDT	0.47	0.55	0.51
DDD	4.60	4.33	4.54
Lindane	2.60	—	—
BHC	2.80	—	—
PPDDE	0.95	1.07	0.85
Phenol	3.22	2.20	1.84
Dichlorophenol	1.06	1.03	1.21
Dichlorophenol acetic acid	3.40	3.71	4.22
*Not tested			

Table 3 — EC₅₀ (% v/v) for Organic Solvents at 5 min for Different Species of Luminous Bacteria

Solvent	<i>Vibrio harveyi</i>	<i>Vibrio fischeri</i>	<i>Photobacterium leiognathi</i>
Acetone	1.52	1.27	1.41
Hexane	2.83	2.00	2.60
Ethyl alcohol	3.24	3.66	3.96
Decanol	5.34	6.46	4.55
Methyl alcohol	2.48	2.02	2.26

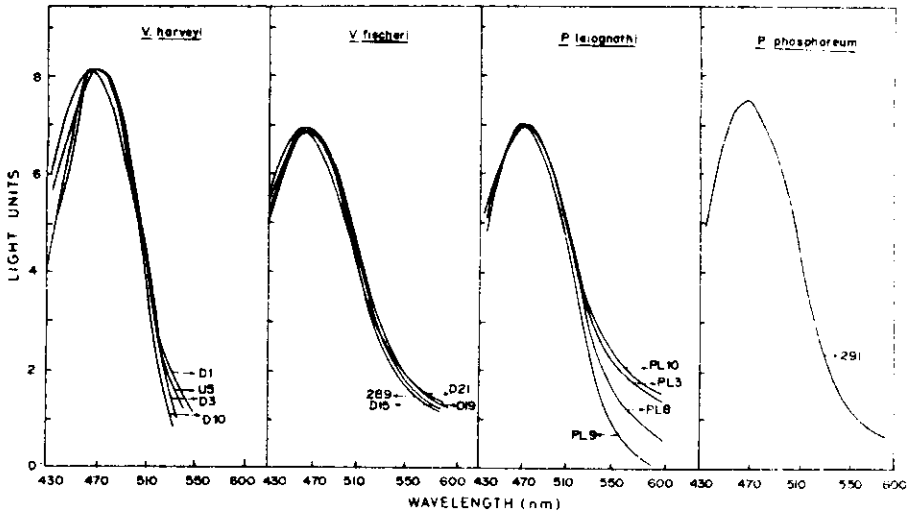


Fig.4 — Peak emission wavelengths of various strains of *Vibrio harveyi*, *Vibrio fischeri*, *Photobacterium leiognathi* and *P. phosphoreum* as measured with a nano second fluorescence spectrophotometer at the Regional Sophisticated Instrumentation Centre, Bombay

Table 4 — EC₅₀ for Antibiotics and Various Other Chemicals at 5 min for Different Species of Luminous Bacteria

Chemical	<i>Vibrio harveyi</i>	<i>Vibrio fischeri</i>	<i>Photobacterium leiognathi</i>
Chloramphenicol ($\mu\text{g}.\text{l}^{-1}$)	23.59	27.00	18.93
Penicillin ($\mu\text{g}.\text{l}^{-1}$)	34.51	30.00	43.27
Streptomycin ($\mu\text{g}.\text{l}^{-1}$)	36.41	151.00	142.74
Nalidixic acid ($\mu\text{g}.\text{l}^{-1}$)	22.87	20.40	20.74
α -Naphthalamine (ppm)	5.94	6.52	8.12
Diaminonaphthalamine (ppm)	3.35	4.10	3.25
Ammonium pyrrolidine dithiocarbamate (ppm)	0.20	0.25	0.20
Tritriplex (ppm)	4.15	3.35	3.55
Sodium arsenate (ppm)	12.93	5.17	6.67
Arsenous oxide (ppm)	11.09	13.70	12.39
Sodium hydroxide (%w/v)	1.04	1.25	1.41
Potassium hydroxide (%w/v)	1.00	1.14	1.05
Formaldehyde (%v/v)	0.20	0.20	0.20

for prolonged periods of 30-50 min. Very low concentrations of ethyl alcohol (0.1% v/v), DDD (0.5 ppm and below) and aqueous extracts of crude petroleum did not affect the light adversely. For that matter, addition of distilled water or aged seawater did not affect light output in any way.

