Chapter 6

Summary, general discussion and outlook
Development and validation of an on-line water toxicity sensor based on genetically modified, luminescent bacteria

Summary
As described in chapter 1, there is a high demand for safe drinking water in present day's society, which has to comply with ever increasing quality standards. Several discoveries in the past two decades have led to an increasing public awareness of the risks of water pollution to both the human population and the environment. These include the discovery of the presence of endocrine disruptors, pharmaceuticals, drug residues and NDMA in surface and sometimes drinking water. As a result, there is an increasing pressure on the government and drinking water utilities to monitor the water quality and safety. This can be done by determination of the chemicals present in the water, but also by measuring the toxicity with cell assays or whole organisms. The introduction of whole organism biomonitors represents a first step towards a stronger focus on the use of toxicity as a measurement parameter in on-line surface water monitoring. These biomonitors are used as alarms by the drinking water companies, to close the surface water intake for drinking water production in case of a toxic event. However, the current biomonitors are only sensitive to compounds that have an acute toxic effect in the organism used, and are not able to respond to compounds with certain chronic and specific toxic effects, such as endocrine disruptors and genotoxic compounds. For this reason, there is a need to develop monitoring systems that can detect these specific toxic effects.

The main aim of the work described in this thesis was to construct, validate and apply a new on-line biosensor for the monitoring of specific toxic effects in surface water. The sensor contained genetically modified bacteria which responded to a specific effect by luminescence. The strain used in the work described here responded to genotoxic compounds. Various aspects of the sensor and the bacteria in the sensor have been studied and results were
discussed in the various chapters, which are summarized in this section. At the end of the summary, an outlook on future perspectives is given for this type of sensors.

Chapter 2 presented an overview of the genetically modified bacterial strains available that have been described in the literature. All strains reviewed contained the bacterial luxCDABE genes for luciferase and its substrate, coupled to a promoter gene that determined the effect or compound they respond to. Based on these promoter genes, they were clustered in groups of strains for the detection of DNA damage, protein damage, membrane damage, oxidative stress, organic pollutants or heavy metals. In general, the more specific strains were also more sensitive, in particular the strains for heavy metals, which showed detection limits in the µg/l to ng/l range. The DNA damage strains appeared to be the most sensitive effect-specific strains with detection limits in the µg/l range. Apart from a few mercury-sensing strains, the sensitivity of these bacteria was not sufficient to measure individual compounds at drinking water target levels. Nevertheless, they might be used to detect sudden peak levels of contaminants, or mixtures of compounds with an additive effect.

Based on this overview, a DNA damage and membrane damage strain were initially selected to be used in the sensor, as these were the strains detecting toxic effects with the highest added value to the already existing biomonitors. The bacterial strains specific for other toxic effects, such as protein damage and oxidative stress are more related to cytotoxicity, which is also detected by the currently available biomonitors, especially those applying Aliivibrio fischeri. Although membrane damage is also related to cytotoxicity, it was thought that this effect might also be caused by neurotoxicants, which are also of interest regarding water quality control monitoring. After a laboratory experiment with these strains, using a 96-well plate format, it became clear that the response of the strain specific for membrane damage was influenced too much by bacterial growth to be able to reliably distinguish a response caused by the presence of a toxicant. Therefore, it was decided to continue with the DNA damage strain, DPD2794, only.

In chapter 3, a study was performed in 96-well plates to characterize the response of the selected DNA damage strain. The bacteria were exposed to a concentration range of a training set of known genotoxic and non-genotoxic compounds, as well as a number of confirmed environmental pollutants.
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Although useful from a mechanistic point of view and for widening the scope of detection, no metabolic activation was included. This was mainly decided because metabolic activation, usually by addition of rat liver enzymes, is not feasible in the type of on-line sensor that was constructed. Therefore, only the three direct genotoxicants (mitomycin C, nalidixic acid, and 4-NQO) showed a measurable positive response. One false positive response was obtained for chloramphenicol, which was actually cytotoxic instead of genotoxic. This compound caused a shift in the background luminescence of the bacteria, which was registered as a response. However, since there was no actual rise in signal, it is very unlikely that chloramphenicol will give a response in the newly constructed on-line sensor. None of the environmental pollutants gave a positive response. This was not unexpected, since the most well-known environmental contaminants are not directly genotoxic. It was thus confirmed that this strain responds reliably to a training set of genotoxic compounds and showed a low probability for false positives.

