Chapter 1

General introduction
Chapter 1

Surface water monitoring
At present day, there is a growing worldwide demand for clean and safe drinking water. At the same time, there is an increasing pressure on the quality of the drinking water sources. Several issues and incidents have led to an increased awareness of the risks of chemical pollution of fresh surface waters in many countries (1). Not only are these pollutants harmful to the environment, but they also have to be removed if this water is used for the production of drinking water.

In the Netherlands, about 37.5 percent of the drinking water is derived from surface water, mainly from the rivers Meuse and Rhine (2). The quality of the water in these rivers is not constant, but changes due to natural and human influences. Periods of draught, accidents with ships, runoffs from fields, or discharges from factories can all lead to a (temporary) increase in the contamination in these rivers (3). Some of the water utilities that derive their drinking water from surface water, therefore continuously monitor the water quality upstream of their water intake (4, 5). By doing so, they can close the water intake when there is a sudden increase in undesirable compounds in the water. This follows the philosophy of choosing the cleanest water source available for the production of drinking water, meant to prevent the introduction of contaminants in the production chain. Such prevention reduces the strain on the treatment installations and lowers the risk of contamination of the final drinking water.

Water monitoring techniques
The methods that are used for water monitoring can be divided in three groups: chemical analysis, biomonitors, and in vitro bioassays (4, 6). Chemical analysis is used to identify and quantify individual compounds in the water. The most common methods used in water monitoring are HPLC (High-Performance Liquid Chromatography) and GC-MS (Gas Chromatography-Mass Spectrometry). HPLC with UV diode array-detection (HPLC-UV) has been applied on-line for continuous monitoring of Rhine and Meuse water for several years (the SAMOS system). Besides this continuous monitoring, collected batch samples are regularly analyzed with e.g. LC-MS and GC-MS, but these analyses take too long to close the water inlet in time. With these methods it is possible to determine the exact concentration of specific compounds. Unfortunately however, only compounds with already known structures
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(“target compounds”) can be detected in this way. With advanced techniques, such as the Orbitrap mass spectrometer and UV diode array-detection, it is also possible to detect unknown compounds in a so-called broad-screening exercise (7). This produces very valuable data. However, the identification and quantification of these unknown compounds is not always straightforward. For this reason, the use of broad screening for routine monitoring is limited, especially for continuous, on-line monitoring for inlet guarding (8). Another disadvantage is that the toxicity of compounds cannot be determined with chemical analysis. This is an important issue, because there is a large difference in toxic potency for different compounds (9).

The second group of monitoring methods consists of the whole organism biomonitors. A whole organism biomonitor is a device in which the behaviour of living organisms is monitored to detect changes in the water quality. The organisms used in biomonitors include luminescent bacteria (Aliivibrio fischeri), algae, water fleas (Daphnia magna), Quagga mussels (Dreissena bugensis), and fish (various, i.e. zebra fish, Danio rerio) (10). The typical behaviours that are used as indicator of changing water quality include luminescence, photosynthesis, swimming velocity, shell closing, and swimming location, amongst others. With whole organism biomonitors, it is possible to measure the toxicity of the water continuously and unattended, with fast response times that enable inlet guarding. Unknown toxic compounds can thus be detected, and the total toxicity of the mixture of present compounds is determined. This is limited however, to compounds exerting acute toxic effects that result in behavioural changes in the biomonitoring species that is used. Compounds that cause chronic or specific toxicity, for example genotoxicity or hormonal disruption are usually not detected by these biomonitors.

The last group of techniques used for water monitoring consists of the in vitro bioassays. These assays make use of mammalian or microbial cells, sometimes genetically manipulated to increase their responsiveness, to determine a specific toxic effect in a sample. Examples are the ER CALUX assay for estrogenic compounds (11), or the Ames test for mutagenicity (12). With in vitro bioassays, it is possible to detect compounds that cannot be detected with the whole organism biomonitors, but are very relevant from a human perspective, because they can cause long-term adverse effects at low concentrations. The
problem with \textit{in vitro} bioassays is that they are mostly used for batch-wise monitoring and there are no bioassay systems developed as yet that can be applied for on-line water quality monitoring in field situations, such as at a monitoring station \cite{10}.

\section*{On-line water toxicity sensors based on luminescent bacteria}

What is still missing is a method that can be used for on-line monitoring of human-relevant toxic effects that are not detected by the biomonitors. In order to fill this gap, several on-line biosensors have been developed, based on genetically modified luminescent bacteria. A biosensor is defined as a sensor in which a biological recognition element, in this case the luminescent bacteria, is coupled to a transducer. These bacteria are usually \textit{Escherichia coli}, in which a gene from a specific stress response pathway has been coupled to the genes for luciferase by genetic modification. As a result, these bacteria emit light after exposure to certain compounds, such as certain heavy metals, or compounds with a specific effect, for example DNA damage (Figure 1). An extensive overview of these bacteria and their modifications can be found in Chapter 2.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{A picture taken of strain DPD2794, which is sensitive to compounds causing DNA damage in a 96-well plate, five and a half hours after induction with different doses of mitomycin C.}
\end{figure}
To be able to use these genetically modified luminescent bacteria for on-line water monitoring, there are a number of conditions that have to be fulfilled. The most important consideration is that the bacteria have to remain in the sensor for a prolonged time enabling them to respond to an increase in toxicity, which is at least one hour for most strains. Several methods to achieve this have been developed and described by different research groups. Roughly, these methods can be divided in sensors using bacteria in suspension, and sensors using immobilized bacteria.

The sensors with bacteria in suspension are usually bioreactors, in which the bacteria are cultured continuously. These bacteria are mixed with a flow of sample water, either in the same reactor as where the bacteria are grown (13-15), or in a separate reactor (16-18). The advantages of suspended bacteria are that there is no risk of an effect of the immobilization (matrix) on the response and that there is a constant supply of fresh bacteria. The downside is that the reactor volume has to be relatively large compared to the flow rate of the water sample to prevent a wash-out of the bacteria. This means the sensitivity and speed of these sensors will be hindered by dilution of the sample (19). It also means there is a constant outflow of water that contains high concentrations of genetically modified organisms (GMO's), which creates complications with respect to the strict legislation on use of GMO's in the field in most countries. For example, in The Netherlands, the use and disposal of GMO's is bound to strict rules and regulations. The purpose of these regulations is to prevent contamination of people or the environment with GMO's. An important consequence of these rules is that all the water that comes out of a sensor has to be disinfected prior to its disposal. For this reason, it is desirable to limit the risk of release of bacteria to the water in the outflow of the sensor.

By immobilizing the bacteria in a sensor, it is possible to reduce the size of the flow chamber considerably, to limit the outflow of bacteria, and to use more strains of bacteria simultaneously. Bacteria have been immobilized successfully on surfaces coated with antibodies (20, 21), or in matrices of alginate (22), agar (23-25), agarose (26), latex (27, 28), or silica (sol-gel) (29, 30), amongst others. Alginate, an organic polymer derived from brown seaweed, has been used to immobilize these bacteria on the tips of optical fibers, with a light detector on the other end of the fiber. It has been shown that the response of immobilized
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bacteria to toxic compounds was similar to bacteria in suspension (22, 31, 32). An early prototype of a sensor was built with bacteria immobilized on optical fibers, which was used to establish the proof of principle of this technique (33). However, this device appeared not to be very robust and offered limited control over various parameters.

Aims and outline of the thesis
This main aim of the work described in this thesis was to construct and develop a novel on-line biosensor using luminescent bacteria and to test and validate its performance both under laboratory and under field conditions. After this general introduction, the following chapters address these different aspects of the novel biosensor:

In chapter 2, an overview is presented of the various genetically modified luminescent bacteria available, based on literature data. The strains are clustered, based on the effect or compounds they respond to. Particular attention is given to the merits of the different strains for water monitoring. The ultimate choice for the DNA damage strain DPD2794 for use in the sensor was mainly based on this overview.

In chapter 3, the performance of the DNA damage strain was tested under laboratory conditions using a training set of compounds in a 96-well plate format. These included known genotoxic and non-genotoxic compounds to test the sensitivity and predictability of the strain, and a number of known environmental pollutants. The advantage of using well plates was that they enabled the testing of a range of concentrations in each experiment.

In chapter 4, the construction and setup of the hardware for the new sensor are described and the results of the first experiments are shown, which were used to establish the proof of principle. All experiments in this chapter were performed with tap water in the laboratory at KWR in Nieuwegein, the Netherlands.

In chapter 5, the on-line biosensor, constructed and tested under laboratory conditions, was installed in a field situation at monitoring station Keizersveer along the river Meuse. Here, the sensor was tested for a period of two months,
in which a normal background was established and the limit of detection was determined, among others. The results of these field experiments are presented in this chapter, as well as a few additional laboratory experiments to test the effect of filtration and the effectiveness of an UV-lamp positioned after the sensor for the continuous disinfection of the sensor outflow water.

In chapter 6 the work in the thesis is summarized, conclusions are derived and an outlook is given to future developments
References


The response of a bacterial strain for the detection of DNA damage to concentration series of mitomycin C and nalidixic acid, two genotoxic compounds
Chapter 2

Are luminescent bacteria suitable for on-line detection and monitoring of toxic compounds in drinking water and its sources?

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Abstract
Biosensors based on luminescent bacteria may be valuable tools to monitor the chemical quality and safety of surface and drinking water. In this review, an overview is presented of the recombinant strains available that harbour the bacterial luciferase genes luxCDABE, and which may be used in an on-line biosensor for water quality monitoring. Many bacterial strains have been described for the detection of a broad range of toxicity parameters, including DNA damage, protein damage, membrane damage, oxidative stress, organic pollutants, and heavy metals. Most lux strains have sensitivities with detection limits ranging from mg/l to μg/l, usually with higher sensitivities in compound-specific strains.
While the sensitivity of lux strains can be enhanced by various molecular manipulations, most reported detection thresholds are still too high to detect levels of individual contaminants as they occur nowadays in the European drinking waters. However, lux strains sensing specific toxic effects have the advantage of being able to respond to mixtures of contaminants inducing the same effect, and thus could be used as sensor for the sum effect, including the effect of compounds that are as yet not identified by chemical analysis. An evaluation of the suitability of lux strains for monitoring surface and drinking water is therefore provided.
Introduction

To ensure the chemical quality and safety of drinking water, it is essential to monitor the surface water sources as well as critical points in the distribution network. Currently, the presence of toxic chemicals in water is investigated by chemical analysis, by using aquatic organisms as biomonitors, and by in vitro toxicity assays (1). Chemical analysis is quantitative, sensitive, and highly selective, but only target compounds are detected. The biomonitoring methods, using mussels, *Daphnia*, algae, or natural bacteria are able to detect the total, mostly systemic, acute toxic effects of compounds such as herbicides and heavy metals. However, the toxic effects in these organisms have little predictive value for possible hazards for human individuals. In addition, these biomonitors do not react to non-systemic, specific toxic effects of compounds such as genotoxicants and endocrine disruptors. In vitro toxicity assays, using human or other mammalian cell lines, provide information on hazards relevant for human toxicity and can detect the sum effect of the whole mixture of toxicants present. For real-time monitoring of toxicants in water, there is currently no suitable system available that provides relevant information about human hazards. This gap may be filled by a type of biosensor that employs genetically modified luminescent bacteria which provide a rapid, easily measurable response in the presence of relevant toxic (mixtures of) compounds. A rapidly growing number of luminescent bacteria have already been constructed and described, which may be applicable for toxicity detection in water.

In this paper, an overview is provided of available bacterial *luxCDABE* strains and an evaluation and concurrent selection of strains which might be used in a biosensor for water quality monitoring. Lowe defined a biosensor as “an analytical device, which converts the concentration of the target substance into an electrical signal through a combination of a biological recognition system associated with a physico-chemical transducer” (2). For a toxic compound to elicit a measurable response in bacterial cells in a biosensor, it first has to cross the cell wall and cell membrane. Then, it has to trigger a sensing element, in most cases a promoter linked to a reporter gene, leading to the production of easily measurable reporter proteins. Detailed reviews have been written by van der Meer et al., which explain the mechanisms involved in the cellular transport and activation mechanisms of analytes (3, 4).
Currently, the most commonly used reporter proteins for optical detection in microbial systems are GFP for fluorescence and bacterial luciferase for luminescence. Bioluminescence offers the advantages of faster response times and higher short-term sensitivity (seconds to minutes). Fluorescent proteins may keep accumulating for many hours and due to their high stability, allow detection even after cell death (5-7). GFP also does not require substrate or ATP, thereby lowering the burden on the cells (3). For on-line monitoring of water, sensitivity and fast response times are more important factors than reporter stability. Therefore, luminescence is the detection method of choice for on-line monitoring, and this overview will thus focus on available luminescent bacterial reporter strains.

Bioluminescent bacteria express luminescence through the production of luciferase, either bacterial (lux) or firefly (luc). The latter has the advantage of a higher quantum yield, but requires the constant addition of luciferine. As a result, bacterial luciferase is favoured in most cases (8). Bacterial luciferase catalyses the oxidation of a long-chain aliphatic aldehyde (R-CHO and a reduced flavin mononucleotide (FMNH$_2$). In this reaction, free energy is emitted in the form of light with a wavelength of 490 nm:

$$\text{FMNH}_2 + \text{RCHO} + \text{O}_2 \rightarrow \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{light (490 nm)}$$

As this reaction depends on a functional electron transport system, it only functions in viable cells (9). Of the bacterial luciferase operon, only the luxAB genes are required for luminescence; however in this case a substrate has to be administered externally. More practical for on-line monitoring is the use of the luxCDABE genes, in which luxCDE code for the (re)generation of the substrate (10). Thus, no substrate addition is necessary and the luciferase reporter can operate independently. This overview will thus be limited to strains with the five bacterial luciferase genes, luxCDABE (lux strains).

Assays using bioluminescent bacteria can be divided into two groups, namely systems with constitutive and systems with inducible expression. Bacteria with constitutive expression have normally a high expression of luminescence, which decreases under toxic conditions (‘lights off’). They are usually natural
bioluminescent bacteria, such as *Aliivibrio fischeri* (until recently known as *Vibrio fischeri*), and are often used to detect acute (cyto)toxicity, as the response is not compound-specific. The use of these bacteria is categorized under biomonitors, as it involves natural aquatic organisms displaying a cytotoxic response to the presence of toxic compounds. These organisms and assays are therefore not included in this overview.

Inducible systems, in contrast, have a low baseline luminescence, which increases after exposure to specific compounds ('lights on'). In these systems, both promoter and reporter genes may be inserted from other bacteria to give an optimal response to the compounds of interest. It should be noted however, that many inducible strains emit a low level of background luminescence when they are not induced. This enables the detection of high acute toxicity or other types of severe stress that may compromise the survival of the bacteria, as the background luminescence will then disappear (11). However, when the background luminescence is on the high side, this tends to lower the sensitivity as it makes it harder to distinguish the signal (12).

Based on the promoters used in the construction of such “lights on” bioreporters, the inducible systems can be divided into effect- and compound-specific strains. The former respond to a specific type of toxicity, for example DNA damage, oxidative stress, or protein damage (heat shock). The compound-specific strains detect a single compound or group of compounds with similar chemical characteristics or mode of action, like specific metals, alkanes, or BTEX compounds (Figure 1) (11, 13).

In summary, the aim of this review is to provide an overview of inducible, genetically engineered luminescent bioreporter bacteria, harbouring the *luxCDABE* genes (*lux* strains), which can potentially be integrated into a biosensor device. Several strains of these luminescent bacteria will be categorized and compared based on their inducers (and corresponding response). An evaluation is made of the characteristics that are important for water quality monitoring, e.g., sensitivity, response time, robustness, and pathogenicity.
Chapter 2

**Effect-specific lux strains**

The effect-specific lux strains include strains that detect DNA, protein, and membrane damage, and oxidative stress. These strains are constructed by coupling the luxCDABE gene to a promoter that is involved in a specific stress response. As a result, the response of these strains is directly correlated with the total amount and potency of compounds that induce a specific type of stress.

**DNA damage**

Damage to DNA in bacteria can trigger at least two repair systems, the ada-controlled adaptive response specific to damage by alkylation, and the recA-dependent, lexA-controlled SOS response.

DNA damage by alkylation activates the ada-gene, which leads to transcription of the ada, alkA, alkB, and aid genes (14). The SOS response can be induced by many chemicals that damage DNA, arrest DNA synthesis or arrest cell division, including mitomycin C (MMC), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and methyl methane sulfonate (MMS). More than 40 genes are activated in the SOS response, including recA, lexA, uvrA and umuDC (14, 15). When the promoters of these genes are coupled to the lux gene, the extent of DNA damage can be easily measured as an increase in luminescence. In the majority
of *lux* strains, *recA* is used as promoter. An overview of the strains used in the following studies can be found in Table 1.

Vollmer et al. (1997) compared three *lux* strains, DPD2794, DPD2818, and DPD2844, containing the promoters of the *recA*, *uvrA*, or *alkA* genes, respectively, fused to the *A. fischeri luxCDABE* genes. The best results were gained with DPD2844 (*alkA*), which showed the highest sensitivity (detection limit of <0.01 mg/l MNNG) and shortest response time (40-50 minutes). An important cause for the very high sensitivity was the very low background luminescence in this strain. It should be mentioned however, that not all strains were tested with the same compounds. For DPD2844, only results with MNNG were given, while for DPD2794 and DPD2818 detection limits for MMC and UV were displayed (16).

Davidov et al. (2000) conducted a study to improve the sensitivity of *recA* based DNA damage strains. The use of *luxCDABE* genes of *A. fischeri* resulted in a more sensitive, but slower response then *luxCDABE* from *Photorhabdus luminescens*. The sensitivity was also improved by insertion of a single copy of the *recA::lux* fusion into the chromosome, instead of in multi-copy plasmids, and by a mutation in the *tolC* gene. Insertion in the chromosome resulted in a lower background luminescence, thus enhancing the ability to distinguish the signal. A *tolC* mutation impairs the ability of the cell to excrete toxicants, which leads to higher intracellular concentrations. These two adaptations resulted in strains with detection limits of 0.1 µg/l for MMC. In addition to the positive controls MMC, MNNG, and H$_2$O$_2$, also a group of other genotoxic and non-genotoxic compounds was tested. No false negatives were found, and except for one, all positives had been tested as potential genotoxicants in other bioassays (12).

