

Applications of Luminous Bacteria on Environmental Monitoring

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Preliminary communication

Received: July 20, 2001

Accepted: March 19, 2002

The present study belongs to the field of “environmental monitoring” and specifically refers to the natural biomarkers that are normally present in the seawater ecosystem: the luminous bacteria.

The application of traditional macro-biomarkers (fishes, invertebrates; e.g.: mussel watching) represents an important integration to the classic laboratory methodologies, above all for the high sensitivity that can be reached. Unfortunately, these methods have some drawbacks due to the need of placement of the specimens in situ, the waiting time and the final processing with quite complicated analysis procedure.

The luminous bacteria (e.g. *Vibrio fischeri*) and the correlated detection technologies, give a new promising method for assessing the water pollution, because they are:

- easy to cultivate and preserve;
- easy to be detected (the nowadays available luminosity detectors are highly sensitive);
- endemic in sea or salty waterbodies;
- applicable also to non salty waterbodies.

Progresses can be made on the automatic devices that can handle and automatize their use; this paper illustrates a prototype that may work on field in near-real-time, opening the way to future applications of along-the-path mapping and multitemporal comparison.

Key words:

Bioluminescence; biomarkers; chemoluminescence; environmental and automatic monitoring; luminous bacteria; *Vibrio fischeri*.

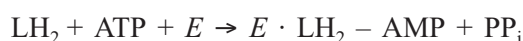
Introduction

The present study deals with the water monitoring made with the natural micro-biomarkers that are normally endemic in sea-water ecosystems: the luminous bacteria.

The chemoluminescence or bioluminescence is an aerobic bio-oxidation process alternative to normal bacterial respiration, realized by some species of *Epi* or *Vibrio* bacteria (known as luminous bacteria; e.g.: *Pseudomonas fluorescens*, *Vibrio fischeri*).¹

The luminous bacteria produce a specific substance, the *auto-inductor*, which, during their growth and multiplication is accumulated and, if above a critical level, induces the enzyme *luciferase* to generate the luminous emission.²

The reactions that bring to luminescence are the following:



In nature and, in particular, in the seawater ecosystem, bacteria appear luminous if a sufficient

quantity of *auto-inductor* accumulates; therefore, the luminescence appears only in favourable conditions for the development of high density of population.³

This phenomenon can be found in the photogenic organum of the invertebrate *Pholas dactylus* and in some species of fishes as the *Photinus pyralis* or the *Chauliodus*; in this case luminous bacteria find an optimal micro-environment to develop, multiply and produce the luminescence.⁴

Luminous bacteria (like other bacteria) represent a useful indicator for the biological description of sea-water status and they belong to the biomarkers category, that corresponds to “sentinel” organisms useful to assess the pollution level in close relationship with the induction or the suppression of some essential enzymatic activities or other relevant biochemical modifications.⁵

The emitted luminescence is a function of the bacteria total number present in the sample and the specific single bacterium luminescence (proportional to its vitality/ metabolism), according to the following relationship:

$$\begin{aligned} \text{Total Sample Luminescence} &= \\ &= \text{BTN} \times \sum_i^{\text{BTN}} [\text{Specific Luminescence}]_i \end{aligned}$$

Where BTN is the Bacteria Total Number

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Their superiority with respect to other non luminous bacterial species for the automatic monitoring lies in the fact, that they can be automatically detected by means of photoelectronic amplifying devices, and that the level of sensitivity is linked to the luminosity variation that is far above the survival threshold (traditional methods using bacteria deal only with surviving bacterial colonies count through microscopical observation).

Several studies and experiments led to the following conclusions:

- high luminous bacteria concentrations are normally correlated to high nutrient and organic concentration, in standard environmental conditions;⁶

- toxic substances in small quantities can inhibit the biochemical process of light emission, therefore their effect may be detected with high sensitivity by means of residual luminous bacteria.⁶

The luminous bacteria concentration and the relevant luminosity factor lead to the introduction of an “indicator” that expresses the deviation from standard conditions.⁷

The present study deals with the design, implementation and test of an automatic continuous monitoring system to assess the presence of this “indicator” in seawater.