The effect of arginine on bioluminescence was checked in greater detail. About 50 μ l of arginine at a concentration of 0.01M was added several times, one dose each at the end of every 300 sec, to 50 μ l of *V. harveyi* cell suspension in a

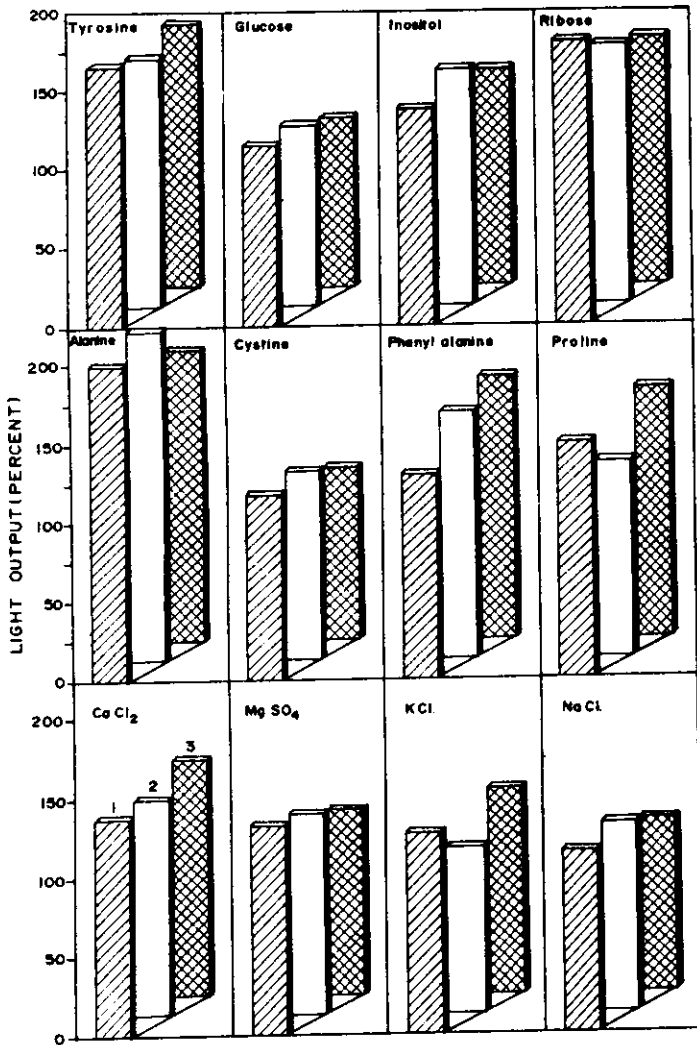


Fig. 5 — Effect of different salts, amino acids and sugars on the light output of 1) *Vibrio harveyi*, 2) *Vibrio fischeri* and 3) *Photobacterium leiognathi*

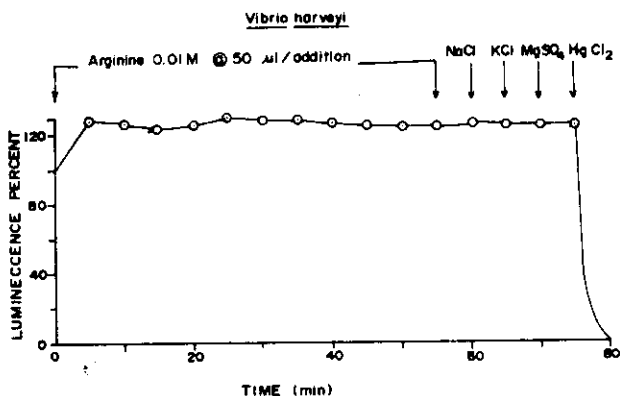


Fig.6 — Effect of arginine on the enhancement and maintenance of luminescence in *Vibrio harveyi* [Note the reduction of light soon after mercuric salt is added]