As an alternative for the *E. coli* based strains, Elasri and Miller (1998) developed strain RM4440, based on *Pseudomonas aeruginosa*. *P. aeruginosa* was chosen because it is a natural resident of water and soil, and as such, it was expected to be more robust than *E. coli* for on-line water monitoring. Strain RM4440 contained the *recA* and *luxCDABE* genes and was exposed to UV light. No limit of detection was mentioned, but lag times were about 30 minutes (17). In a follow-up study by the same group, the same strain was exposed to 17 chemicals, of which eight are known SOS inducers. The cells were exposed in alginate beads to fixate them, as well as to simulate their own natural alginate
biofilms. The *P. aeruginosa* reporter strain reacted to all compounds known to induce SOS in *E. coli* except H$_2$O$_2$ (18). Suggested explanations were protection by the alginate or the ability of *P. aeruginosa* to actively degrade H$_2$O$_2$ (19). A drawback of *P. aeruginosa* for use in environmental monitoring is that it is a known opportunistic human pathogen.

**Table 1:** Literature overview of *lux* strains for the detection of DNA damage and their detection limits.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ref.</th>
<th>promoter</th>
<th>receiving strain</th>
<th>LOD</th>
<th>setup</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPD2794</td>
<td>(21)</td>
<td>recA</td>
<td>RFM443</td>
<td>480 μg/l H$_2$O$_2$</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(22)</td>
<td></td>
<td></td>
<td></td>
<td>portable sensor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(12)</td>
<td></td>
<td></td>
<td>2 μg/l MMC</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td></td>
<td></td>
<td>500 μg/l trimethoprim</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(24)</td>
<td></td>
<td></td>
<td>10 μg/l MMC</td>
<td>bioreactor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(25)</td>
<td></td>
<td></td>
<td>50 μg/l MMC</td>
<td>bioreactor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td></td>
<td></td>
<td>5 μg/l MMC, 0.013 μg/l benzo[a]pyrene</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(28)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A-body plates</td>
</tr>
<tr>
<td></td>
<td>(16)</td>
<td></td>
<td></td>
<td>100 μg/l MMC</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>DPD2797</td>
<td>(12)</td>
<td>recA</td>
<td>DE112</td>
<td>0.1 μg/l MMC</td>
<td>culture plate</td>
<td>tolC mutant</td>
</tr>
<tr>
<td></td>
<td>(30)</td>
<td></td>
<td></td>
<td></td>
<td>optical fibre</td>
<td></td>
</tr>
<tr>
<td>DPD1718</td>
<td>(12)</td>
<td>recA</td>
<td>DPD1692</td>
<td>100 μg/l MMC</td>
<td>culture plate</td>
<td>Chr.</td>
</tr>
<tr>
<td></td>
<td>(31)</td>
<td></td>
<td></td>
<td></td>
<td>optical fibre</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(32)</td>
<td></td>
<td></td>
<td>25 μg/l MMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD1710</td>
<td>(33)</td>
<td>recA</td>
<td>RFM443</td>
<td>1 μg/l MMC</td>
<td>sol-gel wells</td>
<td>Chr.</td>
</tr>
<tr>
<td>DPD1714</td>
<td>(12)</td>
<td>recA</td>
<td>DM800</td>
<td></td>
<td>culture plate</td>
<td>Chr.</td>
</tr>
<tr>
<td>DPD1709</td>
<td>(12)</td>
<td>recA</td>
<td>DM803</td>
<td></td>
<td>culture plate</td>
<td>Chr.</td>
</tr>
<tr>
<td>DPD3063</td>
<td>(12)</td>
<td>recA</td>
<td>W3110</td>
<td>0.1 μg/l MMC</td>
<td>culture plate</td>
<td>Chr.</td>
</tr>
<tr>
<td>Sal94</td>
<td>(12)</td>
<td>recA</td>
<td>WG49</td>
<td>250 μg/l 4-nitrophenol</td>
<td>culture plate</td>
<td><em>S. Typhimurium</em></td>
</tr>
<tr>
<td>RM4440</td>
<td>(17)</td>
<td>recA</td>
<td>FRD1</td>
<td></td>
<td>culture plate</td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>(18)</td>
<td></td>
<td></td>
<td></td>
<td>alginate beads</td>
<td></td>
</tr>
<tr>
<td>DPD2818</td>
<td>(16)</td>
<td>uvrA</td>
<td>RFM443</td>
<td>10 μg/l MMC</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>DPD2844</td>
<td>(16)</td>
<td>alkA</td>
<td>RFM443</td>
<td>&lt;10 μg/l MMC</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>BBTNrdA</td>
<td>(20)</td>
<td>NrdA</td>
<td></td>
<td>156.3 μg/l MNNG</td>
<td>culture plate</td>
<td></td>
</tr>
</tbody>
</table>

All strains are *E. coli* based with *luxCDABE* as reporter, unless stated otherwise under notes. chr. = genes are inserted in the chromosome, instead of plasmids. LOD: Limit of Detection.

Hwang et al. (2008) constructed strain BBTNrdA, which is an *E. coli* based *lux* strain that has the *nrdA* gene as promoter. The *nrdA* gene is activated in DNA
Are luminescent bacteria suitable for on-line detection and monitoring

synthesis, but is not regulated by the SOS response. The strain reacted on all four DNA damaging agents tested (nalidixic acid, MMC, MNNG, and 4-nitroquinolin N-oxide). Also four phenolic compounds and four oxidative compounds were tested, but only one of these (H$_2$O$_2$) induced luminescence in this strain (20).

**Conclusion**

A large variety of DNA damage strains has been developed in the last decades, based upon a number of different promoters involved in DNA damage repair. The most sensitive lux strains found in this study were two modified recA strains, namely DPD2797 and DPD3063 (12).

**Protein damage**

A mechanism found in cells of all organisms to counteract protein damage is the heat shock response. This response can be triggered by high temperatures, viral infections, exposure to various chemicals that react with proteins, and abnormal proteins resulting from other processes. Van Dyk et al. (1994) used two promoters to develop lux strains for the detection of protein damage, namely the grpE gene and the dnaKp gene. The gene dnaK encodes for Hsp70, a heat-shock protein that has an important cellular function in protein folding and re-naturation. The gene grpE encodes for Hsp60, which has a similar function as Hsp70.

Both promoters responded to generally the same compounds, namely ethanol, methanol, copper sulphate, phenol and derivates, and 2,4-dichlorophenoxyacetic acid, but grpE proved to be about 5- to 10-fold more sensitive. The grpE strain also had a higher basal luminescence level, and therefore it showed a lights-off effect at higher concentrations. Both lights-on and lights-off effects at high concentrations were enhanced in cells with a tolC mutation (34).

The grpE strain TV1061 has also been used in later studies, often incorporated in various setups (see Table 2). It has been shown to respond to a wider range of toxicants than any other lux strain, including phenols, halomethanes, oxidants, phosdrin, chlordimeform, sodium cyanide, and MNNG. However, it is less sensitive than for example DNA damage strains, with LOD’s in the mg/l range. Human toxicants that did not induce TV1061 include colchicine, trimethylolpropane phosphate, nickel chloride, sodium selenite, and lindane (21, 30).
Chapter 2

Conclusion
Two promoter gene have been used to generate lux strains that are sensitive to protein damage. Of these, *grpE* gave the most sensitive strains (TV1061/TV1076), responded to a wide range of toxicants and has been used most often.

Table 2: Summary of strains for the detection of protein damage and their corresponding detection limits.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ref.</th>
<th>promoter</th>
<th>receiving strain</th>
<th>LOD</th>
<th>setup</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>TV1061</td>
<td>(21)</td>
<td><em>grpE</em></td>
<td>RFM443</td>
<td>0.1 mg/l</td>
<td>1-bromophenol</td>
<td>culture plate</td>
</tr>
<tr>
<td></td>
<td>(22)</td>
<td></td>
<td></td>
<td>&lt;1% ethanol</td>
<td>portable sensor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(23)</td>
<td></td>
<td></td>
<td>5 mg/l rifampicin</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(24)</td>
<td></td>
<td></td>
<td>300 mg/l phenol</td>
<td>optical fibre</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(25)</td>
<td></td>
<td></td>
<td>0.3% ethanol</td>
<td>sol-gel wells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td></td>
<td></td>
<td></td>
<td>optical fibre</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(27)</td>
<td></td>
<td></td>
<td></td>
<td>A-body glass/gold plates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(28)</td>
<td></td>
<td></td>
<td>±12 mg/l pentachlorophenol 1% ethanol</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>WM1202</td>
<td>(36)</td>
<td><em>dnaKp</em></td>
<td>RFM443</td>
<td>4% ethanol/19 mg/l pentachlorophenol</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>WM1302</td>
<td>(36)</td>
<td><em>dnaKp</em></td>
<td>DE112</td>
<td>50 mg/l phenol</td>
<td>culture plate</td>
<td>tolC mutant</td>
</tr>
<tr>
<td>TV1076</td>
<td>(36)</td>
<td><em>grpE</em></td>
<td>DE112</td>
<td>±0.07 mg/l pentachlorophenol</td>
<td>culture plate</td>
<td>tolC mutant</td>
</tr>
</tbody>
</table>

All strains are *E. coli* with luxCDABE as reporter.

Cell membrane damage
For compounds that cause cell membrane damage, bacteria can be used that contain the *fabA* gene, coupled to the full lux gene. The *fabA* gene codes for β-hydroxydecanoyl-ACP dehydrase and is responsible for the formation of double bonds in fatty acids which are used in the cell membrane. The *fabA* gene is activated by binding of the FadR protein, a process that is inhibited by long chain acyl-CoA thioesters. In case of membrane damage, fatty acid starvation will occur and long chain acyl-CoA thioester levels will be low, resulting in a high induction of *fabA*.

Strain DPD2540 containing *fabA* and luxCDABE was used in a study by Choi and Gu (1999) and was tested with several compounds. As expected, *fabA* was induced by membrane damaging agents, such as ethanol, phenol, and...
Are luminescent bacteria suitable for on-line detection and monitoring

cerulenin. More surprisingly however, DPD2540 also responded to the DNA damaging agent MMC and the oxidative agent H$_2$O$_2$. The reason for this is probably that these agents also cause damage to the membrane as a secondary effect. In these cases the response was delayed from around 60 minutes for ethanol to 150 minutes for MMC and H$_2$O$_2$. Unfortunately, no detection limits were given in this study. (37). For more studies that used this strain and other fabA strains, see Table 3.

A comparison of several fabA based lux strains was made by Bechor et al. (2002). The strains used included a tolC mutant (DPD2543), fadR mutant (DPD2549), and a strain with fabA::lux inserted in the chromosome (DPD1674). The fadR mutant gave almost no response, proving that the luminescence was indeed induced via fabA. The tolC mutant strain (DPD2543) was more sensitive for most of the tested chemicals than the non-mutant strain (DPD2544). However, this was not true for all membrane damaging substances. For example, DPD2543 was more sensitive to many phenol derivatives, but not to phenol itself. The chromosomal insertion resulted in a drop in background luminescence of about a factor 100. DPD1674 had a higher response ratio for ethanol than DPD2544, but this was slightly lower for phenol. Whether the detection limit also differed was not clarified. Compounds that induced luminescence in fabA strains included alcohols, phenol and derivatives, halo-methanes, aromatics and detergents (38).

In comparisons between effect-specific lux strains, the fabA strains showed a close similarity to the protein damage strain (TV1061) in the compounds it responded to. Only in case of bromodichloromethane, paraquat, cumene hydroperoxide, and sodium cyanide, TV1061 was induced while the fabA strains failed to respond (21, 30).

**Conclusion**

Several strains have been developed for the detection of membrane damage, all based on the fabA promoter. The amount of information on the sensitivity and specificity of these strains is limited, thus no preference for a strain could be entertained. Like the protein damage strains, they react on a broad range of compounds with detection limits in the mg/l range.
Chapter 2

Table 3: Literature overview of lux strains for the detection of cell membrane damage and their detection limits.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ref.</th>
<th>promoter</th>
<th>receiving strain</th>
<th>LOD</th>
<th>setup</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPD2544</td>
<td>(38)</td>
<td>fabA</td>
<td>W3110</td>
<td>0.19 mg/l</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(29)</td>
<td></td>
<td></td>
<td>Triton X-100</td>
<td>A-body glass/gold plates</td>
<td></td>
</tr>
<tr>
<td>DPD2540</td>
<td>(38)</td>
<td>fabA</td>
<td>RFM443</td>
<td>0.14 mg/l</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(21)</td>
<td></td>
<td>RFM444</td>
<td>4-Bromophenol</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(37)</td>
<td></td>
<td></td>
<td></td>
<td>portable sensor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(22)</td>
<td></td>
<td></td>
<td></td>
<td>bioreactor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(25)</td>
<td></td>
<td></td>
<td>100 mg/l phenol</td>
<td>bioreactor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td></td>
<td></td>
<td></td>
<td>bioreactor</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(35)</td>
<td></td>
<td></td>
<td></td>
<td>bioreactor</td>
<td></td>
</tr>
<tr>
<td>DPD2543</td>
<td>(38)</td>
<td>fabA</td>
<td>DE112</td>
<td>0.08 mg/l</td>
<td>culture plate</td>
<td>toIC mutant</td>
</tr>
<tr>
<td></td>
<td>(30)</td>
<td></td>
<td></td>
<td>4-nitrophenol</td>
<td></td>
<td>optical fibre</td>
</tr>
<tr>
<td>DPD2546</td>
<td>(38)</td>
<td>fabA</td>
<td>DC530</td>
<td></td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>DPD2549</td>
<td>(38)</td>
<td>fabA</td>
<td>MH163</td>
<td></td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>DPD1674</td>
<td>(38)</td>
<td>fabA</td>
<td>W3110</td>
<td></td>
<td>culture plate</td>
<td>Chr.</td>
</tr>
</tbody>
</table>

All strains are E. coli with luxCDABE as reporter. Chr. = genes are inserted in the chromosome, instead of by plasmids.

Oxidative stress

Active oxygen species are a serious threat to cells as they are capable of damaging proteins, nucleic acids, lipids, and membranes. Oxidative stress occurs naturally in cells as a result of respiration, or may be caused by toxic compounds. These toxicants can either generate peroxides ($H_2O_2$), superoxides ($O_2^-$) or hydroxyl radicals (OH$^-$). Peroxides lead to the activation of the E. coli OxyR regulon, while superoxides induce SoxRS. Despite the fact that $O_2^-$ can also trigger the formation of OH$^-$/$H_2O_2$, most promoters are only activated by one of the two groups (39). A large number of promoters from these pathways have been used to form lux strains that detect oxidative stress. In Table 4 a literature overview of these strains is given.

One of the genes under control of the OxyR regulon is katG (catalase hydroperoxidase I) (40). Belkin et al. (1996) have introduced the katG promoter coupled to the full luxCDABE gene of Vibrio fischeri in E. coli resulting in strain DPD2511. Luminescence induction was found for several oxidative compounds, including $H_2O_2$, organic

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peroxides, paraquat, menadione, xanthine, xanthine oxidase, and cigarette smoke. It also responded to ethanol and showed a synergistic response to the combination of ethanol and H$_2$O$_2$ (41). Additionally, the same strain has been reported to respond to cadmium chloride, ethidium bromide, and bisphenol A. No response was seen after exposure to potassium dichromate (42).

DK1, a similar strain with katG as promoter also responded on H$_2$O$_2$ and menadione, but not on paraquat or structural analogs of paraquat (43).

The defence mechanism against superoxides, the SoxRS regulon, acts in two steps. Superoxide generating compounds are first detected by SoxR, which then induces transcription of SoxS, a transcriptional activator of 16 other genes. These include sodA (Mn-superoxide dismutase), nfo (DNA repair endonuclease IV), zwf (glucose-6-phosphate dehydrogenase), acnA (aconitase), fumC (stable fumarase), fpr (ferredoxin reductase), acrAB (efflux pumps), micF (antisense RNA for the ompF porin mRNA), and fur (repressor of iron transport) (40).

Several superoxide sensitive lux strains have been reported that are based on promoters from the SoxRS regulon, including DPD2515 (micF), EBSoxS (soxS), EBHJ (sodA), ZWF (zwf), DP1 (pqi-5), DS1 (sodA), EBFumC (fumC), and FPR (fpr) (21, 42-45). Reported inducers of these strains include paraquat (methyl viologen) and its structural analogs ethyl viologen, benzyl viologen and heptyl viologen. Exposure to hydroxyl radical or peroxides, like H$_2$O$_2$, gave much lower or no responses (21, 43, 44). Unfortunately, little is known about the sensitivity of these strains, although for DPD2515 and EBSoxS detection limits have been reported of about 0.01 mg/l paraquat (21, 42).

In addition to the aforementioned strains, EBHmp (hmp) and PGRFM (pgi) have been developed, which contain promoters that belong neither to the OxyR or SoxRS pathway. PGRFM has been reported to respond to both superoxides and peroxides, with a lowest LOD of 0.6 mg/l for paraquat (39). However, in another study both strains responded only to superoxides, namely paraquat, ethyl viologen, and heptyl viologen (43).

**Conclusion**

For the detection of all oxidative agents, both a superoxide and a peroxide sensing strain are needed. For peroxides, only katG based strains are available and relatively well documented. For superoxides, a large array of strains has been constructed, but there is very limited information both on their sensitivity and selectivity.
Table 4: Literature overview of lux strains for the detection of oxidative stress and their detection limits.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ref.</th>
<th>reacts on</th>
<th>promoter</th>
<th>receiving strain</th>
<th>LOD</th>
<th>setup</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPD2511</td>
<td>(21)</td>
<td>peroxides</td>
<td>katG</td>
<td>RFM443</td>
<td>0.1 mg/l H₂O₂, 0.0006% H₂O₂ ~ 6 mg/l, 0.1 mg/l bisphenol A</td>
<td>culture plate, portable sensor, culture plate, optical fibre, A-body glass/gold plates</td>
</tr>
<tr>
<td></td>
<td>(22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(23)</td>
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<td>(24)</td>
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<td>(25)</td>
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<td>(26)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPD2515</td>
<td>(21)</td>
<td>superoxides</td>
<td>micF</td>
<td>W3110</td>
<td>0.01 mg/l paraquat</td>
<td>culture plate, A-body glass/gold plates</td>
</tr>
<tr>
<td></td>
<td>(29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DP1</td>
<td>(27)</td>
<td>superoxides</td>
<td>pgi-S</td>
<td>RFM443</td>
<td>0.1 mg/l paraquat, 2.5% ethanol, 7.8 mg/l paraquat</td>
<td>bioreactor, sol-gel wells</td>
</tr>
<tr>
<td></td>
<td>(33)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGRFM</td>
<td>(43)</td>
<td>superoxides peroxides</td>
<td>pgi</td>
<td>RFM443</td>
<td>0.6 mg/l paraquat</td>
<td>culture plate</td>
</tr>
<tr>
<td></td>
<td>(39)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DK1</td>
<td>(27)</td>
<td>superoxides peroxides</td>
<td>katG</td>
<td>RFM443</td>
<td>10 mg/l H₂O₂, 0.15% isopropanol, 0.8 mg/l H₂O₂</td>
<td>bioreactor, sol-gel wells plate</td>
</tr>
<tr>
<td></td>
<td>(33)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(46)</td>
<td></td>
<td></td>
<td></td>
<td>0.88 μM (0.03 mg/l) H₂O₂</td>
<td>micro-fluid chip</td>
</tr>
<tr>
<td>EBSoxS</td>
<td>(45)</td>
<td>superoxides</td>
<td>soxS</td>
<td>RFM443</td>
<td></td>
<td>cell chip</td>
</tr>
<tr>
<td>DS1</td>
<td>(45)</td>
<td>superoxides</td>
<td>sodA</td>
<td>RFM443</td>
<td></td>
<td>cell chip</td>
</tr>
<tr>
<td>ZWF</td>
<td>(43)</td>
<td>superoxides</td>
<td>zwf</td>
<td>RFM443</td>
<td></td>
<td>cell chip</td>
</tr>
<tr>
<td>FPR</td>
<td>(43)</td>
<td>superoxides</td>
<td>fpr</td>
<td>RFM443</td>
<td></td>
<td>cell chip</td>
</tr>
<tr>
<td>EBFumC</td>
<td>(43)</td>
<td>superoxides</td>
<td>fumC</td>
<td>RFM443</td>
<td></td>
<td>cell chip</td>
</tr>
<tr>
<td>EBHmp</td>
<td>(43)</td>
<td>superoxides</td>
<td>hmp</td>
<td>RFM443</td>
<td></td>
<td>cell chip</td>
</tr>
<tr>
<td>EBHJ</td>
<td>(42)</td>
<td>superoxides</td>
<td>sodA</td>
<td>RFM443</td>
<td>0.015 mg/l paraquat</td>
<td>culture plate</td>
</tr>
</tbody>
</table>

All strains are E. coli with luxCDABE as reporter.