Material and methods

The laboratory scale experimental hardware, named A002, a prototype of a multifunctional instrument for bioluminescence measurements, that can be configured in different modes, is schematically shown in Figure 1.

A central processing unit drives the hydraulic components (pumps, electrovalves), controlling the flows and the measurements; an amplified photomultiplier is utilized as luminescence detector in a measurement cell.

The automation of the equipment is also intended to reduce the measurement sampling periods; the objective is to have a nearly real-time equipment; sampling times may be shortened with respect to the traditional laboratory equipment test times.

The built-in configuration flexibility allows to implement two main modes of operation, illustrated in the following paragraphs:

- Activated Bacteria Sample Exposure (ABSE) mode;
- Integrated Natural Bacteria (INB) mode.

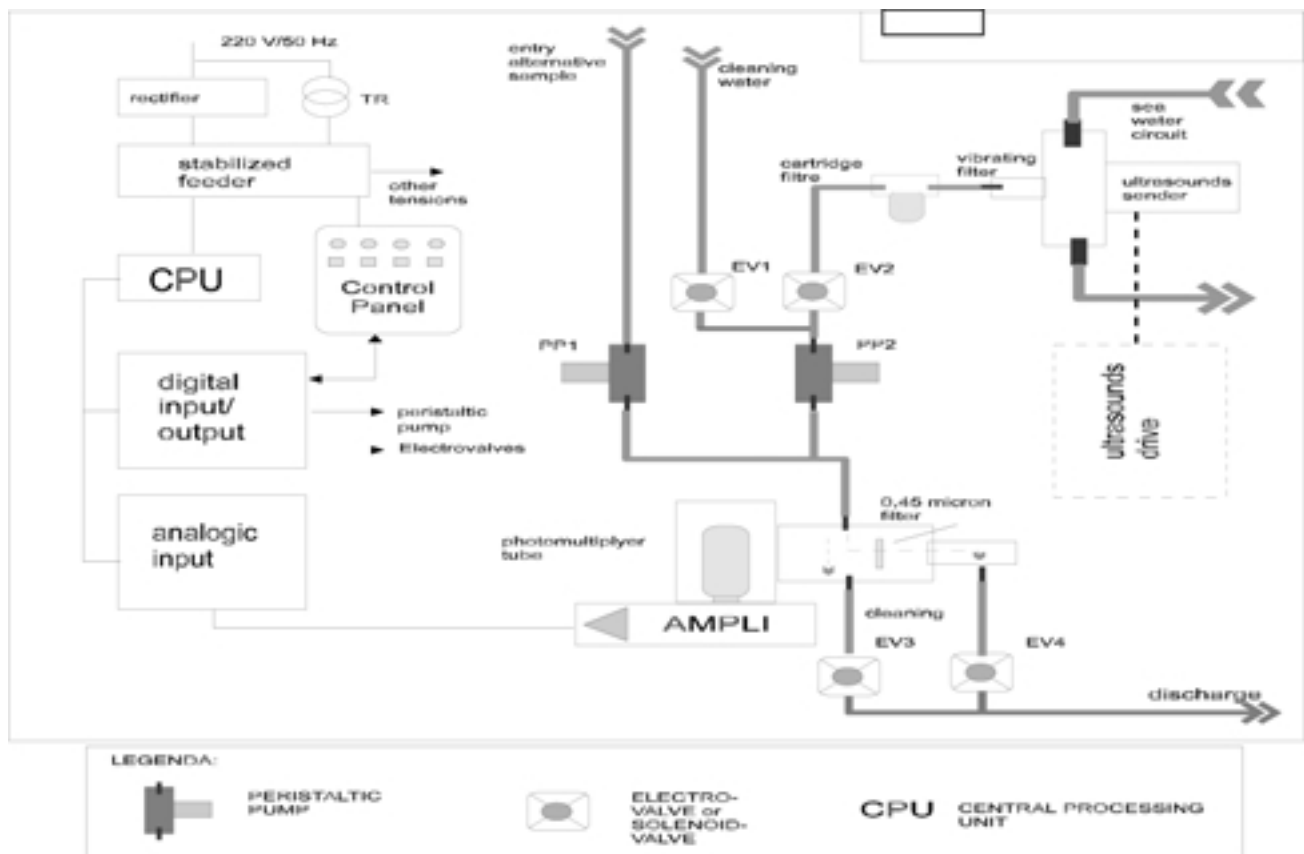


Fig. 1 – General functional scheme of the multifunctional bioluminescence measurement apparatus A002.

Activated Bacteria Sample Exposure (ABSE) mode

In the ABSE mode the bacteria are preliminary prepared in significant concentrations and preserved in a liquid solution that is stored at 3 °C; when a measure is needed, they are transferred to another recipient kept at higher temperature (32 °C), where a nutrient solution is added; a quick oxygenation is useful to reach high sensitivity performances. This part of the process was manually implemented but can be easily automatized.

The activated bacteria are introduced in the measurement cell (through the peristaltic pump PP1) and a first luminosity measurement is made (blank luminosity value measurement = V_b).

The sample (any kind of liquid matrix, not only seawater), filtered through a coarse and fine filter is also introduced into the cell, through EV2 and PP2 pump.

The mixing effect may result in a luminosity variation, positive or negative in sign, depending on the prevailing conditions of the sample (nutrient, neutral or toxic substances); a second measurement gives a luminosity value with sample exposure: V_{se} ; the variation is: $V_r = V_{se} - V_b$ and represents the effect of the exposed sample on the bacterial solution, inhibiting or increasing the relevant luminosity.

