Using bioluminescent biosensors for hazard analysis and critical control point (HACCP) in wastewater control

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Abstract Starting from a new approach for water pollution control and wastewater treatment plant management, the hazard analysis and critical control point (HACCP) quality concept, the interest for the development of new rapid and sensitive methods such as bioluminescence-based methods is evident. After an introduction of the HACCP procedure, a bibliographic study of the bioluminescence potentiality is presented and discussed.

Keywords Bioluminescence; HACCP; lux-biosensor; pollution control; wastewater

Introduction
Water-related contamination constitutes an important health problem world-wide. (McMeekin et al., 2002). A microbiological and chemical risk assessment of diseases should comprise the entire water treatment management system and take into account the hazard analysis and critical control point (HACCP) concept which provides specific critical control points (CCPs) relating to each step of a wastewater treatment plant (WWTP). The HACCP concept was initially introduced to food microbiology as a proactive, preventive system of quality control. (Hartmann, 1997). In the initial report from the United States, the National Advisory Committee on Microbiological Criteria for foods (NACMCF) endorsed HACCP as an effective and rational means of assuring food safety from harvest to consumption. Seven basic principles are employed in the development of a HACCP system (conduct a hazard analysis, identify the CCPs in the process, establish critical limits, monitoring requirements, corrective action, record keeping and procedure for verification that the HACCP is working correctly) (NACMCF, 1992). With the development of these seven principles and the international standardization of approach (Codex Alimentarius, 1993) the HACCP concept has been gaining increasing international acceptance. The regulation is being introduced in many countries (EEC, 1993; FDA, 1995) (Khandke and Mayes, 1998). In the environmental domain, the HACCP model has been applied to the management of drinking water by Havelaar (1994), Dewettinck et al. (2001), Kistemann (2001) and Casani and Knochel (2002).

In this frame, the choice of relevant measurement techniques is important. As the initial topic of the HACCP was microbiological risk monitoring, and as some of the determination methods can also be envisaged for toxicity or ecotoxicity measurement, the choice of a versatile solution for both purposes must be preferable. It is the reason why this study reviews a part of the applications of bioluminescent methods and discusses the HACCP for biological WWTP control, which could be performed in a chemical or petrochemical industry. This includes information about toxic compounds and pathogens of potential importance in the wastewater process and sludge-borne microorganisms. Furthermore, following the active or viable biomass and the chemical toxicity would allow us to control and improve the WWTP’s efficiency.
Hazard identification

Concerning the general problem of wastewater quality, a survey of several parameters, either aggregate (BOD, COD, TSS, …) or specific (nitrogen compounds, metals, organics, …) is needed. According to the directive (91/271), only the limit values of the global parameters (COD, TSS, BOD) should be respected when treated wastewater is not discharged in sensitive zones. For the sensitive zones, the concentrations of nitrogenous and phosphorous compounds are also regulated. Moreover, a list of 33 priority toxic compounds (EC, 2001) has been drawn up. Organic compounds (polycyclic aromatic hydrocarbons (PAH), organohalogenated substances, pesticides, endocrine disruptors) and metal compounds (mercury, lead, organotins) are included in this list.

Raw wastewater is moreover a potential carrier of pathogenic microorganisms and may pose a health risk in the case of aerosolisation during the wastewater treatment aeration step. (Bauer et al., 2002). The major source of pathogens in domestic wastewater is faecal material of infected individuals. The quantification of these pathogens or toxic chemical compounds in wastewater treated by various techniques are sometimes considered as the main risk. Moreover, the sludge can contain a large variety of chemical and/or biological toxins. Indeed, 10% of organisms stay in the effluent while the majority are concentrated in the sludge. (Tsai et al., 1998). Due to the epidemic risk, sludge or wastewater use to amended soils is controlled by regulation in Europe (Journal Officiel, 1998) and the USA (CFR 40 503, 1997). Estimating the microbiological risk connected to the use of sludge or the discharge of effluent from WWTP in the environment would be necessary in order to evaluate their biological hazard, which would allow us to control the desired safety level.