Compound specific strains

In this section an overview is given of lux strains that respond specifically to certain types of compounds. These strains have been constructed by combining the luxCDABE reporter with promoter genes from bacteria that have developed an enhanced resistance to specific toxic compounds. Included are strains that respond to several types of organic pollutants and heavy metals. An overview of the strains for the detection of organic compounds is given in Table 5, and for metal sensing strains in Table 6.
Are luminescent bacteria suitable for on-line detection and monitoring

**BTEX compounds (Benzene, Toluene, Ethylbenzene, and Xylene)**

For the monitoring of toluene and trichloroethylene (TCE) co-metabolism, strain B2 based on *Pseudomonas putida* was constructed by Applegate et al. (1997). *P. putida* is a non-pathogenic soil bacterium that is capable of utilizing and degrading organic solvents. This strain harbours the *tod-lux* complex that enables the detection of toluene and co-metabolised TCE. TCE is co-metabolic in the sense that it can not be used as a carbon source, but is degraded as a side effect. The *tod* operon encodes for a suite of enzymes that mediate the metabolism of toluene via the toluene dioxygenase complex.

The strain had a limit of detection of 0.1 mg/l for toluene and a response time of 90 minutes. A strong response was also observed when the strain was exposed to jet fuel containing toluene (47).

Additionally to the aforementioned strain, the same group also constructed a strain with the same *tod-lux* complex inserted in the chromosome (TVA8). This resulted in higher sensitivity with a LOD of 30 μg/l, but a longer response time of 120 minutes. It was also responsive to benzene, ethylbenzene and *m*- and *p*-xylene, indicating that strains with the *tod* promoter can be used as a general BTEX monitor (48).

**Naphthalene and salicylate**

Naphthalene belongs to the polycyclic aromatic hydrocarbons (PAHs) and is a widely spread environmental pollutant. For the detection as well as the catabolism of naphthalene and its degradation intermediate salicylate, *Pseudomonas fluorescens* strain HK44 was developed. *P. fluorescens* is a non-pathogenic bacterium that lives on plants, in water, and in soil. This strain harbours a fusion of the promoter of its own *nahG* gene for naphthalene degradation to *luxCDABE*. It responded in a dose dependant manner to both naphthalene and salicylate, with a LOD for naphthalene of 45 μg/l (49, 50).

In a comparative study by Trogl et al. (2005) strain HK44 was exposed to 32 components other than naphthalene and salicylate. From the 32 compounds, it responded only to 2-aminobenzoic acid, salicylaldehyde, 4-methylsalicylic acid and, 4-chlorsalicylic acid (51).

The same strain, while immobilized in a sensor, also responded to jet fuel and contaminated soil extracts that contained naphthalene. Response times of 8 to 24 minutes were recorded in this setup. Toluene did not induce any measurable effects in these bacteria (52).
Two other strains based on a different naphthalene degradation pathway have been developed by Mitchell & Gu (2005). Both strains contain the nagR-nagAa gene promoters which are up-regulated by salicylate, coupled to luxCDABE. The first strain, called DNT5, has E. coli as host organism. The second strain, NAGK-1768, has P. putida as host organism which also possesses the ability to degrade naphthalene via the nah and sal operons. It was shown that, of the two strains, NAGK-1768 had the more favourable response characteristics with higher luminescence and a lower LOD for salicylic acid (4 µg/l, versus 331 µg/l in DNT5). NAGK-1768 also responded on 13 of 25 tested salicylic acid derivatives (also called salicylates) and naphthalene. DNT5 reacted on 5 of 25 salicylic acid derivatives (53).

In an additional study on the performance of DNT5 a slightly lower detection limit was reached then before (164 µg/l). The strain was also responsive to benzoic acid and two of its derivatives. Benzene, naphthalene, and phenol failed to induce a response (54).

**Polychlorinated biphenyls (PCBs)**
Layton et al. (1998) tested the PCB sensitive strain Ralstonia eutropha ENV307(pUTK60) containing the orf0-bphA1 genes coupled to luxCDABE. R. eutropha is a non-pathogenic bacterium that lives in soil and water and can degrade chloroaromatic compounds. The bacteria were exposed to biphenyl, monochlorinated biphenyls (CB) and Aroclor 1242 (PCB mixture) solutions. As the water-solubility of PCBs is very low, non-ionic surfactants were used to reach measurable concentrations in water. The highest sensitivity was reached with 4-chlorobiphenyl, which gave a detection limit of 0.15 mg/l. For Aroclor 1242 however, the detection limit was 1.5 mg/l, while the aqueous solubility without surfactants is <1 mg/l (55). As these results indicate that as the concentrations PCBs in water will always be below LOD, the usefulness of this bacterial strain for in situ PCB monitoring in water is highly questionable.

**Phenols**
A strain for the detection of phenol and its methylated derivatives was developed by Leedjarv et al. (2006). The dmpR gene for catabolism of phenols was linked to luxCDABE and introduced in P. fluorescens. The lowest detection limits were found for 2-methylphenol (0.03 mg/l) and phenol (0.08 mg/l). Different phenols applied in a mixture caused an additive effect on the induction of the bacterial strain (56).
Are luminescent bacteria suitable for on-line detection and monitoring

Table 5: Compound-specific lux strains for selective groups of organic chemicals and their detection limits.

<table>
<thead>
<tr>
<th>Strain</th>
<th>species</th>
<th>Ref.</th>
<th>reacts on</th>
<th>promoter</th>
<th>LOD</th>
<th>setup</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2</td>
<td><em>P. putida</em></td>
<td>(47)</td>
<td>toluene and TCE</td>
<td>todC1C2BA</td>
<td></td>
<td>vials and alginate beads</td>
<td></td>
</tr>
<tr>
<td>TVA8</td>
<td><em>P. putida</em></td>
<td>(48)</td>
<td>all BTEX</td>
<td>tod</td>
<td>30 µg/l toluene</td>
<td>vials</td>
<td>Chr.</td>
</tr>
<tr>
<td>HK44</td>
<td><em>P. fluorescens</em></td>
<td>(50)</td>
<td>naphthalene, salicylate</td>
<td>nahG</td>
<td>45 µg/l naph.</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>550 µg/l naph.</td>
<td>sensor probe</td>
<td></td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1200 µg/l naph,</td>
<td>sol-gel plates</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>500 µg/l sal</td>
<td></td>
</tr>
<tr>
<td>NAGK-1768</td>
<td><em>P. putida</em></td>
<td>(53)</td>
<td>naphthalene, salicylate</td>
<td>nagR-nagAa</td>
<td>4 µg/l salicylic acid</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>DNT5</td>
<td></td>
<td>(54)</td>
<td></td>
<td></td>
<td>164 µg/l salicylic acid</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td>(53)</td>
<td>salicylate</td>
<td>nagR-nagAa</td>
<td>331 µg/l salicylic acid</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>ENV307 (pUTK60)</td>
<td><em>R. eutropha</em></td>
<td>(59)</td>
<td>PCBs</td>
<td>orf0-bphA1</td>
<td>150 µg/l 4-CB</td>
<td>culture plate</td>
<td></td>
</tr>
<tr>
<td>OS8 (pDNdm-pRlux)</td>
<td><em>P. fluorescens</em></td>
<td>(56)</td>
<td>phenols</td>
<td>dmpR</td>
<td>30 µg/l 2-methylphenol, 80 µg/l phenol</td>
<td>culture plate</td>
<td></td>
</tr>
</tbody>
</table>

All strains have luxCDABE as reporter, unless stated otherwise under notes. Chr. = genes are inserted in the chromosome, instead of plasmids.

Heavy metals

For the construction of metal sensing strains, the operons for metal resistance that some natural occurring bacteria possess are often used as promoters. For example the mer operon enables cells to convert Hg(II) to the less toxic Hg(0). The ars operon on the other hand, provides the ability to transport antimonite and arsenite out of the cell and to reduce arsenate to arsenite (60).

Unfortunately, no study was found in which an arsenic sensing strain was used that contains the complete luciferase gene. Since it is a very relevant metal for water monitoring, it was decided to include some studies that use strains that contain luxAB instead.

Stocker et al. (2003) used a regulatory gene from the ars operon, the arsR gene, combined with luxAB to construct a strain for the detection of arsenite. For this compound, it had a limit of detection of 4 µg/l and a response time of 30 minutes. The strain also responded to arsenate and antimony (61). In a later
study, the same strain was also used to determine arsenite in groundwater samples from Vietnam. The sensitivity in these samples was reduced somewhat by the presence of iron, which binds arsenite. It was determined that with 20 mg/l Fe the lowest detectable concentration was 7.5 µg/l, which is still below the WHO guideline value of 10 µg/l. In total 194 samples were tested, of which 112 samples were deemed safe. 8.0% was tested false negative and 2.4% false positive when compared with chemical analysis (62).

Two *E. coli* strains for the detection of various heavy metals were tested by Riether *et al.* (2001). The *pZNT:*lux strain proved to be sensitive to Cd(II), Pb(II), Zn(II), Hg(II), and to a lesser degree to Co(II), Ni(II), SbO₂⁻, CrO₄²⁻, and Cr₂O₇²⁻. The strongest inducer of this strain was cadmium, with a detection limit of 10 nM (1.1 µg/l). The other strain tested was *pCOP:*lux, which reacted only to copper and silver, with detection limits of respectively 0.1 and 0.3-1 µM (11 and 19-64 µg/l). It was also verified that EDTA, and probably also other chelating components, lower the bioavailability of metals (26).

Three *E. coli* HB101 variants for the detection of mercury (HgII) were tested by Lyngberg *et al.* (1999). The largest dynamic range of detection for HgCl₂ was found with strain HB101(pRB28), which contains merR and a truncated form of merT (merT'). The highest sensitivity was found in cells immobilized in latex, which gave a detection limit of 0.1 nM HgCl₂ (27.2 ng/l) after 15 hours of induction. The detection limit was a factor 10 higher in suspended culture (1 nM or 0.27 µg/l), but the response time was reduced to one hour for the lowest concentration. In all cases, the lag time was drastically shortened at higher concentrations, although the suspended cells remained the fastest responders. It was also possible to enhance the response and sensitivity by adding cysteine, which increases mercury uptake. This resulted in a detection limit of 0.05 nM (13.6 ng/l) in immobilized cells (63).

Another strain for the detection of mercury which contains the *merR:*luxCDABE genes was constructed by Hakkila *et al.* (2002). The response time of this strain was 30 minutes. A limit of detection was not mentioned in this study (6). The same strain was also tested during the EILATox-Oregon Workshop while it was immobilized in an alginate gel on the tip of a fibre. It reacted on mercury chloride, a river sample, and slightly on sodium cyanide (64).
Are luminescent bacteria suitable for on-line detection and monitoring?

<table>
<thead>
<tr>
<th>Strain</th>
<th>species</th>
<th>Ref.</th>
<th>reacts on</th>
<th>promoter</th>
<th>LOD</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHSα (pJAMA-arsR)</td>
<td><em>E. coli</em></td>
<td>(61)</td>
<td>As, Sb</td>
<td><em>arsR</em></td>
<td>4 μg/l As</td>
<td><em>luxAB</em></td>
</tr>
<tr>
<td>MG1655 (pZNT-lux)</td>
<td><em>E. coli</em></td>
<td>(66)</td>
<td>Cd, Pb, Zn, Hg</td>
<td><em>zntA</em></td>
<td>1.1 μg/l Cd</td>
<td></td>
</tr>
<tr>
<td>MG1655 (pCOP-lux)</td>
<td><em>E. coli</em></td>
<td>(66)</td>
<td>Cu(II), Ag(I)</td>
<td><em>copA</em></td>
<td>11 μg/l Ag</td>
<td></td>
</tr>
<tr>
<td>HB101 (pRB28)</td>
<td><em>E. coli</em></td>
<td>(63)</td>
<td>Mercury</td>
<td><em>merR-merT</em></td>
<td>0.0136 μg/l HgCl₂</td>
<td>latex immob.</td>
</tr>
<tr>
<td>MC1061 (pmerRluxCDABE)</td>
<td><em>E. coli</em></td>
<td>(6)</td>
<td>Hg</td>
<td><em>merR</em></td>
<td>optical fibre</td>
<td></td>
</tr>
<tr>
<td>MC1061 (pmerR_BPmerlux)</td>
<td><em>E. coli</em></td>
<td>(65)</td>
<td>Hg, Cd</td>
<td><em>merRB</em></td>
<td>0.002 μg/l CH₃HgCl</td>
<td></td>
</tr>
<tr>
<td>MC1061 (pSLzntR/pDNPzntAlux)</td>
<td><em>E. coli</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>zntRA</em></td>
<td>3 μg/l CdCl₂</td>
<td></td>
</tr>
<tr>
<td>MC1061 (pSLcueR/pDNPcopAlux)</td>
<td><em>E. coli</em></td>
<td>(65)</td>
<td>Cu, Ag</td>
<td><em>copA</em></td>
<td>20 μg/l CuSO₄</td>
<td></td>
</tr>
<tr>
<td>OS8 (pDNmerR_BPmerlux)</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Hg, Cd</td>
<td><em>merRB</em></td>
<td>0.03 μg/l CH₃HgCl</td>
<td></td>
</tr>
<tr>
<td>OS8 (pDNpbrRPbrAlux)</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>pbrRA</em></td>
<td>40 μg/l HgCl₂</td>
<td></td>
</tr>
<tr>
<td>OS8:KnmerR_BPmerlux</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>cadRA</em></td>
<td>10 μg/l CdCl₂</td>
<td></td>
</tr>
<tr>
<td>OS8:KnmerR_BPmerlux</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Hg, Cd</td>
<td><em>merRB</em></td>
<td>0.8 μg/l HgCl₂</td>
<td></td>
</tr>
<tr>
<td>OS8::KnmerR_BPmerlux</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Cu</td>
<td><em>copA</em></td>
<td>8000 μg/l CuSO₄</td>
<td></td>
</tr>
<tr>
<td>OS8::KnzntRPzntAlux</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>zntRA</em></td>
<td>20 μg/l CdCl₂</td>
<td></td>
</tr>
<tr>
<td>OS8::KnpbrRPbrAlux</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>pbrRA</em></td>
<td>8 μg/l HgCl₂</td>
<td></td>
</tr>
<tr>
<td>OS9::KncadRPcadAlux</td>
<td><em>P. fluorescens</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>cadRA</em></td>
<td>5 μg/l HgCl₂</td>
<td></td>
</tr>
<tr>
<td>BR151 (pcadCPcadAlux)</td>
<td><em>S. aureus</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>cadCA</em></td>
<td>3 μg/l HgCl₂</td>
<td></td>
</tr>
<tr>
<td>BR151 (pcadCPcadAlux)</td>
<td><em>B. subtilis</em></td>
<td>(65)</td>
<td>Hg, Cd, Zn, Pb</td>
<td><em>cadCA</em></td>
<td>2 μg/l CdCl₂</td>
<td></td>
</tr>
</tbody>
</table>

All strains have *luxCDABE* as reporter, unless stated otherwise under notes. With two exceptions, all experiments were performed on culture plates, for the exceptions, see notes. Chr. = genes are inserted in the chromosome, instead of plasmids.

A large comparative study was performed by Ivask *et al.* (2001) with 13 newly constructed metal-inducible strains with *luxCDABE* as reporter. Also 6 unspecific constitutive strains were tested as controls, to be able to recognize general toxic effects of the compounds. Special attention was given to the effect of other types of bacteria as host organisms: three strains were *E. coli*, eight strains *P. fluorescens*, one *Staphylococcus aureus*, and one *Bacillus subtilis* (Table 6). LODs were mostly in the microgram per litre range, with two mercury strains in the nanogram per litre range. Of the Gram-negative bacteria, *E. coli*
was slightly more sensitive than *P. fluorescens*. Chromosomal insertion in the latter reduced background luminescence, but this did not lead to a higher sensitivity. Gram-positive bacteria were more difficult to modify, requiring a helper plasmid to prevent loss of the plasmid. They yielded quite similar LODs as the Gram-negative strains (65).

**Discussion**

Biosensors based on luminescent bacteria may prove to be a valuable additional tool for the monitoring of water quality and safety. By combining different stress-responsive promoters with the bioluminescence genes, strains have been constructed that react with varying degrees of specificity to toxic effects (e.g., DNA damage), or specific groups of chemicals (e.g., heavy metals). The main purpose of this review is to evaluate available *lux* strains and their perspective for use in on-line water quality and safety monitoring. A preliminary selection was made upfront for strains with the bacterial *luxCDABE* genes, as this enables a fast response without the need for substrate addition.

An important factor in the evaluation of the strains is whether the measured effect or compound(s) can be expected to cause toxic effects in humans and whether they have additional value above already existing techniques. Since all strains in this review are genetically modified, strains should preferably be based on non-pathogenic bacteria to be allowed for use outside a laboratory. Another aspect that is taken into account is the sensitivity of the strains, which is compared to actual levels of contaminants that have been found in Dutch rivers, as well as drinking water standards. Unfortunately, in most cases little information is available on selectivity and specificity, which limits the possibility to compare strains on these parameters. The same lack of information exists for the robustness and stability of the bacteria when they are used over prolonged periods in natural water.