From that aspect the instrument is not very different from the existing and known Microtox-like commercial instrumentation, the only practical difference being that it can be supplied with a longer lasting reserve of bacteria (several days if refrigerated), that can be activated with nutrients, and automatically transferred in small quantities to the test cell only when needed.

Integrated Natural Bacteria (INB) mode

In seawater one can find a variety of mineral, chemical and living matter in terms of:

- macro-components: such as plancton, all kind of macro-organisms and floating inert substances;
- micro-components: dissolved chemical elements (among which pollutants), suspended mineral particles, bacteria, protozoa.

The luminescent bacteria are endemic in natural seawater but their luminosity is not easy to detect even with modern light amplifiers because the signal-to-noise ratio is very low, due to the masking factor of all non-bacterial components; accumulation is needed because they must reach a certain concentration above the auto-induction threshold; moreover, the seawater may contain some macro-organisms that exhibit photo-emitting properties

and whose presence cannot be correlated to our purposes.

In the INB mode the instrument has been utilized to integrate (or accumulate) a significant biomass of luminescent bacteria, and to separate it from all other components of seawater; the longer the integration time the higher the sensitivity, but, in the other hand, the real-time performance may fall below the expectations.

The bacterial accumulation has been obtained through a *selective micro-filtering* process; this process implies that seawater passes sequentially through different filtering membranes with micro-pore dimensions sequentially decreasing (50 μm , 5 μm , 0.45 μm).

Obviously, the first filtering step that should reject the coarse components (over 50 μm : macro-components and plancton) is the most difficult, because the rejected matter quickly obstructs the filter pores and should be frequently removed. This has been implemented utilizing a metallic, microperforated membrane that is periodically cleaned by means of ultrasonic induction. The main flow of sampled water is pumped parallel to the filter surface to improve the removal of the deposited matter.

After that, it is necessary to separate the higher dimension microorganisms and particles from the small sized bacteria, in particular *Vibrio fischeri* that is smaller than 5 μm ; therefore a second intermediate filter has been used.