luminometer vial and the luminescence recorded. By adding arginine, it was possible to increase and maintain the luminescence to around 120% for a prolonged period (Fig.6). It is important to note that such repeated additions decreased the salinity to as low as 2.18 ppt. Towards the end of this exercise, additions of 50 µl each of 10 µM NaCl, KCl, CaCl₂ and MgSO₄ did not affect the light levels. However, additions of 25 µl of 100 nM solution of HgCl₂ to the same vial reduced the light drastically to less than 40% within 30 sec. This experiment clearly signifies the sensitivity of bioluminescence to the toxicants even in the presence of a stimulating (e.g. arginine) agent(s).

DISCUSSION

One major advantage of bacterial bioluminescence over other currently used toxicity assays is that the instrumentation reduces the chance of human error. In bioluminescence assays, problems inherent in many of the cell systems in attempting to determine the extent of cellular alteration do not arise. The data output being the function of the instrument used, pipetting is the principal source of laboratory error which can be eliminated easily by paying little more attention. For absolute values of toxicity, the monitoring time after adding the chemical solution to the bacterial suspension is critical. Hence, short-term assays adopted during this study certainly assure the lower limits of toxicity. Apart from being extremely suitable and far more advantageous, growing and handling these non-pathogens (to humans) is fairly simple and the live bacterial cells can be handled almost as chemical reagents.

The physiological properties of luminous bacterial strains recorded during this study, though similar to the ones reported by many workers [10,34 and references

therein], indicated that all the tested strains, but for those of *P. phosphoreum*, are well suited for maintenance and handling in tropical conditions. Of particular importance for the aims and objectives of the application of bioluminescence in marine pollution monitoring is the fact that prolonged availability of substantial levels of light for over a week from all the 3 species. It is reported that accumulation of secondary metabolites in the medium represses bioluminescence [16]. Indeed, in our earlier studies, we collected strains from environs of the Arabian Sea that can luminesce on agar medium for over 15-30 days [20,21]. We have selected such strains and used them in this study for assessing the toxicity or nontoxicity of various chemicals. As far as the sensitivity to different toxicants is concerned, among different species of bacteria tested in this study, the extent of variation was, for most cases, within 10%.

In the present study, the light output of *V. harveyi*, *V. fisheri* and *P. leiognathi* has been studied to evaluate EC_{50} of various toxicants, most of which reach the marine environment through one or the other source. Information on the EC_{50} for different toxicants can very well serve to closely monitor and decide upon the disposal of wastes after treating the effluent in question. The speed of response of bacterial luminescence to the action of toxicants brings close to reality the prospect of online assays in the handling of industrial wastes.

Many currently employed short-term or long-term bioassays in marine pollution assessment studies have been found to be inadequate and unreliable owing to the adaptive responses shown by many assay organisms [12,27], whereas such problems are avoided by the bacteria since the response in terms of light output in the cell suspensions are recorded for a very short time.

The protocol we propose for pollution monitoring programmes in the tropical seas is outlined in Fig.7. Any sample (e.g. seawater, sediment) suspected to be originating from the polluted region or any industrial effluent at various stages of treatment can be subjected to quick prescreening following a few simple steps. Small amounts (25 or 50 μ l) of these samples are carefully transferred to a luminometer vial containing 25 or 50 μ l luminous bacterial suspension and the response in terms of light output is measured in a luminometer. Once this step is completed, measures required for dilution of effluent can be worked out or any other measure can be decided upon to reduce the toxic effect of the effluent. In several earlier studies environmental samples have been evaluated for pollution, based on the principle adopted in this study [13,25,26,31]. Although it would be difficult to pinpoint the source and type of pollutants, which is also the case with other bioassays, bioluminescence test would certainly help in expeditious recognition of the presence of toxicants in the marine environment. Great attention needs to be paid to effluents with high toxicity, those reducing the light to less than 20% within 5 min. Since this bioluminescence assay allows us to determine the dilution factors required (software programmes on personal computers would aid in arriving accurately at such factors), decision-making becomes easier. Further, as the order of toxicity determined by bacterial bioluminescence parallels that

reported for mammalian cells [15] or, many times, is far more sensitive [11,14,18], it would be best to attain the dilutions that allow at least 80% bioluminescence at the time of discharge. Larger dilutions and dispersals in the marine environment would certainly take care of such low levels of toxicity.