**Effect-specific strains**

Of all effects that can be detected by bacterial strains, DNA damage is probably the most relevant for humans. Because it is an effect that causes little acute damage, existing biomonitors are not very sensitive to compounds causing this effect. However, it can have severe consequences in humans on the long term. A number of strains have been developed for the detection of DNA damage by coupling DNA repair genes to *luxCDABE*. To improve performance, some strains also contain different adjustments, like a *tolC* mutation or insertion of
Are luminescent bacteria suitable for on-line detection and monitoring

the genes in their chromosome. The most sensitive lux strains found in this study were two modified recA strains, namely DPD2797 and DPD3063 (12).

An important consideration when it comes to DNA damage that is rarely addressed is that many agents require metabolic activation before they become harmful to the DNA. This may be (partially) solved by adding rodent-derived cytochrome P450 (59). However, in an on-line biosensor this is very impractical. Another option would be to incorporate some of these enzymes in the lux strains themselves as has been done already in Salmonella typhimurium for the umu-test (for an overview see (14)). These modifications are very appealing, but since there is a large variety of P450 enzymes involved and still very little experience with such strains it is unlikely that they will be used in on-line biosensors in the near future.

Both protein and membrane damage strains are also referred to as strains for general damage or stress, as they are relatively unspecific and have overlapping target components. As a result, these strains are expected to have less additional value above the existing biomonitors than for example DNA damage strains. Additionally, compounds that also cause other types of damage (e.g. MNNG, paraquat) can usually be detected more sensitively with strains that react specifically on these effects. On the other hand, in a system that employs multiple strains they may be very useful, as they detect toxicants that diminish the response in other, more specific strains (21, 30).

For the detection of protein damage, strain TV1061 (grpE::luxCDABE) has probably the most favourable combination of sensitivity, specificity, and user experience (36). As all membrane damage strains have the fabA gene as promoter, differences in sensitivity, selectivity, and response times between these strains are minor (38).

Oxidative stress differs somewhat from the aforementioned effects, as it causes damage in itself to DNA, proteins, and membranes. Thus it can be expected that compounds that cause oxidative stress will be detected to some extent by the aforementioned strains. However, by using genes from specific defence regulons as promoters, strains have been generated that have an enhanced sensitivity for oxidative compounds. These strains can be divided in peroxide and superoxide sensing strains. Both types showed LODs of around 0.1 mg/l for respectively H$_2$O$_2$ and paraquat (45).
What might limit the usefulness of oxidative stress sensing strains in the field is the unstable nature of oxidative compounds. As no experimental field data is available on the occurrence of such compounds, it still has to be shown whether biosensors with these strains are of use.

**Compound-specific strains**

Additional to measuring a certain effect, one can also use a promoter that is activated in the presence of a specific compound or group of compounds. The advantage is that the detection limit for these compounds is usually lower in a compound-specific strain then in a strain that reports only the effect, often in the µg/l range. Because of their high specificity, they are generally less suitable for the monitoring of ‘normal’ surface or drinking water, as they are likely to miss many contaminations. However, in cases where only a specific type of contamination is of interest, they may be used as a fast and convenient detection method. Examples of such situations are after an incident or on (former) industrial sites (5). The choice for a certain strain will in such situation mainly depend on the toxicants involved.

**General remarks: comparability of studies referenced**

A comment that needs to be made when attempting to compare the performance of such a diverse selection of reporter strains is that it is practically impossible to directly compare levels of luminescence between studies. Depending on the instrumentation used, different units are used to express light intensity. These are often completely arbitrary, and presented as instrument-specific RLU (Relative Light Unit), Ampere or SBL (Specific Bioluminescence in nAmp/OD) (30, 37, 48, 67). Another complicating factor is that the number of photons that corresponds with 1 RLU differs per photomultiplier, even if they are of the same type and manufacturer.

However, most performance criteria can be calculated regardless of the units of luminescence used. The most common way to do this is by using response ratios to quantify the signal, which are defined as the ratio of luminescence relative to the un-induced control (21). The disadvantage of this method is that response ratios are strongly influenced by the background luminescence. As a result, the relationship between response ratios and actual luminescence differs per strain, with in general lower maximal response ratios in strains with high background luminescence (65, 68). Most studies with response ratios also give a graph with the luminescence levels in RLU's, to give some insight in the absolute difference
between signal and background (12). Another possibility is to use the absolute difference in luminescence between background and induced cells (68).

The Limit of Detection has also been determined in several different manners. If response ratios are used, the LOD is usually defined as the concentration that leads to a two fold induction of luminescence over the background, i.e. a response ratio of two (38). In studies that express light intensity in RLUs, the LOD is often defined as the concentration that leads to a significantly higher luminescence compared with the control (69). It should be mentioned that the LOD is not only dependent on the strain, but also the inducer, cell concentration, bioavailability, measurement time and operational protocol. These differences in determining induction and LODs should be kept in mind when comparing studies, especially when the differences are relatively small. In this study, the LODs given have been determined by the original authors of the studies. However, for the comparisons between studies only differences in LOD of a factor of ten or more for the same component are considered relevant.

A similar observation may be made concerning the determination of response or lag times, which are highly dependent on the concentrations used. Nevertheless it can be said that, on average most lag times are around 60 minutes, with a few fast responders of 30 minutes or less and slow responders of 90 minutes or more. In case of an on-line biosensor that is used as an early warning system in surface water, a response time of around one hour will usually be fast enough for detecting passing contamination peaks. There will then be enough time to react to an alarm from the sensor, before the contaminated water reaches the treatment plant inlet or ultimately the consumers as drinking water. For alarm monitoring of drinking water in distribution networks, naturally, response times will have to be much shorter.

**Biosensing pollutants in surface water**

The most important question that remains is whether these bacteria can actually detect the levels of toxicants that occur in reality. To truly answer this question, it would be necessary to perform field studies. However, an estimate can be made by comparing the LODs with peak levels of compounds of concern that have been found recently in surface water, and with the target values for contaminants of drinking water.
A large number of studies have been performed to determine levels of pollutants in surface water, of which a few examples will be given. A survey was performed by Loos et al. (2009) to determine the occurrence of polar organic pollutants in European rivers. The highest concentrations measured were 31 µg/l ibuprofen, 39.8 µg/l caffeine, 19.4 µg/l tolyltriazole, and 11.6 µg/l carbamazepine (70).

In the Netherlands, measurements have also been performed after large spills. There were 49 large spills reported in 2008 in the Rhine, of which most involved BTEX compounds, MTBE (methyl tertiary butyl ether), ETBE (ethyl tert-butylether), or a combination of these substances. The highest concentration measured after an incident was 91 µg/l trichloromethane. The peak concentrations measured in routine measurements were generally lower, for example 6.0 µg/l MTBE (Nieuwegein), 2.58 µg/l ETBE (Lobith), 1.3 µg/l toluene (Nieuwegein), and 7.8 µg/l lead (Nieuwegein) (71).

In two recent literature surveys, emerging contaminants and trace pollutants were evaluated on their occurrence and toxicity (72, 73). The highest priority was given to compounds that are both a human health hazard and are frequently detected in surface water. In table 7 a summary of the highest ranking compounds is displayed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Common use</th>
<th>Max conc</th>
<th>Location</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>Anticonvulsant</td>
<td>12</td>
<td>EU</td>
<td>(70)</td>
</tr>
<tr>
<td>1,4-dioxane</td>
<td>Solvent</td>
<td>10</td>
<td>EU</td>
<td>(73)</td>
</tr>
<tr>
<td>17 α-ethinyl estradiol (EE2)</td>
<td>Hormone</td>
<td>0.83</td>
<td>US</td>
<td>(74)</td>
</tr>
<tr>
<td>17 β-estradiol</td>
<td>Hormone</td>
<td>0.2</td>
<td>US</td>
<td>(74)</td>
</tr>
<tr>
<td>Estrone</td>
<td>Hormone</td>
<td>0.11</td>
<td>US</td>
<td>(74)</td>
</tr>
<tr>
<td>Perfluorooctanoic acid (PFOA)</td>
<td>Water proofing</td>
<td>19</td>
<td>US</td>
<td>(75)</td>
</tr>
<tr>
<td>Perfluorooctanesulfonic acid (PFOS)</td>
<td>Water proofing</td>
<td>1.4</td>
<td>EU</td>
<td>(70)</td>
</tr>
<tr>
<td>Bis(2-ethylhexyl)phthalate</td>
<td>Plasticizer</td>
<td>98</td>
<td>EU</td>
<td>(76)</td>
</tr>
<tr>
<td>Diazinon</td>
<td>Insecticide</td>
<td>1.1</td>
<td>US</td>
<td>(77)</td>
</tr>
<tr>
<td>Methoxychlor</td>
<td>Herbicide</td>
<td>1.7</td>
<td>EU</td>
<td>(78)</td>
</tr>
<tr>
<td>Dieldrin</td>
<td>Insecticide</td>
<td>0.21</td>
<td>US</td>
<td>(74)</td>
</tr>
<tr>
<td>N,N-diethyl-meta-tolumide (DEET)</td>
<td>Insect repellant</td>
<td>1.1</td>
<td>US</td>
<td>(74)</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Antiseptic</td>
<td>2.3</td>
<td>US</td>
<td>(74)</td>
</tr>
<tr>
<td>Acetaminophen</td>
<td>Pain reliever</td>
<td>10</td>
<td>US</td>
<td>(74)</td>
</tr>
</tbody>
</table>

All concentrations are in µg/l.
The WHO (World Health Organization) has set guideline values for drinking water for some chemicals, including arsenic (10 µg/l), cadmium (3 µg/l), mercury (6 µg/l), and toluene (700 µg/l) (79). These levels can be detected with strains specific for these compounds. However, for the majority of the detected compounds, no specific guideline values have been determined yet. With analytical methods becoming more and more sensitive, the number of contaminants detected in drinking water is increasing, while their levels are mostly not of any health concern. Recently, target values have been set by the Dutch drinking water utilities to define what levels of contaminants are acceptable from both a human health perspective, as well as from an ethical or esthetical perspective. This latter perspective stems from the philosophy that contaminants do not belong in drinking water. Based on the Thresholds of Toxicological Concern (TTCs) set for food additives and on the opinions of Dutch drinking water experts of what is ethically acceptable, target values of 0.01 µg/l for genotoxic contaminants and 0.1 µg/l for other contaminants have been derived (1, 73, 80).

When compared to the TTC derived target values for drinking water, the detection thresholds of both effect- and compound-specific strains are generally too high to be used for monitoring. The only exceptions are the strains for the detection of mercury with a lowest LOD of 0.002 µg/l for methyl mercury (65).

However, the peak concentrations that have been measured in surface water are much higher, as is also expected to be the case after intentional water poisoning. In these cases, sensors based on lux strains may be very useful to detect spills in an early stage, provide an early warning of such events and prevent the intake of contaminated water.

Additionally, there are also various efforts being undertaken to improve the sensitivity of luminescent strains by using different or additional modifications. The insertion of the promoter-reporter genes in the chromosome and the addition of a tolC mutation have already been mentioned. In an article in this same special issue, Yagur-Kroll and Belkin demonstrate that both the sensitivity and response times can also be improved by splitting the luxCDABE genes in two separately controlled units. The best effect was gained when luxAB (the luciferase enzyme) was inducible by a promoter and luxCDE (the aldehyde substrate) was expressed constitutively (81).
What also should be noted is that contaminations often consist of mixtures of compounds. Compounds exerting a similar type of effect through a similar mode of action often behave additively in mixtures, but sometimes synergism and antagonism can also occur with compounds with a different mode of action, interfering with the effects observed. Depending on the strain and compounds involved, this can lead to either improvement or reduction of the ability of the bacteria to detect the collective activity of a complex mixture as a sum parameter. At this moment, little is known about the significance of these mixture reactions for lux strains.

Another issue that is of great importance for the application of biosensors is the ability of the bacteria to remain alive and active in natural water. This is of course also dependent on the design of the sensor and the matrix in which the bacteria are contained. Nevertheless, differences in robustness between species of bacteria can be expected.

So far, most studies have been performed with E. coli K12, as these are non-pathogenic and relatively easy to modify. Since E. coli is originally an inhabitant of the intestine, it is not very robust when exposed to natural water. Several studies have shown that especially the presence of other microorganisms is detrimental for the survival of E. coli in unsterilized water (82-84).

However, very few other species have been used to construct lux strains. Only for the detection of organic pollutants like toluene and naphthalene it is common to use different bacteria, like the natural soil bacteria P. putida and P. fluorescence (47, 52, 85). P. fluorescence has also been used in the development of metal sensing strains, while cadA combined with luc or luxCDABE has been brought into B. subtilis and S. aureus. Of these two, S. aureus is a common resident of human skin, while B. subtilis is a non-pathogenic soil bacterium (65, 86).

Although these bacteria are generally no more sensitive than E. coli, they might be more robust as water is often their natural habitat, which makes it likely they will remain active over a longer time. However, it still has to be confirmed whether they indeed yield better results in an on-line sensor.

**Selection of bacterial strains**

A choice had to be made out of the available strains for implementation in a biosensor for the monitoring of the chemical water quality of surface water in the Netherlands.
The most interesting strains for continuous water screening are those that detect toxic effects that are relevant for humans, such as genotoxicity and endocrine disruption. Strains for general toxicity, like protein-damage strains or unmodified bacteria have a lower priority, as they overlap in sensitivity and selectivity with the biomonitors. On the other hand, they can be very useful in a multi-strain sensor or on a location where there are no other biomonitors yet. Compound-specific strains are usually more sensitive than the effect specific-strains. In comparison with chemical analysis, they are less sensitive and accurate, but faster and more suitable for on-line or field monitoring. As a result of their limited range of detectable compounds, compound specific strains will usually not be the first choice for use in an on-line biosensor. They may be used when certain compounds are of specific interest or in a system that employs an array of strains.

For the monitoring of Dutch surface water the highest demand is for strains that detect effects that are relevant for humans, therefore an initial choice has been made for strains that detect DNA damage and membrane damage. Membrane damage was included because these strains were expected to respond also to neurotoxicants. Unfortunately, no strains were available that respond to endocrine disruptors, otherwise these would also have been viable options. Since no alternative, more robust, non-pathogenic alternatives are available, both selected strains had *E. coli* as host species.

**Conclusion**

A large number of luminescent bacteria have been developed for the detection of various toxic effects and compounds. Although most studies so far have been performed in the laboratory, implementation in a biosensor in the field holds the highest promise for these bacteria. The currently existing strains have detection thresholds that range from mg/l to µg/l. In general, more specific strains tend to be more sensitive. Since peak concentrations after incidents in Dutch rivers are generally in the low µg/l range, only a few strains will be capable of detecting individual compounds. Sensitivity improvement is therefore necessary. Nevertheless, these strains may have added value above existing techniques in the detection of mixtures of toxicants and in early warning systems. Other issues that need to be addressed in case of field use include response times, robustness, signal quantification, and pathogenicity.
Acknowledgements

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Are luminescent bacteria suitable for on-line detection and monitoring

References


Chapter 2


Are luminescent bacteria suitable for on-line detection and monitoring

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Are luminescent bacteria suitable for on-line detection and monitoring

The intake of Meuse water at monitoring station Keizersveer
Chapter 3

Responsiveness of the genetically modified bacterial strain DPD2794 for the detection of genotoxic compounds

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Abstract
The aim of this study was to investigate the applicability of the genetically modified Escherichia coli strain DPD2794 for ultimate use in on-line sensors for the detection of direct-acting genotoxic compounds in water. For this purpose, the response of strain DPD2794 to 27 chemicals was analyzed using a 96-well microplate assay setup. This was done without a metabolic activation system, because at present it is impossible to include metabolic activation in on-line sensors. The compounds tested included a list of 13 genotoxic and non-genotoxic compounds recommended by the European Centre for the Validation of Alternative Methods (ECVAM) for the performance assessment of new genotoxicity tests, as well as a list of 14 environmental pollutants. Three of the 13 tested ECVAM-recommended chemicals gave a positive response, namely nalidixic acid, mitomycin C, and 4-NQO. This was in accordance with results reported for these compounds in the Ames mutagenicity assay, without metabolic activation. The compounds that required metabolic activation, namely cyclophosphamide, 2,4-diaminotoluene and p-chloroaniline, did not induce a response in strain DPD2794. There was one false positive result for chloramphenicol, which was due to cytotoxicity, rather than a genuine genotoxic response. None of the other negative compounds induced a response in strain DPD2794. None of the 14 environmental pollutants showed a positive response using strain DPD2794 in the microplate assay. This is in accordance with Ames test results reported in literature for several of these compounds. In conclusion, the bacterial strain DPD2794 appears to be reliable in detecting direct-acting genotoxic substances.
Introduction

Although several in vitro mutagenicity assays exist based on bacterial or mammalian cells (1), there are no methods available for the on-line detection of genotoxic compounds in water. Such on-line monitoring is of interest at drinking water inlet points of surface water, for example, where the water quality can change quickly, e.g. in case of chemical spills. Fast detection of the presence of harmful compounds enables operators to temporarily close the inlet in time to prevent that water with a spill of these harmful chemicals is used for drinking water production. Several biomonitors, based on, for example, Daphnia or algae, are already used for this purpose, but these can not detect genotoxic compounds at low concentrations. Luminescent bacteria are promising for on-line monitoring, since luminescence can be measured quickly on-line at high sensitivity. In addition, bacteria are more robust in changing environmental conditions than e.g., mammalian cells (2-4).

In recent years, much effort has been invested in the development of genetically modified bacteria that respond to specific groups of pollutants by emitting luminescence (5). Often, these bacteria have a gene for bacterial luciferase, coupled to a promoter gene that is part of a stress response. Bacterial strains of high interest for drinking water inlet monitoring are strains for the detection of DNA damage (6).

One important matter however, for which there has been limited attention, is the sensitivity and selectivity of these bacteria for wide range of compounds. So far, experiments with these bacteria have mainly focussed on providing a 'proof of principle' and included only a limited number of positive and negative control compounds (7-11). However, little is known about the ability of these bacterial strains to detect compounds that can be encountered in field situations. When these bacteria are to be used in a sensor in the field, more information is necessary on their response to various chemical substances.