The third and last filter allow to trap *Vibrio fischeri*, as well as other bacteria and particles of comparable size; therefore the luminescence detector is in a better signal-to-noise condition, because the ratio of luminescent bacteria vs. other components is much greater than in the original seawater condition.

It is worthwhile to remark that all non-luminescent bacterial organisms are filtered before the second step.

In other words, this multi-step filtration process allows the selective increase of bacterial population density vs. the particles and microorganisms of greater dimensions.

The membrane with porosity of 50 μm is embodied in an ultrasonically-cleaned filter periodically and frequently (even continuously) activated, without stopping the process.

The second membrane is composed by a pass-through filter that can periodically be self-cleaned in a cleaning off-line cycle (a “vortex” type cartridge filter was utilized).

The third one (0.45 μm), can be cleaned off-line with a flow of pure water parallel to its surface and has to be periodically substituted.

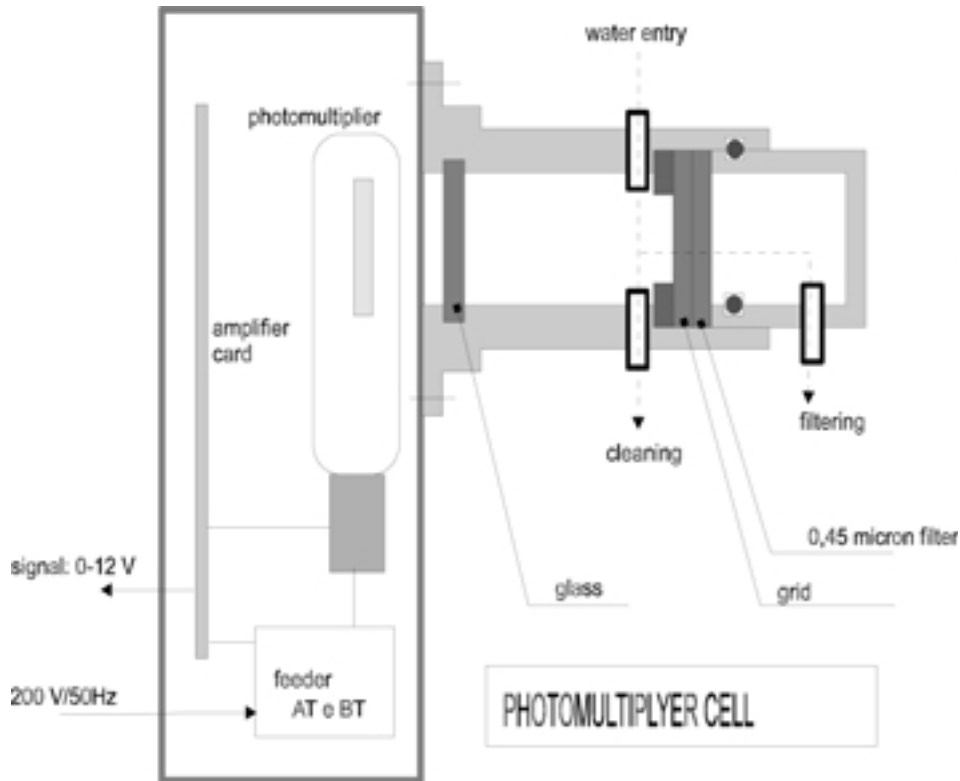


Fig. 2 – Scheme of the measurement cell

The operation is discontinuous and the normal procedure is the following:

- a) collection of the water sample;
- b) filtering it for a preset period (some minutes), then stop the process;
- c) luminosity measurement;
- d) cleaning cycle.

The measurement cell (Figure 2) is constituted of a cylindrical tube, at one end of which is located a sealed transparent window that is the optical interface to the photomultiplier; the latter is contained in a small light-proof box in front of it; at the other end of the tube, a filter and an output collector, for exhaust water discharge, are inserted.

The normal water flow is from the side sample input to the filter/collector output (EV1 electrovalve Off, EV2 On, EV3 Off, EV4 On).