ON-LINE BACTERIAL BIOLUMINESCENCE ASSAY

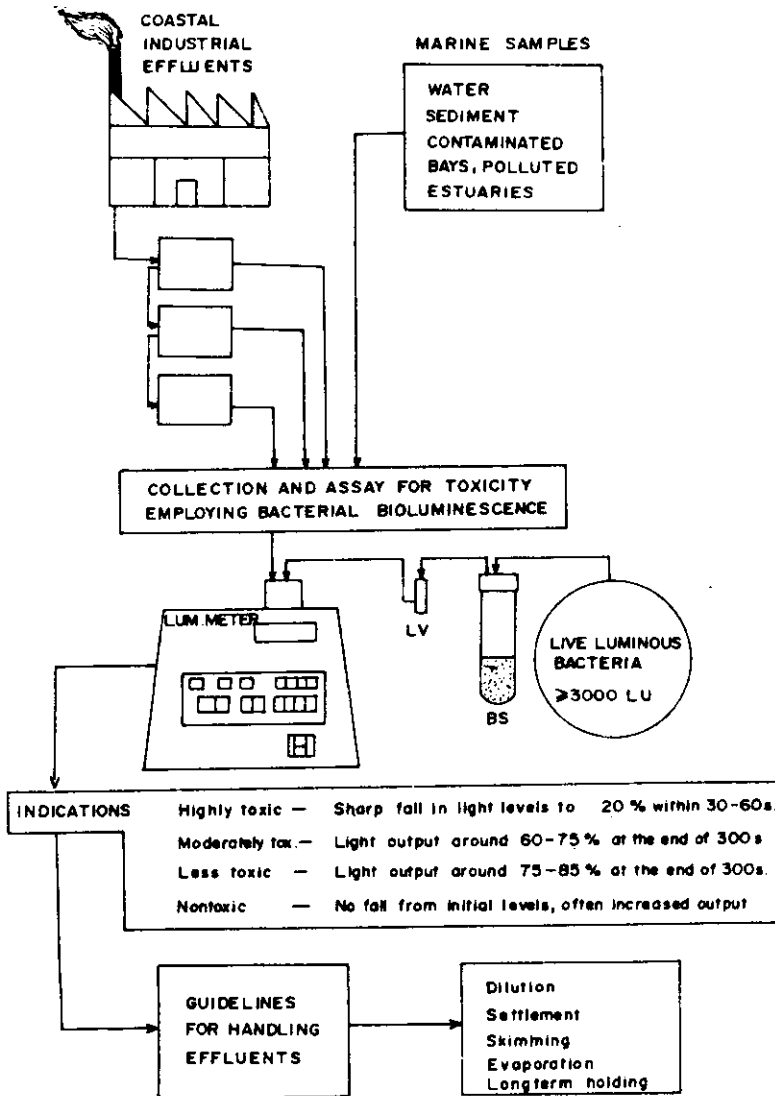


Fig.7 — Protocol of bacterial luminescence bioassay for prescreening for pollution in the marine environment [BS = bacterial suspension, LV= luminometer vial, LU= light units]

The fact that bioluminescent bacteria are of marine origin was thought to have limited relevance in many industrial settings. However, the successful transfer of *lux* genes from *V. fischeri* into *Escherichia coli* by Belas *et al.* [3] widened the application of bioluminescence in the detection of environmentally toxic agents for many terrestrial and freshwater systems [14]. The simple expedient of transferring these genes into the normally dark terrestrial microorganisms which are either particularly sensitive to specific toxicants or specifically relevant to an industrial process has been particularly useful. Although we have not carried out any gene transfer studies, the freedom to exploit luminous bacteria and their luminescence as the reliable biosensors for detecting the presence of extremely low levels of toxicants in the environment and industrial discharges is a particularly exciting and practical prospect.

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