For this reason, a response characterization was performed for strain DPD2794, a commonly used strain for the detection of DNA damage. DPD2794 is a genetically modified Escherichia coli, in which the recA promoter is coupled to the bacterial luciferase gene, luxCDABE (7, 12). Activation of the recA gene normally leads to the production of a RecA filament, which activates the auto
cleavage of the LexA repressor. This process results in the activation of the SOS response, which is a bacterial DNA damage repair system (13). In strain DPD2794, activation of the recA gene is also coupled to the production of bacterial luciferase and its aldehyde substrate, which in turn generates the production of light with a wavelength of 490 nm.

In this study, strain DPD2794 was exposed to concentration ranges of 27 compounds, without the addition of a metabolic activation system. The reason for this is that we are aiming to apply strain DPD 2794 in an on-line sensor system, and at present it is impossible to use a metabolic activation step in conjunction with an on line sensor setup. The compounds tested can be divided in two groups. The first group of compounds was based on the lists of European Centre for the Validation of Alternative Methods (ECVAM) for genotoxic and non-genotoxic compounds, which are recommended for assessing the performance of new genotoxicity tests (14). A selection of 13 compounds was made from these lists with, as recommended at least one compound from each group (table I). These included mutagenic carcinogens that should be positive, non-DNA reactive compounds that should be negative, and compounds that should be negative, but have been reported positive due to chromosomal aberrations.

Many mutagenic compounds in the list require metabolic activation, of which several were included in this study. To increase the number of positive controls, three genotoxic compounds were added which are not listed by Kirkland et al. (2008), but had been used previously as positive controls for the same bacterial strain (15).

The second group consisted of 14 compounds that have often been found as contaminants in Dutch surface water used for drinking water production and thus may be encountered when testing in the field, using the on-line sensor (table II) (16, 17). For this reason it was very relevant to determine whether these compounds induced a response and if so, at which concentration. In this study, the compounds were not tested in an on-line sensor, but in 96-well plates, to increase the efficiency of testing multiple compounds at multiple doses in several replicates. This had the disadvantage that a static exposure, with limited nutrient availability, was applied, which would be different in an on-line sensor. However, as the aim was to obtain an impression of the ability of the bacterial strain to respond to genotoxic substances, this
Responsiveness of the genetically modified bacterial strain DPD2794 disadvantage was not deemed too important. The bacteria were exposed, and their response was measured, for 24 hours, as it was aimed to have the bacteria exposed for at least 24 hours in the on-line sensor, too. Their response over time was therefore of interest. The results have been compared with the results of other genotoxicity tests, with emphasis on the Ames test. Based on this comparison, the usefulness of these bacteria for a field sensor was evaluated.

Materials and Methods

Chemicals
Ampicillin, mitomycin C, 4-nitroquinoline-N-oxide (4-NQO), cyclophosphamide, 2,4-diaminotoluene, p-chloroaniline, sodium saccharin, amitrole, p-nitrophenol, carbamazepine, nitrilotriacetic acid (NTA), tetrachloroethane, benzotriazole, and metham sodium were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands).
Peptone, glycerol, lysogeny broth (also known as Luria-Bertani medium or LB), agar, dimethylsulfoxide (DMSO), nalidixic acid, cyclohexane, tertiary-butylhydroquinone (TBHQ), urea, di(2-ethylhexyl)phthalate (DEHP), methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE), glyphosate, aminomethylphosphonic acid (AMPA), diuron, and atrazine were purchased from Boom (Meppel, The Netherlands).
Chloramphenicol was purchased from Fagron BV (Capelle aan de IJssel, The Netherlands). Iomeprol was purchased as a solution of 612.4 mg/ml from a local pharmacy and produced by ALTANA Pharma BV (Hoofddorp, The Netherlands).
Dimethylnitrosamine-D6 (NDMA-D6\textsuperscript{1}) was purchased from Buchem BV (Apeldoorn, The Netherlands). All chemicals except iomeprol were of analytical grade and stored as suggested by the manufacturers instructions.
Stock solutions of mitomycin C, nalidixic acid, 4-NQO, tetrachloroethane, diuron, p-chloroaniline, p-nitrophenol, cyclophosphamide, cyclohexane, MTBE, urea, chloramphenicol, ETBE, atrazine, TBHQ, carbamazepine, DEHP, amitrole, sodium saccharin, iomeprol, 2′4-diaminotoluene, NDMA-D6, and metham sodium were prepared in DMSO and stored at -80 °C.
Stock solutions of amitrole, NTA, benzotriazole, glyphosate, and AMPA were prepared in ultrapure water because of the poor solubility in DMSO and stored

\textsuperscript{1} Deuterated NDMA was used since NDMA was not available
at 4-7 °C. The concentrations of the stock solutions were a 100 times higher than the highest concentration tested, see table I and II.

**Bacteria and growth conditions**
The genetically modified *E. coli* strain DPD2794 was obtained as a kind gift from R. Marks (Hebrew University, Jerusalem, Israel). DPD2794 is based on *E. coli* strain RFM443, in which the plasmid pRecAlux3 has been incorporated. This plasmid contains the *recA* promoter coupled to the *luxCDABE* genes, as well as resistance genes for ampicillin and kanamycin.

Stock cultures of bacteria were stored at -80 °C in 25% (v/v) glycerol medium. Colonies for daily use were maintained at 4 °C on LB-agar plates, supplemented with 100 mg/l ampicillin. Before each experiment, bacteria were grown overnight at 37 °C in an incubator (ETK combi, Elbanton, Kerkdriel, The Netherlands) on a rotary shaker (KS-500, Kika-werk) at 100 rpm in LB which contained 100 mg/l ampicillin. The culture was then diluted to an OD$_{600}$ of 0.2 as determined by a spectrophotometer (Jenway 6300 VIS, Staffordshire, England).

**Experimental procedure**
For all tested substances, concentration series were made of 9 to 14 concentrations per compound in DMSO (see tables 1 and 2). Before each experiment, the solutions were further diluted 25-fold by adding 4 µl of each concentration to 96 µl sterile tap water.

All experiments were performed in white 96-well plates (Nunc, Roskilde, Denmark). In each well, 25 µl diluted aqueous solution of the compounds was mixed with 25 µl LB and 50 µl bacterial culture, thus reaching a total dilution of a hundred times. When DMSO was used as solvent, all negative controls received a similar 1% DMSO. All concentrations were tested in triplicate. The tests with mitomycin C, 4-NQO, sodium saccharin, cyclophosphamide, glyphosate, carbamazepine, and ETBE were repeated on a different day as an independent control. The tests with nalidixic acid, chloramphenicol, and benzotriazole were repeated four times on different days. These repeats were used to determine the variation between different experiments for this assay.

Luminescence measurements were taken every 15 minutes for 23 hours in a Luminoskan Ascent (Thermo Fisher Scientific, Breda, The Netherlands) plate luminometer. During the experiments the temperature was 26 °C and the
plates were shaken with 60 rpm. The experiments were performed by 26 °C, because the luciferase enzyme becomes inactive above 30 °C.

Data analysis
As can be seen in figure 2A-E, the bacteria always gave a background response, which was similar between experiments. For the determination of the limit of detection (LOD), the mean values of the background curves of the compound series that were repeated five times (n=15) plus three times the standard deviation were plotted for each time point. Above these absolute values, a response can be seen as deviating significantly from the background. As the mean and standard deviation varied in time, it was more convenient to convert these absolute values to ratio's and take the worst case scenario. Therefore, the resulting absolute value at each time point (mean + 3*stdev of background) was then divided by the mean value at the same time point, which resulted in a set of ratio's over time. Between two and ten hours (the timeframe where the genotoxic compounds produced a peak in luminescence, and thus the most relevant timeframe); the highest value for this ratio was 1.9.

The mean of the responses to a sample (n=3) was also divided by the mean of the background measured in the same series (n=3), at the time point where the difference between the background and exposed bacteria was the largest. Due to the different characteristics and concentrations of the compounds this point was not fixed, but always lied between two and ten hours from the start of the experiment. The lowest concentration that induced a ratio of more than 1.9 was considered the limit of detection, because this was the lowest concentration of which the response was observed to deviate significantly from the variable background response.

In figure 1, the results of nalidixic acid are used as an example. It can be seen that a concentration of 0.5 mg/l gave a rise in luminescence of more than 1.9 times the background after 2 hours, while the response on a concentration of 0.1 mg/l remained below this level. Thus the limit of detection for nalidixic acid was determined to be 0.5 mg/l.
Figure 1: An example of the determination of the LOD for nalidixic acid. The concentration of 0.5 mg/l gave a response with a response ratio of more than 1.9 times the background, and was thus considered the detection limit for this compound.

Table 1: Overview of the results of the selected compounds recommended for validation of genotoxicity tests.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CAS nr.</th>
<th>DPD2794 response</th>
<th>LOD (mg/l)</th>
<th>Conc. range tested (mg/l)</th>
<th>Max absolute induced response</th>
<th>Ames result (18)</th>
<th>IARC classification (19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nalidixic acid</td>
<td>389-08-02</td>
<td>+</td>
<td>0.5</td>
<td>0.001 - 10</td>
<td>1081</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>mitomycin C</td>
<td>50-07-07</td>
<td>+</td>
<td>0.0001</td>
<td>0.0001 - 1</td>
<td>2263</td>
<td>+</td>
<td>2B</td>
</tr>
<tr>
<td>4-NQO</td>
<td>56-57-5</td>
<td>+</td>
<td>0.5</td>
<td>0.001 - 10</td>
<td>814</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>cyclophosphamide</td>
<td>6055-19-2</td>
<td>-</td>
<td>-</td>
<td>1 - 50</td>
<td>-</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>chloramphenicol</td>
<td>56-75-7</td>
<td>+</td>
<td>0.75</td>
<td>0.05 - 10</td>
<td>76</td>
<td>-</td>
<td>2A</td>
</tr>
<tr>
<td>urea</td>
<td>57-13-6</td>
<td>-</td>
<td>-</td>
<td>0.1 - 1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cyclohexane</td>
<td>110-82-5</td>
<td>-</td>
<td>-</td>
<td>1 - 100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TBHQ</td>
<td>1948-33-0</td>
<td>-</td>
<td>-</td>
<td>0.5 - 50</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sodium Saccharin</td>
<td>128-44-9</td>
<td>-</td>
<td>-</td>
<td>5 - 100</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>amitrole</td>
<td>61-82-5</td>
<td>-</td>
<td>-</td>
<td>5 - 100</td>
<td>-</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>2, 4 – diaminotoluene</td>
<td>95-80-7</td>
<td>-</td>
<td>-</td>
<td>0.5 - 200</td>
<td>+</td>
<td>(59)</td>
<td>2B</td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td>100-02-7</td>
<td>-</td>
<td>-</td>
<td>0.01 - 10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>P-Chloroaniline</td>
<td>106-47-8</td>
<td>-</td>
<td>-</td>
<td>0.01 - 10</td>
<td>+</td>
<td>(59)</td>
<td>2B</td>
</tr>
</tbody>
</table>

1) The absolute highest difference between the response and the background
2) Metabolic activation was required for a response in the Ames test
Responsiveness of the genetically modified bacterial strain DPD2794

The repeatability between experiments was determined for all positive measurements and expressed as the relative standard deviation (n=2-5) of the mean absolute response of each replicate experiment at the time of the highest response and at the concentration closest to the EC\textsubscript{50} concentration. Also the average variation, expressed as relative standard deviation between triplicates within each experiment, was calculated for the same time and concentration.

Table 2: Overview of the results of the environmental compounds. ND = No Data

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CAS nr.</th>
<th>DPD2794 response</th>
<th>Conc. range tested (mg/l)</th>
<th>Use</th>
<th>Ames test result (18)</th>
<th>IARC classification (19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>carbamazepine</td>
<td>298-46-4</td>
<td>-</td>
<td>0.01 - 10</td>
<td>Drug</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>iomeprol</td>
<td>78649-41-9</td>
<td>-</td>
<td>7.5 - 100</td>
<td>Drug</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>DEHP</td>
<td>117-81-7</td>
<td>-</td>
<td>0.01 - 10</td>
<td>Plasticizer</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MTBE</td>
<td>1634-04-4</td>
<td>-</td>
<td>7.5 - 100</td>
<td>Gasoline additive</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>ETBE</td>
<td>637-92-3</td>
<td>-</td>
<td>7.5 - 100</td>
<td>Gasoline additive</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>glyphosate</td>
<td>1071-83-6</td>
<td>-</td>
<td>0.01 - 10</td>
<td>Herbicide</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AMPA</td>
<td>1066-51-9</td>
<td>-</td>
<td>0.001 - 10</td>
<td>Glyphosate metabolite</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NTA</td>
<td>139-13-9</td>
<td>-</td>
<td>0.001 - 10</td>
<td>Chelating agent</td>
<td>-</td>
<td>2B</td>
</tr>
<tr>
<td>diuron</td>
<td>330-54-1</td>
<td>-</td>
<td>0.001 - 10</td>
<td>Herbicide</td>
<td>+ (S9)</td>
<td>-</td>
</tr>
<tr>
<td>atrazine</td>
<td>1912-24-9</td>
<td>-</td>
<td>0.01 - 50</td>
<td>Herbicide</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>benzotriazole</td>
<td>95-14-7</td>
<td>-</td>
<td>0.01 - 100</td>
<td>Corrosion inhibitor</td>
<td>+ (S9)</td>
<td>-</td>
</tr>
<tr>
<td>metham sodium</td>
<td>137-42-8</td>
<td>-</td>
<td>0.01 - 21</td>
<td>Fungicide</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>NDMA-D6</td>
<td>17829-05-9</td>
<td>-</td>
<td>7.5 - 250</td>
<td>Industrial chemical</td>
<td>ND</td>
<td>-</td>
</tr>
<tr>
<td>tetrachloroethane</td>
<td>79-34-5</td>
<td>-</td>
<td>1 - 10</td>
<td>Solvent</td>
<td>ND</td>
<td>3</td>
</tr>
</tbody>
</table>

Results and Discussion

Luminescence response of selected group of recommended compounds

The first group of compounds tested was a selection from lists of genotoxic and non-genotoxic compounds that are recommended by ECVAM for the validation of new genotoxicity assays (14). The advantage of using these lists is that these compounds are relatively well characterized with respect to their mutagenic and carcinogenic potential. The most important disadvantage in
this particular case is that most compounds from these lists require metabolic activation before they become mutagenic. Since it is currently not feasible to use metabolic activation in an on-line sensor, the decision was made to test the performance of the bacteria without metabolic activation. Thus, three compounds were added which have been reported positive in the Ames test without metabolic activation and have been used previously as positive controls for DPD2794 (15, 18): nalidixic acid, mitomycin C, and 4-NQO. A summary of the results is given in table I.
Responsiveness of the genetically modified bacterial strain DPD2794

Figure 2: Average response of DPD2794 to positive and negative controls: (a) mitomycin C (n=6), (b) an enlargement of the lower concentrations of mitomycin C, (c) 4-NQO (n=6), (d) nalidixic acid (n=15), and (e) cyclophosphamide (n=15). All experiments were performed over 23 hours and the concentrations are the final concentrations in the wells.
In figure 2, the response of DPD2794 is shown for the positive compounds mitomycin C, 4-NQO, and nalidixic acid, and for the negative compound, cyclophosphamamide. It was observed that the DPD2794 strain always emitted a certain amount of background luminescence, especially in the first five hours. This is probably caused by the normal metabolic and reproductive activity of the bacteria. In the beginning of the response curve, the bacteria always showed a steep rise in luminescence. In case of mutagenic compounds, a response became visible about one hour after the start of the exposure, although the exact time and pattern depended on the concentration and the compound. The negative compounds either did not show any deviation from the background response, or showed a decrease in luminescence below the normal background curve. (e.g. figure 2E, note that the maximum response is only about 80 relative light units, i.e. much lower than in the case of exposure to positive compounds)

Of the compounds that were reported negative in the Ames test, or that required metabolic activation, only chloramphenicol induced a positive response. This was unexpected, since chloramphenicol has been reported as negative in the Ames test, Vitotox, RadarScreen, and GreenScreen HC assays. The latter three of these assays used reporter genes coupled to DNA repair responses in respectively Salmonella typhimurium, yeast, and human cells (20, 21). The Vitotox test is based on the SOS response, which is the same DNA repair response as used in DPD2794. Chloramphenicol has been reported as positive in chromosomal aberrations tests and the micronucleus test (14). It may be possible that compounds causing chromosomal aberrations induce the SOS response in bacteria, but since the Vitotox test was negative, this is not likely. When looking at the response at different concentrations of chloramphenicol as depicted in figure 3, there is a clear decrease in luminescence at the highest concentrations, probably due to cytotoxicity. This can be easily explained since chloramphenicol is widely used as an antibiotic and inhibits the total protein synthesis in most bacteria (22). At concentrations of 2.5 and 0.75 mg/l, the standard initial peak is at the same level as the background, but is delayed. This might be caused by some degree of cytotoxicity or by a decrease in growth rates of the bacteria. However, this results in the situation that the peaks at these concentrations occur at a time when the background has already dropped to lower levels. This may have caused the response ratio’s to be more than 1.9
Responsiveness of the genetically modified bacterial strain DPD2794

(see section 2.4), thus indicating genotoxicity, while the response might not be due to genotoxicity. It is unknown what causes the initial peak in all curves, including the background curves. We have the impression it is caused by a growth explosion due to the fresh medium, with the growth and thus duplication of DNA leading to more DNA repair (to enable correct duplication) and thus luminescence. This could give artefacts in any genotoxicity assay based on DNA repair induction, which could be identified with time-plots of the response as produced here.

It should be mentioned that, although chloramphenicol is probably falsely positive in wells plates, it is very unlikely to give a positive response in a field sensor. In a sensor, no standard time point can be applied for the determination of a positive response. Instead, the signal will have to increase above a certain continuous level to trigger an alarm. Chloramphenicol however, is expected to give a decrease in signal and as such is expected to be identified as cytotoxic rather than genotoxic.

Probable cytotoxicity was also perceived after exposure to TBHQ, an antioxidant. In contrast to chloramphenicol, the response curve of this compound decreased in a concentration dependent manner without a shift in time. Thus, the response ratio never increased over 1.9. The reason for this difference in inhibition is unknown.
In summary, the three compounds expected to give a positive response in this bacterial strain were indeed positive. Of the ten compounds expected to give a negative response nine were indeed negative and one false positive.

Environmental compounds
The second group of tested compounds consisted of 14 chemicals that have been detected as pollutants in water used for drinking water production (23, 24). Agents with different uses and structures were selected, including drugs, herbicides, and industrial chemicals (table II). None of these compounds induced a response which was positive within the definitions of this study (see section 2.4). This was according to expectation, as available data for these compounds also do not indicate any genotoxic properties of these compounds (table II). Metham sodium gave a concentration dependent decrease of the background luminescence, probably due to cytotoxicity.