During the cleaning cycle, pure water is withdrawn from a tank (EV1 electrovalve On, EV2 off, EV3 On, EV4 Off), through the same input, but the output is on the opposite side of the tube, to remove the deposited matter by means of the parallel water flow.

To compensate for the possible variations of the photomultiplier sensitivity, amplifier gain, residuals of bacteria in the filter surface, after the cleaning cycle, a measure of the detector background

level (noise) can be made, and the result utilized to correct the next active measurement.

Experimental results

Laboratory and in situ measurements allowed to correlate the effects of different kind of substances on luminous bacteria that can be present in marine environment, to give a qualitative indication on these microorganisms sensitivity.⁸

Tests in ABSE mode

In the first mode of utilization of the instrument, according to the above description, it was possible to notice a luminescent answer directly proportional to the concentration and to the type (toxic or nutrients) of introduced substance (Figures 3 and 4), confirming the correct behaviour of the prototype.

This mode was briefly experimented because it is similar to what is reported in literature,⁸ concerning devices similar to Microtox. It was addressed to get in touch with the already known experiences and to demonstrate the flexibility of the instrument, in order to better focus on the second, hereafter illustrated mode.

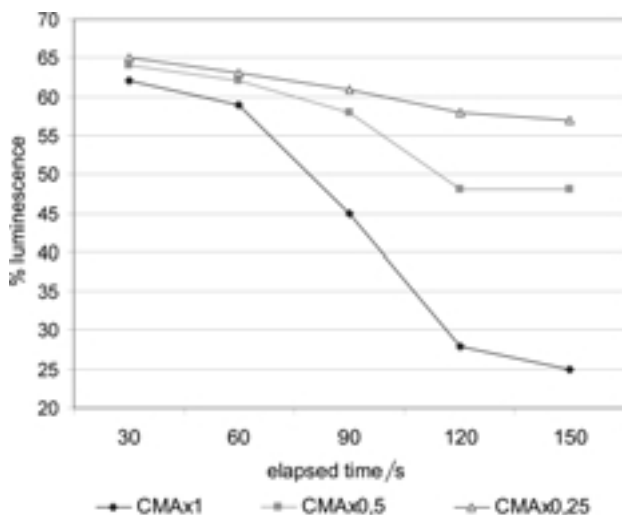


Fig. 3 – Mercury test – luminescence (percent ratio of measured to max (12V) photomultiplier voltage) vs. time for different solutions at different concentrations (elapsed time from the exposition start of sample to bacteria)

CMA = Concentration Max. Admissible = 0,005 mg l⁻¹

CMAx1	CMAx0,5	CMAx0,25	Elapsed Time (s)
7.44	7.68	7.80	30
7.08	7.44	7.56	60
5.40	6.96	7.32	90
3.36	5.76	6.96	120
3.00	5.76	6.84	150

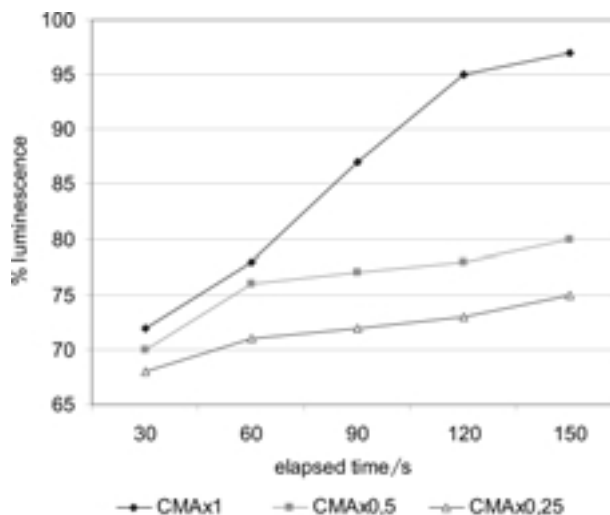


Fig. 4 – Glucose test – luminescence (percent ratio of measured to max (12V) photomultiplier voltage) vs. time for different solutions at different concentrations (elapsed time from the exposition start of sample to bacteria)