Identification of CCPs

Urban wastewater treatment plants with biological sludge production include pre-treatment, activated sludge aeration tank, settling tank, evacuation of treated waters and sludge treatment (digestion, drying …). The identification of CCPs is an important issue in HACCP because the major effort in process control monitoring will be directed towards these steps (Dewettinck et al., 2001). CCPs depend on many factors such as the nature of the effluent (urban, industrial, agricultural), the atmospheric circumstances (temperature, atmospheric pressure, rainfall), the type of treatment process (trickling filter, bio-filtration, wetland, activated sludge…), the sludge quality (primary sludge, mix sludge, activated sludge), the stabilisation process, the monitoring scheme (on-line measurement versus sampling and analysis). For the last point, the sampling is regulated (Directive 91/271/1991) according to the size of WWTP. It is necessary to take all these parameters into account in order to define control points.

Establishing critical limits for preventive measures associated with each identified CCP

The third principle of HACCP is to establish critical limits for control measures at the identified CCPs. In a WWTP, the process control can be carried out by indirect measurements, as for example, the redox potential or the dissolved oxygen. According to the results obtained at the different steps of the process, some actions can be taken such as changing the operating conditions (recycling rate, aeration schedule, management of sludge in excess). In this study, bioluminescence-based tests are discussed, in order to improve the pollution control and the treatment process. The detection limits remain to be defined and standardized according to the various methods used.

Monitoring by bioluminescence

When using bioluminescent-based sensors at the selected CCPs, two different functions
depending on the purpose of the measurement can be considered. If the purpose is to guarantee the WWTP control and alarm system, a large number of sensors will be necessary. This will generate a large amount of data where only the values of order and the mean values will be stored and used for the control of the WWTP. On the other hand, if the CCPs are used to ensure the management of the WWTP in real time, the measurement should be fast and only the relevant information in the diagnosis of a function action will be studied to define what action can be taken.

The normalised methods used today to determine the limit values of different parameters such as $\text{BOD}_5$ measurement, micro-pollutant analysis, and pathogen organisms determination are time-consuming and thus, do not allow us to control the risks of pollution in real time. The ideal way to use CCPs is to monitor on-line to enable corrective action to be taken by a direct feedback procedure. This requires the development of rapid methods that are adapted to meet the criteria needed at the control points. Bioluminescent-based tests using microorganisms are envisaged because of their simple operation, rapid response, and low cost. Furthermore, the bioluminescent-based tests can be used to quantify the toxicity of the toxic compound (unique or mixing) and for the detection of the pathogenic organism.

The phenomenon of bioluminescence has been observed for several different organisms (invertebrates, vertebrates, and bacteria) (Campbell, 1989; Hastings, 1986). The molecular biology of the enzymatic systems producers of light-producing organism has been object of numerous works (Meighen, 1988, 1991, 1993; Brolin and Wettermark, 1991; Kricka, 1993) where the biological and clinical applications of the bioluminescence were studied. The reactions of bioluminescence most frequently used for analytical applications are the systems employing the luciferase enzyme from marine bacterium ($\text{Vibrio fischeri}$) or firefly ($\text{Photinus pyralis}$). In the firefly–luciferase reaction, the luciferase catalyses the reaction between luciferin, ATP and oxygen, leading to the emission of light proportionally to the ATP concentration. The marine bacterial bioluminescent system catalyses a reaction between oxygen, a reduced flavin phosphate and an aldehyde (C8 to C16 straight chain). Bioluminescence-based bacterial biosensors have been particularly exploited for toxicity testing, using naturally luminescent bacteria ($\text{V. fischeri}$) such as the Microtox® test.