Parallel to the tests with the bacteria in the 96-well plates, the mechanical part of the sensor was designed and constructed and sol-gel was tested as a matrix to immobilize the bacteria. The design of the sensor and the results of the first experiments with the DNA damage strain in this sensor were shown in chapter 4. The bacteria were immobilized in the sensor in a sol-gel, which was applied on the surface of an optical fiber and a glass slide. The fiber and slide were positioned in a stainless steel measurement chamber, and the light of the bacteria was measured with two photomultipliers (PMTs), one for the fibre and one for the slide. The water flowed continuously through the chamber, and medium was added to sustain the bacterial response. All parts of the sensor were assembled in an incubator, which ensured a stable temperature of 26 °C of the water in the measurement chamber. The bacteria responded to spikes of genotoxic compounds with a limit of detection (LOD) for mitomycin C of 0.01 mg/l for the glass slide and 0.1 mg/l for the optical fiber. The difference in sensitivity between the two setups was caused by the higher variation between experiments in case of the optical fiber. The bacteria were capable of responding to spikes up to at least 22 hours after the start of an experiment. However, due to a rapid growth of other microorganisms in the sensor (fouling), which clearly deteriorated the bacterial response, it was not possible to do experiments on subsequent days without cleaning the sensor in between.
In chapter 5, firstly the effects were described of two filters, which were installed in front of the sensor to reduce the amount of contaminating microorganisms in the sensor. Although this gave an improvement in the stability of the background response, and was probably necessary for experiments with surface water, it remained impossible to do subsequent experiments without in-between cleaning.

A UV-unit with an 18-Watt UV-C lamp was installed behind the sensor, to inactivate the bacteria in the waste water and prevent the release of GMO's without the use of chlorine. Although this unit was sufficiently effective in the laboratory with tap water, some bacteria were still found after the UV treatment when surface water was used. Therefore, chlorine remained necessary for a complete disinfection of the waste water during field experiments.

The sensor was tested on a field location for two months at a monitoring station along the river Meuse in The Netherlands. At first, there was a very high peak in response after 10-17 hours, possibly due to rapid growth of the luminescent bacteria, a problem which could be solved by reducing the medium concentration. The sensor did respond to spikes of genotoxic compounds dosed to the water inlet of the sensor with an LOD of 0.1 mg/l mitomycin C. However, there were no responses above background when the sensor, with the DNA damage strain, was used for continuous monitoring of Meuse water, even though there were some occasions of alarms in the Daphnia and mussel monitors at the same station and in the same time window for monitoring. This, however, may be explained by the fact that the biosensors respond to species-specific overall toxicity, while the strain in the bacterial sensor is focussed on detecting genotoxicants. During the test period, the sensor proved to be robust in terms of operation, with hardly any technical problems.

In conclusion, the developed on-line biosensor was found to be promising as a valuable addition to the existing water monitoring techniques.
General discussion and outlook
Although the newly constructed on-line sensor, using immobilized luminescent bacteria, has shown potential to become a useful addition to the current water monitoring techniques, there are still some limitations to be improved. Firstly, the fact that the sensitivity of the sensor is not sufficient to enable monitoring at the target level of 10 ng/L for genotoxic substances (1), remains a recurring issue. This holds not only for this sensor, but also for similar sensors (2, 3), and is linked to the limitations posed by the sensitivity of the bacteria themselves. For this reason some researchers have attempted, and also succeeded, in making the bacteria more sensitive by additional genetic modifications (4-6). However, this is a time-consuming process, and tends to make the bacteria even more vulnerable and thus more difficult to sustain in a sensor. Another way of improving the sensitivity of the sensor would be to reduce the variation between experiments. In this way, it should be possible to bring the detection limit (LOD) of the sensor closer to the detection limits obtained in the 96-well plates. Finally, on-line solid phase-extraction (SPE) could be installed in front of the sensor to concentrate the sample, just as SPE is used for water analyses with the Ames (fluctuation) test to gain sufficient sensitivity. In that case, it will be necessary to use a sensor which analyzes small volume batches of water, as on-line SPE is actually a semi on-line system, producing regular batches of extracts. In such a batch system, it also becomes possible to add a module including rat liver extract for metabolic activation, which would increase the range of compounds that can be detected. An example of a sensor analyzing small batch samples will be described later.