CMA = Concentration Max. Admissible= 500 mg l⁻¹

CMAx1	CMAx0,5	CMAx0,25	Elapsed Time (s)
8.64	8.40	8.16	30
9.36	9.12	8.52	60
10.44	9.24	8.64	90
11.40	9.36	8.76	120
11.64	9.60	9.00	150

INB Tests

The in-site experimentation, utilizing the INB mode, was possible during the implementation of a monitoring campaign on the French coastal zone that encompasses Nice and St. Laurent du Var.

In particular, it was monitored with the prototype instrument installed on board of a small boat in the sea area near to the coast, corresponding to some interesting spots of the coast: the outfall of the Var river (A), the Magnan beach (B, Nice coastal centre) and Nice harbour (C).

The three examined sites are of almost interest for assessing the qualitative conditions and trophic-status of seawaters: this particular area has in fact been subjected, in recent years, to several studies; the pollution increases during the turistic season, and should be kept under constant observation; in particular, the possible ecotoxicological pollution, even in minimal quantities, may give interesting clues to the local water management agencies to investigate on the sources and to keep them under control.

The results (Figure 5) compare too badly with other turistic coastal sites of the Mediterranean; however the constant surveillance with this kind of monitoring campaigns may reveal the changing trends, leading to timely interventions, if needed.

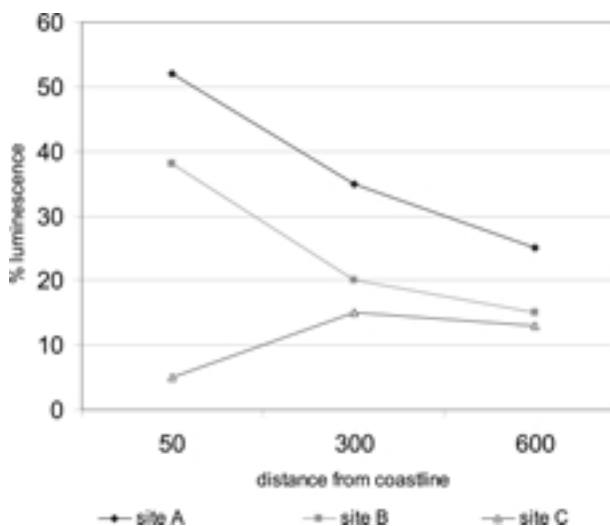


Fig. 5 – Monitoring Test – luminescence (percent ratio of measured to max (12V) photomultiplier voltage) vs. distance in meter from coastline

Site A	Site B	Site C	Distance from coastline (m)
6.24	4.56	0.60	50
4.20	2.40	1.80	300
3.00	1.80	1.56	600

Conclusions – Future projections

A prototype of this system has demonstrated the feasibility of the concept and the flexibility of the design; more improvements are needed to produce an industrial scaled level instrumentation.

Projecting it to future applications, a possible implementation can exploit its near-real-time capabilities on seawaters, conceiving an automatic instrument that could be installed on board of a patrol boat to perform monitoring campaigns; the measurements, taken periodically can be geo-referenced through a GPS (Global Positioning System), producing bacterial luminescence data base along the navigation path, radio-transmitting data to a supervision center in case of emergency, or collecting data for a post processing.

The post processing may consist of a multi temporal comparison in order to detect significant differences with respect to previous campaigns.

The use of short term, along-the-path measurements to verify abnormal variations may lead to an emergency pollution surveillance system, whilst the multitemporal comparison may represent a very useful tool to reveal the medium and long term ecological seawater status modifications.

ACKNOWLEDGEMENT

We wish to thank the French Company I.M.G. (Institut Méditerranéen de Géosciences) and in particular Dr. Roger Dèmosthène Casanova (General Manager) for the support in the work development.

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