Steinberg et al. (1995) have reviewed the in vivo and in vitro bioluminescence methods which were used to detect a specific compound, determine acute respiratory toxicity, estimate potential genotoxicity or quantify bio-indicators such as ATP or NADH. The ATP-based methods have been used as a measure of cell proliferation and cytotoxicity because ATP plays a central role in bioenergetics, as it is a high energy compound found in all viable organisms. When the cells die, the ATP is quickly destroyed by the metabolites (ATPase) of the cells which have died, and thus, contrary to NADPH, ATP is no longer present when the cells are lysed. ATP bioluminescence cannot give an indication of the specific pathogens present, as it measures the total level of ATP in the samples. The ATP bioluminescence technique has been applied to monitor the amount of active biomass in biological wastewater by Chiu et al. (2001); Patterson et al. (1970); Roy et al. (1983); Weddle and Jenkins (1971) and Guwy et al. (1998). The method tends to be simple, fast, and inexpensive. Dalzell and Christofi (2002) have used ATP luminescence to determine the ATP content of organisms in activated sludge during contact with different toxics (3,5 DCP, Cr, Zn) and in real effluent. In a complex matrix, ATP is still not considered as a numeration of the micro-organism parameter since the production of ATP changes with the physiological state of the organisms and their growth phase (Hansen and Karl, 1978). This phenomenon has important implications since the answer is amplified when the cellular rates of ATP are used to estimate the effect of toxic matter. On the contrary, the pool of intracellular and extracellular adenylic nucleotides (AN) (ATP, AMP, ADP) is constant during growth, but the rate of intracellular NA is correlated with the microbial growth. The total nucleotides
(AMP, ADP, ATP) are quantified by their transformation into ATP, in a simple and direct way in the culture medium (Brovko et al., 1994). Thus, the quantification of the AN by the enzymatic ATP-based method can be operated. Furthermore, this alternative method increases the sensibility compared to the ATP-based method.

Nowadays, with the development of biological molecular methods, some bacteria have been transformed with luxCDABE genes (from bacteria) encoding for bioluminescence and used for both chemical toxicity and pathogen organisms detection. Typically, two types of promoters are employed, a stress promoter for the specific toxicity and a constitutive promoter to measure the global toxicity. Specific compounds activate the stress promoter and the luxCDABE genes encoding for luciferase and their substrate (flavin phosphate and an aldehyde) of enzymatic reaction which leads to the emission of light. When the emission of light is constitutive (constitutive promoter), and the cells are altered by the toxicity, the emission of light decreases. Among the bioluminescent-based methods on-line toxicity monitoring has been developed. For example, the Microtox-based tests such as ToxAlert®, LUMISmini, have been commercialised for in situ global chemical toxicity measurement.

It is the same application for the commercialised ATPtox test. The SOS-chromotest® or Mutatox® have been used for genotoxicity. Recently, Choi and Gu (2002) have used E. coli recombinant to develop a portable biosensor kit for toxicity detection. Several studies have developed a bioluminescent biosensor with lux genes (Table 1), the principal bacteria used being E. coli, P. putida and P. fluorescens. In the WWTP control, the environmental bacteria are more used because there are specific for the sludge medium and the treatment activity (e.g. nitrate reducing bacteria). Many of the bacteria present in sludge have not been isolated due to the difficulty in cultivating them in a known cultural medium.

The potential applications of bioluminescence to detect pathogen bacteria (M. tuberculosis, M. avium, M. paratuberculosis, Listeria sp, enteric bacteria) became obvious with the development by Billard and DuBow (1998) of a luciferase reporter gene. The genes encoding luciferase were introduced into the genome by a bacterial virus and the light emission was then a measure of the number of bacteria. The applications of the bioluminescence-based assays for detection and characterization of bacteria and chemicals technique have been reported by Ulitzur and Kuhn (2000). Living cells are complex systems, and the light output of a bioluminescent biosensor depends not only on the chemical complexity of the sample such as the type and quantity of inducers present, but also on the physiological state of cells at the time of measurement. In spite of these limitations, whole cell biosensors hold a great deal of promise for continuous on-line monitoring of toxic compounds in environmental applications (Keane et al., 2002). For bioluminescent applications, biosensors need to be developed for both the screening of a group of compounds (e.g. PCB) and the identification of individual compounds within a group (individual PCB) simultaneously. The goal is that biosensors should be capable of detecting all types of toxicity (chemical and biological) which may have an impact.