Another more practical limitation is that the bacteria have to be changed and the system cleaned every day. Apart from the fact that this makes the operation of the sensor very labour-intensive, it also results in the typical day-to-day pattern shown in Chapter 5. The daily fluctuations in background signal compromise the option to use a single value as limit for an alarm and there is a period every day during which the sensor cannot be used. The daily background curve consists of an increase in luminescence, which is thought to be due to bacterial growth, and then a decrease in response. The reason for the loss of response remains a complex subject, especially since it seems to be linked to several factors. What has become clear is that the growth of other microorganisms in the sensor is an important aspect. These native water bacteria reproduce.
rapidly in the sensor, especially after the point where the medium is added to the water. It is expected that they compete with the luminescent *E. coli* for nutrients, oxygen, and space. As described in Chapter 5, an attempt was made to reduce the disturbance by fouling with other organisms by placing filters. Although this was not unsuccessful, i.e., the experiments with surface water would most likely have been impossible without filters, they were not sufficient to entirely prevent contamination of the system. To really prevent contamination with other microorganisms, the setup of the sensor must be changed.

Another aspect is thought to be the loss of growth of the luminescent bacteria themselves. Although it was unfortunately not possible to measure the density of the bacteria in the gel during the experiments, it was observed that the gel, which was clear at the beginning, became opaque in the course of an experiment. Measurements of bacteria in the waste water of the sensor also showed an increase in the concentration of *E. coli* in the waste water during the course of the experiment. This showed that the bacteria were dividing in the gel during the experiments and could escape from the gel into the water. However, since bacteria only replicate until a certain density is reached, it is expected that the division of the bacteria slows down or even stops when a certain density in the gel has been reached. When this happens, there is less DNA replication, therefore less DNA quality control, less DNA damage repair and at the end less induction of luminescence (7, 8). This could be a reason for the observed reduction in background luminescence, but also for the observed decrease in response over time: at later time points, a smaller portion of the DNA damaged by the toxicant will be repaired, because there are less cell divisions. This issue can also not be solved with the sensor setup described in this thesis; a different setup is necessary.

A few alternative sensors have been designed to deal with these issues. A logical solution for the problem of the shift in growth phase by increasing density would be a sensor in which a continuous supply of fresh bacteria is provided and excess bacteria are removed. The mini-bioreactor sensors already mentioned in the Introduction were clearly designed with this in mind. In these sensors, the bacteria are grown continuously in a bioreactor and exposed to the sample, either in the same reactor or a second reactor. By also culturing the bacteria on-line, measurements of several days to a week have been reported with a stable concentration of bacteria (3, 7). The most important disadvantages
have also been mentioned, namely the dilution rate of the sample water and the high concentration of GMO's in the waste water. The dilution rate is the flow rate divided by the reactor volume and indicates the rate at which the water in the reactor is refreshed. The dilution rate of both the normal and mini-two stage reactors was 0.8 h\(^{-1}\) \((3, 9)\), which is much less than the dilution rate of 23 h\(^{-1}\) of the water in the measurement chamber of the new sensor. The bioreactors also require filtration of the incoming water to prevent biofilm formation in the reactor and tubing. These limitations make also this design cumbersome for environmental monitoring, especially when more strains are used.