However, one compound warrants further discussion, namely benzotriazole. As can be seen in figure 4, the response curve of benzotriazole is similar to the curve of a negative compound, with exception of the period between 7 and 18 hours. Within this period, a response ratio was obtained of more than 1.9. However, this was not occurring at the moment of the highest difference between background and response, which occurred in the first five hours. Moreover, this response only occurred at concentrations of at least 80 mg/l, which is so high that its relevance is questionable. Nevertheless, it was concentration dependent and consistent over all five replicate experiments. It should also be mentioned that this effect was not observed with any of the other compounds.

Unfortunately, the amount of genotoxicity data on benzotriazole in literature is very limited. Some mutagenicity and carcinogenicity studies have been performed, but also these studies show contradicting results. Both positive and negative results have been reported for the Ames test with S. typhimurium and E. coli, both with and without metabolic activation (25).

Thus, there are indications that benzotriazole has some mutagenic potential, but if this is the case, it is very weak. As such, it is highly unlikely that this compound can be detected by a sensor employing genetically modified bacteria in the field, as the concentrations in the environment are generally too low to induce a response. It would be interesting, nevertheless, to determine if benzotriazole is really genotoxic and to test the effect of benzotriazole when it is administered in a mixture with stronger inducers.
Figure 4: (a) Average response in time for 23 hours exposure of DPD2794 to different concentrations of benzotriazole (n=15). (b) An enlargement of the part of the curve between 7 and 18 hours.

3.3 Sensitivity and repeatability
The strain 2794 appeared to be most sensitive for mitomycin C, which already induced a response at a concentration of 0.0001 mg/l. This limit of detection is lower than those reported in earlier studies with the same strain (7, 8, 15). This can be explained by the fact that at the concentrations below 0.001 mg/l, the response is equal to the control until approximately seven hours after the start of the induction (Figure 2B), while in most studies the response is not measured for such long periods of time (7, 8, 11). The reason for this response pattern is not clear; it is interesting to see that the higher concentrations all give double
peaks in their responses and that the second peak coincides with the response of the low concentrations. Also noteworthy is that this pattern seems to be typical for mitomycin C, as it is not observed in the responses for nalidixic acid or 4-NQO, which implies there is an association of the response pattern with the mode of action of the compounds. Mitomycin C is used as an antimicrobial and antitumor agent and causes DNA damage by a combination of DNA alkylation and the formation of reactive oxygen species (26). Nalidixic acid binds to DNA gyrase in bacteria and thus stabilizes normally transient DNA-gyrase complexes formed during cell division, which can subsequently lead to DNA breaks (27). 4-NQO reacts with DNA and forms bulky adducts (28). To determine whether these differences in mechanism are indeed the cause of the differences in the appearance of the response, it would be necessary to test more compounds with similar effects.

A consequence of the difference in potency of the compounds is that if this strain is used in a sensor in the field, quantitative measurements are probably not feasible. In that case the focus will be on a qualitative determination of the occurrence of mutagenicity. When a positive signal is measured, additional experiments can be performed to determine the exact compound and concentration.

The repeatability between and within experiments was determined for the positive compounds nalidixic acid, mitomycin C, and 4-NQO. The concentrations closest to the EC\textsubscript{50} were used, namely 1 mg/l for nalidixic acid and 4-NQO, and 0.1 mg/l for mitomycin C. The repeatability was calculated at the time point where these concentrations on average yielded the highest response and was expressed as relative standard deviation. The variation between experiments for mitomycin C was 2.16% (n=2), for nalidixic acid 18.39% (n=5), and for 4-NQO 13.16% (n=2). The average variation between triplicates within the same experiment was 18.30% for mitomycin C, 17.35% for nalidixic acid, and 11.55% for 4-NQO. From these numbers it can be concluded that the variation between experiments was very acceptable for a biological system.

**Conclusions**

The response of the genetically modified *E. coli* strain DPD2794 for the detection of DNA damage was determined for 27 compounds: 13 listed genotoxicity test validation compounds and 14 compounds detected regularly in surface waters.
The three compounds expected to give a positive response in this bacterial strain were indeed positive. No metabolic activation was applied, because the purpose was to determine the suitability of the strain for use in an on-line sensor. As a result, the three genotoxic compounds that require metabolic activation were negative. The Ames negative compounds were indeed negative, except for chloramphenicol, which in turn out to be positive, probably due to an effect of cytotoxicity rather than a genuine genotoxic compound. This is unlikely to be an issue in an on-line sensor, since it will initially give a decrease in luminescence. It may therefore be concluded that this limited dataset indicates that DPD2794 functions reliably. The environmental compounds were all negative, as expected, even though benzotriazole showed a very light response, which was finally not considered significant. These results show that this strain is not likely to respond to the individual, major well-known compounds detected in surface water, because these are not mutagenic. However, as there may be unknown genotoxic substances present in the water, there may be spills, and the response to environmental mixtures is unknown, a sensor with this strain can still be useful.

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References

Responsiveness of the genetically modified bacterial strain DPD2794

The sensor under construction in the workshop of KWR (Nieuwegein, The Netherlands)
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Construction of an on-line water toxicity sensor based on immobilized genetically modified bacteria

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Abstract
Despite the existence of a number of commercially available tools to monitor the presence of water contaminants, there are few, if any, that measure human-relevant toxicants in surface water on-site. To fill this need, a new on-line water toxicity sensor was developed in which genetically modified bacteria are continuously exposed to the water. These bacteria emit luminescence in response to a toxic effect. In this study, *E. coli* strain DPD2794 was used, which responds to genotoxicity. The sensor was built to be robust and easy to clean, with inert materials, temperature control and nutrient feed for the reporter organisms. The bacteria were immobilized in sol-gel on either an optical fiber or a glass slide and then continuously exposed to tap water. Both showed increased luminescence when genotoxic compounds were spiked in the water. The device indeed proved to be robust, with hardly any operational problems. The sensitivity of the sensor was higher with the slide (LOD of 0.01 mg/l mitomycin C) than with the fiber (LOD of 0.1 mg/l mitomycin C). Exposure after 22 hours still resulted in a response, albeit decreased in amplitude. A more drastic decrease in response was observed if the sensor was used on subsequent days without cleaning, in which case a strong growth of contaminating microorganisms occurred in the sensor. The proof of principle of the constructed on-line toxicity sensor was demonstrated and it appears to be a valuable addition to current methods of surface water monitoring.
Introduction

To prevent contamination plumes from reaching drinking water stations, several drinking water utilities monitor the quality of the water upstream of surface water inlet points. The currently applied monitoring routine relies mostly on chemical analysis and biomonitors. Although chemical analysis is valuable in many ways, it is, however, only useful in identifying chemicals and not their toxicity. Moreover, it may miss unknown compounds. Biomonitors with whole organisms, such as algae and Daphnia, give an indication of the total toxicity (1). However, many harmful compounds that are very relevant from a human perspective, such as genotoxicants, can not be detected by biomonitors. Therefore, there is a need for on-line biosensors that can detect these human-relevant toxic compounds and mixtures in water in real-time.

The most convenient reporter organisms to apply in on-line sensors are probably genetically modified bacteria in which a promoter gene for a specific stress response is coupled to genes for luciferase (lux). By using different promoter genes, strains have been constructed for the detection of DNA-, protein-, or membrane-damage, oxidative stress, organic pollutants and various heavy metals (2, 3). The use of a full luxCDABE operon enables the organisms to provide for their own substrate without need for external addition (4) making them very suitable for use in an on-line biosensor.

Various configurations of bioreporter-based biosensors have been built and tested, such as miniature bioreactors with bacteria in suspension (5, 6), a sensor with freeze-dried bacteria (7), biochips (8), a sensor with bacteria immobilized in a disposable card (9), and self-contained sensors with bacteria immobilized onto optical fiber tips (10-12), later adapted as a flow-through sensor system (10). The latter was promising, but not yet capable of long term measurements while exhibiting limitations in sensitivity and sturdiness. For this reason, a new prototype and improved version was built with some major adjustments. A dual immobilization system was integrated enabling the comparison of a larger glass slide conformation with that of the fibre optic. As only a small portion of the emitted light reaches the photomultiplier through the fiber (12), it was expected that the capture of the emitted light of a larger surface would increase the sensitivity.

We observed that alginate did not attach to a flat glass surface. We therefore
tried an inorganic sol-gel which forms a porous and transparent silica layer. Indeed, it has been shown that bacteria remain viable (13-16), and their response is not disrupted after immobilization in sol-gel (17).

In addition, new features were integrated, such as control over temperature, flow speed, concentration of growth medium, and the addition of positive controls. The instrument was built with inert materials, to prevent biofouling, leaching of toxic compounds, loss of spiked compounds, or memory effects from the sensor or tubing. Finally, in order to ensure no living genetically modified bacteria would be released into the environment, the efflux water was sterilized with chlorine. The new device was tested with the *E. coli* strain DPD2794 (18), which responds to DNA damage, and which is therefore one of the most relevant strains to use in such a device (3).

**Materials and methods**

**Chemicals**
Mitomycin C, ampicillin, and tetramethyl orthosilicate (TMOS) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Nalidixic acid, Lysogeny broth (LB) powder, agar, dimethylsulfoxide, acetone, glycerol, HCl, and NaOH were purchased from Boom (Meppel, The Netherlands). All chemicals were of analytical grade and stored as suggested by the manufacturer’s instructions.

**Bacteria and growth conditions**
The bacterial strain used for testing the system was DPD2794 (kindly donated by Prof. S. Belkin through prof. Marks [Ben Gurion University, Beer Sheva, Israel]). DPD2794 is a genetically modified (GM) *Escherichia coli* strain, which contains a plasmid with the *recA* promoter, coupled to the *luxCDABE* genes. The *recA* gene is part of the bacterial SOS response, which utilizes DNA damage repair (18). The *luxCDABE* genes are derived from *Aliivibrio fischeri* and code for the enzyme luciferase and the synthesis of its substrate. As a result of the coupling of these genes, DPD2794 generates light with a wavelength of 490 nm when DNA damage is induced (4). Stock cultures of bacteria were stored at -80 °C in 25% (v/v) glycerol medium. Colonies for daily use were maintained at 4 °C on LB-agar plates, supplemented with 100 mg/l ampicillin. Before each experiment, bacteria were grown overnight in an incubator at 26 °C (Friocell, MMM, Planegg, Germany). Bacteria were grown in LB broth.
which contained 100 mg/l ampicillin. The cultures were diluted to an OD$_{600}$ of 0.2 as determined by a spectrophotometer (Jenway 6300 VIS, Staffordshire, England).

**Immobilization**

The sol-gel was prepared by mixing 4 ml TMOS, 2.4 ml ultrapure water, and 0.68 ml of 0.1 M HCl. This mixture was left for 24 hours at 4°C to pre-polymerize. The glass slides were standard microscope slides (Waldemar Knittel, Braunschweig, Germany) purchased from Boom, Meppel. The optical fibers used were HUV-H pigtail fibers (Ceramoptec, Bonn). They had a core of pure, synthetic, fused silica with a diameter of 1800 µm and a numerical aperture of 0.48 ±0.2. The cladding was of hardpolymer with a diameter of 1900 µm. The tip on one end was polished; on the other end a SMA-standard connector was attached.

The pre-polymerized gel was first mixed with 0.05 M NaOH and then immediately mixed with the bacterial culture in a ratio of 1:1:4 sol-gel:NaOH:bacteria. One millilitre of the sol-bacteria mixture was immediately brought on the surface of the glass slide in a rectangular area of approximately 10 cm$^2$ and with a thickness around 1 mm. A drop with a height of approximately 3 mm was attached to the tip of the fiber by keeping the fiber upside down and letting the drop flow to the tip from a pipette (Figure 1). Both slide and fiber were left for 5 minutes to let the gel polymerize.
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Figure 1: The bacteria are immobilized on the tip of an optical fiber and a microscope slide

System setup

The glass plate and optical fiber with the attached sol gel-bacterial mixture were fixed in a stainless steel measurement chamber, see figure 2.

Figure 2: Photographs of the measurement chamber as seen from the outside (a) and from the inside (b) of the pulled-out chamber side. The numbers correspond with the following parts: 1. photomultiplier tubes, 2. pH and temperature sensor inlet, 3. light shutters, 4. water outlet, 5. air outlet, 6. plate, 7. fiber.
Figure 3: An overview of the sensor. When the water entered the cooled incubator, it was brought to 26 °C in the heat exchanger. The flow of the water and the addition of medium and toxic compounds were regulated by three separate pumps. After the addition of medium and test compounds, the water passed a static mixer to ensure an even distribution of these compounds. The water then reached the measurement chamber, which is displayed in the middle, while the enlargement shows the inside of the chamber. The water entered the measurement chamber from the bottom and flowed out from the side at the top (on the left in the enlargement). The bacteria were immobilized on a glass slide and a plate, which were attached to a removable side of the chamber (on the left in the main figure, on the right in the enlargement). The fiber was attached behind the slide (not visible in the picture). The light of the bacteria was measured with two photomultipliers, which were protected by shutters. After the chamber, the water passed a flow meter and a pressure meter and was collected in a waste barrel.

The side of the chamber was easily removable to enable efficient changing of the probes (Figure 2+3). This chamber was designed by 2M Sensors (Eindhoven) and the assemblage was done at KWR Watercycle Research Institute (Nieuwegein).
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The inner volume of the chamber was reduced as much as possible, down to a volume of 65 ml. This was done to prevent a reduction or delay of the response by dilution of a plume of compounds in the chamber. The inner corners of the chamber were rounded both to reduce the volume and to ease the removal of biofilm. Tap water flowed from bottom to top through the chamber, which was equipped with an air outlet at the top and a tap at the bottom to be able to drain the water. Two photomultiplier tubes (H-7467, Hamamatsu Photonics, Shimokanzo) were connected to measure both the plate and fiber light signal outputs separately. The fiber was placed behind the slide holder so both were shielded from each other’s light. Both photomultipliers were protected from overstimulation (e.g. when the chamber was opened to change the slide and fiber) by manually operated light shutters. The luminescence was measured four times per minute with an integration time of 10 milliseconds. All data points presented are averages over five minutes.

An overview of the entire sensor is provided in figure 3. Before the water entered the chamber, LB medium was added by an independent pump to optimize the response of the bacteria. To be able to verify the response of the bacteria and to characterize the system, compounds could be added at will through a third independent pump. To ensure an even distribution of these additions, a static mixer (SS tube mixer, Cole-Parmer, Vernon Hills) was installed before the measurement chamber. As an extra safety measure, the water passed through a flow meter (M-21, Tecfluid, Sant Just Desvem) and pressure meter (Ceraphant, Endress+Hauser, Reinach) after the measurement chamber. All tubing in the system, apart from the dosing of LB, was of either stainless steel or Teflon to prevent loss of compounds and contaminating ‘memory’ effects. Masterflex PharMed tubing was used instead of Teflon for the addition of LB broth, as Teflon was too inflexible for this pump for low volumes and the high LB stock concentration would not be influenced by the tubing. All aforementioned parts were placed in a cooled incubator (Friocell 222, MMM, Planegg) to stabilize the temperature at 26 °C. The temperature of 26 °C was applied because the luciferase enzyme becomes inactive above 30 °C. The water that entered the sensor was first brought to 26 °C with a heat exchanger inside the incubator.

During experiments the total water flow through the sensor was maintained at 25 ml/minute. LB broth was added as a nutrient source in concentrations...
of 7.5% or 2%. Nalidixic acid and mitomycin C were used as positive controls and added to the water after the medium. Exposure to positive controls was always for a period of one hour. Mitomycin C was used as a positive control when it was necessary to use the most potent genotoxin (e.g. for sensitivity determination). In all other cases, nalidixic acid was used as this compound was less hazardous, facilitating working conditions and waste removal.

**Cleaning protocol**
Because of regulations of the waste disposal of water containing GM-bacteria, water leaving the sensor was collected in a 50 litre barrel to which one litre household bleach was added after each experiment. The free chlorine level in the bleach was about 5%. Plate counts confirmed that this was sufficient to incapacitate all bacteria in the wastewater. The disinfected barrel was emptied directly into the sewage. To clean the entire system, the chamber and tubing in the sensor were flushed with household bleach diluted with tap water in a 3:7 ratio for 15 minutes. Afterwards, the system was thoroughly flushed with tap water for 1 hour.

**Electronics and control**
All instruments that were part of the sensor were connected to a programmable automation controller (CompactRIO, National Instruments). This controller includes a real-time operating system, which enables it to run independently and without delays. The user input and data handling were performed on a Windows PC, which was connected to the controller. The program that was used to control the sensor and gather the data was developed in Labview (version 2011, National Instruments).

**Results and discussion**

**Determination of the effect of nutrient concentration on the response**
The first priority after the construction of the new sensor was to establish a stable and reliable signal. An essential factor in achieving this was adjustment of the concentration of nutrients available to the bacteria. In a previous study, it has already been shown that this strain of genetically modified *E. coli* did not respond without the addition of medium to the water (10). The optimum concentration found in that study was 7.5% LB medium, which was used as starting point in the current study.
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The response with 7.5% LB medium showed very strong fluctuations in luminescence after about 5 hours in this sensor, both as background signal and after exposure of the bacteria to 0.1 mg/l mitomycin C. The reason for the strong fluctuations is not clear. These fluctuations were accompanied by a strong increase in background luminescence, which might have been caused by growth of the bacteria. We speculate that growth of these bacteria leads to luminescence as cell division includes DNA duplication, which includes DNA repair when DNA damage is detected. There always is some background DNA damage (i.e. spontaneous mutations) present in organisms, thus cell division will always lead to some DNA repair. In these modified bacteria, this will lead to some luminescence, even if no genotoxic substance is present in the water. Therefore, a high nutrient level, leading to increased growth of bacteria, could cause the observed increase in background luminescence.

![Figure 4: The effect of different nutrient concentrations on the response of the bacteria immobilized on a glass slide (left) or fiber (right). In two curves, mitomycin C was added between 1 and 2 hours from the start of the experiment in a concentration of 0.1 mg/l.](image_url)
For this reason, the LB concentration was reduced to two percent. As can be seen in figure 4, a remarkable reduction of the fluctuations was observed at this concentration, without loss of the response on the plate. On the fiber, there was some reduction in response in the beginning of the curve, but the response was still clearly distinguishable. Also, the contrast between the background and the response levels increased as a result of a decrease in background luminescence. Only with the fiber a peak remained after about ten hours. The cause of this peak remains unclear, especially since it did not occur on the plate. The observed lag-time, the time between the start of the dosing and the start of the response, remained the same as with 7.5% LB, namely about one hour. An additional advantage of a lower medium concentration was that it delayed the manifestation of biofouling in the system. In all the following experiments, 2% LB was therefore used as nutrient concentration.