**Establishing corrective action to be taken when monitoring indicates that there is a deviation from an established critical limit**

After identification, the potential hazards (toxicity and pathogen agent) are grouped according to common properties, for example their sensibility to additional treatment of secondary effluent and sludge. Secondary effluent of a WWTP cannot be reused directly, in general, due to its insufficient quality, which requires additional treatment (e.g. sand filtration, activated carbon adsorption, ozone/hydrogen peroxide treatment (advanced oxidation process (AOP)), microfiltration (MF), disinfection or reverse osmosis (RO)). For the sludge, the main processes of decontamination used are: incineration, aerobic digestion, drying process, liming (alkaline treatment), and thermal conditioning. Some other treat-
ments such as composting, pasteurisation, AOP can be envisaged as treatment processes for sludge. Generally, the action of inactivation and the stabilization processes vary depending on the type of microorganism. For example, the enteric viruses can multiply in the environment and are sensitive to treatment. Among bacteria, the faecal Streptococcus is more resistant than E. coli and than Salmonella. Ooysts of helminths cannot multiply in the environment but are very resistant to the treatment processes.

After the establishment of corrective action, the approved HACCP plan and associated records must be on file at the environmental establishment. The major infusion of science in a HACCP system focuses on the proper identification of pollution, critical control point, critical limits, and instituting proper verification procedures (NACMCF, 1992).

Table 1  Microorganisms genetically modified for the detection of environmental pollution