Another alternative is a batch setup, in which samples are taken at regular intervals and each sample is added to a new batch of bacteria. There is already a commercial biomonitor that uses such a batch setup with *Aliivibrio fischeri*, namely the ToxControl (Microlan). A good example of such a setup with genetically modified bacteria is the Lumisens IV, which contained a multi-well plate with lyophilized bacteria for the detection of metals \((10)\). Because the bacteria were lyophilized before use, they were always in the same growth phase at the time of exposure. Also fouling was not so much an issue, since a new well was used for every measurement. An additional advantage was that the large number of wells makes it relatively easy to use multiple strains at the same time. It has been proven that with this setup, it was possible to measure environmental samples for ten days with a very low variation. Of course, the obvious disadvantage of such a device is that it is arguable whether it is really an ‘on-line’ sensor, since the bacteria are not continuously exposed. The maximum length of a measurement will in this case be determined by the number of wells, the number of strains used, and the measuring frequency. It will depend on these factors and the purpose for which the sensor is used whether batch sampling is truly a limitation or not.

The last example of an alternative design is a sensor that was recently developed in the SAWA (Sensors and water) project by 2M Sensors, with the limitations of the sensor described in this thesis in mind. This sensor is currently tested and optimized at Waterlaboratorium Noord. In this new device, the bacteria are kept between dialysis membranes with the sample water on one side and the medium on the other side. Thus, there is no mixing of medium and sample water before it reaches the luminescent bacteria, which is expected to cause much less fouling in the sensor. The membranes also prevent direct contact between the bacteria in the water and the luminescent bacteria, and help to avoid the
release of GMO’s in the waste water. The design also enables a relatively easy addition of more measurement units with different strains of bacteria. Whether this new design is indeed more stable and easily usable during longer periods of on-line monitoring, is a question that will be answered in the near future. In any case, it is not suitable to couple to “on-line” SPE, thus the sensitivity remains a problem with this setup, and the bacterial density can still reach a maximum in the limited space between the dialysis membranes.

Theoretically, the best design for the purpose of surface water inlet monitoring for drinking water production, seems to be a semi-on-line device, with a semi-online SPE unit coupled to it, which concentrates the water 1000-10,000 fold. The SPE will remove the foreign micro-organisms from the surface water simultaneously, which should decrease the problem of fouling. The SPE extracts are then best analyzed on fresh batches of (lyophilized) bacteria, e.g. in a device like the Lumisens IV. In such a setup, it might also be easier to compare a response to a water sample to that of a blank, as tap water samples can be extracted and analyzed at the same time as the surface water samples.

Regardless of the exact design, this new (semi)-on-line sensor will have the highest added value if it contains multiple strains that can measure several different effects and/or compounds simultaneously. The choice for the strains can depend on the purpose for which the sensor is used. For surface water monitoring, DNA damage strains are useful because this effect can not be measured by the biomonitors. A strain for more general stress, such as protein damage, can be useful as control for toxicity. The strains specific for certain metals or organic pollutants might also be used when there is a specific interest in these compounds.

Such a sensor can be a valuable addition to the current methods for surface water inlet monitoring, but also other applications can be imagined, where a relatively fast and simple screening method would be useful. For example, after an accident in which toxic chemicals have been released to the water and the spread of the pollution has to be determined. Or when there is a suspicion of pollution of the ground water or a lake, these bacteria could be used as a quick first test. For these applications, there is no need for on-line monitoring and also a simpler array setup might serve this purpose. The added value of this test above the current methods is the speed: a response is visible after one
hour, while current methods need at least 24 hours (e.g. CALUX tests, Ames (fluctuation) test, comet assay). Nevertheless, the incorporation of the bacteria in a sensor can be useful to reduce the variation in handling in the field, for example by temperature stabilization and automatic standardization of the dosing of the samples. In any case, this device should also provide a way to ensure no bacteria are released to the environment, either by disabling the bacteria or by preventing their release completely. This will be necessary to comply with the strict regulations regarding GMO’s, which have to be taken into account for a biosensor, based on these bacteria, to become successful.
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References