**Determination of sensitivity**

An important aspect of the sensor is the achieved sensitivity, as the device can only be useful in the field if it can detect the relevant contamination levels. To test the sensitivity of the sensor device, the bacteria were exposed to several concentrations of mitomycin C, the most potent compound found so far for this strain, between 1 and 2 hours from the start of the experiment. The signal started to differ from the background about 1 hour after the start of the exposure, thus about 2 hours from the start of the experiment. To determine the limit of detection, the responses of the exposed bacteria were compared with the average of three background curves. For the concentrations of 0.01 mg/l and 0.001 mg/l, the average of two experiments was plotted.

The choice was made not to use a response ratio (response divided by the background) of two as the limit of detection, as is usually done. The reason was that this can render an analysis unnecessarily insensitive, in case of high backgrounds with relatively small variation, or, in cases with high variation, it can render an analysis more sensitive than realistic. In this respect, Takanashi and Uranako (1998) and Kim and Margolin (1999) considered the application of the conventional twofold rule to the data of Ames tests as too conservative (i.e. few samples are evaluated as genotoxic) (19, 20).

A common evaluation method in analytical chemistry, which is suitable for normally distributed data, is to define a response as significantly different from
the background when it exceeds the average response of the background + three times the standard deviation of the background response. This method is also compulsory to comply with the ISO standard for the specification and performance tests of new on-line sensors and analyzing equipment for water (21). For normally distributed data, there is then 99% certainty that the sample response is significantly different from the background. We have therefore chosen to apply an adapted version of this latter method.

To correct for the variation between experiments, a curve was made of the average background of three experiments plus three times the standard deviation (see figure 5). This curve was then divided by the average background to obtain a response ratio. The highest response ratio between 2.5 and 10 hours was determined as a significance limit independent of the point in time; this was 1.55 for the plate and 2.19 for the fiber. The curve of the average background response times this highest response ratio was the significance limit (Mean x RR in figure 5 and 6). The Limit of Detection (LOD) was defined as the lowest concentration that gave a response that was above this significance limit (i.e. 1.55 or 2.19 times the average background) anywhere in the time period between 2.5 and 10 hours of monitoring time, as visualized in figure 6. Using this definition, the LOD for detecting Mitomycin C was estimated to be 0.01 mg/l for the plate setup and 0.1 mg/l for the fiber configuration of the sensor. The higher LOD of the fiber was mainly caused by the variation in the background peak of this setup. Especially the variation in the time at which this peak occurred reduced the sensitivity of the fiber. The variation on the plate was relatively low compared to the background, resulting in a higher sensitivity. The newly added plate setup clearly is an improvement to the design of an on-line bacterial toxicity sensor using these modified bacteria. The detection of 10 µg/l of a genotoxic substance by the plate setup lies in the same range as the sensitivity of other biomonitors. The sensitivity of the Daphnia monitor lies between 1 and 10 µg/l for the insecticides it is most sensitive for. The algae monitor has limit of detection for herbicides of 0.5-2 µg/l, depending on the compound (22). The current sensitivity of the sensor is sufficient to detect heavy plumes of contamination in surface water. Unfortunately, it is not yet sensitive enough to detect compounds at the target values for drinking water, which are 10 ng/l for genotoxic compounds and 0.1 µg/l for other compounds (23). Additional improvement is necessary, possibly achievable through reduction
of the variation in the response by further standardization or through on-line sample preconcentration.

It is also noteworthy that the response to Mitomycin C endured long after the inducing compound was released from the system, especially after exposure to a concentration of 0.1 mg/l. HPLC analysis of samples from the sensor showed that no mitomycin C was detectable 30 minutes after stopping the input of the toxicant. This indicates that the signal was caused by a prolonged biochemical reaction of the DNA damage repair system. The implication for field monitoring is that once a positive signal has been induced, either the bacteria have to be replaced or a long lag period must be taken until the bacteria return to their normal state.

Figure 5: An overview of the average background of three experiments, the background plus three times standard deviation and the average times the highest response ratio (RR) of the previous two curves between two and ten hours. This ratio was 1.7 for the glass slide and 4 for the fiber. On the left is the response of the bacteria on the glass slide, on the right the response of the fiber.
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Figure 6: The response to different concentrations of mitomycin C, which was administered between 1 and 2 hours, and the curve of the average background response times the response ratio of respectively 1.55 for the slide and 2.19 for the fiber. On the left is the response of the bacteria on the glass slide, on the right the response of the fiber.

Delayed exposure
As the sensor should be able to detect a contamination at any point in time, the ability of the bacteria to respond at a later time point was tested by dosing 1 mg/l nalidixic acid after 5, 18 or 22.5 hours in separate experiments. A response was observed both on the fiber and the glass slide at all time points (Figure 7). Very striking was the reduction in response on the plate, which showed much higher levels of luminescence at 5 hours than at later time points. Further enhancement of the bacterial response after these longer running times is desirable, future research would be necessary on this aspect. This trend was different for the fiber, which gave a very low response at 5 hours compared to the background, but performed much better at later time points. It may be possible that the same processes that result in generation of the background...
peak between 5 and 15 hours on the fiber are also responsible for the lower response during this time period.

Another observation regarding the glass slides was the occurrence of typical fluctuations in the response, as also seen at higher nutrient levels (Figure 4). These were already present before the addition of the positive controls, but became more pronounced during the time interval of the response peak. One suggestion is that this might be caused by the visually observed growth of other microorganisms on the gel during the experiment, which might hinder the luminescent *E. coli*. The relation between observed fluctuations and growth of the bacteria was also apparent in the case of higher nutrient levels. The mechanism of this apparent relation remains unclear. The exact cause of the observed differences between both setups, which entail the same bacteria and sol-gel, also remains a question to be answered.

![Graph](image)

**Figure 7:** The response of the bacteria on the glass slide (left) and fiber (right) to 1 mg/l nalidixic acid administered for one hour after 18 or 22.5 hours.
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The effect of biofouling
Prior to all previously shown experiments, the sensor was always cleaned with diluted bleach to remove biofouling from the system. However, some experiments were also performed without prior cleaning before the start of a new measurement. These experiments were not shown in this paper, except one example in figure 8. In these cases, the signals generated on both the fiber and glass slide were drastically altered in comparison with the signals gained from a cleaned system.

Figure 8: The difference in response when using the sensor directly after a previous experiment and when the system has been cleaned in between. The bacteria were exposed to 1 mg/l nalidixic acid between 5 and 6 hours. The response of the glass slide is depicted left and the response of the fiber on the right.

In figure 8, an example is given of an experiment where nalidixic acid was administered as a positive control between 5 and 6 hours. In one case, the system had been cleaned and in the second case the experiment was
performed directly following another 24 hour experiment, without any in-between cleaning or flushing. In the first case, there was a normal background, followed by a sharp increase in signal in response to the positive control. Without cleaning, the bacteria on the plate gave a relatively low and short peak, after which the response decreased. Also on the fiber, the response was lower and without the characteristic peak between 600 and 800 minutes. Other experiments without cleaning showed similar results, with a much lower or even absent response. Also very typical in these instances was the occurrence of strong fluctuations in luminescence, especially on the glass slide.

Culturing of water and biofilm samples from the sensor on LB agar plates showed that high numbers of contaminating bacteria, probably originating from the tap water, were present in the sensor when several experiments were run in succession without cleaning. This was in particular the case from the point where LB broth was added to the water. Also visual inspection confirmed the formation of biofilm in the Teflon tubing as well as on the sol-gel on the glass slide. Although only 2% medium was used, apparently the nutrient concentration was sufficiently high to accommodate a rapid growth of bacteria. This effect was probably enhanced by the temperature of 26 °C. It therefore seems that fast growing native water bacteria may cause a decrease in the signal of the reporter bacteria. There may be many speculative explanations for this phenomenon, but additional studies are necessary to further understand this observation. In order to solve this problem in a practical way, the system can be disinfected regularly with bleach, which is easy to perform and relatively cheap on the short term, but not practical for longer measurements and environmentally problematic. Alternatively, one could apply pre-filtration of the water to remove native microorganisms. However, a possible drawback may be that the latter approach may result in adsorption and loss of analytes in the filter, as well as possible problems by pressure build-up and the need for frequent filter changes. Pre-treatment of the input water using UV may limit contaminating microorganisms in their capacity to form biofilms, but can also break down compounds of interest.

**Evaluation immobilization methods**

The bacteria were immobilized on both a glass slide and an optical fiber, which were used simultaneously to compare their relative performance. Fibers have
been used in previous studies, thus they were already known as a viable platform for immobilization of bacteria and guidance of the produced light signal to a photodetector (10, 24). In this study, fibers were used with a larger diameter than previously, namely 1800 µm instead of 400 µm. The motivation behind this choice was that with a larger surface of the tip, more bacteria are directly in front of the fiber. Therefore a larger portion of the emitted light is coupled into the fiber. It was also for this reason that glass slides were tested as immobilization platform, as they have a larger surface area than fibers. The idea was that by being able to gather more light, the resolution of the signal as well as the sensitivity would increase. Indeed, one can see that in all figures, the signal from the plate is often more than a factor hundred higher than that of the fiber. This is easily explained by the surface area differences (fiber-optic 5 mm² and flat plate 1000 mm²).

More unexpected was the difference in shape of the background response from the bacteria. The bacteria on the plate always started with a steep rise in response, followed by a slow decrease after about ten hours. The response of the bacteria on the fiber increased more slowly and showed a characteristic peak between 10 and 14 hours from the start of the experiment. This pattern remained when a mutagenic compound was added before the peak, although an increase in light production was observed one hour after the start of the exposure. In comparison, the response of the plate was sharper and probably easier to distinguish when the exact time of the dosing is not known.

More importantly, the LOD of the plate was lower, namely 0.01 mg/l versus 0.1 mg/l for the fiber. This difference was caused by the large variation in the amplitude and time of the background peak of the fiber. It should be noted though, that the response of the fiber improved, when the exposure took place later in time, after the background response of the fiber had flattened out. As can be seen in figure 7, exposure after 18 hours gave a much sharper response in the fiber than with the plate at that time or with the fiber at an earlier time. Nevertheless, if one setup would have to be chosen for use in the field, based on these results, the plate is the better choice due to its higher sensitivity and predictability.

It would be interesting to see whether the relatively low response of the plate in longer experiments is related to fouling with foreign microorganisms. In this case, it would be expected that the response improves when the natural bacteria are removed by, for example, filtration. Furthermore, there still remain additional issues to be solved to optimize the sensor, such as to understand which underlying mechanisms determine the size and shape of background
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luminescence or what is the maximal life span of the probe batches and the influence of the immobilization matrix.

Conclusions

A new biosensor was developed and tested for the on-line monitoring of genotoxic compounds in water using luminescent bacteria. In this sensor, bacteria for the detection of DNA damage were immobilized on both an optical fiber and a glass slide. The light signal from the bacteria on the glass slide was much stronger than from the fiber, as a result of the larger surface area. Moreover, the variation between experiments was higher with the fiber, which reduced its sensitivity. The detection limit was 0.1 mg/l for the fiber and 0.01 mg/l for the glass slide.

A response could still be measured after 22 hours. However, the amplitude of the response, as well as the background luminescence, decreased over time. This was probably caused by interference of other microorganisms. High numbers of contaminating bacteria were observed to interfere with the luminescent bacteria sensor signals when the system was used for more than one day without cleaning. If this problem of interfering microorganisms is solved, this sensor could be a useful addition to the current biomonitors for the screening of surface water meant for drinking water production.

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References


Construction of an on-line water toxicity sensor

Monitoring station Keizersveer seen from the water intake point
Chapter 5

Application of a sensor with immobilized luminescent bacteria for on-line surface water monitoring

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Abstract
Surface water used for drinking water production is frequently monitored in The Netherlands using whole organism biomonitors, with for example *Daphnia magna* or mussels, that respond to changes in the water quality. However, not all human-relevant toxic compounds can be detected by these biomonitors. Therefore, we have developed a new on-line biosensor, containing immobilized genetically modified bacteria, which respond to genotoxicity in the water by emitting luminescence. In this study, the performance of the sensor, which was tested before under laboratory conditions, was now further tested, under field conditions at a monitoring station along the river Meuse in The Netherlands.

During continuous on-line monitoring sessions with Meuse water there was no increase in luminescence above background in the monitoring period of one month. In the same month, the *Daphnia* and mussel monitors did give some alarms. This can be explained by the difference in the type of compounds and the type of toxicity that is detected between the biomonitors (acute systemic toxicity) and the immobilized bacterial sensor (genotoxicity). The sensor did respond to spikes of genotoxic compounds in the water with a minimal detectable concentration of 0.1 mg/l mitomycin C. With further optimization, the sensor has the potential to become a useful addition to the current biomonitors, showing a low potential for false alarms.
Introduction
Contamination of surface water with various pollutants is a well known worldwide problem (1). It poses a particular challenge for the drinking water companies that use surface water as a source for drinking water (2). Accidents with ships, runoffs from fields or discharges from factories can cause sudden peaks in the concentrations of pollutants in rivers (3). To prevent the intake of these plumes of contaminants, the water quality is monitored upstream of the drinking water inlet points in some countries, including the Netherlands. The devices which are in use for on line monitoring can be divided in three categories. The first are sensors that measure chemical or physical parameters, such as turbidity, pH, or oxygen content. The second group measures several specific organic pollutants by chemical analysis such as on-line HPLC-UV. The third group consists of biological systems, for example biomonitors with *Daphnia*, algae, or mussels, which respond to the toxicants in the water (4-6). If the concentration of a compound or the response of a biomonitor reaches a certain critical value, an alarm is generated and the water intake is closed.

However, not all compounds that can have adverse effects in humans are detected by the panel of current biomonitors or on-line chemical analysis instruments. Especially compounds that are not detected by UV (by lack of a conjugated system in the molecule), or do not affect the organisms used in the biomonitors, or only cause long term effects in low concentrations are easily missed. It is for this reason that a new flow-through biosensor was developed based on genetically modified bacteria that generate luminescence in response to specific groups of toxic compounds (7). The bacteria in this sensor were immobilized in a sol-gel on an optical fiber and a glass slide and exposed continuously to a flow of water. Although several luminescent bacteria are available for use in this device, we have so far used *Escherichia coli* strain DPD2794, which contains the *luxCDABE* genes for luciferase coupled to the *recA* promoter gene from the SOS response, which is activated by DNA damage (8). This strain was found to be the most relevant of the available strains, for the purpose of surface water inlet monitoring for drinking water production (9).

In a previous publication (7), the sensor was constructed and tested for proof-of-principle in the laboratory. In the study presented here, the goal was to further optimize and test the sensor and particularly to determine the sensors' performance in field studies, e.g. in surface water monitoring at a monitoring station. Since the first tests showed that the glass slide setup gave the most
reproducible signal and because the gel did not always remain attached to the fiber, only the glass slide setup was used in this study. The first tests from the studies performed in the laboratory \(7\) showed that the bacteria indeed responded to spikes of genotoxic compounds when these were added to tap water. Unfortunately, even in tap water, the formation of a layer of bacterial fouling was an issue. This was further stimulated by the rich conditions for bacterial growth in the sensor compartment, i.e. 2 % growth medium at a temperature of 26 °C that was maintained in the sensor. Therefore, daily cleaning with chlorine was necessary to prevent a loss of response. To prevent the interference of too much fouling in the sensor and possibly make the daily cleaning unnecessary, two filtration units (0.5 µm and 30 nm) were installed and first tested in the laboratory using tap water. Also, a UV-C unit was installed behind the sensor for on-line disinfection of the waste water, as an alternative to the chlorination of collected batches of waste water. The reason for using UV instead of chlorine was not only that it can be used on-line, but also because the disposal of large amounts of chlorine is undesirable and the handling of large barrels with chlorinated water posed occupation risks. Next, a few preliminary experiments were performed with river water that was collected and tested in the laboratory. Eventually the sensor was transported to the field monitoring station Keizersveer (Hank) along the river Meuse in the Netherlands and tested on site for a period of two months. During this time, the background signal was established and several spike experiments were performed. The signal of the sensor was compared with the alarms of the *Daphnia* and mussels monitors at the same station, and with the measurements of the chemical and physical sensors that were active during this period.

**Materials and Methods**

**Chemicals**

Mitomycin C, ampicillin, and tetramethyl orthosilicate (TMOS) were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). Nalidixic acid, Lysogeny broth (LB) powder, agar, dimethylsulfoxide, acetone, glycerol, NaCl, KCl, NaHPO\(_4\)·2H\(_2\)O, KH\(_2\)PO\(_4\), HCl, and NaOH were purchased from Boom (Meppel, The Netherlands). All chemicals were of analytical grade and stored as suggested by the manufacturer’s instructions.
Bacteria and growth conditions
The bacterial strain used in the sensor was DPD2794 (kindly donated by Prof. S. Belkin through prof. Marks (Ben Gurion University, Beer Sheva, Israel)). DPD2794 is a genetically modified *Escherichia coli* strain, which contains a plasmid with the *recA* promoter, coupled to the lux*CDABE* genes. The *recA* gene is part of the bacterial SOS response, which generates DNA damage repair (8). The lux*CDABE* genes are derived from *Aliivibrio fischeri* and code for the enzyme luciferase and the synthesis of its substrate. As a result of the coupling of these genes, DPD2794 generates light with a peak wavelength of 490 nm when DNA damage is induced (10), without the need of adding a substrate. Stock cultures of bacteria were stored at -80 °C in 25% (v/v) glycerol medium. Colonies for daily use were maintained at 4 °C on LB-agar plates, supplemented with 100 mg/l ampicillin. Before each experiment, bacteria were grown overnight in an incubator at 26 °C (Friocell, MMM, Planegg, Germany). Bacteria were grown in LB broth which contained 100 mg/l ampicillin. The cultures were diluted to an OD$_{600}$ of 0.2 as determined by a spectrophotometer (Jenway 6300 VIS, Staffordshire, England).

Immobilization
The sol-gel was prepared by mixing 4 ml TMOS, 2.4 ml ultrapure water, and 0.68 ml of 0.1 M HCl. This mixture was left for 24 hours at 4 °C to pre-polymerize. The glass slides were standard microscope slides (Waldemar Knittel, Braunschweig, Germany) purchased from Boom, Meppel. The pre-polymerized gel was first mixed with 0.05 M NaOH, and then immediately mixed with the fresh bacterial culture with a respective ratio of 1:1:4. One millilitre of the sol gel-bacteria mixture was immediately applied on the surface of the glass slide, where it dispersed until it filled a rectangular area of approximately 10 cm$^2$. The slides were left for 5 minutes to let the gel polymerize.