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Toxic compounds</th>
<th>Source of reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. eutrophus</td>
<td>chr-::luxCDABE pEBZ</td>
<td>Chromate</td>
<td>Peitzsch et al. (1998)</td>
</tr>
<tr>
<td>Burkholderia, P. fluorescens</td>
<td>Lux genes</td>
<td>Phenols</td>
<td>Boyd et al. (2001)</td>
</tr>
<tr>
<td>E. coli</td>
<td>precALuxCDABE a</td>
<td>Mitomycin C; irradiation Phenols; chloroform</td>
<td>Vollmer et al. (1997); Davidov et al. (2000)</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>flicC::luxAB</td>
<td>Aluminium</td>
<td>Guzzo et al. (1992)</td>
</tr>
<tr>
<td>E. coli</td>
<td>fcb:: luxCDABE pAUS</td>
<td>Benzoic acid</td>
<td>Rozen et al. (1999)</td>
</tr>
<tr>
<td>E. coli</td>
<td>mer-::luxCDABE</td>
<td>Inorganic mercury</td>
<td>Selinova et al. (1993); Rasmussen et al. (1997); Ivask et al. (2002)</td>
</tr>
<tr>
<td>E. coli</td>
<td>dnaKp::luxCDABE pRY002</td>
<td>Pentachlorophenol</td>
<td>Van Dyk et al. (1994)</td>
</tr>
<tr>
<td>E. coli</td>
<td>fabA'::luxCDABE pFablux6 b</td>
<td>Alcohols; phenol; Halomethanes; detergent</td>
<td>Bechor et al. (2002)</td>
</tr>
<tr>
<td>E. coli</td>
<td>lac::luxCDABE pLITE2</td>
<td>Benzene</td>
<td>Gil et al. (2002)</td>
</tr>
<tr>
<td>E. coli</td>
<td>alkB::luxAB</td>
<td>Alkanes; petroleum; hydrocarbons; benzene</td>
<td>Sticher et al. (1997)</td>
</tr>
<tr>
<td>E. coli</td>
<td>pGrcELux c pKatGLux d</td>
<td>Ethanol; phenol</td>
<td>Van Dyk et al. (1995); Belkin et al. (1996); Gu et al. (2002)</td>
</tr>
<tr>
<td>E. coli</td>
<td>pfabALux b</td>
<td>EDCs g</td>
<td>Heitzer et al. (1994, 1998); King et al. (1991)</td>
</tr>
<tr>
<td>P. fluorescens</td>
<td>nahG-::luxCDABE</td>
<td>Naphthalene</td>
<td>Weitz et al. (2001)</td>
</tr>
<tr>
<td>P. putida</td>
<td>Mini-Tn5 luxCDABE pUCD 607</td>
<td>Copper; zinc; 3,5-DCP g; toluene</td>
<td>Lajoie et al. (2002)</td>
</tr>
<tr>
<td>P. putida</td>
<td>tod-::luxCDABE dmpR-::luxAB</td>
<td>BTEX g; Phenols</td>
<td>Applegate et al. (1998); Shingler and Moore (1994)</td>
</tr>
<tr>
<td>P. putida</td>
<td>pLOW2-mer-::luxCDABE pRB28; pUT-mer-::luxAB</td>
<td>Tetracyclines</td>
<td>Hansen and Sorensen (2000)</td>
</tr>
<tr>
<td>P. putida</td>
<td>xylR-::luxCDABE</td>
<td>Toluene; xylene</td>
<td>De Lorenzo et al. (1993)</td>
</tr>
<tr>
<td>P. putida</td>
<td>mini-Tn5::luxAB-KmR</td>
<td>Bacteria in soil</td>
<td>Ramos et al. (2000)</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>bph-::luxCDABE</td>
<td>PCB g</td>
<td>Layton et al. (1998)</td>
</tr>
<tr>
<td>R. eutropha</td>
<td>tfd-::luxCDABE</td>
<td>2,4-DCP g</td>
<td>Hay et al. (2000)</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>pLS–1Luxb- e</td>
<td>Heavy metal</td>
<td>Rabbo et al. (2002)</td>
</tr>
<tr>
<td>S. aureus, E. coli</td>
<td>ars-::luxAB</td>
<td>Arsenic</td>
<td>Corbiers et al. (1993)</td>
</tr>
<tr>
<td>Sphingomonas sp.</td>
<td>pUTmini-Tn5-::luxAB-tet pRK2013</td>
<td>Hydrocarbon f</td>
<td>Bastiaens et al. (2001)</td>
</tr>
<tr>
<td>Synechococcus</td>
<td>smta-::luxCDABE</td>
<td>Zinc</td>
<td>Erbe et al. (1996)</td>
</tr>
</tbody>
</table>

a: DNA damage; b: sensitive to membrane damage; c: sensitive to protein damage; d: sensitive to oxidative damage; e: operon of Photobacterium leiognathi; f: fluorene, acenaphthene, naphthalene, phenanthrene, fluoranthene, pyrene, dibenzoanthene, biphenyl, diesel fuel, n-pentane, hexane, heptane, nonane, decane, undecane, dodecane; g: EDCs: Endocrine Disrupting Chemicals; DCP: Dichlorophenol; BTEX: benzene, toluene, ethylbenzene, and xylene; PCB: polychlorinated biphenyls

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Conclusions
The HACCP concept can be used to improve the survey of the network (upstream (source of toxicity) and downstream (release in environment) of the WWTP management). Rapid and simple tests have been (or would be) developed and used to screen potentially hazardous matter and to protect WWTP, environment and consumer from the impact and effect of potentially dangerous contaminants. Whole-cell bioluminescent biosensors can be used to indicate the presence and effect of contaminants on the species encountered in the WWTP communities. Among bioluminescent-based tests, on-line monitoring tests have been developed. Such assays are rapid and inexpensive and thus offer great potential for routine monitoring. Whole-cell culture assays, in combination with other assays, can be incorporated into a battery of tests for the screening assays. According to the identified dangers, it is important to adapt the legislation and the monitoring methods. The on-line monitoring allows us to quickly open the necessary corrective actions and to determine the zone of danger according to the points of control. Afterwards, it will then be possible to anticipate the potential dangers and so decrease the risks of pollution in the environment.

References


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