System setup
The sensor has been previously described in detail (7), so only a short overview will be given here. The glass slide with the immobilized bacteria was fixed in a stainless-steel measurement chamber. The light emitted by the bacteria was measured using a photomultiplier (PMT) (H-7467, Hamamatsu Photonics, Shimokanzo) on the side of the chamber, which was protected by a manually operated light shutter. The integration time of the PMT was 10 milliseconds.
during the laboratory experiments and 100 ms during the field tests. Four measurements were taken per minute and all data were averaged over 5 minutes. The measurement chamber was mounted in an incubator (Friocell 222, MMM, Planegg), which was set at 26°C, together with the other parts of the sensor (Figure 1). These included a heat exchanger, three peristaltic pumps (07523-80 L/S Digital Drive, Masterflex, Schiedam), a static mixer (SS tube mixer, Cole-Parmer, Vernon Hills), flow meter (M-21, Tecfluid, Sant Just Desvem), and a pressure meter (Ceraphant, Endress+Hauser, Reinach). The water that entered the sensor was first brought to 26 °C in the heat exchanger. Then LB medium and control compounds were added and mixed through the water in the static mixer. The water then entered the measurement chamber from the bottom, passed the glass slide with the immobilized bacteria, and left at the top. Afterwards it passed the flow meter and pressure meter and then left the incubator. All tubing in the sensor was made of either stainless-steel or Teflon, apart from the dosing of the LB medium, which was done with Masterflex PharMed tubing. The flow speed of the water during all experiments was 25 ml/minute. The sensor was controlled and the data was collected with Labview software (version 2011, National Instruments).

To reduce the formation of fouling in the sensor, two filters were installed upflow of the incubator. The first was a 0.5 µm cartridge filter, the second a membrane filter with a 30 nm polyvinylidene membrane (Compact PVC module with membrane F4385, Norit X-Flow, Enschede). In the laboratory experiments, a separate pump (7523-57 L/S Digital Drive, Masterflex, Schiedam) was used to push the water over the filters, after which it was gathered in a 50L barrel (Graf, Teningen).

The water that left the sensor was collected in a waste barrel, where one litre of bleach was added for disinfection (40 gram chlorine per litre). In some experiments, the water went through an 18-Watt UV-C water clarifier (AquaCristal Series II, JBL, Neuhofen) which was tested as an alternative method for disinfection. The clarifier had three obstacles around the UV-lamp to prevent the water from taking the shortest route, thus reducing the chance that a portion of the water would not be irradiated. It was installed between the sensor and the waste barrel (visible at the bottom of figure 1b).
To clean the entire system, the chamber and tubing in the sensor were flushed with household bleach diluted with tap water in a ratio of respectively 3:7 for approximately 15 minutes every day. Afterwards, the system was thoroughly flushed with tap water for 1 hour.

**Determination of the performance of the UV-disinfection**

The effectiveness of the disinfection was determined by collecting samples before and after the UV unit. The 0.1 ml samples taken before the UV-unit were diluted a hundred times, plated on LB agar plates and incubated overnight. Three 0.1 ml samples taken after the UV unit were directly plated on LB agar plates. One 100 ml sample taken after the UV unit was filtered over a 0.2 µm filter, which was also incubated on an agar plate.

**Surface water experiments in the laboratory**

Before the sensor was moved to the field, the performance of the sensor with river water samples was tested in the laboratory. The purpose was to compare the response of the bacteria with the results previously obtained for tap water and to confirm their proper functioning in surface water. The water was collected from the Lekkanaal in Nieuwegein, The Netherlands, which originates from the river Rhine. The water was filtered and tested in the sensor on the same day as it was collected.

**Field tests**

For the field experiments, the sensor was moved to monitoring station Keizersveer in Hank, The Netherlands. This monitoring station is situated along the river Meuse and is managed by drinking water company Evides. Other monitors that are employed at the station include the *Daphnia* monitor (bbe-Moldaenke, Kiel) and the Musselmonitor (AquaDect, Brouwershaven), (containing the Quagga Mussel, *Dreissena bugensis*). Both monitors were also running during the test period. The sensor was tested from the 2nd of July, 2012 until the 7th of September, 2012. At the monitoring station, on-line sensors for temperature, oxygen content, turbidity, conductivity and acidity are used for a general water quality assessment. Besides a regular chemical monitoring program, based on the regulation for drinking water, once a week samples are screened for chemical pollutants using GC-MS and HPLC-UV-DAD by the water laboratory, Aqualab Zuid (Werkendam, the Netherlands).
Figure 1: a) A schematic overview of the sensor, the inside of the measurement chamber is shown in the enlargement. b) Photograph of the sensor at monitoring station Keizersveer. The filters were attached to the side of the sensor (on the right). The UV-unit is visible on the bottom left.
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The water at the monitoring station was taken directly from the Meuse with a pipe and first went through a 1.0 mm filter (D-74613, Mahle, Flintbek), before being distributed to the various monitors in the station. Then it was led through the aforementioned 0.5 µm and 0.03 µm filters for the bacterial sensor specifically and subsequently entered the sensor.

Because the equipment at the monitoring station was limited, the bacteria had to be immobilized at the laboratory located in Nieuwegein and transported to Hank by car. During the transport, the slide with the immobilized bacteria was submerged in phosphate buffered saline (PBS), with an initial temperature of ±4 °C, and placed in a cool box. All slides were used on the same day as they were prepared. The time between the immobilization of the bacteria and their insertion in the sensor was approximately 1.5-2 hours.

Results and Discussion

Laboratory experiments
A small number of laboratory experiments were done in preparation of and prior to performing the field tests. The purpose of these experiments was to test the effectiveness of filtration in reducing biofouling and prolonging the response of the bacteria, and to determine the difference in response between tap and river water.

As can be seen in figure 2a, the use of the two filters (0.5 µm and 30 nm) indeed resulted in a delay of the decline of the average background response (n=3) in tap water. This was an improvement, because a decrease in background usually coincides with a decline of the responsiveness of the bacteria. Indeed, the response of the bacteria to 1 mg/l nalidixic acid, added after 22.5 hours, was more pronounced when the filters were used (Figure 2b). Unfortunately, it was not possible to prevent biofouling sufficiently to enable experiments on subsequent days without cleaning in between, as the ‘not cleaned’ curves clearly show. Since faster formation of fouling of the sensor was expected with surface water, the filters were used in all further experiments.
Figure 2: The effect of filtration on the response of the bacteria is shown. In (a) the background response is compared in both a cleaned sensor and when the sensor was used on subsequent days without cleaning in between. The curves of the cleaned sensor are averages of three measurements, those of the not cleaned sensor single measurements. In (b) the bacteria were exposed to 1 mg/l nalidixic acid after 22.5 hours, for one hour.

Before the sensor was moved to the field location at the river Meuse, a few experiments were performed with water from the Lekkanaal in Nieuwegein. The Lekkanaal originates from the river Rhine, which is known to be much cleaner than the river Meuse (11, 12), but the Lekkanaal water was easier to bring to the laboratory and can be seen as representative for surface waters in general. As can be seen from the results presented in figure 3, the response of the bacteria to spikes of nalidixic acid was very similar in tap and Lekkanaal water. A decrease in height of the response peak was observed, but the response was still well distinguished.
Figure 3: The results of experiments with tap water are compared using water from the Lekkanaal (Nieuwegein). The bacteria were exposed to 1 mg/l nalidixic acid for 1 hour after 5/5.5 hours (a) or after 22.5 hours (b).

Evaluation of the UV-disinfection unit

In several countries, including The Netherlands, the use of genetically modified bacteria is bound to strict Governmental or European regulations. This means that all contaminated material, including the waste water from the sensor, had to be disinfected. The use of chlorine bleach has been established as a valid method for the disinfection of water contaminated with genetically modified E. coli (13). However, this method has the disadvantages that it can not be used on-line and that the use and disposal of large quantities of chlorine is undesirable. Therefore, UV-C irradiation was tested as an alternative method of disinfection.
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The collected agar plate counts from three separate laboratory experiments showed an average concentration of $9.4 \times 10^4$ bacteria per ml in the waste water of the sensor at the end of a normal one-day experiment. No colonies were found on any of the agar plates with the samples taken after installation and activation of the UV unit, including that of the filtered 100-mL sample. This indicated that the UV unit was very effective at the inactivation of the bacteria (>log 6 removal), under laboratory conditions. Unfortunately, during the field experiments there were still some colonies of undefined bacteria found after the UV-lamp, although their numbers were drastically reduced. Therefore, in addition to the UV-unit, bleach was still added to the water to be able to ensure a complete disinfection. In conclusion, the use of UV for disinfection showed promising results, but more research is needed to determine the light intensity and contact time required to ensure a complete inactivation of all bacteria under field conditions.

Field experiments Keizersveer

Following the experiments discussed in the previous paragraphs, the sensor was moved to the field monitoring station Keizersveer in Hank, The Netherlands. There, the sensor was tested with water from the river Meuse. The integration time of the PMT of the glass slide was set to 100 millisecond instead of the 10 millisecond used in the laboratory to reduce the noise of the signal. The bacteria were prepared in the laboratory and transported to Keizersveer by car. Apart from these changes, the first experiments were performed under the same circumstances as in the laboratory, with the same filters installed, a water flow of 25 ml/min. and a medium concentration of 2% LB. As can be seen in figure 4a, in the first few hours, the luminescence signal started high on the glass slide, immediately followed by a sharp decrease. This was probably caused by the sudden change from the PBS-buffer the bacteria were stored in, to the water in the sensor. After the first few hours, the signal showed a similar shape as previously in the laboratory, albeit around a factor 10 higher, which was caused by the longer integration time. After 10-17 hours however, the light intensity increased very drastically, to levels that could no longer be measured with the PMT (>1000000 Relative Light Units). The exact offset time of this increase varied somewhat, but it occurred very consistently. It is noteworthy that another PMT, which was previously used to measure the light from bacteria on a fiber (the fiber was not used in this study), also...
registered an increase in light intensity between 10 and 17 hours (see the ‘fiber’ line in figure 4a). This PMT was attached to the other side of the measurement chamber, at the back of the slide holder, and did normally not receive light from the bacteria on the slide. Of course, there is a possibility that some of the light of the glass slide reached the fiber PMT by reflection on the stainless steel walls of the chamber. If this were the main source however, it would be expected that the signal of this PMT would follow that of the plate PMT but at a lower level. Since this was not the case, it is thought that this drastic increase in signal might be caused by a strong replication of the luminescent bacteria, not just in the gel on the glass slide, but also in the water surrounding the glass slide, thus reaching the space in front of the fiber PMT. Regardless of the cause, the high peak later in time presented a considerable problem, since it was no longer possible to measure toxicity. Several changes in the protocol were tested in order to prevent this peak, including a lower initial number of bacteria in the gel and storage of the glass slides with immobilized bacteria in the refrigerator at Keizersveer overnight, prior to installation in the sensor. Eventually, it was found that the peak disappeared after reducing the medium concentration from 2% to 0.2% LB. As shown in figure 4b, the signal became stable at this medium concentration and the sudden increase did no longer occur at either PMT. Another remarkable observation was that the height of the signal seemed directly proportional to the medium concentration. As a result, the height of the signal with 0.2% LB was similar to the signals in the laboratory with 2% LB, considering the 10-fold difference in integration time. This strong influence of nutrient availability is something to consider very carefully when testing this type of bacterial sensors. It implies that, when different types of water are compared, some form of correction should be used for the difference in nutrient concentration. For example, when waste water is compared with tap or surface water and the waste water gives a higher signal, this might not necessarily mean the water contains more genotoxic compounds, but may be due to a higher nutritional content of the waste water.
Figure 4: The luminescence of the bacteria when exposed to Meuse water with (a) 2% medium or (b) 0.2% medium. The signals of both the PMT in front of the slide (“plate”, integration time 100 ms) and from the back (“fiber”, intgr. time 10 ms) are given. The average of the background for tap water with 2% medium (“laboratory”, intgr. time 10 ms) is given as a reference.

Determination of the limit of detection

The limit of detection (LOD) was determined in Meuse water with mitomycin C, while using a medium concentration of 0.2% LB. To avoid an effect of the glass slide handling at the beginning of the experiments, mitomycin C was added after four hours from the start of the experiment, and for the duration of one hour.

The same method was used for the determination of the limit of detection as in the previous study (7), in which the response to the compound is compared to a reference response ratio (RRR). In this case, the RRR was determined by dividing the mean of five background curves plus three times the standard
deviation by the mean. By taking the highest response ratio between 4 and 20 hours, the RRR when using Meuse water was 3.15.

![Graph](image)

**Figure 5:** (a) shows the mean of five background experiments, the mean + 3 times the standard deviation and the mean x the response ratio of 3.15 (mean x RRR). In (b), the response to three concentrations of mitomycin C, added between 4 and 5 hours, are compared to the mean x RRR.

The lowest concentration that caused a response of more than 3.15 times the mean background was defined as the LOD. In figure 5, the response curves of three concentrations mitomycin C are shown, as well as the curve of the mean background times 3.15. It can be seen that, although there was already a concentration-dependent increase in luminescence at lower concentrations, only 0.1 mg/l mitomycin C was sufficient to cause a significant response. Considering that the 0.1 mg/l concentration far exceeded the limit of 3,15-times mean background, it may well be that the true LOD is somewhat lower, but concentrations between 0.01 and 0.1 mg/l were not tested.
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The higher LOD compared to tap water (LOD tap water was 0.01 mg/l (7)) can mainly be attributed to the higher variation in the background. This is not surprising, since the quality and composition of the Meuse water varies more than that of tap water. It is an important effect that should be kept in mind when determining the LOD of a sensor under field conditions.

Unfortunately, the LOD as determined in this study is still too high to enable the detection of genotoxic compounds at the target value for these compounds in drinking water of 10 ng/l (14). By reducing the variation in background between experiments, by for example standardization and/or automation of the measurements, it is expected that the sensitivity of the sensor will increase. Another option to increase the sensitivity might be pre-concentration of the water. It should be mentioned in this respect that also for in vitro genotoxicity assays, the water samples are usually concentrated to gain sufficient sensitivity (15). However, this can probably not be combined with continuous on-line monitoring and instead would require a (semi-)batch like setup of the sensor.

Comparison with other monitors at Keizersveer

Several other (bio)sensors were also running at Keizersveer for regular water quality monitoring during the period the sensor was tested there. Among them were the Daphnia and mussel monitors for on-line toxicity monitoring, and pH, oxygen, and turbidity sensors. In figure 6, the data of these monitors are given for the period between 31-07-2012 and 16-08-2012, as well as the response of the bacterial sensor during the same period and the response of the sensor after subtraction of the mean background. All experiments with the bacterial sensor shown in this figure were performed with 0.2% LB. The sensor was cleaned and new bacteria were inserted each day. Most of the experiments were done with unaltered Meuse water, except those of 7, 9, and 14 August 2012, when a spike of 1 mg/l nalidixic acid was dosed between 4 and 5 hours. The curve of an experiment performed on 28 August 2012, when 10 mg/l nalidixic acid was added as a spike between 4 and 5 hours, is shown as well.

The last curve in figure 6 shows the mean x RRR, which is the limit that should be passed to obtain a significant response. It can be derived from these curves that 10 mg/l nalidixic acid caused a very strong response in the sensor, while 1 mg/l failed to give a distinguishable increase in signal.
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Figure 6: The response of the sensor, compared to other (bio)monitors at monitoring station Keizersveer, Hank. For the Daphnia and mussel monitor only the alarms are shown. All bacterial sensor measurements were performed with 0.2% LB and continuous exposure to Meuse water. The vertical bars indicate when spikes of nalidixic acid were added to the water in the sensor. The duration of the spikes was always one hour. Graph a) shows the unaltered signal of the sensor, while in graph b) the mean background was subtracted from the original response. The signals from the other (bio)monitors are shown in graph c).
Clearly visible in this graph is a typical daily pattern in the response of the sensor, with an increase in luminescence followed by a decrease. The increase is probably due to replication of the bacteria, while the decrease is thought to be caused by a combination of a reduction of the growth of the luminescent bacteria and fouling with other microorganisms. To prevent the reduction in signal and enable longer measurements would require a different setup of the sensor. One way to keep the bacteria at a constant density and growth phase is by keeping them in a continuous culture, as has been done with a few bioreactor sensors (16, 17). The disadvantages of these reactors however, include a relatively high dilution rate of the sample water and a high concentration of genetically modified bacteria in the waste water. Another, more practical alternative, might be a (semi)batch design, with bacteria in wells plates and automated dosing. Thus, new bacteria are used for every measurement (18). Such a system has the additional advantage that it can be coupled to on-line SPE to increase the sensitivity of the sensor.

The pH, oxygen concentration, and turbidity varied little during the test period of the sensor and no influence of these parameters was observed on the sensor signal. The *Daphnia* and mussel monitors both gave several alarms during the same time period, none of which coincided with an increase or decrease of the signal of the bacterial sensor beyond the normal variation. HPLC and GC-MS analysis showed that several industrial chemicals and pesticides were present in the low µg/l range on the days of the alarms, as well as unknown compounds (unpublished data). Since none of these measured compounds are known to have a strong genotoxic effect, it is not surprising that the bacterial sensor did not show an increase in luminescence above the background. Also a decrease in response due to cytotoxicity has only been observed in this strain of bacteria after exposure to concentrations in at least the mg/l range (19).

Although the period during which the sensor was tested in the field was relatively short, based on the results obtained and the knowledge available of the compounds that occur in surface water, it is expected that a positive signal will not occur often. However, many water pollutants are not known yet or even detected and identified with standard analytical methods. This is clearly shown by the alarms generated by the *Daphnia* and mussel monitors, which are often caused by compounds that can not be identified by standard HPLC.
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analysis (6). It should also be kept in mind that if there is a release of a genotoxic contaminant, an on-line sensor has a far higher probability of detecting such a release than routine sampling. As the experiments with nalidixic acid and mitomycin C spikes show, the sensor responded well to such sudden increases in genotoxic compounds in surface water.

Conclusions

A new on-line biosensor with immobilized, luminescent bacteria for the detection of genotoxicants was tested in a field monitoring station along the river Meuse. The sensor responded well to spikes of genotoxic compounds added to the river water, with a limit of detection of 0.1 mg/l for mitomycin C. The concentration of the medium, which was added to the water, directly influenced the height of the luminescent signal. A very strong increase in response was observed with 2% LB medium, which was reason to lower the medium concentration to 0.2%. The *Daphnia* and mussel monitors, which were employed at the same station, generated several alarms during the test period, none of which resulted in a parallel alarm of the bacterial sensor. This was not unexpected, since these monitors respond to different compounds and toxic effects. For future use of this on-line bacterial sensor in field studies it is required to improve the sensitivity (e.g. by reducing the variation between experiments) as well as the time needed for daily maintenance of the sensor. Both might be achieved by further standardization of the methods and simplification and/or automation of the sensor.

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References